ADVANCES IN THE UNDERSTANDING OF THE COMMENSAL EUKARYOTA AND VIRUSES OF THE HERBIVORE GUT

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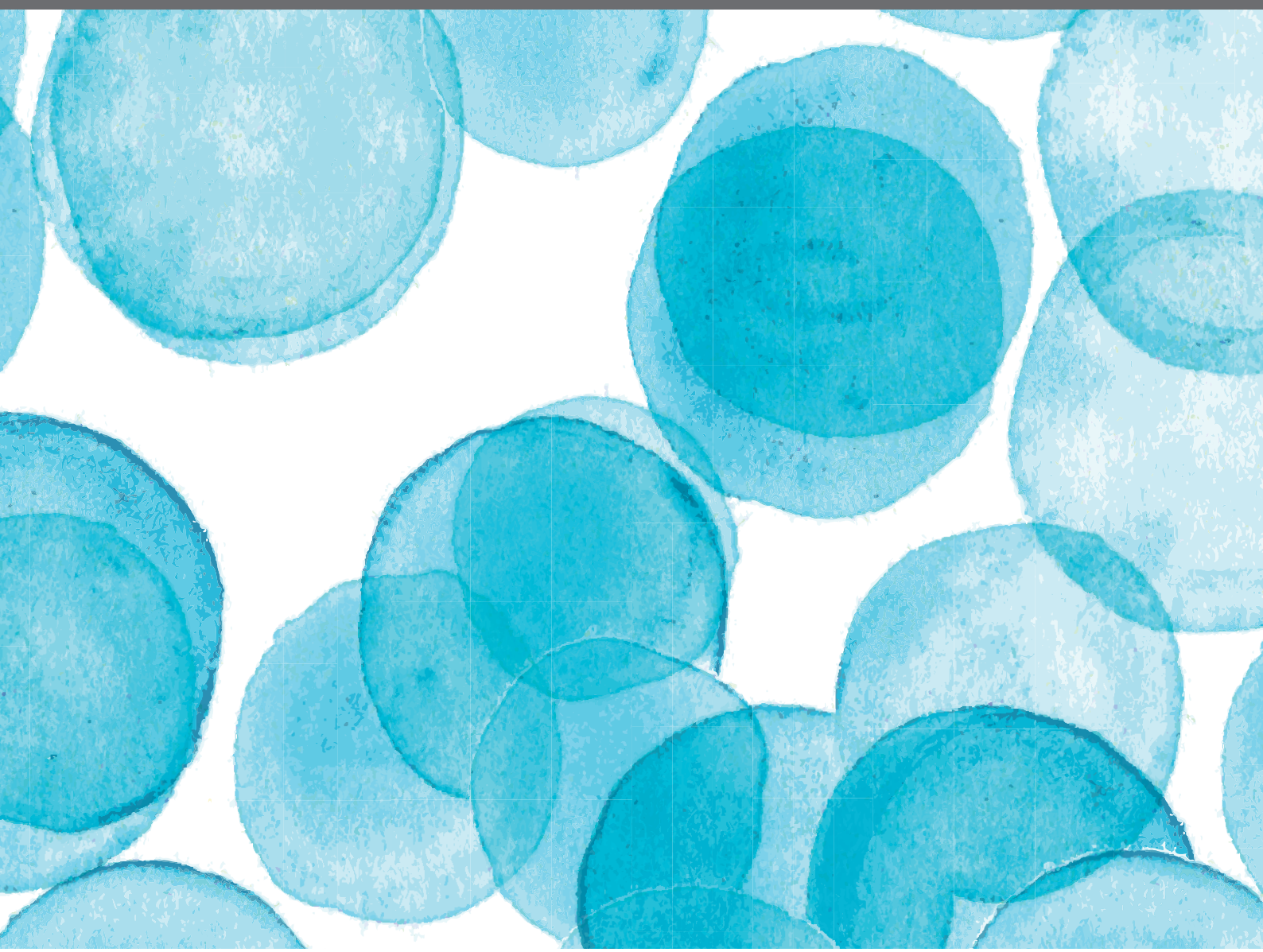

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ADVANCES IN THE UNDERSTANDING OF THE COMMENSAL EUKARYOTA AND VIRUSES OF THE HERBIVORE GUT

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Editorial: Advances in the Understanding of the Commensal Eukaryota and Viruses of the Herbivore Gut

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Editorial on the Research Topic

Advances in the Understanding of the Commensal Eukaryota and Viruses of the Herbivore Gut

Herbivores play an important role in the survival of humanity, contributing food and textiles, as well as social and economic value. For decades, optimizing the productivity, health, welfare, and environmental footprint of herbivorous animals, particularly ruminant livestock, has been the subject of an extensive, global research effort. Much of this research effort has focused on the herbivore gut. The specialized nature of the herbivore digestive tract and its resident microbes enables the breakdown of highly fibrous plant materials, which are unable to be utilized by omnivores and carnivores. In recent years, the bacteria and methanogenic archaea have been the major focus of research efforts, with the other gut microbes being understudied in comparison.

The eukaryotic anaerobic fungi and ciliate protozoa represent up to half of the herbivore gut microbial biomass. They are generally recognized as mutualistic symbionts in ruminant animals where they have been most extensively studied to date. These eukaryotic microbes produce a wide range of highly potent, cellulolytic, hemicellulolytic, and amylolytic enzymes, which play a key role in feed degradation for the host. This is particularly true of anaerobic fungi, which are the most powerful fiber degraders in the known biological world. These specialist fungi initiate a physical and enzymatic attack on plant fiber that benefits the microbial community as a whole, not only by degrading highly complex carbohydrates but also by increasing the accessibility of substrates and facilitating biofilm formation. In addition, due to the production of hydrogen in their hydrogenosomes, anaerobic fungi and ciliate protozoa provide micro-habitats for hydrogen-scavenging bacteria and methanogenic archaea.

The herbivore gut microbiome also contains a dense and diverse population of viruses. The majority of these viruses actively infect and replicate within the microbes resident in the gut (for example, bacteriophages and archaeal viruses), and viral genomes (prophages) can often be found integrated into the genomes of gut microbes. Viruses contribute to gene transfer and cause microbial lysis, resulting in the release of microbial enzymes and modulation of microbial community diversity.

Despite the clear importance of the anaerobic fungi, ciliate protozoa, and viruses to our understanding of herbivore gut function, only bacteria and archaea are commonly characterized in the majority of herbivore gut studies. This Research Topic, therefore, has focused on reviewing

current knowledge and reporting original research and technical advances in our understanding of the roles of commensal eukaryotes (anaerobic fungi and ciliate protozoa) and viruses in the herbivore gut.

Historical and current research, along with future perspectives, were detailed in three comprehensive reviews focused on anaerobic fungi (Hess et al.), ciliate protozoa (Firkins et al.) and viruses (Gilbert et al.). Each of these reviews represents a collaborative effort, drawing on contributions from multiple international research groups. Therefore, they provide a unique snapshot of collective knowledge for each of the respective microbial taxa. These reviews also provide a valuable learning resource, although of particular note is the extensive image library of rumen protozoa (https://ansci.osu.edu/our-people/jeffrey-l-firkins). This library was collated by the laboratory of Burk Dehority and has been made available electronically to aid researchers in understanding protozoal morphology for evaluation and identification purposes (Firkins et al.).

Interactions between the anaerobic fungus *Pecoramyces* sp. F1 and methanogenic archaea were comprehensively investigated using multiple molecular-based approaches (genomic, transcriptomic and proteomic) by Li et al. These tools allowed the authors to provide a detailed mechanistic understanding of the metabolism of the anaerobic fungus-methanogen syntrophic co-culture.

Metatranscriptomics and protein expression techniques were used to show that rumen protozoa produce several carbohydrate-active enzymes (i.e., glycosyl hydrolases 5 and 11, polysaccharide lyases, deacetylases, and xylanases) as well as enzymes active against pectin, mannan and chitin (Williams et al.). Therefore, this study highlighted the predatory capacity of ruminal protozoa as well as the significant contribution these eukaryotes make to carbohydrate breakdown and fermentation in the rumen.

A significant gap in current knowledge of gut viruses was addressed by the isolation and sequencing of novel viruses (phages) infecting the rumen bacterial genus *Butyrivibrio* (Friedersdorff et al.). In addition to this new fundamental knowledge, the addition of sequences for rumen-sourced phage isolates to publicly available sequence databases represents an important and essential step forward for the research community. This will increase the accuracy of viral gene taxonomic and functional annotation, facilitating future advances in the understanding of gut viral communities.

Original research and technical perspectives were provided by two studies investigating the use of alternative techniques for determining the taxonomy and ecology of anaerobic fungal (Edwards et al.) and protozoal communities (Cedrola et al.) in the herbivore gut. Both these studies provided guidance for researchers unfamiliar with the technologies used, and highlighted the challenges and opportunities associated with the study of these specialist eukaryote communities.

As well as reviewing and advancing our understanding of eukaryota and viruses in the herbivore gut, the compilation of the published articles in this e-book, highlights the significant and inspirational efforts made by several research groups, particularly those who focused on the early studies of anaerobic fungi [Professor Colin Orpin and the late Professor Anthony (Tony) Trinci] and protozoa (the late Professor Burk Dehority). The ground-breaking work of Colin Orpin in the early 1970's was a paradigm shift in the understanding of fungal biology, as until his work, all fungi were believed to respire aerobically (Orpin, 1975, 1984; Mountfort and Orpin, 1994). From a long career commencing in the 1960's, Tony Trinci also significantly contributed to current understanding of both anaerobic fungi and mycology in general, exploring fungal physiology, enzymology and providing the conceptual foundations of fungal multicellular growth (Trinci, 1974; Lowe et al., 1987; Trinci et al., 1994). Between 1957 and 2013, Burk Dehority pioneered methodology and research into the synergism of prokaryotic and eukaryotic microbial species for the digestion of plant fiber (Dehority, 1984, 1993, 2003). He shed light onto the vast diversity, function, and beauty of ciliate protozoa, in both domesticated and non-domesticated herbivorous animals, and described no less than 21 new species.

While many historical, current and new advances are detailed in this e-book, collectively this body of work emphasizes the urgent need to adopt more holistic approaches to the study of the herbivore gut microbiome. Only then can the complex interactions within the herbivore gut microbiome be better understood, facilitating the development of novel and sustainable approaches to benefit the nutrition and health of the host animal, whilst mitigating the ecological impact and environmental footprint of livestock-based agriculture.

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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REFERENCES

Dehority, B. A. (1984). Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *Appl. Environ. Microbiol.* 48, 182–185. doi: 10.1128/AEM.48.1.182-185.1984

Dehority, B. A. (1993). *Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa*. Boca Raton, FL: Taylor & Francis.

Dehority, B. A. (2003). *Rumen Microbiology*. Nottingham: Nottingham University Press.

Lowe, S. E., Griffith, G. G., Milne, A., Theodorou, M. K., and Trinci, A. P. J. (1987). The life cycle and growth kinetics of an anaerobic rumen fungus. *Microbiology* 133, 1815–1827. doi: 10.1099/00221287-133-7-1815

Mountfort, D. O., and Orpin, C. G. (1994). *Anaerobic Fungi: Biology, Ecology, and Function*. New York, NY: M. Dekker.

Orpin, C. G. (1975). Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* 91, 249–262. doi: 10.1099/00221287-91-2-249

Orpin, C. G. (1984). The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. *Animal Feed Sci. Tech.* 10, 121–143. doi: 10.1016/0377-8401(84)90003-8

Trinci, A. P. J. (1974). A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. *J. Gen. Microbiol.* 81, 225–236. doi: 10.1099/00221287-81-1-225

Trinci, A. P. J., Davies, D. R., Gull, K., Lawrence, M. I., Bonde Nielsen, B., Rickers, A., et al. (1994). Anaerobic fungi in herbivorous animals. *Mycol. Res.* 98, 129–152. doi: 10.1016/S0953-7562(09)80178-0

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ORIGINAL RESEARCH
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Combined Genomic, Transcriptomic, Proteomic, and Physiological Characterization of the Growth of *Pecoramyces* sp. F1 in Monoculture and Co-culture With a Syntrophic Methanogen

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In this study, the effects of a syntrophic methanogen on the growth of *Pecoramyces* sp. F1 was investigated by characterizing fermentation profiles, as well as functional genomic, transcriptomic, and proteomic analysis. The estimated genome size, GC content, and protein coding regions of strain F1 are 106.83 Mb, 16.07%, and 23.54%, respectively. Comparison of the fungal monoculture with the methanogen co-culture demonstrated that during the fermentation of glucose, the co-culture initially expressed and then down-regulated a large number of genes encoding both enzymes involved in intermediate metabolism and plant cell wall degradation. However, the number of up-regulated proteins doubled at the late-growth stage in the co-culture. In addition, we provide a mechanistic understanding of the metabolism of this fungus in co-culture with a syntrophic methanogen. Further experiments are needed to explore this interaction during degradation of more complex plant cell wall substrates.

Keywords: anaerobic fungus, methanogen, metabolism, genome, RNAseq, iTRAQ

INTRODUCTION

In the rumen, microorganisms, which are mainly composed of anaerobic fungi, bacteria, archaea, and protozoa, have coevolved for millions of years, making the rumen one of the most effective and highly evolved systems regarding degradation of recalcitrant lignocellulosic plant material in nature (Russell and Rychlik, 2001; Weimer et al., 2009). Within this system, the diverse microbial communities cooperate efficiently in the digestion and conversion of plant biomass in feeds to various compounds crucial for body maintenance and performance (Kim et al., 2011; Mao et al., 2016). Anaerobic fungi, bacteria, and protozoa degrade and ferment ingested plant biomass and

release hydrogen during this process (Akin et al., 1988). However, the accumulation of hydrogen is energetically unfavorable and can inhibit the fermentation of ingested feed (Ungerfeld, 2015). Ruminal methanogens are effective hydrogen utilizers and can use the hydrogen generated to reduce carbon dioxide (which is also a product of primary fermentation) to methane, thereby keeping the steady-state hydrogen concentration low and the rumen operating more efficiently (Janssen and Kirs, 2008). Thus, trophic interactions exist between the methanogenic archaea and the hydrogen-producing microorganisms that includes both anaerobic fungi and bacteria.

Anaerobic fungi assigned to the phylum Neocallimastigomycota play key roles in the decomposition of recalcitrant plant lignocellulosic materials in the rumen. Since the identification of anaerobic fungi by Orpin (1975), 11 genera assigned to the phylum Neocallimastigomycota have been described: *Neocallimastix* (Heath et al., 1983), *Caecomyces* (Gold et al., 1988), *Piromyces* (Gold et al., 1988), *Orpinomyces* (Barr et al., 1989), *Anaeromyces* (Breton et al., 1990), *Cyllamyces* (Ozkose et al., 2001), *Buwchfawromyces* (Callaghan et al., 2015), *Oontomyces* (Dagar et al., 2015), *Pecoramyces* (Hanafy et al., 2017), *Feramyces* (Hanafy et al., 2018), and *Liebetanzomyces* (Joshi et al., 2018). Despite their potent capacities for lignocellulose degradation, anaerobic fungi and their enzymes are yet to be exploited in biotechnological processes. This is largely due to their obligately anaerobic lifestyle and a poor understanding of their growth requirements and metabolic characteristics. Anaerobic fungi can ferment a wide range of fermentable sugars, such as glucose, fructose, xylose, and cellobiose as energy sources. These are utilized to produce H_2, CO_2, formate, acetate, lactate, and ethanol as the major fermentation end products (Lowe et al., 1987; Teunissen et al., 1993). In their natural habitat in the rumen and hind-gut of large mammalian herbivores, anaerobic fungi grow together in communities with other microbes. Anaerobic fungi and closely associated methanogens can be isolated from mixed microbial communities and can be cultured in stable co-culture in media that do not contain appreciable amounts of compounds that methanogens need to grow (Cheng et al., 2009). Anaerobic fungal-methanogen co-cultures have been shown to be stable with robust growth evident over long periods of time (Bauchop and Mountfort, 1981; Cheng et al., 2009). Additionally, in co-cultures, as a consequence of inter-species hydrogen transfer, the metabolite profile of the anaerobic fungus alters, shifting away from more reduced products, such as lactate and ethanol, toward acetate and formate. The formate and hydrogen, end products of fungal fermentation, are used by the methanogens to produce methane (Cheng et al., 2009; Jin et al., 2011; Li et al., 2016). Meanwhile, the fiber-degrading ability of the anaerobic fungus in co-cultures was improved (Jin et al., 2011). Thus, the metabolic profile of anaerobic fungi in the co-culture is comparable to that of their counterparts in the rumen, where hydrogen and formate are known to be transient and low (Hungate, 1967; Hungate et al., 1970), and the fiber-degrading ability is known to be high (Krause et al., 2003). Thus, investigating the interaction between anaerobic fungi and co-cultured methanogen might provide insights into the complex microbial interactions in the rumen.

In recent years, omics-based techniques have been used to study the diversity, ecology, and biology of anaerobic fungi. Five genomes of anaerobic fungal strains have been reported, including *Piromyces* sp. E2, *Pecoramyces ruminantium* C1A, *Anaeromyces robustus*, *Neocallimastix californiae*, and *Piromyces finnis* (Youssef et al., 2013; Haitjema et al., 2017). The transcriptomes of *Pecoramyces ruminantium* C1A, *Piromyces finnis*, *Neocallimastix californiae*, *Caecomyces churrovis*, *Anaeromyces mucronatus*, *Neocallimastix frontalis*, *Orpinomyces joyonii*, *Piromyces rhizinflata*, and *Anaeromyces robustus* have been described (Couger et al., 2015; Solomon et al., 2016; Henske et al., 2017; Gruninger et al., 2018). To our knowledge, there are no studies that apply functional genomic, transcriptomic, and proteomic approaches to interrogate the effect of co-culturing a methanogen on the metabolism, including expression of fiber-degrading enzymes, of an anaerobic fungus.

In the present study, we used genomic, transcriptomic, and metabolomic data of the anaerobic fungal monoculture to draw a metabolic pathway of the fungus. The mRNA expression profile of the anaerobic fungus *Pecoramyces* sp. F1 in the presence and absence of its syntrophic methanogen, *Methanobrevibacter thaueri*, was also investigated. By combining the foregoing analysis with the anaerobic fungal proteome dynamics and analysis of the metabolites induced by growth with the methanogen, we reveal the effects of the archaeon on the metabolism of the anaerobic fungus.

MATERIALS AND METHODS

Maintenance of Anaerobic Fungal Monoculture and Co-culture

The anaerobic fungus *Pecoramyces* sp. F1, formerly described as *Piromyces* sp. F1, and its symbiotic methanogen, *Methanobrevibacter thaueri*, were isolated and identified from goat rumen by Jin et al. (2011). The culture was maintained in rumen fluid media (Davies et al., 1993) with 1% (w/v) rice straw as substrate and transferred every 3 days. The media was prepared according to Cheng et al. (2009) and 90 ml media was dispensed into 160 ml serum bottle with 1 g rice straw as substrate. At each transfer, 10 ml of 3-day-old culture was inoculated into 90 ml of fresh media and incubated at 39°C for 3 days. The fungal monoculture was obtained by adding chloramphenicol (50 mg l^{-1} final concentration) to inhibit the growth of the associated methanogen (Cheng et al., 2009). The relative abundance of methane in the head-space gas of the monoculture was analyzed by GC-TCD (Agilent 7890B, Agilent, Santa Clara, CA, United States) to ensure that no methane was being produced by the culture to confirm that the methanogen was no longer present in fungal pure culture studies.

Experimental Design and Sample Collection

In the current study, the medium used for experiments was a modified medium M2 (Barichevich and Calza, 1990)

with 2.16 g l^{-1} (12 mM) glucose as substrate. The medium was prepared and dispensed under anaerobic conditions into serum bottles (90 ml/bottle), with pH adjusted to 6.8 (Li et al., 2016). For anaerobic fungal genome sequencing, 40 bottles of *Pecoramyces* sp. F1 monoculture were incubated at 39°C for 72 h without shaking. The fungal cells were then harvested by centrifugation at 10,000 × *g* for 15 min.

To investigate the effects of co-culturing with *M. thaueri* on the metabolism of *Pecoramyces* sp. F1, the anaerobic fungus was grown alone (monoculture) and also in co-culture with the methanogen at 39°C without shaking. A total of 72 bottles were used for the experiment; details of the protocol information are shown in **Supplementary Figure 1**. Samples were collected from each replicate for transcriptomic, proteomic, and metabolite analysis. The total volume of gas accumulated in each culture over the incubation period was also measured using the pressure transducer technique (Theodorou et al., 1994). After each reading, the head-space was vented to return the pressure to ambient conditions. Furthermore, the gas drawn was analyzed for CH_4 and H_2 content. Samples from the cultures were collected at approximately 50% and 100% of maximum gas production (i.e., mid- and late-growth stages) as determined from previously generated gas accumulation curves. The pH was measured at each time point immediately upon removing crimp-seals and stoppers from the serum vials. Aliquots of 5 ml supernatant were then collected and stored at −20°C for subsequent analysis of water-soluble metabolites. The rest of the culture was then centrifuged at 8,000 × *g* for 15 min, and 1 ml of supernatant was used for the analysis of residual glucose with a commercial glucose kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China). The cells from the remaining six bottles, representing each replicate, were then mixed and split into two parts for RNAseq and iTRAQ analysis. Two bottles of each replicate were used for the analysis of gas, glucose, pH, and water-soluble metabolites.

DNA Extraction, Sequencing, Genome Assembly, and Gene Calling and Annotation

Genomic DNA was extracted from a 3-day-old anaerobic fungal monoculture with the CTAB method (Cheng et al., 2017). Briefly, the culture was centrifuged and ground in liquid nitrogen. CTAB buffer was added to dissolve the powder and phenol/chloroform/isoamyl alcohol (25:24:1) was then used to purify the DNA. Three libraries with insert sizes of 170 bp, 350 bp, and 6,000 bp were prepared at BGI (Beijing Genomics Institute, Shenzhen, China) according to the manufacturer's instructions (Illumina). Paired-end sequencing was conducted on an Illumina Hiseq 2000 platform (BGI, Shenzhen, China). A total of 28.67 Gb in 159,302,966 quality-filtered paired-end reads were used for assembly (**Supplementary Table 1**). The quality-filtered reads were assembled with SOAPdenovo V1.05 (Li et al., 2008, 2010) using a kmer value of 43. The assembly was then optimized by the paired-end and overlap relationship of reads through mapping reads to assembled contigs. Gene calling was then conducted using a combination of Augustus V2.6.1 and Genemarkes V2.3e (Ter-Hovhannisyan et al., 2008; Keller et al., 2011). Transposable elements (TEs) were identified by RepeatMasker (Repbase) and RepeatProteinMasker[1]. Tandem repeats were identified by Tandem Repeat Finder (TRF) (Benson, 1999). The number of simple sequence repeats (SSRs) were calculated using the results of TRF according to Youssef et al. (2013). The rRNAs and tRNAs were identified using RNAmmer 1.2 (Lagesen et al., 2007) and tRNAscan-SE 1.23 (Lowe and Eddy, 1997), respectively. BLAST was used for the annotation of gene models against KEGG, GO, CAZy, Uniprot_Swissprot and non-redundant (NR) databases (Bard and Winter, 2000; Kanehisa et al., 2004; Cantarel et al., 2009; The UniProt Consortium, 2015). The genome assembly and gene calling and annotation were conducted by BGI (Shenzhen, China). The raw data was submitted to SRA under the accession number: PRJNA517297.

RNAseq Mapping and Differentially Expressed Gene Analysis

The RNA for RNAseq analysis were isolated from the mid- and late-growth stages of the anaerobic fungal monoculture and co-culture. The RNAseq libraries, which included only mRNA, were generated according to the Illumina TruSeq RNA sample protocol. The mRNA was enriched using oligo-dT (Rio et al., 2010). Paired-end sequencing was conducted on an Illumina Hiseq 2000 platform (BGI, Shenzhen, China). All quality-filtered reads were mapped to the genome and genes by BWA (Li and Durbin, 2009) and Bowtie (Langmead et al., 2009), respectively. The number of reads produced per sample and the mapping results are provided in **Supplementary Table 2**. The quantification of gene expression was calculated in fragments per kilobase of transcript per million mapped reads (FPKM) with the RSEM package (Li and Dewey, 2011). To assess variability between biological replicates, the coefficient of determination R^2 was calculated between biological replicate pairs using RSEM-generated FPKM values (**Supplementary Table 3**). The raw data was submitted to SRA under the accession number: PRJNA517315. Differentially expressed genes were screened with the NOISeq package (Tarazona et al., 2015) according to the following criteria: fold change $> \pm 2$ and divergence probability > 0.8.

Isobaric Tags for Relative and Absolute Quantization (iTRAQ) Analysis of Proteins

Proteins for iTRAQ analysis were collected from the mid- and late-growth stages of the anaerobic fungal monoculture and co-culture. The cells were digested and labeled according to Yan et al. (2016). One biological replicate from each sample (four samples in total) was then mixed as one iTRAQ set resulting in three iTRAQ sets that were analyzed. The mixed

[1]http://repeatmasker.org

fractions were then separated by liquid chromatography (LC) and analyzed by two-step mass spectrometry (MS) (Yan et al., 2016). All procedures were conducted at BGI (Shenzhen, China). The MGF files, converted from the raw data using a 5600 msconverter, were used for protein identification with the Mascot search engine (Matrix Science, London, United Kingdom; version 2.3.02) against the fungal transcriptome containing 17,639 sequences (Yan et al., 2016). The identification of proteins in the three sets is shown in **Supplementary Table 4**. The proteomic dataset was deposited in the iPROX database under the accession number IPX0001499000. The criteria for differential expression of proteins was a P-value < 0.05 and fold change $> \pm 1.2$ in at least two iTRAQ sets.

Nucleotide Sequencing of 28S rRNA Gene and ITS Sequences and Phylogenetic Analysis

The genomic DNA from the anaerobic fungal monoculture was used to amplify the 28S rRNA gene using the primer pair AF-LSU (5′-GCTCAAAYTTGAAATCTTMAAG-3′) and AF-LSU (5′-CTTGTTAAMYRAAAAGTGCATT-3′) (Dollhofer et al., 2016). To amplify the ITS sequence, primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used (White et al., 1990). The PCR reaction (20 μl) consisted of 0.5 μl of each primer, 1 μl of the template DNA and 10 μl of PCR Master Mix. For 28S rRNA gene amplification, after initial denaturation at 94°C for 3 min, 36 cycles of amplification were performed, with 94°C for 20 s denaturation, 61°C for 45 s annealing, 72°C for 45 s extension, and a final extension of 72°C for 10 min. For the amplification of ITS sequence, we performed an initial denaturation at 95°C for 3 min, followed by 39 cycles of amplification with 95°C for 30 s denaturation, 52°C for 1 min annealing, 72°C for 1 min extension, and a final extension of 72°C for 5 min. The sequences were deposited at the GenBank under accession numbers MG250475 and MG250482 for 28S rRNA gene and ITS sequences, respectively. 28S rRNA gene and ITS sequences from representatives of the anaerobic fungal genera were retrieved and used to construct phylogenetic trees with MEGA 6 (Tamura et al., 2013).

Chemical Analysis

The head-space gas of the culture was collected and analyzed for relative abundances of H_2 and CH_4 using GC-TCD (Agilent 7890B, Agilent, Santa Clara, CA, United States) according to Li et al. (2016). The volumes of H_2 and CH_4 were then calculated according to the total gas production. The concentration of ethanol was measured by GC-TCD using a method described by Li et al. (2016). The concentrations of formate, acetate, lactate, malate, citrate, and succinate were analyzed by HPLC (1220 Infinity LC system, Agilent, Santa Clara, CA, United States) with a reversed phase column ZorbaxSB-Aq (Agilent, Santa Clara, CA, United States) according to Li et al. (2016). The statistical analysis of glucose, gas, pH, and fermentation end products was conducted in RStudio[2] and a significant effect was declared at $P < 0.05$.

RESULTS

The Genome of Anaerobic Fungus *Pecoramyces* sp. F1

The anaerobic fungus in the present study was isolated in co-culture with the methanogen, *M. thaueri* and the results were published by Jin et al. (2011). Based on the fungal morphology, particularly the monocentric fungal thallus and presence of monoflagellated zoospores, the fungal component of the co-culture was assigned to the genus *Piromyces* (Jin et al., 2011). Subsequently it became apparent that the newly discovered genus, *Pecoramyces* was morphological similar to some *Piromyces* isolates (Hanafy et al., 2017). To obtain a more accurate identification of our fungal isolate, we applied molecular techniques based on the amplification and sequencing of the gene encoding the 28S rRNA and its ITS sequences. Using these sequences information, two phylogenetic trees were constructed based on the 28S rRNA gene sequence and the ITS sequence, respectively (**Supplementary Figure 2**). Both phylogenetic trees confirmed that the fungus isolated in co-culture with *M. thaueri* (Jin et al., 2011) is a member of the newly described anaerobic fungal genus, *Pecoramyces* (Hanafy et al., 2017), and is subsequently referred to as *Pecoramyces* sp. F1.

The genome of *Pecoramyces* sp. F1 was sequenced using paired-end Illumina technology with approximately 268× coverage. Results estimated the genome size of this fungus to be 106.83 Mb (**Table 1**). As observed in previously reported anaerobic fungal genomes (Youssef et al., 2013; Haitjema et al., 2017), *Pecoramyces* sp. F1 exhibited low GC content (16.07%) with a very low proportion of the genome used in coding for proteins (23.54%). From the data, it was estimated that the genome encoded 17,740 genes with an average length of 1,918 bp. A comparison of the *Pecoramyces* sp. F1 genome with five published anaerobic fungal genomes is shown in **Table 2**. The implications relating to genome structure are discussed later. The putative pathway for metabolism of glucose by *Pecoramyces*

[2]http://www.rstudio.org

TABLE 1 | The assembly of the genome of *Pecoramyces* sp. F1.

Items	Scaffold	Contig
Total number	10,442	19,426
Total length (bp)	106,834,627	98,707,616
N50 (bp)	40,524	10,106
N90 (bp)	2,916	2,011
Max length (bp)	272,868	156,300
Min length (bp)	1,000	200
Sequence GC (%)	16.07	16.07

Three libraries with insert sizes of 170 bp, 350 bp, and 6,000 bp were prepared. A total of 28.67 Gb in 159,302,966 quality-filtered paired-end reads was used for assembly with SOAPdenovo V1.05 using a kmer value of 43.

TABLE 2 | A comparison of genomes of *Pecoramyces* sp. F1 and other anaerobic fungi.

Items	*Pecoramyces* sp. F1	*Pecoramyces ruminantium*[#]	*Piromyces* sp. E2[$]	*Piromyces finnis*[$]	*Anaeromyces robustus*[$]	*Neocallimastix californiae*[$]
Estimated genome size (Mb)	106.83	100.95	71.02	56.46	71.69	193.03
Number of scaffolds	10,442	32,574	1,656	232	1035	1,819
Protein coding (%)	23.54	20.60	23.90	30.35	27.41	15.22
Number of genes	17,740	16,347	14,612	11,314	13,081	21,028
Average gene length (bp)	1,918	1,623	1,675	2,278	2,350	2,216
Number of exons	66,993	52,044	45,130	54,796	60,136	86,802
GC content (%)	16.07	17.00	21.80	21.18	16.30	18.20

[#]*The data were reported by Youssef et al. (2013).* [$]*The data were reported by Haitjema et al. (2017).*

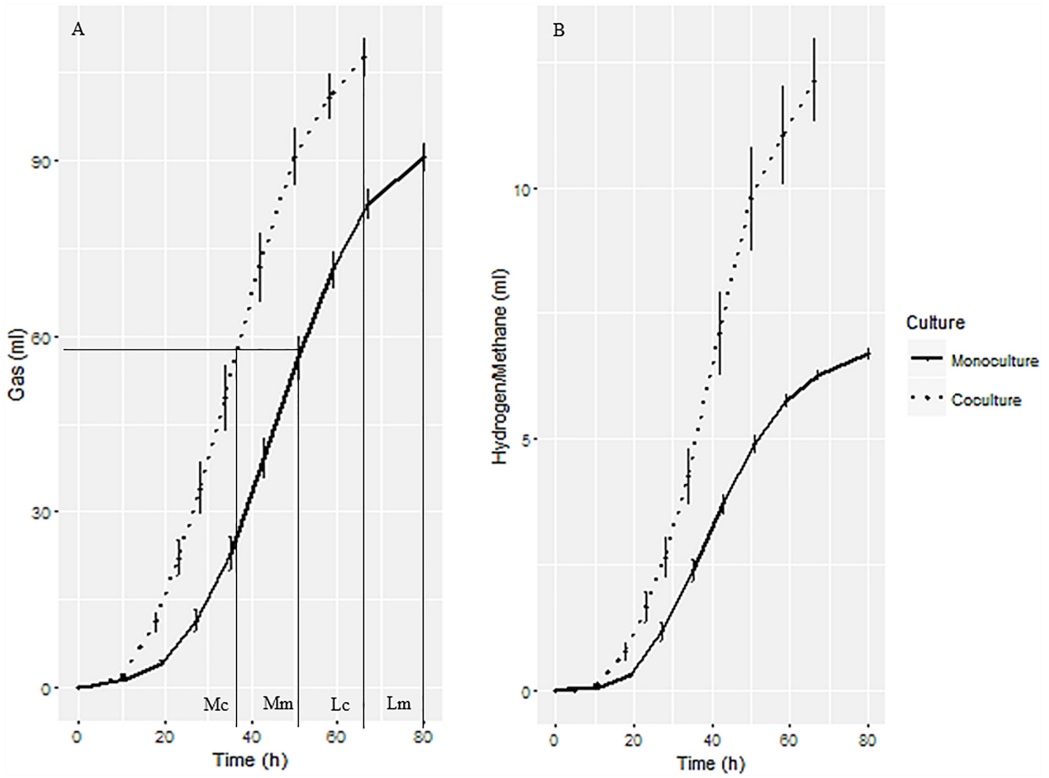

FIGURE 1 | Gas production of anaerobic fungal monoculture and fungal-methanogen co-culture. **(A)** Cumulative gas production curve showing the sampling time for transcriptomic, proteomic, and fermentation end products analysis. Mc, mid-growth stage of co-culture; Mm, mid-growth stage of monoculture; Lc, late-growth stage of co-culture; Lm, late-growth stage of monoculture. **(B)** H_2 and CH_4 production from fungal monoculture and co-culture, respectively.

sp. F1 was demonstrated in **Supplementary Figure 3** based on genomic and transcriptomic data. Comparison of the genomes of anaerobic fungi and aerobic fungi was demonstrated in **Supplementary Figure 4**.

Effect of Co-culturing With a Methanogen on the Metabolism of *Pecoramyces* sp. F1 at Mid-Growth Stages

The gas production curves of the anaerobic fungal monoculture and co-culture with the methanogen are shown in **Figure 1**. The co-culture grew more rapidly and produced more gas, reaching mid- and late-growth stages sooner than the corresponding axenic cultures (**Figure 1A**). A total gas volume of 107 ml in the anaerobic fungus/methanogen co-culture was measured after 66 h of cultivation compared with 90 ml after a longer incubation time of 80 h of the monoculture. Large amounts of H_2 accumulated in the monoculture whereas it was undetectable in the co-culture. As expected, CH_4 accumulated in the anaerobic fungus/methanogen co-culture (**Figure 1B**). For further molecular analysis, samples were taken at mid- and late-growth stages.

Based on transcriptional analysis (mRNA data), at the mid-growth stage 12,262 ± 171 and 12,176 ± 311 genes were expressed in the monoculture and co-culture, respectively

($P > 0.05$). In comparison to the monoculture, it was observed that 62 and 121 genes were up-regulated and down-regulated, respectively, in the co-culture (**Supplementary Table 5**). The top 10 up-regulated and down-regulated genes and their functional annotations are shown in **Table 3**. Half of the top 10 up-regulated genes were annotated as fiber-degrading enzymes. The number of genes undergoing alternative splicing were examined and 8,281 ± 878 and 7,727 ± 169 alternatively spliced genes were detected in the monoculture and co-culture, respectively ($P > 0.05$).

In addition to the transcriptional analysis, proteomic analysis was carried out on total proteins at mid- and late-growth stages using the iTRAQ approach. A total of 2,149 proteins were identified (MASCOT) and quantified in all three replicates in both cultures at the mid- and late-growth stages. In comparison with the monoculture, it was observed that 117 and 162 proteins were up-regulated and down-regulated, respectively, in the co-culture at the mid-growth stage. The top 10 up-regulated and down-regulated proteins and their functions are shown in **Table 4**. It is significant that many of the transcripts and proteins that were highly up-regulated or down-regulated had no matches in the databases included in this study (**Tables 3**, **4**), however, a large number of proteins associated with cellular-binding and transmembrane activities were moderately up-regulated (>2 and <100 folds) (**Supplementary Table 6**).

The pH value of the co-culture, although not very different, was significantly higher (6.5 ± 0.03) than that of the monoculture (6.4 ± 0.03) ($P < 0.05$) at the mid-growth stage. Metabolites, including formate, lactate, acetate, ethanol, succinate, malate, and citrate were detected in the supernatant of the monoculture; formate, lactate, succinate, malate, and citrate concentrations were significantly decreased when measured in the anaerobic fungus/methanogen co-culture when compared with the fungal monoculture (**Figure 2**).

The effects of the methanogen on the metabolism of *Pecoramyces* sp. F1 in co-culture at the mid-growth stage is presented in **Supplementary Figure 5**. The expression levels of aconitase and NADH dehydrogenase genes were down-regulated in the co-culture, while no significant differences were observed at the protein level. The expression levels of lactate dehydrogenase and pyruvate formate lyase (PFL) genes were not affected by co-culturing the fungus with the methanogen, although they were up-regulated at the protein level. In the case of aldehyde/alcohol dehydrogenase, it was found to be down-regulated at both the transcription and protein levels.

Effect of Co-culturing With a Methanogen on the Metabolism of *Pecoramyces* sp. F1 at Late-Growth Stages

Measurements made at the late-growth stage showed that 11,978 ± 237 and 10,010 ± 348 genes were expressed in the monoculture and co-culture, respectively ($P < 0.05$). Relative to the monoculture, 42 and 852 of the expressed genes were up-regulated and down-regulated, respectively, in the co-culture. It

TABLE 3 | The top 10 up-/down-regulated genes of anaerobic fungus *Pecoramyces* sp. F1 at mid- and late-growth stages.

Stages	Up-regulated genes	Annotation (NCBInr)	Dow-regulated genes	Annotation (NCBInr)
Mid-growth stage	A07452	–	A15543	–
	A03863	–	A06045	–
	A11553	–	A03640	PREDICTED: LRR receptor-like serine
	A14137	Hypothetical protein	A14257	F5/8 type C domain protein, partial
	A04599	Sugar transporter	A18279	Extracellular alpha amylase
	A00805	Aldo/keto reductase diketogulonate reductase	A03239	Chitin binding protein, partial
	A14029	Putative cellulase	A16618	Rubrerythrin
	A06074	Cellobiohydrolase II-like cellulase Cell	A17342	Circumsporozoite protein
	A08689	Putative cellulase	A16337	Conserved hypothetical protein
	A06592	Putative cellulase	A06176	–
Late-growth stage	A15892	Hypothetical protein Haur_1598	A14983	–
	A08101	Lectin-B	A18355	–
	A15439	PREDICTED: CCR4-NOT transcription complex subunit 1-like	A11982	Putative uncharacterized protein
	A00782	Hypothetical protein Haur_1598	A12645	Endo-1,3-1,4-beta-glucanase
	A01657	Hypothetical protein PFL1_01810	A10479	Cellulase
	A12240	Conserved hypothetical protein	A13908	Hypothetical protein BATDEDRAFT_27702
	A03105	Circumsporozoite protein	A13113	Beta-glucosidase
	A00753	Hypothetical protein RO3G_04189	A05614	Alpha-amylase
	A15083	Hypothetical protein Haur_1598	A14764	Pyruvate kinase, partial
	A10600	Circumsporozoite protein	A06138	Endoglucanase B

TABLE 4 | The top 10 up-/down-regulated proteins of anaerobic fungus *Pecoramyces* sp. F1 at mid- and late-growth stages.

Stages	Up-regulated proteins	Annotation (Uniprot_Swissprot)	Dow-regulated proteins	Annotation (Uniprot_Swissprot)
Mid-growth stage	A10870	–	A08105	Endochitinase A
	A11918	Ubiquinone/menaquinone biosynthesis methyltransferase ubiE	A16996	Probable isoprenylcysteine alpha-carbonyl methylesterase ICMEL1
	A10968	–	A00985	Glutaredoxin-C1
	A01230	–	A04792	Mannan endo-1,4-beta-mannosidase B
	A17203	–	A11779	26 kDa endochitinase 1
	A15978	Uncharacterized symporter ynaJ	A15646	Enamine/imine deaminase
	A11958	ATP-binding cassette sub-family A member 1	A11651	–
	A08103	D-Xylose-proton symporter	A07589	Transcriptional activator HAP5
	A14498	ABC transporter A family member 1	A12393	–
	A15678	–	A04227	Zinc-type alcohol dehydrogenase-like protein PB24D3.08c
Late-growth stage	A15978	Uncharacterized symporter ynaJ	A04324	60S ribosomal protein L27a (fragment)
	A01843	Uncharacterized symporter ynaJ	A12393	–
	A08103	D-Xylose-proton symporter	A09942	Peptidyl-prolyl *cis–trans* isomerase pin1
	A10870	–	A08105	Endochitinase A
	A06767	–	A18633	–
	A07467	Extracellular matrix protein FRAS1	A04839	Adenine phosphoribosyltransferase
	A01230	–	A07269	Guanylate kinase
	A14498	ABC transporter A family member 1	A06738	Histidinol-phosphate aminotransferase
	A06268	Tubulin-specific chaperone A	A12012	–
	A17203	–	A15624	–

was observed that most of the highly up-regulated genes at the transcriptional level in the co-culture (RNA fold change $> \pm 100$) were related to binding activities in the cell (**Supplementary Table 7**). The top 10 up-regulated and down-regulated genes and their functional annotations are shown in **Table 3**. In comparison to the mid-growth stage, at the late-growth stage, fewer genes were alternatively spliced. Thus, we observed 5,908 $\pm$ 603 and 2,061 $\pm$ 226 genes were alternatively spliced in the monoculture and the co-culture, respectively ($P < 0.05$).

In the late-growth stage, the number of proteins up-regulated was double that at the mid-growth stage cultures (276 versus 117). In the case of the down-regulated proteins, however, there was no difference in the numbers observed for the mid- and late-growth stage cultures (168 versus 162). Most of the highly up-regulated proteins (protein ratio $> \pm 2$) were related to sporulation, transmembrane, and cellular-binding activities (**Supplementary Table 8**). The top 10 up-regulated and down-regulated proteins and their functions are shown in **Table 4**.

As observed in the mid-growth stage, the pH value of the co-culture (6.53 $\pm$ 0.002) was significantly higher than that of the monoculture (6.24 $\pm$ 0.01) ($P < 0.05$) at the late-growth stage. Relative to the monoculture, the concentrations of formate, lactate, malate, and citrate were significantly decreased in the co-culture ($P < 0.05$), while the concentrations of acetate and succinate were significantly increased in the co-culture ($P < 0.05$). In contrast, the concentration of ethanol did not vary between the monoculture and the co-culture (**Figure 3**).

The effects of co-culturing the methanogen with *Pecoramyces* sp. F1 on metabolism at the late-growth stage are shown in **Supplementary Figure 6**. At the gene expression level, all of the enzymes, except for fumarase, involved in the metabolism of glucose in the anaerobic fungus were down-regulated when cultured with the methanogen, while only glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase (PGM), PFL, and aldehyde/alcohol dehydrogenase (ADH) were also down-regulated at the protein level.

Effect of Co-culturing With a Methanogen on the Expression of Carbohydrate-Targeting Enzymes of *Pecoramyces* sp. F1 at the Mid- and Late-Growth Stages

The top 20 differentially expressed fiber-degrading enzymes at the mid- and late-growth stages were examined (**Table 5**). Importantly, all of these genes were down-regulated at the late-growth stage. Although it was not anticipated that polysaccharide-degrading enzymes are required for the metabolism of glucose, our search for the top 20 fiber-degrading enzymes showed that several genes coding for such enzymes

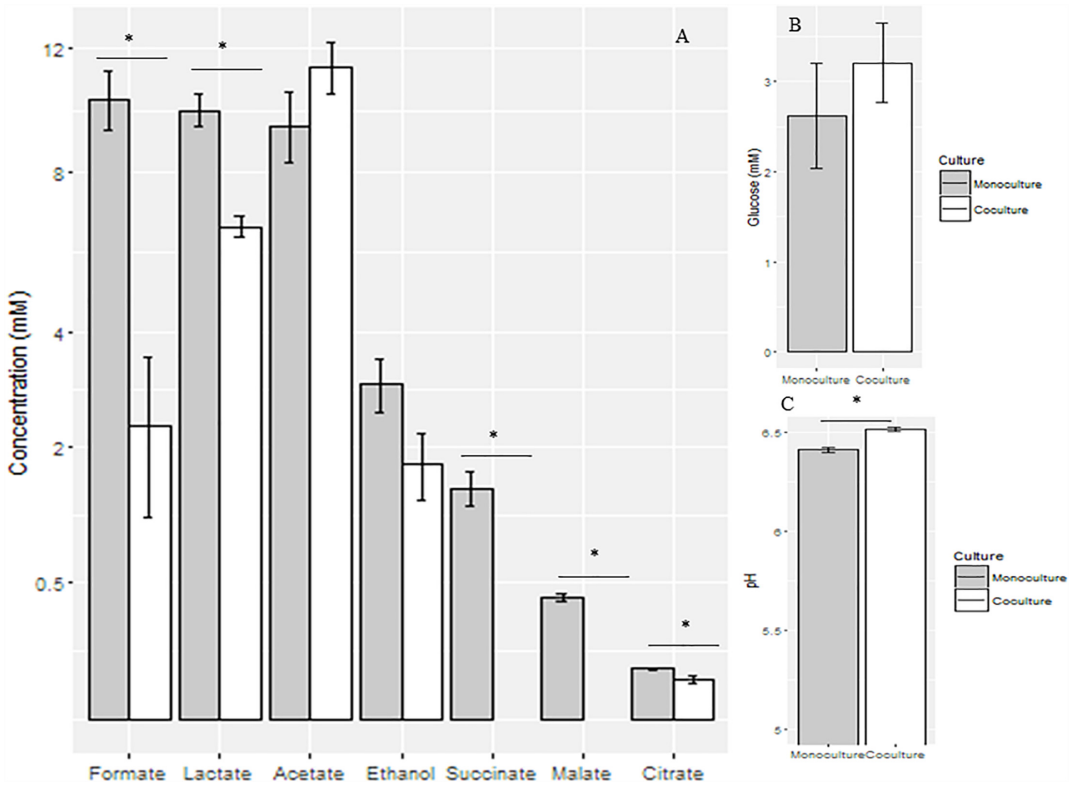

FIGURE 2 | Concentrations of fermentation end products **(A)** and glucose **(B)** and pH values **(C)** in the supernatant of anaerobic fungal monoculture and co-culture at the mid-growth stage. *Means significant difference between two groups.

were up-regulated in the mid-growth stage. The up-regulated genes included the afore-mentioned putative cellulase and others with encoded polypeptides annotated as cellulases, endoxylanases, alpha-amylases and a feruloyl esterase. Thus, a broad range of polysaccharide-degrading enzymes were released during the early stages of glucose metabolism in co-culturing of *Pecoramyces* sp. F1 with its syntrophic methanogen. However, these polysaccharide targeting enzymes were down-regulated during the late-growth stage.

DISCUSSION

Isolation and maintenance of anaerobic fungi requires a relatively complex, strictly anaerobic culture methodology limiting their study to relatively few research groups worldwide. Consequently, we do not have a good understanding of the diversity and taxonomy of these unique fungi. According to classical taxonomy, zoospore ultrastructure and to a lesser extent, fungal morphology were used to assign generic and specific names to isolates (Theodorou et al., 1996; Ozkose et al., 2001). More recently, molecular techniques based on the amplification and sequencing of genes encoding the 28S rRNA gene and ITS sequences have been used to aid classification. In the current work, molecular techniques were used to reassign the isolate *Piromyces* sp. F1 to *Pecoramyces* sp. F1. To date, only one species of *Pecoramyces* (*P. ruminantium*) has been described (Youssef et al., 2013; Hanafy et al., 2017). To date, a limited number of publications have studied fungal/methanogen interactions (Mountfort et al., 1982; Nakashimada et al., 2000; Jin et al., 2011; Li et al., 2016). In much of the original work, fungi and methanogens were isolated separately from different ruminal environments (Mountfort et al., 1982; Nakashimada et al., 2000). In working with the new isolate of *Pecoramyces* and its syntrophically associated methanogen, *M. thaueri*, we studied the metabolism of this isolate on glucose to obtain primary information about a *Pecoramyces* strain grown in monoculture and in co-culture with a syntrophic methanogen.

The estimated genome size of *Pecoramyces* sp. F1 matched that of the previously reported estimate for *Pecoramyces ruminantium*, as shown in **Table 2** (Youssef et al., 2013). This observation shows that *Pecoramyces* has a larger genome size compared with the *Piromyces* and *Anaeromyces* genera, although the estimated genome size of a *Neocallimastix* is double the size of *Pecoramyces*. In contrast, the data further demonstrates that the anaerobic fungal genomes are consistently AT-rich (GC% content range from 16 to 22; **Table 2**). The genera *Piromyces* and the *Anaeromyces* appear to have fewer genes (∼13,000) compared to the genus *Pecoramyces* (∼17,000), while the gene number reported for *Neocallimastix* is almost twice that for *Piromyces* and *Anaeromyces*. While *Piromyces finnis*

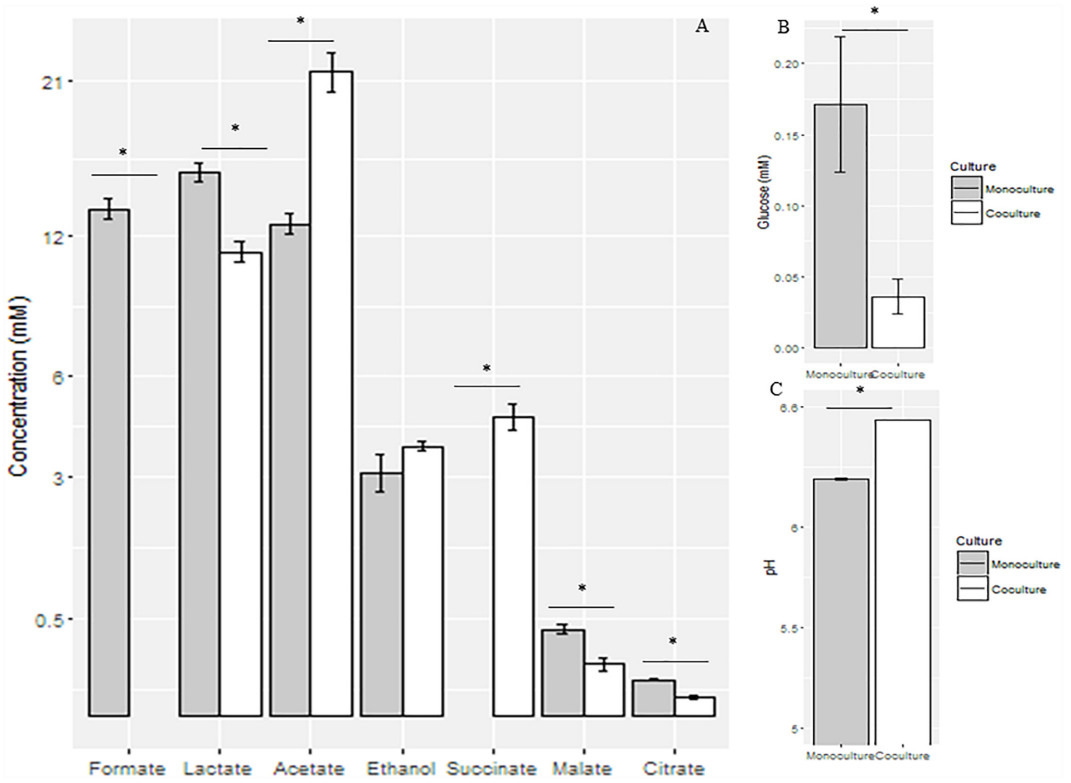

FIGURE 3 | Concentrations of fermentation end products **(A)** and glucose **(B)** and pH values **(C)** in the supernatant of anaerobic fungal monoculture and co-culture at the late-growth stage. *Means significant difference between two groups.

codes for approximately 11,000 genes, the number of genes coded by *Piromyces* sp. E2 is not very different from that of *Pecoramyces ruminantium* (Youssef et al., 2013). The number of genes coded by *Neocallimastix californiae* (Haitjema et al., 2017) shows that this fungal species uses about twice the genome of the *Pecoramyces* strains to encode a number of genes only slightly higher than that of the *Pecoramyces* strains. Therefore, the protein coding percentage of the genome of the reported *Neocallimastix* strain is very low in comparison with the other genera discussed in this manuscript.

The results from this study show that alternative splicing occurs in *Pecoramyces* sp. F1, as reported in the aerobic fungi (Grutzmann et al., 2014). Meanwhile, the average alternative splicing rates of *Pecoramyces* sp. F1 (∼45% and 22% at the mid- and late-growth stages, respectively) seem higher than the aerobic fungi, which was 6.4% on average (Grutzmann et al., 2014). Furthermore, it was observed that alternative splicing in the co-culture of the fungus with the methanogen was significantly lower than the fungal monoculture at the late-growth stage. The decreased splicing might be due to the limitation of the substrate in the culture or a slower growth rate associated with substrate depletion; Birch et al. (1995) reported that differential splicing in *Phanerochaete chrysosporium* might regulate the specificities of substrate of this fungus.

As observed in previous reports (Cheng et al., 2009; Jin et al., 2011; Li et al., 2016) during co-culturing of anaerobic fungi with methanogens, total gas production exceeded that of the gut fungal culture alone and the rate of gas production was faster. This observation confirms the increased efficiency with which the anaerobic fungi ferment substrates in the presence of the hydrogen-utilizing methanogen. Sampling at the mid-growth stage showed that total mRNA expression was not different between the monoculture and co-culture and the number of up-regulated genes was half the number of the down-regulated genes. However, by the late-growth phase, mRNA expression of the co-culture was significantly lower than that of the monoculture and the ratio of the up-regulated and down-regulated genes were dramatically decreased. The mRNA expression profiles suggest that on encountering a glucose energy source, *Pecoramyces* sp. F1 secretes a large number of polysaccharide degrading enzymes including endoglucanases, chitinases, amylases, and licheninases. In the case of the monoculture, this enzyme secretion appears to continue throughout growth, perhaps due to comparatively inefficient substrate utilization. On coupling the fermentation of the anaerobic fungus with the methanogen, the efficiency of the fermentation increased, leading to a down-regulation of the expression of the polysaccharide degrading enzymes. As shown in **Table 5**, this is particularly so for the putative enzymes involved in cellulose metabolism, including about six putative cellulases, likely reflecting the hydrolysis of the cellulose backbone. The efficiency of the fermentation in anaerobic fungus/methanogen co-culture increases is likely

TABLE 5 | Top 20 differentially expressed fiber degrading enzymes at mid- and late-growth stages.

Gene ID	Mid-growth stage[#]	Late-growth stage	Annotation (NCBInr)
A16134	5.57	−7.07	Putative cellulase
A11665	1.86	−8.19	Alpha-amylase
A13113	−0.03	−9.81	Beta-glucosidase
A16053	5.82	−3.81	Putative cellulase
A18358	0.17	−9.45	AmyE alpha-amylase
A07248	−0.66	−9.61	Feruloyl esterase
A17896	−0.07	−8.51	Endo-1,4-beta-xylanase
A17801	2.11	−6.20	Alpha-amylase G-6
A02885	4.94	−3.27	Endoxylanase
A14736	2.63	−5.03	Pectate lyase D
A07671	−0.52	−8.13	Cellulase Cel48A precursor
A03809	1.09	−6.49	Alpha-amylase MalS
A11825	4.54	−2.81	Putative cellulase
A10479	−2.64	−9.98	Cellulase
A16521	3.54	−3.66	Putative cellulase
A02179	4.09	−2.81	Putative cellulase
A17482	4.22	−2.59	Glucan endo-1,3-beta-D-glucosidase
A09998	0.44	−6.36	1,4-beta-D-glucan-4-glucanohydrolase
A08700	0.66	−6.09	Alpha-amylase
A07525	5.17	−1.55	Putative cellulase

[#] *The values are log_2 of (co-culture FPKM/monoculture FPKM).*

due to the removal of H_2 through interspecies transfer to the syntrophic methanogen to produce CH_4. In the fungal cell, the oxidization of NADH into NAD^+ and H^+ is associated with the production of acetic acid. This pathway is likely to be favorable for obtaining higher amounts of ATP, compared with the more reduced electron sinks end-products (e.g., lactate, ethanol) used by anaerobic fungi to regenerate NAD^+ for glycolysis (Bauchop and Mountfort, 1981; Marvin-Sikkema et al., 1990).

The results in the present study are in agreement with the observation that during syntrophic interactions between several ruminal organisms with hydrogen-removing methanogens, a shift in the metabolism occurs leading to extra ATP gain by the organism co-cultured with the methanogen (Bauchop and Mountfort, 1981; Marvin-Sikkema et al., 1990). Unlike the transcriptomic data, major shifts in the proteomic data were not observed in the present study. This may be due to the fact that the proteins in the cell have a much longer lifetime than that of the mRNAs. A genome-wide study showed that the lifetime of mRNAs in *Escherichia coli* were between 3 and 8 min (Bernstein et al., 2002). However, the rate of intracellular protein degradation in *E. coli* was 4 h (Koch and Levy, 1955).

The changes in the metabolites observed in the present study are similar to the results observed in our previous studies (Li et al., 2016, 2017). In brief, the pH value increased significantly at the late-growth stage as formate was utilized by the co-cultured methanogen and the lactate decreased due to a reduced demand for electron sink products for regeneration of reducing equivalents. Finally, acetate increased significantly because metabolism in the hydrogenosome became more efficient.

Combining the data reported in the present study and previous reports on anaerobic fungi and methanogens co-culture (Cheng et al., 2009; Li et al., 2016, 2017), we found that in the early growth stage of the co-culture, the metabolism in the fungal cell improved and large amounts of end products were produced. At this growth stage, the substrate was adequate and only H_2 was used by co-cultured methanogens to reduce the gas pressure, which could inhibit the microbial growth (Li et al., 2016). At the late-growth stage, the substrate was inadequate for anaerobic fungi to produce enough H_2 and methanogens would use formate to produce methane, which increased the pH value of the culture. The metabolic interaction between the two organisms would help both of them to be competitive in the rumen. For the anaerobic fungus, the fiber-degrading ability was improved and feedback inhibition (both gas pressure and water-soluble metabolites) was eliminated. For the methanogen, it could obtain H^+ as soon as it was produced.

In summary, in the present report we have used modern molecular approaches to assign phylogenetic placement to a new anaerobic fungal isolate and concomitantly provided a mechanistic understanding of its intermediary metabolism in co-culture with a syntrophic methanogen. We look forward to future experiments that explore interactions during degradation of more complex substrates.

AUTHOR CONTRIBUTIONS

YC and WZ conceived and designed the experiments. WJ, YfL, and YqL performed the experiments. YfL, YqL, WJ, YC, TS, RM, and IC generated and analyzed the data. YC, TS, RM, IC, and WZ wrote and revised the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00435/full#supplementary-material

REFERENCES

Akin, D. E., Borneman, W. S., and Windham, W. R. (1988). Rumen fungi: morphological types from Georgia cattle and the attack on forage cell walls. *Biosystems* 21, 385–391. doi: 10.1016/0303-2647(88)90037-8

Bard, J., and Winter, R. (2000). Gene Ontology: tool for the unification of biology. *Nat. Genet.* 25, 25–29. doi: 10.1038/75556

Barichevich, E. M., and Calza, R. E. (1990). Supernatant protein and cellulase activities of the anaerobic ruminal fungus *Neocallimastix frontalis* EB188. *Appl. Environ. Microbiol.* 56, 43–48.

Barr, D. J. S., Kudo, H., Jakober, K. D., and Cheng, K. J. (1989). Morphology and development of rumen fungi: *Neocallimastix* sp., *Piromyces communis*, and *Orpinomyces bovis* gen. nov., sp. nov. *Can. J. Bot.* 67, 2815–2824. doi: 10.1139/b89-361

Bauchop, T., and Mountfort, D. O. (1981). Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl. Environ. Microbiol.* 42, 1103–1110.

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res.* 27, 573–580. doi: 10.1093/nar/27.2.573

Bernstein, J. A., Khodursky, A. B., Lin, P. H., Lin-Chao, S., and Cohen, S. N. (2002). Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9697–9702. doi: 10.1073/pnas.112318199

Birch, P. R. J., Sims, P. F. G., and Broda, P. (1995). Substrate-dependent differential splicing of introns in the regions encoding the cellulose binding domains of two exocellobiohydrolase I-like genes in *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 61, 3741–3744.

Breton, A., Bernalier, A., Dusser, M., Fonty, G., Gaillard-Martinie, B., and Guillot, J. (1990). *Anaeromyces mucronatus* nov. gen., nov. sp. A new strictly anaerobic rumen fungus with polycentric thallus. *FEMS Microbiol. Lett.* 70, 177–182. doi: 10.1111/j.1574-6968.1990.tb13974.x

Callaghan, T. M., Podmirseg, S. M., Hohlweck, D., Edwards, J. E., Puniya, A. K., Dagar, S. S., et al. (2015). *Buwchfawromyces eastonii* gen. nov., sp. nov.: a new anaerobic fungus (*Neocallimastigomycota*) isolated from buffalo faeces. *MycoKeys* 9, 11–28. doi: 10.3897/mycokeys.9.9032

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, D233–D238. doi: 10.1093/nar/gkn663

Cheng, Y., Wang, Y., Li, Y., Zhang, Y., Liu, T., Wang, Y., et al. (2017). Progressive colonization of bacteria and degradation of rice straw in the rumen by Illumina sequencing. *Front. Microbiol.* 8:2165. doi: 10.3389/fmicb.2017.02165

Cheng, Y. F., Edwards, J. E., Allison, G. G., Zhu, W. Y., and Theodorou, M. K. (2009). Diversity and activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on lignocellulose in consecutive batch culture. *Bioresour. Technol.* 100, 4821–4828. doi: 10.1016/j.biortech.2009.04.031

Couger, M. B., Youssef, N. H., Struchtemeyer, C. G., Liggenstoffer, A. S., and Elshahed, M. S. (2015). Transcriptomic analysis of lignocellulosic biomass degradation by the anaerobic fungus isolate *Orpinomyces* sp. strain C1A. *Biotechnol. Biofuels* 8:208. doi: 10.1186/s13068-015-0390-0

Dagar, S. S., Kumar, S., Griffith, G. W., Edwards, J. E., Callaghan, T. M., Singh, R., et al. (2015). A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*). *Fungal Biol.* 119, 731–737. doi: 10.1016/j.funbio.2015.04.005

Davies, D. R., Theodorou, M. K., Lawrence, M. I., and Trinci, A. P. (1993). Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. *J. Gen. Microbiol.* 139, 1395–1400. doi: 10.1099/00221287-139-6-1395

Dollhofer, V., Callaghan, T. M., Dorn-In, S., Bauer, J., and Lebuhn, M. (2016). Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi. *J. Microbiol. Methods* 127, 28–40. doi: 10.1016/j.mimet.2016.05.017

Gold, J. J., Heath, I. B., and Bauchop, T. (1988). Ultrastructural description of a new chytrid genus of caecum anaerobe, *Caecomyces equi* gen. nov., sp. nov., assigned to the Neocallimasticaceae. *Biosystems* 21, 403–415. doi: 10.1016/0303-2647(88)90039-1

Gruninger, R. J., Nguyen, T. T. M., Reid, I. D., Yanke, J. L., Wang, P., Abbott, D. W., et al. (2018). Application of transcriptomics to compare the carbohydrate active enzymes that are expressed by diverse genera of anaerobic fungi to degrade plant cell wall carbohydrates. *Front. Microbiol.* 9:1581. doi: 10.3389/fmicb.2018.01581

Grutzmann, K., Szafranski, K., Pohl, M., Voigt, K., Petzold, A., and Schuster, S. (2014). Fungal alternative splicing is associated with multicellular complexity and virulence: a genome-wide multi-species study. *DNA Res.* 21, 27–39. doi: 10.1093/dnares/dst038

Haitjema, C. H., Gilmore, S. P., Henske, J. K., Solomon, K. V., Groot, R., Kuo, A., et al. (2017). A parts list for fungal cellulosomes revealed by comparative genomics. *Nat. Microbiol.* 2:17087. doi: 10.1038/nmicrobiol.2017.87

Hanafy, R. A., Elshahed, M. S., Liggenstoffer, A. S., Griffith, G. W., and Youssef, N. H. (2017). *Pecoramyces ruminantium*, gen. nov., sp. nov., an anaerobic gut fungus from the feces of cattle and sheep. *Mycologia* 109, 231–243. doi: 10.1080/00275514.2017.1317190

Hanafy, R. A., Elshahed, M. S., and Youssef, N. H. (2018). *Feramyces austinii*, gen. nov., sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild Barbary sheep and fallow deer. *Mycologia* 110, 513–525. doi: 10.1080/00275514.2018.1466610

Heath, I. B., Bauchop, T., and Skipp, R. A. (1983). Assignment of the rumen anaerobe *Neocallimastix frontalis* to the Spizellomycetales (Chytridiomycetes) on the basis of its polyflagellate zoospore ultrastructure. *Can. J. Bot.* 61, 295–307. doi: 10.1139/b83-033

Henske, J. K., Gilmore, S. P., Knop, D., Cunningham, F. J., Sexton, J. A., Smallwood, C. R., et al. (2017). Transcriptomic characterization of *Caecomyces churrovis*: a novel, non-rhizoid-forming lignocellulolytic anaerobic fungus. *Biotechnol. Biofuels* 10:305. doi: 10.1186/s13068-017-0997-4

Hungate, R. E. (1967). Hydrogen as an intermediate in the rumen fermentation. *Arch. Microbiol.* 59, 158–164. doi: 10.1007/BF00406327

Hungate, R. E., Smith, W., Bauchop, T., Yu, I., and Rabinowitz, J. C. (1970). Formate as an intermediate in the bovine rumen fermentation. *J. Bacteriol.* 102, 389–397.

Janssen, P. H., and Kirs, M. (2008). Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74, 3619–3625. doi: 10.1128/AEM.02812-07

Jin, W., Cheng, Y. F., Mao, S. Y., and Zhu, W. Y. (2011). Isolation of natural cultures of anaerobic fungi and indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. *Bioresour. Technol.* 102, 7925–7931. doi: 10.1016/j.biortech.2011.06.026

Joshi, A., Lanjekar, V. B., Dhakephalkar, P. K., Callaghan, T. M., Griffith, G. W., and Dagar, S. S. (2018). *Liebetanzomyces polymorphus* gen. et sp. nov., a new anaerobic fungus (*Neocallimastigomycota*) isolated from the rumen of a goat. *MycoKeys* 40, 89–110. doi: 10.3897/mycokeys.40.28337

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004). The KEGG resource for deciphering the genome. *Nucleic Acids Res.* 32, D277–D280. doi: 10.1093/nar/gkh063

Keller, O., Kollmar, M., Stanke, M., and Waack, S. (2011). A novel hybrid gene prediction method employing protein multiple sequence alignments. *Bioinformatics* 27, 757–763. doi: 10.1093/bioinformatics/btr010

Kim, H., Lee, I., Kwon, Y., Kim, B. C., Ha, S., Lee, J. H., et al. (2011). Immobilization of glucose oxidase into polyaniline nanofiber matrix for biofuel cell applications. *Biosens. Bioelectron.* 26, 3908–3913. doi: 10.1016/j.bios.2011.03.008

Koch, A. L., and Levy, H. R. (1955). Protein turnover in growing cultures of *Escherichia coli*. *J. Biol. Chem.* 217, 947–957.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T., et al. (2003). Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27, 663–693. doi: 10.1016/S0168-6445(03)00072-X

Lagesen, K., Hallin, P., Rodland, E. A., Staerfeldt, H. H., Rognes, T., and Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* 35, 3100–3108. doi: 10.1093/nar/gkm160

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25. doi: 10.1186/gb-2009-10-3-r25

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323. doi: 10.1186/1471-2105-12-323

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler Transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Li, R., Li, Y., Kristiansen, K., and Wang, J. (2008). SOAP: short oligonucleotide alignment program. *Bioinformatics* 24, 713–714. doi: 10.1093/bioinformatics/btn025

Li, R., Zhu, H., Ruan, J., Qian, W., Fang, X., Shi, Z., et al. (2010). De novo assembly of human genomes with massively parallel short read sequencing. *Genome Res.* 20, 265–272. doi: 10.1101/gr.097261.109

Li, Y., Jin, W., Cheng, Y., and Zhu, W. (2016). Effect of the associated methanogen *Methanobrevibacter thaueri* on the dynamic profile of end and intermediate metabolites of anaerobic fungus *Piromyces* sp. F1. *Curr. Microbiol.* 73, 434–441. doi: 10.1007/s00284-016-1078-9

Li, Y., Jin, W., Mu, C., Cheng, Y., and Zhu, W. (2017). Indigenously associated methanogens intensified the metabolism in hydrogenosomes of anaerobic fungi with xylose as substrate. *J. Basic Microbiol.* 57, 933–940. doi: 10.1002/jobm.201700132

Lowe, S. E., Theodorou, M. K., and Trinci, A. P. (1987). Growth and fermentation of an anaerobic rumen fungus on various carbon sources and effect of temperature on development. *Appl. Environ. Microbiol.* 53, 1210–1215.

Lowe, T. M., and Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* 25, 955–964. doi: 10.1093/nar/25.5.955

Mao, S. Y., Huo, W. J., and Zhu, W. Y. (2016). Microbiome–metabolome analysis reveals unhealthy alterations in the composition and metabolism of ruminal microbiota with increasing dietary grain in a goat model. *Environ. Microbiol.* 18, 525–541. doi: 10.1111/1462-2920.12724

Marvin-Sikkema, F. D., Richardson, A. J., Stewart, C. S., Gottschal, J. C., and Prins, R. A. (1990). Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. *Appl. Environ. Microbiol.* 56, 3793–3797.

Mountfort, D. O., Asher, R. A., and Bauchop, T. (1982). Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. strain RA1 and *Methanosarcina barkeri*. *Appl. Environ. Microbiol.* 44, 128–134.

Nakashimada, Y., Srinivasan, K., Murakami, M., and Nishio, N. (2000). Direct conversion of cellulose to methane by anaerobic fungus *Neocallimastix frontalis* and defined methanogens. *Biotechnol. Lett.* 22, 223–227. doi: 10.1023/A:1005666428494

Orpin, C. G. (1975). Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* 91, 249–262. doi: 10.1099/00221287-91-2-249

Ozkose, E., Thomas, B. J., Davies, D. R., Griffith, G. W., and Theodorou, M. K. (2001). *Cyllamyces aberensis* gen. nov. sp. nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. *Can. J. Bot.* 79, 666–673. doi: 10.1139/b01-047

Rio, D. C., Ares, M. Jr., Hannon, G. J., and Nilsen, T. W. (2010). Enrichment of poly(A)+ mRNA using immobilized oligo(dT). *Cold Spring Harb. Protoc.* 7:db.rot5454. doi: 10.1101/pdb.prot5454

Russell, J. B., and Rychlik, J. L. (2001). Factors that alter rumen microbial ecology. *Science* 292, 1119–1122. doi: 10.1126/science.1058830

Solomon, K. V., Haithema, C. H., Henske, J. K., Gilmore, S. P., Borges-Rivera, D., Lipzen, A., et al. (2016). Early-branching gut fungi possess a large, comprehensive array of biomass-degrading enzymes. *Science* 351, 1192–1195. doi: 10.1126/science.aad1431

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Tarazona, S., Furio-Tari, P., Turra, D., Pietro, A. D., Nueda, N. J., Ferrer, A., et al. (2015). Data quality aware analysis of differential expression in RNA-seq with NOISeq R/Bioc package. *Nucleic Acids Res.* 43:e140. doi: 10.1093/nar/gkv711

Ter-Hovhannisyan, V., Lomsadze, A., Chernoff, Y. O., and Borodovsky, M. (2008). Gene prediction in novel fungal genomes using an ab initio algorithm with unsupervised training. *Genome Res.* 18, 1979–1990. doi: 10.1101/gr.081612.108

Teunissen, M. J., de Kort, G. V., Op den Camp, H. J., and Vogels, G. D. (1993). Production of cellulolytic and xylanolytic enzymes during growth of anaerobic fungi from ruminant and nonruminant herbivores on different substrates. *Appl. Biochem. Biotechnol.* 39–40, 177–189. doi: 10.1007/BF02918988

The UniProt Consortium (2015). UniProt: a hub for protein information. *Nucleic Acids Res.* 43, D204–D212. doi: 10.1093/nar/gku989

Theodorou, M. K., Williams, B. A., Dhanoa, M. S., Mcallan, A. B., and France, J. (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim. Feed Sci. Technol.* 48, 185–197. doi: 10.1016/0377-8401(94)90171-6

Theodorou, M. K., Zhu, W. Y., Rickers, A., Nielsen, B. B., Gull, K., and Trinci, A. P. J. (1996). "Biochemistry and ecology of anaerobic fungi," in *The Mycota: VI Human and Animal Relationship*, eds D. H. Howard and J. D. Miller (New York, NY: Springer-Verlag), 265–295.

Ungerfeld, E. M. (2015). Limits to dihydrogen incorporation into electron sinks alternative to methanogenesis in ruminal fermentation. *Front. Microbiol.* 6:1272. doi: 10.3389/fmicb.2015.01272

Weimer, P. J., Russell, J. B., and Muck, R. E. (2009). Lessons from the cow: what the ruminant animal can teach us about consolidated bioprocessing of cellulosic biomass. *Bioresour. Technol.* 100, 5323–5331. doi: 10.1016/j.biortech.2009.04.075

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (San Diego, CA: Academic Press), 315–322.

Yan, G., Lestari, R., Long, B., Fan, Q., Wang, Z., Guo, X., et al. (2016). Comparative proteomics analysis reveals L-arginine activates ethanol degradation pathways in HepG2 cells. *Sci. Rep.* 6:23340. doi: 10.1038/srep23340

Youssef, N. H., Couger, M. B., Struchtemeyer, C. G., Liggenstoffer, A. S., Prade, R. A., Najar, F. Z., et al. (2013). The genome of the anaerobic fungus *Orpinomyces* sp. strain C1A reveals the unique evolutionary history of a remarkable plant biomass degrader. *Appl. Environ. Microbiol.* 79, 4620–4634. doi: 10.1128/AEM.00821-13

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Assessment of the Accuracy of High-Throughput Sequencing of the ITS1 Region of Neocallimastigomycota for Community Composition Analysis

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Anaerobic fungi (Neocallimastigomycota) are common inhabitants of the digestive tract of large mammalian herbivores, where they make an important contribution to plant biomass degradation. The internal transcribed spacer 1 (ITS1) region is currently the molecular marker of choice for anaerobic fungal community analysis, despite its known size polymorphism and heterogeneity. The aim of this study was to assess the accuracy of high-throughput sequencing of the ITS1 region of anaerobic fungi for community composition analysis. To this end, full-length ITS1 clone libraries from five pure cultures, representing the ITS1 region size range, were Sanger sequenced to generate a reference dataset. Barcoded amplicons of the same five pure cultures, and four different mock communities derived from them, were then sequenced using Illumina HiSeq. The resulting sequences were then assessed in relation to either the reference dataset (for the pure cultures) or the corresponding theoretical mock communities. Annotation of sequences obtained from individual pure cultures was not always consistent at the clade or genus level, irrespective of whether data from clone libraries or high-throughput sequencing were analyzed. The detection limit of the high-throughput sequencing method appeared to be influenced by factors other than the parameters used during data processing, as some taxa with theoretical values >0.6% were not detected in the mock communities. The high number of PCR cycles used was considered to be a potential explanation for this observation. Accuracy of two of the four mock communities was limited, and this was speculated to be due to preferential amplification of smaller sized ITS1 regions. If this is true, then this is predicted to be an issue with only six of

the 32 named anaerobic fungal clades. Whilst high-throughput sequencing of the ITS1 region from anaerobic fungi can be used for environmental sample analysis, we conclude that the accuracy of the method is influenced by sample community composition. Furthermore, ambiguity in the annotation of sequences within pure cultures due to ITS1 heterogeneity reinforces the limitations of the ITS1 region for the taxonomic assignment of anaerobic fungi. In order to overcome these issues, there is a need to develop an alternative taxonomic marker for anaerobic fungi.

Keywords: Neocallimastigomycota, anaerobic fungi, internal transcribed spacer 1 region, high-throughput sequencing, clone library, size polymorphism

INTRODUCTION

Neocallimastigomycota are an important class of strictly anaerobic fungi that are commonly found in herbivore gut ecosystems, particularly that of large mammals. Anaerobic fungi have been most extensively studied in ruminants, where they can increase fiber degradation and feed intake by 7–9% and up to 40%, respectively (Gordon and Phillips, 2005). Due to their potent fiber degrading enzymes (Solomon et al., 2016), anaerobic fungi are also of great biotechnological interest. Eleven anaerobic fungal genera are currently characterized (Edwards et al., 2017; Hanafy et al., 2018; Joshi et al., 2018), with evidence for the existence of more genera from cultivation independent analysis of environmental samples (Liggenstoffer et al., 2010; Nicholson et al., 2010; Kittelmann et al., 2012, 2013). Using the ITS1 region of anaerobic fungi, a taxonomic framework and associated curated database have been developed, which classifies ITS1 sequences to characterized genera and as yet uncultured genus- or species-level clades (Koetschan et al., 2014). This is a valuable resource for the analysis of sequences obtained from environmental samples, particularly when using HTS of barcoded amplicons, which has become the method of choice for determining anaerobic fungal community composition (Liggenstoffer et al., 2010; Kittelmann et al., 2012, 2013). However, it has since been recognized that the forward primer site based within the ITS1 region (primer MN100F) used is not conserved in all anaerobic fungi (Kittelmann et al., 2013; Callaghan et al., 2015).

Due to the lack of conserved priming sites within the ITS1 region for anaerobic fungal specific amplification, primers targeting the more conserved 18S and 5.8S rRNA genic flanking regions are recommended. The primers previously developed for anaerobic fungal specific automated ribosomal intergenic spacer analysis (ARISA), which generate a ∼350–440 bp amplicon, are such an example (Edwards et al., 2008). Based on full-length sequences in the database of Koetschan et al. (2014), the ITS1 region ranges in size from 192–282 bases. Whilst this ITS1 size polymorphism is valuable for ARISA, it is problematic for creating a stable ITS1 phylogeny unless sequence alignments are improved using secondary structure information (Edwards et al., 2017).

Internal transcribed spacer 1 size heterogeneity exists not only between anaerobic fungal pure cultures, but also within them (Edwards et al., 2008). As such, it is perhaps not surprising that within a single culture multiple cloned ITS1 sequences can vary as much as 13% between ITS1 repeats (Callaghan et al., 2015). However, the implication that this has for the interpretation of high-throughput sequencing data generated from pure cultures and environmental samples is not known. The objective of this study was, therefore, to assess the accuracy of HTS of the ITS1 region of anaerobic fungi based on the previously published ARISA primers (Edwards et al., 2008), using anaerobic fungal pure cultures and defined mock communities of different composition and complexity. This is important not only in terms of data quality control, but also to identify issues associated with polymorphism and heterogeneity within the ITS1 region. Following current debate about the value of the ITS1 region for anaerobic fungal analysis (Edwards et al., 2017), the findings of this study will provide a clear evidence base regarding the strengths and limitations of its use as an anaerobic fungal taxonomic marker.

Abbreviations: AF-ITS1, anaerobic fungal ITS1; HTS, high throughput sequencing; ITS1, internal transcribed spacer 1; NTC, non-template control; OTU, operational taxonomic unit; PCR, polymerase chain reaction; PE, paired end; SD, standard deviation; SNP, single nucleotide polymorphism.

MATERIALS AND METHODS

Pure Cultures and DNA

The five pure culture DNA extracts used in this study were kindly provided by Dr. Tony M. Callaghan and Veronika Dollhofer (Bavarian State Research Center for Agriculture, Freising, Germany), and were obtained as previously described (Dollhofer et al., 2016). *Neocallimastix frontalis* strain RE1 and *Orpinomyces* sp. SR2 (also known as *Orpinomyces* sp. OUS1) were isolated from the sheep rumen (Stewart and Richardson, 1989; Brookman et al., 2000). *Anaeromyces* sp. 28xy was isolated from feces of a Highland cow (Callaghan, 2014). *Piromyces* sp. CaDo16a was isolated from digester sludge of a Bavarian biogas plant (Dollhofer et al., 2017). *Caecomyces* sp. CaDo13a was isolated from rumen fluid of a wild alpine goat (personal communication, Callaghan and Dollhofer). Available ITS region sequence data from one of the five pure cultures, *Piromyces* sp. CaDo16a, is assigned to the species hypothesis code SH1571620.08FU in the UNITE database (Nilsson et al., 2018).

Clone Library Based Sequencing of Pure Cultures

For each of the five pure cultures an ITS1 reference dataset was created using a clone library approach. A PCR amplicon comprising the partial 18S rRNA gene (∼310 bp), full ITS1 region and partial 5.8S rRNA gene (116 bp) was amplified using the forward primer 5′-CAT CCT TGA TCG GRA GGT CC-3′ (i.e., the AF-SSU reverse primer of Dollhofer et al. (2017) in the forward orientation), and the reverse primer "Neo QPCR Rev" (5′-GTG CAA TAT GCG TTC GAA GAT T-3′, Edwards et al., 2008). PCR was performed in triplicate for each culture using 50 μL reactions containing 1 × HF buffer (Finnzymes, Vantaa, Finland), 1 μL dNTP Mix (10 mM; Promega, Leiden, Netherlands), 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Finnzymes), 500 nM of each primer, and 2 ng of DNA. The cycling conditions consisted of an initial denaturation at 98°C for 3 min followed by 40 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 6 min. Successful amplification was confirmed by agarose gel electrophoresis on a 2% (w/v) agarose gel containing 1 × SYBR® Safe (Invitrogen, Carlsbad, CA, United States). A NTC reaction was also performed and generated no PCR product. A pooled PCR product for each of the five pure cultures was purified using HighPrep™ (MagBio Europe Ltd., Kent, United Kingdom), and quantified using a Qubit fluorometer in combination with the dsDNA BR Assay Kit (Invitrogen). PCR products were then A-tailed and cloned using the pGEM-T easy vector system (Promega). Using blue/white screening, transformed white clones were randomly selected (19–20 per pure culture) and sent for Sanger sequencing using both the M13F and M13R priming sites within the vector (GATC-Biotech, Cologne, Germany). The quality of reads was manually verified, and consensus reads prepared for each clone.

Preparation of Mock Community Template DNA

Four different mock communities (Mock_1 to Mock_4) were prepared by combining the cleaned and quantified PCR amplicons used for clone library preparation. PCR amplicons were used rather than genomic DNA as the *rrn* operon copy number of the cultures used was not known. PCR amplicons were combined based on the amount of DNA, giving a total of 500 ng in a 50 μl volume. Mock_1 was composed of 250 ng of both *N. frontalis* RE1 and *Anaeromyces* sp. 28xy. Mock_2 was composed of 100 ng of each of the five pure cultures. Mock_3 was composed of 250 ng of *N. frontalis* RE1, 100 ng of *Orpinomyces* sp. SR2, 75 ng of *Piromyces* sp. CaDo16a, 50 ng of *Caecomyces* sp. CaDo13a, and 25 ng of *Anaeromyces* sp. 28xy. Mock_4 was composed of 88.89 ng of *N. frontalis* RE1, 10 ng of *Caecomyces* sp. CaDo13a, 1 ng of *Piromyces* sp. CaDo16a, 0.1 ng of *Orpinomyces* sp. SR2, and 0.01 ng of *Anaeromyces* sp. 28xy. Each mock community was prepared in duplicate, and then pooled to minimize variation associated with pipetting. The theoretical composition of each mock community was then determined taking account of the molarity of each culture PCR amplicon in the mock community. This was done by calculating the number of PCR amplicons in the amount of DNA from each culture present in the mock, using the amount of DNA added (as indicated above) and the average of the size of the clones from the corresponding clone library. Percentage relative abundances were then derived from these values for each mock community.

Illumina High-Throughput Sequencing

Barcoded amplicons comprising the partial 18S rRNA gene (∼130 bp), full ITS1 region, and partial 5.8S rRNA gene (∼31 bp) were generated for the five pure cultures and four mock communities using a 2-step PCR strategy with a Labcycler (SensoQuest, Göttingen, Germany). This preparation was repeated three times, as all samples were independently run in three different libraries (A, B, and C). Furthermore, mock community samples were also sequenced in duplicate within one library A (i.e., A1 and A2).

The first PCR step was performed using the previously published ARISA primers (Edwards et al., 2008) with the addition of UniTag adapters (underlined): Neo 18S For 5′-GAGCCGTAGCCAGTCTGCAATCCTTCGGATTGGCT-3′ and Neo 5.8S Rev 5′-GCCGTGACCGTGACATCGCGAGAACC AAGAGATCCA-3′. PCR was performed in a total volume of 25 μL containing 1 × HF buffer, 1 μL dNTP Mix (10 mM), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of each primer, and 2 ng of pure culture or mock community DNA. The cycling conditions consisted of an initial denaturation at 98°C for 3 min followed by 40 cycles of 98°C for 10 s, 58°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 6 min. Triplicate PCR reactions were prepared for each sample, along with NTC reactions. The presence of PCR products from samples, and their absence in the NTC, was confirmed by agarose gel electrophoresis on a 2% (w/v) agarose gel containing 1 × SYBR® Safe. Pooled triplicate reactions, as well as the negative individual NTC reactions, were then purified using HighPrep™. NTC reactions were further processed and sequenced in the same manner as the samples so that any OTU (Blaxter et al., 2005) clearly associated with any of the NTC reactions could be manually removed during processing of the resulting sequence data.

The second PCR step was then employed to add an eight nucleotide sample specific barcode to the 5′- and 3′- end of the PCR products as previously described (van Lingen et al., 2017). Each PCR reaction, with a final volume of 100 μL, contained 5 μL of the purified first step PCR product, 5 μL each of barcoded forward and reverse primers (10 μM), 2 μL dNTP Mix (10 mM), 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, and 1 × HF buffer. Amplification consisted of an initial denaturation at 98°C for 30 s followed by five cycles of 98°C for 10 s, 52°C for 20 s, and 72°C for 20 s, and a final extension at 72°C for 10 min. Barcoded PCR products were then purified using the HighPrep™, and quantified using a Qubit in combination with the dsDNA BR Assay Kit. Purified sample PCR products were then pooled in equimolar amounts, with the exception of the purified NTC PCR

products which were included based on the maximum volume of purified sample PCR product used in the equimolar pool. Pools then underwent adaptor ligation followed by sequencing on the Illumina HiSeq platform using 300 PE chemistry (GATC-Biotech, Konstanz, Germany, now part of Eurofins Genomics Germany GmbH).

Theoretical Mock Community Sequence Files

Theoretical fastq files (forward and reverse) for each mock community were created (T_Mock_1 to T_Mock_4) based on the clone library reference data. These files served multiple purposes. The optimal parameters for bioinformatics processing of the anaerobic fungal HTS data were determined using this reference dataset (**Supplementary Figure S1**). In addition, comparison of the theoretical mock communities to the actual sequenced mock communities (Mock_1 to Mock_4) enabled determination of whether any biases found were likely to be associated with the generation of the HTS data itself, or its subsequent bioinformatics processing. The files were prepared as follows. For each culture, all the cloned sequences from the reference dataset were first aligned using ClustalW version 2.1 (Larkin et al., 2007) and then trimmed *in silico* to generate ends that matched the PCR primers used for the HTS using GeneDoc version 2.6 (Nicholas and Ncholas, 1997). Using the knowledge of the theoretical composition of each mock community (see above), the trimmed sequences for the pure cultures were then combined in the appropriate proportions to create forward and reverse fastq files each containing a total of 200,000 reads. Different unique barcodes were then added to each theoretical mock community, enabling the corresponding fastq files to be processed in exactly the same way as the sequenced mock community samples during bioinformatics processing. No taxonomic information was included in the files as they were processed in exactly the same manner as samples during data analysis. The script used for generating the theoretical mock communities fastq files, as well as the associated theoretical mock community data used in this study, is available at https://gitlab.com/wurssb/gen_fake_mocks.

Analysis of High-Throughput Sequence Data

Raw Illumina sequence data and theoretical mock community fastq files were processed using NG-Tax (version NGTax-2.jar[1]). Using an open reference approach, NG-Tax defines OTUs as unique sequences that are above a user-defined minimum abundance threshold (Ramiro-Garcia et al., 2016). NG-Tax filters the PE libraries to contain only read pairs with perfectly matching barcodes, with the details of the sample barcodes and library files used in this study provided in **Supplementary Table S1**. NG-Tax was performed with the following parameters: PE read length 150 bases (as beyond this length the mean read quality scores deteriorated), ratio OTU abundance 2.0, minimum abundance threshold was set at 0.6% (**Supplementary Figure S1**), identity level 100%, and error correction of 1 mismatch (99.33%). As the PCR amplicon primers used were not within the AF-ITS1 database (version 3.3[2]) used for OTU annotation (which is a requirement for annotation by NG-Tax), an empty database file (emptydb.fasta.gz[3]) was used and the OTUs then subsequently annotated manually.

Fasta files of the OTUs from the NG-Tax generated biom file[4] were extracted using the script otuseq_export.py[5]. The OTUs were annotated using BLASTN searches against the AF-ITS1 database using default settings with "-num_alignments 10" (BLAST version 2.4.0). For OTUs that could not be annotated by the AF-ITS1 database, BLASTN searches were performed against the NCBI database. Cut-off levels for OTU annotations were determined based on the mean percentage similarities of full-length sequences in the AF-ITS1 database within clade and within genus. These cut-off levels were >98% for clade and >95% for genus. Based on the study of Koetschan et al. (2014), the term clade is defined as a known species or an uncultivated subgroup within a monophyletic lineage that has been identified using secondary structure informed analysis of ITS1 region sequence data. As previously noted by Koetschan et al. (2014), it is not known if some of the uncultivated subgroups represent new species or potentially new genera. The NG-Tax generated biom file was converted to a tab-delimited table to enable OTU annotations to be added. The OTUs that were clearly associated with the NTC samples were also manually removed from the tab-delimited table at this stage. The resulting tab delimited table was then converted back to a biom file[6].

Plots were created using ggplot2 (Wickham, 2009) in R version 3.4.0. Accuracy of the sequencing of the mock communities was determined by calculating Pearson correlation values (Pearson, 1909) and pairwise weighted UniFrac distances (Lozupone et al., 2011) between the sequenced mock communities and the corresponding theoretical mock community. To test for differences in accuracy between the mock communities, the data (as described above) for all the mock communities was analyzed by ANOVA and a Tukey *post hoc* test performed (Genstat, 19th edition, VSN International Ltd.). Probability values <0.05 were considered to be significant.

Data Availability

The Sanger sequenced clone library data is deposited in NCBI under the following accession numbers: *N. frontalis* RE1 (MK036660-MK036676), *Orpinomyces* sp. SR2 (MK036677-MK036695), *Piromyces* sp. CaDo16a (MK036696-MK036714), *Caecomyces* sp. CaDo13a (MK036715-MK036728), and *Anaeromyces* sp. 28xy (MK036729-MK036744). The HTS data is deposited in the European Nucleotide Archive under the study accession number PRJEB29131.

[1] http://download.systemsbiology.nl/ngtax/

[2] www.anaerobicfungi.org

[3] http://download.systemsbiology.nl/ngtax/databases/

[4] https://gitlab.com/wurssb/gen_fake_mocks/tree/master/paper_data/TestMock_150_06.biom

[5] https://gitlab.com/wurssb/gen_fake_mocks/master/paper_data/otuseq_export.py

[6] https://gitlab.com/wurssb/gen_fake_mocks/tree/master/paper_data/TestMock_150_06_TAX_FINAL_hdf5.biom

RESULTS AND DISCUSSION

Clone Library Based Analysis of Anaerobic Fungal Pure Culture Taxonomy and ITS1 Size Polymorphism

Pure cultures of five morphologically distinct anaerobic fungal genera were used to generate a reference ITS1 dataset using cloning and Sanger sequencing. The five anaerobic fungi were *N. frontalis* RE1, *Anaeromyces* sp. 28xy, *Orpinomyces* sp. SR2, *Piromyces* sp. CaDo16a, and *Caecomyces* sp. CaDo13a. All five pure cultures generated ribosomal operon fragments (partial 18S rRNA gene, full ITS1 region, and partial 5.8S rRNA gene), which varied in size both within and between cultures (**Table 1**). This is consistent with previously published ARISA analysis of anaerobic fungal pure cultures (Edwards et al., 2008).

Findings from the BLAST based annotation of the complete ITS1 region of the cloned sequences against the AF-ITS1 database showed that full and consistent annotation at the clade level, for all clones, only occurred with *N. frontalis* RE1 (clade *Neocallimastix 1*). With *Anaeromyces* sp. 28xy, only seven of the 16 clones could be reliably annotated to the clade level (*Anaeromyces 1*). For *Orpinomyces* sp. SR2, 18 of the 19 clones were annotated as clade *Orpinomyces 1a* whilst one sequence was annotated as clade *Orpinomyces 1b*. This raises a question regarding the validity of the sub-division of the *Orpinomyces 1* clade (Koetschan et al., 2014).

None of the *Piromyces* sp. CaDo16a clones could be annotated at either the clade or genus level using the AF-ITS1 database due to having <90.5% identity. Therefore, in this study sequences matching to *Piromyces* sp. CaDo16a were annotated as "CaDo16a; NA." The low identity of *Piromyces* sp. CaDo16a to other *Piromyces* sequences in the AF-ITS1 database is not entirely unexpected, as this strain has recently been suggested to represent a new clade within this genus based on phylogenetic analysis of its 28S rRNA gene (Dollhofer et al., 2017). With *Caecomyces* sp. CaDo13a, 11 of the 14 clones were annotated as clade *Caecomyces 1*, whereas two of the clones could only be annotated to the genus level. Interestingly, one of the 14 clones was annotated at the genus level as *Cyllamyces*. This adds weight to the current speculation as to whether *Cyllamyces* and *Caecomyces* are (Ozkose et al., 2001; Paul et al., 2018) or are not (Callaghan et al., 2015; Wang et al., 2017) distinct genera.

Within pure cultures, cloned sequences that were annotated differently varied in size relative to other clones (**Table 1**). As the 18S rRNA and 5.8S rRNA genic flanking regions were consistent in size, the variation in the amplicon size was associated only with the ITS1 region. However, in *N. frontalis* RE1 the large range in ITS1 size did not result in different annotations. This likely is a reflection of size differences in the *N. frontalis* RE1 clones being due to insertions rather than deletions within the ITS1 region. These findings highlight the need to sequence multiple clones from individual pure cultures to further refine current ITS1 based taxonomic frameworks for anaerobic fungi (Koetschan et al., 2014; Paul et al., 2018). For example, based on the full-length ITS1 sequences in the clone libraries, a 98% identity cut-off for clade (this study) or species equivalent (Paul et al., 2018) seems reasonable based on the average identity value within each clone library (**Supplementary Table S2**). However, when the minimum identity is considered within each clone library, then this cut-off value is only valid for one of the five pure cultures (*Piromyces* sp. CaDo16a). This is a limitation with the use of ITS1 as a taxonomic marker that cannot be easily circumvented, particularly when interpreting sequence data from cultivation independent analysis of environmental samples.

All of the cloned sequences fully matched the primers used for the HTS. The clone library sequence data was used to predict the sizes of the amplicons that would be theoretically generated using HTS (**Figure 1**). The ITS1 region size range of the pure cultures (200–279 bases) was representative of the size range of the full-length ITS1 region sequences present within the AF-ITS1 database (192–282 bases).

High-Throughput Sequence Analysis of the ITS1 Region of Anaerobic Fungal Pure Cultures in Terms of OTUs and Their Taxonomy

After processing of anaerobic fungal Illumina HiSeq data, the average number of reads per pure culture sample was 391,807

TABLE 1 | Sequence size and taxonomy of cloned sequences (partial 18S rRNA gene – full ITS1 region – partial 5.8S rRNA gene) generated from anaerobic fungal pure cultures.

Genus	Strain	No. of unique sequences*	Sequence size (bases)#	Taxonomic classification (Genus; Clade)$
Neocallimastix	RE1	7 (of 17)	681–705 (17)	*Neocallimastix*; *Neocallimastix 1*
Orpinomyces	SR2	6 (of 19)	631–632 (18)	*Orpinomyces*; *Orpinomyces 1a*
			637 (1)	*Orpinomyces*; *Orpinomyces 1b*
Piromyces	CaDo16a	3 (of 19)	658–660 (19)	NA; NA+
Caecomyces	CaDo13a	8 (of 14)	625 (1)	*Cyllamyces*; NA
			657 (2)	*Caecomyces*; NA
			658 (11)	*Caecomyces*; *Caecomyces 1*
Anaeromyces	28xy	10 (of 16)	671–672 (9)	*Anaeromyces*; NA
			674–675 (7)	*Anaeromyces*; *Anaeromyces 1*

*Numbers in parentheses indicate the total number of clones. #Numbers in parentheses indicate the number of clones with the taxonomic classification indicated. $NA, not annotated. +In subsequent analysis in this study, sequences from the Piromyces sp. CaDo16a were annotated as "CaDo16a; NA."

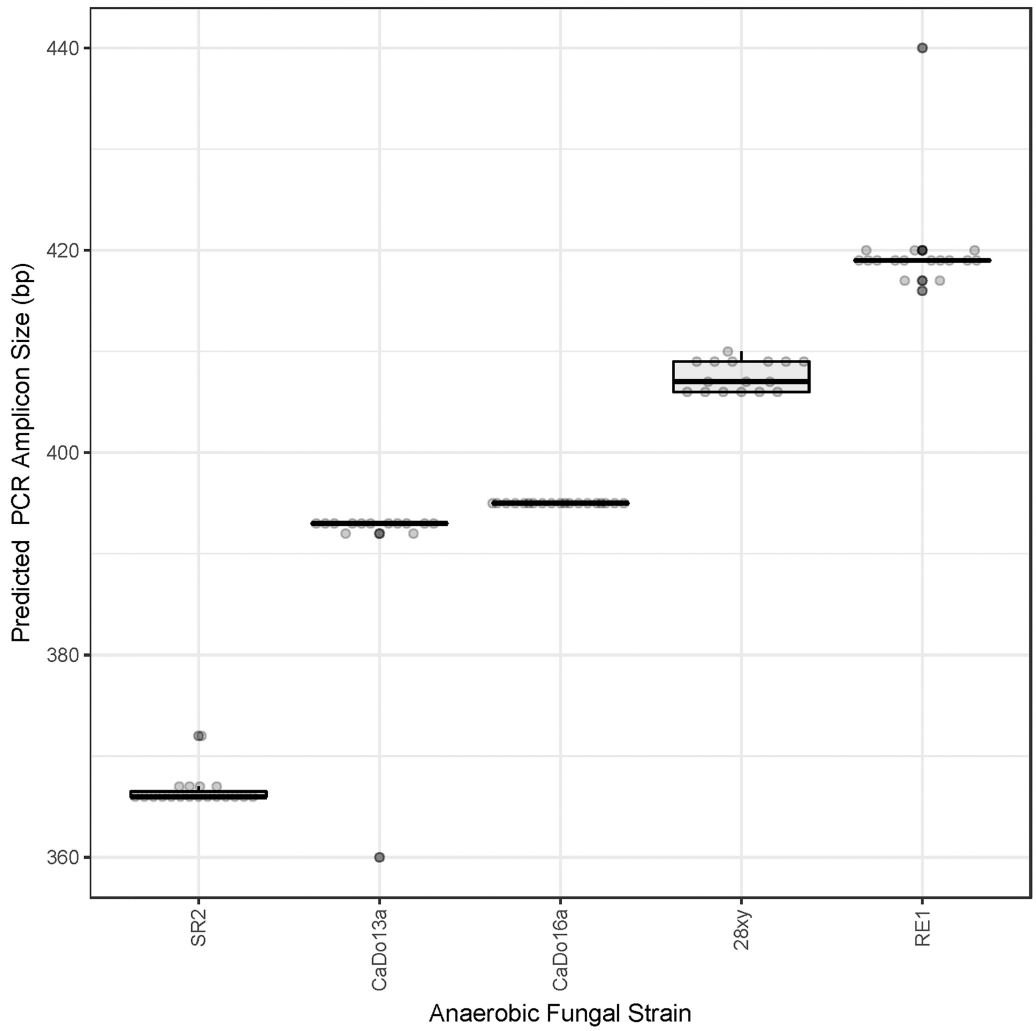

FIGURE 1 | Prediction of the barcoded amplicon sizes. Clone library sequences (see **Table 1**) were used to predict *in silico* the amplicon size (excluding UniTag adapters and barcodes) that would be generated with the barcoded amplicon primers for each of the pure cultures: *N. frontalis* RE1 (n = 17), *Orpinomyces* sp. SR2 (n = 19), *Piromyces* sp. CaDo16a (n = 19), *Caecomyces* sp. CaDo13a (n = 14), and *Anaeromyces* sp. 28xy (n = 16).

(SD 149,721) (**Supplementary Table S1**). Analysis of the pure culture HTS data indicated that the number of OTUs detected was generally consistent among sample replicates (n = 3), but varied greatly among the cultures: *Anaeromyces* sp. 28xy (28–29 OTUs), *Orpinomyces* sp. SR2 (14–15 OTUs), *N. frontalis* RE1 (12 OTUs), *Piromyces* sp. CaDo16a (3–7 OTUs), and *Caecomyces* sp. CaDo13a (3–5 OTUs). The number of OTUs was mostly higher (e.g., *Anaeromyces* sp. 28xy, *Orpinomyces* sp. SR2, *N. frontalis* RE1, and *Piromyces* sp. CaDo16a), but in one case lower (*Caecomyces* sp. CaDo13a), than the number of unique sequences detected in clone libraries (**Table 1**). More OTUs being detected was expected due to the increased sequencing depth ($>10^4$ × higher coverage per pure culture) of the HTS method compared to the clone libraries. However, the detection of fewer OTUs was unexpected. The reason for this occurring with *Caecomyces* sp. CaDo13a was that a SNP was present in the partial 5.8S rRNA gene in an area that was not included in the barcoded amplicon. Due to this, the two different sequence types could not be distinguished using amplicon sequencing and resulted in a lower number of OTUs compared to the clone library.

When summarized at the clade level, the BLAST based annotation of the OTUs was not always consistent with that predicted from the corresponding clone libraries (**Figure 2**). In two of the pure cultures, *N. frontalis* RE1 and *Orpinomyces* sp. SR2, fewer OTUs could be reliably assigned to the clade level compared to the clone libraries. Both of these cultures had more OTUs detected compared to the number of unique sequences in the corresponding clone libraries. In *Anaeromyces* sp. 28xy, the opposite was observed with a greater proportion of the OTUs that could be reliably assigned to the clade level compared to the clone library. The opposite differences in response between these genera is likely to be due to differences in terms of where variation between ITS1 copies is located

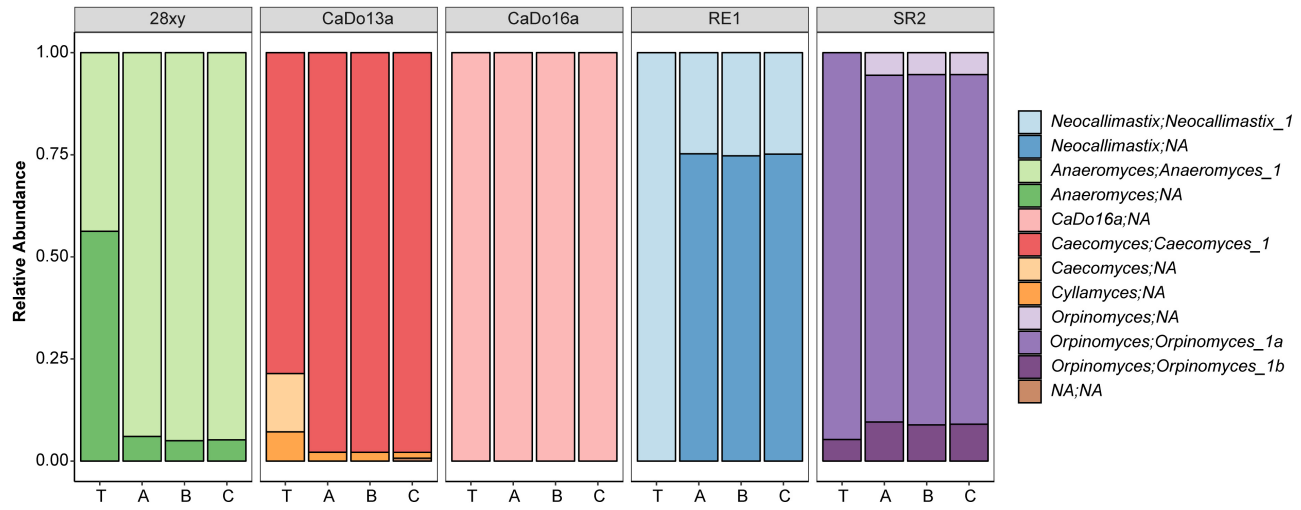

FIGURE 2 | Taxonomic annotation of anaerobic fungal pure cultures based on high-throughput sequencing. The five different pure cultures (indicated by strain name) replicated by library (A, B, and C) are shown compared to the theoretical relative abundance (T), which was determined based on the corresponding clone library data (see **Table 1**). Annotations are indicated in terms of both genus and uncultured genus- or species-level clade. NA indicates that the clade or genus level could not be annotated within the family *Neocallimastigaceae*.

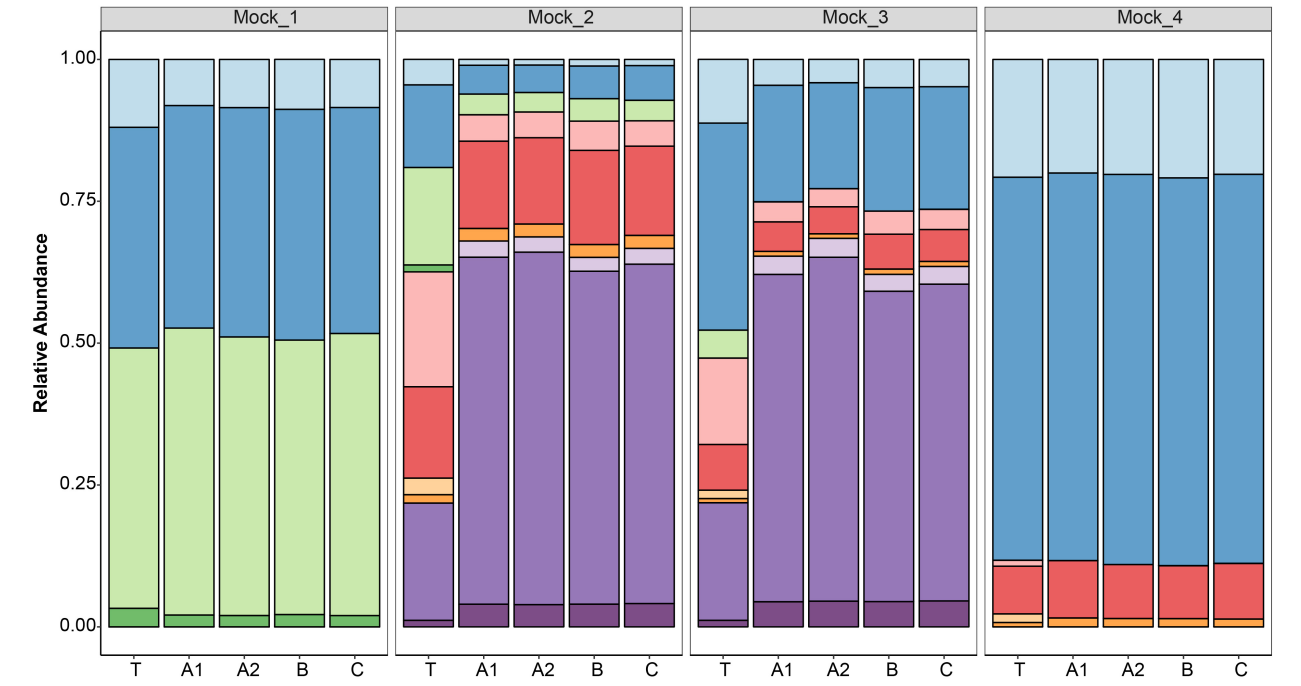

FIGURE 3 | Taxonomic annotation of four defined anaerobic fungal mock communities (Mock_1 to Mock_4) based on high-throughput sequencing. Replicates were sequenced in three different libraries (A, B, and C), and duplicates were also prepared for one library (A1 and A2). The results of the replicated samples were compared to the relative abundances of the theoretical mock communities (T). The color key for the taxonomic annotations is the same as **Figure 2**.

within the ITS1 region, as the ITS1 region was only partially sequenced in the barcoded amplicons compared to being fully sequenced in the clone libraries. However, in all of the cultures the annotation at the genus level was consistent with that of the clone libraries. With *Caecomyces* sp. CaDo13a, the relative abundance of the *Caecomyces* and *Cyllamyces* genus annotations differed compared to that determined for the clone libraries. The higher sequencing depth with the HTS method is likely the reason for this, as the change in relative abundance is the opposite of what would be expected if the smaller sized *Cyllamyces* OTU was preferentially amplified (**Figure 1** and **Table 1**).

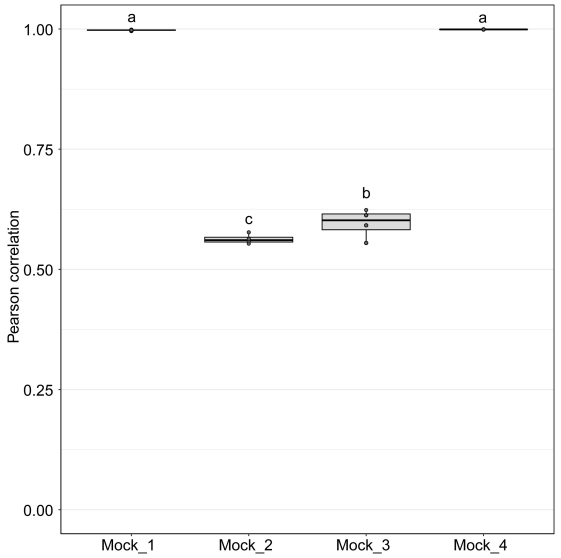

FIGURE 4 | Pearson correlation values between sequenced mock communities (Mock_1 to Mock_4) and the corresponding theoretical mock communities. A value of 1 indicates a perfect match to the corresponding theoretical mock community. Mock communities with different letters significantly differ from each other in terms of Pearson correlation values ($P < 0.001$).

Assessment of Accuracy of High-Throughput Sequencing of the ITS1 Region of Anaerobic Fungi Using Mock Communities

Four mock communities (Mock_1 to Mock_4) that differed in composition were prepared. In general, these mock communities were representative of the anaerobic fungal community composition previously reported in the herbivore gut (Liggenstoffer et al., 2010). Mock_1 and Mock_2 were prepared using similar amounts of PCR amplicons from either two or five of the pure cultures, respectively. Mock_3 and Mock_4 were both composed of DNA from all five of the pure cultures, but in different proportions compared to Mock_2. Mock_3 had staggered proportions of each of the five pure cultures, whereas Mock_4 had several of the pure cultures at low abundances (i.e., 1, 0.1, and 0.01%). These four mock communities were then used for HTS in order to assess the accuracy of the method when applied to samples differing in complexity and diversity.

After data processing, the average number of reads per mock community sample was 211,817 (SD 58,041) (**Supplementary Table S1**). All the replicates (n = 4) of the sequenced mock communities generated similar profiles (**Figure 3**). Mock_1 and Mock_4 compared well to the theoretical composition of the corresponding mock community (**Figure 4**). However, in Mock_4 there was no OTU associated with *CaDo16a*; NA or *Caecomyces*; NA detected despite it being present at 1.1 and 1.5%, respectively, in the theoretical mock community (**Table 2**). This seems to contradict the detection of *Cyllamyces*; NA which was present at 0.8% in the theoretical mock community (**Table 2**).

TABLE 2 | Comparison of the percentage deviation[a] of sequenced mock communities (n = 4) relative to the theoretical mock communities.

Taxonomic Annotation[b] (Genus; Clade)	Mock_1		Mock_2		Mock_3		Mock_4	
	Theor. Rel. Abund.	Deviation (SD)	Theor. Rel. Abund.	Deviation (SD)	Theor. Rel. Abund.	Deviation (SD)	Theor. Rel. Abund.	Deviation (SD)
Neocallimastix; Neocallimastix_1	0.120	−29.2 (2.28)	0.045	−76.0 (1.66)	0.112	−58.9 (3.32)	0.208	−1.99 (1.77)
Neocallimastix; NA	0.389	2.9 (1.67)	0.146	−62.7 (4.07)	0.365	−43.4 (3.91)	0.675	1.49 (0.32)
Anaeromyces; Anaeromyces_1	0.459	7.8 (2.04)	0.172	−78.6 (1.52)	0.049	ND*	0.000	–
Anaeromyces; NA	0.033	−37.1 (2.56)	0.012	ND*	0.000	–	0.000	–
CaDo16a; NA	–	–	0.202	−76.8 (1.52)	0.152	−76.4 (2.36)	0.011	ND*
Caecomyces; Caecomyces_1	–	–	0.161	−2.4 (3.83)	0.081	−32.7 (7.25)	0.084	15.5 (4.10)
Caecomyces; NA	–	–	0.029	ND*	0.015	ND*	0.015	ND*
Orpinomyces; NA	–	–	–	D#	–	D#	–	–
Orpinomyces; Orpinomyces_1a	–	–	0.207	192.3 (7.30)	0.207	176.0 (12.44)	0.000	–
Orpinomyces; Orpinomyces_1b	–	–	0.011	248.2 (6.67)	0.012	289.9 (5.53)	0.000	–
Cyllamyces; NA	–	–	0.015	54.7 (2.51)	0.007	17.8 (8.04)	0.008	91.64 (9.98)

[a] *A deviation value of 0% indicates an identical match to the theoretical mock community. Mean deviation values are shown along with SD (in parentheses).* [b] *Taxa in the table are arranged by decreasing amplicon size (as predicted from the clone library data), and NA indicates that the clade level could not be annotated.* **ND indicates that the taxon was not detected in the sequenced mock community although it was present in the analyzed theoretical mock community.* [#] *D indicates that the taxon was detected in the sequenced mock community (**Figure 3**), as well as the HiSeq sequenced pure cultures (**Figure 2**), but not present in the theoretical mock community (which was derived from the clone library data).*

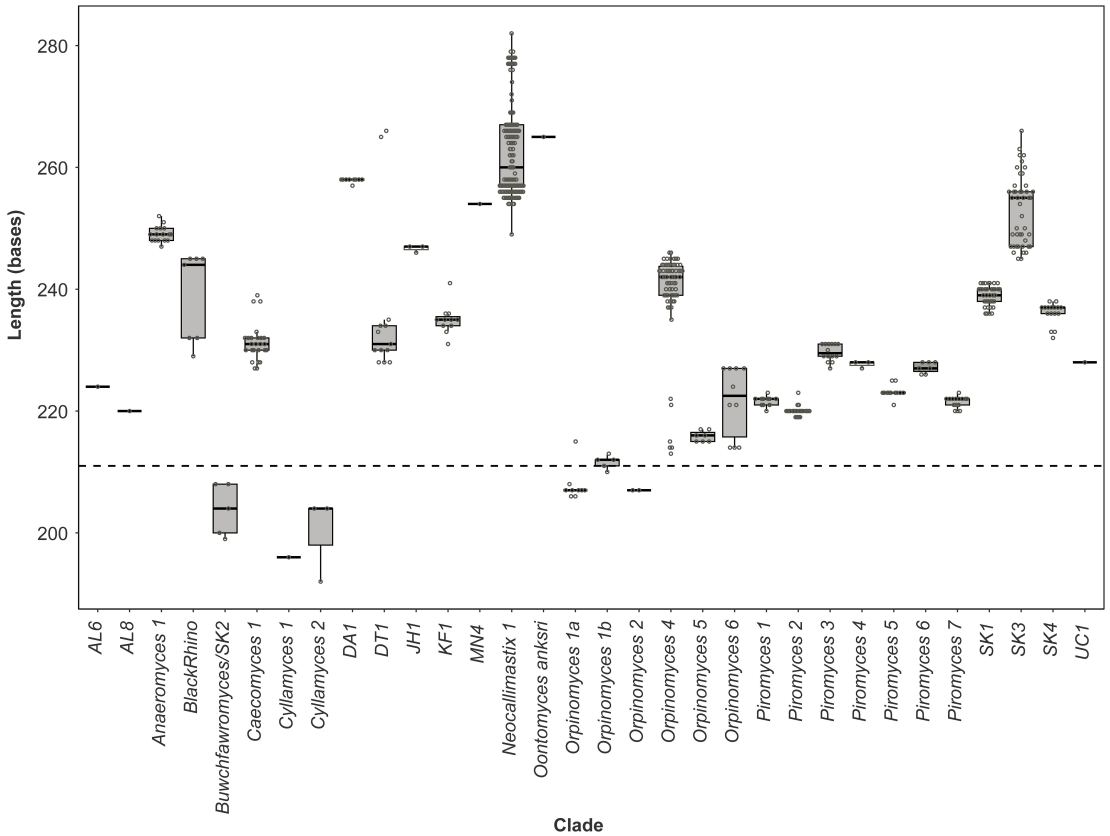

FIGURE 5 | Size distribution of the anaerobic fungal ITS1 region. Boxplot of the size distribution of the full-length ITS1 region sequences present in the anaerobic fungal ITS1 database (version 3.3) that are associated with named clades. The dashed line indicates the size from which preferential amplification was observed in the mock communities (i.e., 211 bases is the ITS1 region length for the *Orpinomyces* sp. SR2 clone that was annotated as *Orpinomyces* 1b).

These observations indicate that the taxon detection limit of the method is not a "hard-line," and is influenced by something other than the 0.6% minimum abundance threshold used during data processing. The high number of PCR cycles used to generate the barcoded amplicon, as with other studies (Liggenstoffer et al., 2010; Kittelmann et al., 2013), may offer a potential explanation for this. Under these conditions, minor taxa can be underrepresented if preferential amplification occurs or other templates are more abundant.

Pearson correlation values between the sequenced and theoretical mock communities were significantly higher for Mock_1 and Mock_4 compared to both Mock_2 and Mock_3 ($P < 0.001$) (**Figure 4**). Pearson correlation values for Mock_3 were also significantly higher than for Mock_2 ($P < 0.001$) (**Figure 4**). A significant difference between Mock_2 and Mock_3 compared to Mock_1 and Mock_4 ($P < 0.001$) was also found using weighted UniFrac distances (**Supplementary Figure S2**). In both Mock_2 and Mock_3 the relative abundances of *Orpinomyces*; *Orpinomyces 1a*, *Orpinomyces*; *Orpinomyces 1b*, and *Cyllamyces*; NA were much higher than expected (**Figure 3** and **Table 2**). These three taxa represent the smallest of the barcoded amplicons predicted from the clone library data (**Figure 1** and **Table 1**). Therefore, it is speculated that their higher relative abundance may be due to preferential amplification of these smaller amplicons during PCR. Analysis of the ITS1 region size in the AF-ITS1 database indicated that five of the 32 named clades were the same size or smaller than *Orpinomyces 1b* (**Figure 5**). Discrimination against longer PCR products has been previously reported when universal fungal primers were used for the entire ITS region (Ihrmark et al., 2012). In another study, no evidence of size bias in the ITS1 region was found when a mock community was analyzed using universal fungal primers, however, it was not stated what ITS1 size range the mock community represented (Tedersoo et al., 2015).

As amplicon sequencing data is inherently compositional, measurements of individual taxa are not independent (Gloor et al., 2017). Therefore, if the relative abundance of several taxa is higher than expected, the relative abundance of others is lower. This was clearly the case for Mock_2 and Mock_3. In Mock_2, taxa belonging to *Neocallimastix*, *Anaeromyces*, and CaDo16a were 0.6–0.8 fold lower than expected. In Mock_3, *Neocallimastix* and CaDo16a taxa were 0.4–0.8 fold lower than expected, and *Anaeromyces* was not detected at all despite accounting for 4.9% of the theoretical mock community.

From the poor match of Mock_2 and Mock_3 to the theoretical mocks, relative to Mock_1 and Mock_4, it can be

concluded that the accuracy of the method is influenced by sample community composition. Consequently, there is a need to develop an alternative taxonomic marker for anaerobic fungi and associated curated database to ensure accurate analysis of environmental samples. In general, it has been reported that the ITS2 region is similar (Blaalid et al., 2013) or better (Yang et al., 2018) than the ITS1 region as a taxonomic marker for the fungal kingdom. Tuckwell et al. (2005) also showed that anaerobic fungal subgroups identified using ITS2 were broadly the same as subgroups identified using ITS1. However, in some cases Tuckwell et al. (2005) found for individual cultures sequence differences in the ITS1 region but not the ITS2 region, and vice versa. As a consequence of this, and the limited amount of ITS2 sequence data available for anaerobic fungi, it is perhaps not surprising that the anaerobic fungal research community has focused its attention on the 28S rRNA gene as an alternative to ITS1 (Edwards et al., 2017).

For anaerobic fungi, the D1/D2 region of the 28S rRNA gene appears to have a taxonomic resolution similar to the ITS1 region (Wang et al., 2017). As such, it has the potential to generate a more stable phylogenetic backbone for anaerobic fungi than ITS1 due to its more conserved size and, therefore, more limited heterogeneity within individual cultures. Anaerobic fungal specific primers targeting the D1/D2 region of the 28S rRNA gene have been developed (Dollhofer et al., 2016), and also used in conjunction with clone libraries to study the anaerobic fungal community composition of environmental samples (Dollhofer et al., 2017). However, reference sequences of this region for previously characterized taxa are currently limited (Wang et al., 2017). There is also a challenge in terms of how to relate 28S rRNA gene sequences to the uncultivated genus- or species level clades that have only been characterized to date based on environmentally derived ITS1 region sequences. Furthermore, contrasting findings have recently been reported when ITS1 and 28S rRNA gene clone libraries were both used to analyze anaerobic fungi in an environmental sample (Mura et al., 2018). Therefore, for now at least, it is likely that ITS1 will still be used to assess anaerobic fungal diversity and community structure in environmental samples until an alternative taxonomic marker, and associated taxonomic scheme and database (analogous to that currently available for ITS1), has been developed and evaluated.

CONCLUSION

The findings of this study indicate that whilst HTS of the ITS1 region of anaerobic fungi can be used for environmental sample analysis, e.g., to detect differences between host species, diets, treatments groups etc., the accuracy of the method is influenced by sample community composition. Furthermore, ambiguity in the annotation of sequences within pure cultures due to ITS1 heterogeneity reinforces the limitations of the ITS1 region for the taxonomic assignment of anaerobic fungi. In order to overcome these issues, there is a need to develop an alternative taxonomic marker for anaerobic fungi.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI database (MK036660-MK036676, MK036677-MK036695, MK036696-MK036714, MK036715-MK036728, and MK036729-MK036744) and the European Nucleotide Archive (PRJEB2913).

AUTHOR CONTRIBUTIONS

JE initiated the study, participated in the study design, conducted the lab work, analyzed and interpreted the data, drafted the manuscript, and obtained funding. GH and HS participated in the study design, interpretation of data, and drafting of the manuscript. BN and SK were involved in data analysis and interpretation, and drafting of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02370/full#supplementary-material

REFERENCES

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., and Kauserud, H. (2013). ITS1 versus ITS2 as DNA metabarcodes for fungi. *Mol. Ecol. Resour.* 13, 218–224. doi: 10.1111/1755-0998.12065

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., et al. (2005). Defining operational taxonomic units using DNA barcode data. *Philos Trans. R. Soc. B Biol.Sci.* 360, 1935–1943. doi: 10.1098/rstb.2005.1725

Brookman, J. L., Mennim, G., Trinci, A. P. J., Theodorou, M. K., and Tuckwell, D. S. (2000). Identification and characterization of anaerobic gut fungi using

molecular methodologies based on ribosomal ITS1 and 18S rRNA. *Microbiology* 246, 393–403. doi: 10.1099/00221287-146-2-393

Callaghan, T. M. (2014). *Developing Tools for the Identification and Taxonomic Placement of the Neocallimastigales*. Ph.D. Thesis, Penglais, Aberystwyth University.

Callaghan, T. M., Podmirseg, S. M., Hohlweck, D., Edwards, J. E., Puniya, A. K., Dagar, S. S., et al. (2015). Buwchfawromyces eastonii gen. nov., sp. nov.: a new anaerobic fungus (*Neocallimastigomycota*) isolated from buffalo faeces. *MycoKeys* 9, 11–28. doi: 10.3897/mycokeys.9.9032

Dollhofer, V., Callaghan, T. M., Dorn-In, S., Bauer, J., and Lebuhn, M. (2016). Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi. *J. Microbiol. Methods.* 127, 28–40. doi: 10.1016/j.mimet.2016.05.017

Dollhofer, V., Callaghan, T. M., Griffith, G. W., Lebuhn, M., and Bauer, J. (2017). Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants. *Bioresour. Technol.* 235, 131–139. doi: 10.1016/j.biortech.2017.03.116

Edwards, J. E., Forster, R. J., Callaghan, T. M., Dollhofer, V., Dagar, S. S., Cheng, Y., et al. (2017). PCR and omics based techniques to study the diversity, ecology and biology of anaerobic fungi: insights, challenges and opportunities. *Front. Microbiol.* 8:1657. doi: 10.3389/fmicb.2017.01657

Edwards, J. E., Kingston-Smith, A. H., Jimenez, H. R., Huws, S. A., Skøt, K. P., Griffith, G. W., et al. (2008). Dynamics of initial colonization of nonconserved perennial ryegrass by anaerobic fungi in the bovine rumen. *FEMS Microbiol. Ecol.* 66, 537–545. doi: 10.1111/j.1574-6941.2008.00563.x

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., and Egozcue, J. J. (2017). Microbiome datasets are compositional: and this is not optional. *Front. Microbiol.* 6:2224. doi: 10.3389/fmicb.2017.02224

Gordon, G. L. R., and Phillips, M. W. (2005). The role of anaerobic gut fungi in ruminants. *Nutr. Res. Rev.* 11, 133–168. doi: 10.1079/nrr19980009

Hanafy, R. A., Elshahed, M. S., and Youssef, N. H. (2018). Feramyces austinii, gen. nov., sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild barbary sheep and fallow deer. *Mycologia* 110, 513–525. doi: 10.1080/00275514.2018.1466610

Ihrmark, K., Bödeker, I. T. M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., et al. (2012). New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol. Ecol.* 82, 666–677. doi: 10.1111/j.1574-6941.2012.01437.x

Joshi, A., Lanjekar, V. B., Dhakephalkar, P. K., Callaghan, T. M., Griffith, G. W., and Dagar, S. S. (2018). Liebetanzomyces polymorphus gen. et sp. nov., a new anaerobic fungus (*Neocallimastigomycota*) isolated from the rumen of a goat. *MycoKeys* 40, 89–110. doi: 10.3897/mycokeys.40.28337

Kittelmann, S., Naylor, G. E., Koolaard, J. P., and Janssen, P. H. (2012). A proposed taxonomy of anaerobic fungi (class Neocallimastigomycetes) suitable for large-scale sequence-based community structure analysis. *PLoS One* 7:e36866. doi: 10.1371/journal.pone.0036866

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., et al. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One* 8:e47879. doi: 10.1371/journal.pone.0047879

Koetschan, C., Kittelmann, S., Lu, J., Al-Halbouni, D., Jarvis, G. N., Müller, T., et al. (2014). Internal transcribed spacer 1 secondary structure analysis reveals a common core throughout the anaerobic fungi (*Neocallimastigomycota*). *PLoS One* 9:e91928. doi: 10.1371/journal.pone.0091928

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., Mcgettigan, P. A., McWilliam, H., et al. (2007). Clustal W and clustal X version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

Liggenstoffer, A. S., Youssef, N. H., Couger, M. B., and Elshahed, M. S. (2010). Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. *ISME J.* 4, 1225–1235. doi: 10.1038/ismej.2010.49

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., and Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *ISME J.* 5, 169–172. doi: 10.1038/ismej.2010.133

Mura, E., Edwards, J., Kittelmann, S., Kaerger, K., Voigt, K., Mrázek, J., et al. (2018). Anaerobic fungal communities differ along the horse digestive tract. *Fungal Biol.* 123, 240–246. doi: 10.1016/j.funbio.2018.12.004

Nicholas, K. B., and Ncholas, H. B. (1997). *GeneDoc: a tool for Editing and Annotating Multiple Sequence Alignments. Version 1.1.004, Distributed by the Author*.

Nicholson, M. J., McSweeney, C. S., Mackie, R. I., Brookman, J. L., and Theodorou, M. K. (2010). Diversity of anaerobic gut fungal populations analysed using ribosomal ITS1 sequences in faeces of wild and domesticated herbivores. *Anaerobe* 16, 66–73. doi: 10.1016/j.anaerobe.2009.05.003

Nilsson, R. H., Larsson, K.-H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., et al. (2018). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* 47, 259–264. doi: 10.1093/nar/gky1022

Ozkose, E., Thomas, B. J., Davies, D. R., Griffith, G. W., and Theodorou, M. K. (2001). *Cyllamyces aberensis* gen.nov. sp.nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. *Can. J. Bot.* 79, 666–673. doi: 10.1139/cjb-79-6-666

Paul, S. S., Bu, D., Xu, J., Hyde, K. D., and Yu, Z. (2018). A phylogenetic census of global diversity of gut anaerobic fungi and a new taxonomic framework. *Fungal Divers.* 89, 253–266. doi: 10.1007/s13225-018-0396-6

Pearson, K. (1909). Determination of the coefficient of correlation. *Science* 30, 23–25. doi: 10.1126/science.30.757.23

Ramiro-Garcia, J., Hermes, G. D. A., Giatsis, C., Sipkema, D., Zoetendal, E. G., Schaap, P. J., et al. (2016). NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. *F1000Research* 5:1791. doi: 10.12688/f1000research.9227.1

Solomon, K. V., Haitjema, C. H., Henske, J. K., Gilmore, S. P., Borges-Rivera, D., Lipzen, A., et al. (2016). Early-branching gut fungi possess large, comprehensive array of biomass-degrading enzymes. *Science* 351, 1192–1195. doi: 10.1126/science.aad1431

Stewart, C. S., and Richardson, A. J. (1989). Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and lasalocid in the presence of methanogenic bacteria. *J. Appl. Bacteriol.* 66, 85–93. doi: 10.1111/j.1365-2672.1989.tb02458.x

Tedersoo, L., Anslan, S., Bahram, M., Põlme, S., Riit, T., Liiv, I., et al. (2015). Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. *MycoKeys* 10, 1–43. doi: 10.3897/mycokeys.10.4852

Tuckwell, D. S., Nicholson, M. J., McSweeney, C. S., Theodorou, M. K., and Brookman, J. L. (2005). The rapid assignment of ruminal fungi to presumptive genera using ITS1 and ITS2 RNA secondary structures to produce group-specific fingerprints. *Microbiology* 151, 1557–1567. doi: 10.1099/mic.0.27689-0

van Lingen, H. J., Edwards, J. E., Vaidya, J. D., van Gastelen, S., Saccenti, E., van den Bogert, B., et al. (2017). Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. *Front. Microbiol.* 8:425. doi: 10.3389/fmicb.2017.00425

Wang, X., Liu, X., and Groenewald, J. Z. (2017). Phylogeny of anaerobic fungi (phylum Neocallimastigomycota), with contributions from yak in China. *Antonie Van Leeuwenhoek* 110, 87–103. doi: 10.1007/s10482-016-0779-1

Wickham, H. (2009). *Ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Yang, R. H., Su, J. H., Shang, J. J., Wu, Y. Y., Li, Y., Bao, D. P., et al. (2018). Evaluation of the ribosomal DNA internal transcribed spacer (ITS), specifically ITS1 and ITS2, for the analysis of fungal diversity by deep sequencing. *PLoS One* 13:e0206428. doi: 10.1371/journal.pone.0206428

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Trichostomatid Ciliates (Alveolata, Ciliophora, Trichostomatia) Systematics and Diversity: Past, Present, and Future

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The gastrointestinal tracts of most herbivorous mammals are colonized by symbiotic ciliates of the subclass Trichostomatia, which form a well-supported monophyletic group, currently composed by ∼1,000 species, 129 genera, and 21 families, distributed into three orders, Entodiniomorphida, Macropodiniida, and Vestibuliferida. In recent years, trichostomatid ciliates have been playing a part in many relevant functional studies, such as those focusing in host feeding efficiency optimization and those investigating their role in the gastrointestinal methanogenesis, as many trichostomatids are known to establish endosymbiotic associations with methanogenic Archaea. However, the systematics of trichostomatids presents many inconsistencies. Here, we stress the importance of more taxonomic works, to improve classification schemes of this group of organisms, preparing the ground to proper development of such relevant applied works. We will present a historical review of the systematics of the subclass Trichostomatia highlighting taxonomic problems and inconsistencies. Further on, we will discuss possible solutions to these issues and propose future directions to leverage our comprehension about taxonomy and evolution of these symbiotic microeukaryotes.

Keywords: Entodiniomorphida, integrative taxonomy, Macropodiniida, symbiotic ciliates, Vestibuliferida

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INTRODUCTION

The gastrointestinal tracts of most herbivorous mammals are colonized by symbiotic ciliates of the subclass Trichostomatia Bütschli, 1889 (**Supplementary Video S1**). These play a central role for the efficient fermentative process in the host intestinal tract and also contribute to the degradation process of proteins, lipids, nitrogen compounds and carbohydrates, such as cellulose, hemicellulose and starch (Dehority, 1986; Wright, 2015). These microeukaryotes form a well-supported monophyletic group, currently composed of ∼1,000 species, 129 genera, and 21 families (**Supplementary Material S1**) that are distributed across three orders: Entodiniomorphida Reichenow, in Doflein & Reichenow, 1929, including species with ciliary zones restricted to tufts or bands, and infraciliatures organized as polybrachykineties, Macropodiniida Lynn, 2008 and Vestibuliferida de Puytorac et al., 1974, including ciliates all covered by cilia and with a densely ciliated vestibulum (Lynn, 2008; Cedrola et al., 2015; Gao et al., 2016). In recent years,

trichostomatid ciliates have been playing a part in many relevant functional studies, such as those focusing on host feed efficiency optimization (Newbold et al., 2015) and those investigating their role in gastrointestinal methanogenesis, as many trichostomatids are known to establish endosymbiotic associations with methanogenic Archaea (Embley et al., 2003). Methanogenesis from ciliate associated methanogens may account for up to 60% of methane emissions into the Earth's atmosphere (Intergovernmental Panel on Climate Change [IPCC], 2019; Malmuthuge and Guan, 2017). However, the systematics of trichostomatids presents many inconsistencies. Here, we stress the importance of more taxonomic works, to improve classification schemes of this group of microorganisms. This will provide a sound basis for ciliate community structure assessment. We present a historical review of the systematics of the subclass Trichostomatia highlighting taxonomic problems and inconsistencies. We also discuss possible solutions and propose future directions to broaden our understanding of the taxonomy and evolution of these symbiotic microeukaryotes.

PAST

Trichostomatid ciliates were discovered in the first half of the 19th century by Gruby and Delafond (1843). However, the authors, presented only a brief and succinct report about high densities of "animaculous" inhabiting the stomach and intestine of domestic cattle and horses. The first illustrations of trichostomatid ciliates are attributed to Colin (1854) while the author studied domestic mammals. G. Colin performed live observations of many species, possibly including members of the genera *Blepharocorys* Bundle, 1895, *Bundleia* da Cunha and Muniz, 1928, *Cycloposthium* Bundle, 1896, *Diplodinium* Schuberg, 1888 and *Entodinium* Stein, 1859. The first author to publish a formal taxonomic work on trichostomatid ciliates was F. Stein (1858) describing, although superficially, species of the genera *Entodinium*, *Isotricha*, and *Ophryoscolex* and the family Ophryoscolecidae. Following, several novel species were described from many geographic locations and from different host species. In this period, beginning with the work of F. Stein (1858) until the late 1970s, more than 400 species were described, indicating that trichostomatid ciliates may constitute a diverse group of microorganisms (Fiorentini, 1889; Bundle, 1895; Poche, 1913; Da Cunha, 1914a,b; Gassovsky, 1919; Buisson, 1923a,b,c, 1924; Crawley, 1923; Dogiel, 1925a,b, 1926a,b, 1927, 1928, 1932, 1934, 1935; Fantham, 1926; Becker and Talbot, 1927; Hsiung, 1930, 1935a,b, 1936; Kofoid and MacLennan, 1930, 1932, 1933; Jirovec, 1933; Kofoid and Christenson, 1933; Kofoid, 1935; Wertheim, 1935; Fonseca, 1939; Moriggi, 1941; Sládeček, 1946; Bush and Kofoid, 1948; Lubinsky, 1957a, 1958a,b; Latteur, 1966a,b, 1967, 1968, 1969, 1970; Wolska, 1967b, 1968, 1969). Most of these studies were done based only on live observations and by using simple ciliatological techniques, such as hematoxylin and iodine staining methods, which were the available tools at that time. Nevertheless, many morphological characters, such as skeletal plates (Dogiel, 1923; Schulze, 1924, 1927; Dogiel and Fedorowa, 1925), contractile vacuoles (Kraschnninikow, 1929; MacLennan, 1933), concretion vacuoles (Dogiel, 1929), and paralabial organelles (Bretschneider, 1962) could be clearly characterized, allowing the inclusion of these microeukaryotes into the phylum Ciliophora, orders Entodiniomorphida and Vestibuliferida (for history of classification, see **Supplementary Material S2**). In this same period, the first studies appeared that proposed hypotheses on the evolution of this group of microorganisms. According to Dogiel (1947) and Lubinsky (1957a,b,c), within the family Ophryoscolecidae, subfamily Entodiniinae could be considered ancestral due to its characteristic single ciliary zone, single contractile vacuole, poorly developed caudal spines and lack of skeletal plates. The Ophryoscolecinae is considered to be the most recent group for presenting two ciliary zones, large number of vacuoles and skeletal plates, and developed caudal projections. Diplodiniinae is considered an intermediate group.

The development of silver impregnation techniques in 1930s (Bodian, 1936, 1937), which can reveal in details infraciliary and other argentophilic structures patterns, represented a great revolution in the systematics of Ciliophora (Lynn, 2008). They were initially applied to trichostomatids by Noirot-Timothée (1956a,b) where the infraciliary band patterns of *Epidinium* Crawley, 1923 and *Ophryoscolex* Stein, 1858 were described. Further studies were performed by several authors and contributed to our understanding of infraciliary band patterns in various trichostomatid ciliate species (Noirot-Timothée, 1960; Grain, 1962, 1963a,b, 1964, 1965; Batisse, 1966). However, the greatest contribution was achieved by M. Wolska in a series of seminal works (Wolska, 1963, 1964, 1965, 1966a,b, 1967a,b, 1968, 1969, 1970, 1971a,b, 1978a,b,c,d, 1979, 1985, 1986), which described infraciliary band patterns and morphogenetic processes in ciliates of the families Buetschliidae Poche, 1913, Blepharocorythidae Hsiung, 1929, Spirodiniidae Strelkow, 1939, Pseudoentodiniidae Wolska, 1985 (Entodiniomorphida), Isotrichidae Bütschli, 1889 and Paraisotrichidae Da Cunha, 1915 (Vestibuliferida). As a result of these detailed investigations, a hypothesis on the evolutionary relationship within the Trichostomatia was proposed by Wolska (1971b). According to the descriptions there are several patterns of infraciliary bands in Trichostomatia in which are composed by at least one of these bands: adoral polybrachykinety, dorsal polybrachykinety, dorso-adoral polybrachykinety, kinety loop, paralabial kineties, vestibular polybrachykinety, and vestibular kineties (**Supplementary Figure S1**).

Ultrastructural works also impacted the systematics of trichostomatid ciliates. Bonhomme (1989), after collecting data on the ultrastructure of many Entodiniomorphina (order Entodiniomorphida) representatives, suggested that this suborder could be classified into two groups, according to their cortex ultrastructure information. The first is composed of ciliates with the cortex lacking dense longitudinal cords (genus *Cycloposthium* Bundle, 1895; Ophryoscolecidae Stein, 1859 and Troglodytellidae Corliss, 1979), and the second is composed of ciliates with dense longitudinal cords (genus *Tripalmaria* and Spirodiniidae Strelkow, 1939).

Further, based on a compilation of structural and ultrastructural data, Small and Lynn (1981) proposed

Trichostomatia as a subclass of the class Litostomatea, and as a sister group of the subclass Haptoria Corliss, 1974.

Over the last 30 years, after a long period of scarce taxonomic data being produced, many taxonomic inventories of trichostomatids isolated from several mammalian host species, domestic and wild, from different geographic locations (**Supplementary Table S1**) started to appear in the literature, leading to the characterization of a series of novel species, including trichostomatids inhabiting the gastrointestinal tracts of Australian marsupials (Dehority, 1996; Cameron et al., 2000a,b, 2001a,b, 2002, 2003; Cameron and O'Donoghue, 2001, 2002a,b,c, 2003a,b,c, 2004a,b). These ciliates present several exclusive morphological features among trichostomatids. For this reason, Lynn (2008) proposed the creation of a new order to include them, Macropodiniida. This period was also characterized by the establishment of new silver impregnation techniques for trichostomatid ciliates, such as the adaptations of ammoniacal silver carbonate impregnation proposed by Ito and Imai (1998) and Rossi et al. (2016) and the adaption of Protargol's impregnation for vestibuliferids proposed by Ito and Imai (2000). These techniques allowed the development of several studies describing the infraciliature and morphogenetic process in different trichostomatid species (Ito et al., 1997, 2001, 2002, 2006, 2008, 2010, 2011, 2014, 2017, 2018; Ito and Imai, 1998, 2003, 2005, 2006; Gürelli and Ito, 2014; Cedrola et al., 2016, Cedrola et al., 2017a,b, 2018a,b; Gürelli and Akman, 2016; Gürelli, 2018, 2019; Ito and Tokiwa, 2018), which were very important to understand the evolutionary relationships within the Trichostomatia.

A novel view on the systematics of trichostomatid ciliates emerged in the late 1990s with the advent of molecular techniques. The first molecular phylogenies (Wright and Lynn, 1997a,b,c; Wright et al., 1997) corroborated the initial morphological studies placing trichostomatids as a monophyletic group within the Litostomatea. Starting from early 2000s and with the increasing availability of 18S rRNA gene sequences of members of the subclass Trichostomatia in public repositories (Cameron et al., 2001a, 2003; Cameron and O'Donoghue, 2004b; Strüder-Kypke et al., 2007; Ito et al., 2010, 2014; Pomajbíková et al., 2010, 2013; Snelling et al., 2011; Chistyakova et al., 2014; Moon-Van der Staay et al., 2014; Grim et al., 2015; Kittelmann et al., 2015; Rossi et al., 2015; Bardele et al., 2017; Cedrola et al., 2017, 2019), the internal phylogenetic relationships within the subclass began to be elucidated. This caused a revolution in their systematics and revealed several taxonomic incongruences, mainly with respect to Entodiniomorphida and Vestibuliferida, for which the grouping based on morphological features does not seem to hold.

PRESENT

Currently, the subclass Trichostomatia consists of three major orders, Entodiniomorphida, Macropodiniida, and Vestibuliferida. Macropodiniida is the only group for which multidisciplinary taxonomic approaches were applied (Cameron and O'Donoghue, 2001, 2002a,b,c, 2003a,b,c, 2004a,b; Cameron et al., 2000a,b, 2001a,b, 2002, 2003). Their representatives are distributed in three monophyletic families all with well-supported internal nodes (**Figure 1** and **Supplementary Figure S2**). However, most of the species diversity of Trichostomatia occurs within the Entodiniomorphida and Vestibuliferida, which are extremely neglected groups concerning taxonomic studies. According to 18S rRNA gene reconstructions (**Figure 1** and **Supplementary Figure S2**; Ito et al., 2014; Kittelmann et al., 2015), the order Entodiniomorphida is not monophyletic, emerging in the tree as two independent clades, one containing representatives of the families Blepharocorythidae Hsiung, 1929, Parentodiniidae Ito et al., 2002, Pseudoentodiniidae Wolska, 1986, Cycloposthiidae Poche, 1913, Spirodiniidae Strelkow, 1939, Polydiniellidae Corliss, 1960, Troglodytellidae Corliss, 1979, and Ophrysocolecidae Stein, 1859; and another containing members of the family Buetschliidae Poche, 1913. Moreover, many of these families do not constitute natural groups, such as Blepharocorythidae, Cycloposthiidae, and Spirodiniidae; and for those that are monophyletic, such as Ophryoscolecidae, the internal branching is poorly supported, as detected in previous works (Ito et al., 2014; Kittelmann et al., 2015; Rossi et al., 2015; Cedrola et al., 2017). Many inconsistencies can also be observed in the order Vestibuliferida with representatives distributed in three distinct clades (**Figure 1** and **Supplementary Figure S2**; Ito et al., 2014; Kittelmann et al., 2015), in which the families Balantididae and Paraisotrichidae do not constitute natural groups. Moreover, 18S rRNA gene sequences are only available from representatives of 16 out of the 21 currently recognized families of Trichostomatia. The families with no molecular data are: Gilchristinidae (Ito et al., 2014), Rhinozetidae Van Hoven et al., 1988, Telamonididae Latteur and Dufey, 1967 (Entodiniomorphida), Protocaviellidae Grain and Corliss, 1979, Protohallidae Cunha and Muniz, 1927 (Vestibuliferida). Still, many of the existing families of which molecular data are available, such as Polydiniellidae Corliss, 1960, Troglodytellidae Corliss, 1979 (Entodiniomorphida) and Pycnotrichidae Poche, 1913 (Vestibuliferida) have only one representative with its 18S rRNA gene sequenced, limiting the power of phylogenetic reconstructions within the whole group. The scarcity and absence of consistent morphological data from many trichostomatid groups is also of concerns, for example, there are no structural (infraciliary pattern and morphogenesis) and ultrastructural data described for many cycloposthiids, troglodytelids, and spirodinids, which makes it impossible to establish homology hypotheses on trichostomatids. Moreover, the lack of detailed morphological data contributes to taxonomic inconsistencies and hinders the development of novel classifications schemes that reflect evolutionary divergences.

FUTURE

Despite the great advances obtained after implementing silver staining, ultrastructural and molecular methods, it is clear that huge gaps are still preventing a cohesive systematic scheme of Trichostomatia, especially when we compare the existing

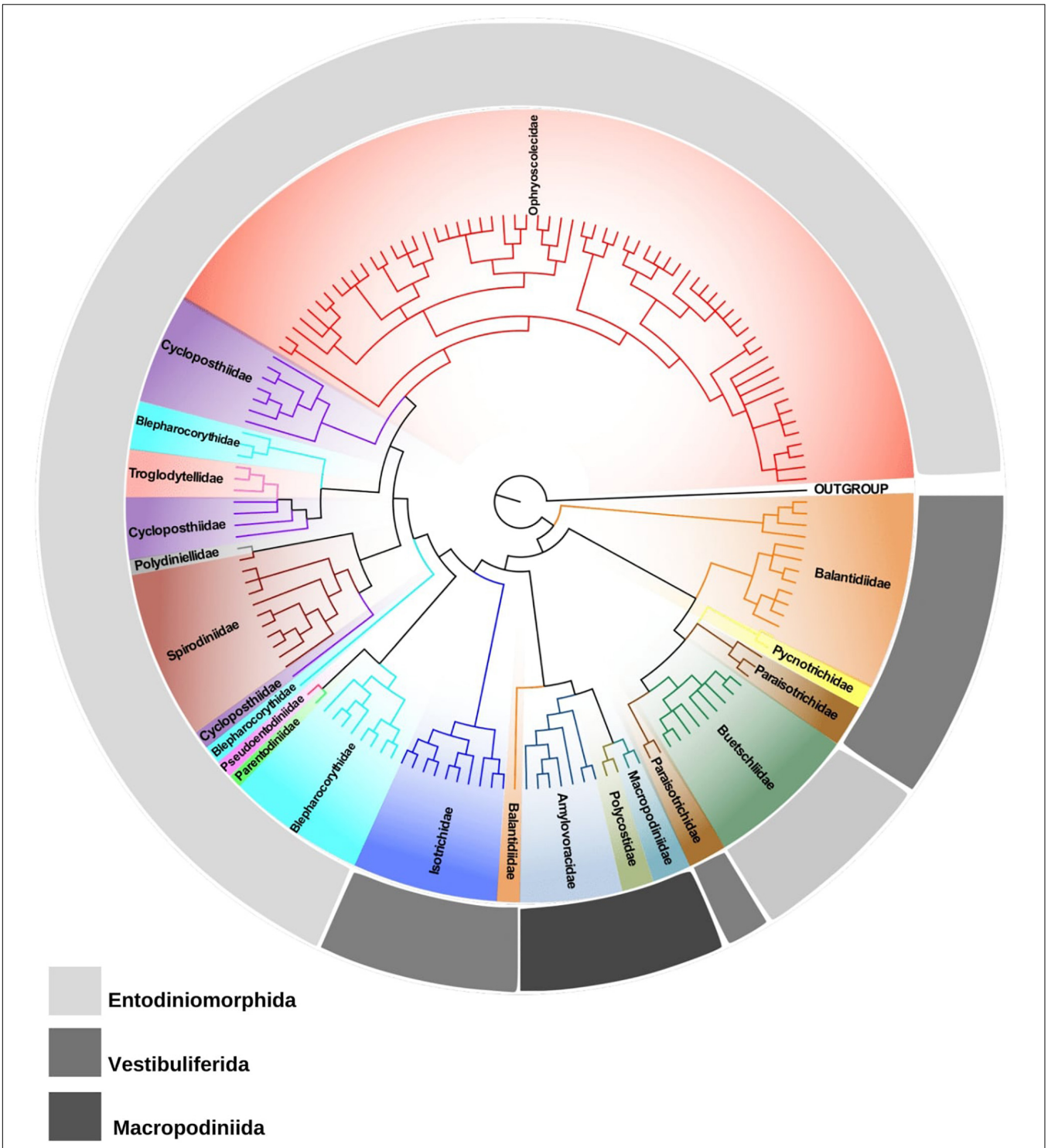

FIGURE 1 | Phylogenetic tree of trichostomatid ciliates (Ciliophora, Litostomatea, and Trichostomatia) estimated by Bayesian Inference and based on 18S rRNA gene data. *Spathidium papilliferum* was chosen as out group.

data with other Ciliophora groups (Warren et al., 2017). In the forthcoming years, we need to invest more in detailed descriptions and redescriptions of infraciliary band patterns and morphogenesis, on 18S rRNA gene sequencing, and in depth ultrastructure characterizations. Using these methods, we need to study trichostomatids from a wide variety of hosts especially in so far neglected geographical regions such as, e.g., neotropical areas, with emphasis on Entodiniomorphida and Vestibuliferida.

We should further expand this work to trichostomatid families such as the Protocaviellidae and Protohallidae from domestic and wild rodents and Gilchristinidae, Rhinozetidae, and Telamonididae from elephants, rhinos and wild pigs, respectively. Moreover, improvements to trichostomatid cultivation techniques, which are still poorly developed (Williams and Coleman, 1992; Dehority and Wright, 2014; Newbold et al., 2015; Belzecki et al., 2016), would be of great importance to obtain suitable samples for morphology and molecular characterization approaches. Collectively, this information will contribute to develop more robust phylogenetic hypotheses, to elaborate taxonomic reformulations, contributing to elucidate the many taxonomic incongruences presented above and to establish new classification schemes that reflect evolutionary divergences within Trichostomatia.

Apart from 18S rRNA genes, it is time to obtain data on other informative loci from pure/axenic cultures, such as the internal transcribed spacer region and 28S ribosomal RNA genes, to further improve our understanding of the phylogenetic relationships within the Litostomatea (Rajter and Vďačný, 2017). In addition, it is possible to identify new macronuclear regions, using genomic information of Trichostomatia representatives (Park et al., 2018), and to obtain hydrogenosomal sequences, such as those from 16S and Fe-Hydrogenase. Also, it is possible to use the next generation sequencing techniques to perform phylogenomic reconstruction, as done for other Ciliophora groups within the last decade (Feng et al., 2015; Gentekaki et al., 2017; Jiang et al., 2019). This data could be used in macro-evolutionary approaches to reveal divergence times and the mode of evolution in trichostomatid ciliates. The timescale and evolutionary dynamics of these symbiotic ciliates are yet to be determined (Newbold et al., 2015). Molecular dating studies are restricted to Wright and Lynn (1997c) and Vďačný (2015, 2018), which employed different molecular dating methods, taxon sampling and calibration data, using mostly the fossil record of hosts and the posterior ages estimated from previous studies as calibration priors for ciliates time tree. Baele et al. (2006) provided evidence for the presence of numerous heterotachous sites (sites in which its substitution rates can vary with time) within the 18S rRNA gene of ciliates, which may result in the introduction of bias. Thus, further improvements to the calculation and resolution of trichostomatid phylogenies are needed through the use of evolutionary models, such as, for example, the mixture of branch lengths (MBL) (Zhou et al., 2007).

DATA AVAILABILITY STATEMENT

The datasets generated for the phylogenetic analyses are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FC, PF, and MD collected the data. FC, MS, and RD participated in the conception of the study. FC, MS, and MR participated in the manuscript writing. FC, MR, and PF prepared the figures and supplementary material. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02967/full#supplementary-material

FIGURE S1 | Oral infraciliary bands pattern of Trichostomatid ciliates. **A–D**. Order Vestibuliferida. **A**, Isotrichidae; **B**, Paraisotrichidae; **C**, Protocaviellidae; **D**, Protohallidae; **E–L**, Order Entodiniomorphida; **E**, Buetschliidae; **F**, Blepharocorythidae; **G**, Cycloposthiidae; **H**, Gichristinidae; **I**, Ophryoscolecidae; **J**, Parentodiniidae; **K**, Pseudoentodiniidae; **L**, Spirodiniidae; **M–O**, Order Macropodiniida; **M**, Amylovoracidae; **N**, Polycostidae; **O**, Macropodiniidae; AP, adoral polybrachykinety; CB, cytopharyngeal basket; DAP, dorso-adoral polybrachykinety; PVP, perivestibular polybrachykinety, and VK, vestibular kineties.

FIGURE S2 | Phylogenetic tree of trichostomatid ciliates (Ciliophora, Litostomatea, and Trichostomatia) based on 18S rRNA gene data. *Spathidium papilliferum* was chosen as out group. The black dots in the nodes indicate bootstrap (ML) or posterior probability (BI) values >80/0.8. The scale bar corresponds to four substitutions per 100 nucleotides positions.

TABLE S1 | Hosts where Trichostomatia ciliates were registered.

MATERIAL S1 | Trichostomatid families and genera.

MATERIAL S2 | History of classification of subclass Trichostomatia.

VIDEO S1 | Trichostomatid domestic cattle rumen ciliates under live observation.

REFERENCES

Baele, G., Raes, J., Van der Peer, Y., and Vansteelandt, S. (2006). An improved statistical method for detecting heterotachy in nucleotide sequences. *Mol. Biol. Evol.* 23, 1397–1405. doi: 10.1093/molbev/msl006

Bardele, C. F., Schultheib, S., Wright, A.-D. G., Dominguez-Bello, M. G., and Obispo, N. E. (2017). Aviistricha hoazini n. gen., n. sp., the morphology and molecular phylogeny of an anaerobic ciliate from the crop of the hoatzin (*Opisthocomus hoazin*), the cow among the birds. *Protist* 168, 335–351. doi: 10.1016/j.protis.2017.02.002

Batisse, A. (1966). Quelques infusoires holotriches parasites du coecum de l'hydrochaire (*Hydrocheirus capybara*, L.). *Protist* 2, 39–52.

Becker, E. R., and Talbot, M. (1927). The protozoan fauna of the rumen and reticulum of American cattle. *Iowa State Coll. J. Sci.* 1, 345–371.

Belzecki, G., Miltko, R., Michalowski, T., and McEwan, N. R. (2016). Methods for the cultivation of ciliated protozoa from the large intestine of horses. *FEMS Microbiol. Lett.* 363, 1–4. doi: 10.1093/femsle/fnv233

Bodian, D. (1936). The new ethod for staining nerve fibers and nerve endings in mounted paraffin sections. *Anat. Rec.* 69, 153–162.

Bodian, D. (1937). The staining of paraffin sections of nervous tissues with activated protargol. The role of fixatives. *Anat. Rec.* 70, 153–162. doi: 10.1002/ar.1090690205

Bonhomme, A. (1989). Etude ultrastructurale de Troglodytella gorillae, cilié de l'intestin des gorilles. *Eur. J. Protistol.* 24, 225–237. doi: 10.1016/s0932-4739(89)80059-8

Bretschneider, L. H. (1962). Das Paralabialorgan der Ophryoscoleciden. *Proc. Natl. Acad. Sci. Amsterdam* 65, 423–452.

Buisson, J. (1923a). Infusoires nouveux parasites d'antilopes africaines. *C. R. Séances Soc. Biol.* 89, 1217–1219.

Buisson, J. (1923b). *Les Infusoires Ciliés du Tube Digestif de L'homme et des Mammifères*. Ph. D. Thèse. Paris Le Gall: Paris.

Buisson, J. (1923c). Sur quelques infusoires nouveux ou peu connus parasites de mammifères. *Ann. Par. Hum. Comp.* 1, 209–246. doi: 10.1051/parasite/2019003

Buisson, J. (1924). Quelques infusoires parasites d'antilopes africaines. *Ann. Par. Hum. Comp.* 2, 155–160. doi: 10.1051/parasite/1924022155

Bundle, A. (1895). Ciliate infusorien in coecum des Pferdes. *Z. Wiss. Zool.* 60, 284–350.

Bush, M., and Kofoid, C. A. (1948). Ciliates from the Sierra Nevada bighorn (*Ovis canadensis* sierra Grinnell). *Univ. Calif. Publ. Zool.* 53, 237–261.

Cameron, S., O'Donoghue, P., and Adlard, R. (2000a). First record of *Cycloposthium edentatum* Strelkow, 1928 from the black-striped wallaby, *Macropus dorsalis*. *Parasitol. Res.* 86, 158–162. doi: 10.1007/s004360050025

Cameron, S. L., O'donoghue, P. J., and Adlard, R. D. (2000b). Novel isotrichid ciliates endosymbiotic in Australian macropodid marsupials. *Syst. Parasitol.* 46, 45–57. doi: 10.1023/a:1006208802110

Cameron, S. L., and O'Donoghue, P. J. (2001). Stomatogenesis in the ciliate genus Macropodinium Dehority, 1996 (Litostomatea: Macropodiniidae). *Eur. J. Protistol.* 37, 199–206. doi: 10.1078/0932-4739-00819

Cameron, S. L., and O'Donoghue, P. J. (2002a). The ultrastructure of Amylovorax dehorityi comb. nov. and erection of the Amylovoracidae fam. nov. (Ciliophora: Trichostomatia). *Eur. J. Protistol.* 38, 29–44. doi: 10.1078/0932-4739-00841

Cameron, S. L., and O'Donoghue, P. J. (2002b). The ultrastructure of Macropodinium moiri and revised diagnosis of the Macropodiniidae (Litostomatea: Trichostomatia). *Eur. J. Protistol.* 38, 179–194. doi: 10.1078/0932-4739-00861

Cameron, S. L., and O'Donoghue, P. J. (2002c). Trichostome ciliates from Australian marsupials. I. Bandia gen. nov. (Litostomatea: Amylovoracidae). *Eur. J. Protistol.* 38, 405–429. doi: 10.1078/0932-4739-00889

Cameron, S. L., and O'Donoghue, P. J. (2003a). Trichostome ciliates from Australian marsupials. II. Polycosta gen. nov. (Litostomatea: Polycostidae fam. nov.). *Eur. J. Protistol.* 39, 83–99. doi: 10.1078/0932-4739-00890

Cameron, S. L., and O'Donoghue, P. J. (2003b). Trichostome ciliates from Australian marsupials. III. Megavestibulum gen. nov.(Litostomatea: Macropodiniidae). *Eur. J. Protistol.* 39, 123–137. doi: 10.1078/0932-4739-00891

Cameron, S. L., and O'Donoghue, P. J. (2003c). Trichostome ciliates from Australian marsupials. IV. Distribution of the ciliate fauna. *Eur. J. Protistol.* 39, 139–147. doi: 10.1078/0932-4739-00892

Cameron, S. L., and O'Donoghue, P. J. (2004a). Morphometric and cladistic analyses of the phylogeny of Macropodinium (Ciliophora: Litostomatea: Macropodiniidae). *Acta Protozool.* 43, 43–53.

Cameron, S. L., and O'Donoghue, P. J. (2004b). Phylogeny and biogeography of the "Australian" trichostomes (Ciliophora: Litostomata). *Protist* 155, 215–235. doi: 10.1078/143446104774199600

Cameron, S. L., Adlard, R. D., and O'Donoghue, P. J. (2001a). Evidence for an independent radiation of endosymbiotic litostome ciliates within Australian marsupial herbivore. *Mol. Phylogenet. Evol.* 20, 302–310. doi: 10.1006/mpev.2001.0986

Cameron, S. L., O'donoghue, P. J., and Adlard, R. D. (2001b). Four new species of Macropodinium (Ciliophora: Litostomatea) from Australian wallabies and pademelons. *J. Eukaryot. Microbiol.* 48, 542–555. doi: 10.1111/j.1550-7408.2001.tb00190.x

Cameron, S. L., O'Donoghue, P. J., and Adlard, R. D. (2002). Species diversity within Macropodinium (Litostomatea: Trichostomatia): endosymbiotic ciliates from Australian macropodid marsupials. *Mem. Queensl. Mus.* 48, 49–69.

Cameron, S. L., Wright, A. D. G., and O'Donoghue, P. J. (2003). An expanded phylogeny of the Entodiniomorphida (Ciliophora: Litostomatea). *Acta Protozool.* 42, 1–6.

Cedrola, F., Dias, R. J. P., Martinele, I., and D'Agosto, M. (2017a). Description of *Diploplastron dehorityi* sp. nov. (Entodiniomorphida, Ophryoscolecidae), a new rumen ciliate from Brazilian sheep. *Zootaxa* 4258, 581–585. doi: 10.11646/zootaxa.4258.6.8

Cedrola, F., Dias, R. J. P., Martinele, I., and D'Agosto, M. (2017b). Polymorphism and inconsistencies in the taxonomy of Diplodinium anisacanthum da Cunha, 1914 (Ciliophora, Entodiniomorphida, Ophryoscolecidae) and taxonomic notes on the genus Diplodinium. *Zootaxa* 4306, 249–260.

Cedrola, F., Fregulia, P., D'Agosto, M., and Dias, R. J. P. (2018a). Intestinal ciliates of Brazilian capybara (*Hydrochoerus hydrochaeris* L.). *Acta Protozool.* 57, 61–67.

Cedrola, F., Rossi, M. F., Martinele, I., D'Agosto, M., and Dias, R. J. P. (2018b). Morphology and description of infraciliary bands pattern in four Metadinium Awerinzew and Mutafowa, 1914 species (Ciliophora, Entodiniomorphida, Ophryoscolecidae) with taxonomic notes on the genus. *Zootaxa* 4500, 574–580. doi: 10.11646/zootaxa.4500.4.6

Cedrola, F., Martinele, I., Dias, R. J. P., and D'Agosto, M. (2016). Rumen ciliates in Brazilian sheep (*Ovis aries*) and redescription of Entodinium contractum (Ciliophora, Entodiniomorphida, Ophryoscolecidae). *Zootaxa* 4088, 292–300. doi: 10.11646/zootaxa.4088.2.10

Cedrola, F., Rossi, M. F., Dias, R. J. P., Martinele, I., and D'Agosto, M. (2015). Methods for taxonomic study of rumen ciliates (Alveolata, Ciliophora): a brief review. *Zool. Sci.* 32, 8–15. doi: 10.2108/zs140125

Cedrola, F., Senra, M. V. X., D'Agosto, M., and Dias, R. J. P. (2017). Phylogenetic analyses support validity of genus *Eodnium*. *J. Eukaryot. Microbiol.* 64, 242–247. doi: 10.1111/jeu.12355

Cedrola, F., Senra, M. V. X., D'Agosto, M., and Dias, R. J. P. (2019). Helmet-shaped body of entodiniomorphid ciliates (Ciliophora, Entodiniomorphida), a synapomorphy or a homoplasy? *J. Eukaryot. Microbiol.* 0, 1–4. doi: 10.1111/jeu.12749

Chistyakova, L. V., Kostygov, A. Y., Kornilova, O. A., and Yurchenko, V. (2014). Reisolation and description of *Balantidium duodeni* Stein, 1867 (Litostomatea, Trichostomatia). *Parasitol. Res.* 113, 4207–4215. doi: 10.1007/s00436-014-4096-1

Colin, G. (1854). *Traité de Physiologie Comparée des Animaux; Considérée dans ses Rapports Avec les Sciences Naturelles, la Médecine, la Zootechnie, et L'économie Rurale*. Paris: Baillière.

Crawley, H. (1923). Evolution in the family Ophryoscolecidae. *Proc. Natl. Acad. Sci. U.S.A.* 75, 393–412.

Da Cunha, A. M. (1914a). Sobre os ciliados do estomago dos ruminantes no Brasil. *Mem. Inst. Oswaldo Cruz.* 6, 58–69.

Da Cunha, A. M. (1914b). Sobre os ciliados intestinaes dos mammiferos. *Mem. Inst. Oswaldo Cruz.* 7, 139–145. doi: 10.1590/s0074-02761915000200001

Dehority, B. A. (1986). Protozoa of the digestive tract of herbivorous mammals. *Insect Sci. Appl.* 7, 279–296. doi: 10.1017/s1742758400009346

Dehority, B. A. (1996). A new family of Entodiniomorph protozoa from the marsupial forestomach, with descriptions of a new genus and five new species. *J. Protozool.* 4, 285–295. doi: 10.1111/j.1550-7408.1996.tb03991.x

Dehority, B. A., and Wright, A.-D. (2014). Studies on the in vitro cultivation of ciliate protozoa from the kagaroo forestomach. *Eur. J. Protistol.* 50, 395–401. doi: 10.1016/j.ejop.2014.04.001

Dogiel, V. A. (1923). Cellulose ais Bestandteil des Skeletts bei einigen infusioren. *Biol. Zent. Bl.* 43, 289–291.

Dogiel, V. A. (1925a). Neue parasitische infusorien aus dem Magen des Renntiers (*Rangifer tarandus*). *Rusk Arkh Protist.* 4, 43–65.

Dogiel, V. A. (1925b). Nouveaux infusoires de la famille des ophryoscolécidés parasites d'antilopes africaines. *Ann. Parasitol. Hum. Comp.* 15, 116–142. doi: 10.1051/parasite/1925032116

Dogiel, V. A. (1926a). Sur quelques infusoires nuveaux habitant l'estomac du dromedaire (*Camelus dromedarius*). *Ann. Parasitol. Hum. Comp.* 4, 241–271. doi: 10.1051/parasite/1926043241

Dogiel, V. A. (1926b). Une nouvelle espèce du genre Blepharocorys, *B. bovis* n. sp. habitant l'estomac do boeuf. *Ann. Parasitol. Hum. Comp.* 4, 61–64. doi: 10.1051/parasite/1926041061

Dogiel, V. A. (1927). Monographie der familie Ophryoscolecidae. *Arch. Protistenkd.* 59, 1–288.

Dogiel, V. A. (1928). La faune d'infusoires inhabitant l'estomac du buffle et du dromedaire. *Ann. Parasitol.* 6, 323–338. doi: 10.1051/parasite/1928063323

Dogiel, V. A. (1929). Die sog. "Konkrementenvakuole" der infusoiren al eine statocyste 11 betrachtet. *Arch. Protistenk.* 63, 319–348.

Dogiel, V. A. (1932). Bueschreibung einiger neuer vertreter der familie Ophryoscolecidae aus afrikanishen Antilopen nebst. Revision der infusioren fauna afrikanischer wiederkäuer. *Arch. Protistenkd.* 77, 92–107.

Dogiel, V. A. (1934). Angaben ber die Ophryoscolecidae des wildchafs aus kamtschatka des elches und des yaks, nebst deren zoogeographischen verwertung. *Arch. Protistenkd.* 82, 144–148.

Dogiel, V. A. (1935). Eine notiz ber die infusioren des renntiermagens. *Trans. Arct. Inst.* 24, 144–148.

Dogiel, V. A. (1947). The phylogeny of the stomach-infusorians of ruminants in the light of palaeontological and parasitological data. *Q. J. Microsc. Sci.* 88, 337–342.

Dogiel, V. A., and Fedorowa, T. (1925). Uber den Bau und die Funktion des inneren Skeletts der Ophryoscolesciden. *Zool. Anz.* 62, 97–107.

Embley, T. M., van der Giezen, M., Horner, D. S., Dyal, P. L., Bell, S., and Foster, P. G. (2003). Hydrogenosomes, mitochondria and early eukaryotic evolution. *Life* 55, 387–395. doi: 10.1080/15216540310001592834

Fantham, H. B. (1926). Some parasitic protozoa found in South Africa. *S. Afr. J. Sci.* 23, 560–570.

Feng, J. M., Jiang, C. Q., Warren, A., Tian, M., Cheng, J., Liu, G. L., et al. (2015). Phylogenomic analyses reveal subclass Scuticociliata as the sister group of subclass Hymenostomatia within class Olygohymenophorea. *Mol. Phylogenet. Evol.* 90, 104–111. doi: 10.1016/j.ympev.2015.05.007

Fiorentini, A. (1889). *Intorno ai Protisti Stomaco dei Bovini*. Pavia: Frat Fusi.

Fonseca, F. (1939). Ciliado gigante, Muniziella cunhai, gen. n., sp. n., parasite de Hydrochoerus capybara (Holotricha, Pycnothrichidae). *Mem. Inst. Butantan.* 12, 165–173.

Gao, F., Warren, A., Zhang, Q., Gong, J., Miao, M., Sun, P., et al. (2016). The all-data-based evolutionary hypothesis of ciliated protists with a revised classification of the phylum Ciliophora (Eukaryota, Alveolata). *Sci. Rep.* 6:24874. doi: 10.1038/srep24874

Gassovsky, G. (1919). On the microfauna of the intestine of the horse. *Travaux de la Soc. des Naturalistes de Pétrograd.* 48, 20–37.

Gentekaki, E., Kolisko, M., Gong, Y., and Lynn, D. (2017). Phylogenomics solves a long-standing evolutionary puzzle in the ciliate world: the subclass Peritrichia is monophyletic. *Mol. Phylogenet. Evol.* 106, 1–5. doi: 10.1016/j.ympev.2016.09.016

Grain, J. (1962). L'infraciliature d'Isotricha intestinalis Stein, cilié trichostome de la panse des ruminants. *C. R. Acad. Sci.* 254, 2221–2223.

Grain, J. (1963a). L'infraciliature d'Isotricha prostoma Stein, cilié trichostome de la panse des ruminants. *C. R. Acad. Sci.* 256, 3885–3888.

Grain, J. (1963b). Sur Dasytricha ruminantium Schuberg, cilié de la panse des ruminants. *Arch. Zool. Exp. Gen.* 102, 183–188.

Grain, J. (1964). La stomatogenèse chez les ciliés trichostomes *Isotricha intestinalis* et Paraisotricha colpoidea. *Arch. Zool. Exp. Gen.* 104, 85–93.

Grain, J. (1965). Premières observtions sur les systèmes fibrillaires chez quelques ciliés des ruminants et des equidés. *Arch. Zool. Exp. Gen.* 105, 185–190.

Grim, N., Jirku-Pomajbíková, J., and Ponce-Gordo, F. (2015). Light microscopic morphometrics, ultrastructure, and molecular phylogeny of the putative pycnotrichid ciliate, Buxtonella sulcata. *Eur. J. Protistol.* 51, 425–436. doi: 10.1016/j.ejop.2015.06.003

Gruby, D., and Delafond, O. (1843). Recherches sur des animalcules se développant en grand nombre dans l'estomac et dans l'intestin, pendant la digestion des animaux herbivores et carnivores. *C. R. Acad. Sci. Hebd. Seances Acad. Sci.* 17, 1304–1308.

Gürelli, G. (2018). Infraciliature of *Eudiplodinium maggi*, *E. dolobum*, and *E. rostratum*. *Comm. J. Biol.* 2, 16–18.

Gürelli, G. (2019). New entodiniomorphid ciliates, Buetschlia minuta n. sp., B. cirrata n. sp., Charonina elephanti n. sp., from Asian elephants of Turkey. *Zootaxa* 4545, 419–433. doi: 10.11646/zootaxa.4545.3.6

Gürelli, G., and Akman, F. T. B. (2016). Rumen ciliate biota of domestic cattle (*Bos taurus* taurus) in Stambul, Turkey and infraciliature of Metadinium medium (Entodiniomorphida, Ophryoscolecidae). *Acta Protozool.* 53, 173–182. doi: 10.5152/tpd.2012.55

Gürelli, G., and Ito, A. (2014). Intestinal ciliated protozoa of the Asian elephant Elaphas maximus Linnaeus, 1758 with the description of Triplumaria izmirae n. sp. *Eur. J. Protistol.* 50, 25–32. doi: 10.1016/j.ejop.2013.10.002

Hsiung, T.-S. (1930). A monograph on the protozoa of the large intestine of the horse. *Iowa State Coll. J. Sci.* 4, 359–423.

Hsiung, T.-S. (1935a). Notes on the known species of Triadinium with the description of a new species. *Bull. Fan Mem. Inst. Biol.* 6, 21–32.

Hsiung, T.-S. (1935b). On some new species from the mule, with the description of a new genus. *Bull. Fan Mem. Inst. Biol.* 6, 81–94.

Hsiung, T.-S. (1936). A survey of the ciliates of Chinese equines. *Bull. Fan Mem. Inst. Biol.* 6, 289–304.

Intergovernmental Panel on Climate Change [IPCC] (2019). *Refinement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories*. Geneva: IPCC.

Ito, A., Arai, N., Tsutsumi, Y., and Imai, S. (1997). Ciliate protozoa in the rumen of sassaby antelope, *Damaliscus lunatus* lunatus, including the description of a new species and form. *J. Eukaryot. Microbiol.* 44, 586–591. doi: 10.1111/j.1550-7408.1997.tb05964.x

Ito, A., Eckardt, W., Stoinski, T. S., Gillespie, T. R., and Tokiwa, T. (2017). Gorilloflasca africana n. g., n. sp., (Entodiniomorphida) from wild habituated Virunga mountain gorillas (*Gorilla beringei beringei*) in Rwanda. *Eur. J. Protistol.* 60, 68–75. doi: 10.1016/j.ejop.2017.06.002

Ito, A., Eckardt, W., Stoinski, T. S., Gillespie, T. R., and Tokiwa, T. (2018). Three new Troglodytella and a new Gorilloflasca ciliates (Entodiniomorphida) from mountain gorillas (*Gorilla beringei beringei*) in Rwanda. *Eur. J. Protistol.* 65, 42–56. doi: 10.1016/j.ejop.2018.05.002

Ito, A., Honma, H., Gürelli, G., Göçmen, B., Mishima, T., Nakai, Y., et al. (2010). Redescription of Triplumaria selenica Latteur et al., 1970 (Ciliophora, Entodiniomorphida) and its phylogenetic position based on the infraciliary bands and 18SSU rRNA gene sequence. *Eur. J. Protistol.* 46, 180–188. doi: 10.1016/j.ejop.2010.01.005

Ito, A., and Imai, S. (1998). Infraciliary bands in the rumen Ophryoscolecid ciliate *Ostracodinium gracile* (Dogiel, 1925), observed by light microscopy. *J. Eukariot. Microbiol.* 45, 628–636. doi: 10.1111/j.1550-7408.1998.tb04559.x

Ito, A., and Imai, S. (2000). Ciliates from the cecum of capybara (*Hydrochoerus hydrochaeris*) in Bolivia 1. The families Hydrochoerellidae n. fam., Protohallidae, and Pycnotrichidae. *Eur. J. Protistol.* 36, 53–84. doi: 10.1016/s0932-4739(00)80023-1

Ito, A., and Imai, S. (2003). Light microscopical observation of infraciliary bands of *Eodinium posterovesiculatum* in comparison with *Entodinium bursa* and *Diplodinium dentatum*. *J. Eukariot. Microbiol.* 50, 34–42. doi: 10.1111/j.1550-7408.2003.tb00103.x

Ito, A., and Imai, S. (2005). Infraciliature and morphogenesis in three rumen Diplodinium ciliates, Diplodinium polygonale, Diplodinium leche, and Diplodinium nanum, observed by light microscopy. *J. Eukariot. Microbiol.* 52, 44–51. doi: 10.1111/j.1550-7408.2005.3312r.x

Ito, A., and Imai, S. (2006). Infraciliary band pattern of rumen ophryoscolecid ciliates. *Endocytobiosis Cell Res.* 17, 103–110.

Ito, A., Mishima, T., Nataami, K., Ike, K., and Imai, S. (2011). Infraciliature of eight Triplumaria species (Ciliophora, Entodiniomorphida) from Asian elephants with the description of six new species. *Eur. J. Protistol.* 47, 256–273. doi: 10.1016/j.ejop.2011.06.002

Ito, A., Ishihara, M., and Imai, S. (2014). Bozasella gracilis n. sp. (Ciliophora, Entodiniomorphida) from Asian elephant and phylogenetic analysis of entodiniomorphids and vestibuliferids. *Eur. J. Protistol.* 50, 134–152. doi: 10.1016/j.ejop.2014.01.003

Ito, A., Miyazaki, Y., and Imai, S. (2001). Light microscopic observations of infraciliature and morphogenesis in six species of rumen Ostracodinium ciliates. *J. Eukariot. Microbiol.* 48, 440–448. doi: 10.1111/j.1550-7408.2001.tb00177.x

Ito, A., Miyazaki, Y., and Imai, S. (2002). Descriptions of new Parentodinium ciliates in the family Parentodiniidae n. fam. From *Hippopotamus amphibius* in comparison with some entodiniomorphs from horses and cattle. *Eur. J. Protistol.* 37, 405–426. doi: 10.1078/0932-4739-00828

Ito, A., and Tokiwa, T. (2018). Infraciliature of Opisthotrichum janus, *Epidinium ecaudatum*, and *Ophryoscolex purkynjei* (Ciliophora, Entodiniomorphida). *Eur. J. Protistol.* 62, 1–10. doi: 10.1016/j.ejop.2017.10.001

Ito, A., Van Hoven, W., Miyazaki, Y., and Imai, S. (2006). New entodiniomorphid ciliates from the intestine of the wild African white rhinoceros belong to a new family, the Gilchristidae. *Eur. J. Protistol.* 42, 297–307. doi: 10.1016/j.ejop.2006.07.006

Ito, A., Van Hoven, W., Miyazaki, Y., and Imai, S. (2008). Two new entodiniomorphid Triplumaria ciliates from the intestine of the wild African white rhinoceros. *Eur. J. Protistol.* 44, 149–158. doi: 10.1016/j.ejop.2007.11.003

Jiang, C. Q., Wang, G. Y., Xiong, J., Yang, W. T., Sun, Z. Y., Feng, J. M., et al. (2019). Insights into the origin and evolution of Peritrichia (Oligohymenophorea, Ciliophora) based on analyses of morphology and phylogenomics. *Mol. Phylogenet. Evol.* 132, 25–35. doi: 10.1016/j.ympev.2018.11.018

Jirovec, O. (1933). Beobachtungen über die fauna des rinderpansen. *Z. Parasitenkd.* 5, 584–591. doi: 10.1007/bf02121364

Kittelmann, S., Devente, S. R., Kirk, M. R., Seedorf, H., Dehority, B. A., and Janssen, P. H. (2015). Phylogeny of intestinal ciliates, including Charonina ventriculi, and comparison of microscopy and 18S rRNA gene pyrosequencing for rumen ciliate community structure analysis. *Appl. Environ. Microbiol.* 81, 2433–2444. doi: 10.1128/AEM.03697-14

Kofoid, C. A. (1935). Two remarkable ciliate protozoa from the caecum of the Indian elephant. *Proc Natl Acad Sci U.S.A.* 21, 501–506. doi: 10.1073/pnas.21.7.501

Kofoid, C. A., and Christenson, J. F. (1933). Ciliates from Bos-gaurus H. *Smith. Univ. Calif. Publ. Zool.* 39, 341–391.

Kofoid, C. A., and MacLennan, R. F. (1930). Ciliates from Bos indicus Linn. I. The genus Entodinium. *Stein. Univ. Calif. Publ. Zool.* 33, 471–544.

Kofoid, C. A., and MacLennan, R. F. (1932). Ciliates from Bos indicus Linn. II. A revision of Diplodinium Schuberg. *Univ. Calif. Publ. Zool.* 37, 53–152.

Kofoid, C. A., and MacLennan, R. F. (1933). Ciliates from Bos indicus Linn. 3. Epidinium Crawley, Epiplastron gen. nov., and Ophryoscolex Stein. *Univ. Calif. Publ. Zool.* 39, 1–34.

Kraschnninikow, S. (1929). Zur frage des lipoidenexkretionsapparats einiger infusorienarten aus der familie Ophryoscolecidae. *Z. F. Zellforsch. Mikr. Anat.* 8, 470–483. doi: 10.1007/bf00587501

Latteur, B. (1966a). *Diplodinium archon* n. sp. Ciliate Ophryoscolecidae du rumen de l'antelope *Tragelaphus scriptus* Pallas. *Ann. Soc. Belge Med. Trop.* 46, 727–740.

Latteur, B. (1966b). Epidinium dactylodonta n. sp. Ciliate Ophryoscolecidae du rumen de l'antelope *Tragelaphus scriptus* Pallas. *Bull. Inst. R. Sci. Nat. Bel.* 42, 1–27.

Latteur, B. (1967). Helicozoster indicus n. gen., n. sp., ciliate holotriche du caecum de l'elephant des indes. *Acta Zool. Pat. Ant.* 43, 93–106.

Latteur, B. (1968). Revision systematique de la famille des *Ophryoscolecidae* Stein, 1858: sous-Famille des *Entodiniinae* Lubinsky, 1957. Genre *Entodinium* Stein, 1858. *Ann. Soc. R. Zool. de Belgique* 98, 1–41.

Latteur, B. (1969). Revision systematique de la famille des *Ophryoscolecidae* Stein, 1858: sous-famille des *Epidiniinae* Lubinsky, 1957. Genre *Epidinium* Stein, 1958. *Ann. Soc. R. Zool. de Belgique* 99, 3–25.

Latteur, B. (1970). Revision systematique de la famille des *Ophryoscolecidae* Stein, 1858: sous-famille des *Diplodiniinae* Lubinsky, 1957. Genre *Diplodinium* (Schuberg, 1888). *sensu novo. Ann. Soc.R. Zool. de Belgique* 100, 275–312.

Lubinsky, G. (1957a). Studies on the evolution of the Ophryoscolecidae (Ciliata: Oligotricha) I. A new species of *Entodinium* with "caudatum", "lobosospinosum", and "dubardi" forms, and some evolutionary trends in the genus *Entodinium*. *Can. J. Zool.* 35, 111–133. doi: 10.1139/z57-007

Lubinsky, G. (1957b). Studies on the evolution of the Ophryoscolecidae (Ciliata: Oligotricha) II. On the origin of the higher Ophryoscolecidae. *Can. J. Zool.* 35, 135–140. doi: 10.1139/z57-008

Lubinsky, G. (1957c). Studies on the evolution of the Ophryoscolecidae (Ciliata: Oligotricha) III. Phylogeny of the Ophryoscolecidae based on their comparative morphology. *Can. J. Zool.* 35, 141–159. doi: 10.1139/z57-009

Lubinsky, G. (1958a). Ophryoscolecidae (Ciliata: Entodiniomorphida) of reindeer (*Rangifer tarandus* L.) from the Canadian Arctic. I. Entodiniinae. *Can. J. Zool.* 36, 819–835. doi: 10.1139/z58-068

Lubinsky, G. (1958b). Ophryoscolecidae (Ciliata: Entodiniomorphida) of reindeer (*Rangifer tarandus* L.) from the Canadian Arctic. II. Diplodiniinae. *Can. J. Zool.* 36, 937–959. doi: 10.1139/z58-080

Lynn, D. H. (2008). *The Ciliated Protozoa. Characterization, Classification, and Guide to the Literature*. Dordrecht: Springer.

MacLennan, R. F. (1933). The pulsatory cycle of the contractile vacuoles in the Ophryoscolecidae, ciliates from the stomach of cattle. *Univ. Calif. Publ. Zool.* 39, 205–249.

Malmuthuge, N., and Guan, L. L. (2017). Understand the gut microbiome of dairy calves: opportunities to improve early-life gut health. *J. Dairy Sci.* 100, 5996–6005. doi: 10.3168/jds.2016-12239

Moon-Van der Staay, S. Y., Van Der Staay, G. W. M., Michalowski, T., Jouany, J.-P., Pristas, P., Javorsky, P., et al. (2014). The symbiotic intestinal ciliates and the evolution of their hosts. *Eur. J. Protistol.* 50, 166–173. doi: 10.1016/j.ejop.2014.01.004

Moriggi, M. (1941). Protozoa ciliate parasitica. *Rev. Biol.* 10, 103–134.

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., and McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6:1313. doi: 10.3389/fmicb.2015.01313

Noirot-Timothée, C. (1956a). Les structures infraciliaires chez les Ophryoscolecidae. I. Étude du genre Epidinium Crawley. *C. R. Acad. Sci.* 242, 1076–1078.

Noirot-Timothée, C. (1956b). Les structures infraciliaires chez les Ophryoscolecidae. II. Étude du genre Ophryoscolex Stein. *Ibid.* 242, 2865–2867.

Noirot-Timothée, C. (1960). Etude d'une famille de ciliés: les Ophryoscolecidae. Structures et ultrastructures. *Ann. Sci. Nat. Zool. Biol. An.* 2, 527–718.

Park, T., Wijeratne, S., Meulia, T., Firkins, J., and Yu, Z. (2018). Draft macronuclear genome sequence of the ruminal ciliate Entodinium caudatum. *Microbiol. Resour. Announc.* 7, 1–2. doi: 10.1128/MRA.00826-18

Poche, F. (1913). Das system der protozoa. *Arch. Protistenkd.* 30, 125–321.

Pomajbíková, K., Oborník, M., Hórak, A., Petrzelková, K. L., Grim, J. N., and Levecke, B. (2013). Novel insights into the genetic diversity of Balantidium and Balantidium-like cyst-forming ciliates. *PLoS Negl. Trop. Dis.* 7:3. doi: 10.1371/journal.pntd.0002140

Pomajbíková, K., Petrzelkova, K. J., Profousova, I., and Modry, D. (2010). Discrepancies in the occurrence of Balantidium coli between wild and captive African great apes. *J. Parasitol.* 96, 1139–1144. doi: 10.1645/GE-2433.1

Rajter, L., and Vďačný, P. (2017). Constrains on phylogenetic interrelationships among four free-living Litostomatean lineages inferred from 18S rRNA gene-ITS region sequences and secondary structure of the ITS2 molecule. *Acta Protozool.* 56, 255–281.

Rossi, M., Dias, R. J. P., Senra, M. V. X., Martinele, I., Soares, C. A. G., and D'Agosto, M. (2015). Molecular phylogeny of the family Ophryoscolecidae (Ciliophora, Litostomatea) inferred from 18S rDNA sequences. *J. Eukaryot. Microbiol.* 62, 584–590. doi: 10.1111/jeu.12211

Rossi, M. F., Cedrola, F., Dias, R. J. P., Martinele, I., and D'Agosto, M. (2016). Improved silver carbonate impregnation for rumen ciliate protozoa. *Rev. Bras. Zooc.* 17, 33–40.

Schulze, P. (1924). Der nachweiss und die verbreitung des chitins mit einem anhang über das komplizierte verdauungsystem der ophryoscoleciden. *Z. Morphol. Okol. Tiere* 2, 643–666. doi: 10.1007/bf01254877

Schulze, P. (1927). Noch einmal die "skelettplaten" der ophryoscoleciden. *IBID* 7, 670–689.

Sládeček, F. (1946). Ophryoscolecidae z bachoru jelena (*Cervus elaphus* L.), dan'ka (Dama dama L.) a srnce (*Capreolus capreolus* L.). *Vestnik Ceskoslovenske Zoologicke Spolecnosti.* 10, 201–231.

Small, E. B., and Lynn, D. H. (1981). A new macrosystem for the Phylum Ciliophora Doflein, 1901. *BioSystems* 14, 387–401. doi: 10.1016/0303-2647(81)90045-9

Snelling, T., Pinloche, E., Worgan, H. J., Newbold, J., and McEwan, N. R. (2011). Molecular phylogeny of Spirodinium equi, Triadinium caudatum and Blepharocorys sp. from the equine hindgut. *Acta Protozool.* 50, 319–326.

Stein, F. (1858). Über mehrere neue im pansen der wiederkäuer lebende infusionstiere. *Abhandlungen der k. Böhmischen Gesellschaft der Wissenschaften* 10, 69–70.

Strüder-Kypke, M. C., Kornilova, O. A., and Lynn, D. H. (2007). Phylogeny of trichostome ciliates (Ciliophora, Litostomatea) endosymbiotic in the Yakut

Horse (Equus caballus). *Eur. J. Protistol.* 43, 319–328. doi: 10.1016/j.ejop.2007.06.005

Vďačný, P. (2015). Estimation of divergence times in litostomatean ciliates (Ciliophora: Intramacronucleata), using Bayesian relaxed clock and 18S rRNA gene. *Eur. J. Protistol.* 51, 321–334. doi: 10.1016/j.ejop.2015.06.008

Vďačný, P. (2018). Evolutionary associations of endosymbiotic ciliates shed light on the timing of Marsupial-Placental split. *Mol. Biol. Evol.* 35, 1757–1769. doi: 10.1093/molbev/msy071

Warren, A., Patterson, D. J., Dunthorn, M., Clamp, J. C., Achiles-Day, U. E. M., Aescht, E., et al. (2017). Beyond the "code": a guide to the description and documentation of biodiversity in ciliated protists (Alveolata, Ciliophora). *J. Eukaryot. Microbiol.* 64, 539–554. doi: 10.1111/jeu.12391

Wertheim, P. (1935). A new ciliate, Entodinium bovis n. sp., from the stomach of *Bos taurus* L. with the revision of Entodinium exiguum, E. nanellum, E. simplex, E. dubardi dubardi and E. parvum. *Parasitology.* 27, 226–230. doi: 10.1017/s0031182000015092

Williams, A. G., and Coleman, G. S. (1992). *The Rumen Protozoa*. New York, NY: Springer.

Wolska, M. (1963). Morphology of the buccal apparatus in Balantidium coli (Malmsten, 1857). *Acta Protozool.* 1, 147–152.

Wolska, M. (1964). Infraciliature of Didesmis ovalis Fior. and Blepharozoum trizonum (Hsiung) - fam. Buetschliidae (Ciliata, Rhabdophorina). *Acta Protozool.* 2, 153–157.

Wolska, M. (1965). Studies on the representatives of the family Paraistrochidae Da Cunha (Ciliata, Trichostomata). III. Division morphogenesis in the genus Paraisotricha Fior. and Rhizotricha Wolska. *Acta Protozool.* 3, 27–36.

Wolska, M. (1966a). Application of the ammonium-silver impregnation method to the investigation of ciliates from the rumen. *Acta Protozool.* 4, 105–108.

Wolska, M. (1966b). Study on the family Blepharocorythidae Hsiung I. Preliminary remarks. *Acta Protozool.* 4, 97–103.

Wolska, M. (1967a). Study on the family Blepharocorythidae Hsiung II. Charonina ventriculi (Jamenson). *Acta Protozool.* 4, 279–284.

Wolska, M. (1967b). Study on the family Blepharocorythidae Hsiung III. Raabena bella gen. nov., sp. n. from the intestine of the Indian elephant. *Acta Protozool.* 4, 284–294.

Wolska, M. (1968). Study on the family Blepharocorythidae Hsiung IV. Pararaabena dentata gen. n., sp. n. from the intestine of Indian elephant. *Acta Protozool.* 5, 219–225.

Wolska, M. (1969). Triadinium minimum Gassovsky. Its phylogenetic significance. *Rev. Soc. Mex. Hist. Nat.* 30, 65–78.

Wolska, M. (1970). Spirocorys indicus Wolska, 1969 ciliate holotriche from the intestine of Indian elephant, its systematic position. *Acta Protozool.* 8, 143–148.

Wolska, M. (1971a). Studies on the family Blepharocorythidae Hsiung. V. A review of genera and species. *Acta Protozool.* 9, 23–43.

Wolska, M. (1971b). Studies on the family Blepharocorythidae Hsiung VI. Phylogenesis of the family and description of the new genus *Circodinium* gen. n. with the species C. minimum (Gassovsky, 1918). *Acta Protozool.* 9, 170–195.

Wolska, M. (1978a). Light and electron microscope studies on Ochoterenaia appendiculata Chavarria (Ciliata, Blepharocorythina). *Acta Protozool.* 17, 483–492.

Wolska, M. (1978b). Triadinium caudatum Fiorent. Electron microscope observations. *Acta Protozool.* 17, 445–454.

Wolska, M. (1978c). Tripalmaria dogieli Gass., 1928 (Ciliata, Entodiniomorphida) structure and ultrastructure. Part I. Light-microscope investigations. *Acta Protozool.* 17, 13–20.

Wolska, M. (1978d). Tripalmaria dogieli Gasso., 1928 (Ciliata, Entodiniomorphida). Structure and ultrastructure. Part II. Electron-microscope investigations. *Acta Protozool.* 17, 21–30.

Wolska, M. (1979). Circodinium minimum (Gassovsky, 1918), electron-microscope investigations. *Acta Protozool.* 18, 223–229.

Wolska, M. (1985). A study of the genus Spirodinium Fiorentini. Ciliata, Entodiniomorphida. *Acta Protozool.* 24, 1–11.

Wolska, M. (1986). Pseudoentodinium elephantis gen. nov., sp. n. from the order Entodiniomorphida. Proposition of the new family Pseudoentodiniidae. *Acta Protozool.* 25, 139–146.

Wright, A. D. (2015). "Rumen protozoa," in *Rumen Microbiology: From Evolution to Revolution*, eds A. K. Punyia, R. Singh, and D. N. Kamra, (Cham: Springer), 113–120. doi: 10.1007/978-81-322-2401-3_8

Wright, A. D., Dehority, B. A., and Lynn, D. H. (1997). Phylogeny of the rumen ciliates Entodinium, Epidinium and Polyplastron (Litostomatea, Entodiniomorphida) inferred from small subunit ribosomal RNA sequences. *J. Eukaryot. Microbiol.* 44, 61–67. doi: 10.1111/j.1550-7408.1997.tb05693.x

Wright, A. D. J., and Lynn, D. H. (1997a). Maximum ages of ciliate lineages estimated using a small subunit rRNA molecular clock, crown eukaryotes date back to the paleoproterozoic. *Arch. Protist.* 148, 329–341. doi: 10.1016/s0003-9365(97)80013-9

Wright, A. D., and Lynn, D. H. (1997b). Monophyly of the trichostome ciliates (Phylum Ciliophora: Class Litostomatea) tested using new 18S rRNA sequences from vestibuliferids, Isotricha intestinalis and Dasytricha ruminantium and haptorian Didinium nasutum. *Eur. J. Protistol.* 33, 305–315. doi: 10.1016/s0932-4739(97)80008-9

Wright, A. D., and Lynn, D. H. (1997c). Phylogenetic analysis of the rumen ciliate family Ophryoscolecidae based on 18S ribosomal RNA sequences, with new sequences from Diplodinium, Eudiplodinium and Ophryoscolex. *Can. J. Zool.* 75, 963–970. doi: 10.1139/z97-117

Zhou, Y., Rodrigue, N., Lartillot, N., and Philippe, H. (2007). Evaluation of the models handling heterotachy in phylogenetic inference. *BMC Evol. Biol.* 7:206. doi: 10.1186/1471-2148-7-206

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REVIEW
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Extending Burk Dehority's Perspectives on the Role of Ciliate Protozoa in the Rumen

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Dr. Burk Dehority was an international expert on the classification and monoculture of ruminal ciliated protozoa. We have summarized many of the advancements in knowledge from his work but also in his scientific way of thinking about interactions of ruminal ciliates with the entire rumen microbial community and animal host. As a dedication to his legacy, an electronic library of high-resolution images and video footage catalogs numerous species and techniques involved in taxonomy, isolation, culture, and ecological assessment of ruminal ciliate species and communities. Considerable promise remains to adapt these landmark approaches to harness eukaryotic cell signaling technology with genomics and transcriptomics to assess cellular mechanisms regulating growth and responsiveness to ruminal environmental conditions. These technologies can be adapted to study how protozoa interact (both antagonism and mutualism) within the entire ruminal microbiota. Thus, advancements and limitations in approaches used are highlighted such that future research questions can be posed to study rumen protozoal contribution to ruminant nutrition and productivity.

Keywords: rumen protozoa, protozoal taxonomy, rumen protozoal monoculture, isotrichid, entodiniomorphid, rumen protozoal 18S rRNA genes

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INTRODUCTION

Rumen ciliated protozoa enhance methanogenesis (Newbold et al., 2015) and contribute to proteolysis and particularly to intra-ruminal recycling of microbial protein (Hartinger et al., 2018; Firkins and Mackie, 2020). These negative aspects are countered by benefits in limiting the post-prandial depression in ruminal pH through engulfing starch and metabolizing lactate, and some ciliates contribute significantly to fiber degradation (Newbold et al., 2015). Hence, untargeted protozoal suppression can lead to repercussions in the complex ruminal ecosystem and variable efficacy in suppression of methanogenesis (Hristov et al., 2013) and probably for suppression of intra-ruminal recycling to limit nitrogen excretion (Firkins and Mackie, 2020).

The classical ecology of ruminal ciliates was described decades ago (Williams and Coleman, 1992). The rumen ciliates remain a partial enigma today with respect to weighing their pros vs. cons, although they now are less enigmatic thanks to the nearly six decades of pioneering research by our colleague, Dr. Burk Dehority. He was an avid swimmer for decades. Hence, the 2013 symposium in his honor at the joint American Society of Animal Science/American Dairy Science Association meeting in Indianapolis, IN was aptly entitled: "Swimming in the Rumen with Burk Dehority." What made him a good "rumen swimmer" was his broad thinking. For example, when invited to talk on the role of protozoa in the rumen, he once excerpted part of that title for his main theme

that, rather than defining their role, he focused on "protozoa are in the rumen because they can be." When researchers focus excessively on defining their role from the top–down (teleological vantage), he questioned if they missed opportunities to actually better understand their role from the bottom up (biologically).

Burk Dehority helped shape our own views that ruminal ciliates are in the rumen "because they can be." For example, should we continue to study inhibiting agents without studying how the protozoal community adapts to them (and how they do it)? Indeed, *Entodinium caudatum* expressed eukaryotic stress response pathways when the inhibitors monensin and wortmannin were introduced (Wang et al., 2020). We questioned if protozoal autolysis in the rumen is less than that projected from cultures (Diaz et al., 2014). We also questioned if ruminal ciliates can be cultured without prokaryotes (Park et al., 2017; Park and Yu, 2018a). Of course, protozoal cultures have led to critical advances in our understanding of the growth rate and function of various species, but Dr. Dehority always conditioned observations made *in vitro*. For example, were increased protozoal numbers in monocultures a direct stimulation by the treatment imposed or a result of indirect inhibition of the uncharacterized prokaryotes in co-culture? Hence, there are some important similarities in prokaryotic diversity in ciliate monocultures compared with *in vivo*, but there also are major differences (Park and Yu, 2018b). Dehority (2003) contextualized the difference between protozoal counts (cells per milliliter) and protozoal pool size (total cells) in the rumen, their ruminal passage or lack thereof (sequestration), and their adaptation to host and diet based on function and taxonomy from the bottom up. Unfortunately, protozoal pool size has rarely been measured (see later discussion).

Dr. Dehority's scientific passion intersected with his artistic side; his drawings of rumen protozoa led to more sophisticated images in his books, which were the basis for the corresponding author's (JLF) rumen microbiology class (and used in other classes across different universities). For the current perspective, these images seeded the image database as a lasting legacy to our colleague. Readers are referred to the Acknowledgments section to access this image library. In addition to his artistic talents, his scholarship on the study of ruminal ciliates was also a passion few got to witness; even after retirement, stretching out maintenance procedures to avoid tending them over weekends (Dehority, 2008) was not a reflection of the joy he derived from tending his cultures daily—these revisions were for others to follow. Rather than accepting the standard explanation, he always sought to test hypotheses—typically in elegantly simple ways such as incubating protozoa in the rumen within an apparatus that prevented entry or exit of protozoa across the screen (Ankrah et al., 1990). He successfully defaunated animals but did not rely on defaunation as the main means of explaining a role based on the "because they can" philosophy; he always questioned how the faunation vs. defaunation comparison removed the positive and negative interactions of protozoa with bacteria and fungi.

Our current objective is to review his work and connect his findings with current and especially future research objectives to further our understanding of interactions among protozoa and other microbes in the ruminal pool from a microbiology-nutrition interface.

LANDMARK FINDINGS ON CILIATED PROTOZOA

Swimming in the Rumen

Dehority (1970) described *Buetschlia parva* (family *Buetschliidae*, order *Entodiniomorphida*) in his first PubMed-indexed paper on protozoa in January of 1970. The family *Ophryoscolecidae* is diverse and has the major role with respect to how ciliates influence rumen function by most ruminants fed most production diets. Ophryoscolecids primarily have cilia only near their oral region, although some have other patches (Dehority, 2003). We will maintain the term 'entodiniomorphid' to describe this group. The order *Vestibuliferida* has two main families typically identified in the rumen, with the predominant one being *Isotrichidae* (Cedrola et al., 2015). We will maintain the term 'isotrichid' to represent this main group, which is fully ciliated; however, we abandon the previous term 'holotrichs' because cilial pattern and location does not taxonomically distinguish minor members such as the *Buetschliidae*.

A few months after his first paper, Dehority and Purser (1970) described factors influencing isotrichid counts in the sheep rumen. Dr. Purser and his colleague, Dr. Moir, had important landmark papers on ruminal ciliates from the 1960s. This particular paper was the first among many of Dr. Dehority's fruitful ventures with international scientists (in this case, Australia). Purser (1961) noted: "Several outstanding biochemical differences between Oligotrichs [i.e., entodiniomorphids] and Holotrichs [i.e., isotrichids] have now been established *in vitro*. However, *in vitro* work can delineate biochemical capabilities only. It cannot predict with certainty the activity of these organisms *in vivo*." This theme guided Dr. Dehority's thinking so that even his culture-based studies were designed to reflect important *in vivo* responses.

Only a relatively few experts were truly accepted internationally to verify a new protozoal species or differentiate some closely related species based on morphological features. Therefore, numerous scientists across the globe traveled to the Dehority laboratory or invited him to theirs, and he has coauthored numerous indexed papers with multiple authors using multiple animals from multiple continents. He studied rumen protozoa in relation to feed intake, forage:concentrate ratio, feeding frequency, ruminal pH (Dehority, 2003), and even osmolality (Dehority and Males, 1974). Toward the end of his career, he helped establish protozoal adaptations to body temperature differences between Australian macropods and ruminants (Dehority and Wright, 2014). Based on this work, one of our final exchanges with Burk was when he suggested to briefly decrease the temperature to eliminate protozoa from continuous cultures. Curiously, our continuous culture conditions sometimes greatly increased the relative abundance of *Charonina* (Wenner, 2016), which is often overlooked during typical counting and therefore might contribute more to

protozoal ecology than currently known (Wenner et al., 2018). Dehority and Mattos (1978) noted that this genus had an ecology more closely resembling that of entodiniomorphids even though it was then classified with the isotrichids, perhaps envisaging its reclassification in *Entodiniomorphida* (Cedrola et al., 2015). Discussing flagellated protozoa in the rumen and distinguishing a myriad of ciliate species in numerous herbivores across many continents is beyond our current scope, his international legacy can be readily appreciated by searching his indexed publications.

Microscopic Characterization of Protozoa

Dr. Dehority was particularly adept at culturing bacteria, protozoa, and fungi using a gassing station and characterizing protozoa based on their morphology. Originating with notes shared with colleagues and progressing to a widely used laboratory manual (Dehority, 1993), he was then requested to turn his laboratory-based class and his artfully drawn illustrations into his book (Dehority, 2003). In both resources, Dr. Dehority explained how to effectively count protozoa with both high accuracy and precision.

Size is a relative distinction among species, but relatively little gain in knowledge has been made despite the long-held concept that generic or even species distinctions do not explain changes in protozoal biomass or activity (Whitelaw et al., 1984). Although not well-understood in the literature, he emphasized the concept that increasing grain inclusion in a diet increases protozoal cell density by a combination of more substrate to stimulate cell growth but also a lower ruminal fluid volume to concentrate those cells (Dehority, 2003). He reasoned that cell counts should be multiplied by ruminal volume to derive a rumen pool. The nutritional need to move from cell counts to ruminal and duodenal biomass led to our development of a qPCR technique that relies on collecting a protozoal standard (Sylvester et al., 2005) and our development of a videographic technique (Wenner et al., 2018) to adapt counts to volume. Both counts and protozoal 18S rRNA gene copies are useful (Kittelmann et al., 2015), but both also have pros and cons that will be described in a subsequent section.

Speciation of protozoa based on visual morphology is challenging. An excellent example is the case when Dehority (1994) exhaustively characterized and offered a biological explanation why different species should be collapsed into differing morphological types of the same species, *Entodinium dubardi*. Prior to the Dehority symposium, the corresponding author (JLF) asked him what prompted this paper, and his reply was "sheer frustration." This response was from a scientist who described and named 21 new species and had at least two species named after him. This textbook case documents why some morphological characteristics are stronger indicators of taxonomic distinction of species than others. *Entodinium caudatum* has three main forms of spination (from none to very long), assuming spination is a defense against predation by larger protozoa and is not needed in monocultures (Dehority, 2003). Similar changes in morphology were demonstrated with extended culture of *Ophryoscolex* (Miltko et al., 2006).

Visual Library

Based on Dr. Dehority's tutelage, many researchers classified protozoa into the genera *Isotricha*, *Dasytricha*, *Entodinium*, and the subfamilies *Diplodiniinae* and *Ophryoscolecinae* (with further classification of the genera *Ophryoscolex* and *Epidinium*). However, as Burk's health declined, we prioritized our goal of creating a photographic library of a broad range of protozoal species to leave a lasting framework for future researchers (see Acknowledgments for directions to access the image library). Burk agreed to serve as a collaborator in the fall of 2014, when one of the coauthors (JEP) began to collect rumen fluid samples to supplement existing images of confirmed species. *Dasytricha ruminantium* images and *Isotricha intestinalis* videos were added. As discussed previously, Burk distinguished *Charonina ventriculi*, and images are now better represented in the library to help future researchers distinguish this atypical protozoan's unusual features (Wenner et al., 2018).

Collecting a sample and preparing the specimen for proper resolution requires attention to detail, patience, and experience—especially for some genera or species that are very small (such as *C. ventriculi*) or for which morphology types are very similar (and might require a high-resolution microscope). Iodine staining highlights skeletal plates, a morphological property differentiating species in the *Ophryoscolecinae* subfamily. To avoid the obfuscation from staining both skeletal plates and intracellular starch or glycogen, Burk recommended sampling from donor cows fed an all forage or high fiber diet or withholding feed for 24 h prior to collection. Due to ongoing research projects in our dairy herd (presumably a limitation for numerous researchers), these suggestions could not be followed. Collection before feeding might seem a disadvantage for taxonomic approaches based on morphological characteristics compared with molecular approaches (discussed subsequently), but we also note that large particles also typically are screened even when DNA is extracted from rumen contents containing particulates. Our best option was to sample immediately prior to the morning feeding (cows were fed twice daily but eating at *ad libitum*). Flocculation and aspiration of the top layer reduced the concentration of particulates that also stained with iodine, but we also noted that large protozoa were sometimes inadvertently removed from the sample. During this process, we captured images that were confirmed by Dr. Dehority to be *Ostracodinium gracile* and *O. obtusum*.

Aware of our challenges to find rare images, Burk generously offered to donate additional materials. Electronic presentations entitled 'Classification and Morphology,' 'Counting Methods,' 'Species Identification,' and 'Difficulties with *Entodinium*' are insightful and included in the image library. Burk was helping us replace a lost monoculture of *Epidinium caudatum* that had failed to revive after extended cryopreservation (in which he also was experienced through collaborations with others). Two of the coauthors (JEP and TP) collected fluid from a rumen-cannulated cow in Columbus, Ohio that was previously confirmed to have moderate abundance of *Ep. caudatum*. Unfortunately, due to the then low concentrations of *Epidinium*, no monocultures were confirmed. Despite declining health, Burk found a more promising donor in Wooster, OH, United States. He isolated and

confirmed a clone culture for our subsequent usage. As was his decades-old practice, he invited two coauthors (JEP and TP) to Wooster to isolate a single cell for clone culture, leading to one of the videos in the visual library.

On January 15, 2016, one of the coauthors (JEP) emailed Burk a short video clip and a few still photographs of a specimen previously undocumented by our laboratory. On the 18th, our last exchange, Burk's return email expressed the enthusiasm that marked his scientific career; although he had cataloged this rare species, it was the first image in our collection confirmed as *Buetschlia parva*. Our final efforts also identified images as *Entodinium rostratum, Ostracodinium trivesiculatum, Epidinium quadricaudatum,* and *Ep. parvicaudatum*. The authors thank Dr. Svetlana Kisidayova (Slovak Academy of Sciences, Košice, Slovakia) and Raul Franzolin (University of São Paulo, Brazil), who graciously identified more protozoa in the unlabeled photographs of our collection that had not yet been classified because of his swift decline and passing on February 9, 2016.

Outline for Mentoring the Technique for Protozoal Counting

Dr. Dehority's technique is captured in a PowerPoint presentation in the image library (see Acknowledgments for directions to access the link), with some of our own examples from his mentoring highlighted below.

(1) Pass ruminal samples only through one or two layers of cheesecloth to remove large particles of feed before staining (for good viewing). Larger protozoa can be entrapped and under-represented using multiple (i.e., more than 2) layers (Dehority, 1984). He demonstrated and patiently evaluated sampling and subsampling approaches, and use of a glass open-bore pipette was especially critiqued by Dr. Dehority. We note that, aspiration of flocculated particles can help remove contamination, as described above, but this process could differentially remove some of the larger protozoal species.

(2) Researchers should first learn to differentially count *Isotricha*, *Dasytricha*, *Epidinium*, *Ophryoscolex*, *Entodinium*, and subfamily *Diplodiniinae*. More details and practice are needed to distinguish the subfamilies *Entodiniinae* (which includes only *Entodinium*), *Diplodiniinae*, and *Ophryoscolecinae* (Dehority, 2003). For greater depth, readers are referred to Dr. Dehority's PowerPoint on distinguishing *Entodinium* in the image library.

(3) Either unstained or stained samples, as described in his manual (Dehority, 1993), researchers should focus up and down the cilial patterns to distinguish *Isotricha* (longitudinal patterning) from *Dasytricha* (cilia spiraling around the cell); *I. prostoma* is distinguished from *I. intestinalis* by the vestibule. If using iodine, visualize as soon as possible, and its concentration might need to vary based on the sample (especially if varying in starch).

(4) Genera can be further distinguished based on cilial zone patterns, the number of contractile vacuoles, the number and shape of skeletal plates, and the shape and location of the macronucleus. However, sometimes smaller *Diplodiniinae* can be distinguished using methylene blue or iodine staining only at a higher magnification on separate slides with cover slips; their percentage can subsequently be multiplied by a normalizing count from a matched subsample.

(5) Caudal spine location has some value in taxonomy, but spines should not be used in length measurement, and spination can vary with culture conditions of some entodinia (see above discussion).

Clone Cultures of Entodiniomorphids

Monocultures made important contributions to ruminal protozoology since the 1950s, but Dr. Dehority established numerous conditions and improvements that standardized approaches used to define the roles of substrate source, how the substrates were processed, and deviations of temperature (Fondevila and Dehority, 2001a; Dehority, 2010); he also verified inhibition by decreasing pH (Dehority, 2005). Protozoal cultures typically are fed daily but can be maintained over weekends without feeding (Dehority, 2008). In contrast, the generation time (the typical term Burk used to represent protozoal doubling time) of several species studied, including large and slower growing strains, can be shortened from $>$ 2 days to approximately 12 h by shortening transfer interval (Dehority, 1998, 2004). This relationship appears to hold over different protozoal species such that the larger ciliates occupy a similar mass in the culture tube even if proportionately lower in numbers (Sylvester et al., 2009; Dehority, 2010).

The competition between protozoa and bacteria for substrate has led to standardized methods (Dehority, 2008) that help to maintain higher counts in monocultures. However, protozoa can grow rapidly on a mass basis when one considers that numerous generations of co-cultured bacteria would be needed to regenerate the same amount of biomass in a single dividing protozoan. Even the numbers of bacteria derived from most-probable number, which typically underestimates true abundance of bacteria (Firkins and Yu, 2006), were $> 10^3$ the counts of protozoa in the 'protozoal' monocultures (Dehority, 2008). Although not expressly measured, Burk commented that prokaryotes make a significant contribution to the total microbial biomass. Despite the apparent competition by prokaryotes and protozoa for the same substrate, protozoal cultures grew best with live rather than dead (autoclaved) bacteria (Fondevila and Dehority, 2001a), which might be a result of continuous recruitment of endosymbionts, as discussed later.

One of the authors (JLF) questioned Dr. Dehority if protozoa overshooting growth in culture can lead to subsequent lysis if substrate or growth factors are subsequently depleted. This discussion shifted our thinking that slowing growth rate "because they can" might maintain a competitive advantage against bacteria if feed intake and ruminal passage rate are low (Firkins et al., 2007). Based on principles derived from the non-rumen ciliate, *Paramecium* (Berger, 2001), we reasoned that an upshift in protozoal growth rate associated with increasing nutrient supply can be countered by a downshift in growth

rate with decreasing nutrient supply as mediated by an arrested eukaryotic cell cycle.

Protozoal cultures adapt to monensin after a few transfers (Sylvester et al., 2009). After abrupt introduction to monensin, protozoal growth was apparently stunted (as assessed by protozoal 18S rDNA copies per unit of total nucleic acids) similarly to the effects of not feeding. Even after longer adaptation, monensin increased the generation time of mixed protozoa by 5 to 6 h in continuous culture (Ye et al., 2018). Yet, protozoal abundance was not diminished when monensin was fed to dairy cattle (Oelker et al., 2009; Reveneau et al., 2012), supporting our supposition that generation time being shorter than retention time is not the only factor that limits the abundance of protozoa in the rumen. Decades prior, Potter and Dehority (1973) reasoned that decreasing feed intake decreases the passage rate of ruminal fluid and diminishes its role in affecting protozoal generation time. At that time, attachment to feed particles and therefore passage with the particulate phase was not yet emphasized.

Although entodiniomorphid adhesion to fiber particles was assumed based on observed attachment by *Epidinium* (Bauchop and Clarke, 1976), the attachment is passive and weak except when associated with feeding (Jouany and Ushida, 1999). Our experience has been that *Epidinium* counts vary considerably among cows (Sylvester et al., 2005) even though this genus was curiously resistant to toxicity by coconut oil (Reveneau et al., 2012). The intimate association with plant matter was not exhibited in all strains of *Ep. caudatum* monocultures (Dehority, 2010), and the mechanical disruption of plant material that can be visualized microscopically (and as described as 'shredding' in conversations with Burk) for these strains of *Epidinium* should not be extrapolated to all of the entodiniomorphids, anyway (Jouany and Ushida, 1999). Isotricha has a specialized ability to attach to feed particles and the ventral reticulorumen wall (Jouany and Ushida, 1999). However, based on their ecology (below), attachment would seem more important for sequestration in the ventral rumen than for gaining access to feed particles.

ECOLOGICAL DIFFERENCES BETWEEN ENTODINIOMORPHIDS AND ISOTRICHIDS

Isotrichid Ecology

That the isotrichids were fully ciliated and had a different ecology than the entodiniomorphids has been long known (Purser, 1961). They typically establish after the entodiniomorphids in young ruminants (Yáñez-Ruiz et al., 2015) even after inoculation from a fully faunated donor (Cersosimo et al., 2019). Isotrichids become opaque after rapidly converting sugars and very small starch granules into glycogen, and their increased density causes them to sink to the ventral reticulorumen (Dehority, 2003). Diaz et al. (2014) argued that sinking would be facilitated by intentional swimming behavior to pass ventrally through the fibrous rumen mat and even by contorting their cells to move around particulates. Concentrating ventrally below the reticulo-omasal orifice allows isotrichids to evade passage and explains their lower numbers recovered from samples collected near a ruminal cannula (i.e., they migrated to the ventral rumen and reticulum) within a few hours post-feeding. The more feedings per day, the more cyclical patterns in isotrichid counts as measured from these dorsal sampling locations (Abe et al., 1981). However, a pivotal study (Dehority and Tirabasso, 1989) started with a simple but novel question: what would happen to isotrichid counts if the animal was not fed at its regular interval? The isotrichid counts in the ruminal samples (near the cannula) peaked right on schedule coinciding with their 'trained' feeding pattern even when the animals were not fed. Migratory behavior to swim dorsally seemingly would have to precede chemotaxis toward an increasing gradient of nutrients in the dorsal area. Dr. Dehority reasoned that chemotaxis would be reduced when isotrichids were repleted with glycogen; however, with glycogen-depletion, chemotaxis would be enhanced by some signaling mechanism coinciding with dorsal migration.

Dr. Dehority was never able to maintain long-term cultures of the isotrichids (genera *Isotricha* and *Dasytricha*), although he cited the importance of controlling excess glycogenesis as one of the factors for culturing *Dasytricha* (Dehority, 2003). Diaz et al. (2014) hypothesized that increased glycogen storage capacity decreased isotrichid chemotaxis. Although this supposition remains unconfirmed, improved glycogen quantification methods (Hall, 2019) could be integrated with cell signaling measurements (later section) to better understand cellular mechanisms underpinning their ecology.

Although generation times have not been well-studied in isotrichids compared with entodiniomorphids, hydrogenosomal function and aerotolerance have been better described for isotrichids (Williams and Coleman, 1992). Oxygen enters the rumen via feed and water (i.e., near the rumen mat) and by diffusion from the blood across the rumen wall (where they sequester); consequently, electron transport-linked ATP generation from ingested glucose and stored glycogen would be enhanced if they can use O_2 as a terminal electron acceptor (Williams and Coleman, 1992). Although hydrogenosomes have been linked with O_2 consumption (Newbold et al., 2015), to our knowledge, the actual mechanism of O_2 usage has not been clarified since it was summarized by Williams and Coleman (1992). Further study is needed to distinguish hydrogenosomal synthesis of ATP via succinyl CoA, which could not be verified using biochemical techniques (Williams and Coleman, 1992) but was predicted using metatranscriptomics (Qi et al., 2011). Extra ATP yield could offset extra ATP usage for glycogenesis of ingested sugars and potential glycogen cycling (Teixeira et al., 2017). Williams and Coleman (1992) noted glycogen cycling in isotrichids but also suggested that isotrichids collect near the reticulorumen wall to metabolize glycogen with greater efficiency of ATP production through O_2 respiration. This migration away from sugars leaching from freshly ingested feed also could improve energetic efficiency by decreasing glycogen cycling.

After discussion with Dr. Dehority, we questioned if the high expected protozoal autolysis (Dijkstra and Tamminga, 1995) is inflated as a result of necessarily relying on measurements that were derived *in vitro*. In this case, Prins and Van Hoven (1977) dosed a relatively large amount of substrate to a previously starved *Isotricha* monoculture at the same time as antibiotic was introduced, thus likely abruptly inhibiting lactilytic bacteria in co-culture. Accumulation of lactate (pH sometimes < 5.0) in that study would not reflect normal conditions in monoculture, let alone *in vivo*. The diurnal rhythm of migration and subsequent sedimentation might be heavily entrenched in isotrichid ecology. Hence, Diaz et al. (2014) postulated that extensive autolysis by isotrichids in culture tubes could result from their inability to swim away from lytic conditions as would occur when they migrate to and sequester in the ventral rumen.

Acquisition of Substrate

Cilial pattern and coordination in beating pattern are intricately linked to the ecology of isotrichids. They rapidly consume glucose, fructose, and sucrose and rapidly store glycogen; however, they do not appear to be capable of using lactose (Dehority, 2003). They also can produce lactate (Williams and Coleman, 1992), as noted above. Those latter authors documented swimming behavior and chemotaxis to soluble nutrients. However, Diaz et al. (2014) explained how their swimming behavior, including rotation like a screw and mechanical contortion, allows them to move around obstacles as they migrate between the rumen wall and mat. Isotrichids are highly chemotactic toward glucose and xylose (Diaz et al., 2014) and almost certainly other sugars. Peptides can be both chemoattractive and chemorepellent to isotrichids (Diaz et al., 2014; Roman-Garcia et al., 2019). Hence, uncontrolled glycogenesis *in vitro* (Jouany and Ushida, 1999; Hall, 2011) probably is lessened *in vivo* because of much less abrupt increase in sugar availability. Williams and Coleman (1992) also refuted excess sugar uptake and glycogenesis as a mechanism for isotrichid autolysis in favor of toxicity resulting from a buildup of acidic endproducts *in vitro*.

In contrast to the isotrichids, the entodiniomorphids ingest fibrous particles and larger starch granules and appear to be important lactate consumers, not producers (Williams and Coleman, 1992; Jouany and Ushida, 1999). Ingestion of particles through the vestibulum using specialized cilia has been described (Jouany and Ushida, 1999). The adoral cilial zone of entodiniomorphids probably helps both with ingestion and locomotion (Dehority, 2003). Although less responsive than isotrichids, the entodiniomorphids exhibited chemotaxis toward glucose, xylose, and peptides (Diaz et al., 2014). Those authors presented a model in which entodiniomorphids maintain moderate but constant chemotaxis toward soluble nutrients leaching from freshly ingested or rapidly degrading plant particles. Consistent chemotaxis should maintain the entodiniomorphids swimming freely in fluid but without long-term attachment while passing with the particulate phase (see earlier discussion). Association with particles should lessen rate of protozoal outflow that would otherwise occur with the faster passage of fluid (Orpin, 1985). Also, entodiniomorphids probably are more bacterivorous and proteolytic than isotrichids (Newbold et al., 2015; Firkins and Mackie, 2020), and the majority of bacteria are in the particulate phase (Sok et al., 2017).

Cell Signaling

Like other eukaryotic organisms, ruminal ciliated protozoa presumably can respond to external and internal factors in a regulated manner to control their cell cycle, and signal transduction is essential to these responses (Firkins et al., 2007). Although well-described for the environmental ciliate models *Paramecium tetraurelia*, *Paramecium multimicronucleatum*, and *Tetrahymena thermophila* (Plattner, 2017), signal transduction in rumen protozoa was first indirectly demonstrated using wortmannin [an inhibitor of phosphoinositide 3-kinase (PI3K)], insulin (a growth factor), genistein (inhibitors of receptor tyrosine kinase), and U73122 (an inhibitor of phospholipase C) using a chemotaxis assay and quantifying engulfment of fluorescent beads that mimic bacteria in size and surface charge (Diaz et al., 2014). Trafficking of vesicles typically involves second messengers such as Ca^{++} and specific phosphoinositides.

Understanding of cellular signaling by ruminal ciliates was expanded using commonly available eukaryotic inhibitors and activators. The PI3K inhibitor, wortmannin, depressed chemotaxis in isotrichids but increased chemotaxis to glucose in entodiniomorphids (Diaz et al., 2014). Protein kinase G activation by cyclic GMP (based on the nitric oxide stimulator, sodium nitroprusside) was projected to activate chemotactic directional turning for entodiniomorphids but not for isotrichids. Roman-Garcia et al. (2019) suggested that increased concentration of NO_3^{2-} disrupted protein kinase G-stimulated chemotaxis by entodiniomorphids to peptides but not to glucose. Cellular receptors and the associated signaling mechanisms need much more research attention in ruminal ciliates.

In the transcriptome of *En. caudatum*, at least 25 different putative signal transduction pathways were recorded (Wang et al., 2020). Those transcripts relatively highly expressed included well-described eukaryotic pathways MAPK, Ras, calcium, cGMP-PKG, cAMP, FoxO, phosphatidylinositol, sphingolipid, TOR, PI3K-Akt, AMPK, Wnt, and Apelin. The expression of these pathways reflects the ability of *En. caudatum* to regulate its transcription, translation, ribosome biogenesis, cell growth, proliferation and differentiation, cytoskeletal organization and dynamism, chemotaxis, metabolism, secretion, calcium homeostasis, cell fate, gene transcription, apoptosis, cell-cycle control, oxidative stress resistance, etc. The functionality of these signal transduction pathways may also play an important role in their fitness and overall contribution to rumen function. Key signaling differences between isotrichids and entodiniomorphids need further verification using more specific techniques such as specific antibodies, which should become more feasible with whole genome sequencing (Park et al., 2018).

QUANTIFYING PROTOZOAL CONTRIBUTIONS TO SUPPLY OF PROTEIN

Ruminal Pool Size and Passage Rate: From Culture to Cow

Turnover of the ruminal protozoal pool is the net of growth relative to passage and recycling. Growth involves primarily cytokinesis associated with mitosis, although conjugation (meiosis) is sporadically observed in ruminal ciliates (Williams and Coleman, 1992). Little progress has been made on the molecular events regulating growth of ruminal ciliates, although complexity can be expected based on extrapolation from non-rumen ciliate models (Wang et al., 2017). Clearly, the relatively rich source of nutrients and relatively fast passage rate from the rumen would be unique for ruminal ciliates compared with such conditions influencing environmental ciliates. These conditions also support horizontal transfer of prokaryotic genes into protozoa (Newbold et al., 2015).

Dr. Dehority emphasized that generation time (net of cell growth and lysis) was highly related to transfer interval (Dehority, 1998, 2004, 2008). At each transfer, an abrupt halving of cell numbers would coincide with fresh substrate, and growth would respond quickly and as coordinated with decreasing transfer interval until a minimum generation time could be derived. With faster growth, we would expect a larger percentage of dividing forms and more nucleic acid and N per cell; even so, the observed percentage of dividing forms was not always clearly related to transfer interval (Sylvester et al., 2009). The ciliate's cellular signaling mechanisms should be coordinated with its cell cycle (Firkins et al., 2007; Diaz et al., 2014), which is supported by transcriptomics profiling in *En. caudatum* (Wang et al., 2020). These same eukaryotic cell cycle controls should both increase growth and decrease growth rate, depending on stage of incubation. At 30 h after the previous feeding (i.e., 6 h past the scheduled 24-h feeding that was interrupted), protozoal cultures already started losing cell numbers (Sylvester et al., 2009); abrupt changes in feeding pattern presumably precluded a downshift in cell cycle control that was not quick enough to avoid autolysis.

Faster passage rate in the rumen is typically associated with increasing feed intake, which stimulates protozoa to replenish their numbers but also coincides with increased substrate supply. Potter and Dehority (1973) noted that ruminal turnover rate (which is positively correlated with feed intake) was suggested to be the dominant factor associated with protozoal counts when feed intake was high. Czerkawski (1987) suggested that the mean residence time for protozoa would be approximated by the retention time of the particulate phase in the rumen. Measurement of omasal outflow of cells avoids the destruction by the acidic abomasum and, combined with ruminal pool size of cells, allows quantification of generation time of total and individual taxa (Karnati et al., 2007). Those authors noted that protozoal generation time approximated the ruminal retention time of Yb-labeled forage. A model parameterized based on cell counts emphasized loss of cells in the omasum (Hook et al., 2017). However, cell counts in the omasum should be expected to be lower than in the rumen (Czerkawski, 1987) because omasal counts can be diluted by bypass of drinking water that does not mix with the rumen contents and by destruction of omasal cells from abomasal backwash resulting from relaxation of sphincters following euthanization (Firkins and Yu, 2006). Those authors explained that ruminal outflow (rumen volume multiplied by fluid dilution rate) typically overestimates cellular outflow and should be avoided.

Passage of ruminal ciliates needs context to connect prior expectations with more current thinking. First, we argue that, unlike many of those publications and based on numerous discussions with Dr. Dehority, the predominant entodiniomorphids do not sequester in the rumen as do the isotrichids. Some of the expectation for sequestration by entodiniomorphids is based on interpretation of 'attachment.' In contrast with intimate and long-term attachment by other important ruminal microbes such as by cellulolytic bacteria, Dehority (2010) defined attachment as being "closely associated with the insoluble particulate matter." Second, protozoa traditionally have been expected to be about 50% of the microbial biomass in the rumen and even as high as 70% (Jouany, 1996). This high a pool size is inconsistent with the autolyzing argument—how can the protozoal pool size be so high if they have extensive autolysis? In contrast, if the pool size is so high, then autolysis would have to be high to explain the much slower ruminal outflow of protozoa compared with bacteria if the predominant entodiniomorphids do not sequester. We queried this apparent quandary during useful discussions with Burk.

The ruminal pool size of protozoa has rarely been measured at the same time and using the same technique as that used to measure their ruminal outflow (Firkins et al., 2007). Those authors explained why exogenous ^{14}C-choline almost certainly overestimated protozoal ruminal pool size yet typically was not used to quantify ruminal outflow of protozoal N. Because of the difficulty in collecting a purified sample of choline, specific activity was instead related to radioactivity of ^{14}C per unit of N in samples that were theoretically pure protozoa, whereas those samples were likely significantly contaminated with bacterial N (bacterial cells have virtually no choline). In addition, removal of protozoa for short-term culture needed to enrich them with ^{13}C-choline followed by washing likely would introduce stress responses that would increase their generation time. This perturbation likely disturbed the assumption that ^{14}C-choline in protozoa turns over the same as unlabeled choline. That is, the ^{14}C-choline specific activity would not be diluted as rapidly as it would have without perturbation. These types of studies should be repeated using better procedures such as a quantitative PCR assay (Sylvester et al., 2005). Those authors emphasized the need to assess recovery of 18S rDNA copies just like one would use an internal standard for any chemical assays, whereas few users of this approach seem to be evaluating recovery.

Very early expectation for such high contribution of protozoal biomass (at least 50% of the microbial biomass) were at least in part derived from calculations based on protozoal volume and converted to biomass. However, these prior protozoal volumes derived using the geometric formula for cylinders were probably overestimated by 25 to 40% (Wenner et al., 2018). Based on these

arguments, rather than 50%, perhaps a more accurate expectation should be 25% of the microbial biomass being derived from protozoa in the rumen compared with 15% in the ruminal outflow for lactating dairy cattle (Ahvenjärvi et al., 2018). Similar contributions to outflow in dairy cattle (about 17%) were derived using various approaches methods (Sok et al., 2017; Fessenden et al., 2019) that were not based on faulty microbial marker systems (Firkins et al., 1998). More studies are needed to assess factors influencing rumen protozoal N pool size and outflow with animals under production situations.

Protozoal Contribution to the Host's Nutrition

The ruminal outflow of protozoa deserves future study because protozoa influence the amino acid profile (especially lysine) of microbial protein (Sok et al., 2017). Moreover, high intake and fast ruminal passage rate presumably increase the efficiency of protozoal protein synthesis just as is expected for bacteria (Firkins et al., 2007). With respect to intra-ruminal N recycling, there is a high flux of rapidly turning over N that equilibrates with ruminal ammonia-N and likely has minimal effect on the efficiency of microbial protein synthesis compared with lysis of microbial protein (Oldick et al., 2000). Lysozyme and protease inhibitors (Park et al., 2019) offer potential to better ascertain mechanism and quantitative estimates of protozoa-mediated degradation of dietary protein and recycling of bacterial protein. Greater intake and faster ruminal passage might lessen intra-ruminal recycling caused by protozoa compared with source data mostly from low producing animals (Firkins et al., 2007). Therefore, protozoal autolysis and predation-derived bacterial lysis need better quantitative estimates to help improve feed efficiency and lessen environmental impact of ruminant enterprises under normal feeding conditions (Firkins and Mackie, 2020).

INTERACTIONS OF PROTOZOA WITH OTHER COMMUNITY MEMBERS

Interactions With Bacteria

Predation based on qualitative (i.e., which bacterial types are preferred prey) and quantitative (i.e., loss of dosed bacterial cells) bases has been summarized by Williams and Coleman (1992) mostly from their own classical studies. When numbers of dosed planktonic bacteria consumed by monocultures of protozoa are extrapolated to *in vivo*, protozoa can theoretically clear the entire ruminal pool of bacteria in just a few hours (Hristov and Jouany, 2005). Those authors also noted that bacterial consumed by protozoa that were collected *in vivo* is less than corresponding bacterial consumption from long-term monocultures. In addition, feeding particulate matter will fill protozoal internal space and limit their bacterial engulfment compared with monocultures that were starved before dosing bacteria without feeding. Those authors concluded that bacterial cell walls are more slowly degraded, so protozoa degraded Gram-positive bacteria more completely compared with Gram-negative bacteria. Hence, significant amounts of partially digested fragments are released into the ruminal fluid and contribute to intra-ruminal N recycling. Firkins and Mackie (2020) emphasized the need for more studies like that performed by Belanche et al. (2012) to assess the importance of protozoal predation under situations that better represent high producing animals.

The findings and limitations of defaunation studies were extensively detailed by Newbold et al. (2015). More evaluation of defaunation perturbations such as length of adaptation time and potential shifts in prokaryotic composition are needed in short-term studies such as that carried out by Morgavi et al. (2012). Relatively few studies have evaluated the effect of defaunation using animals fed at relatively high feed intakes (Firkins et al., 2007). Moreover, some studies must be qualified based on the choice of markers used. For example, those authors explained why diaminopimelic acid (DAP) can be a biased bacterial marker, typically inflating ruminal outflow of bacterial N; because DAP is converted to lysine by protozoa (Martin et al., 1996), this inflation is probably worse in defaunated animals. Discerning readers should discount some reports from which bacterial N flows derived using DAP approached the magnitude of their respective non-ammonia N flows (inferring unreasonably low rumen-undegraded protein).

Quantitative measures often require collection of a respective standard representing the bacterial and protozoal communities. Washing without filtering does a poor job of removing contaminating bacteria (Sylvester et al., 2005). Typically, such cells could be fixed with a low concentration of formaldehyde. However, formaldehyde could disrupt amino acid (especially lysine) profile (Sok et al., 2017). Although multiple washes reduce bacterial contamination, the greater stress likely promotes autophagy and even autolysis at rates greater than would be occurring in undisturbed cells in the rumen (Firkins et al., 2007), so harvesting technique is critical. Less stress from better short-term fractionation (Teixeira et al., 2017) or culturing with a much more limited contribution of bacteria is needed to understand the role of protozoa with less conditional qualification of results.

When considering fresh ruminal isolates or longer term monocultures of protozoa, the co-cultured bacteria in protozoal monocultures do not necessarily have a community structure that would be typical of the rumen (Park and Yu, 2018b). These prokaryotes probably have been naturally selected over time for an ability to survive predation while also withstanding typical feeding intervals of 24 h. Protozoal formation (the main storage form of reserve carbohydrate) is much more extensive than bacterial reserve carbohydrate (Teixeira et al., 2017). Although particularly extensive for isotrichids consuming sugars (earlier discussion), entodiniomorphids also rapidly convert ingested starch to glycogen (Bełżecki et al., 2017). Glycogen accumulation in *En. caudatum* was associated with inhibition by antibiotics (Park et al., 2017). Glycogenesis was probably an indicator, not a cause. For these reasons, in addition to prior discussion on the benefit of protozoal glycogen formation to limit low pH troughs, future research on glycogen regulation is recommended.

Protozoal communities fall into distinctive categories probably because of antagonism among species (Kittelmann et al., 2016). Protozoal community structure is important

also for bacterial community composition because of the potential for selective predation of bacteria by different protozoal genera (Park and Yu, 2018b). Even so, researchers need to consider the difference between direct predation (i.e., assuming bacteria are the target) and indirect 'grazing' (i.e., bacteria that adhere to engulfed particulate matter). None of these concepts has been well-studied in the rumen other than by *post hoc* association. For example, bacteria were visualized to collect after advancing stage of fibrous particle degradation (Bohatier et al., 1990). Hence, protozoal predation and contribution to intra-ruminal N recycling deserve further attention but with a more holistic perspective (Firkins and Mackie, 2020). Researchers need to prevent oversimplification of results because of the complexity of microbial community webs. We still have poor representation of ruminal bacteria, particularly some clades of the Bacteroidetes, so functionality of these phylogenetic associations needs further attention (Firkins and Yu, 2015).

Interactions With Fungi and Archaea

Like bacteria, fungi compete with protozoa for substrate but also need to avoid predation of their zoospores (Edwards et al., 2017). Those authors described the high activity of rumen fungi against recalcitrant fiber. Thus, one might expect defaunation (removal of protozoa) to increase fiber digestibility if fungi expand into the void resulting from defaunation. In contrast, defaunation was also associated with a decrease in anaerobic fungal counts and the 16S rDNA copies of some important cellulolytic bacteria, suggesting an overall benefit to the entire fibrolytic consortium for improved NDF digestibility associated with presence of protozoa (Newbold et al., 2015). Dehority and Tirabasso (2000) determined that the competition for substrate by bacteria might be enhanced by bacterial antibiosis (presumably a heat- and protease-stable agent) against fungi. Dr. Dehority was involved in research explaining how bacteriocins target other bacteria (Chan and Dehority, 1999), which could also be affected by the protozoal community if they have a major role shaping the bacterial community. Bacteriocin research leaves unanswered questions for animal production (Firkins, 2010).

Because of interspecies H_2 transfer from protozoa, fungi, and some bacteria to hydrogenotrophic archaea, further research is needed to explain why protozoal inhibition was not recommended as a CH_4 mitigation strategy (Hristov et al., 2013). Many of the methanogens are extracellular and therefore are not specific to H_2 source. Moreover, not all ruminal protozoa have hydrogenosomes (Hackstein and Tielens, 2010). For example, *En. caudatum* lacks a hydrogenosome (Park et al., 2017) and does not consistently shift fermentation toward butyrate as shown with mixing monofaunated cultures (Zeitz et al., 2013). Defaunation *in vivo* is typically associated with a decrease in molar ratio of butyrate (Newbold et al., 2015). *En. caudatum* still expresses hydrogenases (Wang et al., 2020), whereas hydrogenosome-linked hydrogenases in other protozoa might be more constitutively linked with fermentation pathways because some fermentative enzymes (especially if sensitive to O_2) also might be hydrogenosomal, as documented for *Dasytricha* and *Isotricha* (Williams and Coleman, 1992). Although counts of mixed protozoa were positively associated with methanogenesis (Guyader et al., 2014), a decrease in protozoal counts also was associated with a decrease in NDF digestibility and in dry matter intake. Decreased NDF digestibility *per se* should decrease the relative fermentation through acetate or butyrate and thereby also depress methanogenesis. Depressed dry matter intake would require more days on feed for growing ruminants and more animals to maintain milk production, thus eroding the benefit of protozoal suppression strategies from a systems perspective. Variation among ciliates and their associated methanogens, makeup of the carbohydrate, and potential non-additive responses associated with defaunation are complicating factors limiting our understanding of the role of protozoa in methane production (Hristov et al., 2013; Zeitz et al., 2013). Newbold et al. (2015) discussed the differing relationships between protozoal species and methanogens; among those apparent differences, compared with entodiniomorphids, the isotrichids probably support more methanogenesis because of greater O_2 consumption. Both dissolved concentrations of O_2 and H_2 (entry with feed or production, respectively, minus their consumption) influence redox potential, which is associated with and likely influences microbial community and function (Huang et al., 2018).

MOLECULAR APPROACHES TO STUDY PROTOZOAL ECOLOGY

Protozoa-Specific PCR-Based Analysis

The diversity and abundance of rumen ciliates have been examined using morphology- or cultivation-independent molecular approaches, primarily DNA-based and 18S rRNA-targeting methods employing PCR amplification. The early molecular approaches include cloning and sequencing of 18S rRNA gene amplicons (Karnati et al., 2003; Huang and Li, 2018), denaturing gradient gel electrophoresis (DGGE) of such amplicons (Regensbogenova et al., 2004; Sylvester et al., 2005), and quantification of 18S rDNA copies using real-time PCR (Sylvester et al., 2004; Skillman et al., 2006; Saminathan et al., 2017) using protozoa-specific primers. Technically, these molecular approaches circumvent some of the previously described limitations of the morphology-based microscopic identification, including lack of expertise, length of time to count numerous samples, potential misidentification, and morphological variations of the same taxa. In recent years, qPCR for quantification of total protozoa and amplicon sequencing, nearly exclusively with a next-generation sequencing (NGS) technology (primarily MiSeq of Illumina, Inc., San Diego, CA, United States), has been the primary molecular approaches used in compositional and diversity analyses of protozoal communities in the rumen. Protozoa-specific primers are required for accurate and reliable analyses using both approaches.

Several sets of universal rumen protozoal primers have been used to amplify either the nearly full-length or a region of the protozoal 18S rRNA gene. As in the case of prokaryotic

analyses based on 16S rRNA gene amplicons, choice of the primers and the hypervariable regions of the marker gene targeted can substantially affect the accuracy and reliability of the community analysis (Bonk et al., 2018). This potential limitation certainly applies to PCR amplicon-based analysis of rumen protozoa. Among the protozoa-specific primers targeting the 18S rRNA genes, most studies used primers RP841F (5′-GACTAGGGATTGGAGTGG-3′) and Reg1302R (5′-AATTGCAAAGATCTATCCC-3′) targeting the V5–V8 regions (Regensbogenova et al., 2004; Kittelmann and Janssen, 2011) and P-SSU-316F (5′-GCTTTCGWTGGTAGTGTATT-3′) and GIC758R (5′-CAACTGTCTCTATKAAYCG-3′) covering two signature regions of rumen ciliates (Sylvester et al., 2004; Ishaq and Wright, 2014). The RP841F/Reg1302R primer set was developed initially for DGGE analysis and designed from only the 18S rDNA sequence of rumen ciliates (Kittelmann and Janssen, 2011). This primer set allows detection of 12 major rumen ciliate genera, which represent over 99% of total protozoal abundance across 742 samples from 32 species of ruminants (Henderson et al., 2015). The P-SSU-316F/GIC758R primer set was designed for amplicon sequencing using NGS. It was evaluated for its specificity for rumen protozoa against protozoal reference 18S rRNA gene sequences, including those of non-ruminal ciliates that were available in NCBI (Ishaq and Wright, 2014). Both primer sets allowed detection and identification of the major ruminal ciliates at the genus taxonomic rank and detected similar diversity as microscopic identification; however, rather different protozoal relative abundance resulted from the two primer sets.

Accurate quantification of rumen protozoal abundance can be difficult to achieve using qPCR or amplicon sequencing with NGS for several reasons. First, the 18S rRNA genes in rumen protozoa (as in non-rumen ciliate protozoa) reside in both micronuclei and macronuclei. In the latter, its copy number is very large, ranging from 1,010 to 6,210 copies per cell of a mixed protozoal community (Sylvester et al., 2005). In general, small entodiniomorphids have fewer 18S rRNA gene copies per cell than larger cells (Sylvester et al., 2009). Therefore, the absolute abundance (copies of 18S rRNA genes per unit of weight or volume of samples) and relative abundance (% of total protozoal sequences) of small entodinia can be underestimated by qPCR and amplicon sequencing, respectively, whereas those with a large cell, such as *Polyplastron* and *Epidinium*, are overestimated (Ishaq and Wright, 2014; Kittelmann et al., 2015). As such, 18S rRNA gene-targeting qPCR probably does not allow accurate quantification of protozoal abundance *per se* but might be associated more positively with activity if we can assume that larger cells have greater activity on a cell basis (Wenner et al., 2018). Kittelmann et al. (2015) showed that microscopic counting was more accurate than high-throughput sequencing using primer set RP841F/Reg1302R in determining protozoal abundance. Equally challenging is the determination of protozoal biomass using qPCR or amplicon sequencing with NGS because of the unknown copy numbers of 18S rRNA genes per cell for all the genera and species of rumen protozoa. Regulation of copy number of 18S rRNA genes in ruminal ciliates also is poorly understood (other than the obvious replication before cytokinesis).

Primer Coverage and Specificity for Protozoal Community Analysis

Individual protozoal genera and species contribute differently to the overall rumen function both qualitatively and quantitatively. Thus, quantification of individual ciliate genera, if not species, is needed to assess their associations with dietary interventions. Hereto, at least 15 genera, all of which belong to the subclass *Trichostomatia*, of rumen ciliates have been identified based on 18S rRNA genes (**Table 1**). Because the 18S rRNA gene is highly conserved among different genera of protozoa, genera- or species-specific primers have been difficult to design. Only one study reported one pair of *Entodinium*-specific and one pair of *Dasytricha ruminantium*-specific primers, which allowed quantification of these two taxa (Skillman et al., 2006). Based on our *in silico* evaluation using TestPrime 1.0[1] as documented by Klindworth et al. (2013), both primer sets are specific for their targets (as assumed for up to one mismatch), but the *Entodinium*-specific primer set could only achieve 51.7% coverage (data not shown). Clearly, further research is needed to improve upon the nearly 50% of the missing *Entodinium* spp. sequences and to verify specificity with one or even no mismatch allowed. To overcome the limitation of microscopic counting and morphologic identification (described above), specific primers for at least the major genera of rumen protozoa need to be developed and adequately validated; until then, we recommend for cell counting to be continued.

To make this section more useful to some readers who need to choose a primer set in analyzing rumen protozoa, we evaluated the primers that have been used commonly with respect to their coverage and specificity using *in silico* evaluation. Briefly, using TestPrime 1.0, we compared the sequences of each primer set against the recent SILVA non-redundant SSU reference dataset (SSU r132), which contains over 400 reference 18S rRNA gene sequences representing 15 genera and 29 species of protozoa, including *C. ventriculi* (Kittelmann et al., 2015) and *Eodinium posterovesiculatum* (Cedrola et al., 2017), within subclass *Trichostomatia*, which covers all rumen ciliates. Coverage and specificity of each primer set were calculated as below (Raymann et al., 2017):

$$\text{Coverage}(\%) = (\text{number of sequences matched} \div \text{the number of eligible target sequences}) \times 100$$

$$\text{Specificity}(\%) = 100 - [(\text{number of outgroup matched sequences} \div \text{number of outgroup matchable sequences}) \times 100]$$

Table 1 summarizes the evaluation results of the primer sets. All those tested primer sets are specific for ciliates within the phylum *Ciliophora*, except the GIC1080F/GIC1578R set, which

[1]https://www.arb-silva.de/search/testprime/

TABLE 1 | Evaluation of 18S rRNA published gene-targeting primer pairs using the SILVA database 18S rRNA genes of rumen protozoa[1].

	RP841F/Reg1302R (Kittelmann et al., 2015)			P-SSU-316F/GIC758R (Ishaq and Wright, 2014)			GIC1080F/GIC1578R (Ishaq and Wright, 2014)			P-SSU-54F/P-SSU-1747R (Sylvester et al., 2004)			P.324f/P.1747r_2 (Zhang et al., 2015)		
Genus targeted	**Eligible target sequences[2]**	**Coverage (%)[3]**	**Specificity (%)[4]**	**Eligible target sequences**	**Coverage (%)**	**Specificity (%)**	**Eligible target sequences**	**Coverage (%)**	**Specificity (%)**	**Eligible target sequences**	**Coverage (%)**	**Specificity (%)**	**Eligible target sequences**	**Coverage (%)**	**Specificity (%)**
Charonina	3	0	0	2	50.0	100	3	33.3	99.9	2	100	99.9	3	33.3	100
Dasytricha	22	72.7	99.9	12	100	100	20	75.0	99.9	5	100	99.9	16	37.5	100
Diplodinium	34	100	99.9	17	88.2	100	33	54.5	99.9	15	100	99.9	0	0	NA
Diploplastron	2	100	99.9	1	100	100	2	100	99.9	1	100	99.9	0	0	NA
Enoploplastron	1	100	99.9	1	100	100	1	100	99.9	1	100	99.9	0	0	NA
Entodinium	201	89.1	100	38	92.1	100	196	80.1	99.9	16	100	99.9	175	20.0	100
Eodinium	3	100	99.9	3	100	100	0	0	NA	0	0	NA	0	0	NA
Epidinium	3	100	99.9	2	100	100	3	100	99.9	2	100	100	0	0	NA
Eremoplastron	5	40.0	99.9	5	80.0	100	5	80.0	99.9	4	75.0	99.9	0	0	NA
Eudiplodinium	36	100	99.9	34	100	100	36	33.3	99.9	33	97.0	100	0	0	NA
Isotricha	8	0	0	6	100	100	8	75.0	99.9	6	100	99.9	0	0	NA
Metadinium	6	100	99.9	4	100	100	6	66.7	99.9	4	100	99.9	0	0	NA
Ophryoscolex	43	93.0	99.9	31	96.8	100	43	41.9	99.9	30	100	100	46	2.2	100
Ostracodinium	5	100	99.9	3	100	100	5	100	99.9	3	100	99.9	0	0	NA
Polyplastron	27	88.9	99.9	21	90.5	100	27	22.2	99.9	20	95.0	99.9	30	6.7	100
uncultured	17	94.1	99.9	0	0	NA	17	94.1	99.9	0	0	NA	17	64.7	100
Total	416	88.2[5]	99.9	180	94.4	100	405	66.2	99.9	142	97.9	99.9	287	19.5	100

[1] TestPrime 1.0 was used to evaluate the primer pairs (Klindworth et al., 2013).[2] Eligible target seqs = number of target sequences with corresponding regions of the primer pairs.[3] Coverage (%) = (number of sequences matched/number of eligible target sequences) × 100. Total coverage mean (bottom row of data) among all genera is weighted for the numbers of sequences within each genus.[4] Specificity (%) = 100 - (outgroup matched sequences/outgroup matchable sequences) × 100. NA = not available because it could be calculated only when at least one sequence was matched. NA values were excluded from the total mean.[5] With one mismatch allowed (and not compromising specificity), coverage of this primer pair equals 95.9%.

TABLE 2 | Relative sequence abundance of major bacterial taxa exclusively found in single cells of freshly isolated rumen ciliates[1].

Phylum	Ciliate genera							
Lowest taxa assigned[2]	***Dasytricha***	***Diplodinium***	***Diploplastron***	***Entodinium***	***Epidinium***	***Isotricha***	***Ophryoscolex***	***Polyplastron***
Acidobacteria	1.72	1.86	1.48	1.38	1.03	0.92	0.98	1.68
Actinobacteria								
o_*Acidimicrobiales*	3.41	3.58	3.49	3.16	1.98	2.84	4.12	1.66
Bacteroidetes								
f_*Chitinophagaceae*	4.90	3.94	4.04	4.25	4.05	4.84	5.10	2.15
g_*Sediminibacterium*	4.78	3.63	3.62	3.53	3.54	4.22	4.70	2.14
Proteobacteria								
o_*Ellin329*	2.34	1.57	1.00	1.84	2.86	1.20	1.71	2.20
o_*Rhizobiales*	1.36	2.23	1.37	1.06	1.36	1.65	1.74	1.71
f_*Bradyrhizobiaceae*	1.09	1.43	0.91	0.85	0.69	1.46	1.29	1.45
f_*Rhodospirillaceae*	2.88	3.60	1.26	1.90	2.27	2.08	1.88	1.60
g_*Limnobacter*	1.51	2.51	2.06	1.01	1.77	2.84	1.68	1.71
c_ε-*Proteobacteria*	1.88	1.37	1.96	2.94	1.43	0.33	0.79	1.55
o_*Xanthomonadales*	4.36	3.52	2.26	5.55	3.66	3.11	3.81	1.65
f_*Sinobacteraceae*	4.25	2.56	1.92	4.77	3.10	2.97	3.64	1.53
g_*Nevskia*	2.74	1.56	1.36	2.29	1.41	1.55	1.49	1.06

[1]Relative abundance (% of total prokaryotic sequences) retrieved from isolated single cells of rumen ciliates extracted from fresh rumen fluid. No archaeal sequences were recovered that were exclusive to protozoa. Excerpted from Park and Yu (2018b). [2]The lowest classified taxa are class (c), order (o), family (f), or genus (g).

matched one algal sequence. However, none of the primer sets evaluated could achieve complete inclusive coverage (i.e., 100%), thus probably leaving some genera undetected. Among the three primer sets that can generate amplicons with a length suitable for qPCR and NGS (RP841F/Reg1302R, P-SSU-316F/GIC758R, and GIC1080F/GIC1578R), the P-SSU-316F/GIC758R primer pair achieved the highest average coverage (94.4% total coverage). With one mismatch allowed for the primer evaluation, RP841F/Reg1302R achieved 95.9% total coverage with all 15 rumen protozoal genera detected (data not shown). Of the two primer sets that can generate nearly full-length amplicons of protozoal 18S rRNA genes, the primer set P-SSU-54F/P-SSU-1747R achieved a coverage totaling 97.9%. Until new specific primers with improved coverage are developed, we recommend for these three primer sets to be used. Two primer sets, RP841F/Reg1302R and P-SSU-316F/GIC758R, are most suitable for qPCR and preparation of amplicon libraries of 18S rRNA genes of rumen microbiota, whereas P-SSU-54F/P-SSU-1747R is more suitable for generating nearly full-length amplicons for sequencing using the third generation sequencing (TGS) technologies, such as PacBio[2].

The NGS technologies have enabled comprehensive analysis of diverse and complex microbial communities since 2004 (Handelsman, 2004; Jovel et al., 2016). They are the primary technologies used in metagenomic studies of prokaryotic microbiota (Jovel et al., 2016), including rumen microbiota (Denman et al., 2018). Building on previous functional information based largely on monocultures (Williams and Coleman, 1992) and biochemical analyses (Béra-Maillet et al., 2005), metatranscriptomics techniques have emphasized the importance of protozoa to ruminal fiber degradation (Dai et al., 2015; Comtet-Marre et al., 2017), thus more closely relating protozoal functions to production situations. Gene sequencing approaches are being used more routinely to assess protozoal community structure *in vivo*. The first study that used NGS in sequencing rumen protozoal 18S rRNA genes to analyze the protozoal, prokaryotic, and fungal microbiota was reported by Kittelmann et al. (2013) after which Ishaq and Wright (2014) designed and evaluated the primers for specific analysis of rumen protozoal communities using NGS. These primers have enabled comprehensive and efficient analysis of the rumen protozoal populations in the rumen microbial community, including variations caused by different diets (Ishaq et al., 2017; Zhang et al., 2017; Bainbridge et al., 2018), breed and lactation stage in dairy cattle (Cersosimo et al., 2016), and geographical regions among moose samples (Ishaq et al., 2015).

Most of the 18S rRNA gene sequences of rumen protozoa archived in public databases are partial sequences. Improvement of current primers and design of new primers with desirable specificity and coverage require more reference sequences with accurate taxonomic identification, ideally at the species or OTU (operational taxonomic unit; currently at 97% similarity) rank. Reference sequences need to be full- or nearly full-length to allow proper primer design and *in silico* evaluation. None of the NGS platforms in use can sequence the full-length 18S rRNA gene of protozoa. However, the TGS techniques, such as PacBio RS II, produces sequence reads averaging over 10,000 base pairs. TGS can sequence the entire rRNA operon, which is composed of one 18S rRNA gene, one internal transcribed spacer (ITS1), one 5.8S rRNA gene, one ITS2, and one 28S rRNA gene. All but the 5.8S rRNA gene are useful phylogenetic markers. Full-length sequences of the entire operon allow not only the design of universal primers specific for rumen protozoa, but also the design of genera- and species-specific

[2]https://www.pacb.com/

primers. Indeed, ITS1, ITS2, and the 28S rRNA gene are better markers for ciliate identification (Abraham et al., 2019). Species-specific primers would allow researchers to reliably identify rumen protozoal species of interest using minimal expertise based on morphological features and to determine dietary shifts in the relative abundance of individual rumen protozoal species.

Although many researchers have attempted to develop protozoal axenic cultures (having no influence by prokaryotes, which contrasts with protozoal monocultures), no laboratory maintainable axenic cultures have been established (Park et al., 2017). The lack of an axenic culture of any ruminal ciliate species has hindered understanding of their metabolism, physiology, and ecology; thus, their actual roles in the rumen could only be inferred from indirect evidence (Coleman, 1962; Hino and Kametaka, 1977; Onodera and Henderson, 1980; Fondevila and Dehority, 2001b). Genomics and transcriptomics, empowered by NGS and TGS technologies, would be enabling approaches to help gain new insights into the fundamental biological features of rumen protozoa. Genomic (Park et al., 2018) and transcriptomic (Wang et al., 2020) studies of *En. caudatum* are providing starting points for future studies investigating roles of prokaryotes or interactions such as in defaunation studies (discussed previously).

Protozoa-Associated Bacteria

Rumen protozoa interact intensively with other members of the rumen microbiota. Predation, symbiosis, and cross-feeding are among the major interactions between protozoa and other members of the rumen microbiota. These interactions can shape the rumen microbiota (diversity, composition, and dynamics) and its functions and therefore are key to advancing our understanding of rumen microbial ecosystem. The most obvious and well-documented interaction is predation of bacteria (Diaz et al., 2014; Newbold et al., 2015; Rossi et al., 2019). Although still debatable if rumen ciliates do select their preys (Dehority, 2003; Belanche et al., 2011), the enriched intracellular *Proteobacteria* populations in other free-living ciliates in marine or freshwater environments might correlate to the symbiotic relationship between bacteria and host ciliates (Görtz and Brigge, 1998; Gong et al., 2016). Selective predation of rumen bacteria by rumen protozoa, at least *En. caudatum*, is likely (Park and Yu, 2018b); however, more research is needed to determine the prey selections by individual species of rumen protozoa and how prey selectivity is achieved.

Live bacteria are required as sources of essential nutrients that cannot be produced or obtained from other sources (Fondevila and Dehority, 2001b; Park et al., 2017). In addition to endosymbionts, live bacteria (and possibly archaea) might be sources of prey or symbionts for recurrent recruitment. External bacteria consumed by or harvested with protozoa are difficult to distinguish from endosymbiont prokaryotes. As generated from washed single rumen ciliate cells, the 16S rRNA gene amplicons of protozoa-associated prokaryotic community seem to have a composition that is different from that of free-living prokaryotes of the rumen (Irbis and Ushida, 2004; Levy and Jami, 2018; Park and Yu, 2018b). Some taxa (ranging from genus to order) were exclusively associated with rumen ciliate cells (**Table 2**). However, the detection of "protozoa-associated" species of bacteria probably reflects both the selective predation and presence of symbionts (Gong et al., 2016; Park and Yu, 2018b). In contrast with typical ruminal bacteria (primarily in the phyla *Firmicutes* and *Bacteroidetes*), some species of *Proteobacteria* (mostly within α- and γ-*Proteobacteria* classes) have been exclusively found inside various ciliates (Gong et al., 2016). A few of them, such as *Polynucleobacter* spp., are obligate endosymbionts in several species of ciliates (Soldo, 1987; Görtz and Brigge, 1998). Therefore, some of the α- and γ-*Proteobacteria* that were associated with rumen protozoa appear to be true endosymbionts. No archaeal sequences were detected exclusively in protozoa (i.e., they were also detected in the free-living pool) in the study of Park and Yu (2018b). Although archaea can interact intimately with protozoa, some methanogens interact extracellularly with ciliates, and some intracellular methanogens might be continually replaced by ingestion rather than being true endosymbionts (Ushida, 2010). Future research can further identify specific prokaryotes and reveal their potential mutualistic interactions with rumen ciliates. As suggested previously (Park and Yu, 2018b), single-cell microbiomics coupled with controlled starvation of protozoa cells is one effective approach to distinguish continually ingested prokaryotes from endosymbionts.

CONCLUSION

From monocultures to *in vivo* approaches, our colleague Burk Dehority was the consummate gentleman scholar. He always considered the most parsimonious approach to test a hypothesis, yet he always considered the "because they can" interpretation of results (avoiding teleology) within the larger context of advancing our ability to better define roles for ruminal protozoa with respect to positives and negatives both qualitatively and quantitatively. The topics discussed herein often stem from many discussions, particularly those traveling back from meetings that stimulated new questions, techniques, and approaches to science. This review was written in his honor "because we could."

AUTHOR CONTRIBUTIONS

JF initiated the review and wrote a first draft. ZY, TP, and JP made contributions in content and added to the draft.

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REFERENCES

Abe, M., Iriki, T., Tobe, N., and Shibui, H. (1981). Sequestration of holotrich protozoa in the reticulo-rumen of cattle. *Appl. Environ. Microbiol.* 41, 758–765. doi: 10.1128/aem.41.3.758-765.1981

Abraham, J. S., Sripoorna, S., Maurya, S., Makhija, S., Gupta, R., and Toteja, R. (2019). Techniques and tools for species identification in ciliates: a review. *Int. J. Syst. Evol. Microbiol.* 69, 877–894. doi: 10.1099/ijsem.0.003176

Ahvenjärvi, S., Vaga, M., Vanhatalo, A., and Huhtanen, P. (2018). Ruminal metabolism of grass silage soluble nitrogen fractions. *J. Dairy Sci.* 101, 279–294. doi: 10.3168/jds.2016-12316

Ankrah, P., Loerch, S. C., and Dehority, B. A. (1990). Sequestration, migration and lysis of protozoa in the rumen. *J. Gen. Microbiol.* 136, 1869–1875. doi: 10.1099/00221287-136-9-1869

Bainbridge, M. L., Saldinger, L. K., Barlow, J. W., Alvez, J. P., Roman, J., and Kraft, J. (2018). Alteration of rumen bacteria and protozoa through grazing regime as a tool to enhance the bioactive fatty acid content of bovine milk. *Front. Microbiol.* 9:904. doi: 10.3389/fmicb.2018.00904

Bauchop, T., and Clarke, R. T. (1976). Attachment of the ciliate Epidinium Crawley to plant fragments in the sheep rumen. *Appl. Environ. Microbiol.* 32, 417–422. doi: 10.1128/aem.32.3.417-422.1976

Belanche, A., Abecia, L., Holtrop, G., Guada, J. A., Castrillo, C., de la Fuente, G., et al. (2011). Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. *J. Anim. Sci.* 89, 4163–4174. doi: 10.2527/jas.2010-3703

Belanche, A., de la Fuente, G., Moorby, J. M., and Newbold, C. J. (2012). Bacterial protein degradation by different rumen protozoal groups. *J. Anim. Sci.* 90, 4495–4504. doi: 10.2527/jas2012-5118

Bełżecki, G., McEwan, N. R., Kowalik, B., Michałowski, T., and Miltko, R. (2017). Effect of *Entodinium caudatum* on starch intake and glycogen formation by *Eudiplodinium maggii* in the rumen and reticulum. *Eur. J. Protistol.* 57, 38–49. doi: 10.1016/j.ejop.2016.09.007

Béra-Maillet, C., Devillard, E., Cezette, M., Jouany, J.-P., and Forano, E. (2005). Xylanases and carboxymethylcellulases of the rumen protozoa *Polyplastron multivesiculatum*, *Eudiplodinium maggii*, and *Entodinium* sp. *FEMS Microbiol. Lett.* 244, 149–156. doi: 10.1016/j.femsle.2005.01.035

Berger, J. D. (2001). Riding the ciliate cell cycle–A thirty-five-year prospective. *J. Eukaryot. Microbiol.* 48, 505–518. doi: 10.1111/j.1550-7408.2001.tb00186.x

Bohatier, J., Sénaud, J., and Benyahya, M. (1990). In situ degradation of cellulose fibres by the entodiniomorph rumen ciliate *Polyplastron multivesiculatum*. *Protoplasma* 154, 122–131. doi: 10.1007/bf01539839

Bonk, F., Popp, D., Harms, H., and Centler, F. (2018). PCR-based quantification of taxa-specific abundances in microbial communities: quantifying and avoiding common pitfalls. *J. Microbiol. Methods* 153, 139–147. doi: 10.1016/j.mimet.2018.09.015

Cedrola, F., Rossi, M., Dias, R. J. P., Martinele, I., and D'agosto, M. (2015). Methods for taxonomic studies of rumen ciliates (Alveolata: Ciliophora): a brief review. *Zool. Sci.* 32, 8–15. doi: 10.2108/zs140125

Cedrola, F., Senra, M. V. X., D'agosto, M., and Dias, R. J. P. (2017). Phylogenetic analyses support validity of genus *Eodinium* (Ciliophora, Entodiniomorphida, Ophryoscolecidae). *J. Eukaryot. Microbiol.* 64, 242–247. doi: 10.1111/jeu.12355

Cersosimo, L. M., Bainbridge, M. L., Wright, A.-D. G., and Kraft, J. (2016). Breed and lactation stage alter the rumen protozoal fatty acid profiles and community structures in primiparous dairy cattle. *J. Agric. Food Chem.* 64, 2021–2029. doi: 10.1021/ACS.JAFC.5B05310

Cersosimo, L. M., Radloff, W., and Zanton, G. I. (2019). Microbial inoculum composition and pre-weaned dairy calf age alter the developing rumen microbial environment. *Front. Microbiol.* 10:e01651. doi: 10.3389/fmicb.2019.01651

Chan, W. W., and Dehority, B. A. (1999). Production of *Ruminococcus flavefaciens* growth inhibitor(s) by *Ruminococcus albus*. *Anim. Feed Sci. Technol.* 77, 61–71. doi: 10.1016/S0377-8401(98)00234-X

Coleman, G. S. (1962). The preparation and survival of almost bacteria-free suspensions of *Entodinium caudatum*. *J. Gen. Microbiol.* 28, 271–281. doi: 10.1099/00221287-28-2-271

Comtet-Marre, S., Parisot, N., Lepercq, P., Chaucheyras-Durand, F., Mosoni, P., Peyretaillade, E., et al. (2017). Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. *Front. Microbiol.* 8:67. doi: 10.3389/fmicb.2017.00067

Czerkawski, J. W. (1987). Reassessment of the contribution of protozoa to the microbial protein supply to the host ruminant animal. *J. Theor. Biol.* 126, 335–341. doi: 10.1016/j.jtbi.2016.12.007

Dai, X., Tian, Y., Li, J., Su, X., Wang, X., Zhao, S., et al. (2015). Metatranscriptomic analyses of plant cell wall polysaccharide degradation by microorganisms in the cow rumen. *Appl. Environ. Microbiol.* 81, 1375–1386. doi: 10.1128/aem.03682-14

Dehority, B. A. (1970). Occurrence of the ciliate protozoa *Bütschlia parva* schuberg in the rumen of the ovine. *Appl. Microbiol.* 19, 179–181. doi: 10.1128/aem.19.1.179-181.1970

Dehority, B. A. (1984). Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *Appl. Environ. Microbiol.* 48, 182–185. doi: 10.1128/aem.48.1.182-185.1984

Dehority, B. A. (1993). *Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa*. Boca Raton, FL: CRC Press, Inc.

Dehority, B. A. (1994). Rumen ciliate protozoa of the blue duiker (*Cephalophus monticola*), with observations on morphological variation lines within the species *Entodinium dubardi*. *J. Eukaryot. Microbiol.* 41, 103–111. doi: 10.1111/j.1550-7408.1994.tb01481.x

Dehority, B. A. (1998). Generation times of *Epidinium caudatum* and *Entodinium caudatum*, determined in vitro by transferring at various time intervals. *J. Anim. Sci.* 76, 1189–1196. doi: 10.2527/1998.7641189x

Dehority, B. A. (2003). *Rumen Microbiology*. Nottingham: Nottingham University Press.

Dehority, B. A. (2004). In vitro determination of generation times for *Entodinium exiguum*, *Ophryoscolex purkynjei*, and *Eudiplodinium maggii*. *J. Eukaryot. Microbiol.* 51, 333–338. doi: 10.1111/j.1550-7408.2004.tb00575.x

Dehority, B. A. (2005). Effect of pH on viability of *Entodinium caudatum*, *Entodinium exiguum*, *Epidinium caudatum*, and *Ophryoscolex purkynjei* in vitro. *J. Eukaryot. Microbiol.* 52, 339–342. doi: 10.1111/j.1550-7408.2005.00041.x

Dehority, B. A. (2008). Improved in vitro procedure for maintaining stock cultures of three genera of rumen protozoa. *J. Anim. Sci.* 86, 1395–1401. doi: 10.2527/jas.2007-0238

Dehority, B. A. (2010). Physiological characteristics of several rumen protozoa grown in vitro with observations on within and among species variation. *Eur. J. Protistol.* 46, 271–279. doi: 10.1016/j.ejop.2010.05.002

Dehority, B. A., and Males, J. R. (1974). Rumen fluid osmolality: evaluation of its influence upon the occurrence and numbers of Holotrich protozoa in sheep. *J. Anim. Sci.* 38, 865–870. doi: 10.2527/jas1974.384865x

Dehority, B. A., and Mattos, W. R. (1978). Diurnal changes and effect of ration on concentrations of the rumen ciliate *Charon ventriculi*. *Appl. Environ. Microbiol.* 36, 953–958. doi: 10.1128/aem.36.6.953-958.1978

Dehority, B. A., and Purser, D. B. (1970). Factors affecting the establishment and numbers of holotrich protozoa in the ovine rumen. *J. Anim. Sci.* 30, 445–449. doi: 10.2527/jas1970.303445x

Dehority, B. A., and Tirabasso, P. A. (1989). Factors affecting the migration and sequestration of rumen protozoa in the family Isotrichidae. *J. Gen. Microbiol.* 135, 539–548. doi: 10.1099/00221287-135-3-539

Dehority, B. A., and Tirabasso, P. A. (2000). Antibiosis between ruminal bacteria and ruminal fungi. *Appl. Environ. Microbiol.* 66, 2921–2927. doi: 10.1128/AEM.66.7.2921-2927.2000

Dehority, B. A., and Wright, A.-D. G. (2014). Studies on the in vitro cultivation of ciliate protozoa from the kangaroo forestomach. *Eur. J. Protistol.* 50, 395–401. doi: 10.1016/j.ejop.2014.04.001

Denman, S. E., Morgavi, D. P., and McSweeney, C. S. (2018). Review: the application of omics to rumen microbiota function. *Animal* 12, s233–s245. doi: 10.1017/S175173111800229X

Diaz, H. L., Barr, K. N., Godden, K. R., Plank, J. E., Zapata, I., Schappacher, A. N., et al. (2014). Eukaryotic inhibitors or activators elicit responses to chemosensory compounds by ruminal isotrichid and entodiniomorphid protozoa. *J. Dairy Sci.* 97, 2254–2269. doi: 10.3168/jds.2013-7698

Dijkstra, J., and Tamminga, S. (1995). Simulation of the effects of diet on the contribution of rumen protozoa to degradation of fibre in the rumen. *Br. J. Nutr.* 74, 617–634. doi: 10.1079/bjn19950166

Edwards, J. E., Forster, R. J., Callaghan, T. M., Dollhofer, V., Dagar, S. S., Cheng, Y., et al. (2017). PCR and omics based techniques to study the diversity, ecology and biology of anaerobic fungi: insights, challenges and opportunities. *Front. Microbiol.* 8:1657. doi: 10.3389/fmicb.2017.01657

Fessenden, S. W., Hackmann, T. J., Ross, D. A., Block, E., Foskolos, A., and Van Amburgh, M. E. (2019). Rumen digestion kinetics, microbial yield, and omasal flows of nonmicrobial, bacterial, and protozoal amino acids in lactating dairy cattle fed fermentation by-products or urea as a soluble nitrogen source. *J. Dairy Sci.* 102, 3036–3052. doi: 10.3168/jds.2018-15448

Firkins, J. L. (2010). Reconsidering rumen microbial consortia to enhance feed efficiency and reduce environmental impact of ruminant livestock production systems. *R. Bras. Zootec.* 39, 445–457. doi: 10.1590/S1516-35982010001300049

Firkins, J. L., Allen, M. S., Oldick, B. S., and St-Pierre, N. R. (1998). Modeling ruminal digestibility of carbohydrates and microbial protein flow to the duodenum. *J. Dairy Sci.* 81, 3350–3369. doi: 10.3168/jds.S0022-0302(98)75901-6

Firkins, J. L., and Mackie, R. I. (2020). "Ruminal protein breakdown and ammonia assimilation," in *Improving Rumen Function*, eds C. S. McSweeney, and R. I. Mackie, (Cambridge, MA: Burleigh Dodds).

Firkins, J. L., and Yu, Z. (2006). "Characterisation and quantification of the microbial populations in the rumen," in *Ruminant Physiology, Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress*, eds K. Sejrsen, T. Hvelplund, and M. O. Nielsen, (Wageningen: Wageningen Academic Publishers), 19–54.

Firkins, J. L., and Yu, Z. (2015). How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *J. Anim. Sci.* 93, 1450–1470. doi: 10.2527/jas2014-8754

Firkins, J. L., Yu, Z., and Morrison, M. (2007). Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. *J. Dairy Sci.* 90(Suppl. 1), E1–E16. doi: 10.3168/jds.2006-518

Fondevila, M., and Dehority, B. A. (2001a). *In vitro* growth and starch digestion by *Entodinium exiguum* as influenced by the presence or absence of live bacteria. *J. Anim. Sci.* 79, 2465–2471. doi: 10.2527/2001.7992465x

Fondevila, M., and Dehority, B. A. (2001b). Preliminary study on the requirements of *Entodinium exiguum* and *E. caudatum* for live or dead bacteria when cultured *in vitro*. *Reprod. Nutr. Dev.* 41, 41–46. doi: 10.1051/rnd:2001110

Gong, J., Qing, Y., Zou, S., Fu, R., Su, L., Zhang, X., et al. (2016). Protist-bacteria associations: *Gammaproteobacteria* and *Alphaproteobacteria* are prevalent as digestion-resistant bacteria in ciliated protozoa. *Front. Microbiol.* 7:498. doi: 10.3389/fmicb.2016.00498

Görtz, H.-D., and Brigge, T. (1998). Intracellular bacteria in protozoa. *Sci. Nat.* 85, 359–368. doi: 10.1007/s001140050517

Guyader, J., Eugene, M., Noziere, P., Morgavi, D. P., Doreau, M., and Martin, C. (2014). Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. *Animal* 8, 1816–1825. doi: 10.1017/s1751731114001852

Hackstein, J. H. P., and Tielens, A. G. M. (2010). "Hydrogenosomes," in *(Endo)Symbiotic Methanogenic Archaea*, ed. J. H. P. Hackstein, (Heidelberg: Springer-Verlag), 175–206.

Hall, M. B. (2011). Isotrichid protozoa influence conversion of glucose to glycogen and other microbial products. *J. Dairy Sci.* 94, 4589–4602. doi: 10.3168/jds.2010-3878

Hall, M. B. (2019). Short communication: gelatinization and enzymatic hydrolysis characteristics relevant to digestion and analysis of glycogen granules isolated from ruminal protozoa. *J. Dairy Sci.* 102, 4205–4208. doi: 10.3168/jds.2018-15758

Handelsman, J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. Rev.* 68, 669–685. doi: 10.1128/mmbr.68.4.669-685.2004

Hartinger, T., Gresner, N., and Südekum, K.-H. (2018). Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. *J. Anim. Sci. Biotechnol.* 9:33. doi: 10.1186/s40104-018-0249-x

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Hino, T., and Kametaka, M. (1977). Gnotobiotic and axenic cultures of a rumen protozoan, *Entodinium caudatum*. *J. Gen. Appl. Microbiol.* 23, 37–48. doi: 10.2323/jgam.23.37

Hook, S. E., France, J., and Dijkstra, J. (2017). Further assessment of the protozoal contribution to the nutrition of the ruminant animal. *J. Theor. Biol.* 416, 8–15. doi: 10.1016/J.JTBI.2016.12.007

Hristov, A. N., and Jouany, J.-P. (2005). "Factors affecting the efficiency of nitrogen utilization in the rumen," in *Nitrogen and Phosphorus Nutrition of Cattle and Environment*, eds A. N. Hristov, and E. Pfeffer, (Wallingford: CAB International), 117–166. doi: 10.1079/9780851990132.0117

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., and Waghorn, G. (2013). Special topics—mitigation of methane and nitrous oxide emissions from animal operations. I. A review of enteric methane mitigation options. *J. Anim. Sci.* 91, 5045–5069. doi: 10.2527/jas.2013-6583

Huang, J., and Li, Y. (2018). Rumen methanogen and protozoal communities of Tibetan sheep and Gansu Alpine Finewool sheep grazing on the Qinghai–Tibetan Plateau, China. *BMC Microbiol.* 18:212. doi: 10.1186/s12866-018-1351-0

Huang, Y., Marden, J. P., Julien, C., and Bayourthe, C. (2018). Redox potential: an intrinsic parameter of the rumen environment. *J. Anim. Physiol. Anim. Nutr.* 102, 393–402. doi: 10.1111/jpn.12855

Irbis, C., and Ushida, K. (2004). Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa. *J. Gen. Appl. Microbiol.* 50, 203–212. doi: 10.2323/jgam.50.203

Ishaq, S. L., Alzahal, O., Walker, N., and Mcbride, B. (2017). An investigation into rumen fungal and protozoal diversity in three rumen fractions, during high-fiber or grain-induced sub-acute ruminal acidosis conditions, with or without active dry yeast supplementation. *Front. Microbiol.* 8:1943. doi: 10.3389/fmicb.2017.01943

Ishaq, S. L., Sundset, M. A., Crouse, J., and Wright, A.-D. G. (2015). High-throughput DNA sequencing of the moose rumen from different geographical locations reveals a core ruminal methanogenic archaeal diversity and a differential ciliate protozoal diversity. *Microb. Genom.* 1:e000034. doi: 10.1099/mgen.0.000034

Ishaq, S. L., and Wright, A.-D. G. (2014). Design and validation of four new primers for next-generation sequencing to target the 18S rRNA genes of gastrointestinal ciliate protozoa. *Appl. Environ. Microbiol.* 80, 5515–5521. doi: 10.1128/AEM.01644-14

Jouany, J.-P. (1996). Effect of rumen protozoa on nitrogen utilization by ruminants. *J. Nutr.* 126, 1335S–1346S. doi: 10.1093/jn/126.suppl_4.1335S

Jouany, J. P., and Ushida, K. (1999). The role of protozoa in feed digestion - review. *Asian Australas. J. Anim. Sci.* 12, 113–128. doi: 10.5713/ajas.1999.113

Jovel, J., Patterson, J., Wang, W., Hotte, N., O'keefe, S., Mitchel, T., et al. (2016). Characterization of the gut microbiome using 16S or shotgun metagenomics. *Front. Microbiol.* 7:459. doi: 10.3389/fmicb.2016.00459

Karnati, S. K. R., Sylvester, J. T., Noftsger, S. M., Yu, Z., St-Pierre, N. R., and Firkins, J. L. (2007). Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. *J. Dairy Sci.* 90, 798–809. doi: 10.3168/jds.S0022-0302(07)71564-3

Karnati, S. K. R., Yu, Z., Sylvester, J. T., Dehority, B. A., Morrison, M., and Firkins, J. L. (2003). Technical note: specific PCR amplification of protozoal 18S rDNA sequences from DNA extracted from ruminal samples of cows. *J. Anim. Sci.* 81, 812–815. doi: 10.2527/2003.813812x

Kittelmann, S., Devente, S. R., Kirk, M. R., Seedorf, H., Dehority, B. A., and Janssen, P. H. (2015). Phylogeny of the intestinal ciliates, including *Charonina ventriculi*, and comparison of microscopy and 18S rRNA gene pyrosequencing for rumen ciliate community structure analysis. *Appl. Environ. Microbiol.* 81, 2433–2444. doi: 10.1128/aem.03697-14

Kittelmann, S., and Janssen, P. H. (2011). Characterization of rumen ciliate community composition in domestic sheep, deer, and cattle, feeding on varying diets, by means of PCR-DGGE and clone libraries. *FEMS Microbiol. Ecol.* 75, 468–481. doi: 10.1111/j.1574-6941.2010.01022.x

Kittelmann, S., Pinares-Patino, C. S., Seedorf, H., Kirk, M. R., McEwan, J. C., and Janssen, P. H. (2016). Natural variation in methane emission of sheep fed on a lucerne pellet diet is unrelated to rumen ciliate community type. *Microbiology* 162, 459–465. doi: 10.1099/mic.0.000245

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., and Gordon, J. I. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *Plos One* 8:e47879. doi: 10.1371/journal.pone.0047879

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Levy, B., and Jami, E. (2018). Exploring the prokaryotic community associated with the rumen ciliate protozoa population. *Front. Microbiol.* 9:2526. doi: 10.3389/fmicb.2018.02526

Martin, C., Bernard, L., and Michalet-Doreau, B. (1996). Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents. *J. Anim. Sci.* 74, 1157–1163.

Miltko, R., Michalowski, T., Pristas, P., Javorsky, P., and Hackstein, J. H. P. (2006). Factors influencing morphological variability of rumen ciliates from the genus *Ophryoscolex*. *J. Anim. Feed Sci.* 15, 35–38. doi: 10.22358/jafs/70137/2006

Morgavi, D. P., Martin, C., Jouany, J.-P., and Ranilla, M. J. (2012). Rumen protozoa and methanogenesis: not a simple cause-effect relationship. *Br. J. Nutr.* 107, 388–397. doi: 10.1017/S0007114511002935

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., and McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6:1313. doi: 10.3389/fmicb.2015.01313

Oelker, E. R., Reveneau, C., and Firkins, J. L. (2009). Interaction of molasses and monensin in alfalfa hay- or corn silage-based diets on rumen fermentation, total tract digestibility, and milk production by Holstein cows. *J. Dairy Sci.* 92, 270–285. doi: 10.3168/jds.2008-1432

Oldick, B. S., Firkins, J. L., and Kohn, R. A. (2000). Compartmental modeling with nitrogen-15 to determine effects of degree of fat saturation on intra-ruminal N recycling. *J. Anim. Sci.* 78, 2421–2430. doi: 10.2527/2000.7892421x

Onodera, R., and Henderson, C. (1980). Growth factors of bacterial origin for the culture of the rumen oligotrich protozoon, *Entodinium caudatum*. *J. Appl. Bacteriol.* 48, 125–134. doi: 10.1111/j.1365-2672.1980.tb05214.x

Orpin, C. G. (1985). Association of rumen ciliate populations with plant particles in vitro. *Microb. Ecol.* 11, 59–69. doi: 10.1007/BF02015109

Park, T., Meulia, T., Firkins, J. L., and Yu, Z. (2017). Inhibition of the rumen ciliate *Entodinium caudatum* by antibiotics. *Front. Microbiol.* 8:1189. doi: 10.3389/fmicb.2017.01189

Park, T., Wijeratne, S., Meulia, T., Firkins, J., and Yu, Z. (2018). Draft macronuclear genome sequence of the ruminal ciliate *Entodinium caudatum*. *Microbiol. Resour. Announc.* 7, 300826–300818. doi: 10.1128/mra.00826-18

Park, T., Yang, C., and Yu, Z. (2019). Specific inhibitors of lysozyme and peptidases inhibit the growth of the rumen protozoan Entodinium caudatum without decreasing feed digestion or fermentation in vitro. *J. Appl. Microbiol.* 127, 670–682. doi: 10.1111/jam.14341

Park, T., and Yu, Z. (2018a). Aerobic cultivation of anaerobic rumen protozoa, *Entodinium caudatum* and *Epidinium caudatum*. *J. Microbiol. Methods* 152, 186–193. doi: 10.1016/j.mimet.2018.08.006

Park, T., and Yu, Z. (2018b). Do ruminal ciliates select their preys and prokaryotic symbionts? *Front. Microbiol.* 9:1710. doi: 10.3389/fmicb.2018.01710

Plattner, H. (2017). Signalling in ciliates: long- and short-range signals and molecular determinants for cellular dynamics. *Biol. Rev.* 92, 60–107. doi: 10.1111/brv.12218

Potter, E. L., and Dehority, B. A. (1973). Effects of changes in feed level, starvation, and level of feed after starvation upon the concentration of rumen protozoa in the ovine. *Appl. Microbiol.* 26, 692–698. doi: 10.1128/aem.26.5.692-698.1973

Prins, R. A., and Van Hoven, W. (1977). Carbohydrate fermentation by the rumen ciliate *Isotricha prostoma*. *Protistologica* 13, 549–556.

Purser, D. B. (1961). A diurnal cycle for Holotrich protozoa of the rumen. *Nature* 190, 831–832. doi: 10.1038/190831a0

Qi, M., Wang, P., O'toole, N., Barboza, P. S., Ungerfeld, E., Leigh, M. B., et al. (2011). Snapshot of the eukaryotic gene expression in muskoxen rumen—A metatranscriptomic approach. *PLoS One* 6:e20521. doi: 10.1371/journal.pone.0020521

Raymann, K., Moeller, A. H., Goodman, A. L., and Ochman, H. (2017). Unexplored archaeal diversity in the Great Ape gut microbiome. *mSphere* 2:e00026-17. doi: 10.1128/mSphere.00026-17

Regensbogenova, M., Pristas, P., Javorsky, P., Moon-Van Der Staay, S. Y., Van Der Staay, G. W. M., Hackstein, J. H. P., et al. (2004). Assessment of ciliates in the sheep rumen by DGGE. *Lett. Appl. Microbiol.* 39, 144–147. doi: 10.1111/j.1472-765X.2004.01542.x

Reveneau, C., Karnati, S. K. R., Oelker, E. R., and Firkins, J. L. (2012). Interaction of unsaturated fat or coconut oil with monensin in lactating dairy cows fed twelve times daily. I. Protozoal abundance, nutrient digestibility, and microbial protein flow to the omasum. *J. Dairy Sci.* 95, 2046–2060. doi: 10.3168/jds.2011-4887

Roman-Garcia, Y., Wenner, B. A., Welty, C. M., Wagner, B. K., Plank, J. E., Meller, R. A., et al. (2019). Rumen microbial responses to supplemental nitrate. I. Yeast growth and protozoal chemotaxis in vitro as affected by nitrate and nitrite concentrations. *J. Dairy Sci.* 102, 2207–2216. doi: 10.3168/jds.2018-15274

Rossi, A., Bellone, A., Fokin, S. I., Boscaro, V., and Vannini, C. (2019). Detecting associations between ciliated protists and prokaryotes with culture-independent single-cell microbiomics: a proof-of-concept Study. *Microb. Ecol.* 78, 232–242. doi: 10.1007/s00248-018-1279-9

Saminathan, M., Gan, H. M., Abdullah, N., Wong, C. M. V. L., Ramiah, S. K., Tan, H. Y., et al. (2017). Changes in rumen protozoal community by condensed tannin fractions of different molecular weights from a *Leucaena leucocephala* hybrid *in vitro*. *J. Appl. Microbiol.* 123, 41–53. doi: 10.1111/jam.13477

Skillman, L. C., Toovey, A. F., Williams, A. J., and Wright, A.-D. G. (2006). Development and validation of a real-time PCR method to quantify rumen protozoa and examination of variability between *Entodinium* populations in sheep offered a hay-based diet. *Appl. Environ. Microbiol.* 72, 200–206. doi: 10.1128/AEM.72.1.200-206.2006

Sok, M., Ouellet, D. R., Firkins, J. L., Pellerin, D., and Lapierre, H. (2017). Amino acid composition of rumen bacteria and protozoa in cattle. *J. Dairy Sci.* 100, 5241–5249. doi: 10.3168/jds.2016-12447

Soldo, A. T. (1987). *Parauronema acutum* and its xenosomes: a model system. *J. Protozool.* 34, 447–451. doi: 10.1111/j.1550-7408.1987.tb03212.x

Sylvester, J. T., Karnati, S. K. R., Dehority, B. A., Morrison, M., Smith, G. L., St-Pierre, N. R., et al. (2009). Rumen protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. *J. Dairy Sci.* 92, 256–269. doi: 10.3168/jds.2008-1436

Sylvester, J. T., Karnati, S. K. R., Yu, Z., Morrison, M., and Firkins, J. L. (2004). Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J. Nutr.* 134, 3378–3384. doi: 10.1093/jn/134.12.3378

Sylvester, J. T., Karnati, S. K. R., Yu, Z., Newbold, C. J., and Firkins, J. L. (2005). Evaluation of a real-time PCR assay for measuring the ruminal pool and duodenal flow of protozoal nitrogen. *J. Dairy Sci.* 88, 2083–2095. doi: 10.3168/jds.S0022-0302(05)72885-X

Teixeira, C. R. V., Lana, R. D. P., Tao, J., and Hackmann, T. J. (2017). Comparing the responses of rumen ciliate protozoa and bacteria to excess carbohydrate. *FEMS Microbiol. Ecol.* 93:fix060. doi: 10.1093/femsec/fix060

Ushida, K. (2010). "Symbiotic methanogens and rumen ciliates," in *(Endo)Symbiotic Methanogenic Archaea*, ed. J. H. P. Hackstein, (Heidelberg: Springer-Verlag), 25–34. doi: 10.1007/978-3-642-13615-3_3

Wang, L., Abu-Doleh, A., Plank, J., Catalyurek, U. V., Firkins, J. L., and Yu, Z. (2020). The transcriptome of the rumen ciliate *Entodinium caudatum* reveals some of its metabolic features. *BMC Genomics* 20:1008. doi: 10.1186/s12864-019-6382-x

Wang, Y., Wang, Y., Sheng, Y., Huang, J., Chen, X., Al-Rasheid, K. A. S., et al. (2017). A comparative study of genome organization and epigenetic mechanisms in model ciliates, with an emphasis on *Tetrahymena*, *Paramecium* and *Oxytricha*. *Eur. J. Protistol.* 61, 376–387. doi: 10.1016/j.ejop.2017.06.006

Wenner, B. A. (2016). *Effects of Aqueous Hydrogen on Methane Mitigation in Continuous Culture Fermenters*. Ph.D. thesis, The Ohio State University, Ohio.

Wenner, B. A., Wagner, B. K., and Firkins, J. L. (2018). Using video microscopy to improve quantitative estimates of protozoal motility and cell volume. *J. Dairy Sci.* 101, 1060–1073. doi: 10.3168/jds.2017-13513

Whitelaw, F. G., Eadie, J. M., Bruce, L. A., and Shand, W. J. (1984). Methane formation in faunated and ciliate-free cattle and its relationship with rumen volatile fatty acid proportions. *Br. J. Nutr.* 52, 261–275. doi: 10.1079/bjn19840094

Williams, A. G., and Coleman, G. S. (1992). *The Rumen Protozoa*. New York, NY: Springer-Verlag.

Yáñez-Ruiz, D. R., Abecia, L., and Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6:1133. doi: 10.3389/fmicb.2015.01133

Ye, D., Karnati, S. K. R., Wagner, B., Firkins, J. L., Eastridge, M. L., and Aldrich, J. M. (2018). Essential oil and monensin affect ruminal fermentation and the protozoal population in continuous culture. *J. Dairy Sci.* 101, 5069–5081. doi: 10.3168/jds.2017-13646

Zeitz, J. O., Kreuzer, M., and Soliva, C. R. (2013). In vitro methane formation and carbohydrate fermentation by rumen microbes as influenced by selected rumen ciliate species. *Eur. J. Protistol.* 49, 389–399. doi: 10.1016/j.ejop.2013.02.003

Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., et al. (2017). Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. *Front. Microbiol.* 8:2206. doi: 10.3389/fmicb.2017.02206

Zhang, J., Zhao, S., Zhang, Y., Sun, P., Bu, D., and Wang, J. (2015). New primers targeting full-length ciliate 18S rRNA genes and evaluation of dietary effect on rumen ciliate diversity in dairy cows. *Curr. Microbiol.* 71, 650–657. doi: 10.1007/s00284-015-0898-3

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Metatranscriptomic Profiling Reveals the Effect of Breed on Active Rumen Eukaryotic Composition in Beef Cattle With Varied Feed Efficiency

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Exploring the compositional characteristics of rumen eukaryotic community can expand our understanding of their role in rumen function and feed efficiency. In this study, we applied metatranscriptomics to characterize the active rumen eukaryotic community (protozoa and fungi) in beef cattle (n = 48) of three breeds [Angus (AN), Charolais (CH), and Kinsella Composite (KC)] and with divergent residual feed intake (RFI). The composition of active rumen eukaryotic microbiota was evaluated based on enriched 18S rRNAs from the metatranscriptomic datasets. At the phylum level, a total of four protozoal taxa (*Ciliophora*, *Parabasalia*, unclassified *SAR*, and unclassified *Alveolata*), six fungal taxa (*Neocallimastigomycota*, *Basidiomycota*, unclassified *Fungi*, *Mucoromycota*, *Ascomycota*, and *Chytridiomycota*), and one sister group of kingdom *Fungi* (unclassified *Opisthokonta*) were detected with relative abundances higher than 0.01% and in at least 50% of animals within each breed. Among these, *Ciliophora*, *Parabasalia*, unclassified *Opisthokonta*, and *Neocallimastigomycota* were the top four active eukaryotic phyla. At the genus level, a total of 8 ciliated protozoa, 5 flagellated protozoa, 5 anaerobic fungi, and 10 aerobic fungi taxa were detected, with unclassified *Trichostomatia*, *Tetratrichomonas*, unclassified *Neocallimastigaceae*, and *Pleurotus* being the most predominant taxa of ciliated protozoa, flagellated protozoa, anaerobic fungi, and aerobic fungi, respectively. Differential abundance analysis revealed that breed had a significant effect on the phylogenetic lineages of rumen eukaryotes, and seven fungal taxa were more abundant (linear discriminant analysis score $>$ 2 with $P < 0.05$) in the rumen of KC steers than in the rumen of AN and CH steers. Although principal coordinate analysis (PCoA) revealed that the ruminal active eukaryotic profiles were not distinguishable between high- and low-RFI groups, the diversity indices, including Faith's phylogenetic diversity (PD), observed operational taxonomic units (OTUs), and Shannon index of rumen eukaryotes were higher in low-RFI steers than those in high-RFI steers. Meanwhile, the abundance of genus *Entodinium* and the kingdom *Fungi* was higher in low-RFI steers than that in high-RFI steers. This information on active rumen eukaryotic microbiota and identified differential abundance of taxa between high- and low-RFI animals suggests the possibility of improving feed efficiency through altering rumen eukaryotic microbiota.

Keywords: beef cattle, breed, feed efficiency, eukaryotes, fungi, protozoa, metatranscriptomics

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INTRODUCTION

Ruminant production, especially beef cattle operation, is important for the production of high-quality animal protein (meat). Feed efficiency is one of the most important traits in beef cattle production because the feed cost accounts for approximately 60–70% of the total production cost (Karisa et al., 2014). Therefore, improving feed efficiency in beef cattle can maximize the production efficiency when lower or the same amount of feed is consumed by cattle under current management conditions. In addition, cattle with a high feed efficiency emit lesser enteric methane and excrete lesser feces than cattle with a low feed efficiency (Nkrumah et al., 2006; Hegarty et al., 2007). Therefore, there is an urgent need to develop strategies to improve feed efficiency for enhanced economic and environmental sustainability of beef production.

Many factors can affect feed efficiency, including animal genetics, nutrition, biology, physiology, metabolism, behavior, etc. (Kenny et al., 2018). Although it is not well defined, the digestive activity of animals likely contributes to about 10% of the variation in feed efficiency (Richardson and Herd, 2004). Indeed, recently, studies have shown that rumen function (microbial fermentation and nutrient absorption) can impact residual feed intake (RFI), a measure of feed efficiency (Guan et al., 2008; Kong et al., 2016). Ruminants rely on their symbiotic rumen microorganisms to hydrolyze the plant fiber and generate energy and other nutrients (Puniya et al., 2015). The rumen commensal microbiota consists of bacteria, archaea, protozoa, fungi, and phages (Huws et al., 2018). Within the microbial consortium, the eukaryotic community (protozoa and fungi) has been shown to constitute approximately half of the total microbial biomass (Puniya et al., 2015) and is believed to play a critical role in the degradation of lignocellulosic components of the feed particles (Gruninger et al., 2014; Newbold et al., 2015). To date, many studies have reported significant differences in the relative abundance of several rumen bacterial and archaeal phylotypes between efficient and inefficient cattle (Jewell et al., 2015; Myer et al., 2015; Li et al., 2019a). In addition, it has been observed that cattle with a lower feed efficiency possess more complex and diverse rumen bacterial and archaeal communities than cattle with a high feed efficiency (Zhou et al., 2009; Shabat et al., 2016), thereby suggesting that rumen microbiota may play a role in contributing to cattle feed efficiency. However, most of these studies only focused on the prokaryotic community (bacterial and archaeal) and/or assessed the eukaryotic community at the DNA (genomic) level. To date, our knowledge on the ruminal eukaryotic profile is still limited, especially with respect to function and activity, and the linkage between active rumen eukaryotic community and feed efficiency has not yet been reported.

On the other hand, although previous studies have shown that rumen microbial taxonomic profiles are distinguishable among hosts with different genetic backgrounds (Hernandez-Sanabria et al., 2013; Henderson et al., 2015; Li et al., 2019b), only bacteria and archaea were profiled reported in these studies. Therefore, in the present study, we profiled active rumen eukaryotic communities using the metatranscriptomic data generated from 48 beef steers with divergent RFI from three breeds to identify the active protozoal and fungal communities in the rumen and assess whether they are affected by RFI and breed. The objectives of this study were to holistically explore the active rumen eukaryotic profile in beef cattle and to investigate its relationship with cattle RFI as well as host breed.

MATERIALS AND METHODS

Metatranscriptomic Data

RNA-seq data were obtained from our previously published study (Li et al., 2019a), under accession number PRJNA448333, for the sequences deposited in the Sequence Read Archive of the National Center for Biotechnology Information. These data were generated by extracting total RNA extracted from rumen content samples collected from 48 steers (selected from a herd of 738 beef cattle) from three breeds [two purebreds, Angus (AN) and Charolais (CH), and one crossbred, Kinsella Composite (KC)], with eight animals per RFI category per breed (a total of 16 per breed) who were raised under the same feedlot conditions. Briefly, these cattle were fed with the same finishing diet, consisting of 80% barley grain, 15% barley silage, and 5% rumensin pellet supplement (Killam Tag 849053; Hi-Pro Feeds, Westlock, AB, Canada). The daily dry matter intake (DMI) and average daily gain (ADG) were measured over a test period of 70–73 days using GrowSafe System (GrowSafe Systems Ltd., Airdrie, AB, Canada), and RFI was calculated as described by Basarab et al. (2011). Steers were slaughtered before feeding, and the rumen content samples were collected within half an hour after slaughter. In total, 3,087.41 M paired-end reads [64.32 $\pm$ 0.74 M (mean $\pm$ SEM) per sample] were used for analysis of active rumen eukaryotic microbiota.

Assessment of the Active Rumen Eukaryotic Microbiota Using Metatranscriptomics

The RNA-seq dataset was preliminarily processed as described by Li et al. (2016), and reads after quality control and filtration were used in this study. The 18S rRNA reads were extracted by mapping the filtered reads to the rRNA reference database SILVA_SSU (release 119) using SortMeRNA (version1.9) (Kopylova et al., 2012) and subjected to downstream analysis using Quantitative Insights Into Microbial Ecology (QIIME 2, version 2019.4) (Bolyen et al., 2019). The enriched 18S rRNA paired reads were quality-filtered, merged, dereplicated, and chimera-filtered using the q2-dada2 plugin (Callahan et al., 2016) to obtain representative sequence variants (RSVs) and their frequency distribution tables. This process was achieved without trimming or truncating any base at the beginning or end of the sequences, respectively. The RSVs

were then used to generate compositional profiles of the active rumen eukaryotic communities. Eukaryotic taxonomic classification was performed using the classify-sklearn command (Pedregosa et al., 2011) of the q2-feature-classifier plugin with a pretrained naive Bayesian classifier. The classifier was pretrained on the Silva 18S rRNA database (release 132) using the fit-classifier-Naive–Bayes method from the q2-feature-classifier plugin. Next, to analyze phylogenetic diversity (PD), a phylogenetic tree was generated using the align-to-tree-mafft-fasttree pipeline integrated in the q2-phylogeny plugin. To assess if sample richness has been fully observed, alpha rarefaction plots were generated based on the Shannon index and Faith's PD metrics using the alpha-rarefaction visualizer of the q2-diversity plugin. To comparably analyze the eukaryotic diversity among samples, the sequence count of all samples was standardized by rarefying them to the same number of sequences (the smallest sampling size) using the rarefy command of the q2-feature-table plugin. The rarefied feature table and the phylogenetic tree were then used to compute alpha diversity indices, including the Shannon index, observed operational taxonomic units (OTUs), Faith's PD, and Pielou's evenness and beta diversity metrics, including unweighted and weighted UniFrac distance, using the core-metrics-phylogenetic pipeline integrated in the q2-diversity plugin.

Statistical Analysis

In the present study, microbial taxa with a relative abundance higher than 0.01% in at least one animal were considered as being identified, while microbial taxa with a relative abundance higher than 0.01% and presented in at least 50% of animals within each breed were considered as being detected and used for downstream statistical analysis. Eukaryotic composition profiles were summarized at phylum and genus levels, respectively. Relative abundances of microbial taxa were arcsine square root transformed and then compared among different breeds and RFI categories (differential abundance analysis) using the mixed procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC, United States). A mixed effect model was used as follows: $y_{ij} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \varepsilon_{ij}$, where μ is the intercept and ε_{ij} is the residual error term; α_i, β_j, and $(\alpha \times \beta)_{ij}$ are the fixed effects of ith beef breed (AN, CH, and KC), jth RFI group (high- and low-RFI), and their interaction, respectively. The significance level was defined as $P < 0.05$. The more stringent linear discriminant analysis (LDA) effect size (LEfSe) was performed to further identify differentially abundant eukaryotic taxa, as described by Segata et al. (2011), and taxa with LDA score > 2 and $P < 0.05$ were considered to be significantly different. The difference in alpha diversity indices among the three breeds and between different RFI categories and their interaction was tested with the same mixed effect model using SAS. PCoA was used to visualize rumen eukaryotic communities based on the unweighted and weighted UniFrac distances at the RSVs level. Permutational multivariate analysis variance (PERMANOVA) was used to test the dissimilarity of active rumen eukaryotic profiles among the different breeds and RFI categories using the beta-group-significance command of the q2-diversity plugin (Anderson, 2001).

RESULTS AND DISCUSSION

Rumen Eukaryotic Communities Identified by Enriched 18S rRNA Sequences

From the 3,087.41 M metatranscriptomic dataset, a total of 511.85 M paired-end reads belonging to the 18S rRNA gene (10.66 ± 0.74 M) (16.55%) were enriched. The average number of 18S rRNA was found to be over 10-fold higher than that in a recent rumen metatranscriptomic study (945,524 reads) on dairy cow (Comtet-Marre et al., 2017). After quality control, combining paired-end reads, and filtering chimeras, a total of 149,672,807 high-quality 18S rRNA sequences (3,118,183 ± 209,081) were generated. These sequences were further categorized as 38,064 RSVs of 18S rRNA, with a length of 129.79 ± 22.5 bases. The Good's coverage was >99.99 (±0.0006%), suggesting that the sequencing depth of our metatranscriptome had sufficient coverage for the eukaryotic communities.

Taxonomic profiling revealed a total of 15 eukaryotic taxa at the phylum level as being identified, 12 of which were classified as being detected (**Figure 1**) according to the cutoff defined in the section "Materials and Methods." Each detected eukaryotic taxon was found in at least 45 out of 48 samples (>93.75%), confirming the presence of a core active rumen eukaryotic community, as reported previously for bacteria, archaea, and protozoa at the DNA level (Henderson et al., 2015). Notably, 43.62 ± 0.15% of sequences were assigned to unclassified eukaryotes (**Figure 1** and **Supplementary Table S1**), indicating that knowledge on taxonomic identification of rumen eukaryotes is still very limited. However, to better represent the active rumen eukaryotic communities and to reveal the potential associations between microbial taxa and host phenotype, all detected taxa, including the unnamed and/or unclassified taxa were included in the analysis.

Among the rest of 11 detectable active eukaryotic taxa, the *Ciliophora* (41.47 ± 1.43%), unclassified *SAR* (0.66 ± 0.04%), unclassified *Alveolata* (0.14 ± 0.02%), and *Parabasalia* (5.36 ± 1.35%) were categorized as protozoa (**Supplementary Table S1**), including ciliated (*Ciliophora*) and flagellated (*Parabasalia*) protozoa and their sister groups (Warren and Esteban, 2019). In addition, six taxa belonging to the kingdom *Fungi* were detected (**Supplementary Table S1**), including *Neocallimastigomycota* (2.00 ± 0.44%), *Basidiomycota* (1.07 ± 0.09%), unclassified *Fungi* (0.88 ± 0.20%), *Mucoromycota* (0.55 ± 0.03%), *Ascomycota* (0.09 ± 0.05%), and *Chytridiomycota* (0.008 ± 0.001%), with *Neocallimastigomycota* representing the rumen-specific anaerobic fungi and the others representing the aerobic fungi. In addition, the unclassified *Opisthokonta* was the relative group of kingdom *Fungi* (Torruella et al., 2012).

Although these core taxa were consistently presented among almost all animals, we observed noticeable individual variations in their abundances (coefficient of variation ranged from 2.4 to

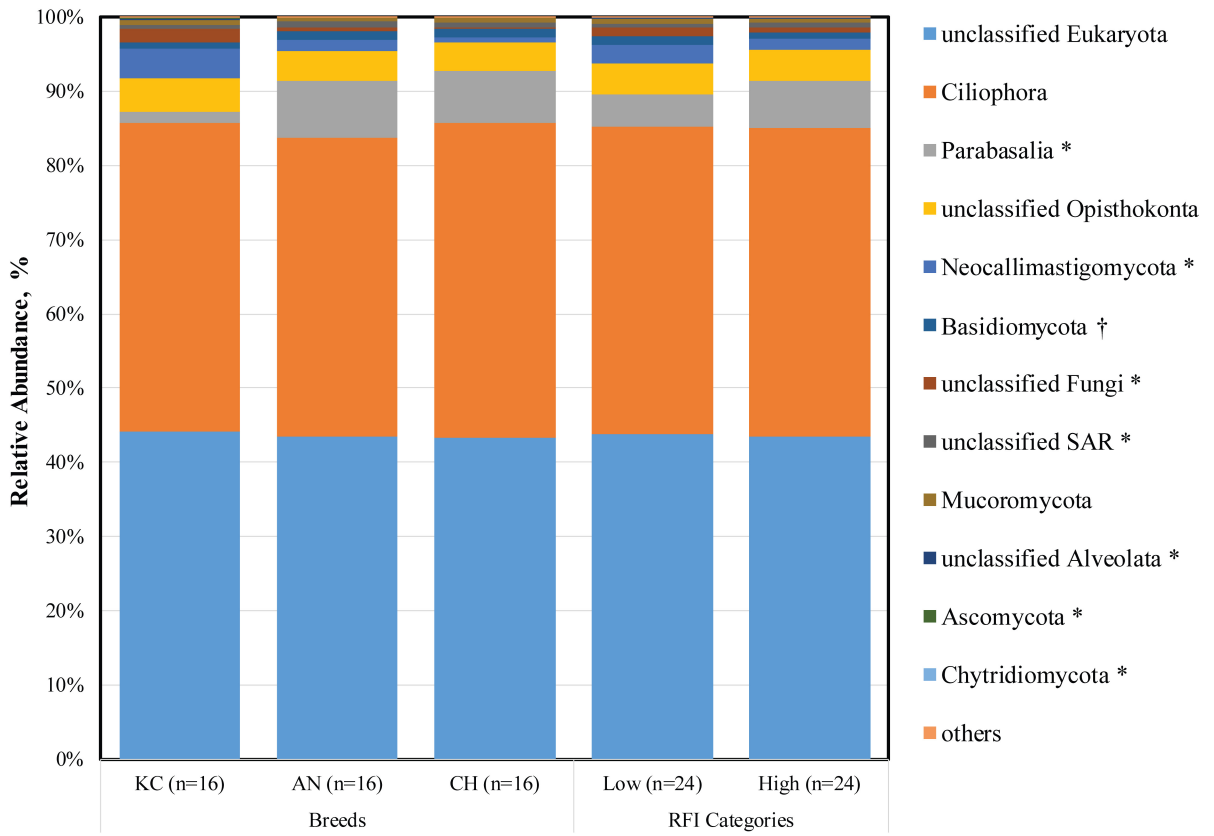

FIGURE 1 | Active rumen eukaryotic composition at the phylum level in beef cattle of three breeds and with divergent residual feed intake (RFI). Taxa name with asterisk (*) represents significant breed effect ($P < 0.05$), and taxa name with dagger (†) represents significant RFI effect ($P < 0.05$).

408.1%). Similar individual variations were reported in a recent study on active rumen prokaryotic microbiota (Li and Guan, 2017). Furthermore, consistent with our findings, Comtet-Marre et al. (2017) reported that the active rumen eukaryotic microbiota was dominated by ciliates (*Intramacronucleata*), flagellates (*Excavata*), and anaerobic fungi (*Neocallimastigomycota*). The authors investigated eukaryotic diversity in rumen samples obtained from a lactating dairy cow using metatranscriptomics, wherein the enriched V3–V4 regions of 18S rRNA were used for taxonomic annotation of eukaryotes. However, these studies did not try to address a potential commensal role of rumen flagellated protozoa or aerobic fungi, which was further explored in our study. This inconsistency might be due to the difference on the fraction of data being analyzed and on the focus of these studies. Earlier studies were mainly focused on rumen ciliates, and the taxonomic assignment was made based on sequence data generated using ciliate-specific marker gene (Kittelmann et al., 2013; Henderson et al., 2015; Kittelmann et al., 2015; Comtet-Marre et al., 2017). Although the rumen ciliate reference database used in these studies was based on a full-length 18S rRNA gene, it was built for the analysis of *Trichostomatia* and thus does not contain reference sequences of flagellated protozoa. Similar for rumen fungi, previous studies were all focused on anaerobic fungi. In our current study, the entirety of 18S rRNA gene sequences was extracted from the metatranscriptomic dataset and used for taxonomic analysis, which was more representative for overall rumen eukaryotic community. However, the metatranscriptome dataset-based taxonomic rumen eukaryota assessment may also be biased due to the depth of sequences, annotated 18S rRNA transcripts, and still limited information of full-length 18S rRNA genes in the SILVA database. To get better understanding of rumen eukaryotes, a combined approach is needed. It is necessary to generate and analyze the metatranscriptome data with SILVA first to understand the overall representation of all eukaryotes, and then the information can be used to discover marker genes for anaerobic fungi and ciliate protozoa in greater detail using the available fine-resolution frameworks.

Compositional Profiles of the Active Rumen Protozoal Community

As described above, *Ciliophora* and *Parabasalia* were the top two active rumen protozoal phyla detected in this study. Further analysis revealed a total of 11 and 7 taxa at the genus level being identified within the *Ciliophora* and *Parabasalia* phylum, respectively, with eight and five taxa being detected, respectively (**Figure 2**). Within the phylum *Ciliophora*, the top five abundant genera were unclassified *Trichostomatia* (26.90 ± 1.09%), *Ophryoscolex* (6.89 ± 0.42%), *Entodinium*

(3.90 ± 0.49%), *Isotricha* (3.72 ± 0.53%), and unclassified *Litostomatea* (1.12 ± 0.14%). Within the phylum *Parabasalia*, *Tetratrichomonas* (4.78 ± 1.26%), unclassified *Trichomonadea* (0.31 ± 0.07%), *Trichomitus* (0.12 ± 0.07%), and unclassified *Parabasalia* (0.10 ± 0.02%) were dominant and prevalent in all 48 samples (**Supplementary Table S1**).

As a phylum of ciliate protozoa, *Ciliophora* is distinguishable from other protozoa by a number of specialized features, including the procession of hair-like cilia, the presence of two types of nuclei at some stage in their life cycle, and a unique form of sexual reproduction called conjugation (Warren and Esteban, 2019). Ciliates are the most abundant protozoa found in the rumen of both domesticated and wild ruminants (Newbold et al., 2015), and they have been extensively studied as representatives of rumen protozoa because of their high prevalence (Newbold et al., 2015; Puniya et al., 2015). The present study further confirmed that ciliates were dominant in the active rumen eukaryotic community. The rumen ciliates were assigned based on the 18S rRNA sequence from the metatranscriptome dataset in this study, and 18S rRNA is the active center of protein synthesis in the 40S ribosomal subunit. Increased numbers of ribosomes, which lead to increased amounts of RNA transcription and protein synthesis, are presumed to be proportional to increases in 18S rRNA (Hayashi, 2014). Therefore, the high proportion of active rumen ciliates might suggest their importance in rumen metabolism.

To date, more than 250 species of ciliates belonging to 1 class (*Litostomatea*), 2 orders (*Vestibuliferida* and *Entodiniomorphida*), 16 families, and at least 25 genera have been identified in the forestomach and large intestine of herbivorous animals (Kittelmann and Janssen, 2011; Puniya et al., 2015). The present study also revealed that 99.99% of the 18S rRNA sequences from the identified active rumen ciliates belonged to the class *Litostomatea*, although sequences belonging to class *Spirotrichea* were also identified in five animals. However, only three genera (*Ophryoscolex*, *Entodinium*, and *Isotricha*) were annotated, and 52.81% of the active ciliates were categorized as the unclassified *Trichostomatia*. These observations might ascribe to the employed reference database (Silva 18S rRNA gene sequences; release 132), which contains very few almost-full-length entries that can serve as references for deeper-level phylogenetic identification of sequencing reads (Kittelmann and Janssen, 2011). A recent survey based on 18S rRNA gene amplicon sequencing revealed that almost all protozoal sequencing data from 742 rumen content samples worldwide were assigned to 12 genus-equivalent protozoal groups, namely, *Anoplodinium-Diplodinium*, *Enoploplastron*, *Entodinium*, *Epidinium*, *Eremoplastron-Diploplastron*, *Eudiplodinium*, *Metadinium*, *Ophryoscolex*, *Ostracodinium*, *Polyplastron*, *Dasytricha*, and *Isotricha* (Henderson et al., 2015). Similar rumen ciliate community structure has also been observed in cattle using high-throughput sequencing and microscopic methods (Kittelmann and Janssen, 2011; Kittelmann et al., 2015). The differences between metatranscriptomic and traditional identification methods further emphasize that more isolated and pure culture-based studies are needed to comprehensively characterize rumen eukaryotic microorganisms.

The *Parabasalia* are a clade of single-celled, anaerobic, flagellated protozoa that have evolved as symbionts or parasites in the digestive tracts of insects and vertebrates. Much of the known parabasalians occur in the gut of termites and their sister lineage, where they contribute to wood digestion as part of a complex microbial community. Compared to ciliated protozoa, the flagellated protozoa have been largely neglected, with very little information available about them (Williams et al., 1992). The present study revealed that active flagellates accounted for 11.45% of the total identified rumen protozoa, although considerable individual variation was observed (from 0.02 to 93.60%). Meanwhile, four taxa of parabasalians at the genus level were observed in all samples, suggesting that they are prevalent in the rumen of beef cattle. In addition, flagellated protozoa of the dominant genus *Tetratrichomonas* were also detected in the bovine rumen using traditional microscopy (Williams et al., 1992). To our knowledge, this is the first study to report the high prevalence of the parabasalians in the rumen of beef cattle. Furthermore, majority of the described parabasalians are obligate symbionts present in the gut of wood-eating insects and help their host to digest cellulose, in cooperation with the other microorganisms present in the intestine (Čepička et al., 2016). Similarly, ruminants also feed on plant structural carbohydrates such as lignin, cellulose, and hemicellulose, which can be hydrolyzed by the symbiotic microbiota *via* producing highly active lignocellulolytic enzymes (Puniya et al., 2015). Therefore, it is speculated that the flagellated protozoa are derived from feed and play an important role in fiber digestion.

Notably, correlation analysis revealed that there was a negative correlation between the relative abundance of phylum *Parabasalia* and *Ciliophora* ($R^2 = -0.83$, $P < 0.0001$). Previous studies have also shown that flagellates can be observed in the calf rumen in early life, but their numbers decrease when ciliated protozoa become established (Williams et al., 1992). These data suggest that both these two groups of protozoa may compete for existence in the rumen, although the mechanism is unclear. Considering the negative correlation between the two protozoal groups, along with the potential role of flagellates in the digestion of plant materials, adequate attention should be paid to the rumen flagellate community in future studies.

Compositional Profiles of the Active Rumen Fungi Community

Rumen fungi are generally considered as anaerobic fungi because they inhabit the rumen and alimentary tract of mammalian herbivores, which are typically deoxygenated. These anaerobic fungi are categorized as a distinct phylum, *Neocallimastigomycota*, which is the earliest diverging lineage unequivocally assigned to the kingdom *Fungi* (Gruninger et al., 2014). The anaerobic fungi are considered to contribute significantly to the overall metabolism of their host by playing a major role in the degradation of lignified plant tissues (Gruninger et al., 2014). Interestingly, in the present study, the phylum *Neocallimastigomycota* only accounted for 43.47% of the total fungal sequences, suggesting that the active rumen fungal community is more diverse than

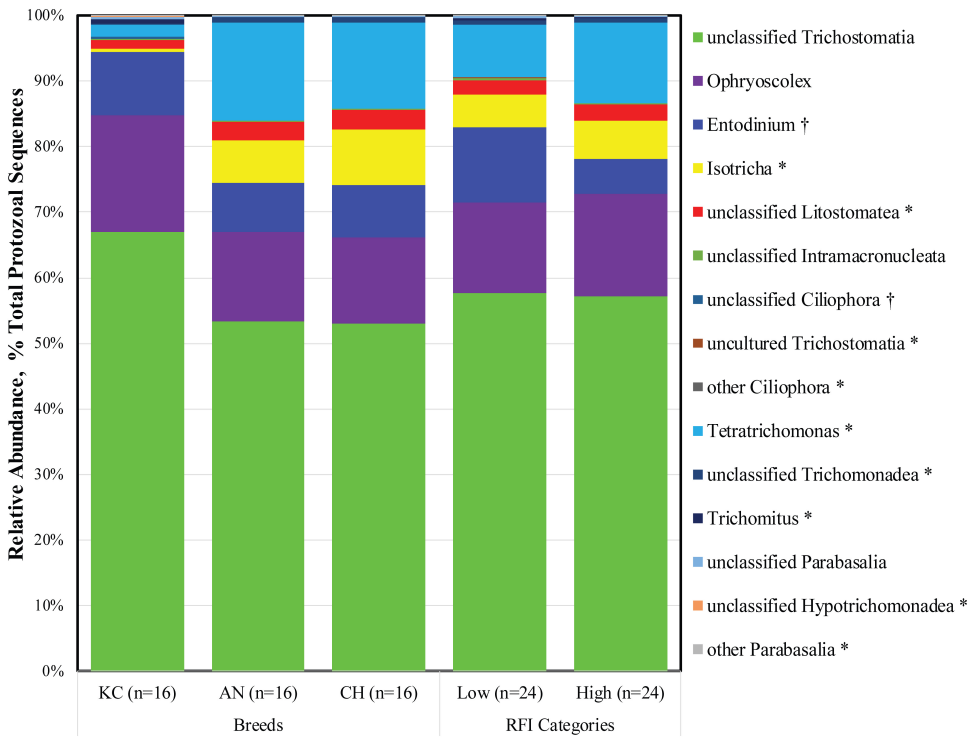

FIGURE 2 | Active rumen protozoal composition at the genus level in beef cattle of three breeds and with divergent residual feed intake (RFI). Taxa name with asterisk (*) represents significant breed effect ($P < 0.05$), and taxa name with dagger (†) represents significant RFI effect ($P < 0.05$).

previously appreciated. Meanwhile, substantial amounts of RSVs were classified as obligate aerobes belonging to the phyla *Basidiomycota, Mucoromycota*, *Ascomycota*, *Chytridiomycota, Mucoromycota*, and unclassified *Fungi*. Although how these aerobic microorganisms survive in the rumen remains unclear, it is speculated that they play a major role in scavenging oxygen entering the rumen. This might have beneficial effects on the anaerobic fermentation process for other obligately anaerobic microbes in the rumen.

At the genus level, a total of 26 fungal taxa were identified, of which 15 were considered as detected (**Figure 3**). Among them, five taxa belonged to the phylum *Neocallimastigomycota*, while others belonged to the aerobic fungal taxa. Unclassified *Neocallimastigaceae* (1.30 ± 0.31%), *Piromyces* (0.40 ± 0.07%), *Cyllamyces* (0.24 ± 0.06%), *Neocallimastix* (0.04 ± 0.01%), *Pleurotus* (1.04 ± 0.09%), unclassified *Fungi* (0.88 ± 0.20%), and *Hesseltinella* (0.55 ± 0.03%) were the top seven active taxa in the rumen fungal community (**Supplementary Table S1**). To date, 10 genera, including monocentric *Piromyces*, *Neocallimastix*, *Caecomyces*, *Buwchfawromyces*, *Oontomyces*, *Pecoramyces*, *Feramyces*, and polycentric *Cyllamyces*, *Orpinomyces*, and *Anaeromyces*, have been described under the phylum *Neocallimastigomycota* according to culture-based and culture-independent approaches (Gruninger et al., 2014; Hanafy et al., 2017, 2018a,b). Among them, four genera, including *Piromyces*, *Neocallimastix*, *Cyllamyces*, and *Pecoramyces*, along with unclassified *Neocallimastigaceae*, were identified as the active fungal taxa present in at least 32 of the total 48 samples in this study.

As the most prevalent rumen aerobic fungi, the genus *Pleurotus* and *Hesseltinella* belonging to the phylum *Basidiomycota* and *Mucoromycota* accounted for 22.66 and 11.91% of the total active rumen fungal sequences, respectively. Although, to the best of our knowledge, this is the first study in which active aerobic fungi were observed in the rumen, we believed that these fungi are true rumen colonizers. First, these fungi were identified from metatranscriptomic rather than metagenomic datasets, suggesting that they are live microorganisms in the rumen rather than dead or inactive microbes from the diets. Second, the prevalence and substantial proportion of *Pleurotus* (1.04%) and *Hesseltinella* (0.55%) in the active rumen eukaryotic microbiome suggested that these fungal sequences were not derived from contingent or chimera sequences generated from RNA-seq. Third, the rumen is not an obligate anaerobic environment as some amounts of oxygen enter the rumen along with the feed (Santra and Karim, 2003), facilitating the survival of these aerobic fungi. Additionally, the genus *Pleurotus* is one of the most efficient white rot fungi lignocellulose degrader and is generally cultivated on non-composted lignocellulosic substrates (Bellettini et al., 2019). The *Hesseltinella* is a genus of fungi under the order *Mucorales*, comprising predominantly saprotrophs inhabiting soil, drug, and dead plant material, as well as several parasites on plants (Walther et al., 2013). Thus,

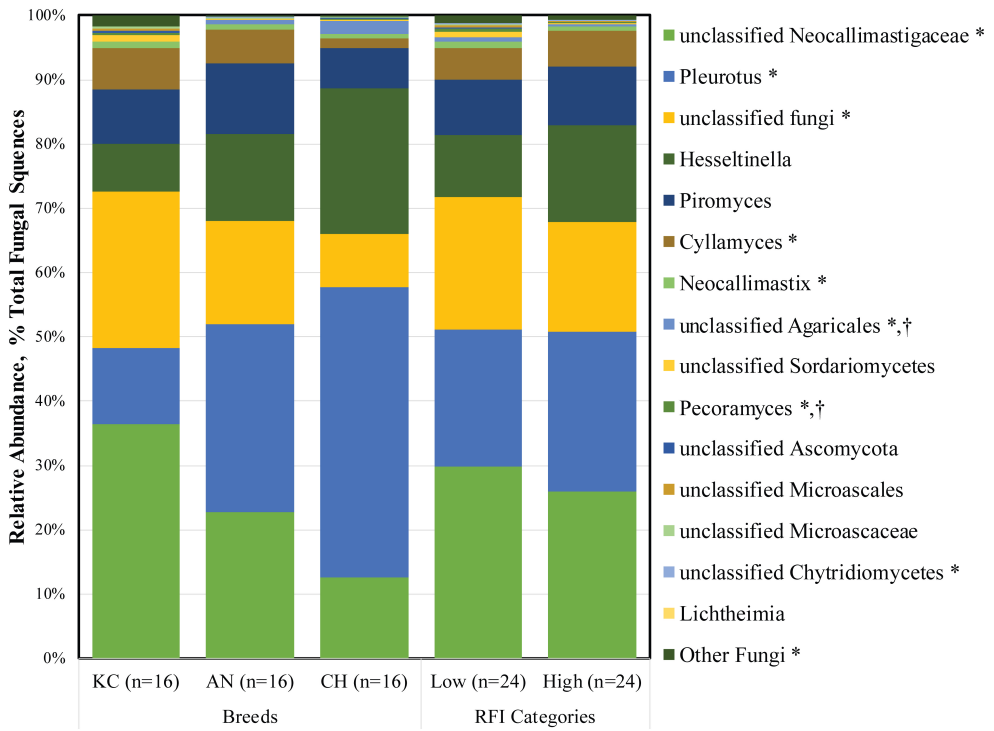

FIGURE 3 | Active rumen fungal composition at the genus level in beef cattle of three breeds and divergent residual feed intake (RFI). Taxa name with asterisk (*) represents significant breed effect ($P < 0.05$), and taxa name with dagger (†) represents significant RFI effect ($P < 0.05$).

these fungi could be ingested by the ruminants along with the plant material, thereby colonizing in the rumen. Moreover, the genus *Pleurotus* has exceptional ligninolytic properties by which it cleavages cellulose, hemicellulose, and lignin from wood (Machado et al., 2016). Mucoralean strains have been used for centuries in the fermentation of traditional Asian and African food (Nout and Aidoo, 2010), and they also play a role in the production of various kinds of cheese. Therefore, it is expected that the presence of active *Pleurotus* and *Hesseltinella* species in the rumen might contribute to the degradation of dietary fiber, although their role in the rumen is currently unknown. Considering their potential role in the degradation of lignocellulosic plant fiber and scavenging of oxygen that enters the rumen, the functionality and metabolism of these aerobic fungi warrant further study.

Effect of Breed on the Rumen Eukaryotic Microbiome

Phenotypic data, including RFI, ADG, and DMI, were obtained from a study by Li et al. (2019a) and were reanalyzed to compare the overall difference among three breeds using the mixed effect model as described in the section "Statistical Analysis." Results showed that no interaction effect between breed and feed efficiency ($P > 0.05$) was observed. The RFI values were not affected by breed ($P = 0.12$). However, KC steers had a lower DMI ($P < 0.05$) than the other two breeds. Meanwhile, AN steers had a higher ADG ($P < 0.05$) than KC steers, but the ADG of both these breeds was not significantly different ($P > 0.05$) from that of CH steers. To comprehensively investigate the effects of breed and RFI on rumen eukaryotic diversity, we treated the eukaryotic microbiota as a whole to combine both protozoal and fungal datasets. The rarefaction curves based on the Shannon index (**Supplementary Figure S1A**) and Faith's PD (**Supplementary Figure S1B**) for each sample appeared to level out as the sampling depth outnumbered 300,000, indicating that the lowest sequence count of the samples (549,095) in this study was sufficient to assess ruminal eukaryotic diversity.

Venn diagram analysis showed that a total of 5,015 RSVs were commonly presented in cattle rumen of all the three breeds, and 15,907, 7,096, and 5,001 RSVs were uniquely identified in cattle rumen of KC, AN, and CH steers, respectively (**Supplementary Figure S2A**). Further analysis showed that breed had no effect on the alpha-diversity indices ($P > 0.05$) (**Table 1**), suggesting that there was no significant difference in active eukaryotic richness among the three breeds. However, PCoA based on unweighted UniFrac distance showed that these samples were clustered by breed (PERMANOVA; $P = 0.001$; **Figure 4A**), indicating that the phylogenetic lineages of rumen eukaryotes among the three breeds were significantly distinct from each other. However, this distinctiveness was reduced when taking the relative abundance of RSVs into consideration, as demonstrated by weighted UniFrac distance PCoA plots (PERMANOVA; $P = 0.02$; **Figure 4B**), where only active rumen eukaryotic

TABLE 1 | Effects of breed and RFI on the alpha diversity indices of rumen eukaryotic communities.

Items	Breed[1]			RFI[2]		SEM	*P*-value		
	AN	CH	KC	High	Low		Breed	RFI	Breed × RFI
Pielou's evenness	0.78	0.79	0.76	0.77	0.78	0.01	0.09	0.32	0.65
Faith's PD	137	126	143	124^{b}	147^{a}	5	0.24	<0.01	0.06
Observed OTUs	1,937	2,106	2,287	1,921^{b}	2,299^{a}	73	0.11	<0.01	0.21
Shannon index	8.60	8.58	8.49	8.40^{b}	8.72^{a}	0.07	0.75	0.02	0.37

[1]AN, CH, and KC represent Angus, Charolais, and Kinsella Composite steers, respectively. [2]OTUs, operational taxonomic units; PD, phylogenetic diversity; RFI, residual feed intake; values within each row with different superscripts are different.

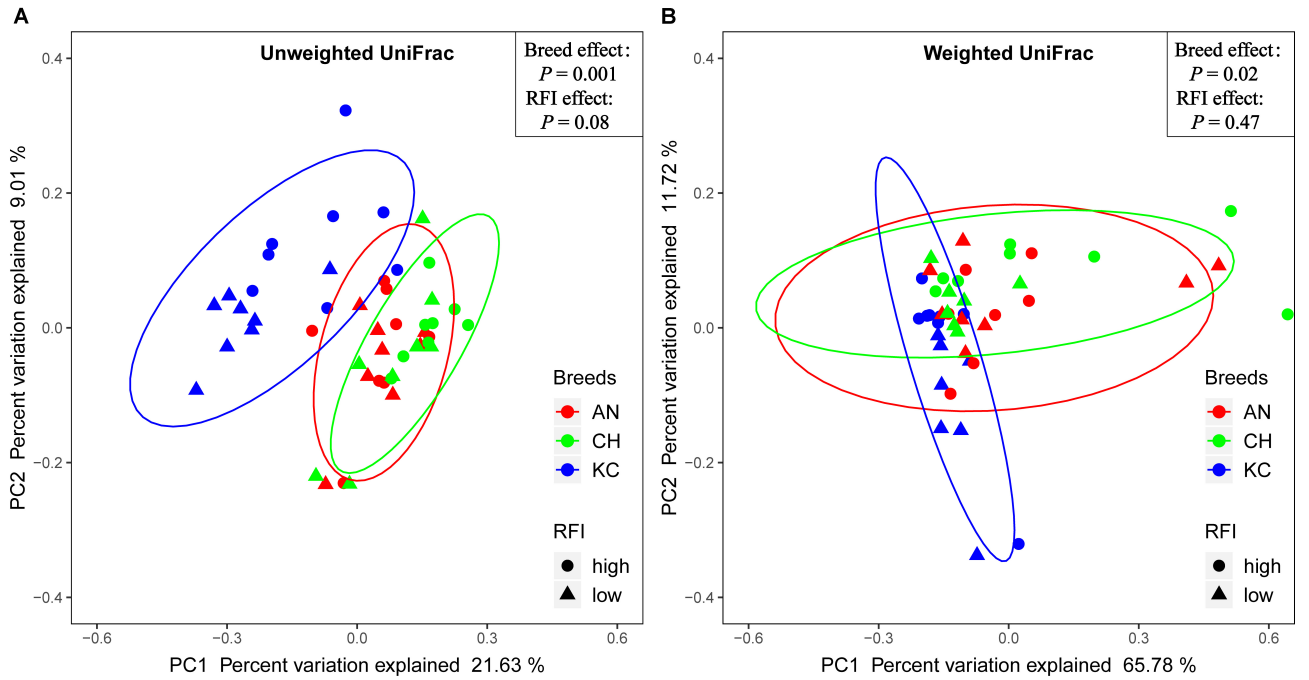

FIGURE 4 | Similarity of the rumen eukaryotic profiles between beef cattle with divergent residual feed intake (RFI) from three breeds. Distance between samples based on similarity in representative sequence variants (RSVs) composition was calculated using unweighted UniFrac **(A)** and weighted UniFrac distance **(B)** and visualized using principal coordinate analysis (PCoA) plots. The impact of breed and RFI on the clustering pattern of microbial communities was tested using PERMANOVA. The AN, CH, and KC represent Angus, Charolais, and Kinsella Composite beef steers, respectively. The ovals in blue, red, and green represent 95% confidence interval of KC, AN, and CH, respectively.

profiles of KC were separated from those of the other two breeds (PERMANOVA; P = 0.0015). This result was expected because unweighted UniFrac distance only considers species presence/absence information in a microbial community, while weighted UniFrac incorporates RSVs abundance information (Chen et al., 2012), and thus the high proportion of the overlapped unclassified eukaryotes across all samples decreases the power over weighted UniFrac distance for detecting the differences among microbial communities (Chen et al., 2012). Hence, our results suggest that breed has no effect on species richness but significantly affects the PD of the rumen eukaryotic community.

Differential abundance analysis showed that the relative abundance of 26 eukaryotic taxa was affected by breed ($P < 0.05$, **Supplementary Table S1**). Considering that most of the active eukaryotic taxa were not affected by the interaction between breed and RFI ($P > 0.05$, **Supplementary Table S1**), a more stringent LEfSe analysis was performed to identify differentially abundant taxa among the three breeds. Results revealed that seven taxa detected at the genus level were affected by breed (LDA score > 2 and $P < 0.05$), with KC steers exhibiting high abundance of unclassified *Neocallimastigaceae*, *Pecoramyces*, and *Cyllamyces*, etc. (**Figure 5A**). Noticeably, all these seven taxa belonged to the rumen fungi, suggesting that the fungal community was more active in the rumen of KC steers than that in the rumen of AN and CH steers.

Similar host genetic background effects on gut microbial profiles have been observed in recent studies based on amplicon sequencing, in which samples from various mammals were investigated (Henderson et al., 2015; Paz et al., 2016;

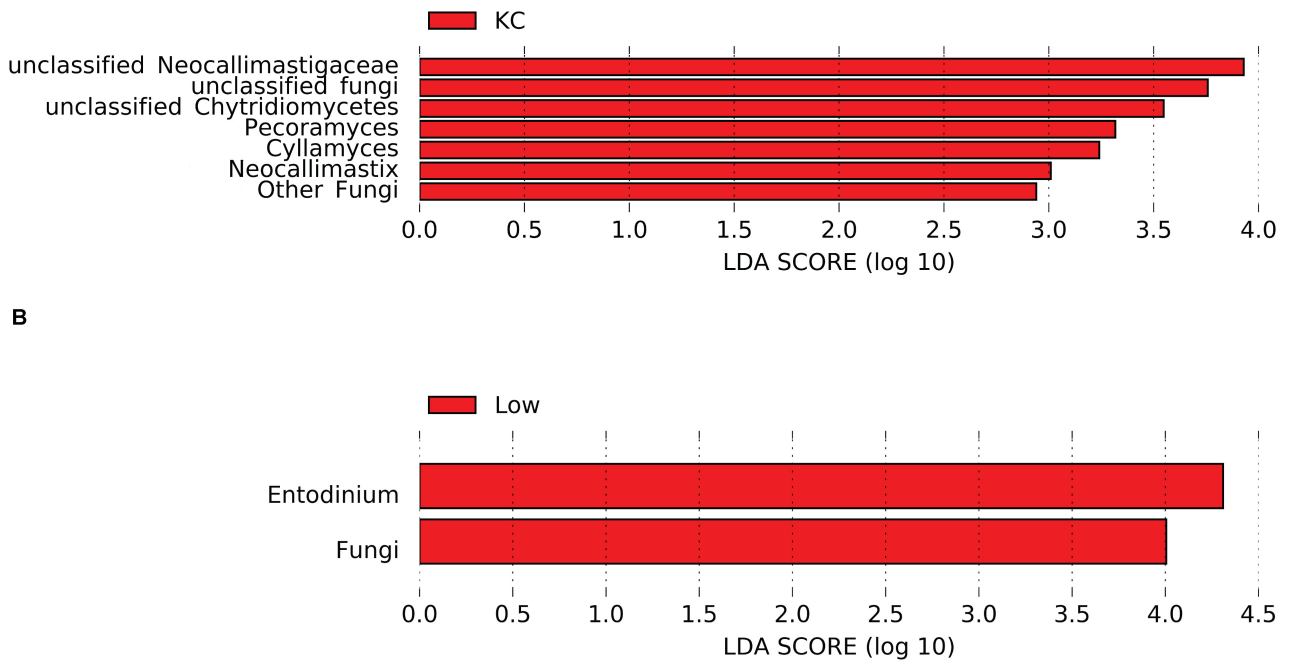

FIGURE 5 | Differential rumen eukaryotic features between the three beef cattle breeds **(A)** and high- and low-residual feed intake (RFI) steers **(B)** by linear discriminant analysis effect size (LEfSe). KC, Kinsella Composite beef steers; Low, steers with low RFI.

Groussin et al., 2017). Furthermore, a recent metatranscriptome-based study also revealed that breed did not influence the richness of active bacterial and archaeal communities, while the active rumen prokaryotic communities in KC were distinct from those in AN and CH steers (Li et al., 2019a) from the same metatranscriptomic datasets. These studies suggest that host genetic background can influence the activity of the entire rumen microbiota, including both prokaryotes and eukaryotes. Nevertheless, our knowledge on the role of host in regulating microbiota is currently limited. Several breed-associated biological factors, such as eating frequency, DMI, and rumen size potentially contribute to the rumen microbiota variations observed among various breeds, as discussed by Li et al. (2019a). However, the mechanisms by which these factors influence the microbial community have not been well described. Therefore, further studies to link these biological factors to eukaryotic compositional profiles are needed to better understand the effect of breed on rumen microbiota.

Effect of Residual Feed Intake on the Rumen Eukaryotic Microbiome

Phenotypic data, including RFI, ADG, and DMI were obtained from a study by Li et al. (2019a) and were reanalyzed to compare the overall difference between high- and low-RFI categories, as mentioned above. Results showed that the RFI values were significantly different between high- and low-RFI steers (0.86 vs. −0.78 kg/day; $P < 0.0001$), and high-RFI animals had a higher DMI than low-RFI individuals (10.73 vs. 9.55 kg/day; $P < 0.0001$). Venn diagram analysis showed that a total of 10,808 RSVs were commonly presented in cattle rumen of all RFI groups, and 16,598 and 10,658 RSVs were uniquely identified in rumen of low- and high-RFI steers, respectively (**Supplementary Figure S2B**). Moreover, majority of the alpha diversity indices were affected by RFI, with Faith's PD, observed OTUs, and Shannon index in low-RFI steers being higher ($P < 0.05$) than those in high-RFI steers (**Table 1**). These results suggest that low-RFI steers exhibit higher eukaryotic richness in the rumen compared with high-RFI steers. However, the PCoA did not show a clear separation of the active rumen eukaryotic community between high- and low-RFI steers based on the unweighted and weighted UniFrac matrices of RSVs (**Figure 4**). In addition, PERMANOVA did not test the statistical differences ($P > 0.05$) in the eukaryotic communities between high- and low-RFI steers, although a trend ($P = 0.08$) of difference was observed between these two groups based on the unweighted UniFrac distance.

Differential abundance analysis showed that the relative abundance of seven eukaryotic taxa was affected by RFI ($P < 0.05$, **Supplementary Table S1**). The more stringent LEfSe analysis further confirmed that the relative abundance of two eukaryotic taxa (kingdom *Fungi* and genus *Entodinium*) was significantly different (LDA score > 2 and $P < 0.05$) between steers with divergent RFI (**Figure 5B**). Low-RFI steers had an approximately twofold higher abundance of

active kingdom *Fungi* and genus *Entodinium* (5.52 ± 1.03 and 5.23 ± 0.74%, respectively) in the rumen than high-RFI steers (3.66 ± 0.84 and 2.58 ± 0.53%, respectively). As discussed above, most of the detected rumen fungal taxa had the ability to degrade the recalcitrant lignocellulosic components of the feed particles by producing all the enzymes necessary for plant material decomposition, including cellulolytic and hemicellulolytic enzymes. These hydrolases enable the rumen fungi to penetrate the plant cell walls, access fermentable substrates not available to surface-acting bacteria, colonize the sturdy plant structures, and weaken and degrade the plant tissues, thus reducing the size of plant particles (Puniya et al., 2015). Meanwhile, the invasive rhizoidal growth of rumen fungi possibly aids substrate decomposition mechanically (Gruninger et al., 2014). Enzymatic and mechanical degradation of plant material by anaerobic fungi provides an increased surface area for bacterial colonization, resulting in an increase in the degradation of plant cell wall (Gruninger et al., 2014). Therefore, the high abundance of active rumen fungi in low-RFI steers may provide increased energy to hosts from the fermentation of structural polysaccharides, leading to a high feed efficiency. As an indirect proof, it has been demonstrated that inclusion of cultures of anaerobic fungi in the diets of various ruminants improved feed intake, animal growth rate, feed efficiency, and milk production (Lee et al., 2000; Saxena et al., 2010; Paul et al., 2011).

Additionally, a high abundance of *Entodinium* was detected in the rumen of low-RFI animals. Inconsistent with the findings of our study, Carberry et al. (2012) reported that the RFI phenotype had no effect on the relative abundance of genus *Entodinium*, as indicated by qPCR analysis. This inconsistency might be due to the differences in genetic material employed (DNA vs. RNA) and targeted microbial populations (several microbial taxa vs. entire active eukaryotic microbiome). Members belonging to the genus *Entodinium* are the smallest, simplest, and most common rumen ciliates and are found in almost all ruminants (Williams et al., 1992). *Entodinium* species are known starch degraders, and starch is essential for their maintenance and growth (Williams et al., 1992). A recent microscopy-based study found that the proportion of *Entodinium* in the total rumen protozoal community could reach 93% in the rumen of beef cattle fed a high concentrate-based diet (Yuste et al., 2019). The starch engulfed by *Entodinium* species was fermented into acetic acid and butyric acids as the major end products (together 80–90% of the total volatile fatty acids), accompanied by the production of lesser amounts of formic acid and propionic acid and some carbon dioxide and hydrogen (Abmu Akkada and Howard, 1960). Furthermore, it was shown that ruminal starch digestibility was increased from 84 (in the absence of protozoa) to 89% (in the presence of *Entodinium* species) (Veira et al., 1983). In the present study, the finishing diet consisted mainly of barley grain and barley silage, which were rich in starch. Therefore, the increased abundance of active *Entodinium* may provide more energy to hosts from the fermentation of starch, resulting in a high feed efficiency. On the other hand, *Entodinium* species have a substantial potential to engulf and degrade bacteria (Belanche et al., 2012) and metabolize lactic acid (Williams et al., 1992). Although the engulfment of bacteria by *Entodinium* species has been proved to be responsible for much of the bacterial protein turnover (Newbold et al., 2015), the loss of bacterial nitrogen due to protozoal activity can be compensated by the increased digestibility of protozoal bodies (Williams et al., 1992). Furthermore, the protozoal fermentation of the ingested starch is less rapid, and a detrimental volatile fatty acid buildup does not occur (Williams et al., 1992). Therefore, a higher abundance of *Entodinium* species may have a significant stabilizing effect on ruminal fermentation and might play an important role in regulating ruminal lactate metabolism and preventing the occurrence of lactic acid acidosis. This is important to animals receiving high-energy diets and contributes to the high feed efficiency of the steers in the current study.

CONCLUSION

In the current study, we investigated the compositional characteristics of active rumen eukaryotic microbiome using metatranscriptomics and revealed a core active eukaryotic community in the rumen of beef steers. Taxonomic analysis revealed that the rumen eukaryotic community was more diverse than previously reported, and the identification of flagellated protozoa and aerobic fungi suggested that these previously unreported eukaryotic microbes could also play a role in rumen function. However, our results also highlight the need to characterize more eukaryotic genomes from rumen to further dissect their exact roles in the rumen microbiome. Further analysis revealed distinguishable rumen eukaryotic microbiota among the three beef cattle breeds. These breed-associated differences represent potential superiorities of each breed, which could further be applied to manipulate the rumen microbiome through selective breeding of the cattle. Moreover, two differentially abundant microbial taxa, the active protozoal genus *Entodinium* and the kingdom *Fungi*, were identified between steers with divergent RFIs, suggesting that these eukaryotes may contribute to variations in the feed efficiency of beef cattle. Overall, these findings extend our understanding on rumen eukaryotic communities and highlight the possibility of improving feed efficiency through altering rumen eukaryotic microbiota.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Sequence Read Archive (SRA) of National Center for Biotechnology Information (NCBI) under accession number PRJNA448333.

ETHICS STATEMENT

The animal study was reviewed and approved by the Livestock Care Committee of the University of Alberta (No. AUP00000882).

AUTHOR CONTRIBUTIONS

YZ, FL, QM, and LG designed this study. FL and YC performed the RNA isolation and sequencing of library construction. YZ, FL, and LG conducted the bioinformatics and statistical analyses and data interpretation. YZ, QM, HW, and LG were responsible for the manuscript writing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Abmu Akkada, A. R., and Howard, B. H. (1960). The biochemistry of rumen protozoa, 3. The carbohydrate metabolism of entodinium. *Biochem. J.* 76, 445–451. doi: 10.1042/bj0760445

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austr. Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Basarab, J. A., Colazo, M. G., Ambrose, D. J., Novak, S., Robertson, K., McCartney, D., et al. (2011). Relationships between residual feed intake and fertility in heifers. *Can. J. Anim. Sci.* 91, 573–584. doi: 10.4141/CJAS2011-010

Belanche, A., de la Fuente, G., Moorby, J. M., and Newbold, C. J. (2012). Bacterial protein degradation by different rumen protozoal groups. *J. Anim. Sci.* 90, 4495–4504. doi: 10.2527/jas.2012-5118

Bellettini, M. B., Fiorda, F. A., Maieves, H. A., Teixeira, G. L., Ávila, S., Hornung, P. S., et al. (2019). Factors affecting mushroom *Pleurotus* spp. *Saudi J. Biol. Sci.* 26, 633–646. doi: 10.1016/j.sjbs.2016.12.005

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Carberry, C. A., Kenny, D. A., Han, S., McCabe, M. S., and Waters, S. M. (2012). Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Appl. Environ. Microb.* 78, 4949–4958. doi: 10.1128/AEM.07759-11

Čepička, I., Dolan, M. F., and Gile, G. H. (2016). "Parabasalia," in *Handbook of the Protists*, eds M. Archibald, A. G. B. Simpson, C. H. Slamovits, L. Margulis, M. Melkonian, D. J. Chapman, et al. (Cham: Springer International Publishing), 1–44. doi: 10.1007/978-3-319-32669-6-9-1

Chen, J., Bittinger, K., Charlson, E. S., Hoffmann, C., Lewis, J., Wu, G. D., et al. (2012). Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics* 28, 2106–2113. doi: 10.1093/bioinformatics/bts342

Comtet-Marre, S., Parisot, N., Lepercq, P., Chaucheyras-Durand, F., Mosoni, P., Peyretaillade, E., et al. (2017). Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. *Front. Microbiol.* 8:67. doi: 10.3389/fmicb.2017.00067

Groussin, M., Mazel, F., Sanders, J. G., Smillie, C. S., Lavergne, S., Thuiller, W., et al. (2017). Unraveling the processes shaping mammalian gut microbiomes over evolutionary time. *Nat. Commun.* 8:14319. doi: 10.1038/ncomms14319

Gruninger, R. J., Puniya, A. K., Callaghan, T. M., Edwards, J. E., Youssef, N., Dagar, S., et al. (2014). Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol. Ecol.* 90, 1–17. doi: 10.1111/1574-6941.12383

Guan, L. L., Nkrumah, J. D., Basarab, J. A., and Moore, S. S. (2008). Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. *FEMS Microbiol. Lett.* 288, 85–91. doi: 10.1111/j.1574-6968.2008.01343.x

Hanafy, R. A., Elshahed, M. S., Liggenstoffer, A. S., Griffith, G. W., and Youssef, N. H. (2017). *Pecoramyces ruminantium*, gen. nov., sp. nov., an anaerobic gut fungus from the feces of cattle and sheep. *Mycologia* 109, 231–243. doi: 10.1080/00275514.2017.1317190

Hanafy, R. A., Elshahed, M. S., and Youssef, N. H. (2018a). *Feramyces austinii*, gen. nov., sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild Barbary sheep and fallow deer. *Mycologia* 110, 513–525. doi: 10.1080/00275514.2018.1466610

Hanafy, R. A., Johnson, B., Elshahed, M. S., and Youssef, N. H. (2018b). *Anaeromyces contortus*, sp. nov., a new anaerobic gut fungal species (Neocallimastigomycota) isolated from the feces of cow and goat. *Mycologia* 110, 502–512. doi: 10.1080/00275514.2018.1465773

Hayashi, R. (2014). "Gene expression and the impact of antioxidant supplements in the cataractous lens," in *Handbook of Nutrition, Diet and the Eye*, ed. V. R. Preedy (San Diego: Academic Press), 517–524. doi: 10.1016/B978-0-12-401717-7.00052-6

Hegarty, R. S., Goopy, J. P., Herd, R. M., and McCorkell, B. (2007). Cattle selected for lower residual feed intake have reduced daily methane production. *J. Anim. Sci.* 85, 1479–1486. doi: 10.2527/jas.2006-236

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Zhou, M., Moore, S. S., and Guan, L. L. (2013). Influence of sire breed on the interplay among rumen microbial populations inhabiting the rumen liquid of the progeny in beef cattle. *PLoS One* 8:e58461. doi: 10.1371/journal.pone.0058461

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., et al. (2018). Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9:2161. doi: 10.3389/fmicb.2018.02161

Jewell, K. A., McCormick, C. A., Odt, C. L., Weimer, P. J., and Suen, G. (2015). Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. *Appl. Environ. Microb.* 81, 4697–4710. doi: 10.1128/AEM.00720-15

Karisa, B., Moore, S., and Plastow, G. (2014). Analysis of biological networks and biological pathways associated with residual feed intake in beef cattle. *Anim. Sci. J.* 85, 374–387. doi: 10.1111/asj.12159

Kenny, D. A., Fitzsimons, C., Waters, S. M., and McGee, M. (2018). Invited review: improving feed efficiency of beef cattle – the current state of the art and future challenges. *Animal* 12, 1815–1826. doi: 10.1017/S1751731118000976

Kittelmann, S., Devente, S. R., Kirk, M. R., Seedorf, H., Dehority, B. A., and Janssen, P. H. (2015). Phylogeny of intestinal ciliates, including charonina ventriculi, and comparison of microscopy and 18S rRNA gene pyrosequencing for rumen ciliate community structure analysis. *Appl. Environ. Microb.* 81, 2433–2444. doi: 10.1128/AEM.03697-14

Kittelmann, S., and Janssen, P. H. (2011). Characterization of rumen ciliate community composition in domestic sheep, deer, and cattle, feeding on varying diets, by means of PCR-DGGE and clone libraries. *FEMS Microbiol. Ecol.* 75, 468–481. doi: 10.1111/j.1574-6941.2010.01022.x

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., et al. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One* 8:e47879. doi: 10.1371/journal.pone.0047879

Kong, R., Liang, G., Chen, Y., Stothard, P., and Guan, L. L. (2016). Transcriptome profiling of the rumen epithelium of beef cattle differing in residual feed intake. *BMC Genom.* 17:192. doi: 10.1186/s12864-016-2935-4

Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28, 3211–3217. doi: 10.1093/bioinformatics/bts611

Lee, S. S., Ha, J. K., and Cheng, K. J. (2000). Influence of an anaerobic fungal culture administration on in vivo ruminal fermentation and nutrient digestion. *Anim. Feed Sci. Tech.* 88, 201–217. doi: 10.1016/S0377-8401(00)00216-9

Li, F., and Guan, L. L. (2017). Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl. Environ. Microb.* 83:e0061-17. doi: 10.1128/AEM.00061-17

Li, F., Henderson, G., Sun, X., Cox, F., Janssen, P. H., and Guan, L. L. (2016). Taxonomic assessment of rumen microbiota using total RNA and targeted amplicon sequencing approaches. *Front. Microbiol.* 7:987. doi: 10.3389/fmicb.2016.00987

Li, F., Hitch, T. C. A., Chen, Y., Creevey, C. J., and Guan, L. L. (2019a). Comparative metagenomic and metatranscriptomic analyses reveal the breed effect on the rumen microbiome and its associations with feed efficiency in beef cattle. *Microbiome* 7:6. doi: 10.1186/s40168-019-0618-5

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., et al. (2019b). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7:92. doi: 10.1186/s40168-019-0699-1

Machado, A. R. G., Teixeira, M. F. S., de Souza Kirsch, L., Campelo, M. D. C. L., and de Aguiar Oliveira, I. M. (2016). Nutritional value and proteases of *Lentinus citrinus* produced by solid state fermentation of lignocellulosic waste from tropical region. *Saudi J. Biol. Sci.* 23, 621–627. doi: 10.1016/j.sjbs.2015.07.002

Myer, P. R., Smith, T. P. L., Wells, J. E., Kuehn, L. A., and Freetly, H. C. (2015). Rumen microbiome from steers differing in feed efficiency. *PLoS One* 10:e129174. doi: 10.1371/journal.pone.0129174

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., and McEwan, N. (2015). The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6:1313. doi: 10.3389/fmicb.2015.01313

Nkrumah, J. D., Okine, E. K., Mathison, G. W., Schmid, K., Li, C., Basarab, J. A., et al. (2006). Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle1. *J. Anim. Sci.* 84, 145–153. doi: 10.2527/2006.841145x

Nout, M. J. R., and Aidoo, K. E. (2010). "Asian fungal fermented food," in *Industrial Applications*, 10th Edn, ed. M. Hofrichter (Berlin: Springer), doi: 10.1007/978-3-642-11458-8-2

Paul, S. S., Deb, S. M., Punia, B. S., Das, K. S., Singh, G., Ashar, M. N., et al. (2011). Effect of feeding isolates of anaerobic fungus *Neocallimastix* sp. *CF* 17 on growth rate and fibre digestion in buffalo calves. *Arch. Anim. Nutr.* 65, 215–228. doi: 10.1080/1745039X.2011.559722

Paz, H. A., Anderson, C. L., Muller, M. J., Kononoff, P. J., and Fernando, S. C. (2016). Rumen bacterial community composition in holstein and jersey cows is different under same dietary condition and is not affected by sampling method. *Front. Microbiol.* 07:1026. doi: 10.3389/fmicb.2016.01206

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., et al. (2011). Scikit-learn: machine learning in python. *J. Mach. Learn. Res.* 12, 2825–2830.

Puniya, A. K., Singh, R., and Kamra, D. N. (2015). *Rumen Microbiology: From Evolution to Revolution*. Berlin: Springer.

Richardson, E. C., and Herd, R. M. (2004). Biological basis for variation in residual feed intake in beef cattle. 2. Synthesis of results following divergent selection. *Aust. J. Exp. Agric.* 44, 431–440. doi: 10.1071/EA02221

Santra, A., and Karim, S. A. (2003). Rumen manipulation to improve animal productivity. *Asian Austr. J. Anim.* 16, 748–763. doi: 10.5713/ajas.2003.748

Saxena, S., Sehgal, J., Puniya, A., and Singh, K. (2010). Effect of administration of rumen fungi on production performance of lactating buffaloes. *Benef. Microb.* 1, 183–188. doi: 10.3920/BM2009.0018

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shabat, S. K., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg, M. M., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. doi: 10.1038/ismej.2016.62

Torruella, G., Derelle, R., Paps, J., Lang, B. F., Roger, A. J., Shalchian-Tabrizi, K., et al. (2012). Phylogenetic relationships within the opisthokonta based on phylogenomic analyses of conserved single-copy protein domains. *Mol. Biol. Evol.* 29, 531–544. doi: 10.1093/molbev/msr185

Veira, D. M., Ivan, M., and Jui, P. Y. (1983). Rumen ciliate protozoa: effects on digestion in the stomach of sheep. *J. Dairy Sci.* 66, 1015–1022. doi: 10.3168/jds.S0022-0302(83)81896-7

Walther, G., Pawłowska, J., Alastruey-Izquierdo, A., Wrzosek, M., Rodriguez-Tudela, J. L., Dolatabadi, S., et al. (2013). DNA barcoding in mucorales: an inventory of biodiversity. *Persoonia* 30, 11–47. doi: 10.3767/003158513X665070

Warren, A., and Esteban, G. F. (2019). "Protozoa," in *Thorp and Covich's Freshwater Invertebrates*, 4th Edn, eds D. C. Rogers and J. H. Thorp (Boston: Academic Press), 7–42. doi: 10.1016/B978-0-12-385024-9.00002-2

Williams, A. G., Coleman, G. S., Brock, T. D., and Service, S. O. (1992). *Rumen Protozoa*. New York, NY: Springer.

Yuste, S., Amanzougarene, Z., de la Fuente, G., de Vega, A., and Fondevila, M. (2019). Rumen protozoal dynamics during the transition from milk/grass to high-concentrate based diet in beef calves as affected by the addition of tannins or medium-chain fatty acids. *Anim. Feed Sci. Tech.* 257:114273. doi: 10.1016/j.anifeedsci.2019.114273

Zhou, M., Hernandez-Sanabria, E., and Guan, L. L. (2009). Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Appl. Environ. Microb.* 75, 6524–6533. doi: 10.1128/AEM.02815-08

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frontiers in Microbiology

REVIEW
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Rumen Virus Populations: Technological Advances Enhancing Current Understanding

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The rumen contains a multi-kingdom, commensal microbiome, including protozoa, bacteria, archaea, fungi and viruses, which enables ruminant herbivores to ferment and utilize plant feedstuffs that would be otherwise indigestible. Within the rumen, virus populations are diverse and highly abundant, often out-numbering the microbial populations that they both predate on and co-exist with. To date the research effort devoted to understanding rumen-associated viral populations has been considerably less than that given to the other microbial populations, yet their contribution to maintaining microbial population balance, intra-ruminal microbial lysis, fiber breakdown, nutrient cycling and genetic transfer may be highly significant. This review follows the technological advances which have contributed to our current understanding of rumen viruses and drawing on knowledge from other environmental and animal-associated microbiomes, describes the known and potential roles and impacts viruses have on rumen function and speculates on the future directions of rumen viral research.

Keywords: rumen, virus, phage, metagenomics, proteomics, biofilm, methanogen

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INTRODUCTION

The presence of viruses in the rumen of domesticated herbivores was first noted in the 1960s (Adams et al., 1966; Hoogenraad et al., 1967) and it was quickly recognized that these viruses were not just those ingested by chance from feed and water. Instead, the majority of viruses in the rumen are those which either co-exist, or actively replicate and predate on, the dense microbial populations of the rumen. While viruses are also thought to infect the eukaryote populations of the rumen (protozoa and fungi), the viruses infecting rumen bacteria, often referred to as bacteriophage or phages, are the most well characterized. More recently, with the increasing focus on enteric methane emissions and rumen methanogenesis, viruses infecting methanogenic archaea (archaeal viruses or archaeaphage) have also been investigated.

Viruses of rumen microbes are an important focus for study, both as a key player in our understanding of rumen microbial community function and as a potential alternative to antibiotics in the face of a potential post-antibiotic era, as either a prophylaxis or treatment for problematic microbes. This review aims to summarize current knowledge of rumen virus populations, encompassing the traditional methods used to study rumen virus populations, as well as new and emerging technologies. We also draw on findings from other, better studied, microbial ecosystems (other animals, water, soils and microbial biofilms) to provide a fresh perspective and improve our understanding of the roles viral populations have in the rumen microbiome.

TECHNOLOGIES FOR STUDYING RUMEN VIRUS POPULATIONS

Developments in rumen virus research have been closely aligned with the technologies available for their study (**Table 1**). While the existence of viruses infecting bacteria were first noted in the early 1900's (Twort, 1915; d'Herelle, 1918), it was the development of electron microscopy (EM) in the 1930's (Haguenau et al., 2003) that showed that phages were actually virus particles and enabled rumen viral populations to be observed and enumerated (Hoogenraad et al., 1967; Paynter et al., 1969). Advances in methodology for the *in vitro* culture of rumen microbes (Hungate, 1969) enabled the cultivation of obligately anaerobic bacteria from the rumen and consequently the isolation of virus particles and determination of their biological characteristics. The advent of molecular biology enabled the preliminary genetic characterization of virus isolates (Klieve et al., 1991) providing an alternative method to TEM for virus particle enumeration and the determination of virus population dynamics (Klieve and Swain, 1993).

In the last 10 years, advances in DNA sequencing has led to the more rapid and accurate detection of viral sequences in prokaryote genome sequences and the sequencing of complete phage genomes (Leahy et al., 2010; Gilbert et al., 2017). A combination of high-throughput DNA sequencing and bioinformatic analysis has facilitated viral metagenomics: the culture-independent analysis of all the viral genetic material in a rumen sample. This has allowed the taxonomic classification of viral populations and determination of the relative abundance of individual viral taxonomic groups (Berg Miller et al., 2012). Sequence analysis of rumen sample RNA (metatranscriptomics) has also been used to identify RNA virus populations (Hitch et al., 2019). In addition, alternative technologies are being applied for the isolation of virus particles (for example, chromatography) and enabling the expression of novel viral proteins *in vitro* (Figura et al., 2011; Krajacic et al., 2017; Altermann et al., 2018). The emerging field of proteomics, is also providing fresh insights into the roles viruses and the proteins produced by viruses, may play within the rumen microbial ecosystem (Solden et al., 2018).

Transmission Electron Microscopy

Electron microscopy is a large research field, with applications in materials science as well as biology. The combination of electron microscopy and specialized staining techniques, enables the visualization of virus particle structures at nanometer scale, which could not otherwise be distinguished using conventional light microscopy. Several different electron microscopy technologies exist including scanning electron microscopy (SEM), transmission electron microscopy (TEM), high voltage electron microscopy, immunoelectron microscopy (IEM) and cryoelectron microscopy (cryoEM) with or without shadowing and three dimensional image reconstruction (reviewed by Ackermann, 2012; Romero-Brey and Bartenschlager, 2015). Of these technologies, TEM is the most widely used technique for the visualization of all types of viruses (Ackermann and Prangishvili, 2012; Popov et al., 2019).

The first use of TEM to visualize virus particles within rumen fluid, was published in 1967 (Hoogenraad et al., 1967). This study initially intended to determine the fate of bacterial cell walls within the rumen of sheep and involved direct examination of rumen samples by TEM. The rumen fluid was filtered through muslin and stained for negative contrast using potassium phosphotungstate. This methodology, however, revealed the presence of large numbers of free virus particles (icosahedral particles and tailed phages) and as the microbes were not removed prior to TEM, the attachment of phage particles to intact bacterial cells, including cocci and spirochetes, was also observed. This study was therefore pioneering not only in its use of the relatively new technique of TEM, but also in its hypothesis of what we now know to be true, that "phage may be a constant feature of the bacterial population of the rumen" (Hoogenraad et al., 1967).

Other studies then followed, employing TEM to conduct morphological surveys of rumen fluid collected from various ruminant species, including sheep, cattle and reindeer (Paynter et al., 1969; Ritchie et al., 1970; Tarakanov, 1972; Klieve and Bauchop, 1988). These surveys sought to enumerate the numbers of different viral morphotypes present, with results ranging from 6 (Paynter et al., 1969), 26 (Klieve and Bauchop, 1988), and more than 40 distinct morphotypes (Ritchie et al., 1970). In all the species of ruminants examined, the most abundant viral morphotypes were those with polyhedral heads and tubular tails, which are now classified within the viral order of tailed phages infecting bacteria, the Caudovirales. This viral order currently contains five families, the Myoviridae, Siphoviridae, Podoviridae, Ackermannviridae, and Herelleviridae, the last two being recently added (Adriaenssens et al., 2012; Barylski et al., 2020a,b). Of these, the short non-contractile tailed phages (family Podoviridae), long contractile-tailed phages (family Myoviridae) and many long non-contractile tailed phages (family Siphoviridae) were observed. These early surveys also sought to quantitate the size of the rumen viral populations using TEM to count the numbers of particles present. While counts varied, possibly as a consequences of differences in animal species as well as differences in sample preparation and counting methodology, virus particle counts ranged from 5×10^7 particles per mL bovine

TABLE 1 | Applications, benefits and limitations of technologies used for the study of rumen viruses.

Method		Application and benefits	Limitations	Example publications
Microscopy	TEM	Visualization of viral particle morphology	Specialized equipment	Hoogenraad et al., 1967; Ritchie et al., 1970; Klieve and Bauchop, 1988
		Estimation of viral numbers	Sample preparation can bias enumeration Time consuming and expensive Cannot determine viral particle viability and biological attributes	
Molecular Biology	PFGE	Provides snapshot of viral community and estimation of viral numbers	Cannot provide taxonomic and functional gene information	Klieve and Swain, 1993; Swain et al., 1996a
Isolation		Confirms viral particle viability Allows viral cultivation and storage in reference collections Enables determination of biological parameters (host range, growth, replication and survival) Allows extraction and sequencing of virus-specific nucleic acids Allows viral protein purification	Requires availability of susceptible microbial host Bias from sample preparation methods (e.g. exclusion of large viral particles)	Klieve, 2005; Gilbert and Klieve, 2015
Sequencing	Viral fraction	Provides snapshot of viral community structure	Requires isolation of intact viral particles from environmental samples	Breitbart et al., 2002; Clokie et al., 2011; Berg Miller et al., 2012; Anderson et al., 2017
		Provides taxonomic and functional gene information Overcomes technical limitations from low concentrations of viral DNA in environmental samples	Bias from sample preparation methods (e.g. exclusion of large viral particles) Bias from any DNA amplification steps High percentage of uncharacterized viral genes limits annotation of gene function and viral taxonomy Sequence assembly bias and challenges Cannot determine viral particle viability and biological attributes Difficult to identify viral lifecycles (e.g. lysogeny) and virus:host interactions	
	Metagenomics	Provides snapshot of viral community structure Captures sequences from intact viral particles and integrated prophages Provides viral taxonomic and functional gene information	Virus sequence numbers relatively low High percentage of uncharacterized viral genes limits annotation of gene function and viral taxonomy Difficult to identify viral lifecycles (e.g. lysogeny) and virus:host interactions Cannot determine viral particle viability and biological attributes Bias toward detection of double stranded DNA phages	Dinsdale et al., 2008; Willner et al., 2009
	Transcriptomics	Identifies actively replicating viral genes Allows detection of viruses with RNA genomes	Virus sequence numbers relatively low Bias toward identification of over-expressed viral genes (e.g. structural proteins)	Hitch et al., 2019

(Continued)

TABLE 1 | Continued

Method		Application and benefits	Limitations	Example publications
			High percentage of uncharacterized viral genes limits annotation of gene function and viral taxonomy	
			Difficult to identify viral lifecycles (e.g. lysogeny) and virus:host interactions	
			Cannot determine viral particle viability and biological attributes	
	Whole genome sequencing	Provides complete viral genome sequences	For lytic viruses requires pure, viable virus isolates	Leahy et al., 2010; Kelly et al., 2014; Gilbert et al., 2017
		Viral reference sequences increase the accuracy of sequence analysis	For lysogenic viruses requires viable prokaryote hosts containing intact, integrated prophage/s	
		Provides structural and functional viral protein information	High percentage of uncharacterized viral genes limits annotation of gene function	
		Indicates mechanisms of virus:host interactions and viral replication		
		Enables assignment of taxonomy and phylogenetic comparison		
Proteomics		Detects proteins produced by actively replicating viruses	Virus proteins found in relatively low concentrations	Solden et al., 2018
			Difficult to identify viral lifecycles (e.g. lysogeny) and virus:host interactions	
			High percentage of uncharacterized viral proteins limits functional annotation	
			Bias toward identification of over-produced viral proteins (e.g. structural proteins)	

rumen fluid (Paynter et al., 1969) to greater than 10^9 particles per mL bovine rumen fluid (Ritchie et al., 1970). It was also noted that the numbers of phages in crude rumen fluid could exceed bacterial numbers ca. 2 to 10:1 (Ritchie et al., 1970).

Images from a more recent TEM morphological survey of a sample obtained from an anaerobic fermentation system started with an inoculum of bovine rumen fluid (**Figure 1**) shows virus particle types representative of those observed in previous studies. This survey also includes examples of non-tailed viral particles and while it can sometimes be difficult to distinguish the small tail structures of the Podoviridae, these particles could belong to other viral families infecting bacteria such as Tectiviridae, Corticoviridae, and Microviridae (Ackermann and Prangishvili, 2012). It is now known that morphological surveys may have technical limitations and operator bias can result in errors in identification (Ackermann, 2013). For example, tail-less virus-like-particles may be confused with tailed phages which have lost their tail structures either naturally, with some phages being more susceptible to tail loss in the environment than others, or as a result of particle concentration and TEM sample preparation (Williamson et al., 2012). In addition, filamentous phages (Inoviridae) can be associated with rumen bacteria (Klieve et al., 1989) and have been observed by morphological survey (Klieve and Bauchop, 1988), however, these long, thin viral particles can be difficult to distinguish by TEM, due to their resemblance to fragments of phage tails and extracellular structures of bacteria (fine pili and flagella).

The use of TEM for estimating rumen viral numbers and population diversity has been largely superseded in favor of metagenomics and molecular methods. Viral genome sequencing can be used to predict viral particle morphology, as specific viral genes are conserved between morphological groups of viruses (Kristensen et al., 2013; Iranzo et al., 2016; Krupovic et al., 2018). However TEM is still widely recognized in virology as an important tool for the detection and morphological characterization of new virus isolates (Cuervo and Carrascosa, 2018; Popov et al., 2019) including those predicted from metagenomics (Shkoporov et al., 2018). The majority of publications to date describing the isolation of phages from the rumen, have used TEM in order to classify new isolates and verify the intact nature of phage particles (Brailsford and Hartman, 1968; Iverson and Millis, 1976a; Styriak et al., 1994; Cheong and Brooker, 2000). Electron microscopy can also be used for visualizing tail fibers involved in host-receptor binding with immunogold labeling, virus:host cell attachment and infection, intracellular viral multiplication and virus particle assembly and release (Ackermann, 1998; Kaelber et al., 2017; Letarov and Kulikov, 2017). TEM and other new, emerging forms of electron microscopy, can therefore be expected to have a continuing, important role in the study of rumen-associated viruses.

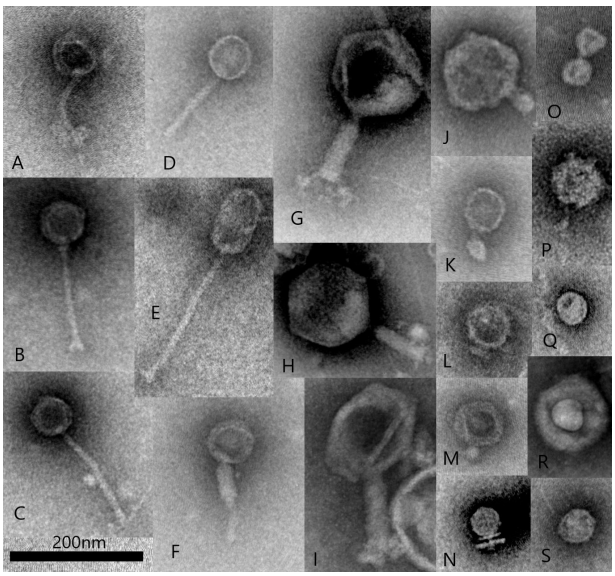

FIGURE 1 | Transmission electron microscopy illustrating examples of common rumen virus morphotypes including Siphoviridae **(A–E)**, Myoviridae **(F–I)**, Podoviridae **(J–O)** and other virus-like-particles including potentially non-tailed phages **(P–S)**, such as Corticoviridae and Microviridae (scale bar 200 nm). All virus particles were purified from a single sample collected from an *in vitro* fermentation system (Infors) started with bovine rumen fluid. Virus particles were fixed with 2.5% glutaraldehyde and stained with 1% ammonium molybdate pH 7.0 and viewed with a JEOL JEM1400 Transmission Electron Microscope. Micrographs were captured using a Gatan Orius CCD camera.

Molecular Biology

In the 1980's and 1990's there were rapid technological advancements in the field of molecular biology enabling: the purification of genetic material (DNA and RNA); charge and size-based separation of nucleic acids (electrophoresis; blotting techniques); the use of restriction enzymes for site-specific cutting of DNA and gene mapping; DNA recombination (DNA cloning and genetic transformation); and DNA sequencing techniques (reviewed by Brody and Kern, 2004; Loenen et al., 2014; Heather and Chain, 2016). Rumen microbiologists readily adopted these technologies (Orpin et al., 1988; Flint, 1997) and the way in which rumen phage isolates and rumen viral populations were studied, changed accordingly. Molecular biology was utilized to characterize new phage isolates, with less emphasis on using culture-based methods to understanding phage growth, survival in rumen fluid and host range, and more focus on genome length and restriction enzyme mapping. These analyses were conducted on phage isolates found to specifically infect several strains of rumen bacteria, including *Fusobacterium necroforum* (Tamada et al., 1985), *Selenomonas ruminantium* (Lockington et al., 1988), *Lactobacillus plantarum* (Nemcova et al., 1993), *Bacteroides ruminicola* (Klieve et al., 1991; Gregg et al., 1994), and *Ruminococcus albus* (Klieve et al., 2004). These phage isolates were mainly classified within the viral families Myoviridae and Siphoviridae however, some examples of Podoviridae and Inoviridae were also characterized in this way (Klieve et al., 2004). At this time, whole genome sequencing was technically very challenging and costly and therefore was not routinely undertaken. These studies, however, provided the first insights into the genetic characteristics and unique attributes of the phages infecting rumen bacteria.

Molecular biology also provided new methodologies that could be used for enumerating and describing viral populations occurring within a rumen sample showing a link between populations of rumen bacteria and virus populations (Klieve and Swain, 1993). These methods could also be applied to the analysis of relatively large numbers of rumen fluid samples derived from *in vivo* experiments (Klieve and Gilbert, 2005). The pulsed field gel electrophoresis (PFGE) technique was used following the purification and concentration of virus particles from relatively small volumes (40–60 mL) of rumen fluid and extraction of viral DNA using agarose-embedding techniques, to enable intact genome lengths to be preserved. Separation of DNA by PFGE then produced a population profile, based on viral genome length (Klieve and Swain, 1993). In addition, as an alternative to TEM viral particle counts, a DNA-based method was developed for the estimation of total viral numbers, based on a combination of conventional DNA extraction and electrophoresis techniques, and laser densitometry (Klieve and Swain, 1993). DNA virus population estimates obtained using this technique were slightly higher than those reported using TEM, with estimates of between 3×10^9 to 1.6×10^{10} particles per mL of ovine rumen fluid reported (Klieve and Swain, 1993).

Using these techniques (Swain et al., 1996a; Swain, 1999) the rumen viral population was found to (1) have variation in viral populations occurring between individual animals maintained on the same diet, as well as variation between different ruminant species (sheep, goats, cattle); (2) result in blooms of phage activity evidenced by sudden increases or bursts of phage particles; (3) have patterns of diurnal (within day) variation viral populations, in sheep fed once a day, with phage numbers showing blooms of phage replication (lysis events) and similar patterns of variation to those previously observed for bacterial populations in once-a-day fed sheep (Leedle et al., 1982); and (4) be affected by plant compounds found in the ruminant diet (Klieve et al., 1998). For example, the hydrolyzable tannin, tannic acid reduced rumen virus populations when added to sheep prior to feeding to give an intra-ruminal concentration of 0.1% wt/vol., suppressing by as much as 50% the increase in phage numbers normally occurring 8–12 h after feeding (Swain et al., 1996b). The mechanism of this reduction was attributed to the protein-binding activity of the tannic acid, and it was hypothesized that viral particles freely circulating in the rumen were being bound by the tannic acid and removed from the liquid phase (Swain, 1999).

These early molecular biology methods provided information about virus genome length (Hallewell et al., 2014), and enabled estimation of the size and relative length profiles of virus populations (Swain et al., 1996a). However, these methods were unable to deliver any DNA or RNA sequence information about the virus populations observed. Only a few studies conducted at this time were able to provide relatively short, gene-specific sequence information (Gregg et al., 1994). Being able to obtain sufficient amounts of genetic information to be able to infer

viral taxonomic classification, was achieved only after significant technological advances in DNA sequencing and the emergence of the field of metagenomics.

Metagenomic Approaches for Studying Rumen Viruses

Sequencing is an important method for understanding virus populations, and there is a wide variety of tools now at our disposal that makes it increasingly easy to generate virus-specific sequence datasets (referred to as viromes). Metagenomics was initially termed as the study of the collective genomes of all microbial species within an environmental sample (Handelsman et al., 1998) and with the advent of next generation sequencing (NGS), direct sequencing of DNA from environmental samples became possible (Escobar-Zepeda et al., 2015). Microbial community sequencing can be subdivided into two major sequencing methods; (i) shotgun metagenomic sequencing and (ii) amplicon sequencing. Shotgun metagenomic sequencing involves fragmentation of the total environmental DNA for sequencing, while amplicon sequencing includes PCR amplification and sequencing of specific genomic regions of interest. For bacterial taxonomy the 16S rRNA gene is used (McGovern et al., 2018) and can be described as metataxonomics (Marchesi and Ravel, 2015). Whilst the communities of bacteria, archaea, protozoa and fungi can be assessed using both amplicon and shotgun sequencing, the viral component can only be studied using shotgun metagenomics, due to the lack of a universally conserved gene across all viral species (Sullivan et al., 2008; Jameson et al., 2011; Wylie et al., 2015). Shotgun sequencing is highly effective in studying virus populations, which have relatively small genomes (∼20–300 kbp) (Wylie et al., 2015). Whilst DNA based sequencing technologies are most commonly undertaken, this approach excludes the potential study of RNA based viruses, which require total RNA metatranscriptomic sequencing for their detection (Hitch et al., 2019).

The marine environment is an easy system on which to develop viral sequencing tools; it is simple to collect liters of water without negatively impacting the environment being sampled, there is no inhibitory chemistry to prevent sequencing and there is minimal debris and organic matter for viruses to adsorb to and be lost. As a result, marine viral communities were one of the very first environments profiled using metagenomic approaches (Breitbart et al., 2002). Through the use of differential filtration, density-dependent gradient centrifugation, shotgun cloning and sequencing, Breitbart et al. revealed a highly diverse, previously unknown marine viral populations and opened the way for the characterization of viral communities in other environments.

Rumen virology is currently lagging behind marine and even human intestinal virology, with fewer studies using metagenomics to describe viral populations (**Table 2**). These studies have varied in the procedures used for sample preparation, however, most of the recent studies have included steps for the concentration of viral particles prior to DNA extraction and shot-gun sequencing to generate a viral-specific (virome) dataset. This is in contrast to the early metagenome studies, which generated viromes by extracting virus-only sequences from whole-rumen metagenome datasets (Dinsdale et al., 2008; Willner et al., 2009). As the available sequencing technologies have progressed from Pyrosequencing and Ion Torrent sequencing systems to the more high-throughput Illumina technologies, the rumen virome datasets obtained have increased in sequencing depth and provide more comprehensive sequence coverage. To date the rumen virome of several ruminant species have been examined, including sheep, goats, buffalo and moose, with cattle, including dairy cows and steers being the most frequently investigated (**Table 2**). Most studies use limited numbers of animals and experimental conditions, such as diet, have remained constant (Berg Miller et al., 2012; Ross et al., 2013).

Currently only one report has used metagenomics to investigate the effect of changing diets on rumen viral populations which showed the total digestible nutrients was the predominant ecological driver of both bacterial and viral response to dietary change (Anderson et al., 2017). In this study, diversity analysis of both the bacterial and viral communities identified that samples clustered based on diet rather than host, confirming that dietary changes led to consistent changes within both communities (Anderson et al., 2017). This study represented a significant advance in our understanding of rumen viral populations. Diet has been shown in many studies, to greatly influence the relative abundance of rumen microbial species (reviewed by Huws et al., 2018). As viral populations are intrinsically linked to the

TABLE 2 | Summary of published rumen viral metagenomics studies including the type of ruminant used for each study (breed description provided in publication), number of individual animals sampled and the DNA sequencing technologies used.

Ruminant description	Number of animals	DNA Sequencing technology	References
Cattle (Angus Simmental Cross steers)	3	Pyrosequencing	Dinsdale et al., 2008
Cattle (Angus Simmental Cross steers)	3	Pyrosequencing	Willner et al., 2009
Cattle (Holstein dairy cows)	3	Pyrosequencing	Berg Miller et al., 2012
Cattle (Holstein dairy cows)	13	Illumina HiSeq	Ross et al., 2013
Sheep (Cross bred rams)	22	Illumina HiSeq	Yutin et al., 2015
Buffalo (Surti breed)	1	Ion torrent	Parmar et al., 2016
Cattle (steers)	5	Ion torrent, Illumina MiSeq	Anderson et al., 2017
Goats (*Caprus hircus*), sheep (*Ovis aries*)	8, 8	Pyrosequencing	Namonyo et al., 2018
Moose (*Alces alces gigas*)	1	Illumina HiSeq	Solden et al., 2018

populations of their microbial hosts (see Lessons to be Learned from Viruses in Other Environments), any changes in the animal diet and consequent availability of specific nutrients for microbial growth in the rumen, can also be expected to cause significance changes the relative abundance of viral populations. While early molecular biology studies eluded to the effects of animal diet on rumen microbial and associated viral populations (Swain et al., 1996a), the advent of viral metagenomics has verified and enhanced our understanding of diet-induced changes on rumen viral populations.

In terms of viral taxonomy the rumen virome varies greatly between individual animals of the same ruminant species and a subset of nearly ubiquitous viral genomic fragments have been identified and proposed to make up a core rumen virome (Anderson et al., 2017). It can also be speculated that these viruses are likely to predate on the bacterial species core to the rumen (Henderson et al., 2015) including the bacterial genera: *Prevotella*, *Butyrivibrio*, *Ruminococcus* and more generally, the families Lachnospiraceae, Ruminococcaceae and Bacteroidales. Metagenomic studies of the rumen have all revealed that the rumen virome is dominated by *Siphoviridae* (32–36%), *Myoviridae* (24–32%), and *Podoviridae* (12–16%), all bacteriophage families belonging to the order Caudovirales (Lefkowitz et al., 2018). As we expect the most abundant rumen viruses to be bacteriophages and public databases are dominated by these, this makes their identification on the basis of genetic homology easier. It is likely that the viral sequencing and identification methods currently adopted, bias against the less common viral groups we anticipate in the rumen, such as archaeal viruses, mycoviruses and other eukaryote viruses.

The dominance of DNA based sequencing methods prevents the study of RNA based viruses including the Totiviridae which infect host-associated protozoa (Grybchuk et al., 2018) and the Partitiviridae, which may exist within the rumen and infect fungi (Hitch et al., 2019). Under these assumptions we may be missing vital functions and relationships. Lower abundance viral families have been identified in the rumen, which have been putatively assigned as large DNA viruses (*Mimiviridae, Phycodnaviridae*), filamentous lysogenic viruses of bacteria and archaea (*Inoviridae*), archaeal viruses (*Bicaudaviridae*) and animal viruses (including; *Baculoviridae,* and *Adenoviridae*) (Berg Miller et al., 2012; Parmar et al., 2016; Anderson et al., 2017). Although they have been detected in rumen virome datasets, the animal viruses identified often represent lineages which would not normally infect rumen microbes. These taxonomic assignments are based on sequence homology to databases of viral sequences available at the time. As both the number of phage genomes in Genbank and publications containing novel viral taxonomies are increasing exponentially, the continuing accumulation of new genetic information, will assist the accuracy of future viral taxonomic classification.

Another application of metagenomics to the study of rumen virus populations involves examining clustered regularly interspaced short palindromic repeats (CRISPR) sequences. The identification of CRISPR-cas arrays and prophages within the cellular component of the rumen microbiome allows for the possible identification of viral-host interactions (Berg Miller et al., 2012). Whilst prophage insertions represent whole viral genomes and are therefore easier to identify and characterize, CRISPR arrays incorporate only a short 25–65 bp region of viral DNA (Shmakov et al., 2017). Despite these short insertions, recent advances have separated and annotated these spacer regions against viral datasets, allowing for viral-host interactions to be identified and studied. Data sets such as the Hungate 1000 rumen collection contain a wealth of data on prophages and viral interactions. The Hungate 1000 collection yielded identifiable CRISPR arrays in over 60% of the isolates (Seshadri et al., 2018). CRISPR arrays can also provide clues to the frequency of aborted lytic viral infections (Sanguino et al., 2015; Edwards et al., 2016). Although these methods have been applied to the rumen microbiome, larger datasets are required to gather enough data for meaningful insights on phage infection (Berg Miller et al., 2012).

Prophages identified from bacterial genomes and microbial metagenome sequencing can help shed light on prophage diversity and the lifestyles of viruses in the rumen. Computational approaches to identify prophages and free viruses in metagenomic data vary depending on the type of data to be analyzed and the prior information available for the virus family of interest. If existing genomic data exists for that viral family, then the direct alignment of reads to the genomes can allow inferences of viral abundances and types in the sample, termed an "aligner" approach (Roux et al., 2015). Another approach, which can utilize reads directly from the sequencer, involves "binning" (separation) of sequences into groups based on characteristics inferred from their sequences. This can be comparing their sequence similarity to known viral sequences (for example Zhao et al., 2017), to more complex approaches which calculate frequency profiles of short strings of DNA, termed "kmers" (for example Willner et al., 2009). This latter approach is based on the observation that different organisms can be identified by the kmer profiles of their genomes and is widely used in metagenomic data analysis (Ren et al., 2017). The resulting "bins" can then be individually assembled into what is hoped to be the genome of a single virus, and these assumptions can be tested by subsequent gene prediction or sequence similarity searches to compare against databases such has those hosted by the NCBI. There is no consensus on which approach or tool is best. The rate at which new tools are emerging (for a current list of tools see Zheng et al., 2019), suggests that using a multi-tool approach may allow balancing of the strengths and weaknesses of each (Anderson et al., 2017). An array of techniques have also been successfully employed on human gut metagenome sequence data to obtain a snapshot of virus communities (Nielsen et al., 2014; Ogilvie and Jones, 2015).

In terms of gene function, the rumen virome has been shown to be relatively consistent across animals of the same ruminant species (Ross et al., 2013). Assigning function to putative viral open reading frames (ORFs) is also difficult, as there is a high percentage of unknown and uncharacterized viral genes (Yooseph et al., 2007). Kyoto Encyclopedia of Genes and Genomes (KEGG) based annotation has suggested that 50–70% of the functional reads within the rumen virome are involved in viral replication (KEGG pathways: nucleotide

metabolism, replication and repair), whilst the remaining reads were functionally diverse and included viral structural genes (head and tail proteins). A subset viral genes have been identified as auxiliary metabolic genes (AMGs), which redirect host metabolism toward reactions favorable to phage replication (Anderson et al., 2017). In this way AMGs encompassed in virome datasets represent one way rumen viral populations may influence the metabolic potential of the rumen microbiome.

Culture-Based Viral Isolations and Genome Sequencing

The first report of culture-based, rumen viral isolation used rumen and non-rumen strains of *Serratia* spp. (facultative anaerobes, family Enterobacteriaceae) to isolate phages, with the intention of demonstrating that phages were prevalent in the rumen of cattle (Adams et al., 1966). As well as successfully isolating phages from rumen fluid, this study also investigated host-specificity (or host range), that is, the ability of these phage isolates to infect multiple strains of bacteria. In this way the study established that phages sourced from the rumen could have limited host-specificity, preferentially infecting *Serratia* host strains sourced from the rumen and being unable to infect non-ruminant *Serratia* strains. Correspondingly, the rumen *Serratia* were not susceptible to infection by *Serratia* phages isolated from soil, water and sewage (Adams et al., 1966). This study was the first to demonstrate that not only were phages prevalent in the rumen, a finding subsequently confirmed using TEM (Hoogenraad et al., 1967), it also demonstrated that the phages found in the rumen differed from those present in other environments.

This primary investigation was followed by numerous studies conducted in laboratories across the globe (Iverson and Millis, 1976a; Tyutikov et al., 1980; Hazlewood et al., 1983; Tamada et al., 1985; Klieve and Bauchop, 1991; Nemcova et al., 1993; Styriak et al., 1994; Ambrozic et al., 2001). The majority of these studies were conducted in the 1980's and 1990's, with very few phage isolations reported in the literature beyond this era. To date, most of the viruses isolated from the rumen have been phages infecting rumen bacteria with only a single, preliminary isolation of a rumen-derived archaeal virus being reported (Baresi and Bertani, 1984).

Bacterial hosts strains used for the isolation of rumen phages have been reviewed elsewhere (Gilbert and Klieve, 2015) and include genera classified within various common phyla associated with the rumen, including Firmicutes (*Ruminococcus*, *Lactobacillus*, *Eubacterium*, *Selenomonas*, *Quinella,* and *Streptococcus*), Bacteroidetes (*Bacteroides*), Proteobacteria (*Serratia*), and Actinobacteria (*Bifidobacterium*). The majority of phages infecting rumen bacteria have a typical tailed morphology (Gilbert and Klieve, 2015) and although originally classified according to the Bradley scheme (Bradley, 1967), the following examples are listed according to the modern international committee on taxonomy of viruses (ICTV) classification scheme (King et al., 2012). Rumen phage isolates with Siphovirus morphology include phage F4 which can infect five strains of *Streptococcus bovis* (Styriak et al., 1994; Nigutova et al., 2008); and a long-tailed phage infecting *Prevotella* (*Bacteroides*) *brevis* strain GA33 (Ambrozic et al., 2001). An example of a rumen phage isolate with Myoviridae morphology is phage FnP1 infecting *Fusobacterium necrophorum* strain FnP1 which interestingly also infected 12 other *F. necrophorum* strains of biovars A and B but none of the *Bacteroides* strains tested (n = 13) (Tamada et al., 1985). A rumen phage isolate with Podoviridae morphology is phage 2BV which infects *Streptococcus bovis* (Iverson and Millis, 1976a). Reports of phage isolates infecting rumen bacteria with a non-tailed morphotypes have been relatively infrequent, for example, the non-tailed, filamentous Inoviridae phages φRa01 and φRa03 infect *R. albus* AR67 (Klieve et al., 2004).

To isolate phages from the rumen, the majority of culture-based studies have used double-layer agar plates for the detection of clearing zones (plaques) within bacterial monolayers (Klieve, 2005). A combination of anaerobic techniques (Hungate et al., 1964) and rumen fluid-based growth medium with modifications are used to ensure the survival and growth of anaerobic bacterial host strains *in vitro* (Klieve et al., 1989). Culture-based plating techniques have also served as important tools for determining the biological properties of phage isolates, particularly those infecting rumen-associated *Streptococcus* species. Biological properties determined in this way include: viral particle viability in rumen fluid; the development of phage resistance; and host range (Iverson and Millis, 1977; Klieve and Bauchop, 1991; Klieve et al., 1999).

Culture-based plating methods tend to favor the isolation of phages which undergo the lytic cycle of replication. Several different phage replication lifestyles exist in nature and the terminology to describe these lifestyles or cycles, may vary (Hobbs and Abedon, 2016). However, when phages only follow the lytic cycle of replication, phage particle attachment and infection of the host cell leads directly to replication and culminates in the bursting, or lysis, of the host cell to release the progeny phage particles. True lytic phages cannot adopt the alternative, lysogenic lifestyle of replication, as they lack the functional genes required for control of replication and/or integration of phage DNA into the host genome. These phage therefore cannot form a dormant, heritable genetic state (prophage) or establish what is sometimes referred to as chronic infection (reviewed by Weinbauer, 2004; Howard-Varona et al., 2017).

While the isolation of phages from the rumen originally began as a way to determine the extent of viral population diversity, phage isolation was later driven by (1) the need to isolate phages for use in potential biocontrol (phage therapy) of problematic bacteria, such as the bacteria implicated in the development of ruminal acidosis, *Streptococcus bovis* (since re-classified as *S. equinus*) (Tarakanov, 1994); and (2) the requirement for molecular tools (vectors) for the stable transformation of rumen bacteria (Lockington et al., 1988; Gregg et al., 1994). As phage therapy is traditionally based on the use of viable lytic phages, several groups established collections of phages infecting *S. bovis* strains (Iverson and Millis, 1976a; Tarakanov, 1976; Klieve and Bauchop, 1991; Styriak et al., 1994; Klieve et al., 1999). The development of cloning vectors for the genetic transformation of rumen bacteria, however, involved utilizing either phage

replication or integration genes, such as those present in lysogenic phages (Gregg et al., 1994; Cheong and Brooker, 1998).

The bacterial genera that were selected for genetic transformation for example *Ruminococcus*, *Bacteroides,* and *Butyrivibrio,* were either those known to be actively involved in the enzymatic breakdown of feed, or examples of common rumen bacteria (Flint, 1997; Wong et al., 2003). Therefore, although many lysogenic phages infecting *S. bovis* were identified (Tarakanov, 1974, 1976; Iverson and Millis, 1976b), only a few phages were genetically characterized (Styriak et al., 2000, 2005). Collectively, however, the numerous isolations of lysogenic phages from the rumen, as well as experiments to chemically induce lysogenic phages from rumen bacteria (Klieve et al., 1989) established, in the absence of DNA sequencing technologies, that a high proportion of rumen bacteria harbor viable lysogenic phages within their genomes.

As DNA sequencing technologies have improved, the whole genome sequencing of bacteria has become more cost-effective and convenient (Didelot et al., 2012). In addition, as bioinformatics methods for genome assembly have become more refined, the detection of integrated prophages has become increasingly possible (Roux et al., 2015; Arndt et al., 2016). Many bacterial and archaeal genomes have been shown to contain fragments or remnants of integrated prophages (Krupovic et al., 2011). For prophage sequences to be considered "intact" (Zhou et al., 2011) and representative of a potentially viable phage, they must contain a full complement of phage genes. These genes are usually clustered into structural and function-related modules including DNA replication and transcriptional regulation, head and tail proteins, DNA and particle packaging, translocation and host cell lysis (Brussow et al., 2004; Roux et al., 2015). While culture-based assessments are required to verify prophage viability, the detection of viral sequences in microbial genome sequences has revolutionized the rate at which novel viruses can be detected.

To date, intact prophages have been detected in the genome sequences of many genera of rumen bacteria (Berg Miller et al., 2012; Gilbert and Klieve, 2015) although the prophage genomes and their homology to known phage sequences, have not been described in detail. Recently prophage sequences detected in the bacterial genome sequences of the ovine rumen isolates *Streptococcus equinus* 2B (∼42 kb, designated φSb2Bpro1) and *Bacteroides ruminicola* ss *brevis* AR29 (∼35 kb, designated φB2bAR29pro1) were described (Gilbert et al., 2017). The latter prophage had been previously examined, with some genetic and biological properties determined, this prophage formed tailed phage particles with a Siphovirus morphology (Klieve et al., 1989; Seet, 2005).

Integrated viral sequences have also been detected in methanogenic archaea isolated from the rumen, including a ∼40 kb prophage of *Methanobrevibacter ruminantium* M1 and a 37 kb prophage of *Methanobacterium formicicum* BRM9 (Attwood et al., 2008; Kelly et al., 2014), although the ability of these prophages to produce intact viral particles has not been reported. The recent international collaborative genome sequencing project, the Hungate 1000 project (Seshadri et al., 2018), analyzed 410 cultured bacterial and archaeal genomes together with their reference genomes and identified virus genes, categorizing these genes according to Pfam numbers (for example pfam03354 Phage Terminase, pfam04860 Phage portal protein and pfam05105 Bacteriophage holin family) and compared them to virus genes found in human intestinal isolates. In this way the study showed that the prophage genes identified differed between the microbes of the herbivore gut and those of the human gut.

Recently whole genome sequences of lytic phages infecting rumen bacterial isolates of *Streptococcus*, *Bacteroides,* and *Ruminococcus* were reported (Gilbert et al., 2017). These phages were of Siphovirus (phages φBrb01 and φBrb02 infecting *B. ruminicola* and phage φSb01 infecting *S. equinus*) and Podovirus morphology (phages φRa02 and φRa04 infecting *R. albus*). Of these phage genomes, the φSb01 sequence had the most genetic homology to other *Streptococcus* phages isolated from non-rumen environments, enabling a more comprehensive annotation of genome and identification of functional modules. In contrast, the Podoviruses φRa02 and φRa04 were more distantly related to known phages, with the few functional genes for which identity could be predicted, all related to genes previously shown to be highly conserved in Podovirus genomes (Iranzo et al., 2016), such as head-tail connector proteins, DNA polymerase and Podovirus encapsidation proteins. Despite this lack of gene annotation, the identified highly conserved phage genes (head-tail connector protein for Podoviridae and phage terminase large subunit (*ter*L) for Siphoviridae) could be employed in phylogenetic analysis to enable the identification of closely related phages. On this basis, φRa02 and φRa04 which infect a *Ruminococcus* host, classified within with the gram positive bacterial phylum Firmicutes, were found to be more highly related to Podoviruses infecting other genera of Firmicutes (for example *Bacillus*, *Clostridium,* and *Streptococcus*), than Podoviruses infecting the gram negative phylum Proteobacteria (for example *Yersinia*, *Pseudomonas,* and *Vibrio*) (Gilbert et al., 2017).

Obtaining the complete genome sequences for rumen virus isolates has many benefits, providing insights into virus:host interactions and facilitating the discovery of novel viral proteins. Most significantly, increasing the number and types of rumen-specific viral genome sequences will greatly enhance the accuracy of viral classification and bioinformatic analysis of viral metagenomics datasets, providing reference sequences and novel viral genes to which meaningful sequence homology can be conferred. Experimental studies with viral isolates is anticipated to remain vital for the determination of important biological properties, such as the determination of virus:host specificity (host range); the characterization of novel viral proteins; determining immunological interactions; and ascertaining viral particle transfer and survival in the gastrointestinal tract and environment.

Interpreting Virome Function Using Metaproteomics

Metaproteomics is the analysis of the entire protein content of a given microbial community at a specific time point (Wilmes and Bond, 2004). Like many other 'omic' technologies,

metaproteomics has experienced rapid development in the past 10 years (Kunath et al., 2019), and is quickly becoming commonplace for the analyses of complex microbiome samples (Hagen et al., 2017; Borton et al., 2018; Heyer et al., 2019). Within the rumen microbiome, metaproteomics is a relatively new field, with only sparse datasets generated in recent years (Deusch et al., 2017; Snelling and Wallace, 2017; Hart et al., 2018; Solden et al., 2018). However, multi-omic approaches that combine both metagenome and metaproteome data are now enabling genome-centric resolution that links active metabolic functions to explicit microbial populations in the rumen (Naas et al., 2018; Solden et al., 2018).

Using functional meta-omics to characterize viral taxonomy and function has quickly shown the impact viruses have on ecosystem processes (Emerson et al., 2018). Metaproteomic analysis of viral communities has been used in bioreactors (Heyer et al., 2019) and ocean communities (Brum et al., 2016), which highlighted the presence of newly annotated functions and indications of phage–host interactions causing cell infection. Within the rumen of Alaskan moose, Solden et al. (2018) used a combined meta-omic approach to identify 1,907 viral metagenomics contigs, which clustered into 810 viral populations. This lead to the detection of 148 viral genera, including 35 previously detected from the cow rumen, although the vast majority (75%) were determined to represent previously unknown viral genera.

The moose metaproteome detected a total of 64 viral proteins from 53 different viral contigs, with most proteins (80%) having no known functions, whereas the remainder were largely structural proteins, such as capsids. Against expectations, very few auxiliary metabolic genes (Emerson et al., 2018) were detected, contrary to previous rumen virus findings (Anderson et al., 2017). Examinations of virus–host interaction dynamics, predicted microbial genome hosts, including direct associations between viruses and some of the most active carbohydyrate-degrading and sugar-fermenting populations sampled from the moose rumen (Solden et al., 2018). In particular, numerically dominant *Prevotella*-affiliated populations that play critical roles in hemicellulose degradation were predicted to be infected, highlighting that rumen viruses are likely to be affecting carbon cycling in a top–down manner, whereby changes in the structure and function of the primary fiber degraders may possibly reverberate through the lower trophic levels of the rumen microbiome (Solden et al., 2018). Much more targeted analyses are required to see if such an effect is indeed the case, or if other versatile populations with similar saccharolytic machinery are capable of either supplementing or contributing to, any newly presented niches in such a scenario.

Expression of Viral Proteins

The study of viral proteins often involves the over-production of the protein of interest *in vitro*. In this way sufficient quantities of protein can be generated to enable analysis using protein chemistry techniques for determination of biological properties. Understanding the biological properties of viral-encoded proteins can lead to an improved understanding of how viruses specifically infect, replicate and interact with host cells and viral proteins have been extensively studied for some well-known, historically important phages for example, the *Escherichia coli* phages lambda (Casjens and Hendrix, 2015) and T4 (Miller et al., 2003); and the Phi29 phages infecting *Bacillus* (Meijer et al., 2001).

The use of viral proteins as new antimicrobials is an area of growing interest, providing an alternative approach to phage therapy, which was traditionally based on the use of whole phage particles to reduce specific microbes (reviewed by Fenton et al., 2010; Abedon, 2012; Shen et al., 2012). Traditionally the use of antibiotics in agriculture was preferred over phage-based therapies for the control of problematic microbes and improving feed efficiency (Russell and Houlihan, 2003). In recent years however, there has been increasing community and regulatory pressure to reduce antibiotic use in agriculture, as well as a drive to reduce enteric methane emissions from ruminant livestock (Hristov et al., 2013; Cameron and McAllister, 2016). One of the antibiotic-free approaches to reduce enteric methane emission harnessed the potential of phage-encoded enzymes to specifically target and lyse rumen methanogens (Klieve and Hegarty, 1999; Leahy et al., 2013). Previously phage-encoded enzymes found in methanogen genomes had only been applied to increase the permeability of methanogen cell walls in order increase the penetration of microscopy stains (Nakamura et al., 2006). Genome sequencing of the rumen methanogen, *M. ruminantium* M1, however, revealed the presence of a prophage gene related to those usually responsible for hydrolyzing the pseudomurein cell walls of methanogens: the endoisopeptidase *pei*R (Attwood et al., 2008) and the potential use of this enzyme was recognized (Leahy et al., 2010).

Recently the *in vitro* expression of *pei*R was reported (Altermann et al., 2018). In this study, initial experiments were conducted to produce this protein using traditional gene cloning, transformation, protein expression in a commercially-available *E. coli* expression strain (Schofield et al., 2015), with the final purified protein employed in lytic activity assays. To further improve the stability and enable practical delivery of the enzyme, an alternative protein expression strategy was adopted. This approach involved the peiR gene being fused to the polyhydroxyalkanoate (PHA) synthase gene which was then expressed in *E. coli* to create PHA bionanoparticles with the lytic enzyme being displayed on the outer surface of the bionanoparticle (Altermann et al., 2018). Using this combination of techniques, sufficient quantities were produced to allow testing for cell-wall degrading effects against several strains of *Methanobrevibacter*, which are usually the most highly abundant methanogen genera found in the rumen (St-Pierre and Wright, 2012), and other methanogenic genera (*Methanobacterium*, *Methanosphaera*, *Methanospirillum,* and *Methanonosarcina*). This study therefore, successfully demonstrated for the first time how viral genes, identified by whole genome sequencing of rumen microbes, can be successfully produced using modern protein expression technologies and developed into a product for activity testing. This study also showed how protein expression techniques are integral to the development of new enzybiotics approaches for the modulation of rumen microbial populations.

Emerging Technologies

Technologies for studying viruses in environmental samples are constantly being developed, and older technologies are being revisited. New technologies, which could be applied in the context of the rumen, include those being developed to address long-standing biological questions. For example, it is currently difficult to link the extensive populations of viruses identified in viral metagenomes, to their respective microbial hosts (Koskella, 2019). In addition, an older technique, being revisited in the context of the rumen, is the use of Chromatography for size-based virus particle fractionation.

Techniques are evolving that enable us to link viruses to their potential hosts using metagenomic information alone, such as CRISPR-spacer regions (Berg Miller et al., 2012). Hi-C or 3C is another promising technique developed to cross-link DNA in genomes that is distant on the DNA strand, but comes into close physical proximity, due to folding and chromosome architecture (Dekker et al., 2002; Lajoie et al., 2015). This method has the potential to cross-link viral and host DNA during infection to identify viral hosts prior to lysis (Marbouty et al., 2017). Single-cell viral tagging is a further tool that has been tested and proven to work on viruses from the human gut and involves fluorescently labeling phage particles, allowing these to attach to hosts, then sorting and sequencing these pairs (Willner and Hugenholtz, 2013; Džunková et al., 2019).

Chromatography is a traditional chemistry technique for the separation of mixtures, and has the potential be applied for the purification of viruses from rumen samples. Currently the use of chromatography in rumen research has been limited to metabolomic and proteomic studies with only a few publications combining chromatography and virus protein research (Schofield et al., 2015; Altermann et al., 2018). Given the proteinaceous nature of virus structures, particularly phages, implementing protein chromatography methods to separate or isolate whole phages directly should be possible. Despite the size difference and more complicated morphology of phages compared to proteins, it is expected that phages would act like proteins in chromatography systems (Oslizlo et al., 2011). Indeed, chromatography for viral purification is not novel, with literature dating back to 1957, where the coliphages T1, T2r and $T2r^{+}$ were purified from host lysates using ECTEOLA cellulose ion exchange chromatography (Creaser and Taussig, 1957). This emerging technology for separating bacteriophages from environmental samples such as rumen fluid, involves the use of coil column separation and high performance counter current chromatography (HPCCC) (Hu and Pan, 2012; Berthod and Faure, 2015); utilizing the size differences of phages and combining this with the centrifugal forces applied in the HPCCC to separate viruses (Friedersdorff et al., 2018). Although this approach is in its infancy, it is anticipated that depending on the resolution of the CCC technique used, viral fractions based on particle size may be obtained from rumen fluid, enabling the study of less-understood viral morphotypes, for example, viral families which form small, non-tailed icosahedral particles.

LESSONS TO BE LEARNED FROM VIRUSES IN OTHER ENVIRONMENTS

Viruses play a pivotal role in microbial ecology and evolution, contributing to global nutrient cycles, pathogenicity and antimicrobial resistance, and influence animal host immune systems (Salmond and Fineran, 2015). The roles viruses play in aquatic, terrestrial and animal-associated environments are vitally important and viruses, particularly phages, have been acknowledged as being responsible for significant bacterial mortality (Clokie et al., 2011). Our understanding of rumen virus ecology is currently inadequate, we must therefore learn from studies undertaken for other environments, where viruses have been shown to affect microbial populations by maintaining microbial diversity; cycling key nutrients; acting as mobile genetic elements to facilitate genetic exchange; infecting and replicating in a host-specific manner (limited host range); interacting with microbial biofilms (**Figure 2**) and the immune systems of animal hosts.

Maintenance of Microbial Diversity

Viruses limit the growth of bacteria in aquatic environments, accounting for an estimated 10–80% of bacterial mortality (reviewed by Weinbauer, 2004). This relentless pressure on bacterial communities shapes their diversity, community structure, function, and temporal dynamics (Letarov and Kulikov, 2009). In the marine environment, the model produced by Thingstad (2000) predicts that viral predation modulates microbial populations via a microbial feedback loop described as the "kill the winner" strategy (i.e., targeting and reducing the size of the most abundant microbial host species). This allows viruses and bacteria to coexist in an equilibrium, thus ensuring microbial diversity. Furthermore, mesocosm experiments by Sandaa et al. (2009) support this model, showing that viruses act as a regulating force, preventing fast growing bacteria from outcompeting slower growers. Viruses have also been shown to control algal blooms (phytoplankton and/or cyanobacteria) occurring in both marine and freshwater environments (reviewed by Weinbauer, 2004).

Within gut-associated microbial communities, viruses are also likely to play a role in modulating microbial populations using predation and microbial feedback loops, particularly during the stages of microbial succession occurring in early life development and at times of microbial community disturbance, e.g., dietary change, dysbiosis and antibiotic use (Lozupone et al., 2012). For example, the "kill the winner" strategy has been observed in the horse gut, where *E. coli* and coliphages co-exist (Golomidova et al., 2007). However, in contrast to the microbial communities found in aquatic microbial ecosystems, the near-theoretical-maximum cell densities combined with the animal immune system lead to temperate phages predominating in animal guts, with the immune system also helping to prevent the over-proliferation of individual gut microbes. In the human gut, phage therapy has been shown to eliminate bacterial pathogens, rather than regulating their numbers, due to the

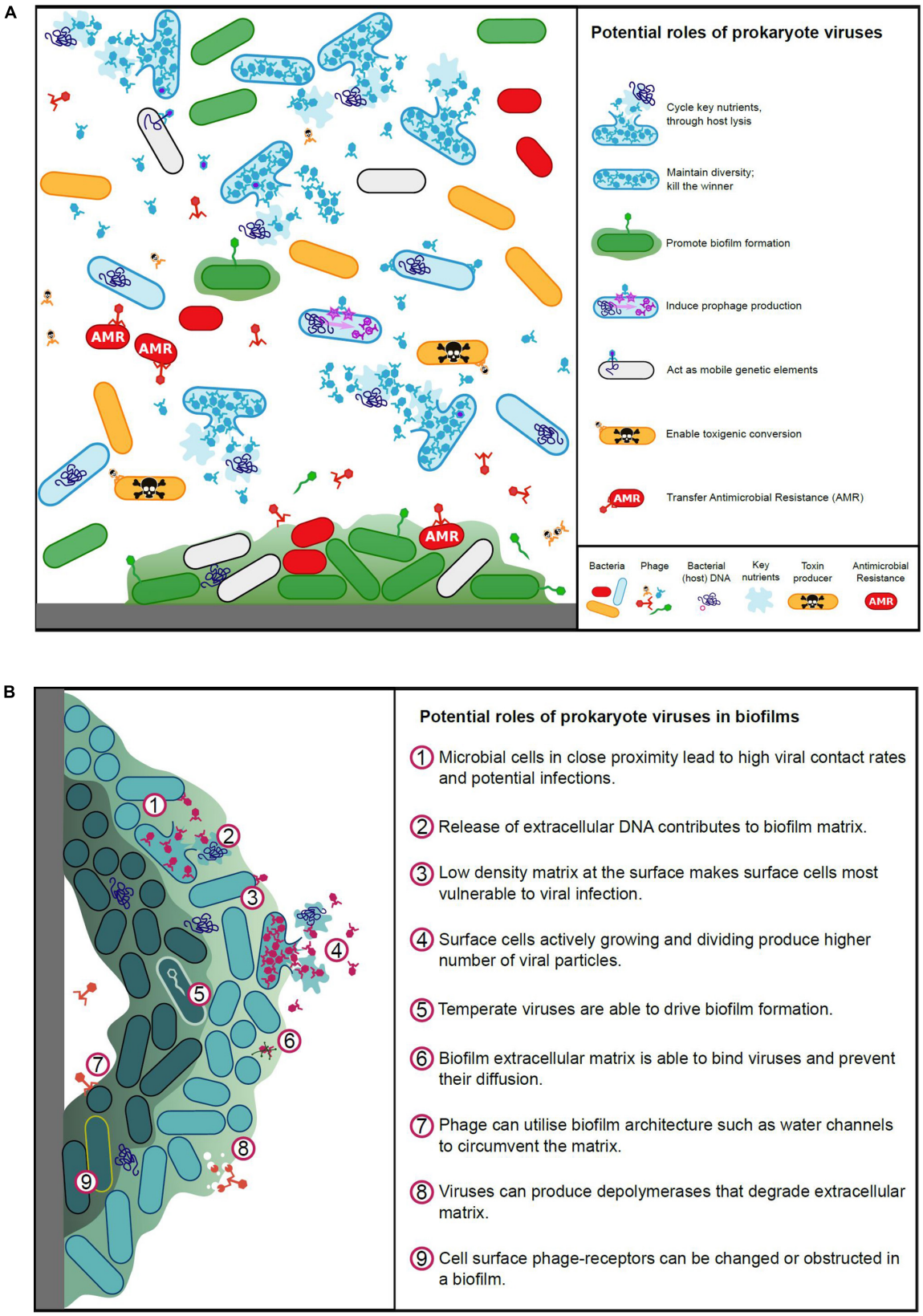

FIGURE 2 | (A) Potential roles of prokaryote viruses in microbial ecology and evolution. **(B)** Potential roles of prokaryote viruses in biofilms.

added selective pressure of the immune system (reviewed by Sulakvelidze, 2005).

Microbial hosts have evolved numerous defense mechanisms to prevent phages from killing the winner. These mechanisms target every stage of infection from receptor binding to phage assembly. These include: (1) modification of phage receptors or hosts only display them during part of life cycle; (2) superinfection exclusion, often associated with lysogenic phages to protect their hosts; (3) bacteriophage exclusion (BREX) is a similar host-encoded defense system that prevents phage DNA replication; (4) restriction modification enzymes to degrade foreign DNA, defense island system associated with restriction–modification (DISARM) is more complex, they are found in bacteria and archaea and restricts phage DNA; (5) methylation and CRISPR/*Cas* elements block phage replication (6), abortive infection/programmed cell death (Abi). In addition bacteria can hijack phages to utilize them as gene transfer agents (GTA) and phage-inducible chromosomal islands (PICI) which repackage host DNA into phage structures (for review see Rostøl and Marraffini, 2019).

For each host defense system microbes have evolved, however, viruses appear to have developed a countermeasure, such as the anti-restriction, anti-CRISPR and anti-toxin systems (Dolgin, 2019; Rollie et al., 2020). While these viral countermeasure systems have been largely described for genera not normally found in the rumen, for example, *Pseudomonas* and *Mycobacterium* (Dedrick et al., 2017; Landsberger et al., 2018). Given the extensive microbial and viral diversity observed in the rumen, and the ecological and evolutionary pressures rumen microbial communities experience, these viral countermeasures are likely to exist and play a role in enabling viral replication and persistence in the rumen.

Cycle Key Nutrients

In aquatic systems viruses have been shown to impact on microbial mortality, causing episodes of microbial lysis, contributing to the recycling of nutrients (Fuhrman and Noble, 1995; Steward et al., 1996; Weinbauer and Höfle, 1998). Viral lysis results in the release of nutrients in the form of dissolved organic matter (DOM) (Shibata et al., 1997). In low nutrient aquatic systems DOM is an essential source of carbon and growth limiting nutrients that is quickly reabsorbed by the microbial community (Bratbak et al., 1990; Proctor and Fuhrman, 1990).

The gut (and rumen) represent much more productive systems than the open ocean, with microbial densities the highest of any studied environment, reaching $\sim 10^{10}$–10^{11} mL^{-1} in the rumen (Russell and Hespell, 1981). Due to this high microbial density, these microbes are in constant competition for nutrients and may be subject to control mechanisms, such as virus predation, that maintain stability and cycle nutrients (Lozupone et al., 2012). Due to the microbial competition, nutrients such as iron and vitamin B_{12} (cobalamin), are also limiting to microbial growth in the human gut (Deriu et al., 2013; Degnan et al., 2014).

Virus-Mediated Genetic Exchange

Viruses infecting microbial populations in various environments have been shown to act as mobile genetic elements, acting as important vectors of horizontal gene transfer and guiding the evolution and diversification of bacteria (Wommack and Colwell, 2000; Koskella and Brockhurst, 2014). Through either generalized transduction (packaging only host bacterial DNA into virus particles) or specialized transduction (mis-packaging host DNA along with virus DNA) viruses contribute to bacterial genetic variability, introducing changes to the genetic make-up of otherwise genetically homogeneous bacterial strains, for example *E. coli* strains causing urinary tract and bloodstream infections (Frost et al., 2005; Ben Zakour et al., 2016). Conversely, the pangenome of dsDNA viruses and prophages infecting a broad range of hosts shows an extensive network of genetic similarity for a subset of viruses, suggestive of a common virus genetic pool and modules of function-related viral genes, resulting in mosaic virus genomes (Hendrix et al., 1999).

Virus genetic exchange from the gut microbiome may have a direct impact on the animal, as well as their bacterial hosts, and virus integrases have been shown to mediate chromosomal integration in human cells (Groth et al., 2000). Lysogenic phages and prophages can influence host phenotype through phage-encoded gene expression, a process termed lysogenic conversion (Brabban et al., 2005). Qin et al. (2010) found that prophages exert a strong influence on the functioning of the human gut microbiome and contribute an astonishing 5% of the conserved microbiome functions observed. Viral DNA can also be incorporated into the genomes of prokaryotes via CRISPR elements: adaptive immune elements that protect against virus infection through targeted nucleases (Barrangou et al., 2007; Koonin et al., 2017). The host-encoded CRISPR spacers can also recombine with incoming virus DNA to perform specialized transduction (Varble et al., 2019).

One of the most studied attributes of viral genetic exchange is their contribution to the development of antibiotic resistance. Antibiotic resistance is a natural bacterial survival strategy, however, the pervasive use of antibiotics in farming and human health-care has led to the development of multidrug-resistant bacterial pathogens (Balcazar, 2014). Antibiotic resistance genes (ARGs) can be subject to virus-mediated horizontal gene transfer (Parsley et al., 2010). Both gram-positive (Ubukata et al., 1975; Mazaheri Nezhad Fard et al., 2011; Goh et al., 2013) and gram-negative (Blahova et al., 1992; Willi et al., 1997; Oliver et al., 2005) bacteria have been shown to carry virus-encoded ARGs. The diversity of ARGs is lower in pathogenic bacteria and environments contaminated with anthropogenic antibiotics, than in unpolluted environments (Muniesa et al., 2013). It has been postulated by Muniesa et al. (2013) that viruses could allow dissemination of these rare ARGs from the natural environment to the human microbiome. A few studies have begun to examine the prevalence and spread of virus-ARGs. In sewage waters, ARGs were detected in virus DNA (Muniesa et al., 2004; Marti et al., 2014). Virus-encoded putative ARGs are also prevalent in healthy human feces, being present in 77% of samples (Minot et al., 2011; Quirós et al., 2014) and have a high carriage rate of in cattle, pigs and poultry (Pereira et al., 1997; Colomer-Lluch et al., 2011).

However, ARGs identified by bioinformatic searches of viral metagenomes of the human and mouse gut may overestimate

ARG abundance, as during experimental testing only a few of the predicted ARGs could confer antibiotic resistance *in vitro* (Enault et al., 2017). Experimental testing is vital to determine the functionality of virus-encoded putative ARGs with low sequence identity to bacterial genes. For example, experimental testing has been used to establish the function of virus-encoded β-lactamases identified in the genomes of human gut viruses (Ogilvie et al., 2013). Interestingly, a high proportion of ARGs are carried on plasmids (Bennett, 2008), meaning that even if viral integration-mediated horizontal gene transfer of ARGs is irrelevant, viruses could still facilitate the spread of ARGs, through the release of intact bacterial plasmids following virus-mediated bacterial lysis (Keen et al., 2017).

Another well-studied example of virus-mediated genetic transfer between bacterial genera, is the movement of virus-encoded toxin genes and the evolution of toxin-producing bacteria. A variety of virus-mediated extracellular toxin genes have been identified (listed in **Table 3**). Conversely, bacteria which develop resistance to phages, rather than allowing the integration of prophage DNA, may become less virulent as a consequence (Leon and Bastias, 2015). In addition to toxicity genes, a number of phage-encoded accessory genes are directly related to pathogenicity and can be expressed alongside toxins. Phage-encoded genes can span an even wider range of functions that increase the fitness of their bacterial hosts including modification of host cell antigens and antibiotic resistance genes (**Table 3**).

TABLE 3 | Examples of genes and other factors conveyed by prokaryote viruses which enhance the virulence, pathogenicity and functional capacity of the infected host.

Toxins and factors encoded by prokaryote virus genes	Example publication
Shiga toxin	Smith and Huggins, 1983
Botulinum neurotoxin	Eklund et al., 1971
Diphtheria toxin	Freeman, 1951
Cholera toxin	Abel et al., 2015
Enterohemolysin	Beutin et al., 1993
Cytotoxin	Nakayama et al., 1999
Superantigens	Beres et al., 2002
Leukocidin	Kaneko et al., 1998
Enterotoxin	Betley and Mekalanos, 1985
Scarlet fever toxin	Smoot et al., 2002
Exfoliative toxin	Yamaguchi et al., 2000
Macro-organism invasion	Hynes and Ferretti, 1989
Serum resistance	Barondess and Beckwith, 1990
Phospholipases	Beres et al., 2002
Bacterial adhesion factors	Karaolis et al., 1999
Intracellular survival	Coleman et al., 1989
Antivirulence factors	Ho and Slauch, 2001
Modification of host-cell surface antigens	Wright, 1971
Type III effector proteins	Mirold et al., 1999
Photosynthesis apparatus	Mann et al., 2003
Defense against phage superinfection	Poullain et al., 2008
Antibiotic resistance genes	Balcazar, 2014

Interactions With the Immune System

In recent years, studies of the human and mouse gastrointestinal tract have provided insights into the establishment of viral populations within the developing gut, and of the interactions occurring between the viruses infecting commensal intestinal microbes and the animal itself. Viruses are rapidly recruited to the human gut within the first few months of life, leading to a diverse viral population dominated by viruses infecting the microbes of the gut, particularly Caudovirales, which then declines in diversity over the following 2 years (Lim et al., 2016). This commensal viral community is an important component of the gut microbiome and plays a role in intestinal inflammation, stimulating a low-level immune response without causing clinical symptoms (Norman et al., 2014).

Growing evidence suggests that viruses have a role in immune defense within the gut mucosa, where they can influence the innate and adaptive immune system (Barr et al., 2015). A number of viruses possess adhesin proteins, with immunoglobulin-like domains, located on the capsid, collar whiskers or tail shaft that are not involved in host attachment, but can facilitate virus attachment to the gut mucosa (Fraser et al., 2007; Barr et al., 2013). Hypervariable regions collocated in either the virus tail fibers and/or immunoglobulin-like genes might help these viruses to evade the adaptive immune system of the gut (Doulatov et al., 2004; Minot et al., 2012). These features allow viruses to attach and persist in the gut mucosa at high concentrations, where they can outnumber bacteria (Barr et al., 2013). Silveira and Rohwer (2016) proposed that the survival strategy employed by virus changes within the mucosa from a lysogenic strategy or "piggyback-the-winner" at the top of the mucosa where hosts are plentiful, to a lytic or "kill-the-winner" strategy deeper in the mucosal layer, where bacterial hosts are scarce.

Although the immune system clears the vast majority of viruses effectively, phages have been shown to be actively transported by transcytosis out of the gut across epithelial cells (Nguyen et al., 2017). This probably accounts for the discovery of phages, delivered to the gastrointestinal tract of mammals for therapy, being found in the blood stream and systemic tissue, where they can trigger both the innate and adaptive immune system (Wolochow et al., 1966; Wolf et al., 1981; Duerr et al., 2004). The translocation of viruses is not always equal among viruses or individuals (Bruttin and Brussow, 2005; Górski et al., 2006).

Biofilm Formation and Virus: Biofilm Interactions

Biofilms are a community of microorganisms aggregated together within a self-produced matrix (Zobell and Anderson, 1936; Caron, 1987). Biofilms are ubiquitous in nature and most bacteria as well as a number of fungal and yeast species, have the potential to form a biofilm. The biofilms that develop are often poly-microbial and inter-kingdom communities (Swidsinski et al., 2013; Smith et al., 2016; Raghupathi et al., 2018).

Within biofilms viruses are often viewed as a predator of bacteria and can therefore offer an alternative to antimicrobial agents against recalcitrant biofilms. The efficacy of viruses against *in vitro* monospecies biofilms has been shown, e.g., in *E. coli* (Doolittle et al., 1995). Not only are viruses are able to prevent biofilm formation (Hibma et al., 1997) and disrupt mature biofilms (Sillankorva et al., 2004; Soni and Nannapaneni, 2010), but they demonstrate synergy with antimicrobial treatments (Sharma et al., 2005; Bedi et al., 2009). The anti-biofilm activity results from not only lysing cells, but also degrading the matrix using depolymerases (Hughes et al., 1998).

Biofilms represent a rich resource for viral production, but the physical nature of biofilms limits the ability of viruses to exploit it (**Figure 2B**). The biofilm environment brings cells close together and structural elements, such as water channels, allow virus particles rapid access to bacterial cells (Wood et al., 2000; Donlan, 2009). The varying metabolic states within a biofilm, along with the matrix itself however, can pose challenges for viral infection (Doolittle et al., 1996; Hanlon, 2007). To combat this, viruses often produce disruptive enzymes to enable access within the matrix (Hughes et al., 1998; Hanlon et al., 2001). Bacteria at the periphery of a biofilm are most vulnerable to virus adsorption, whilst the dense structure at the center of the biofilm can entrap viruses (Abedon, 2016; Bull et al., 2018; Vidakovic et al., 2018). Hydrophobicity and electrostatic charges are important for viral binding, these can be disrupted within biofilms, and furthermore the aggregation of bacteria may obstruct virus surface receptors (Nordstrom and Forsgren, 1974; Sutherland et al., 2004).

To add to this complexity, viruses has been shown to promote biofilm formation for a number of bacterial genera, such as *Bacillus* and *Actinomyces*, via spontaneous induction of prophage and release of extracellular DNA, which contributes to the extracellular matrix (Schuch and Fischetti, 2009; Carrolo et al., 2010; Wang et al., 2010; Shen et al., 2018). Davies et al. (2016) have also demonstrated that temperate viruses can drive the evolution and adaptation of cells within a biofilm through insertional inactivation of genes. Furthermore, viruses may enhance biofilms through promoting host phenotypic variants and viral particles can physically support the biofilm (Rice et al., 2009). As in any natural system, viruses and biofilms have an ill-defined and complex relationship, with the two entities, being simultaneously at war and peace.

ROLES OF VIRUSES IN THE RUMEN

The role and impact of viruses within the rumen microbial ecosystem has been an area of interest for many years, however, relatively few studies have been conducted to directly address this. Several important aspects of rumen virus populations and the biology of individual viral isolates have been established and on the basis of these findings, the effects of viral activity in the rumen can be inferred (**Table 4**). Tailed viruses of prokaryotes, classified within the viral order Caudovirales, have been frequently shown to dominate the rumen viral community. These viruses are always present in high numbers, actively replicating in the dense bacterial populations of the rumen and consequently these prokaryote viruses (bacteriophages) are the most studied and best understood.

Historically, the primary impact of viral populations in the rumen has been their contribution to bacterial cell lysis (Jarvis, 1968; Nolan and Leng, 1972; Swain et al., 1996a). The final stages of viral replication results in the accumulation of intact viral particles inside the cells and fragmentation (lysis) of the cell membrane, causing cell death and the release of progeny viral particles. Lysis also releases remnants of the microbial cell, including cell wall fragments as well as proteins and nucleic acids, which may then be taken up and utilized by other rumen microbes, a process often described as intra-ruminal recycling (Firkins et al., 1992; Hartinger et al., 2018) (**Figure 2A**). The recycling of nutrients amongst microbes was initially regarded as being a negative consequence of viral activity, limiting the amount of microbial protein carried through to the lower intestine where it can be absorbed by the animal (Leng and Nolan, 1984). A more recent study using proteomics (Solden et al., 2018), suggested that virus-mediated cell lysis also releases intact microbial enzymes, including those involved in carbohydrate breakdown, which in turn facilitates and enhances extracellular feed breakdown within the rumen. This study re-enforces the initial hypotheses that virus populations significantly impact on microbial lysis and nutrient recycling within the rumen, but in contrast, has also provided an alternative, more positive perspective on the long standing question of whether viral-mediated microbial lysis is detrimental or beneficial to rumen function.

The second major impact of rumen viral populations is that they modulate microbial populations, notably dominant bacterial populations, causing viral blooms. These viral blooms were first observed in studies monitoring phage population numbers over a 24 h period in once-a-day fed sheep (Swain et al., 1996a). As virus particles, even those sourced from the rumen, are degraded by rumen fluid (Orpin and Munn, 1974; Tarakanov, 1976) there must be constant viral replication to replenish and sustain viral numbers. Blooms of lytic activity may reflect top–down or "kill the winner" activity, with dominant microbial populations being targets of viral predation, however this has never been shown experimentally for the rumen. Given the functional redundancy of rumen microbial populations, whereby multiple microbial species can perform the same function (Henderson et al., 2015; Weimer, 2015), it can be anticipated that blooms of lytic phage activity are unlikely to impact on overall rumen efficiency, with the niche made available by the selective removal of dominant genera, being rapidly filled by a bacterial population with similar fermentative capacity. This turn-over of microbial populations, however, represents the way viruses can drive and maintain microbial diversity in the rumen.

The third proposed impact of rumen viral populations is their capacity to act as mobile genetic elements (MGE). In

TABLE 4 | Roles and characteristics of virus populations within the rumen, as suggested by previous studies of rumen virus populations, rumen microbes (bacteria and archaea) and viruses isolated from the rumen.

Roles and characteristics of virus populations within the rumen	Example publications
Viruses are always present in high numbers and actively replicate in the microbial populations of the rumen.	Adams et al., 1966; Ritchie et al., 1970
Viral replication and action of viral proteins causes lysis of microbial cells and the release of microbial proteins and nucleic acids, contributing to the intra-ruminal recycling of nutrients.	Nolan and Leng, 1972; Orpin and Munn, 1974; Wells and Russell, 1996; Solden et al., 2018
Viruses are mobile genetic elements; they transfer genetic material between microbes and enable the stable integration of virus-encoded (prophage) genes into rumen microbial genomes.	Klieve et al., 1989; Cheong and Brooker, 1998; Attwood et al., 2008; Seshadri et al., 2018
Virus populations drive microbial diversity through predation, causing episodes of microbial lysis and blooms of virus particles.	Hoogenraad et al., 1967; Iverson and Millis, 1977; Klieve and Swain, 1993
Virus infection and development of resistance can cause changes in bacterial growth habit (e.g., increases in bacterial extracellular polysaccharide).	Klieve and Bauchop, 1991; Klieve et al., 1991
Rumen viral population size and composition is dynamic, fluctuating in response to changes in microbial numbers and diet.	Swain et al., 1996a; Klieve et al., 1998; Anderson et al., 2017
Rumen viruses are taxonomically diverse with the prokaryote virus order Caudovirales (tailed phages) being the most abundant.	Ritchie et al., 1970; Klieve and Bauchop, 1988; Berg Miller et al., 2012
Viruses (specifically phages) tend to only infect specific rumen microbes (limited microbial host range).	Iverson and Millis, 1976a; Styriak et al., 1989; Klieve et al., 1999
Virus particles (specifically phage particles) can be degraded with prolonged exposure to rumen fluid.	Iverson and Millis, 1976a; Smith et al., 1987; Nemcova et al., 1993
Virus infection contributes to the development of genetic resistance mechanisms (e.g., CRISPR/*Cas* systems).	Berg Miller et al., 2012; Gilbert et al., 2017
Viruses can interact with cells of the rumen epithelium (demonstrated *in vitro*).	Styriak et al., 1991

this way, viruses can pick up and transfer genetic material between the microbes which they infect and replicate in. The most notable examples include transducing phages, which readily package the host DNA into phage particles (Brussow et al., 2004), and lysogenic phages which form a stable heritable state with the host cell (Weinbauer, 2004; Touchon et al., 2017). Of these, lysogenic phages infecting rumen bacteria and archaea have been most frequently reported (see section "Culture-Based Viral Isolations and Genome Sequencing"). It has also been suggested in a bioinformatics study that lysogenic phages can transfer auxiliary metabolic genes (AMGs) between rumen bacteria, resulting in metabolic re-programming (Anderson et al., 2017). In other microbial environments virus-mediated genetic transfer also contributes to the development of antimicrobial resistance and toxigenic conversion (see section "Virus-Mediated Genetic Exchange").

Although several rumen genera have been shown to produce antimicrobial proteins, for example bacteriocins produced by *Streptococcus* and *Ruminococcus* (Chen and Weimer, 2001; Azevedo et al., 2015), there have been no investigations into the extent of toxin production by rumen microbes and the contribution of viruses to toxigenic conversion is not understood. Rumen microbes have been shown to demonstrate antimicrobial resistance (Mantovani and Russell, 2001; Benahmed et al., 2014) and the use of antibiotics in feed supplements can contribute to the development of this resistance (Russell and Houlihan, 2003; Cameron and McAllister, 2016). The contribution of viruses to the development of antimicrobial resistance in the rumen has not been investigated. Most of the more abundant rumen microbial genera are not recognized as important zoonotic pathogens and if virus-mediated toxigenic conversion does occur, given the lack of published data, it appears to have had little impact on ruminant or human health. Instead the lower intestine of ruminants represents more of a public health concern, because the intestine can harbor zoonotic and highly pathogenic *E. coli* strains, particularly strains that have undergone toxigenic conversion following infection by Shiga-toxigenic phages (Brussow et al., 2004; Bonanno et al., 2016).

The other potential impacts of viruses on microbial populations are in accordance with observations of viral populations in other environments, but have not been extensively investigated. These impacts include: (1) Virus activity facilitating the formation of biofilms, with bacteria changing their physical growth habit, forming clumps and layers of extracellular polysaccharide in response to phage infection and the development of phage resistance (Klieve and Bauchop, 1991). (2) The ruminant gastrointestinal tract may never be free of viruses as commensal microbes and their associated viruses may simultaneously colonize the ruminant gut during early life development. In addition, viruses may be readily introduced into the rumen with between-animal transfer of viruses occurring via aerosols and saliva from close contact (e.g., maternal transfer, especially pre-weaning) and from shared feed and water sources, as well as housing and farm infrastructure (**Figure 3**). (3) Viruses infecting rumen bacteria may interact with the cells of the rumen wall, eliciting an immune response. For all these potential roles, further investigations and technological advances are required, e.g., to link specific virus and host populations, to provide both experimental evidence and new insights, into how rumen viral populations contribute

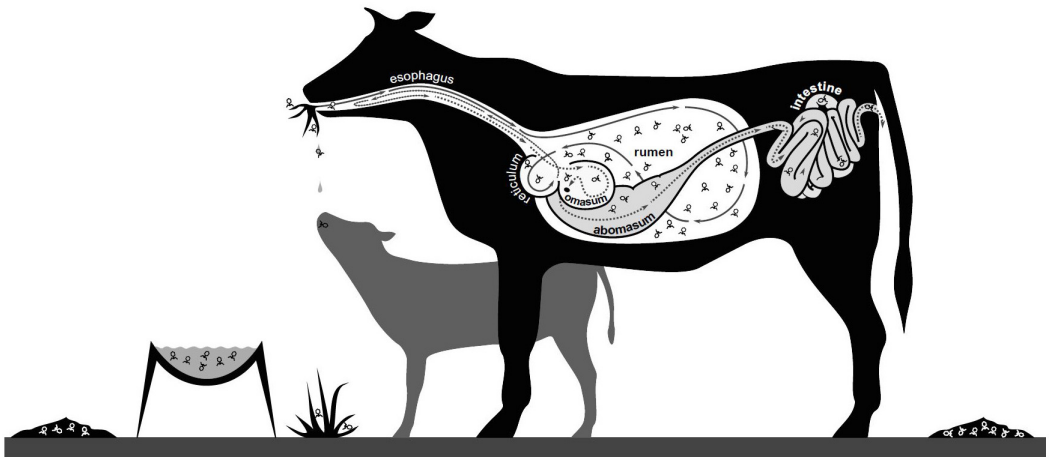

FIGURE 3 | Potential mechanisms for spread of rumen viruses. Viruses may be spread by aerosols, saliva and fecal shedding, with viral particles moving between animals (maternal and within herd interactions) and their environment (water, feed, housing and infrastructure).

to the rumen microbiome, fermentation efficiency and overall ruminant growth and condition.

FUTURE DIRECTIONS AND APPLICATIONS

Our ability to explore microbial communities has expanded with our ability to sequence microbes quickly and cheaply from any given environment. This presents an amazing opportunity to investigate viruses without the requirement to grow them, or their host. Techniques are evolving that enable us to link viruses to their potential hosts using metagenomic information alone; such as matching CRISPR-spacer regions in hosts to homologous sequences in their viruses which also provides clues to the frequency of viral interactions (Berg Miller et al., 2012; Sanguino et al., 2015; Edwards et al., 2016), or, through linking physically close genomic information by proximity ligation, such as infected host genomes and viral progeny prior to release (Marbouty et al., 2017). Our ability to interpret metagenomic sequence data gives us a tantalizing insight into virus-host community dynamics, but a deeper understanding of functionality is hindered by a lack of basic knowledge of the roles and functions of the organisms in the rumen. There is a dearth of basic knowledge of rumen viruses and that is critical to understanding their function; such as prophage induction, auxiliary genes and functional roles, lytic virus virulence, replication rate, host range and burst size. The future of rumen viral work will need to focus on two areas: answering outstanding questions (**Table 5**) and developing practical applications.

To address the outstanding questions, rumen viral research will need to be carried out on three fronts, standard laboratory based *in vitro* work, coupled with *in vivo* rumen studies to inform *in silico* computer modeling which will generate testable hypotheses. Advances in sequencing and proteomics provide vast quantities of data that enable us to characterize, understand and model the role of rumen viruses. Basic knowledge of the rumen microbiome as a whole, including viruses, is essential to allow us to explore the rumen microbiome through modeling (Louca and Doebeli, 2017). Modeling of whole microbial communities opens the possibility to understand community functionality and dynamics (Widder et al., 2016). Rumen modeling has largely concentrated on the impacts of nutrition on emissions and efficiency rather than on the microbes and their functions (Ellis et al., 2009; Mills et al., 2014; Gregorini et al., 2015). A recent study examined a simple rumen microbiome consisting

TABLE 5 | Future rumen virus research: outstanding questions for further investigation.

Outstanding questions
Do viruses infect all of the microbial populations found in the rumen?
How do virus populations impact on biofilm formation, colonization of plant material and rumen fermentation?
To what extent do virus populations impact on microbial lysis and nutrient recycling within the rumen?
What are the biological properties of rumen viruses (e.g., virus:host interactions, host range, replication strategies, virus particle survival and persistence in the environment)?
Do viruses impact on microbial gene transfer in the rumen and what types of genes may they transfer?
Do rumen virus communities change from early life to mature animals and can they change throughout the lifetime of an animal?
Do viruses from the rumen interact with the rumen wall and GI tract and cause immunological effects in the animal?
Can phage therapy (lytic viruses or viral enzymes) be used to remove problematic organisms (e.g., methanogens) from the rumen?

of representatives of three microbial groups: Bacteroidetes, Firmicutes, and Archaea, plus their associated viruses (Islam et al., 2019). This simple model seeks to identify novel metabolic interplay between these microbes, to demonstrate metabolite exchange and the potential importance of viral-encoded auxiliary metabolic genes. Modeling is one tool to understand rumen function and opens the door to explore the use of viruses as alternatives to antibiotics to increase feed efficiency, maintain robust animal health and lower methane production.

The current widespread use of antibiotics as feed additives can be viewed as unsustainable (Economou and Gousia, 2015) and phage therapy shows promise as a targeted approach to control bacterial populations and promote animal health. Natural viruses have been used through phage therapy to remove problematic bacteria, archaea and their biofilms, from plants, animals and food crops, to humans, aquaculture and poultry (Donlan, 2009; Monk et al., 2010; Sarker et al., 2016). More recently engineered viruses have been used clinically to treat human patients with multi-antibiotic resistant infections (Dedrick et al., 2019), however this has been on a compassionate basis, as phage therapy is not currently authorized as a medicinal product. Temperate viruses that integrate into their host genomes also have the potential to confer positive genetic traits, but tend to be avoided due to the negative traits they can also confer (Monteiro et al., 2019). To develop effective phage therapies, we still need to generate rumen-specific information on viral dose size and viral particle longevity, to overcome potential viral inactivation by factors such as tannins, antibodies, macrophages, and clearance rates, as well as undertake basic viral characterization of adsorption efficiency, latency and burst size (Ly-Chatain, 2014). Phage therapy has been applied to ruminants with varying results (Gill et al., 2006; Johnson et al., 2008), however, this has been aimed primarily at the lower intestine (Dini et al., 2012) and has yet to be widely exploited in the rumen.

Phage therapy using intact viruses is highly host specific and presents regulatory issues, the use of virus-derived products or "enzybiotics" could directly replace conventional antimicrobials (O'Mahony et al., 2011; Shen et al., 2012; Louca and Doebeli, 2017). Mechanisms for the delivery and release of viral material from the host often involves enzymes (lysins/holins, peptidases, depolymerases, tail spike proteins), which can be repurposed as enzybiotics (Murphy et al., 1995; Andres et al., 2012; São-José, 2018). Indeed, enzybiotics have been tested in animals (O'Flaherty et al., 2009) and targeted nanoparticles displaying enzybiotics have been demonstrated to reduce methanogenesis by rumen-associated archaea (Altermann et al., 2018). Virus-derived enzybiotics are appealing, due to their scalability and ease of use within existing guidelines, enzybiotics could represent the future direction of phage research.

CONCLUSION

The rumen microbial community is integral in maintaining ruminant health, feed efficiency and is responsible for methanogenesis. Viruses are numerous, ubiquitous components of microbial communities, including those found in the rumen and have been overlooked in the past due to the technical difficulties experienced in their study. From the moment viral particles were first visualized it was clear that rumen viruses deserved investigating. However, direct study was limited to observations and indirect study proved problematic. This has led to rumen viruses being either ignored or overlooked in favor of more rewarding studies. Our current understanding shows that rumen viruses are not only numerically important, but are rapidly turned over. Within the rumen microbiome viruses enable nutrient cycling, genetic exchange and appear to promote functional stability. We can also extrapolate from other environments their likely influence on the rumen, but these require experimental testing.

Fortunately, new technologies are emerging that allow the scientific community to answer many of the fundamental questions pertaining to viruses; what viruses are present, how abundant are they and how do viruses interact with microbes, influence microbial community function and what impact do rumen microbial viruses have on the whole animal? Whole microbiome modeling, metagenome sequencing and metaproteomics are powerful and exciting tools that will enable us to answer these questions and test our predictions. Viruses offer the chance to understand, model and manipulate complex rumen communities. The future potential of viral applications are many and varied and require a more integrative research effort. Viruses are an intrinsic part of rumen microbial communities and are no more or less important than any other component. It is vital that viruses be incorporated into well-designed, large-scale studies that encompass all components of the rumen microbiome for both fundamental and hypothesis driven research. With affordable, powerful tools at our disposal we have a fantastic opportunity to explore rumen viruses and provide practical applications, based on sound knowledge of the whole ruminant.

AUTHOR CONTRIBUTIONS

RG and EJ conceived and designed the manuscript. RG and EJ wrote the majority of the manuscript with ET, TH, JF, CC, and PP contributing specialist sections to the manuscript. EJ, ET, RG, and KC contributed to the figures and tables. RG prepared the rumen viral fraction. KC undertook the electron microscopy. DO critically reviewed the manuscript and contributed to the table improvement. All authors approved the final version of the manuscript.

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REFERENCES

Abedon, S. T. (2012). "Phage-therapy best practices," in *Bacteriophages in Health and Disease*, eds P. Hyman and S. T. Abedon (Wallingford: CAB International), 256–272. doi: 10.1079/9781845939847.0256

Abedon, S. T. (2016). Bacteriophage exploitation of bacterial biofilms: phage preference for less mature targets? *FEMS Microbiol. Lett.* 363:fnv246. doi: 10.1093/femsle/fnv246

Abel, S., Abel Zur, Wiesch, P., Davis, B. M., and Waldor, M. K. (2015). Analysis of bottlenecks in experimental models of infection. *PLoS Pathog.* 11:e1004823. doi: 10.1371/journal.ppat.1004823

Ackermann, H. W. (1998). Tailed bacteriophages: the order caudovirales. *Adv. Virus Res.* 51, 135–201. doi: 10.1016/s0065-3527(08)60785-x

Ackermann, H. W. (2012). Bacteriophage electron microscopy. *Adv. Virus Res.* 82, 1–32. doi: 10.1016/B978-0-12-394621-8.00017-0

Ackermann, H.-W. (2013). Sad state of phage electron microscopy. Please shoot the messenger. *Microorganisms* 2, 1–10. doi: 10.3390/microorganisms2010001

Ackermann, H. W., and Prangishvili, D. (2012). Prokaryote viruses studied by electron microscopy. *Arch. Virol.* 157, 1843–1849. doi: 10.1007/s00705-012-1383-y

Adams, J. C., Gazaway, J. A., Brailsford, M. D., Hartman, P. A., and Jacobson, N. L. (1966). Isolation of bacteriophages from the bovine rumen. *Experentia* 22, 717–718. doi: 10.1007/BF01901335

Adriaenssens, E. M., Ackermann, H.-W., Anany, H., Blasdel, B., Connerton, I. F., Goulding, D., et al. (2012). A suggested new bacteriophage genus: "Viunalikevirus". *Arch. Virol.* 157, 2035–2046. doi: 10.1007/s00705-012-1360-5

Altermann, E., Schofield, L. R., Ronimus, R. S., Beatty, A. K., and Reilly, K. (2018). Inhibition of rumen methanogens by a novel archaeal lytic enzyme displayed on tailored bionanoparticles. *Front. Microbiol.* 9:2378–2378. doi: 10.3389/fmicb.2018.02378

Ambrozic, J., Ferme, D., Grabnar, M., Ravnikar, M., and Avgustin, G. (2001). The bacteriophages of ruminal prevotellas. *Folia Microbiol.* 46, 37–39. doi: 10.1007/bf02825881

Anderson, C. L., Sullivan, M. B., and Fernando, S. C. (2017). Dietary energy drives the dynamic response of bovine rumen viral communities. *Microbiome* 5:155. doi: 10.1186/s40168-017-0374-3

Andres, D., Roske, Y., Doering, C., Heinemann, U., Seckler, R., and Barbirz, S. (2012). Tail morphology controls DNA release in two *Salmonella* phages with one lipopolysaccharide receptor recognition system. *Mol. Microbiol.* 83, 1244–1253. doi: 10.1111/j.1365-2958.2012.08006.x

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387

Attwood, G. T., Kelly, W. J., Altermann, E. H., and Leahy, S. C. (2008). Analysis of the *Methanobrevibacter ruminantium* draft genome: understanding methanogen biology to inhibit their action in the rumen. *Aust. J. Exp. Agric.* 48, 83–88. doi: 10.1071/ea07269

Azevedo, A. C., Bento, C. B. P., Ruiz, J. C., Queiroz, M. V., and Mantovani, H. C. (2015). Distribution and genetic diversity of bacteriocin gene clusters in rumen microbial genomes. *Appl. Environ. Microbiol.* 81, 7290–7304. doi: 10.1128/AEM.01223-15

Balcazar, J. L. (2014). Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS Pathog.* 10:e1004219. doi: 10.1371/journal.ppat.1004219

Baresi, L., and Bertani, G. (1984). Isolation of a bacteriophage for a methanogenic bacterium. *Abstracts Ann. Meeting American Soc. Microbiol.* 28, 133.

Barondess, J. J., and Beckwith, J. (1990). A bacterial virulence determinant encoded by lysogenic coliphage lambda. *Nature* 346, 871–874. doi: 10.1038/346871a0

Barr, J. J., Auro, R., Furlan, M., Whiteson, K. L., Erb, M. L., Pogliano, J., et al. (2013). Bacteriophage adhering to mucus provide a non-host-derived immunity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 10771–10776. doi: 10.1073/pnas.1305923110

Barr, J. J., Auro, R., Sam-Soon, N., Kassegne, S., Peters, G., Bonilla, N., et al. (2015). Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13675–13680. doi: 10.1073/pnas.1508355112

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Barylski, J., Enault, F., Dutilh, B. E., Schuller, M. B., Edwards, R. A., Gillis, et al. (2020a). Analysis of *Spounaviruses* as a case study for the overdue reclassification of tailed phages. *Syst. Biol.* 69, 110–123. doi: 10.1093/sysbio/syz036

Barylski, J., Kropinski, A. M., Alikhan, N.-F., Adriaenssens, E. M., and Ictv Report. (2020b). ICTV Virus taxonomy profile: Herelleviridae. *J. Gen. Virol.* doi: 10.1099/jgv.1090.001392 [Epub ahead of print].

Bedi, M., Verma, V., and Chhibber, S. (2009). Amoxicillin and specific bacteriophage can be used together for eradication of biofilm of *Klebsiella pneumoniae* B5055. *World J. Microbiol. Biotech.* 25, 1145–1151. doi: 10.1007/s11274-009-9991-8

Ben Zakour, N. L., Alsheikh-Hussain, A. S., Ashcroft, M. M., Khanh Nhu, N. T., Roberts, L. W., Stanton-Cook, M., et al. (2016). Sequential acquisition of virulence and fluoroquinolone resistance has shaped the evolution of *Escherichia coli* ST131. *mBio* 7, e347-16. doi: 10.1128/mBio.00347-16

Benahmed, F. H., Gopinath, G. R., Harbottle, H., Cotta, M. A., Luo, Y., Henderson, C., et al. (2014). Draft genome sequences of *Streptococcus bovis* strains ATCC 33317 and JB1. *Genome Ann.* 2, e1012–e1014. doi: 10.1128/genomeA.01012-14

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* 153(Suppl. 1), S347–S357. doi: 10.1038/sj.bjp.0707607

Beres, S. B., Sylva, G. L., Barbian, K. D., Lei, B., Hoff, J. S., Mammarella, N. D., et al. (2002). Genome sequence of a serotype M3 strain of group A *Streptococcus*: phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10078–10083. doi: 10.1073/pnas.152298499

Berg Miller, M. E., Yeoman, C. J., Chia, N., Tringe, S. G., Angly, F. E., Edwards, et al. (2012). Phage–bacteria relationships and CRISPR elements revealed by a metagenomic survey of the rumen microbiome. *Environ. Microbiol.* 14, 207–227. doi: 10.1111/j.1462-2920.2011.02593.x

Berthod, A., and Faure, K. (2015). "Separations with a liquid stationary phase: countercurrent chromatography or centrifugal partition chromatography," in *Analytical Separation Science*, eds V. Pino, J. L. Anderson, A. Berthod, and A. M. Stalcup (Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA), 1177–1206. doi: 10.1002/9783527678129.assep046

Betley, M. J., and Mekalanos, J. J. (1985). Staphylococcal enterotoxin A is encoded by phage. *Science* 229, 185–187. doi: 10.1126/science.3160112

Beutin, L., Stroeher, U. H., and Manning, P. A. (1993). Isolation of enterohemolysin (Ehly2)-associated sequences encoded on temperate phages of *Escherichia coli*. *Gene* 132, 95–99. doi: 10.1016/0378-1119(93)90519-9

Blahova, J., Hupkova, M., Krcmery, V., and Schafer, V. (1992). Imipenem and cefotaxime resistance: transduction by wild-type phages in hospital strains of *Pseudomonas aeruginosa*. *J. Chemother.* 4, 335–337. doi: 10.1080/1120009x.1992.11739187

Bonanno, L., Petit, M.-A., Loukiadis, E., Michel, V., and Auvray, F. (2016). Heterogeneity in induction level, infection ability, and morphology of Shiga toxin-encoding phages (Stx phages) from dairy and human Shiga toxin-producing *Escherichia coli* O26:H11 isolates. *Appl. Environ. Microbiol.* 82, 2177–2186. doi: 10.1128/AEM.03463-15

Borton, M. A., Hoyt, D. W., Roux, S., Daly, R. A., Welch, S. A., Nicora, C. D., et al. (2018). Coupled laboratory and field investigations resolve microbial interactions that underpin persistence in hydraulically fractured shales. *Proc. Natl. Acad. Sci. U.S.A.* 115, E6585–E6594. doi: 10.1073/pnas.1800155115

Brabban, A. D., Hite, E., and Callaway, T. R. (2005). Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer. *Foodborne Pathog. Dis.* 2, 287–303. doi: 10.1089/fpd.2005.2.287

Bradley, D. E. (1967). Ultrastructure of bacteriophages and bacteriocins. *Bacteriol. Rev.* 31, 230–314. doi: 10.1128/mmbr.31.4.230-314.1967

Brailsford, M. D., and Hartman, P. A. (1968). Characterisation of *Streptococcus durans* bacteriophages. *Can. J. Microbiol.* 14, 397–402. doi: 10.1139/m68-063

Bratbak, G., Heldal, M., Norland, S., and Thingstad, T. F. (1990). Viruses as partners in spring bloom microbial trophodynamics. *Appl. Environ. Microbiol.* 56, 1400–1405. doi: 10.1128/aem.56.5.1400-1405.1990

Breitbart, M., Salamon, P., Andresen, B., Mahaffy, J. M., Segall, A. M., Mead, D., et al. (2002). Genomic analysis of uncultured marine viral communities. *Proc. Natl. Acad. Sci. U.S.A.* 99:14250. doi: 10.1073/pnas.202488399

Brody, J. R., and Kern, S. E. (2004). History and principles of conductive media for standard DNA electrophoresis. *Anal. Biochem.* 333, 1–13. doi: 10.1016/j.ab.2004.05.054

Brum, J. R., Ignacio-Espinoza, J. C., Kim, E.-H., Trubl, G., Jones, R. M., Roux, S., et al. (2016). Illuminating structural proteins in viral "dark matter" with metaproteomics. *Proc. Natl. Acad. Sci. U.S.A.* 113, 2436–2441. doi: 10.1073/pnas.1525139113

Brussow, H., Canchaya, C., and Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol. Mol. Biol. Rev.* 68, 560–602. doi: 10.1128/mmbr.68.3.560-602.2004

Bruttin, A., and Brussow, H. (2005). Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. *Antimicrob. Agents Chemother.* 49, 2874–2878. doi: 10.1128/aac.49.7.2874-2878.2005

Bull, J. J., Christensen, K. A., Scott, C., Jack, B. R., Crandall, C. J., and Krone, S. M. (2018). Phage-bacterial dynamics with spatial structure: self organization around phage sinks can promote increased cell densities. *Antibiotics (Basel)* 7:8. doi: 10.3390/antibiotics7010008

Cameron, A., and McAllister, T. A. (2016). Antimicrobial usage and resistance in beef production. *J. Anim. Sci. Biotechnol.* 7:68. doi: 10.1186/s40104-016-0127-3

Caron, D. A. (1987). Grazing of attached bacteria by heterotrophic microflagellates. *Microb. Ecol.* 13, 203–218. doi: 10.1007/BF02024998

Carrolo, M., Frias, M. J., Pinto, F. R., Melo-Cristino, J., and Ramirez, M. (2010). Prophage spontaneous activation promotes DNA release enhancing biofilm formation in Streptococcus pneumoniae. *PLoS One* 5:e15678. doi: 10.1371/journal.pone.0015678

Casjens, S. R., and Hendrix, R. W. (2015). Bacteriophage lambda: Early pioneer and still relevant. *Virology* 47, 310–330. doi: 10.1016/j.virol.2015.02.010

Chen, J., and Weimer, P. (2001). Competition among three predominant ruminal cellulolytic bacteria in the absence or presence of non-cellulolytic bacteria. *Microbiol.* 147, 21–30. doi: 10.1099/00221287-147-1-21

Cheong, J. P., and Brooker, J. D. (1998). Lysogenic bacteriophage M1 from *Selenomonas ruminantium*: isolation, characterisation and DNA sequence analysis of the integration site. *Microbiology* 144, 2195–2202. doi: 10.1099/00221287-144-8-2195

Cheong, J. P., and Brooker, J. D. (2000). Isolation of a virulent bacteriophage from a *Propionibacterium* species in the sheep rumen. *Aust. J. Agric. Res.* 51, 119–123.

Clokie, M. R. J., Millard, A. D., Letarov, A. V., and Heaphy, S. (2011). Phages in nature. *Bacteriophage* 1, 31–45. doi: 10.4161/bact.1.1.14942

Coleman, D. C., Sullivan, D. J., Russell, R. J., Arbuthnott, J. P., Carey, B. F., and Pomeroy, H. M. (1989). *Staphylococcus aureus* bacteriophages mediating the simultaneous lysogenic conversion of beta-lysin, staphylokinase and enterotoxin A: molecular mechanism of triple conversion. *J. Gen. Microbiol.* 135, 1679–1697. doi: 10.1099/00221287-135-6-1679

Colomer-Lluch, M., Jofre, J., and Muniesa, M. (2011). Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS ONE* 6:e17549. doi: 10.1371/journal.pone.0017549

Creaser, E. H., and Taussig, A. (1957). The purification and chromatography of bacteriophages on anion-exchange cellulose. *Virol.* 4, 200–208. doi: 10.1016/0042-6822(57)90057-0

Cuervo, A., and Carrascosa, J. L. (2018). Observation of bacteriophage ultrastructure by cryo-electron microscopy. *Methods Mol. Biol.* 1693, 43–55. doi: 10.1007/978-1-4939-7395-8_5

Davies, E. V., James, C. E., Williams, D., O'brien, S., Fothergill, J. L., Haldenby, S., et al. (2016). Temperate phages both mediate and drive adaptive evolution in pathogen biofilms. *Proc. Natl. Acad. Sci. U.S.A.* 113:8266. doi: 10.1073/pnas.1520056113

Dedrick, R. M., Guerrero-Bustamante, C. A., Garlena, R. A., Russell, D. A., Ford, K., Harris, K., et al. (2019). Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat. Med.* 25, 730–733. doi: 10.1038/s41591-019-0437-z

Dedrick, R. M., Jacobs-Sera, D., Bustamante, C. A., Garlena, R. A., Mavrich, T. N., Pope, W. H., et al. (2017). Prophage-mediated defence against viral attack and viral counter-defence. *Nature Microbiol.* 2:16251. doi: 10.1038/nmicrobiol.2016.251

Degnan, P. H., Barry, N. A., Mok, K. C., Taga, M. E., and Goodman, A. L. (2014). Human gut microbes use multiple transporters to distinguish vitamin B12 analogs and compete in the gut. *Cell Host Microbe* 15, 47–57. doi: 10.1016/j.chom.2013.12.007

Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002). Capturing chromosome conformation. *Science* 295, 1306–1311. doi: 10.1126/science.1067799

Deriu, E., Liu, J. Z., Pezeshki, M., Edwards, R. A., Ochoa, R. J., Contreras, et al. (2013). Probiotic bacteria reduce *Salmonella* typhimurium intestinal colonization by competing for iron. *Cell Host Microbe* 14, 26–37. doi: 10.1016/j.chom.2013.06.007

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., and Seifert, J. (2017). A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. *Front. Microbiol.* 8:1605. doi: 10.3389/fmicb.2017.01605

d'Herelle, F. (1918). Technique de la recherche du microbe filtrant bacteriophage (Bacteriophagum intestinale). *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales* 81, 1160–1162.

Didelot, X., Bowden, R., Wilson, D. J., Peto, T. E. A., and Crook, D. W. (2012). Transforming clinical microbiology with bacterial genome sequencing. *Nat. Rev. Genet.* 13:601. doi: 10.1038/nrg3226

Dini, C., Islan, G. A., De Urraza, P. J., and Castro, G. R. (2012). Novel biopolymer matrices for microencapsulation of phages: enhanced protection against acidity and protease activity. *Macromol. Biosci.* 12, 1200–1208. doi: 10.1002/mabi.201200109

Dinsdale, E. A., Edwards, R. A., Hall, D., Angly, F., Breitbart, M., Brulc, J. M., et al. (2008). Functional metagenomic profiling of nine biomes. *Nature* 452, 629–830. doi: 10.1038/nature06810

Dolgin, E. (2019). The secret social lives of viruses. *Nature* 570, 290–292. doi: 10.1038/s41586-020-1936-2

Donlan, R. M. (2009). Preventing biofilms of clinically relevant organisms using bacteriophage. *Trends Microbiol.* 17, 66–72. doi: 10.1016/j.tim.2008.11.002

Doolittle, M. M., Cooney, J. J., and Caldwell, D. E. (1995). Lytic infection of *Escherichia coli* biofilms by bacteriophage T4. *Can. J. Microbiol.* 41, 12–18. doi: 10.1139/m95-002

Doolittle, M. M., Cooney, J. J., and Caldwell, D. E. (1996). Tracing the interaction of bacteriophage with bacterial biofilms using fluorescent and chromogenic probes. *J. Ind. Microbiol.* 16, 331–341. doi: 10.1007/bf01570111

Doulatov, S., Hodes, A., Dai, L., Mandhana, N., Liu, M., Deora, R., et al. (2004). Tropism switching in Bordetella bacteriophage defines a family of diversity-generating retroelements. *Nature* 431, 476–481. doi: 10.1038/nature02833

Duerr, D. M., White, S. J., and Schluesener, H. J. (2004). Identification of peptide sequences that induce the transport of phage across the gastrointestinal mucosal barrier. *J. Virol. Methods* 116, 177–180. doi: 10.1016/j.jviromet.2003.11.012

Džunková, M., Low, S. J., Daly, J. N., Deng, L., Rinke, C., and Hugenholtz, P. (2019). Defining the human gut host-phage network through single-cell viral tagging. *Nat. Microbiol.* 4, 2192–2203. doi: 10.1038/s41564-019-0526-2

Economou, V., and Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect. Drug Resist.* 8, 49–61. doi: 10.2147/idr.s55778

Edwards, R. A., McNair, K., Faust, K., Raes, J., and Dutilh, B. E. (2016). Computational approaches to predict bacteriophage–host relationships. *FEMS Microbiol. Rev.* 40, 258–272. doi: 10.1093/femsre/fuv048

Eklund, M. W., Poysky, F. T., Reed, S. M., and Smith, C. A. (1971). Bacteriophage and the toxigenicity of *Clostridium botulinum* type C. *Science* 172, 480–482. doi: 10.1126/science.172.3982.480

Ellis, J. L., Kebreab, E., Odongo, N. E., Beauchemin, K., Mcginn, S., Nkrumah, J. D., et al. (2009). Modeling methane production from beef cattle using linear and nonlinear approaches. *J. Anim. Sci.* 87, 1334–1345. doi: 10.2527/jas.2007-0725

Emerson, J. B., Roux, S., Brum, J. R., Bolduc, B., Woodcroft, B. J., Jang, H. B., et al. (2018). Host-linked soil viral ecology along a permafrost thaw gradient. *Nature Microbiol.* 3, 870–880. doi: 10.1038/s41564-018-0190-y

Enault, F., Briet, A., Bouteille, L., Roux, S., Sullivan, M. B., and Petit, M.-A. (2017). Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. *ISME J.* 11:237. doi: 10.1038/ismej.2016.90

Escobar-Zepeda, A., Vera-Ponce, De León, A., and Sanchez-Flores, A. (2015). The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. *Front. Genet.* 6:348. doi: 10.3389/fgene.2015.00348

Fenton, M., Ross, P., Mcauliffe, O., O'mahony, J., and Coffey, A. (2010). Recombinant bacteriophage lysins as antibacterials. *Bioeng. Bugs* 1, 9–16. doi: 10.4161/bbug.1.1.9818

Figura, G., Kopciuch, A., Owczarek, B., Piotrowicz, A., Miernikiewicz, P., Oślizło, A., et al. (2011). Purification of phage display-modified bacteriophage T4 by affinity chromatography. *BMC Biotechnol.* 11:59. doi: 10.1186/1472-6750-11-59

Firkins, J. L., Weiss, W. P., and Piwonka, E. J. (1992). Quantification of intraruminal recycling of microbial nitrogen using Nitrogen-15. *J. Anim. Sci.* 70, 3223–3233. doi: 10.2527/1992.70103223x

Flint, H. J. (1997). The rumen microbial ecosystem—some recent developments. *Trends Microbiol.* 5, 483–488. doi: 10.1016/s0966-842x(97)01159-1

Fraser, J. S., Maxwell, K. L., and Davidson, A. R. (2007). Immunoglobulin-like domains on bacteriophage: weapons of modest damage? *Curr. Opin. Microbiol.* 10, 382–387. doi: 10.1016/j.mib.2007.05.018

Freeman, V. J. (1951). Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae*. *J. Bacteriol.* 61, 675–688. doi: 10.1128/jb.61.6.675-688.1951

Friedersdorff, J., Kingston-Smith, A., Creevey, C., Rooke, D., Bright, C., and West, D. (2018). "Using high performance counter current chromatography for the separation, preparation and purification of bacteriophages from the rumen microbiome" in *Proceedings of the The 10th International Conference on Countercurrent Chromatography* (Braunschweig: Technische Universität Braunschweig), doi: 10.13140/RG.2.2.12796.90246

Frost, L. S., Leplae, R., Summers, A. O., and Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* 3, 722–732. doi: 10.1038/nrmicro1235

Fuhrman, J. A., and Noble, R. T. (1995). Viruses and protists cause similar bacterial mortality in coastal seawater. *Limnol. Oceanogr.* 40, 1236–1242. doi: 10.4319/lo.1995.40.7.1236

Gilbert, R. A., Kelly, W. J., Altermann, E., Leahy, S. C., Minchin, C., Ouwerkerk, D., et al. (2017). Toward understanding phage:host interactions in the rumen; complete genome sequences of lytic phages infecting rumen bacteria. *Front. Microbiol.* 8:2340. doi: 10.3389/fmicb.2017.02340

Gilbert, R. A., and Klieve, A. V. (2015). "Ruminal viruses (Bacteriophages, Archaeaphages)," in *Rumen Microbiology: From Evolution to Revolution*, eds A. K. Puniya, R. Singh, and D. N. Kamra (Berlin: Springer), 121–141. doi: 10.1007/978-81-322-2401-3

Gill, J. J., Pacan, J. C., Carson, M. E., Leslie, K. E., Griffiths, M. W., and Sabour, P. M. (2006). Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical Staphylococcus aureus mastitis in lactating dairy cattle. *Antimicrob. Agents Chemother.* 50, 2912–2918. doi: 10.1128/aac.01630-05

Goh, S., Hussain, H., Chang, B. J., Emmett, W., Riley, T. V., and Mullany, P. (2013). Phage phiC2 mediates transduction of Tn6215, encoding erythromycin resistance, between Clostridium difficile strains. *MBio.* 4:e840-13. doi: 10.1128/mBio.00840-13

Golomidova, A., Kulikov, E., Isaeva, A., Manykin, A., and Letarov, A. (2007). The diversity of coliphages and coliforms in horse feces reveals a complex pattern of ecological interactions. *Appl. Environ. Microbiol.* 73, 5975–5981. doi: 10.1128/aem.01145-07

Górski, A., Ważna, E., Dąbrowska, B.-W., Dąbrowska, K., Świtała-Jeleń, K., and Międzybrodzki, R. (2006). Bacteriophage translocation. *Pathog. Dis.* 46, 313–319. doi: 10.1111/j.1574-695X.2006.00044.x

Gregg, K., Kennedy, B. G., and Klieve, A. V. (1994). Cloning and DNA sequence analysis of the region containing AttP of the temperate phage phiAR29 of *Prevotella ruminicola* AR29. *Microbiology* 140, 2109–2114. doi: 10.1099/13500872-140-8-2109

Gregorini, P., Beukes, P., Waghorn, G., Pacheco, D., and Hanigan, M. (2015). Development of an improved representation of rumen digesta outflow in a mechanistic and dynamic model of a dairy cow. *Molly. Ecolog. Model.* 313, 293–306. doi: 10.1016/j.ecolmodel.2015.06.042

Groth, A. C., Olivares, E. C., Thyagarajan, B., and Calos, M. P. (2000). A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5995–6000. doi: 10.1073/pnas.090527097

Grybchuk, D., Akopyants, N. S., Kostygov, A. Y., Konovalovas, A., Lye, L.-F., Dobson, D. E., et al. (2018). Viral discovery and diversity in trypanosomatid protozoa with a focus on relatives of the human parasite Leishmania. *Proc. Natl. Acad. Sci. U.S.A.* 115:E506. doi: 10.1073/pnas.1717806115

Hagen, L. H., Frank, J. A., Zamanzadeh, M., Eijsink, V. G. H., Pope, P. B., Horn, S. J., et al. (2017). Quantitative metaproteomics highlight the metabolic contributions of uncultured phylotypes in a thermophilic anaerobic digester. *Appl. Environ. Microbiol.* 83:e1955-16. doi: 10.1128/AEM.01955-16

Haguenau, F., Hawkes, P. W., Hutchison, J. L., Satiatjeunematre, B., Simon, G. T., and Williams, D. B. (2003). Key events in the history of electron microscopy. *Microsc. Microanal.* 9, 96–138. doi: 10.1017/S1431927603030113

Hallewell, J., Niu, Y. D., Munns, K., Mcallister, T. A., Johnson, R. P., Ackermann, H.-W., et al. (2014). Differing populations of endemic bacteriophages in cattle shedding high and low numbers of *Escherichia coli* O157:H7 bacteria in feces. *Appl. Environ. Microbiol.* 80, 3819–3825. doi: 10.1128/AEM.00708-14

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., and Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem. Biol.* 5, R245–R249. doi: 10.1016/S1074-5521(98)90108-9

Hanlon, G. W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int. J. Antimicrob. Agents* 30, 118–128. doi: 10.1016/j.ijantimicag.2007.04.006

Hanlon, G. W., Denyer, S. P., Olliff, C. J., and Ibrahim, L. J. (2001). Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 67, 2746–2753. doi: 10.1128/AEM.67.6.2746-2753.2001

Hart, E. H., Creevey, C. J., Hitch, T., and Kingston-Smith, A. H. (2018). Meta-proteomics of rumen microbiota indicates niche compartmentalisation and functional dominance in a limited number of metabolic pathways between abundant bacteria. *Sci. Rep.* 8:10504. doi: 10.1038/s41598-018-28827-7

Hartinger, T., Gresner, N., and Sudekum, K. H. (2018). Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. *J. Anim. Sci. Biotechnol.* 9:33. doi: 10.1186/s40104-018-0249-x

Hazlewood, G. P., Munn, E. A., and Orpin, C. G. (1983). "Temperate bacteriophages of Selenomonas ruminantium and a *Fusobacterium* sp. isolated from the ovine rumen," in *Abstracts Canadian Society Microbiology 33rd Annual Meeting*, Winnipeg, 76.

Heather, J. M., and Chain, B. (2016). The sequence of sequencers: the history of sequencing DNA. *Genomics* 107, 1–8. doi: 10.1016/j.ygeno.2015.11.003

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Hendrix, R. W., Smith, M. C., Burns, R. N., Ford, M. E., and Hatfull, G. F. (1999). Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2192–2197. doi: 10.1073/pnas.96.5.2192

Heyer, R., Schallert, K., Siewert, C., Kohrs, F., Greve, J., Maus, I., et al. (2019). Metaproteome analysis reveals that syntrophy, competition, and phage-host interaction shape microbial communities in biogas plants. *Microbiome* 7, 1–17. doi: 10.1186/s40168-019-0673-y

Hibma, A. M., Jassim, S. A., and Griffiths, M. W. (1997). Infection and removal of L-forms of Listeria monocytogenes with bred bacteriophage. *Int. J. Food. Microbiol.* 34, 197–207. doi: 10.1016/S0168-1605(96)01190-7

Hitch, T. C. A., Edwards, J. E., and Gilbert, R. A. (2019). Metatranscriptomics reveals mycoviral populations in the ovine rumen. *FEMS Microbiol. Lett.* 366:fnz161. doi: 10.1093/femsle/fnz161

Ho, T. D., and Slauch, J. M. (2001). Characterization of grvA, an antivirulence gene on the gifsy-2 phage in *Salmonella enterica* serovar typhimurium. *J. Bacteriol.* 183, 611–620. doi: 10.1128/JB.183.2.611-620.2001

Hobbs, Z., and Abedon, S. T. (2016). Diversity of phage infection types and associated terminology: the problem with 'Lytic or lysogenic'. *FEMS Microbiol. Lett.* 363:fnw047. doi: 10.1093/femsle/fnw047

Hoogenraad, N. J., Hird, F. J. R., Holmes, I., and Millis, N. F. (1967). Bacteriophages in rumen contents of sheep. *J. Gen. Virol.* 1, 575–576. doi: 10.1099/0022-1317-1-4-575

Howard-Varona, C., Hargreaves, K. R., Abedon, S. T., and Sullivan, M. B. (2017). Lysogeny in nature: mechanisms, impact and ecology of temperate phages. *ISME J.* 11, 1511–1520. doi: 10.1038/ismej.2017.16

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., et al. (2013). Special topics -Mitigation of methane and nitrous oxide emissions from

animal operations: I. A review of enteric methane mitigation options. *J. Anim. Sci.* 91, 5045–5069. doi: 10.2527/jas2013-6583

Hu, R., and Pan, Y. (2012). Recent trends in counter-current chromatography. *Trends Anal. Chem.* 40, 15–27. doi: 10.1016/j.trac.2012.07.018

Hughes, K. A., Sutherland, I. W., Clark, J., and Jones, M. V. (1998). Bacteriophage and associated polysaccharide depolymerases–novel tools for study of bacterial biofilms. *J. Appl. Microbiol.* 85, 583–590. doi: 10.1046/j.1365-2672.1998.853541.x

Hungate, R. E. (1969). "A roll-tube method for the cultivation of strict anaerobes," in *Methods in Microbiology*, eds J. R. Norris and D. W. Ribbons (Cambridge, MA: Academic Press), 117–132. doi: 10.1016/s0580-9517(08)70503-8

Hungate, R. E., Bryant, M. P., and Mah, R. A. (1964). The rumen bacteria and protozoa. *Ann. Rev. Microbiol.* 18, 131–166. doi: 10.1146/annurev.mi.18.100164.001023

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., et al. (2018). Adressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present and future. *Front. Microbiol.* 9:2161. doi: 10.3389/fmicb.2018.02161

Hynes, W. L., and Ferretti, J. J. (1989). Sequence analysis and expression in *Escherichia coli* of the hyaluronidase gene of *Streptococcus pyogenes* bacteriophage H4489A. *Infect. Immun.* 57, 533–539. doi: 10.1128/iai.57.2.533-539.1989

Iranzo, J., Krupovic, M., and Koonin, E. V. (2016). The double-stranded DNA virosphere as a modular hierarchical network of gene sharing. *mBio* 7:e978-16. doi: 10.1128/mBio.00978-16

Islam, M. M., Fernando, S. C., and Saha, R. (2019). Metabolic modeling elucidates the transactions in the rumen microbiome and the shifts upon virome interactions. *Front. Microbiol.* 10:2412. doi: 10.3389/fmicb.2019.02412

Iverson, W. G., and Millis, N. F. (1977). Succession of *Streptococcus bovis* strains with differing bacteriophage sensitivities in the rumens of two fistulated sheep. *Appl. Environ. Microbiol.* 33, 810–813. doi: 10.1128/aem.33.4.810-813.1977

Iverson, W. J., and Millis, N. F. (1976a). Characterisation of *Streptococcus bovis* bacteriophages. *Can. J. Microbiol.* 22, 847–852. doi: 10.1139/m76-122

Iverson, W. J., and Millis, N. F. (1976b). Lysogeny in *Streptococcus bovis*. *Can. J. Microbiol.* 22, 853–857. doi: 10.1139/m76-123

Jameson, E., Mann, N. H., Joint, I., Sambles, C., and Muhling, M. (2011). The diversity of cyanomyovirus populations along a north-south atlantic ocean transect. *ISME J.* 5, 1713–1721. doi: 10.1038/ismej.2011.54

Jarvis, B. D. W. (1968). Lysis of viable rumen bacteria in bovine rumen fluid. *Appl. Microbiol.* 16, 714–723. doi: 10.1128/aem.16.5.714-723.1968

Johnson, R. P., Gyles, C. L., Huff, W. E., Ojha, S., Huff, G. R., Rath, N. C., et al. (2008). Bacteriophages for prophylaxis and therapy in cattle, poultry and pigs. *Anim. Health Res. Rev.* 9, 201–215. doi: 10.1017/s1466252308001576

Kaelber, J. T., Hryc, C. F., and Chiu, W. (2017). Electron cryomicroscopy of viruses at near-atomic resolutions. *Ann. Rev. Virol.* 4, 287–308. doi: 10.1146/annurev-virology-101416-041921

Kaneko, J., Kimura, T., Narita, S., Tomita, T., and Kamio, Y. (1998). Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying panton-valentine leukocidin genes. *Gene* 215, 57–67. doi: 10.1016/s0378-1119(98)00278-9

Karaolis, D. K., Somara, S., Maneval, D. R. Jr., Johnson, J. A., and Kaper, J. B. (1999). A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* 399, 375–379. doi: 10.1038/20715

Keen, E. C., Bliskovsky, V. V., Malagon, F., Baker, J. D., Prince, J. S., Klaus, J. S., et al. (2017). Novel "Superspreader" bacteriophages promote horizontal gene transfer by Transformation. *MBio* 8, e02115–e02116. doi: 10.1128/mBio.02115-16

Kelly, W. J., Leahy, S. C., Li, D., Perry, R., Lambie, S. C., and Attwood, G. T. (2014). The complete genome sequence of the rumen methanogen *Methanobacterium formicicum* BRM9. *Stand. Genomic Sci.* 9:15. doi: 10.1186/1944-3277-9-15

King, A. M. Q., Adams, M. J., Carstens, E. B., and Lefkowitz, E. J. (2012). "Order – Caudovirales," in *Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses*, eds A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz (Amstadam: Elsevier), 39–45. doi: 10.1016/B978-0-12-384684-6.00001-X

Klieve, A. V. (2005). "2.2. Bacteriophages," in *Methods in gut Microbial Ecology for Ruminants*, eds H. P. S. Makkar and C. S. McSweeney (Berlin: Springer), 39–46. doi: 10.1007/1-4020-3791-0

Klieve, A. V., Bain, P. A., Yokoyama, M. T., Ouwerkerk, D., Forster, R. J., and Turner, A. F. (2004). Bacteriophages that infect the cellulolytic ruminal bacterium *Ruminococcus albus* AR67. *Lett. Appl. Microbiol.* 38, 333–338. doi: 10.1111/j.1472-765X.2004.01493.x

Klieve, A. V., and Bauchop, T. (1988). Morphological diversity or ruminal bacteriophages from sheep and cattle. *Appl. Environ. Microbiol.* 54, 1637–1641. doi: 10.1128/aem.54.6.1637-1641.1988

Klieve, A. V., and Bauchop, T. (1991). Phage resistance and altered growth habit in a strain of *Streptococcus bovis*. *FEMS Microbiol. Lett.* 80, 155–160. doi: 10.1016/0378-1097(91)90587-z

Klieve, A. V., and Gilbert, R. A. (2005). "4.2 Bacteriophage populations," in *Methods in Gut Microbial Ecology for Ruminants*, eds H. P. S. Makkar and C. S. McSweeney (Berlin: Springer), 129–137. doi: 10.1007/1-4020-3791-0

Klieve, A. V., Gregg, K., and Bauchop, T. (1991). Isolation and characteristics of lytic phages from *Bacteroides* ruminicola ss brevis. *Curr. Microbiol.* 23, 183–187. doi: 10.1007/BF02092277

Klieve, A. V., Heck, G. L., Prance, M. A., and Shu, Q. (1999). Genetic homogeneity and phage susceptibility of ruminal strains of *Streptococcus bovis* isolated in Australia. *Lett. Appl. Microbiol.* 29, 108–112. doi: 10.1046/j.1365-2672.1999.00596.x

Klieve, A. V., and Hegarty, R. (1999). Opportunities for biological control of ruminal methanogenesis. *Aust. J. Agric. Res.* 50, 1315–1319. doi: 10.1071/AR99006

Klieve, A. V., Hudman, J. F., and Bauchop, T. (1989). Inducible bacteriophages from ruminal bacteria. *Appl. Environ. Microbiol.* 55, 1630–1634. doi: 10.1128/aem.55.6.1630-1634.1989

Klieve, A. V., and Swain, R. A. (1993). Estimating ruminal bacteriophage numbers using pulsed field gel electrophoresis and laser densitometry. *Appl. Environ. Microbiol.* 59, 2299–2303. doi: 10.1128/aem.59.7.2299-2303.1993

Klieve, A. V., Turner, A. F., and Heck, G. L. (1998). Dietary influences on bacteriophage numbers in the rumen. *Proc. Aust. Soc. Anim. Prod.* 22:341.

Koonin, E. V., Makarova, K. S., and Wolf, Y. I. (2017). Evolutionary genomics of defense systems in Archaea and Bacteria. *Ann. Rev. Microbiol.* 71, 233–261. doi: 10.1146/annurev-micro-090816-093830

Koskella, B. (2019). New approaches to characterizing bacteria–phage interactions in microbial communities and microbiomes. *Environ. Microbiol. Rep.* 11, 15–16. doi: 10.1111/1758-2229.12706

Koskella, B., and Brockhurst, M. A. (2014). Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol. Rev.* 38, 916–931. doi: 10.1111/1574-6976.12072

Krajacic, M., Ravnikar, M., Štrancar, A., and Gutiérrez-Aguirre, I. (2017). Application of monolithic chromatographic supports in virus research. *Electrophoresis* 38, 2827–2836. doi: 10.1002/elps.201700152

Kristensen, D. M., Waller, A. S., Yamada, T., Bork, P., Mushegian, A. R., and Koonin, E. V. (2013). Orthologous gene clusters and taxon signature genes for viruses of prokaryotes. *J. Bacteriol.* 195, 941–950. doi: 10.1128/JB.01801-12

Krupovic, M., Cvirkaite-Krupovic, V., Iranzo, J., Prangishvili, D., and Koonin, E. V. (2018). Viruses of archaea: Structural, functional, environmental and evolutionary genomics. *Virus Res.* 244, 181–193. doi: 10.1016/j.virusres.2017.11.025

Krupovic, M., Prangishvili, D., Hendrix, R. W., and Bamford, D. H. (2011). Genomics of bacterial and archaeal viruses: Dynamics within the prokaryotic virosphere. *Microbiol. Mol. Biol. Rev.* 75, 610–635. doi: 10.1128/MMBR.00011-11

Kunath, B. J., Minniti, G., Skaugen, M., Hagen, L. H., Vaaje-Kolstad, G., Eijsink, V. G. H., et al. (2019). Metaproteomics: sample preparation and methodological considerations. *Adv. Exp. Med. Biol.* 1073, 187–215. doi: 10.1007/978-3-030-12298-0_8

Lajoie, B. R., Dekker, J., and Kaplan, N. (2015). The Hitchhiker's guide to Hi-C analysis: practical guidelines. *Methods* 72, 65–75. doi: 10.1016/j.ymeth.2014.10.031

Landsberger, M., Gandon, S., Meaden, S., Rollie, C., Chevallereau, A., Chabas, et al. (2018). Anti-CRISPR phages cooperate to overcome CRISPR-Cas immunity. *Cell* 174, 908–916.e12. doi: 10.1016/j.cell.2018.05.058

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, et al. (2010). The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. *PLoS ONE* 5:e8926. doi: 10.1371/journal.pone.0008926

Leahy, S. C., Kelly, W. J., Ronimus, R. S., Wedlock, N., Altermann, E., and Attwood, G. T. (2013). Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. *Animal* 7(Suppl. 2), 235–243. doi: 10.1017/S1751731113000700

Leedle, J. A., Bryant, M. P., and Hespell, R. B. (1982). Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. *Appl. Environ. Microbiol.* 44, 402–412. doi: 10.1128/aem.44.2.402-412.1982

Lefkowitz, E. J., Dempsey, D. M., Hendrickson, R. C., Orton, R. J., Siddell, S. G., and Smith, D. B. (2018). Virus taxonomy: the database of the international committee on taxonomy of viruses (ICTV). *Nucleic Acids Res.* 46, D708–D717. doi: 10.1093/nar/gkx932

Leng, R. A., and Nolan, J. V. (1984). Nitrogen metabolism in the rumen. *J. Dairy Sci.* 67, 1072–1089. doi: 10.3168/jds.S0022-0302(84)81409-5

Leon, M., and Bastias, R. (2015). Virulence reduction in bacteriophage resistant bacteria. *Front. Microbiol.* 6:343. doi: 10.3389/fmicb.2015.00343

Letarov, A., and Kulikov, E. (2009). The bacteriophages in human- and animal body-associated microbial communities. *J. Appl. Microbiol.* 107, 1–13. doi: 10.1111/j.1365-2672.2009.04143.x

Letarov, A. V., and Kulikov, E. E. (2017). Adsorption of bacteriophages on bacterial cells. *Biochemistry (Mosc)* 82, 1632–1658. doi: 10.1134/S0006297917130053

Lim, E. S., Wang, D., and Holtz, L. R. (2016). The bacterial microbiome and virome milestones of infant development. *Trends Microbiol.* 24, 801–810. doi: 10.1016/j.tim.2016.06.001

Lockington, R. A., Attwood, G. T., and Brooker, J. D. (1988). Isolation and characterisation of a temperate bacteriophage from the ruminal anaerobe Selenomonas ruminantium. *Appl. Environ. Microbiol.* 54, 1575–1580. doi: 10.1128/aem.54.6.1575-1580.1988

Loenen, W. A., Dryden, D. T., Raleigh, E. A., Wilson, G. G., and Murray, N. E. (2014). Highlights of the DNA cutters: a short history of the restriction enzymes. *Nucl. Acids Res.* 42, 3–19. doi: 10.1093/nar/gkt990

Louca, S., and Doebeli, M. (2017). Taxonomic variability and functional stability in microbial communities infected by phages. *Environ. Microbiol.* 19, 3863–3878. doi: 10.1111/1462-2920.13743

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

Ly-Chatain, M. H. (2014). The factors affecting effectiveness of treatment in phages therapy. *Front. Microbiol.* 5:51. doi: 10.3389/fmicb.2014.00051

Mann, N. H., Cook, A., Millard, A., Bailey, S., and Clokie, M. (2003). Marine ecosystems: bacterial photosynthesis genes in a virus. *Nature* 424:741. doi: 10.1038/424741a

Mantovani, H. C., and Russell, J. B. (2001). Nisin resistance of *Streptococcus bovis*. *Appl. Environ. Microbiol.* 67, 808–813. doi: 10.1128/AEM.67.2.808-813.2001

Marbouty, M., Baudry, L., Cournac, A., and Koszul, R. (2017). Scaffolding bacterial genomes and probing host-virus interactions in gut microbiome by proximity ligation (chromosome capture) assay. *Sci. Adv.* 3:e1602105. doi: 10.1126/sciadv.1602105

Marchesi, J. R., and Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome* 3:31. doi: 10.1186/s40168-015-0094-5

Marti, R., Tien, Y.-C., Murray, R., Scott, A., Sabourin, L., and Topp, E. (2014). Safely coupling livestock and crop production systems: how rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? *Appl. Environ. Microbiol.* 80, 3258–3265. doi: 10.1128/aem.00231-14

Mazaheri Nezhad Fard, R., Barton, M. D., and Heuzenroeder, M. W. (2011). Bacteriophage-mediated transduction of antibiotic resistance in Enterococci. *Lett. Appl. Microbiol.* 52, 559–564. doi: 10.1111/j.1472-765X.2011.03043.x

McGovern, E., Waters, S. M., Blackshields, G., and McCabe, M. S. (2018). Evaluating established methods for rumen 16S rRNA amplicon sequencing with mock microbial populations. *Front. Microbiol.* 9:1365. doi: 10.3389/fmicb.2018.01365

Meijer, W. J., Horcajadas, J. A., and Salas, M. (2001). Phi29 family of phages. *Microbiol. Mol. Biol. Rev.* 65, 261–287. doi: 10.1128/MMBR.65.2.261-287.2001

Miller, E. S., Kutter, E., Mosig, G., Arisaka, F., Kunisawa, T., and Ruger, W. (2003). Bacteriophage T4 genome. *Microbiol. Mol. Biol. Rev.* 67, 86–156. doi: 10.1128/mmbr.67.1.86-156.2003

Mills, J. A., Crompton, L. A., Ellis, J. L., Dijkstra, J., Bannink, A., Hook, S., et al. (2014). A dynamic mechanistic model of lactic acid metabolism in the rumen. *J. Dairy Sci.* 97, 2398–2414. doi: 10.3168/jds.2013-7582

Minot, S., Grunberg, S., Wu, G. D., Lewis, J. D., and Bushman, F. D. (2012). Hypervariable loci in the human gut virome. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3962–3966. doi: 10.1073/pnas.1119061109

Minot, S., Sinha, R., Chen, J., Li, H. Z., Keilbaugh, S. A., Wu, G. D., et al. (2011). The human gut virome: inter-individual variation and dynamic response to diet. *Genome Res.* 21, 1616–1625. doi: 10.1101/gr.122705.111

Mirold, S., Rabsch, W., Rohde, M., Stender, S., Tschape, H., Russmann, H., et al. (1999). Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic *Salmonella* typhimurium strain. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9845–9850. doi: 10.1073/pnas.96.17.9845

Monk, A. B., Rees, C. D., Barrow, P., Hagens, S., and Harper, D. R. (2010). Under the Microscope, Bacteriophage applications: where are we now? *Lett. Appl. Microbiol.* 51, 363–369. doi: 10.1111/j.1472-765X.2010.02916.x

Monteiro, R., Pires, D. P., Costa, A. R., and Azeredo, J. (2019). Phage therapy: going temperate? *Trends Microbiol.* 27, 368–378. doi: 10.1016/j.tim.2018.10.008

Muniesa, M., Colomer-Lluch, M., and Jofre, J. (2013). Could bacteriophages transfer antibiotic resistance genes from environmental bacteria to human-body associated bacterial populations? *Mob. Genet. Elements* 3:e25847. doi: 10.4161/mge.25847

Muniesa, M., Garcia, A., Miro, E., Mirelis, B., Prats, G., Jofre, J., et al. (2004). Bacteriophages and diffusion of beta-lactamase genes. *Emerg Infect. Dis.* 10, 1134–1137. doi: 10.4161/mge.25847

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, et al. (1995). *Virus Taxonomy, 6th Report of the International Committee on Taxonomy of Viruses*. Berlin: Springer-Verlag.

Naas, A. E., Solden, L. M., Norbeck, A. D., Brewer, H., Hagen, L. H., Heggenes, et al. (2018). "Candidatus Paraporphyromonas polyenzymogenes" encodes multi-modular cellulases linked to the type IX secretion system. *Microbiome* 6:44. doi: 10.1186/s40168-018-0421-8

Nakamura, K., Terada, T., Sekiguchi, Y., Shinzato, N., Meng, X. Y., Enoki, M., et al. (2006). Application of pseudomurein endoisopeptidase to fluorescence in situ hybridization of methanogens within the family Methanobacteriaceae. *Appl. Environ. Microbiol.* 72, 6907–6913. doi: 10.1128/AEM.01499-06

Nakayama, K., Kanaya, S., Ohnishi, M., Terawaki, Y., and Hayashi, T. (1999). The complete nucleotide sequence of phi CTX, a cytotoxin-converting phage of *Pseudomonas aeruginosa*: implications for phage evolution and horizontal gene transfer via bacteriophages. *Mol. Microbiol.* 31, 399–419. doi: 10.1046/j.1365-2958.1999.01158.x

Namonyo, S., Wagacha, M., Maina, S., Wambua, L., and Agaba, M. (2018). A metagenomic study of the rumen virome in domestic caprids. *Arch. Virol.* 163, 3415–3419. doi: 10.1007/s00705-018-4022-4

Nemcova, R., Styriak, I., Stachova, M., and Kmet, V. (1993). Isolation and partial characterisation of three rumen *Lactobacillus plantarum* bacteriophages. *Microbiologica* 16, 177–180.

Nguyen, S., Baker, K., Padman, B. S., Patwa, R., Dunstan, R. A., Weston, T. A., et al. (2017). Bacteriophage transcytosis provides a mechanism to cross epithelial cell layers. *MBio* 8, e1874-17. doi: 10.1128/mBio.01874-17

Nielsen, H. B., Almeida, M., Juncker, A. S., Rasmussen, S., Li, J., Sunagawa, S., et al. (2014). Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nat. Biotech.* 32, 822–828. doi: 10.1038/nbt.2939

Nigutova, K., Styriak, I., Javorsky, P., and Pristas, P. (2008). Partial characterization of Enterococcus faecalis bacteriophage F4. *Folia Microbiol.* 53, 234–236. doi: 10.1007/s12223-008-0033-y

Nolan, J. V., and Leng, R. A. (1972). Dynamic aspects of ammonia and urea metabolism in sheep. *Br. J. Nutr.* 27, 177–194. doi: 10.1079/bjn19720081

Nordstrom, K., and Forsgren, A. (1974). Effect of protein A on adsorption of bacteriophages to *Staphylococcus aureus*. *J. Virol.* 14, 198–202. doi: 10.1128/jvi.14.2.198-202.1974

Norman, J. M., Handley, S. A., and Virgin, H. W. (2014). Kingdom-agnostic metagenomics and the importance of complete characterization of enteric microbial communities. *Gastroenterology* 146, 1459–1469. doi: 10.1053/j.gastro.2014.02.001

O'Flaherty, S., Ross, R. P., and Coffey, A. (2009). Bacteriophage and their lysins for elimination of infectious bacteria. *FEMS Microbiol. Rev.* 33, 801–819. doi: 10.1111/j.1574-6976.2009.00176.x

Ogilvie, L., and Jones, B. (2015). The human gut virome: a multifaceted majority. *Front. Microbiol.* 6:918. doi: 10.3389/fmicb.2015.00918

Ogilvie, L. A., Bowler, L. D., Caplin, J., Dedi, C., Diston, D., Cheek, E., et al. (2013). Genome signature-based dissection of human gut metagenomes to extract subliminal viral sequences. *Nat. Comm.* 4:2420. doi: 10.1038/ncomms3420

Oliver, A., Coque, T. M., Alonso, D., Valverde, A., Baquero, F., and Canton, R. (2005). CTX-M-10 linked to a phage-related element is widely disseminated among *Enterobacteriaceae* in a Spanish hospital. *Antimicrob. Agents Chemother.* 49, 1567–1571. doi: 10.1128/aac.49.4.4567-1571.2005

O'Mahony, J., Fenton, M., Henry, M., Sleator, R. D., and Coffey, A. (2011). Lysins to kill – a tale of viral weapons of mass destruction. *Bioeng. Bugs* 2, 306–308. doi: 10.4161/bbug.2.6.16804

Orpin, C. G., Hazlewood, G. P., and Mann, S. P. (1988). Possibilities of the use of recombinant_DNA techniques with rumen micro-organisms. *Animal Feed Sci. Technol.* 21, 161–174. doi: 10.1016/0377-8401(88)90097-1

Orpin, C. G., and Munn, E. A. (1974). The occurrence of bacteriophages in the rumen and their influence on rumen bacterial populations. *Experentia* 30, 1018–1020. doi: 10.1007/bf01938983

Oslizlo, A., Miernikiewicz, P., Piotrowicz, A., Owczarek, B., Kopciuch, A., Figura, G., et al. (2011). Purification of phage display-modified bacteriophage T4 by affinity chromatography. *BMC Biotechol.* 11:59. doi: 10.1186/1472-6750-11-59

Parmar, N. R., Jakhesara, S. J., Mohapatra, A., and Joshi, C. G. (2016). Rumen virome: an assessment of viral communities and thier functions in the rumen of an Indian Buffalo. *Curr. Sci.* 111, 919–925. doi: 10.18520/cs/v111/i5/919-925

Parsley, L. C., Consuegra, E. J., Kakirde, K. S., Land, A. M., Harper, W. F. Jr., et al. (2010). Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage. *Appl. Environ. Microbiol.* 76, 3753–3757. doi: 10.1128/aem.03080-09

Paynter, M. J. B., Ewert, D. L., and Chalupa, W. (1969). Some morphological types of bacteriophages in bovine rumen contents. *Appl. Microbiol.* 18, 942–943. doi: 10.1128/aem.18.5.942-943.1969

Pereira, M. S., Barreto, V. P., and Siqueira-Junior, J. P. (1997). Phage-mediated transfer of tetracycline resistance in *Staphylococcus aureus* isolated from cattle in Brazil. *Microbiology* 92, 147–155.

Popov, V. L., Tesh, R. B., Weaver, S. C., and Vasilakis, N. (2019). Electron microscopy in discovery of novel and emerging viruses from the collection of the world reference center for emerging viruses and Arboviruses (WRCEVA). *Viruses* 11:v11050477. doi: 10.3390/v11050477

Poullain, V., Gandon, S., Brockhurst, M. A., Buckling, A., and Hochberg, M. E. (2008). The evolution of specificity in evolving and coevolving antagonistic interactions between a bacteria and its phage. *Evolution* 62, 1–11. doi: 10.1111/j.1558-5646.2007.00260.x

Proctor, L. M., and Fuhrman, J. A. (1990). Viral mortality of marine bacteria and cyanobacteria. *Nature* 343, 60–62. doi: 10.1038/343060a0

Qin, J. J., Li, R. Q., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, U59–U70. doi: 10.1038/nature08821

Quirós, P., Colomer-Lluch, M., Martinez-Castillo, A., Miro, E., Argente, M., Jofre, J., et al. (2014). Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. *Antimicrob. Agents Chemother.* 58, 606–609. doi: 10.1128/aac.01684-13

Raghupathi, P. K., Zupancic, J., Brejnrod, A. D., Jacquiod, S., Houf, K., Burmolle, M., et al. (2018). Microbial diversity and putative opportunistic pathogens in dishwasher biofilm communities. *Appl. Environ. Microbiol.* 84:e2755-17. doi: 10.1128/AEM.02755-17

Ren, J., Ahlgren, N. A., Lu, Y. Y., Fuhrman, J. A., and Sun, F. (2017). VirFinder: a novel k-mer based tool for identifying viral sequences from assembled metagenomic data. *Microbiome* 5:69. doi: 10.1186/s40168-017-0283-5

Rice, S. A., Tan, C. H., Mikkelsen, P. J., Kung, V., Woo, J., Tay, et al. (2009). The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *ISME J.* 3, 271–282. doi: 10.1038/ismej.2008.109

Ritchie, A. E., Robinson, I. M., and Allison, M. J. (1970). "Rumen bacteriophage: survey of morphological types," in *Microscopie Électronique*, ed. P. Favard (Paris: Societie Francaise de Microscopie Electronique), 333–334.

Rollie, C., Chevallereau, A., Watson, B. N. J., Chyou, T.-Y., Fradet, O., Mcleod, I., et al. (2020). Targeting of temperate phages drives loss of type I CRISPR–Cas systems. *Nature* 578, 149–153. doi: 10.1038/s41586-020-1936-2

Romero-Brey, I., and Bartenschlager, R. (2015). Viral infection at high magnification: 3D electron microscopy methods to analyze the architecture of infected cells. *Viruses* 7, 6316–6345. doi: 10.3390/v7122940

Ross, E., Petrovski, S., Moate, P., and Hayes, B. (2013). Metagenomics of rumen bacteriophage from thirteen lactating dairy cattle. *BMC Microbiol.* 13:242. doi: 10.1186/1471-2180-13-242

Rostøl, J. T., and Marraffini, L. (2019). (Ph)ighting phages: how bacteria resist their parasites. *Cell Host Microbe* 25, 184–194. doi: 10.1016/j.chom.2019.01.009

Roux, S., Enault, F., Hurwitz, B. L., and Sullivan, M. B. (2015). VirSorter: mining viral signal from microbial genomic data. *Peer J.* 3:e985. doi: 10.7717/peerj.985

Russell, J. B., and Hespell, R. B. (1981). Microbial rumen fermentation. *J. Dairy Sci.* 64, 1153–1169. doi: 10.3168/jds.S0022-0302(81)82694-X

Russell, J. B., and Houlihan, A. J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health. *FEMS Microbiol. Rev.* 27, 65–74. doi: 10.1016/S0168-6445(03)00019-6

Salmond, G. P. C., and Fineran, P. C. (2015). A century of the phage: past, present and future. *Nat. Rev. Microbiol.* 13, 777–786. doi: 10.1038/nrmicro3564

Sandaa, R. A., Gómez-Consarnau, L., Pinhassi, J., Riemann, L., Malits, A., Weinbauer, M. G., et al. (2009). Viral control of bacterial biodiversity – evidence from a nutrient-enriched marine mesocosm experiment. *Environ. Microbiol.* 11, 2585–2597. doi: 10.1111/j.1462-2920.2009.01983.x

Sanguino, L., Franqueville, L., Vogel, T. M., and Larose, C. (2015). Linking environmental prokaryotic viruses and their host through CRISPRs. *FEMS Microbiol. Ecol.* 91:fiv046. doi: 10.1093/femsec/fiv046

São-José, C. (2018). Engineering of phage-derived lytic enzymes: Improving their potential as antimicrobials. *Antibiotics (Basel)* 7:29. doi: 10.3390/antibiotics7020029

Sarker, S. A., Sultana, S., Reuteler, G., Moine, D., Descombes, P., Charton, F., et al. (2016). Oral phage therapy of acute bacterial diarrhea with two coliphage preparations: a randomized trial in children from Bangladesh. *EBioMedicine* 4, 124–137. doi: 10.1016/j.ebiom.2015.12.023

Schofield, L. R., Beattie, A. K., Tootill, C. M., Dey, D., and Ronimus, R. S. (2015). Biochemical characterisation of phage pseudomurein endoisopeptidases PeiW and PeiP using synthetic peptides. *Archaea* 2015:828693. doi: 10.1155/2015/828693

Schuch, R., and Fischetti, V. A. (2009). The secret life of the anthrax agent *Bacillus anthracis*: bacteriophage-mediated ecological adaptations. *PLoS ONE* 4:e6532. doi: 10.1371/journal.pone.0006532

Seet, S. G. M. (2005). *Genome Sequence of Bacetriophage Phi-AR29: A Basis for Integrative Plasmid Vectors.* PhD Thesis, Murdoch University, Perth, WA.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., et al. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotechol.* 36, 359–367. doi: 10.1038/nbt.4110

Sharma, M., Ryu, J. H., and Beuchat, L. R. (2005). Inactivation of *Escherichia coli* O157:H7 in biofilm on stainless steel by treatment with an alkaline cleaner and a bacteriophage. *J. Appl. Microbiol.* 99, 449–459. doi: 10.1111/j.1365-2672.2005.02659.x

Shen, M., Yang, Y., Shen, W., Cen, L., Mclean, J. S., Shi, W., et al. (2018). A linear plasmid-like prophage of *Actinomyces odontolyticus* promotes biofilm assembly. *Appl. Environ. Microbiol.* 84:e1263-18. doi: 10.1128/AEM.01263-18

Shen, Y., Mitchell, M. S., Donovan, D. M., and Nelson, D. C. (2012). "Phage-based enzybiotics," in *Bacteriophages in Health and Disease*, eds P. Hyman and S. T. Abedon (Wallingford: CAB International), 217–239. doi: 10.1079/9781845939847.0217

Shibata, A., Kogure, K., Koike, I., and Ohwada, K. (1997). Formation of submicron colloidal particles from marine bacteria by viral infection. *Mar. Ecol. Prog. Ser.* 155, 303–307. doi: 10.3354/meps155303

Shkoporov, A. N., Khokhlova, E. V., Fitzgerald, C. B., Stockdale, S. R., Draper, L. A., Ross, R. P., et al. (2018). ΦCrAss001 represents the most abundant bacteriophage family in the human gut and infects *Bacteroides* intestinalis. *Nat. Comm.* 9:4781. doi: 10.1038/s41467-018-07225-7

Shmakov, S. A., Sitnik, V., Makarova, K. S., Wolf, Y. I., Severinov, K. V., and Koonin, E. V. (2017). The CRISPR spacer space is dominated by sequences from species-specific mobilomes. *mBio* 8:e1397-17. doi: 10.1128/mBio.01397-17

Sillankorva, S., Oliveira, R., Vieira, M. J., Sutherland, I. W., and Azeredo, J. (2004). Bacteriophage Phi S1 infection of *Pseudomonas* fluorescens planktonic cells versus biofilms. *Biofouling* 20, 133–138. doi: 10.1080/08927010410001723834

Silveira, C. B., and Rohwer, F. L. (2016). Piggyback-the-winner in host-associated microbial communities. *NPJ Biofilms Microbiom.* 2:16010. doi: 10.1038/npjbiofilms.2016.10

Smith, H. W., and Huggins, M. B. (1983). Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. *Microbiology* 129, 2659–2675. doi: 10.1099/00221287-129-10-3121

Smith, H. W., Huggins, M. B., and Shaw, K. M. (1987). Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. *J. Gen. Microbiol.* 133, 1127–1135. doi: 10.1099/00221287-133-5-1127

Smith, K., Collier, A., Townsend, E. M., O'Donnell, L. E., Bal, A. M., Butcher, et al. (2016). One step closer to understanding the role of bacteria in diabetic foot ulcers: characterising the microbiome of ulcers. *BMC Microbiol.* 16:54. doi: 10.1186/s12866-016-0665-z

Smoot, J. C., Barbian, K. D., Van Gompel, J. J., Smoot, L. M., Chaussee, M. S., Sylva, G. L., et al. (2002). Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. *Proc. Natl. Acad. Sci. U.S.A.* 99, 4668–4673. doi: 10.1073/pnas.062526099

Snelling, T., and Wallace, R. (2017). The rumen microbial metaproteome as revealed by SDS-PAGE. *BMC Microbiol.* 17:9. doi: 10.1186/s12866-016-0917-y

Solden, L. M., Naas, A. E., Roux, S., Daly, R. A., Collins, W. B., Nicora, C. D., et al. (2018). Interspecies cross-feeding orchestrates carbon degradation in the rumen ecosystem. *Nat. Microbiol.* 3, 1274–1284. doi: 10.1038/s41564-018-0225-4

Soni, K. A., and Nannapaneni, R. (2010). Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. *J. Food Prot.* 73, 1519–1524. doi: 10.4315/0362-028x-73.8.1519

Steward, G., Smith, D., and Azam, F. (1996). Abundance production of bacteria and viruses in the Bering and Chukchi Seas. *Mar. Ecol. Prog. Ser.* 131, 287–300. doi: 10.3354/meps131287

St-Pierre, B., and Wright, A. D. G. (2012). Diversity of gut methanogens in herbivorous animals. *Animal* 7, 49–56. doi: 10.1017/S1751731112000912

Styriak, I., Galfi, P., and Kmet, V. (1991). Preliminary observations of interaction between bacteriophages and *Streptococcus bovis* bacteria on ruminal epithelium primoculture. *Vet. Microbiol.* 29, 281–287. doi: 10.1016/0378-1135(91)90135-3

Styriak, I., Kmet, V., and Spanova, A. (1989). Isolation and characterisation of two rumen *Streptococcus bovis* bacteriophages. *Microbiologica* 12, 317–322.

Styriak, I., Pristas, P., and Javorsky, P. (2000). Lack of GATC sites in the genome of *Streptococcus bovis* bacteriophage F4. *Res. Microbiol.* 151, 285–289. doi: 10.1016/s0923-2508(00)00148-0

Styriak, I., Spanova, A., Montagova, H., and Kmet, V. (1994). Isolation and characterisation of a new ruminal bacteriophage lytic to *Streptococcus bovis*. *Curr. Microbiol.* 28, 355–358. doi: 10.1007/BF01570201

Styriak, I., Spanova, A., and Zitnan, R. (2005). Partial characterization of two ruminal bacteriophages with similar restriction patterns and different capsids morphology. *Archiv. Tierzucht-Arch. Anim. Breed.* 48, 572–579. doi: 10.5194/aab-48-572-2005

Sulakvelidze, A. (2005). Phage therapy: an attractive option for dealing with antibiotic-resistant bacterial infections. *Drug Discov. Today* 10, 807–809. doi: 10.1016/S1359-6446(05)03441-0

Sullivan, M. B., Coleman, M. L., Quinlivan, V., Rosenkrantz, J. E., Defrancesco, A. S., Tan, G., et al. (2008). Portal protein diversity and phage ecology. *Environ. Microbiol.* 10, 2810–2823. doi: 10.1111/j.1462-2920.2008.01702.x

Sutherland, I. W., Hughes, K. A., Skillman, L. C., and Tait, K. (2004). The interaction of phage and biofilms. *FEMS Microbiol. Lett.* 232, 1–6. doi: 10.1016/S0378-1097(04)00041-2

Swain, R. A. (1999). *Factors Affecting Bacteriophages of the Rumen Ecosystem,* Ph.D Thesis. University of New England, Armidale, NSW.

Swain, R. A., Nolan, J. V., and Klieve, A. V. (1996a). Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. *Appl. Environ. Microbiol.* 62, 994–997. doi: 10.1128/aem.62.3.994-997.1996

Swain, R. A., Nolan, J. V., and Klieve, A. V. (1996b). Effect of tannic aicd on the bacteriophage population within sheep rumen. *Microbiol. Aust.* 17:A87. doi: 10.1128/aem.62.3.994-997.1996

Swidsinski, A., Verstraelen, H., Loening-Baucke, V., Swidsinski, S., Mendling, W., and Halwani, Z. (2013). Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. *PLoS ONE* 8:e53997. doi: 10.1371/journal.pone.0053997

Tamada, H., Harasawa, R., and Shinjo, T. (1985). Isolation of a bacteriophage in *Fusobacterium necrophorum*. *Jpn. J. Vet. Sci.* 47, 483–486. doi: 10.1292/jvms1939.47.483

Tarakanov, B. V. (1972). The electron-microscopy examination of the microflora of reindeer rumen. *Mikrobiologia* 41, 862–870.

Tarakanov, B. V. (1974). Lysogenic cultures of *Streptococcus bovis* isolated from rumen of cattle and sheep. *Microbiologica* 43, 375–377.

Tarakanov, B. V. (1976). Biological properties of *Streptococcus bovis* bacteriophages isolated from lysogenic cultures and sheep rumen. *Microbiologiia* 45, 695–700.

Tarakanov, B. V. (1994). Regulation of microbial processes in the rumen by bacteriophages of *Streptococcus bovis*. *Microbiology* 63, 373–378. (translated from Mikrobiologiya 363, 657–667).

Thingstad, T. F. (2000). Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. *Limnol. Oceanogr.* 45, 1320–1328. doi: 10.4319/lo.2000.45.6.1320

Touchon, M., Moura, De Sousa, J. A., and Rocha, E. P. (2017). Embracing the enemy: the diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Curr. Opin. Microbiol.* 38, 66–73. doi: 10.1016/j.mib.2017.04.010

Twort, F. W. (1915). An investigation on the nature of ultra-microscopic viruses. *Lancet* 2, 1241–1243. doi: 10.1016/S0140-6736(01)20383-3

Tyutikov, F. M., Bespalova, I. A., Rebentish, B. A., Aleksanrushkina, N. N., and Krivisky, A. S. (1980). Bacteriophages of *Methanotropic* bacteria. *J. Bacteriol.* 144, 375–381.

Ubukata, K., Konno, M., and Fujii, R. (1975). Transduction of drug resistance to tetracycline, chloramphenicol, macrolides, lincomycin and clindamycin with phages induced from Streptococcus pyogenes. *J. Antibiot. (Tokyo)* 28, 681–688. doi: 10.7164/antibiotics.28.681

Varble, A., Meaden, S., Barrangou, R., Westra, E. R., and Marraffini, L. A. (2019). Recombination between phages and CRISPR-cas loci facilitates horizontal gene transfer in *Staphylococci*. *Nat. Microbiol.* 4, 956–963. doi: 10.1038/s41564-019-0400-2

Vidakovic, L., Singh, P. K., Hartmann, R., Nadell, C. D., and Drescher, K. (2018). Dynamic biofilm architecture confers individual and collective mechanisms of viral protection. *Nat. Microbiol.* 3, 26–31. doi: 10.1038/s41564-017-0050-1

Wang, X., Kim, Y., Ma, Q., Hong, S. H., Pokusaeva, K., Sturino, J. M., et al. (2010). Cryptic prophages help bacteria cope with adverse environments. *Nat. Commun.* 1:147. doi: 10.1038/ncomms1146

Weimer, P. J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Front. Microbiol.* 6:296. doi: 10.3389/fmicb.2015.00296

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS Microbiol. Rev.* 28, 127–181. doi: 10.1016/j.femsre.2003.08.001

Weinbauer, M. G., and Höfle, M. G. (1998). Significance of viral lysis and flagellate grazing as factors controlling bacterioplankton production in a eutrophic lake. *Appl. Environ. Microbiol.* 64:431. doi: 10.1128/aem.64.2.431-438.1998

Wells, J. E., and Russell, J. B. (1996). Why do many ruminal bacteria die and lyse so quickly? *J. Dairy Sci.* 79, 1487–1495. doi: 10.3168/jds.S0022-0302(96)76508-6

Widder, S., Allen, R. J., Pfeiffer, T., Curtis, T. P., Wiuf, C., Sloan, W. T., et al. (2016). Challenges in microbial ecology: building predictive understanding of community function and dynamics. *ISME J.* 10, 2557–2568. doi: 10.1038/ismej.2016.45

Willi, K., Sandmeier, H., Kulik, E. M., and Meyer, J. (1997). Transduction of antibiotic resistance markers among *Actinobacillus actinomycetemcomitans* strains by temperate bacteriophages Aaφ23. *Cell. Mol. Life Sci.* 53, 904–910. doi: 10.1007/s000180050109

Williamson, K. E., Helton, R. R., and Wommack, K. E. (2012). Bias in bacteriophage morphological classification by transmission electron microscopy due to breakage or loss of tail structures. *Microbiol. Res. Technol.* 75, 452–457. doi: 10.1002/jemt.21077

Willner, D., and Hugenholtz, P. (2013). From deep sequencing to viral tagging: recent advances in viral metagenomics. *Bioessays* 35, 436–442. doi: 10.1002/bies.201200174

Willner, D., Thurber, R. V., and Rohwer, F. (2009). Metagenomic signatures of 86 microbial and viral metagenomes. *Environ. Microbiol.* 11, 1752–1766. doi: 10.1111/j.1462-2920.2009.01901.x

Wilmes, P., and Bond, P. L. (2004). The application of two-dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. *Environ. Microbiol.* 6, 911–920. doi: 10.1111/j.1462-2920.2004.00687.x

Wolf, J. L., Rubin, D. H., Finberg, R., Kauffman, R. S., Sharpe, A. H., Trier, J. S., et al. (1981). Intestinal M cells: a pathway for entry of reovirus into the host. *Science* 212, 471–472. doi: 10.1126/science.6259737

Wolochow, H., Hildebrand, G. J., and Lamanna, C. (1966). Translocation of microorganisms across the intestinal wall of the rat: effect of microbial size and concentration. *J. Infect. Dis.* 116, 523–528. doi: 10.1093/infdis/116.4.523

Wommack, K. E., and Colwell, R. R. (2000). Virioplankton: viruses in aquatic ecosystems. *Microbiol. Mol. Biol. Rev.* 64, 69–114. doi: 10.1128/mmbr.64.1.69-114.2000

Wong, C. M., Klieve, A. V., Hamdorf, B. J., Schafer, D. J., Brau, L., Seet, S. G. M., et al. (2003). Family of shuttle vectors for ruminal *Bacteroides*. *J. Mol. Microbiol. Biotechnol.* 5, 123–132. doi: 10.1159/000069982

Wood, S. R., Kirkham, J., Marsh, P. D., Shore, R. C., Nattress, B., and Robinson, C. (2000). Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. *J. Dent. Res.* 79, 21–27. doi: 10.1177/00220345000790010201

Wright, A. (1971). Mechanism of conversion of the *Salmonella* O antigen by bacteriophage ε34. *J. Bacteriol.* 105, 927–936. doi: 10.1128/jb.105.3.927-936.1971

Wylie, T. N., Wylie, K. M., Herter, B. N., and Storch, G. A. (2015). Enhanced virome sequencing using targeted sequence capture. *Genome Res.* 25, 1910–1920. doi: 10.1101/gr.191049.115

Yamaguchi, T., Hayashi, T., Takami, H., Nakasone, K., Ohnishi, M., Nakayama, K., et al. (2000). Phage conversion of exfoliative toxin A production in *Staphylococcus aureus*. *Mol. Microbiol.* 38, 694–705. doi: 10.1046/j.1365-2958.2000.02169.x

Yooseph, S., Sutton, G., Rusch, D. B., Halpern, A. L., Williamson, S. J., Remington, K., et al. (2007). The sorcerer II global ocean sampling expedition: expanding the universe of protein families. *PLoS Biol.* 5:e16. doi: 10.1371/journal.pbio.0050016

Yutin, N., Kapitonov, V. V., and Koonin, E. V. (2015). A new family of hybrid virophages from an animal gut metagenome. *Biol. Direct* 10:19. doi: 10.1186/s13062-015-0054-9

Zhao, G., Wu, G., Lim, E. S., Droit, L., Krishnamurthy, S., Barouch, D. H., et al. (2017). VirusSeeker, a computational pipeline for virus discovery and virome composition analysis. *Virology* 503, 21–30. doi: 10.1016/j.virol.2017.01.005

Zheng, T., Li, J., Ni, Y., Kang, K., Misiakou, M. A., Imamovic, L., et al. (2019). Mining, analyzing, and integrating viral signals from metagenomic data. *Microbiome* 7:42. doi: 10.1186/s40168-019-0657-y

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., and Wishart, D. S. (2011). PHAST: A fast phage search tool. *Nucl. Acids Res.* 39, W347–W352. doi: 10.1093/nar/gkr485

Zobell, C. E., and Anderson, D. Q. (1936). Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces. *Biol. Bull.* 71, 324–342. doi: 10.2307/1537438

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Rumen Protozoa Play a Significant Role in Fungal Predation and Plant Carbohydrate Breakdown

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The rumen protozoa, alongside fungi, comprise the eukaryotic portion of the rumen microbiome. Rumen protozoa may account for up to 50% of biomass, yet their role in this ecosystem remains unclear. Early experiments inferred a role in carbohydrate and protein metabolism, but due to their close association with bacteria, definitively attributing these functions to the protozoa was challenging. The advent of 'omic technologies has created opportunities to broaden our understanding of the rumen protozoa. This study aimed to utilize these methods to further our understanding of the role that protozoa play in the rumen in terms of their metabolic capacities, and in doing so, contribute valuable sequence data to reduce the chance of mis or under-representation of the rumen protozoa in meta'omic datasets. Rumen protozoa were isolated and purified using glucose-based sedimentation and differential centrifugation, extracted RNA was Poly(A) fraction enriched and DNase treated before use in a phage-based, cDNA metatranscriptomic library. Biochemical activity testing of the phage library showed 6 putatively positive plaques in response to carboxymethyl cellulose agar (indicative of cellulose activity), and no positive results for tributyrin (indicative of esterase/lipase activity) or egg yolk agar (indicative of proteolysis). Direct sequencing of the cDNA was also conducted using the Illumina HiSeq 2500. The metatranscriptome identified a wealth of carbohydrate-active enzymes which accounted for 8% of total reads. The most highly expressed carbohydrate-active enzymes were glycosyl hydrolases 5 and 11, polysaccharide lyases and deacetylases, xylanases and enzymes active against pectin, mannan and chitin; the latter likely used to digest rumen fungi which contain a chitin-rich cell membrane. Codon usage analysis of expressed genes also showed evidence of horizontal gene transfer, suggesting that many of these enzymes were acquired from the rumen bacteria in an evolutionary response to the carbohydrate-rich environment of the rumen. This study provides evidence of the significant contribution that the protozoa make to carbohydrate breakdown in the rumen, potentially using horizontally acquired genes, and highlights their predatory capacity.

Keywords: rumen, protozoa, carbohydrate metabolism, metatranscriptome, eukaryotes, metagenomic library

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INTRODUCTION

Rumen protozoa are classified into 2 groups, namely the entodinomorphs and holotrichs. These protozoa are distinctive in terms of their phenotypic and behavioral adaptations, which allow survival in this harsh anaerobic environment. The rumen is warm (38.4–39.8°C), highly anoxic and contracts frequently to increase the flow of particles through the subsequent chambers of the foregut and into the lower intestines (García et al., 2018). The slow generation time of the protozoa which ranges from 6 to 55 h (Karnati et al., 2007; Sylvester et al., 2009; Denton et al., 2015) has been circumvented by a behavioral adaptation largely resulting in finding niches to hide in during contraction, such as the epithelial wall (Abe et al., 1981).

The rumen protozoa are well known for their fibrolytic activity; however, most enzymatic activity has only been inferred, either by defaunation experiments or in mixed *in vitro* experiments with bacteria or fungi (Forsberg et al., 1984; Orpin, 1984; Varel and Dehority, 1989; Jouany and Ushida, 1999). As such, very few protozoal enzymes have been identified and characterized. The first, and only, draft macronuclear genome sequence of rumen protozoa (*Entodinium caudatum*) was only recently published (Park et al., 2018). In addition to a high A-T bias, rumen protozoa are further complicated by the presence of two types of nuclei: the macro and micronucleus. This lack of reference sequences for rumen protozoa often results in their mis- or under-representation in meta 'omic datasets as well as poor coverage during annotation (Comtet-Marre et al., 2017). Nonetheless, recent developments in the field of 'omics, allow researchers to delve further than ever before into otherwise challenging microbiomes.

Acquisition of functionally important genes via horizontal gene transfer (HGT), from bacteria into protozoa, has also been demonstrated (Ricard et al., 2006). In particular, many genes encoding enzymes involved in carbohydrate transfer and metabolism appeared to have been acquired through HGT from bacteria (<75%), with 35% of these being glycosyl hydrolases, Interestingly, a distinct difference was observed between the enzyme profiles of entodiniomorphids and holotrichs, supporting the hypothesis that these protozoal groups occupy a slightly different niche in the rumen environment and possess varying functionality in terms of rumen metabolism. Ricard et al. (2006) suggests that HGT is an important process in the adaptation of rumen protozoa to a carbohydrate-rich environment. Another method for inferring HGT is to analyse codon usage bias, as the rumen protozoa show a significant skew toward A-T rich amino acids. First observed by Eschenlauer et al. (1998) a later study by Devillard et al. (1999) found that many enzymes isolated from rumen ciliates showed significant codon usage bias e.g., AAG was used to code for lysine in 82% and 67% of bacterial and fungal sequences, respectively, whereas in the protozoal xylanase AAA was used almost exclusively.

Further use of 'omic technologies will allow for a more, in-depth and robust understanding of the rumen protozoa as such techniques reduce the difficulties associated with defining function in a mixed microbial ecosystem. Using next-generation sequencing (NGS) and a meta 'omic approach, this study aimed to investigate the enzymatic activity of the rumen protozoa in terms of carbohydrate, protein and lipid metabolism. The resulting information will significantly enhance and clarify our understanding of rumen protozoal function, whilst providing much-needed sequence data for improvement of gene annotations for these largely understudied eukaryotes. Indeed, the generation of sequence data will enable better coverage of the protozoa in meta 'omic studies of the rumen, reducing the risk of them being mis- and underrepresented, leading to more complete and accurate results.

MATERIALS AND METHODS

All experiments were conducted with the authority of Licenses under the United Kingdom Animal Scientific Procedures Act, 1986. The experiment was also approved by the Local Aberystwyth University Ethics Committee and reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB) in line with University procedure.

Two liters of hand-squeezed and strained rumen fluid was obtained from three non-lactating, cannulated Holstein-Friesian cows. Cows were allowed 24 h access to grazing and free access to water. Samples were collected after 2 h after feeding (withdrawal from pasture and a small (250–500 g) concentrate incentive). Samples were pooled and transferred to an incubator at 39°C until use (samples were used within 1 h of collection; Huws et al., 2009).

Rumen fluid was then aliquoted into two 1 L burettes and the protozoa separated by gravity-fractionation as described by Huws et al. (2009). Per burette, 0.5 g of glucose was added and the apparatus incubated at 39°C for 1 h, during this time, the protozoa and many other microorganisms moved to the bottom of the burette in pursuit of the glucose where they were siphoned off into sterile 50 mL tubes. Samples were centrifuged at 100 × *g* for 10 min to remove the remaining plant material, followed by washing in Coleman's buffer as described by Martin et al. (1994). A small portion of the pellet was diluted 1:10 using ddH_2O and fixed for 2 h at room temperature with an equal volume of MFS solution [3.5% (v/v) formaldehyde and 8 g/L NaCl] before mounting 10 μL onto a slide and examining under a light microscope (Tymensen et al., 2012). The remainder was used for RNA extraction and downstream applications as outlined below (stored at −80°C).

RNA Extraction

The remaining protozoal pellet was thawed and RNA extracted using the FastRNA Pro-Soil Direct kit™ (MP Biomedicals, United Kingdom) according to the manufacturer's guidelines. RNA concentration was quantified using the Epoch™ Micro-Volume Spectrophotometer (Biotek, United States) before use of the Poly(A) Purist™ MAG kit (Life Technologies, United Kingdom) to enrich polyadenylated mRNA, following the manufacturer's guidelines. To remove any contaminating DNA prior to cDNA synthesis, a TURBODNA-free kit (Thermo-Fisher Scientific, United States) was used, following the manufacturer's guidelines. To check for DNA contamination,

a bacterial 16S rDNA (5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-ACGGGCGGTGTGTACAAG-3′) and eukaryotic 18S rDNA (5′-AGCCTGCGGCTTAATTTGAC- 3′ and 5′-CAACTAAGAACGGCCATGCA-3′) polymerase chain reaction (PCR) was carried out. PCR products were analyzed on a 1% agarose gel, with a 1 kB ladder and the gel was visually assessed for the presence of banding using the Gel doc system (Bio-rad, United Kingdom). Absence of bands was observed, indicating that the RNA was low in bacterial and fungal DNA contamination.

Library Construction

The SMART® cDNA library construction kit (Clontech Laboratories. Inc., United States) was used to create the metatranscriptomic library using LDPCR and the Advantage 2 PCR kit (Clontech Laboratories Inc., United States) following the manufacturer's guidelines. The cDNA was digested using the *Sfil* restriction enzyme and size-fractionated using CHROMA SPIN-400 gel filtration columns (Clontech Laboratories. Inc., United States). Fractions containing the largest size and concentration of cDNA were pooled then cleaned via ethanol precipitation. The *Sfil* digested cDNA was then ligated into the λTriplEx2 vector (Clontech Laboratories Inc., United States) according to the manufacturer's instructions.

For the primary titer, Luria Bertani (LB) broth with 10 mM $MgSO_4$ and maltose (0.2%) was added to a liquid culture of *Escherichia coli* XL1-Blue and incubated at 37°C with 200 rpm shaking overnight to achieve an OD_{600} of 2.0. The culture was centrifuged at 5,000 × *g* for 5 min before resuspension in 7.5 mL of 10 mM $MgSO_4$. The vector containing the cDNA was packaged immediately into the λ phage using the MaxPlax™ Lambda Packaging Extract according to the manufacturer's guidelines. To visualize the number of recombinant clones versus non-recombinants, blue-white color screening with 80 μg/mL Xgal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 20 mM IPTG (Isopropyl β-D-1thiogalactopyranoside) was used. LB top agar (0.7% agarose) was combined with 300 μL of 1M $MgSO_4$, X-gal and IPTG. The packaged reactions were diluted 1:10 and 2.5 μL of each was combined with 200 μL of *E.coli* XL1-Blue cells in 10 mM $MgSO_4$ and incubated at 37°C for 15 min. This was added to 4 mL of LB top agar and overlaid onto LB/$MgSO_4$ (10 mM) plates and incubated at 37°C overnight. Plates were visually assessed for blue and white plaques and counted to give a ratio of recombinants to non-recombinants and a pfu/mL.

A culture of XL1-Blue MRF' *E. coli* was grown and resuspended in 10 mM $MgSO_4$ as previously described, then adjusted to OD_{600} 0.5 using sterile 10 mM $MgSO_4$. This was combined (500 μL) with the packaged reaction and incubated at 37°C for 15 min. Cooled LB/$MgSO_4$ (10 mM) top agar was added, mixed and plated onto LB/$MgSO_4$ agar plates. The plates were incubated at 37°C overnight. 1X Lambda dilution buffer (12 mL) was added to each plate followed by storage at 4°C overnight. The plates were then rocked at room temperature for 1 hour (h) before the bacteriophage suspension was recovered and pooled.

Functional Screening

The first screen applied to the library was a functional assay for cellulolytic activity using carboxymethyl cellulose (CMC) as a substrate and Congo red for post-staining. *E. coli* XL1 Blue MRF' cells were prepared and coupled with 2,000 pfu (5 μL) of the amplified library and incubated at 37°C for 20 min. LB top agar (8 mL) with $MgSO_4$ and IPTG was added and mixed then spread onto LB agar with $MgSO_4$. Cellulase (10 μL) from *Aspergillus niger* (Sigma-Aldrich, United States) (8 units/mL) was used as a positive control.

A layer of 0.2% (w/v) CMC, 0.7% (w/v) agarose and 25 mM Potassium phosphate buffer (pH 6.5) was overlaid onto each plate before incubation overnight at 37°C. Plates were then flooded with Congo red dye (1 mg/mL) for 30 min and rocked at 130 rpm. The Congo red dye was removed and 1 M NaCl solution added and rocked at 130 rpm for 20 min. The solution was discarded and the process repeated. Plaques expressing cellulolytic activity would appear yellow on the red background.

The second screen applied to the library was a functional assay for lipolytic activity using Tributyrin as a substrate and Spirit Blue as an indicator dye. Phage and bacterial cells were prepared at described above then spread on Spirit blue plates (Sigma-Aldrich, United Kingdom) supplemented with 1% Tributyrin. Phospholipase A_1 from *Thermomyces lanuginosus* (Sigma-Aldrich) (10 KLU/g) was used as a positive control. The plates were incubated for 24 – 48 h at 37°C. Plaques expressing lipolytic activity would show a clear zone around their circumference.

The third screen applied to the library was a functional assay for protease activity using egg yolk agar, as described by Fu et al. (1997). The phage and bacterial cells were plated onto egg yolk agar and 2 μL of the protease from *Bacillus licheniformis* (Sigma-Aldrich, United States) (2.4 U/g) was used as a positive control. The plates were incubated for 2 – 4 days at 37°C, after which they were examined for plaques expressing proteolytic activity. A positive result would be indicated by off-white precipitation around the plaque.

Sequencing

The protozoal cDNA library was prepared for sequencing using the Nextera® DNA Library Preparation kit (Illumina, United States) according to the manufacturer's instructions. Libraries were pooled and sequenced at 2 × 151 bp using an Illumina HiSeq 2500 rapid run, with samples duplicated over two lanes; procedures followed manufacturer's instructions. Sequences resulting from this work can be found in NCBI's Biosample database under accession number SAMN13506237.

Sequencing data were handled using the Galaxy platform (Version 17.01). Sequences were first aligned to the *Bos taurus* (ARS-UCD1.2) and *Lolium perenne* (ASM173568v1) genome using BowTie2 and any matches were removed from the dataset. The sequences were quality checked using FastQC (Version 0.69; Babraham Bioinformatics) (Andrews, 2010) then trimmed using a sliding window on Trimmomatic (Version 0.36.0) (Bolger et al., 2014) (**Figure 1**). The sliding window size was set at 4 bps and average quality required was set at 20 (given

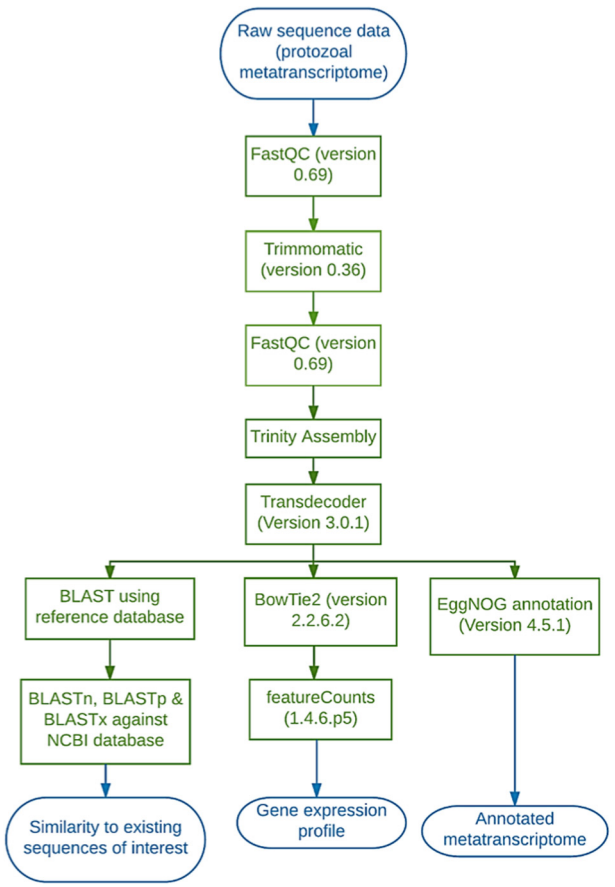

FIGURE 1 | Diagrammatic representation of the workflow employed on the Galaxy platform to analyse the protozoal metatranscriptome.

as a phred33 value this translates to 1 in 100 probability of an incorrect base call i.e., 99% accuracy. Trinity (Version 0.0.1) was used to assemble the sequences (using the paired-end option and default parameters) and then Transdecoder (Version 3.0.1 on default parameters) to identify coding regions. Transdecoder was run using the following parameters: Minimum protein length 100 bps, universal genetic code, retain long ORFs equal to or longer than 900 bps (300 aa) and train with the number of top longest ORFs: 500. EggNOG mapper (Version 4.5.1) was then used to annotate the sequences and Bowtie2 (Version 2.2.6.2) for alignment back to the Trinity assembly (paired library option with a built-in genome index, simple analysis mode and default parameters) (**Figure 1**). FeatureCounts (Version 1.4.6.p5) was used on default parameters to count the number of reads aligned to each functional category (**Figure 1**). The FeatureCounts output was then merged with the EggNOG annotation giving the number of reads per functional category (**Figure 1**). Carbohydrate-active enzymes were manually selected using the appropriate functional category (Carbohydrate Transport & Metabolism, G) and the sequence inputted into BLASTp (NCBI), to gain more information with regards to active domains and enzyme family/subfamily. In doing so, multiple results were produced for the same sequence and all sequences returned matches. As such, the match with the highest% identity and bit score were selected. Any match that fell below the following criteria was discarded: length (100 bp), Bit score (80) and percentage of identical matches (> 0%). The same process was used to investigate the few proteases and lipases that were identified. Carbohydrate-active enzymes that ranked highly (within the top 100 most expressed genes) were also inputted into the dbCAN2 meta server which uses HMMER, DIAMOND and Hotpep to provide more in-depth information on any carbohydrate-binding domains present in the query sequence (Zhang et al., 2018).

To analyse codon usage the CAIcal online tool was used (NCBI). The.cds output file from transdecoder was inputted in FASTA format and the "Mold, protozoan, Coelenterate. Mitochondrial and Mycoplasma/Spiroplasma" genetic code and Codon Usage output was selected alongside default parameters.

The nucleotide sequences of cellulases (annotated as 'cellulase' or 'GH5') and xylanases (annotated as endo-1,4-beta-xylanases, GH10 or GH11) from the sequence data were aligned using MEGA7 (v. 7.0.26; Kumar et al., 2016) with enzymes from the same GH family produced by rumen protozoa, fungi and bacteria (sequences retrieved from GenBank). Phylogenetic trees were constructed using neighbor-joining clustering method (Saitou and Nei, 1987). A bootstrap consensus tree was inferred from 500 replicates, and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site (Tamura et al., 2004). The analysis for xylanase genes involved 30 nucleotide sequences and there was a total of 241 positions in the final dataset. The analysis for cellulase genes involved 32 nucleotide sequences and there was a total of 70 positions in the final dataset. All positions containing gaps and missing data were eliminated.

RESULTS

Protozoal Diversity

Microscopic examination of the protozoal pellet used to extract RNA suggested a mixture of type A and type B protozoa (Eadie, 1962; Dehority, 1993) (**Supplementary Figures S1, S2**). As samples from three different animals were pooled together, animals may have possessed different types. The majority of protozoa in the samples were entodiniomorphids: *Ostracodinium* (species likely to be *dilobum*) (**Supplementary Figures S1A,B**), *Diplodinium* (species *affine*) (**Supplementary Figure S1C**) *Entodinium* (species *furca monolobum*) (**Supplementary Figure S1D**), *Eudiplodinium* (species *maggii*) (**Supplementary Figures S1E,F**) and *Epidinium* (species *caudatum* and *quadricaudatum*) (**Supplementary Figures S1G,H**) (Dehority, 1993; Williams and Coleman, 2012). The holotrichous representatives observed were identified as *Isotricha* (species *intestinalis*) and *Dasytricha* (species *ruminantium*) (**Supplementary Figures S2J,I**) (Dehority, 1993; Williams and Coleman, 2012).

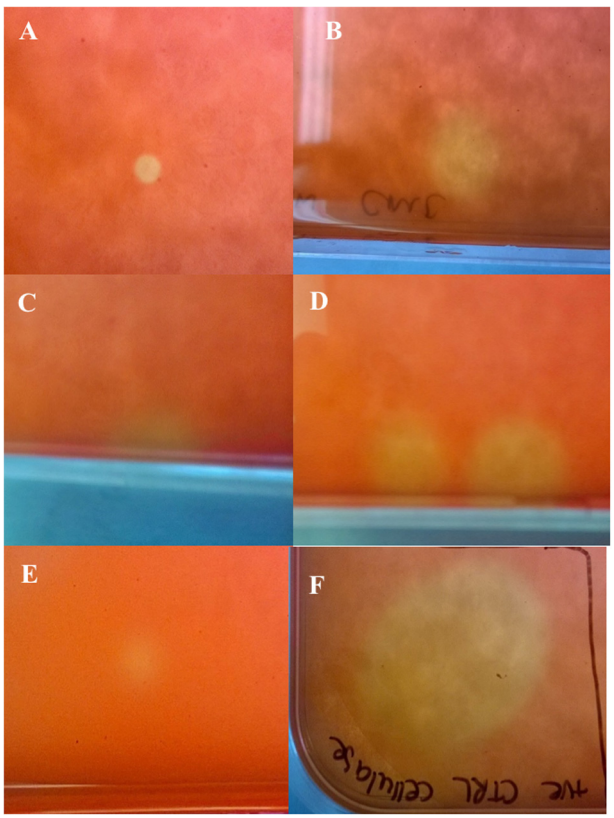

FIGURE 2 | Amplified cDNA library plated on *Escherichia coli* lawn with carboxymethyl cellulose overlay and post-staining with Congo red (Primary assay). **(A–E)** Cellulase positive plaques indicated by yellow coloring; **(F)** Positive control (cellulase from *Aspergillus niger*) indicated by yellow coloring.

RNA Purity

Protozoal RNA was confirmed as sufficiently pure and free from contaminating DNA as evidenced by the absence of PCR products for both the 16S rDNA bacterial and eukaryotic 18S rDNA PCR (**Supplementary Figure S3**).

Functional Phage-Based Assays

The library produced a titer of 6.5×10^6 pfu/mL and recombinant plaques accounted for 15–20% of total plaques. Primary screening of the amplified cDNA library using substrates for cellulase, lipase and protease activities, yielded mixed results. The only putatively positive results were observed in response to CMC substrate, which consistently revealed six positive plaques in addition to the positive control post-staining indicating cellulase activity (**Figure 2**). No positive results were detected using differential media to screen for lipases and proteases. Viral plaques were observed but showed no zones of clearance or colorimetric change in any instances. Positive controls indicated that the assays were functional.

Sequencing and Bioinformatics

Direct sequencing of the metatranscriptome of the protozoal cDNA resulted in approx. 3.3 million raw reads and 3.1 million trimmed reads (**Table 1**). The metatranscriptome sequences were reconstructed using Trinity on Galaxy, which provided 9,101 contigs (**Table 1**). After applying TransDecoder 2,505 genes were predicted, of these EggNOG annotated 968 (**Table 1**).

Use of BowTie2 resulted in 402,226 reads aligned to contigs, which when processed by FeatureCounts resulted in 201,113 reads aligning to predicted genes (**Table 1**). When summarized in terms of hierarchical subsystem functional categories the most highly expressed were: 'translation, ribosomal structure & biogenesis,' 'post-translational modification, protein turnover & chaperones,' 'the cytoskeleton' and 'carbohydrate transport & metabolism' (**Figure 3**). Although, by far the category containing the largest number of expressed reads was 'poorly characterized' in which function was unknown (**Figure 3**).

The total number of carbohydrate-active enzymes was 16,248 (8% of annotated reads) with a large proportion (3,239) being glycosyl hydrolases from an unknown family (20% of all reads involved in carbohydrate metabolism and transport) (**Table 2** and **Figure 4**). Target sequences annotated as carbohydrate-active by EggNOG (in both the functional category and gene description) were further characterized using BLASTp (**Table 2** and **Figure 4**). After selecting results according to criteria laid out in section 2.4, 9 sequences matched a glycoside hydrolase (Family 5) from an uncultured equine cecal ciliate (Sequence ID: CDG46723), 3 sequences matched a xylanase produced by *E. maggii* (Sequence ID: CAL91982), 2 matched a xylanase produced by *P. multivesiculatum* (Sequence ID: CAD56867) and 2 matched to an endo- 1, 4- beta-xylanase also produced by *P. multivesiculatum* (Sequence ID: BAA76395). Other matches were made to various endo-1, 4-beta-xylanases, xylanases, cellulases and endoglucanases from rumen microorganisms (three matches to rumen bacteria and 5 to rumen protozoa; sequence IDs: SCI73846.1; WP_027622710.1; GAE88377; BAA76394; BAA76395; CAL91973; CAL91979; CAH69214).

Using the sequences as queries for a BLASTp alignment against dbCAN helped to give a more comprehensive overview of the glycosyl hydrolases found in this dataset. Only four enzymes were commonly identified by all three tools used by dbCAN, only two of which fell within the top 100 most expressed genes in the dataset and were of some interest. Two of the three tools were able to commonly identify 27 enzymes – 1 by Diamond + HMMER and 26 by Hotpep + HMMER. The two highest-ranking carbohydrate-active enzymes in the dataset (14th and 43rd) were designated CBM79 by HotPep and HMMER and were of most interest here. This protein is produced by

TABLE 1 | Protozoal metatranscriptome sequencing data.

Data	No. sequences
Raw data	3,277,915
Trimmed data	3,125,731
Contigs assembled	9,101
Predicted genes	2,505
Annotated genes	968
No. Reads aligning to contigs	402,226

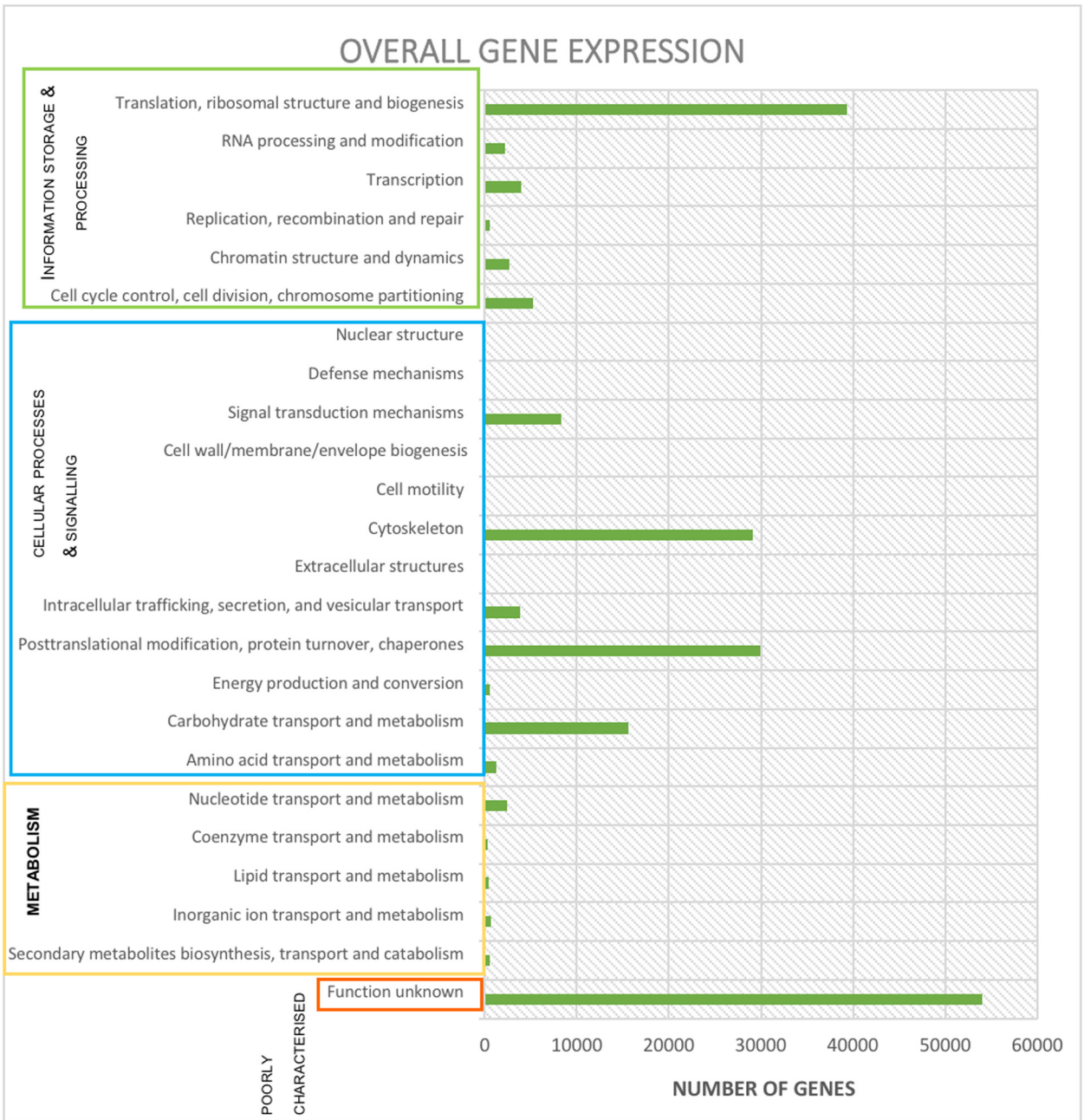

FIGURE 3 | Expression (reads) per functional subcategory from the protozoal metatranscriptome using Bowtie2, FeatureCounts and EggNOG online mapper. Subcategories are collated into the appropriate main category.

Ruminococcus sp. and falls into the GH 9 family, which binds various β-glucans. Other carbohydrate-active enzymes within the top 100 most expressed enzymes were from glycosyl hydrolase (GH) family 11 (xylanases) and 45 (endo-glucanases).

Using BLASTp and dbCAN, putative proteolytic enzymes identified were categorized as Cathepsin B and a cysteine protease (Cathepsin F). Cathepsin B ranked as the 37th most expressed enzyme and showed 67% identity with a hypothetic protein produced by the protozoan parasite *Ichthyophthirius multifiliis* (Sequence ID: XM_004027641). Cathepsin F was ranked at 41 in terms of expression and showed 43% homology with a hypothetical protein from *Halomonas* sp. (Sequence ID: WP_110285651). Two lipases of the GDSL family were identified by BLASTp but were not highly expressed. One showed 53% homology with a lipase from the GDSL family produced by *Prevotella ruminicola* (accession number: EGD48497.1) and the other 51% similarity with a GDSL-family lipase from *Clostridium papyrosolvens* (accession number: ADE81025.1).

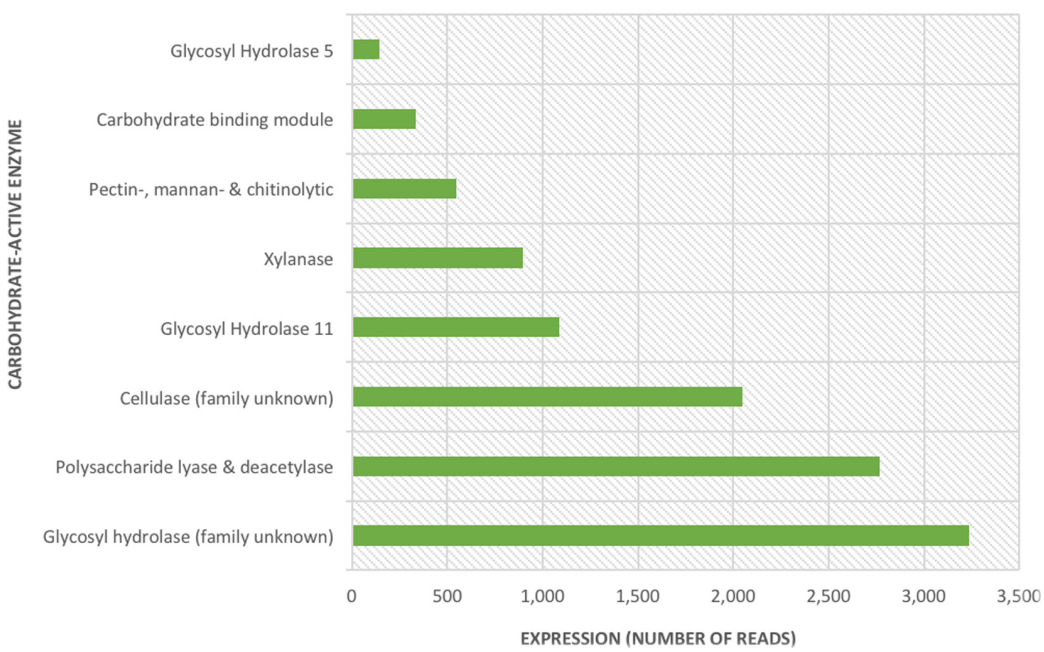

FIGURE 4 | Graphical representation of expression levels (number of reads) of carbohydrate-active enzymes annotated by EggNOG and confirmed using NCBI's online Basic Local Alignment Search Tool.

TABLE 2 | Expression (number of reads) and percentage of total reads per type and family of carbohydrate active enzyme.

Enzyme	Expression (reads)	% of total reads
All carbohydrate-active enzymes	16,248	8
Glycosyl hydrolase (family unknown)	3,239	1.6
Polysaccharide lyase & deacetylase	2,767	1.4
Cellulase (family unknown)	2,046	1
Glycosyl Hydrolase 11	1,090	0.5
Xylanase	898	0.45
Pectin-, mannan- & chitinolytic	546	0.3
Carbohydrate-binding module	337	0.17
Glycosyl Hydrolase 5	146	0.07

Finally, phylogenetic trees constructed from cellulases and xylanases identified from this dataset (target sequences) and sequences retrieved from GenBank (GH5, cellulase, xylanase, GH10 and GH11 sequences from the rumen protozoa, bacteria and fungi) to visualize their similarity. From the phylogenetic tree constructed using cellulase sequences it can be observed that several (7) target sequences cluster together with some similarity shown to AF459452 *Piromyces* sp. Cel9A (**Figure 5**). One target sequence shows some similarity to other fungal enzymes produced by *Anaeromyces* sp. (AF529294) and *Neocallimastix* sp. (HQ386985) (**Figure 5**). The remaining 6 target sequences showed similarity to those produced by *E. ecaudatum* (AJ853911 and AM419212), *E. caudatum* (AB104617) and uncultured bovine rumen ciliates (JN635693) (**Figure 5**). The phylogenetic tree constructed from xylanase sequences shows clustering of fungal genes (GU011968, EU909695, and AF297649) and of a fungal and bacterial gene (M83379 and MH043837) (Murphy et al., 2019) (**Figure 6**). Four target xylanase genes show some, limited similarity to xynD11 and xylanases produced by *P. multivesiculatum* (AB011274, AJ516958, and AJ009828) (**Figure 6**). Five target xylanases showed close similarity to those produced by *E. ecaudatum*, *E. maggii* and an uncultured bovine rumen ciliate (AM419223, AM419225, AM419226, and JN635694) (**Figure 6**).

Codon Usage Bias Analysis

Analysis of codon usage in the data set reveals a clear bias toward AAA, AAT, GAT and GAA all of which are used in >5% of the metatranscriptome (**Table 3**). Codons AAA and GAA were used in 12.61 and 11.5%, respectively, and together account for 27% of all amino acids in this dataset. There is also a bias toward several other A-T rich codons, all of which are used 2 – 5% of the time (i.e., TAT, TTA, TTT, GTT, CAA, ACT, AGA, ATA, and ATT) (**Table 3**). A and T constituted 69% of the genome and G and C accounted for 31%. This is a lower GC content than detected by the FastQC program due to trimming (43% pre-trimming, 31% post-trimming).

DISCUSSION

With the view to developing the current understanding of the role and functions of the rumen protozoa, this study created a

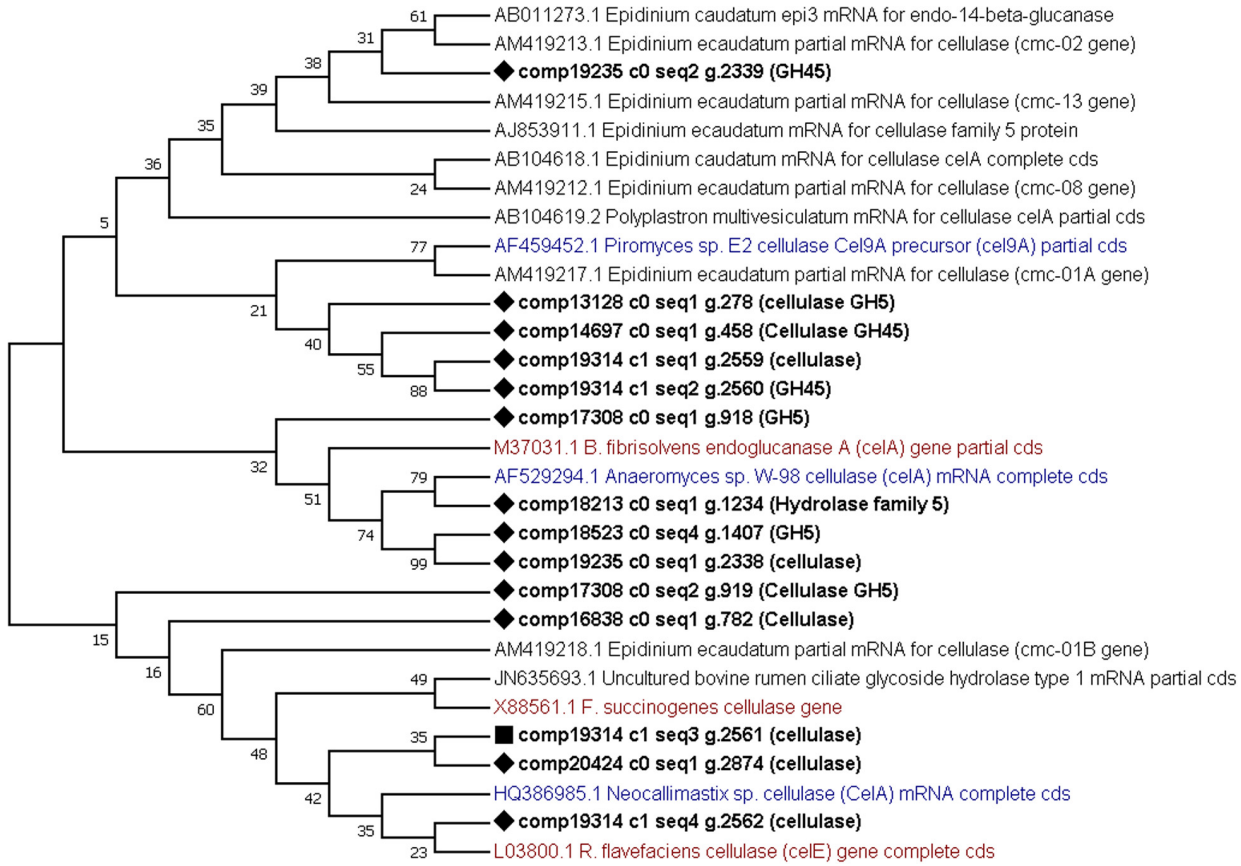

FIGURE 5 | Comparative phylogenetic tree of all putative cellulase (or GH5) genes detected within the protozoal metatranscriptome. Twelve representative protozoal sequences from *Epidinium ecaudatum* (6)*, Epidinium caudatum* (4)*, Polyplastron multivesiculatum,* (1) and an uncultured bovine rumen ciliate (1), three fungal cellulases produced by *Neocallimastix* sp.*, Piromyces* sp. and *Anaeromyces* sp. and three bacterial cellulases from *Ruminococcus flavefaciens, Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* are given for comparison. Target sequences from the metatranscriptome are shown in bold with a diamond shape node marker, the most highly expressed enzyme is marked with a square, protozoal sequences are given in black, bacterial in red and fungal in blue text.

phage-based metatranscriptomic library from purified protozoal cDNA that was functionally screened for lipase, protease and cellulase activity. Six positive plaques were identified in response to CMC agarose, but no activity against tributyrin/spirit blue and egg yolk agar was observed (indicative of lipolytic and proteolytic activity, respectively). Direct sequencing data confirmed the functional screening results by demonstrating that carbohydrate metabolism is a primary function of the rumen protozoa, especially when compared with lipolytic and/or proteolytic activity. The sequencing data also revealed high expression levels of chitinases, which are likely utilized by rumen protozoa to digest rumen fungi which possess chitin-rich cell walls (Morgavi et al., 1994). This study suggests that the primary roles of the rumen ciliates are predation and carbohydrate metabolism.

The majority of studies concerning the carbohydrate-active enzymes of the rumen protozoa span the period between 1960 and 2000, pre-dating the genomic era (Guttmacher and Collins, 2003; Konstantinidis et al., 2006). As such, very little sequence data are available and most research uses faunated vs. defaunated animals or simple agar-based assays with pure cultures to infer activity. The only other published metagenomic library to be constructed exclusively from rumen protozoa was that by Findley et al. (2011) using similar methods to those described here. Major differences between the studies include the kits and protocols used to construct the metagenomic library and the use of *in vivo* excision, which was not possible in the current study. Excising the phagemid vector using *in vivo* excision allows for transformation into eukaryotic cells which would allow functional screening followed by PCR and Sanger sequencing. The use of phage in functional assays is more complex when compared to other vector systems and is not widely used, as any one plaque may contain up to 1 million individual phages, of which only one or two may be expressing the desired activity. On the other hand, using a bacterial or eukaryotic vector allows for more straightforward functional screening in terms of both working conditions and identification of positive colonies. Other studies have utilized cDNA libraries to characterize single species of protozoa, which have produced mostly partial and one complete gene encoding carbohydrate-active enzymes (Wereszka et al., 2004, 2006; Bełżecki et al., 2007). More recently, NGS has allowed some partial mRNA to be deposited in Genbank for GH 5 and 9 as well as uncategorized cellulases and xylanases produced

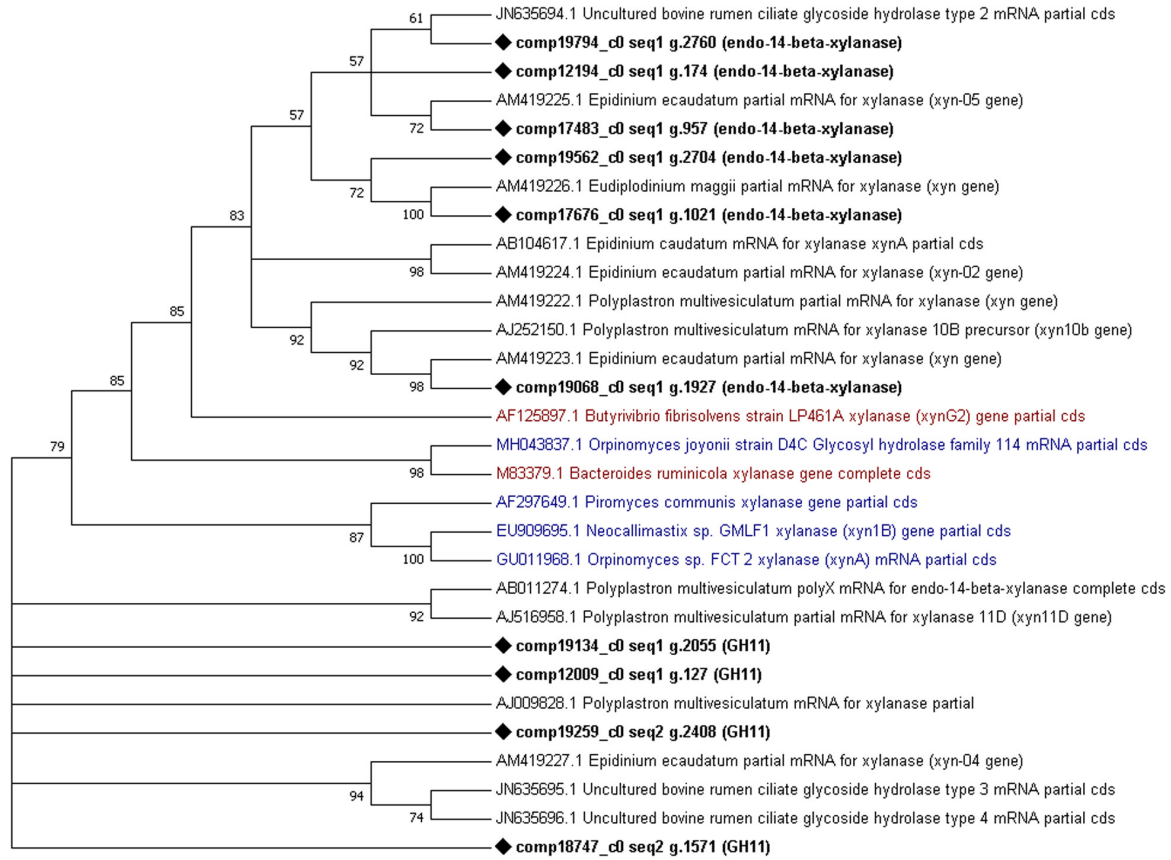

FIGURE 6 | Comparative phylogenetic tree of all putative xylanase (GH10 or GH11) genes detected within the protozoal metatranscriptome. Fourteen representative protozoal sequences from *Epidinium ecaudatum* (4), *Polyplastron multivesiculatum* (5), uncultured bovine rumen ciliates (3) *Epidinium caudatum* (1) and *Eudiplodinium maggii* (1), three fungal cellulases produced by *Neocallimastix* sp., *Piromyces* sp., *Orpinomyces* sp. and *Orpinomyces joyonii* and two bacterial cellulases from *Bacteroides ruminicola* and *Butyrivibrio fibrisolvens* are given for comparison. Target sequences from the metatranscriptome are shown in bold with a diamond shape node marker, the most highly expressed enzyme is marked with a square, protozoal sequences are given.

by ruminant protozoa, but no characterization or further work has been done (e.g., BAC57894; BAC57895; CAL91983; CAL91968). The 2019 study by Wang et al. (2019) provides the first transcriptome of a single rumen ciliate, *Entodinium caudatum*, using similar methods to those detailed here. The study uncovered numerous carbohydrate-active enzymes (including lysozymes and chitinases) in agreement with the present experiment Such studies have provided a basis for the work described here, which utilizes 'omic technologies to elucidate the role and function of the rumen protozoa, provide more in-depth information about the enzymes they produce and to investigate the potential for HGT.

The use of NGS provides many advantages over traditional culture-based methods, the primary benefit being the bypassing of establishing and maintaining a culture of protozoa. Rumen protozoa are notoriously difficult to maintain in culture and although decades of optimization have resulted in improved methods, it remains impossible to maintain them in axenic culture (Newbold et al., 2015). The use of direct sequencing also negates issues with contamination; the rumen protozoa are well-known for engulfing bacterial and fungal cells which can significantly skew assays, rendering results inaccurate and unreliable (Belanche et al., 2012; Williams and Coleman, 2012; Newbold et al., 2015). The use of NGS allows protozoal RNA to be extracted and thoroughly purified (here, Poly(A) enriched, DNase treated and Lithium chloride precipitation was used) as well as assessed for the presence of contaminating DNA using PCR and gel electrophoresis before sequencing. After sequencing, there is also the option to align data to various genomes (host, plant, fungal, bacterial etc.) and bin any matching results which give complete certainty that the sequences retained are purely protozoal. Utilizing these techniques, this study approached the rumen protozoa from both a functional and sequence-based perspective to give a well-rounded picture of their role in the rumen. The main drawback of this approach in terms of the rumen protozoa is the lack of reference sequences available, in large meta' omic datasets this can lead to mis- and underrepresentation, particularly when the concept of horizontal gene transfer is taken into consideration. To combat these limitations, the generation of sequence data and its subsequent publication is key, not only will this improve the accuracy of meta 'omic datasets produced from the rumen but will

TABLE 3 | Codon usage for sequence data obtained for the cDNA library.

CODON	AAA	AAC	AAG	AAT	ACA	ACC	ACG	ACT	AGA	AGC	AGG	AGT	ATA	ATC	ATT	CAA
AMINO ACID	K	N	K	N	T	T	T	T	R	S	R	S	I	I	I	Q
Total	44568	2713	7012	17589	5804	1757	300	9210	12379	1220	977	4926	7723	1968	11518	11781
%	12.61	0.77	1.98	4.99	1.64	0.50	0.08	2.61	3.50	0.35	0.28	1.39	2.19	0.56	3.26	3.33
CODON	CAC	CAG	CAT	CCA	CCC	CCG	CCT	CGA	CGC	CGG	CGT	CTA	CTC	CTG	CTT	GAA
AMINO ACID	H	Q	H	P	P	P	P	R	R	R	R	L	L	L	L	E
Total	638	754	3854	8153	1160	377	5464	258	144	123	837	1127	895	271	4144	40636
%	0.18	0.21	1.09	2.31	0.33	0.11	1.55	0.07	0.04	0.03	0.24	0.32	0.25	0.08	1.17	11.50
CODON	GAC	GAG	GAT	GCA	GCC	GCG	GCT	GGA	GGC	GGG	GGT	GTA	GTC	GTG	GTT	TAC
AMINO ACID	D	E	D	A	A	A	A	G	G	G	G	V	V	V	V	Y
Total	2657	4158	19199	5263	2405	291	8271	13534	936	915	7109	5618	1892	777	8949	1259
%	0.75	1.18	5.43	1.49	0.68	0.08	2.34	3.83	0.26	0.26	2.01	1.59	0.54	0.22	2.53	0.36
CODON	TAT	TCA	TCC	TCG	TCT	TGA	TGC	TGG	TGT	TTA	TTC	TTG	TTT	ATG		
AMINO ACID	Y	S	S	S	S	W	C	W	C	L	F	L	F	M		
Total	9584	5502	1076	319	4750	36	1783	2465	2795	12480	4097	1704	7085	6260		
% Usage	2.71	1.56	0.30	0.09	1.34	0.01	0.50	0.70	0.79	3.53	1.16	0.48	2.00	1.77		

Data shows the number of times a codon occurred in the data and converted to % occurrence.

further our understanding of the rumen protozoa and their role in the rumen.

Illumina sequencing revealed a wealth of carbohydrate-active enzymes in addition to those that specifically target the rumen fungi (and in some cases bacteria). Overall, 8% of all reads from the metatranscriptome were involved in carbohydrate metabolism or transport. However, 20% of carbohydrate-active enzymes identified were annotated as 'GH, family unknown.' Such a result serves to highlight the distinct lack of sequence data available for the rumen protozoa and calls into question whether this result may even be an underestimation in itself (Comtet-Marre et al., 2017). When approaching the metatranscriptome as a whole, this issue becomes more obvious as EggNOG was unable to annotate 27% of predicted genes. Such a result highlights the lack of data available for the rumen protozoa but also adds value to that presented here, which could contribute significantly to our current understanding and future sequence-based work.

A range of glycosyl hydrolases were identified (from families 5, 25, 16, 43, 45, and 97) as well as endo-1, 4-β -xylanases (belonging to the glycosyl hydrolase 11 family). Two of these enzymes: glycoside hydrolase family 11 and polysaccharide deacetylase are ranked in the top 20 most expressed genes in the whole metatranscriptome. Polysaccharide deacetylases accounted for 1.4% of the entire metatranscriptome and were the 18th most expressed gene. Polysaccharide deacetylases fall into carbohydrate esterase family 4 and catalyze the hydrolysis of N- or O-linked acetyl groups from polysaccharide residues (Arnaouteli et al., 2015). These enzymes are active against plant polysaccharides but are also used by bacteria to modify peptidoglycan in their cell walls to adjust to varying environmental conditions (Kobayashi et al., 2012). When subject to BLAST, this protein showed similarity (98%) to a glycosyl hydrolase family protein from *R. flavefaciens*. Analysis using the dbCAN metaserver identified the protein as carbohydrate-binding module 79 (CBM79); found specifically in the *R. flavefaciens* cellulosome, this forms part of a GH 9 catalytic module with endo-glucanase activity. However, with an AT composition of 67% it is highly likely that this enzyme has been horizontally acquired from the rumen bacteria to act against plant material and potentially against rumen bacteria upon engulfment (Garcia-Vallvé et al., 2000; Ricard et al., 2006; Kelly et al., 2009).

Some of the most abundant enzymes were the cellulases, or GH5s. When aligned to known protozoal sequences, several (7) of the sequences grouped away, showing no similarity, instead the phylogenetic tree indicated a relationship with cel9A from *Piromyces* sp. (AF459452) and cellulases from *R. flavefaciens* and *B. fibrisolvens* (L03800 and M37031, respectively). This highlights novelty in the enzymes identified here and supports the hypothesis that many carbohydrate active enzymes utilized by the protozoa have been horizontally acquired.

Xylanases, mostly endo-1, 4-beta-xylanases (EC 3.2.1.8 of the GH11 family), were also highly expressed - this is in keeping with current literature where the protozoa have been shown to produce several, highly active xylanases (Orpin, 1984; Williams and Coleman, 1985; Devillard et al., 1999, 2003; Béra-Maillet et al., 2005). Many of the xylanases identified in this dataset matched those described by McEwan et al. (2008); Findley et al. (2011) and/or Béra-Maillet et al. (2005) which is demonstrated in the phylogenetic trees. Some xylanases (4) did not group with known sequences in the phylogenetic tree, suggesting novelty.

The addition of glucose during fractionation has the potential to skew results and affect the expression of monosaccharide degrading enzymes, however, comparatively few enzymes involved in glucose metabolism were identified. Examples include glucokinase (K00845), aldose 1-epimerase (KO1785) and glucose phosphoglucomutase (K01835), none of which were highly expressed ranking 551st, 863rd and 302nd in the dataset in terms of expression. Far more numerous were those responsible for the breakdown of polysaccharides.

The metatranscriptome also revealed chitin and pectin degrading enzymes, generally thought to be produced by the rumen bacteria and fungi. It has been demonstrated that many enzymes belonging to the rumen microbes have been acquired or shared via horizontal transfer (Garcia-Vallvé et al., 2000; Ricard et al., 2006; Kelly et al., 2009). This is highly likely in the case of the rumen protozoa, as they are prolific in their capture and digestion of rumen bacteria and fungi, placing these microbes in very physical contact which increases the chance of an exchange. Previous studies suggested that some protozoa may produce chitinases. Miltko et al. (2012) found two endo-chitinases and two exochitinases produced by *E. maggii* but the evidence is scarce in comparison to that for the rumen bacteria and fungi. Strains of *Clostridia* have been proven to possess a variety of chitinases, showing activity lyzing the fungal cell wall and inhibiting the fungal β- 1, 4- endoglucanases (Kopečný et al., 1996; Kopečný and Hodrová, 2000). This may be perceived as evidence of direct competition between the rumen fungi and bacteria and in the case of the protozoa as a means of digestion inside the food vacuole (Morgavi et al., 1994). Two chitinases were identified in this dataset, one of which showed similarity to a hypothetical protein produced by *Butyrivibrio* sp. (90%). Another showed 95% identity with the GH18 chitinase-like family of enzymes produced by the *Eubacterium* genus. This result is consistent with the current literature and contributes toward the hypothesis that the rumen protozoa may have acquired chitinolytic enzymes from neighboring bacteria.

On the other hand, the production of pectin-active enzymes can be attributed solely to the need to break down polysaccharides in plant material. This is a largely recalcitrant compound used by the plant to give physical strength and to act as a barrier; as such, this makes it difficult to degrade (Voragen et al., 2009). In a study by Gradel and Dehority (1972), seven rumen bacteria were found to degrade solubilized and purified pectin in pure culture and Dušková and Marounek (2001) found consistent activity against pectin in *P. ruminicola*. In addition to the rumen bacteria, fungal species *Orpinomyces* and *Neocallimastix* produce pectinolytic enzymes: polygalacturonase and pectin lyase (not pectinesterase) (Kopečný et al., 1996). In the present study, sequencing revealed two of the four classes of pectin-active enzymes: pectinesterases and pectate lyases. When subject to BLAST, pectate lyases showed similarity to those found in *Piromyces* sp. *R. flavefaciens* and *Neocallimastix californiae* whilst the pectinesterase identified showed some similarity to that produced by *Ruminococcus albus*.

Of the few proteases identified in this dataset, both ranked in the top 50 most expressed enzymes and were predicted to display lysosomal activity (particularly cathepsin B). The most likely use for this enzyme is in the breakdown of ingested bacteria. This supports the observation that the rumen protozoa predate bacteria and are well adapted to digest them (Newbold et al., 2015). Two lipases of the GDSL family were identified but were not highly expressed (one with just 43 reads and the other with just 22). This lack of both sequences and expression levels may suggest that the rumen protozoa do not directly contribute to ruminal lipid metabolism. Instead, this result supports that reported by Huws et al. (2009) whereby due to the ingestion of chloroplasts the rumen protozoa are rich in polyunsaturated fatty acids, potentially protecting them from biohydrogenation. In terms of protein metabolism, this data suggests that protozoal contribution stem mostly from the breakdown of their fellow microbes. However, it is important to note that the diet has a significant effect on the rumen microbiome (Kim et al., 2008; Huws et al., 2010; Petri et al., 2013; Ross et al., 2013). In this study, animals were allowed 24/7 access to pasture with no silage or concentrates, which may affect the results observed. In general, perennial ryegrass contains a good amount of protein (16-25%), especially when compared to silage, however, concentrates such as soya bean meal contain much higher levels (approx. 47%) (Germinal, 2015). Indeed, the vast majority of lipids in pasture are phospholipids (polyunsaturated fatty acids), whilst lipid supplementation in the TMR varies considerably depending on production values/aims (Elgersma et al., 2003; Rasmussen and Harrison, 2011). As such, there is strong potential for dietary effects to influence the activity of the rumen protozoa – to address the issues, future research may look to profile the protozoa under different dietary conditions and over different time points, for example.

Results from codon usage analysis showed significant usage bias in lysine and glutamic acid. Lysine was coded for by AAA in 12.61% of cases, compared to AAG which was used in just 1.98% of cases. Glutamic acid was coded for by GAA 11.5% of the time, compared to GAG which was used in 1.18% of cases. Bias can also be observed in coding for asparagine, where the AAT codon was used 4.99% of the time, whilst AAC was employed in just 0.77% of cases. Overall, 70% of the total sequences consisted of A and T. This codon bias is demonstrated throughout the metatranscriptome and also persists in genes that show similarity at the protein level to those produced by bacteria, maintaining a 60–70% A-T content in individual genes. This extreme nucleotide bias is observed in both coding and non-coding regions but the reason behind this sway toward AT-rich codons is yet to be elucidated. This skewed codons usage that still follows the "universal" codon usage pattern, are in keeping with previous work (McEwan et al., 2000b). In this previous work it was shown that unlike some ciliates such as *Tetrahymena*, the rumen ciliates do not use TAG and TAA to encode glutamic acid, but rather they use them together with TGA as stop codons. Furthermore, given the high percentage of A-T present in their genomes, TAA is the most abundant stop codon used by these organisms. In addition, beyond the coding region of messages from rumen ciliates, there is a general absence of AAUAAA the "universal" polyadenylation signal. Instead, the short 3' untranslated region (typically < 100

nucleotides) contains a similar consensus sequence AUAAA (McEwan et al., 2000a).

This study has provided valuable insight into the metabolic contribution of the rumen protozoa. Their considerable contribution to carbohydrate breakdown has been observed, as well as some enzymes that were not thought to be commonly produced by rumen protozoa (pectinesterases, cathepsins and some glycosyl hydrolase families). Additional evidence of extensive HGT has been introduced, strengthening the argument that many genes have been acquired from the rumen bacteria and fungi that have enabled the protozoa to adapt to the carbohydrate-rich environment of the rumen. Evidence of adaptation to predation has also been presented, enzymes whose function are most likely in the digestion of rumen fungi and bacteria have been uncovered (pectin-active enzymes and cathepsins), cementing the role of the protozoa as predators in the rumen. The greatest limitation of this study is the use of a single time point, however, this information provides a sound basis for further research in terms of both the results presented and methods described. Nevertheless, this snapshot provides a wealth of new data which will further our understanding of the rumen protozoa, their functions and their role in the rumen environment in addition to providing valuable and much-needed reference sequences. This research may also serve as a guide or starting block for more in-depth research using the latest 'omic techniques to improve our knowledge of the protozoa.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Biosample database under SAMN13506237.

ETHICS STATEMENT

The animal study was reviewed and approved by the local Aberystwyth University Ethics Committee and the Animal Welfare and Ethical Review Body (AWERB) in line with University procedure. Experiments were conducted with the authority of Licenses under the United Kingdom Animal Scientific Procedures Act, 1986.

AUTHOR CONTRIBUTIONS

CW and SH conceived and designed the experiments. CW performed the experiments with support from PR, SH, and NM. CW were carried out the bioinformatic analyses with guidance from CC and BT. CW wrote the manuscript with revisions from SH. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00720/full#supplementary-material

REFERENCES

Abe, M., Iriki, T., Tobe, N., and Shibui, H. (1981). Sequestration of holotrich protozoa in the reticulo-rumen of cattle. *Appl. Environ. Microbiol.* 41, 758–765.

Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. Burlington, MA: ScienceOpen, Inc.

Arnaouteli, S., Giastas, P., Andreou, A., Tzanodaskalaki, M., Aldridge, C., Tzartos, S. J., et al. (2015). Two putative polysaccharide deacetylases are required for osmotic stability and cell shape maintenance in Bacillus anthracis. *J. Biol. Chem.* 22, 13465–13478. doi: 10.1074/jbc.M115.640029

Belanche, A., De la Fuente, G., Moorby, J. M., and Newbold, C. J. (2012). Bacterial protein degradation by different rumen protozoal groups. *J. Anim. Sci.* 90, 4495–4504. doi: 10.2527/jas2012-5118

Bełżecki, G., Newbold, C. J., McEwan, N. R., McIntosh, F. M., and Michałowski, T. (2007). Characterization of the amylolytic properties of the rumen ciliate protozoan *Eudiplodinium maggii*. *J. Anim. Feed Sci.* 16, 590–606. doi: 10.22358/jafs/66817/2007

Béra-Maillet, C., Devillard, E., Cezette, M., Jouany, J. P., and Forano, E. (2005). Xylanases and carboxymethylcellulases of the rumen protozoa *Polyplastron multivesiculatum Eudiplodinium maggii* and *Entodinium* sp. *FEMS Microbiol. Lett.* 244, 149–156. doi: 10.1016/j.femsle.2005.01.035

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Comtet-Marre, S., Parisot, N., Lepercq, P., Chaucheyras-Durand, F., Mosoni, P., Peyretaillade, E., et al. (2017). Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. *Front. Microbiol.* 8:67. doi: 10.3389/fmicb.2017.00067

Dehority, B. A. (1993). *Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa*. Boca Raton, FL: CRC Press.

Denton, B. L., Diese, L. E., Firkins, J. L., and Hackmann, T. J. (2015). Accumulation of reserve carbohydrate by rumen protozoa and bacteria in competition for glucose. *Appl. Environ. Microbiol.* 81, 1832–1838.

Devillard, E., Bera-Maillet, C., Flint, H. J., Scott, K. P., Newbold, C. J., Wallace, R. J., et al. (2003). Characterization of XYN10B, a modular xylanase from the ruminal protozoan *Polyplastron multivesiculatum*, with a family 22 carbohydrate-binding module that binds to cellulose. *Biochem. J.* 373, 495–503. doi: 10.1042/bj20021784

Devillard, E., Newbold, C. J., Scott, K. P., Forano, E., Wallace, R. J., Jouany, J. P., et al. (1999). A xylanase produced by the rumen anaerobic protozoan *Polyplastron multivesiculatum* shows close sequence similarity to family 11 xylanases from gram-positive bacteria. *FEMS Microbial. Lett.* 181, 145–152.

Dušková, D., and Marounek, M. (2001). Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rumen bacterium *Lachnospira multiparus*. *Lett. Appl. Microbiol.* 33, 159–163. doi: 10.1046/j.1472-765x.2001.00970.x

Eadie, J. M. (1962). Inter-relationships between certain rumen ciliate protozoa. *Microbiology* 29, 579–588.

Elgersma, A., Ellen, G., Van der Horst, H., Muuse, B. G., Boer, H., and Tamminga, S. (2003). Comparison of the fatty acid composition of fresh and ensiled perennial ryegrass (*Lolium perenne* L.), affected by cultivar and regrowth interval. *Anim. Feed Sci. Technol.* 108, 191–205.

Eschenlauer, S. C. P., McEwan, N. R., Calza, R. E., Wallace, R. J., Onodera, R., and Newbold, C. J. (1998). Phylogenetic position and codon usage of two centrin genes from the rumen ciliate protozoan, *Entodinium caudatum*. *FEMS Microbiol. Lett.* 166, 147–154. doi: 10.1111/j.15746968.1998.tb13196.x

Findley, S. D., Mormile, M. R., Sommer-Hurley, A., Zhang, X. C., Tipton, P., Arnett, K., et al. (2011). Activity-based metagenomic screening and biochemical characterization of bovine ruminal protozoan glycoside hydrolases. *Appl. Environ. Microbiol.* 77, 8106–8113.

Forsberg, C. W., Lovelock, L. K., Krumholz, L., and Buchanan-Smith, J. G. (1984). Protease activities of rumen protozoa. *Appl. Environ. Microbiol.* 47, 101–110.

Fu, Y., Ibrahim, A. S., Fonzi, W., Zhou, X., Ramos, C. F., and Ghannoum, M. A. (1997). Cloning and characterization of a gene (LIP1) which encodes a lipase from the pathogenic yeast Candida albicans. *Microbiology* 143, 331–340.

García, J. J., Bartolomé, D. J., Posado, R., Zúñiga, J. A., and Montañés, M. (2018). Weather conditions and rumen temperature and pH in lidia cattle. *J. Vet. Sci. Technol.* 9:532. doi: 10.4172/2157-7579.1000532

Garcia-Vallvé, S., Romeu, A., and Palau, J. (2000). Horizontal gene transfer of glycosyl hydrolases of the rumen fungi. *Mol. Biol. Evol.* 17, 352–361.

Germinal (2015). *The Value of Grass*. Available online at: https://www.britishgrassland.com/system/files/uploads/The%20Value%20of%20Grass%202015.pdf (accessed 16/03/2020)

Gradel, C. M., and Dehority, B. A. (1972). Fermentation of isolated pectin and pectin from intact forages by pure cultures of rumen bacteria. *Appl. Environ. Microbiol.* 23, 332–340.

Guttmacher, A. E., and Collins, F. S. (2003). Welcome to the genomic era. *N. Engl. J. Med.* 349, 996–998. doi: 10.1056/NEJMe038132

Huws, S. A., Kim, E. J., Kingston-Smith, A. H., Lee, M. R., Muetzel, S. M., Cookson, A. R., et al. (2009). Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. *FEMS Microbiol. Ecol.* 69, 461–471. doi: 10.1111/j.1574-6941.2009.00717.x

Huws, S. A., Lee, M. R., Muetzel, S. M., Scott, M. B., Wallace, R. J., and Scollan, N. D. (2010). Forage type and fish oil cause shifts in rumen bacterial diversity. *FEMS Microbiol. Ecol.* 73, 396–702. doi: 10.1111/j.1574-6941.2010.00892.x

Jouany, J. P., and Ushida, K. (1999). The role of protozoa in feed digestion-REVIEW, Asian Austral. *J. Anim.* 12, 113–128. doi: 10.5713/ajas.1999.113

Karnati, S. K. R., Sylvester, J. T., Noftsger, S. M., Yu, Z., St-Pierre, N. R., and Firkins, J. L. (2007). Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. *J. Dairy Sci.* 90, 798–809.

Kelly, B. G., Vespermann, A., and Bolton, D. J. (2009). Gene transfer events and their occurrence in selected environments. *Food Chem. Toxicol.* 47, 978–983. doi: 10.1016/j.fct.2008.06.012

Kim, E. J., Huws, S. A., Lee, M. R., Wood, J. D., Muetzel, S. M., Wallace, R. J., et al. (2008). Fish oil increases the duodenal flow of long chain polyunsaturated fatty acids and trans-11 18: 1 and decreases 18: 0 in steers via changes in the rumen bacterial community. *J. Nutr.* 138, 889–896. doi: 10.1093/jn/138.5.889

Kobayashi, K., Sudiarta, I. P., Kodama, T., Fukushima, T., Ara, K., Ozaki, K., et al. (2012). Identification and characterization of a novel polysaccharide deacetylase C (PdaC) from Bacillus subtilis. *J. Biol. Chem.* 287, 9765–9776. doi: 10.1074/jbc.M111.329490

Konstantinidis, K. T., Ramette, A., and Tiedje, J. M. (2006). The bacterial species definition in the genomic era. *Philos. Trans. R. Soc. B* 361, 1929–1940. doi: 10.1098/rstb.2006.1920

Kopečný, J., and Hodrová, B. (2000). Chitinolytic enzymes produced by ovine rumen bacteria. *Folia Microbiol.* 45, 465–468.

Kopečný, J., Hodrová, B., and Stewart, C. S. (1996). The isolation and characterization of a rumen chitinolytic bacterium. *Lett. Appl Microbial.* 23, 195–198. doi: 10.1111/j.1472765X.1996.tb00063.x

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874.

Martin, C., Williams, A. G., and Michalet-Doreau, B. (1994). Isolation and characteristics of the protozoal and bacterial fractions from bovine ruminal contents. *J. Anim. Sci.* 72, 2962–2968. doi: 10.2527/1994.72112962x

McEwan, N., Hackstein, J., Gatherer, D., McIntosh, F., Thomas, N., Nelson, N., et al. (2008). "Fibrolytic genes of the large entodinomorphid rumen ciliates were acquired by lateral gene transfer," in *Proceedings of the Joint Symposium, Clermont-Ferrand: GUT MICROBIOME: Functionality, Interaction With the Host and Impact on the Environment*, France, 46.

McEwan, N. R., Eschenlauer, S. C. P., Calza, R. E., Wallace, R. J., and Newbold, C. J. (2000a). The 3′ untranslated region of messages in the rumen protozoan *Entodinium caudatum*. *Protist* 151, 139–146.

McEwan, N. R., Gatherer, D., Eschenlauer, S. C. P., McIntosh, F. M., Calza, R. E., Wallace, R. J., et al. (2000b). An unusual codon usage pattern in the ciliate family *Ophryoscolecidae* and its implications for determining the source of cloned DNA. *Anaerobe* 6, 21–28. doi: 10.1006/anae.1999.0310

Miltko, R., Belzecki, G., and Michalowski, T. (2012). Chitinolytic enzymes of the rumen ciliate *Eudiplodinium maggii*. *Folia Microbiol.* 57, 317–319.

Morgavi, D. P., Sakurada, M., Tomita, Y., and Onodera, R. (1994). Presence in rumen bacterial and protozoal populations of enzymes capable of degrading fungal cell walls. *Microbiology* 631–636.

Murphy, C. L., Youssef, N. H., Hanafy, R. A., Couger, M. B., Stajich, J. E., Wang, Y., et al. (2019). Horizontal gene transfer as an indispensable driver for evolution of *Neocallimastigomycota* into a distinct gut-dwelling fungal lineage. *Appl. Environ. Microb.* 85, e988–e919.

Newbold, C. J., De La Fuente, G., Belanche, A., Ramos-Morales, E., and McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6:1313. doi: 10.3389/fmicb.2015.01313

Orpin, C. G. (1984). The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. *Anim. Feed Sci. Technol.* 10, 121–143.

Park, T., Wijeratne, S., Meulia, T., Firkins, J., and Yu, Z. (2018). Draft macronuclear genome sequence of the ruminal ciliate *Entodinium caudatum*. *Microbiol. Resour. Announc.* 7:e826-18.

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J., et al. (2013). Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. *PLoS ONE* 8:e83424. doi: 10.1371/journal.pone.0083424

Rasmussen, J., and Harrison, A. (2011). The benefits of supplementary fat in feed rations for ruminants with particular focus on reducing levels of methane production. *ISRN Vet. Sci.* 2011:613172.

Ricard, G., McEwan, N. R., Dutilh, B. E., Jouany, J. P., Macheboeuf, D., Mitsumori, M., et al. (2006). Horizontal gene transfer from Bacteria to rumen Ciliates indicates adaptation to their anaerobic, carbohydrates-rich environment. *BMC Genomics* 7:22. doi: 10.1186/1471-2164-7-22

Ross, E. M., Moate, P. J., Marett, L., Cocks, B. G., and Hayes, B. J. (2013). Investigating the effect of two methane-mitigating diets on the rumen microbiome using massively parallel sequencing. *J. Dairy Sci.* 96, 6030–6046.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sylvester, J. T., Karnati, S. K. R., Dehority, B. A., Morrison, M., Smith, G. L., St-Pierre, N. R., et al. (2009). Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. *J. Dairy Sci.* 92, 256–269.

Tamura, K., Nei, M., and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11030–11035.

Tymensen, L., Barkley, C., and McAllister, T. A. (2012). Relative diversity and community structure analysis of rumen protozoa according to T-RFLP and microscopic methods. *J. Microbiol. Method* 88, 1–6. doi: 10.1016/j.mimet.2011.09.005

Varel, V. H., and Dehority, B. A. (1989). Ruminal cellulolytic bacteria and protozoa from bison, cattle-bison hybrids, and cattle fed three alfalfa-corn diets. *Appl. Environ. Microbiol.* 55, 148–153.

Voragen, A. G., Coenen, G. J., Verhoef, R. P., and Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Struct. Chem.* 20:263. doi: 10.1007/s11224009-9442-z

Wang, L., Abu-Doleh, A., Plank, J., Catalyurek, U. V., Firkins, J. L., and Yu, Z. (2019). The transcriptome of the rumen ciliate *Entodinium caudatum* reveals

some of its metabolic features. *BMC Genomics* 20:1008. doi: 10.1186/s12864-019-6382-x

Wereszka, K., McIntosh, F. M., Michalowski, T., Jouany, J.-P., Nsabimana, E., Macheboeuf, D., et al. (2004). A cellulase produced by the rumen protozoan *Epidinium ecaudatum* is of bacterial origin and has an unusual pH optimum. *Endocytobiosis Cell Res.* 15, 561–569.

Wereszka, K., Michalowski, T., Newbold, C. J., and McEwan, N. R. (2006). Xylanolytic activity of the rumen protozoan *Diploplastron affine*. *J. Anim. Feed Sci.* 15, 43–46. doi: 10.22358/jafs/70139/2006

Williams, A. G., and Coleman, G. S. (1985). Hemicellulose-degrading enzymes in rumen ciliate protozoa. *Curr. Microbiol.* 12, 85–90. doi: 10.1007/BF01567397

Williams, A. G., and Coleman, G. S. (2012). *The Rumen Protozoa*. Berlin: Springer Science & Business Media.

Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., et al. (2018). dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res.* 46, W95–W101. doi: 10.1093/nar/gky418

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ORIGINAL RESEARCH
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The Isolation and Genome Sequencing of Five Novel Bacteriophages From the Rumen Active Against *Butyrivibrio fibrisolvens*

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Although the prokaryotic communities of the rumen microbiome are being uncovered through genome sequencing, little is known about the resident viral populations. Whilst temperate phages can be predicted as integrated prophages when analyzing bacterial and archaeal genomes, the genetics underpinning lytic phages remain poorly characterized. To the five genomes of bacteriophages isolated from rumen-associated samples sequenced and analyzed previously, this study adds a further five novel genomes and predictions gleaned from them to further the understanding of the rumen phage population. Lytic bacteriophages isolated from fresh ovine and bovine fecal and rumen fluid samples were active against the predominant fibrolytic ruminal bacterium *Butyrivibrio fibrisolvens*. The double stranded DNA genomes were sequenced and reconstructed into single circular complete contigs. Based on sequence similarity and genome distances, the five phages represent four species from three separate genera, consisting of: (1) Butyrivibrio phages Arian and Bo-Finn; (2) Butyrivibrio phages Idris and Arawn; and (3) Butyrivibrio phage Ceridwen. They were predicted to all belong to the *Siphoviridae* family, based on evidence in the genomes such as size, the presence of the tail morphogenesis module, genes that share similarity to those in other siphovirus isolates and phylogenetic analysis using phage proteomes. Yet, phylogenomic analysis and sequence similarity of the entire phage genomes revealed that these five phages are unique and novel. These phages have only been observed undergoing the lytic lifecycle, but there is evidence in the genomes of phages Arawn and Idris for the potential to be temperate. However, there is no evidence in the genome of the bacterial host *Butyrivibrio fibrisolvens* of prophage genes or genes that share similarity with the phage genomes.

Keywords: bacteriophage, rumen, lytic phages, phage genome, Butyrivibrio phage, *Butyrivibrio fibrisolvens*

INTRODUCTION

Despite the abundance of viruses in the rumen, little is known about the bacteriophage (phage) population in the rumen microbiome compared to other predominant microbes in this environment (Gilbert et al., 2020). The influences that phages have on ruminal function and digestion are yet to be understood (Anderson et al., 2017). Early studies based on electron microscopy observations of the viral fractions of rumen fluid (Hoogenraad et al., 1967; Paynter et al., 1969; Klieve and Bauchop, 1988) established that a highly diverse phage population exists in high abundance in ruminal fluid and could influence bacterial populations (Orpin and Munn, 1973). It is only now with the application of metagenome sequencing to rumen associated viruses that the full extent of the phage community in this environment is beginning to be uncovered and better understood (Berg Miller et al., 2012; Gilbert et al., 2017).

Rumen phages are those that either target rumen bacterial species, or those that are believed to reside in the rumen, evidenced either by direct sampling of rumen fluid (Klieve and Bauchop, 1988), or though isolation from ruminant associated samples, such as from compacted manure from transport trucks, and abattoir kill-floor run-off (Klieve et al., 2004). Indeed, samples from these two rumen-associated environments (as well as from sewage samples) yielded five phages that are, to date, the only genomes of lytic rumen phages to be sequenced and published in the context of the rumen virome. Of these five, three belonging to the *Siphoviridae* family; two that target the bacterial host *Prevotella ruminicola* and one which targets *Streptococcus bovis*, and another two belonging to the *Podoviridae* family that target *Ruminococcus albus* (Gilbert et al., 2017). These seminal efforts applied and adapted methods for isolating and characterizing lytic phages that infect anaerobic bacteria known to reside in the rumen. However, there is still much to be learned about phages in this environment and the opportunity exists to further investigate their presence in samples taken directly from rumen fluid and fresh feces, as well as exploring phages that target other rumen bacterial hosts.

Not only are individual rumen phages of interest, but the application of metagenomic sequencing on the viral fraction of rumen fluid offers a more holistic approach to observing rumen phage populations. For example, metagenomic analysis of the rumen viromes of three cattle indicated that the phage community is formed of twice as many temperate phages as lytic phages, with the bacterial hosts mostly belonging to the *Firmicutes* and *Proteobacteria* phyla (Berg Miller et al., 2012). Additionally, it was concluded that because the abundance of phage populations tended to correlate with the abundance of the phyla of their prospective bacterial hosts, phages play an important role in the rumen microbiome, manipulating the gene pool through the genetic transfer activity of temperate phages, and bacterial population control through cell lysis activity of the lytic phages (Berg Miller et al., 2012). Another metagenomic study of the viral populations in the rumen of steers fed different diets revealed that although there are changes in viral communities with a change in diet, there were abundant populations of phages found across all samples, indicating a core virome (Anderson et al., 2017). An analysis of DNA viruses in buffalo revealed the order *Caudovirales*, the tailed bacteriophages, formed the largest community, and within this the *Siphoviridae* and *Myoviridae* families were predominant (Parmar et al., 2016). A similar study of rumen fluid from sheep and goats also revealed that of the reads that could be identified, the highest proportion shared similarity to the *Siphoviridae* family, yet the majority of the reads were unidentified (Namonyo et al., 2018). Without representative genomes of the rumen phage population, the ability to make statements about anything more detailed than the viral family level is limited. Therefore, the novel isolation and sequencing of cultured phages from rumen-associated samples, in particular rumen fluid, would be of huge benefit to the research community.

This study presents five novel bacteriophages directly isolated from ovine and bovine rumen fluid and fresh fecal samples, along with their sequenced and annotated genomes. To date, this is the second report of phage genomes to be sequenced from rumen associated samples, and the first to report sequence data of isolated and cultured phages against the bacterial host *Butyrivibrio fibrisolvens*. Previously, filamentous phage-like particles were observed in cultures of *B. fibrisolvens* AR14 cultures when induced with mutagen mitomycin C (Klieve et al., 1989), suggesting the induction of a prophage, but these were not cultured or explored further. As a predominant bacterium in the rumen, *B. fibrisolvens* is thought to contribute to a range of functions, as it has shown abilities such as fiber degradation and xylan fermentation (Cotta and Hespell, 1986), proteolysis (Sales et al., 2000) and biohydrogenation (Kepler et al., 1966). *B. fibrisolvens* has also been isolated from human fecal samples (Brown and Moore, 1960; Rumney et al., 1995), which was shown to be related to ruminal strains and is thought to play a role in butyrate production from starch degradation in the human colon (Barcenilla et al., 2000; Ramsay et al., 2006).

MATERIALS AND METHODS

Bacterial Culturing Methods

Butyrivibrio fibrisolvens DSM 3071 was cultured and maintained using M2 medium (Hobson, 1969); using 10 ml/L sodium lactate solution at 60% (w/v), tryptone (Melford, Ipswich United Kingdom) as the protein source, and minerals (b) consisting of KH_2PO_4, 3.0 g/L; $(NH_4)_2SO_4$, 6.0 g/L; NaCl, 6.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.6 g/L; $CaCl_2 \cdot 2H_2O$, 0.6 g/L.

Rumen-Associated Samples

Samples of rumen fluid and feces were obtained from fistulated sheep and cows. Hand grabs of rumen contents were obtained through the cannulae of three 7-year-old Aberdale cross Texel sheep which were grazing *ad libitum* on grass, with ∼300 g sugar beet and grass nuts supplemented each morning. Rumen fluid samples were obtained in the same way from three 10-year-old Holstein-Friesian dry cows which were grazing *ad libitum* on grass, with ∼500 g dairy concentrates in the morning during sampling. Rumen fluid was obtained under the authority of licenses under the United Kingdom Animal Scientific Procedures

act 1986. All animal associated research is managed according to the Aberystwyth University Animal Welfare and Ethics Review Board[1]. Each of the sheep rumen fluid samples were squeezed and strained through a sieve and then combined into one air-tight pre-warmed container. The same procedure was carried out for the cow rumen fluid and collected in another air-tight pre-warmed container.

Feces were caught in a gloved hand once excreted naturally from three sheep of the same flock and transferred to sterile plastic bags. Cow feces were obtained soon after excretion; a small amount of fresh fecal matter was taken from the field, collected into pots, and transferred into sterile centrifuge tubes. Three fecal samples each for cows and sheep were collected this way and screened for phage presence. Where necessary, fecal samples were refrigerated for less than 1 week before use to avoid a freeze/thaw cycle if stored in a freezer.

Screening Phage Filtrates From Rumen Fluid and Feces

For both sheep and cows, phage filtrates were made from both the rumen fluid and fecal samples as per the methods described previously (Klieve, 2005; Klieve and Gilbert, 2005). The rumen fluid was aliquoted into centrifuge tubes, spun at 15,000 × *g* for 15 min at 4°C, and the supernatants stored on ice and filtered through 0.45 μm pore size low-protein binding PES syringe filters and retained on ice. Around 10 g of feces were weighed out into centrifuge tubes and a volume (in ml) equivalent to the mass (in g) of the sample of Phage Storage Buffer [PSB; 20 mM Tris–HCl, 200 mM NaCl, 20 mM $MgCl_2$, 0.1% (w/v) gelatin (Klieve, 2005)] was added and incubated at room temperature for 1 h with gentle mixing on a tilting laboratory shaker. After spinning at 15,000 × *g* for 15 min at 4°C, the supernatant was filtered through a 0.45 μm pore size syringe filter. All filtrates were wrapped in foil and stored at 4°C.

Viral particles were concentrated from filtrates (and later lysates) by the addition of polyethylene glycol (PEG) and sodium chloride (NaCl) with modifications to methods published previously (Bourdin et al., 2014; Antibody Design Laboratories, 2015; Gutiérrez et al., 2018; Namonyo et al., 2018). To phage solutions, PEG 8000 and NaCl was added to a final concentration of 10% (w/v) and 0.5 M, respectively, and used to precipitate phages overnight at 4°C. Concentrated phages were collected by spinning at 12000 × *g* for 30 min at 4°C. The supernatant was discarded, and the pellet resuspended in a smaller volume (a tenth of the initial volume) of suitable phage buffer, wrapped in foil and refrigerated until further use. Previous studies have shown phage filtrates to remain viable for months or even years at 4°C (Klieve, 2005).

A lawn of host bacteria was achieved by adding 1 ml of overnight (or actively growing) culture of *Butyrivibrio fibrisolvens* DSM 3071 to 3 ml of warm 0.8% (w/v) M2 agar (soft agar), mixing and pouring over set bottom agar [1.5% (w/v) M2 agar] in the anaerobic cabinet with gas mix of 10:10:80 of CO_2, H_2 and N_2 (Whitley A35 Anaerobic Workstation, Don Whitley Scientific) (Klieve, 2005). Once set, a spot test was carried out by spotting 10 μl of each phage filtrate in triplicate onto the host lawn and incubating for >24 h at 39°C, or until adequate lawn growth [similar method that was used by Nale et al. (2016)]. Any resulting areas of lysis or clearing on the bacterial lawn was indicative of phage activity. Positive spots or plaques were scraped using a 5 μl inoculating loop, extracting top soft agar, and mixed well in a small volume of PSB, vortexed briefly and left to stand at room temperature for 30 min and stored at 4°C until testing again on the same host. Where a sample spot contained what appeared to be a very small area of lysis or that resembled a small plaque, these were picked using a pipette tip and all combined into a microcentrifuge tube containing a small volume of PSB, vortexed briefly and left to stand as above, before undergoing further plaque assays. Combining putative plaques in this way from a variety of samples increases the likelihood of phage isolation and reduces consumable use but removes the traceability of subsequent phage isolation to the original fecal or rumen fluid sample, which for this study was deemed acceptable.

Isolation and Purification of Bacteriophages

Upon an initial indication of phage activity in a sample, then in later stages to purify and propagate positive phage samples, a plaque assay was carried out. An aliquot of 10 μl of the sample positive for phage activity was combined with 1 ml of host bacterial culture, left to incubate for no more than 15 min, mixed with 3 ml of soft agar [0.8% (w/v)] and poured over bottom agar [1.5% (w/v) M2 agar] (Klieve, 2005). Plaques with clearly defined, non-overlapping zones of clearance were picked using a pipette tip, placed into a small volume of PSB (∼200 μl) vortexed briefly, left standing for half an hour at room temperature, and tested again. Dilutions of the picked plaques were made using PSB to avoid confluent growth in the early stages of purification, so that single isolated plaques with one morphology type could be observed.

Once the plaque morphology was believed to be homogenous, or at least three rounds of purification had taken place, the phage was eluted from a confluent plaque assay plate by adding 5 ml of PSB to the plate, macerating the agar gently using a spreader, and leaving to incubate at room temperature on a rocker, for at least 30 min. The eluate was aspirated using a pipette tip into a microcentrifuge tube, spun at 15,000 × *g* for 2 min to pellet any agar, and the supernatant was filtered through a 0.45 μm pore size PES syringe filter. This resulted in five phage samples, temporarily termed phage C, D, J, M, and P.

Nucleic Acid Extraction and Sequencing

Phages from 800 μl of eluates were precipitated using PEG/NaCl overnight and resuspended in a volume one tenth of the initial volume, 40 μl of which was then used for nucleic acid extraction. Controls were added to the nucleic acid extraction process to evaluate the extraction process and other downstream applications, such as gel electrophoresis. The controls were comprised of molecular grade water as the negative control, and a "host lysate sample" as a positive control, which was made by taking an aliquot of host bacterial liquid culture, vortexing

[1]https://www.aber.ac.uk/en/rbi/staff-students/ethics/animals/

briefly and boiling at 100°C for 15 min to lyse the cells, before spinning at 15000 × *g* for 10 min, and filtering the supernatant through a 0.45 μm pore size PES syringe filter. Of this, 600 μl was precipitated overnight with PEG/NaCl, as well as the negative control. From each of these controls, 40 μl also underwent DNA extraction alongside the phage samples. The FastDNA™ Spin Kit for Soil (MP Biomedicals, Solon, OH, United States) was used to extract DNA from the lysate samples, using the proprietary FastPrep-24 for 30 s at 6.0 m/s, and spinning at 14,000 × *g* for 5 min at 18°C. DNA was eluted from the column with 30 μl of supplied DES water. The DNA concentration was determined using the Qubit fluorometer (Qubit 3 Fluorometer, Invitrogen by Thermo Fisher Scientific) and the high sensitivity DNA assay. Those samples that had a low concentration were increased using a DNA Speed Vac (DNA 100, Savant), or in the case of phage sample J, was re-extracted using 900 μl of phage eluate and combining this with 300 μl of the supplied sodium phosphate buffer in the first step. DNA was eluted from the column first with 30 μl of DES water, then a second time with 50 μl. The concentration of DNA in the re-extracted and concentrated sample were measured again, and all samples were diluted with nuclease free water to achieve a final concentration of ∼2 ng/μl in 10 μl necessary for sequencing. Quality of the DNA was tested using spectrophotometry and the 260/280 ratio (Epoch, BioTek Instruments) and sequencing was done in-house, where the isolated phage DNA was first diluted to 1 ng/ul, and libraries then prepared using the Illumina NextEra XT protocol as per the manufacturer's instructions (selecting AMPure bead ratio as suggested for 2 × 300 bp reads). Libraries were quantified via Qubit fluorescence spectrophotometry, pooled at an equimolar ratio, and diluted to 6 pM before loading on an Illumina MiSeq platform using a v3 600-cycle kit in 2 × 300 bp format. The five samples are available on NCBI as BioSamples under the BioProject accession code PRJNA613207, and raw reads for these in the Sequence Read Archive (SRA) under the accession code SRP255162.

Quality Control and Assembly

Quality control of the raw reads was carried out using FastQC v.0.11.8 (Andrews, 2010), followed by quality trimming using the paired end default settings in Sickle (Joshi and Fass, 2011) and assembly using SPAdes v3.13.0 on paired and single reads after trimming, with just "assembly-only" option applied (Bankevich et al., 2012), as conducted for phage genomes previously (Rihtman et al., 2016; Sazinas et al., 2018). Contigs were visualized using Bandage v0.8.1 (Wick et al., 2015), and those circularized contigs with the highest coverage for assembled reads of a reasonable length (>10 kbp) were extracted as the phage genomes. The phage genome contigs were visualized manually using Geneious Prime 2020.1.2[2] and repeat regions were identified. A 127 bp length of sequence in each of the genomes was repeated as an overlap of the assembled start and end (as the genomes were circular), which corresponded to the k-mer size used by SPAdes during assembly, and was removed. BWA-MEM v0.7.16a-r11810 (Li, 2013) was then used with default settings to align all reads in a sample to all of the corresponding assembled contigs, and SAMtools v1.5 (Li et al., 2009) was then used to extract the alignments to the phage contig and manage output files. Coverage was assessed using the depth command in SAMtools and calculating the coverage for the entire genome by averaging the coverage for each base in a genome.

The quality of genomes was assessed using Pilon v1.23 (Walker et al., 2014), and then were rearranged using the terminase gene, which was predicted using Prokka v1.12 (Seemann, 2014), supplemented with the Caudovirales database[3]. The genomes were reordered and orientated manually using Geneious Prime, such that the start of the linear sequence for the circularized genome was at the first base of the first gene found downstream of the terminase gene, as recommended previously (Russell, 2018).

Comparing and Annotating the Phage Genomes

The entire genome sequences were queried against the viruses (taxid:10239) nucleotide collection (nr/nt) using blastn (Zhang et al., 2000) and default settings (carried out on 15/05/2020). For comparative genomics, average nucleotide identity (ANI) was calculated using FastANI v1.3 (Jain et al., 2018) using default settings. Genomic synteny was visualized using ProgressiveMauve within Geneious Prime, using default settings.

Open reading frames (ORFs) were then predicted using Glimmer v3.02 (Delcher et al., 2007) with default settings, GeneMarkS-2 (Lomsadze et al., 2018) with Prokaryotic sequence type, genetic code 11 and GFF3 output using the online tool[4], Prodigal v2.6.3 with default settings with output as GFF (Hyatt et al., 2010) and PHANOTATE v1.2.2 (McNair et al., 2019) with default settings. All ORFs were manually curated using Geneious to visualize all predicted ORFs, allocating each putative ORF into one of four groups; (A) those ORFs where all gene callers agreed with presence, start and end; (B) those ORFs where all gene callers agreed with gene presence, but with different predicted starts and/or ends; (C) those ORFs where the majority of gene callers agreed on ORF presence, with same start and ends; (D) those ORFs where the majority of gene callers agreed on ORF presence, but with different predicted starts and/or ends.

Nucleotide and protein sequences for ORFs in all four categories were obtained using gff2bed tool [Bedops; v2.4.37; (Neph et al., 2012)], getfasta [Bedtools; v2.27.1; (Quinlan and Hall, 2010)] and transeq [Emboss; v6.6.0.0; (Rice et al., 2000)]. These were then annotated by searching for homologs with BLAST v2.8.1+ (Altschul et al., 1990; Camacho et al., 2009) and all BLAST hits that achieved an e-value greater than 10^{-5} (Aziz et al., 2018) and query coverage >80%, as used by Prokka (Seemann, 2014) were retained. Custom nucleotide and protein databases comprised of a number of NCBI databases were used, including

[2]https://www.geneious.com

[3]Available at: http://s3.climb.ac.uk/ADM_share/Caudovirales.tar.gz; last modified 20/07/2017

[4]Available at: http://exon.gatech.edu/GeneMark/genemarks2.cgi, accessed 10/03/2020

representative viral reference genomes[5], viral genomes, proteins and non-redundant proteins from the 8th July 2019 RefSeq database[6], against which the full genomes and nucleotide gene sequences were searched; alongside the Caudovirales database[3] and the prokaryotic virus orthologous groups (pVOGs) database supplied with multiPhATE v1.0 (Zhou et al., 2019). Protein databases were obtained from Swissprot[7] and virus specific orthologous groups from EGGNOG[8], annotating the best hits with UniProt IDs. Each ORF for each genome was manually curated and annotated using the best of the retained hits, where multiple hits with equally good scores from different databases were all recorded. The name of the genome containing the gene with the best hit(s) for each ORF was also noted. Where gene callers did not agree on an ORF, the ORF with the best hit was used, considering coverage of query and subject and percentage identity to determine the best.

Protein motifs were also predicted using hmmscan in HMMER v3.1b2 (Eddy, 2011) against pfam[9], TIGRfam[10] (Haft et al., 2003) and HAMAP[11] (Lima et al., 2009) databases. A consensus gene name was then derived manually for each predicted ORF for each phage genome based on the evidence gathered from homology searches. Numbers of overlapping genes were obtained from the GFF files for each of the genomes, and tRNAs were predicted using tRNAscan-SE v2.0[12] assuming a bacterial sequence source and otherwise default settings (Chan and Lowe, 2019) and added to the annotated ORFs. All ORFs were also allocated a functional category, for example Structural or Lysis, depending on the process(es) to which the predicted gene contributes. ORFs that that were not assigned an annotation were allocated to the Hypothetical category, whereas those that shared sequence similarity with another hypothetical gene in another phage genome were allocated to Uncharacterized.

TMHMM v2.0 (Krogh et al., 2001) was run to detect transmembrane regions, einverted was used in Emboss to find inverted repeats and PHACTS[13] (McNair et al., 2012) to determine lifestyle of the phage. Promoters were identified in each genome using PhagePromoter[14] (Sampaio et al., 2019) setting the lifecycle for each phage with the outcome of PHACTS, assuming all phages were in the *Siphoviridae* family, host bacterial genus as other, both directions and a threshold of 0.5. Terminators were predicted using FindTerm[15] (Solovyev and Salamov, 2011), showing all putative terminators with energy threshold value -10, as recommended previously (Aziz et al., 2018). Promoters and terminators were then manually checked, removing terminators present within genes whilst retaining ones present in intergenic regions or in the 3′ end of upstream genes (Aziz et al., 2018), and choosing promoters in the correct orientation and with the highest score, where multiple were present.

Phylogenetics and Phylogenomics

The nucleotide genome sequences of these genomes were uploaded into ViPTree (Nishimura et al., 2017) using default settings, dsDNA nucleic acid type, Prokaryote host categories and a user-defined gene annotation file. From the resulting tree from the ViPTree analysis, the closest related taxa were identified as those in the same clade as the Butyrivibrio phage genomes, of which there were 13, and were downloaded using the NCBI ID supplied by ViPTree. The nucleotide sequences of these 13 genomes, along with the five phage genomes from this study, were then analyzed using the Genome-BLAST Distance Phylogeny (GBDP) method (Meier-Kolthoff et al., 2013) with default settings recommended for prokaryotic viruses in VICTOR[16] (Meier-Kolthoff and Göker, 2017) using the D0 formula.

Assessing Host Interactions

The host genome of the bacterial host *Butyrivibrio fibrisolvens* DSM 3071 was downloaded (NCBI Reference Sequence NZ_FQXK01000003.1) and the GC content and codon usage were summarized in Geneious Prime. Additionally, the presence of prophages was predicted using the PHASTER online tool[17] (Zhou et al., 2011; Arndt et al., 2016). The amino acid sequences of the predicted prophage genes were subjected to a sequence similarity search using BLASTP against the protein sequences for genes predicted in the five phage genomes from this study and filtered using the same thresholds as previous. CRISPR/Cas genes were identified in the reference genome and in 497 genomes of the Hungate1000 collection (Seshadri et al., 2018) using CRISPRCasFinder (v4.2.19) with default settings (Couvin et al., 2018). The resulting CRISPR spacer sequences were queried against the phage genomes from this study using blastn with default settings.

RESULTS AND DISCUSSION

The screening of bacteriophages from a total of eight rumen-associated samples resulted in the isolation of five phage samples active against *Butyrivibrio fibrisolvens* DSM 3071. The implementation of PEG/NaCl to concentrate bacteriophages from fecal and rumen fluid phage filtrates resulted in the observation of plaques which were further purified to create

[5] ftp://ftp.ncbi.nlm.nih.gov/blast/db/ref_viruses_rep_genomes.tar.gz; last updated 23rd August 2019

[6] Release 95; ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/

[7] August 2019 release; ftp://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/complete/uniprot_sprot.fasta.gz

[8] v5.0, http://eggnog5.embl.de/download/eggnog_5.0/e5.viruses.faa

[9] Available at ftp://ftp.ebi.ac.uk/pub/databases/Pfam/current_release/Pfam-A.hmm.gz; last modified 30/08/2018

[10] Release 15, available at https://ftp.ncbi.nlm.nih.gov/hmm/TIGRFAMs/release_15.0/TIGRFAMs_15.0_HMM.tar.gz

[11] HAMAP available with Prokka v1.12 (Seemann, 2014).

[12] http://lowelab.ucsc.edu/tRNAscan-SE/

[13] Available at http://edwards.sdsu.edu/PHACTS/upload.php; accessed on 24/03/2020

[14] Available as a Galaxy tool at https://galaxy.bio.di.uminho.pt/; accessed on 24/03/2020

[15] http://www.softberry.com/berry.phtml?topic=findterm&group=programs&subgroup=gfindb; version v2.8.1, accessed 24/03/2020

[16] Available at: https://ggdc.dsmz.de/victor.php; accessed 09/04/2020

[17] http://phaster.ca/

five phage samples that were seemingly homogenous and morphologically distinct in plaque assays. These were informally labeled as samples C, D, J, M, and P, and correspond to the BioSamples deposited in the NCBI database. From these phage samples, DNA was successfully extracted using a bead beating method and sequenced. After quality control, trimming and assembly, each of the five samples yielded one fully reconstructed circularized phage contig, except for phage sample J, which yielded two contigs each with different genome lengths and coverage. The reconstructed contigs surpassed the recommended minimum of 100-fold coverage recommended for phage genomes (Russell, 2018).

Further analysis of these phage genomes revealed that the phage genome reconstructed from sample J (J-1) with the highest coverage was 100% similar at the nucleotide level to the phage genome reconstructed in phage C sample, as determined by ANI. This genome did not undergo further analysis. The whole phage genomes were queried in the NCBI virus nucleotide database and revealed that there was no significant similarity to viral genomes published and deposited in this database previously. Further searches against the viral representative reference sequence database and viral non-redundant RefSeq database from NCBI also did not reveal similarity to any previously deposited viruses. Therefore, as it is likely that these five phage genomes are novel, they have been named; phage sample C yielded Butyrivibrio phage Bo-Finn, phage sample D yielded Butyrivibrio phage Arawn, phage sample J-2 yielded Butyrivibrio phage Idris and phage sample M yielded Butyrivibrio phage Ceridwen. The summary of the samples, the source of the samples, the genome contigs reconstructed and subsequent names can be seen in **Table 1**.

To determine the similarity between these phage genomes, genome comparisons were first carried out using Mauve (**Figure 1**), then by calculating the ANI between any two genomes that appeared similar. As is apparent in **Figure 1**, Butyrivibrio phage Arian and Butyrivibrio phage Bo-Finn share a similar genome length and genome synteny, and 98.6% ANI. Butyrivibrio phage Arawn and Butyrivibrio phage Idris also share a similar genome length and genome synteny to each other, and 98.96% ANI.

TABLE 1 | Summary table of phage sample and its source, the genomes constructed, coverage and allocated phage name.

Phage Sample	Source	Genome Contigs Reconstructed	Average Coverage Over Entire Genome	Butyrivibrio phage
J	Sheep	J-1	1399.46	N/A
	rumen fluid	J-2	242.56	Idris
C	Cow rumen fluid	C	125.832	Bo-Finn
P	Cow feces	P	754.716	Arian
D	Mixture*	D	1390.5	Arawn
M	Mixture*	M	352.867	Ceridwen

**Mixture – plaques from one sample of cow feces, cow rumen fluid and sheep rumen fluid were combined and tested again to produce the plaques from which these phages were isolated.*

The reconstructed phage genomes ranged from 31 (Arawn) to 39.7 Kbp (Ceridwen) in length (**Table 2**), with numbers of predicted open reading frames (ORFs) ranging from 50 (Arawn) to 73 (Ceridwen). The percentage of ORFs with homology to known sequences ranged from 40% (Ceridwen) to 52% (Arawn) (**Table 2**). All phage genomes have overlapping genes, ranging from 15% of genes in Idris, which has the smallest genome, and up to 30% of the genes in phage Ceridwen, which has the largest genome. Interestingly, this correlation is opposite to that seen across other viruses, where smaller viral genomes were generally found to have more overlapping genes (Brandes and Linial, 2016). All genomes have small intergenic regions, with few areas of

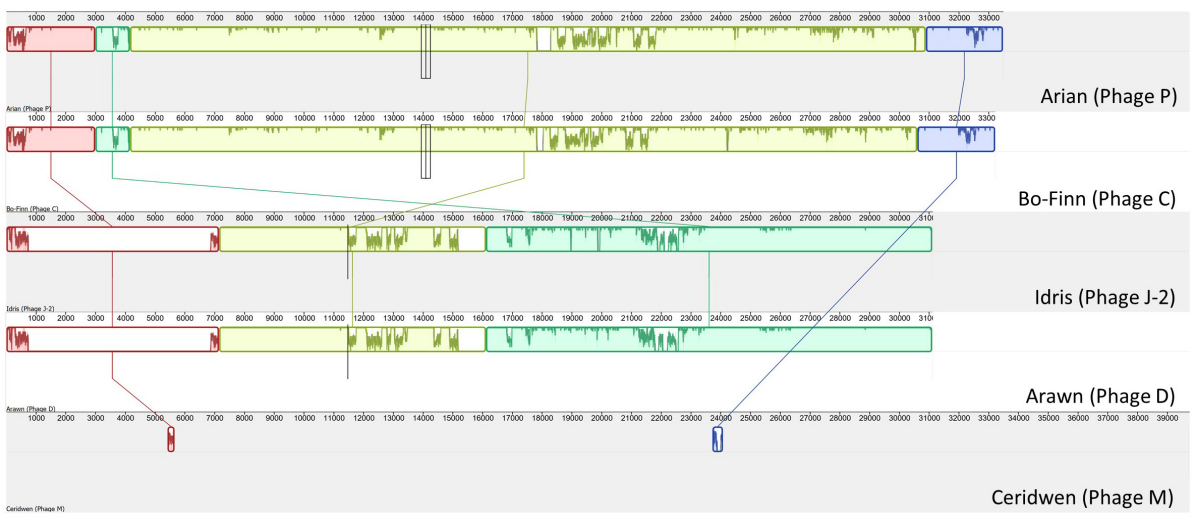

FIGURE 1 | Mauve alignment (Darling et al., 2004) of the five Butyrivibrio phages. Colinear blocks are color-coded, with the similarity graph plotted within the block and lines join similar blocks across genomes.

TABLE 2 | Genome statistics summary.

Butyrivibrio phage	Genome Length (bp)	GC (%)	ORFs	ORFs with best hit homologous proteins	Most common phage name (number of best hit homologous proteins)*
Arawn	31118	46.9	50	26	Paenibacillus phage PG1 (2)
Arian	33499	39.7	54	25	N/A
Bo-Finn	33227	39.7	51	24	N/A
Ceridwen	39745	40.8	73	29	Clostridium phage phiCP13O (2), Clostridium phage phiCP26F (2)
Idris	31128	46.9	55	25	Paenibacillus phage PG1 (2)

**Using the most common phage name with the number of best hit homologous proteins was a method used previously with rumen phages (Gilbert et al., 2017). N/A indicates that no single phage genome shared more than one best hit homologous protein.*

the genome that are not populated by ORFs, and many ORFs overlap, characteristics which are common in phage genomes (McNair et al., 2019).

Phages Bo-Finn and Arian

Butyrivibrio phages Bo-Finn and Arian are different isolates that belong to the same species and genus. A score of 98.6% ANI is greater than the 95% nucleotide sequence identity threshold indicative of the same species, as set by the International Committee on Taxonomy of Viruses (ICTV) (Adriaenssens and Brister, 2017). The two genomes are syntenous and share high sequence similarity across their entire genome (**Figure 2A**). The most pronounced difference is around 18 Kbp, where a subsequence is present in each genome that is not found in the other genome, as shown by the gray boxes in **Figure 2A**. Both phages have tail genes that correspond to those found in most members of the *Siphoviridae*; tail terminator, major tail protein, two chaperones, tape measure protein (TMP), distal tail protein, tail-associated lysozyme and baseplate/tip proteins, which together form a functional 'tail morphogenesis module' (Veesler and Cambillau, 2011). **Figure 2B** shows these genes in Butyrivibrio phage Arian, including a promoter and terminator, suggesting that these genes can be regulated together. Not all genes here showed homology to previously identified proteins, but if the tail morphogenesis module is conserved, these hypothetical proteins may be involved in the tail formation. Additionally, of the predicted ORFs that were homologous to genes in other phages, 10 in the Bo-Finn genome and 11 in the Arian genome shared homology to phages of the *Siphoviridae* family, six in each genome to phages of *Myoviridae,* and one each to a phage of *Podoviridae*. Along with the absence of any tail sheath proteins which are common to *Myoviridae* (Adriaenssens and Cowan, 2014), and the majority of genes being similar to other *Siphoviridae* phages, it is likely that these two phages belong to the *Siphoviridae* family.

Butyrivibrio phage Bo-Finn was predicted by PHACTS non-confidently to undergo the temperate lifecycle, whereas Arian was predicted non-confidently to undergo the lytic lifecycle. This suggests that there was some uncertainty with predicting the lifecycle using the proteomes of these two phages and that this outcome was not statistically supported (McNair et al., 2012). The major capsid proteins in these two phage genomes were predicted using protein motifs, resembling those in the HK97 family of major capsid proteins, populated by temperate phages (Haft et al., 2003). This suggests that these phages could undergo the temperate lifecycle, yet there is no further evidence in the genome to suggest this ability; no immunity repressors, partitioning genes or integration and excision enzymes were annotated, which are common functions associated with temperate phages (Mavrich and Hatfull, 2017; Stark, 2017). Additionally, these two phages were observed to undergo the lytic cycle *in vitro* and phage Arian harbors an endolysin (QHJ73651.1), and phage Bo-Finn an endolysin (QHJ73713.1) and putative holin (QHJ73715.1). Moreover, a tRNA gene for the amino acid glutamine was predicted in these genomes, which is used in 3.6% of coding genes in genome of phage Arian and 4.4% in Bo-Finn, compared to 2.7% of coding genes in the bacterial host genome (**Supplementary Table S1**). These codon usage statistics suggest that this codon is utilized more often in the phage genome than the host genome and therefore encoding this tRNA would increase efficiency and virulence, something that has been noted previously in lytic phages (Bailly-Bechet et al., 2007). Because these genomes are so highly similar, it is likely that they would both undergo the same lifecycle, which is not reflected in the PHACTS results. Evidence so far would therefore indicate that these phages are virulent, however, with some ORFs remaining uncharacterized, there may be novel proteins associated with the temperate lifecycle. The full annotated genomes can be seen for phage Arian and Bo-Finn in **Supplementary Figures 1B,C**, respectively.

A blastn search for the entire genome sequences of phage Arian and Bo-Finn against the NCBI virus genome database did not reveal any significant alignments covering more than 8% of the query genome.

Phages Arawn and Idris

Butyrivibrio phages Arawn and Idris also share high sequence similarity (98.96% ANI). The two genomes are syntenous, with highly similar genome sequences at the nucleotide level. The most noticeable difference between the two genomes is at 21.3 Kbp, where the similarity is low, and a different sequence in phage Arawn results in three ORFs predicted in this region (**Figure 3A**). The series of tail proteins present in Arawn (**Figure 3B**) also resembles the tail morphogenesis module common to phages in the *Siphoviridae* family (Veesler and Cambillau, 2011). Unlike phage Arian, Arawn has a terminator predicted after the major tail protein (QHJ73553.1) and a promoter located in the 3′ end

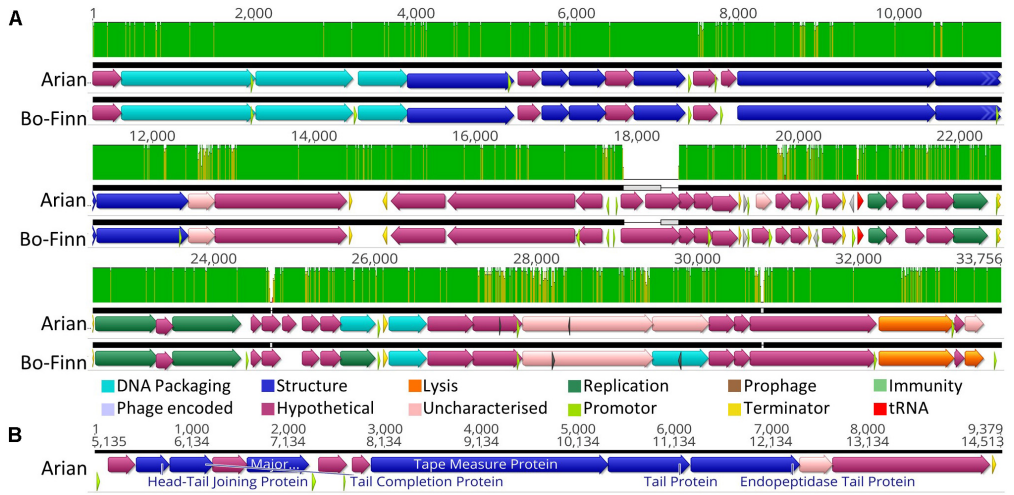

FIGURE 2 | Analysis of the Butyrivibrio phage Arian and Bo-Finn genomes. **(A)** Mauve alignments (Darling et al., 2004) of the two genomes showing annotated ORFs, with directionality and function according to color. Nucleotide similarity is expressed as a graph, with green indicating 100%, yellow > 30%, red < 30%. **(B)** The organization of the tail morphogenesis module in phage Arian. Uncharacterized proteins indicate where the protein was homologous to one reported previously, but had an unknown function, whereas hypothetical proteins did not show similarity above the set thresholds to any known proteins. Phage encoded proteins are those where there was uncertainty about their function.

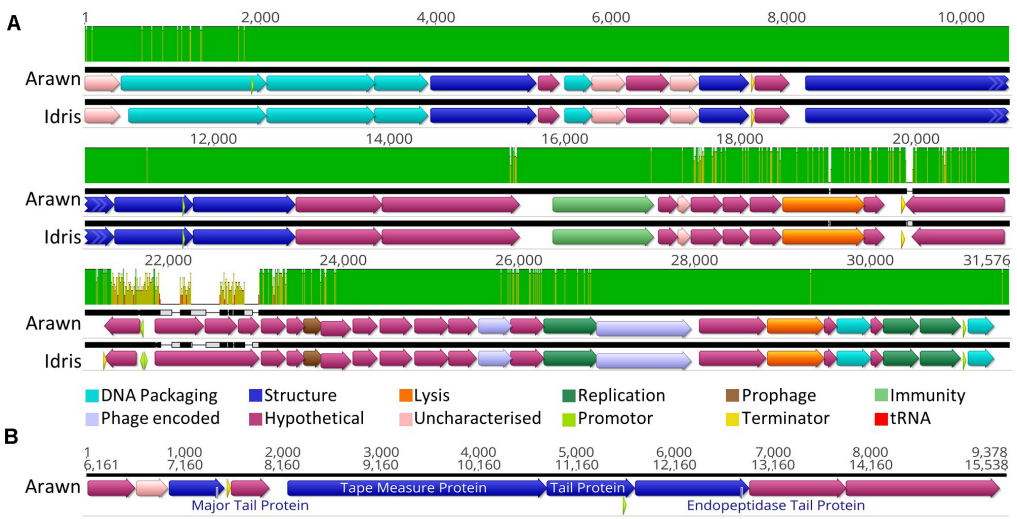

FIGURE 3 | Analysis of the Butyrivibrio phage Arawn and Idris genomes. **(A)** Mauve alignments (Darling et al., 2004) of the two genomes showing annotated ORFs, with directionality and function according to color. Nucleotide similarity is expressed as a graph, with green indicating 100%, yellow > 30%, red < 30%. **(B)** The organization of the tail morphogenesis module in phage Arawn. Uncharacterized proteins indicate where the protein was homologous to one reported previously, but had an unknown function, whereas hypothetical proteins did not show similarity above the set thresholds to any known proteins. Phage encoded proteins are those where there was uncertainty about their function.

of a tail protein (QHJ73556.1). As with the tail morphogenesis module of Arian and Bo-Finn, not all genes in the genomes of Arawn and Idris showed homology to previously identified proteins, but these hypothetical proteins may be involved in the tail formation. Additionally, of the predicted ORFs that were homologous to genes in other phages, 16 in the Arawn genome and 17 in Idris genome shared homology to phages of the *Siphoviridae* family, and two in each genome to phages of *Myoviridae*. Along with the absence of any tail sheath proteins which are common to *Myoviridae* (Adriaenssens and Cowan, 2014), and the majority of genes being similar to other *Siphoviridae* phages, it is likely that these two phages also belong to the *Siphoviridae* family.

Both phage Arawn and Idris were predicted confidently by PHACTS to undergo the temperate lifecycle. The major capsid proteins are similar to those in the temperate phages Streptococcus phage phiD12 and Streptococcus phage phi-SsUD.1 (Tang et al., 2013). A putative excisionase (xis)

gene was predicted in both Arawn (QHJ73575.1) and Idris (QHJ73845.1), after identification of a DNA binding domain in the excisionase family. The presence of a protein homologous to a reverse transcriptase in phage Arawn (QHJ73560.1) and Idris (QHJ73832.1) suggests a possible mechanism of prophage immunity; previously shown to have an abortive effect on lytic phage infection and prolong lysogeny (Odegrip et al., 2006). Although these phages were observed *in vitro* undergoing the lytic cycle, the presence of these genes suggests the potential for Butyrivibrio phages Arawn and Idris to undergo the temperate lifecycle. The fully annotated genomes of phage Arawn and Idris can be seen in **Supplementary Figures S1A,E**, respectively.

As with phages Arian and Bo-Finn, a blastn search for the entire genome sequences of phage Arawn and Idris against the NCBI virus genome database did not reveal any significant alignments covering more than 8% of the query genome.

Phage Ceridwen Genome

The longest of the Butyrivibrio phage genomes belongs to phage Ceridwen, which also has the densest genome, with more predicted ORFs in the genome proportional to the length (**Table 2**). Of these ORFs, ∼40% shared similarity with a viral sequence or protein in one of the databases searched. This genome shows similar organization to the other five phage genomes, with packaging, structural proteins and DNA modification related genes mostly found together (**Supplementary Figure S1D**). As with the other genomes, there is evidence of a tail morphogenesis module, formed of a putative tape measure protein (QHJ73746.1), tail protein (QHJ73747.1), and endopeptidase tail protein (QHJ73748.1). Of the predicted ORFs that were homologous to genes in other phages, 20 shared homology to phages of the *Siphoviridae* family, and one of *Podoviridae*. Therefore, it is likely that this phage belongs to the *Siphoviridae* family.

Phage Ceridwen was predicted non-confidently as having a lytic lifecycle by PHACTS, but the presence of an ORF similar to an antirepressor (QHJ73772.1) suggests lysogenic abilities. This antirepressor may act on the gene upstream of it; a gene homologous to the XRE family of translational inhibitors (QHJ73771.1). The most characterized phage repressors belong to this family (Durante-Rodríguez et al., 2016), which act as the main repressor, mediated by the antirepressor to trigger downstream lysis genes (Argov et al., 2019), which in this case could be the neighboring putative lysin (QHJ73773.1). Despite this, there is no evidence of excisionase genes, and this phage was only observed to undergo the lytic lifecycle *in vitro*.

A blastn search for the entire genome sequences of phage Ceridwen against the NCBI virus genome database did not reveal any significant alignments covering more than 1% of the query genome.

Similarities and Differences in the Butyrivibrio Phages

The five phages in this study were observed *in vitro* to undergo the lytic lifecycle, infecting the same *B. fibrisolvens* host strain. The phages likely belong to the *Siphoviridae* family, as evidenced through genes encoded, genome organization, size, and similarity to other phages belonging to this family. All five phages possess genomes with sizes > 20 Kbp and <125 Kbp, and tail morphogenesis modules typical of siphoviruses (Hatfull, 2008; Veesler and Cambillau, 2011).

Despite all targeting the same bacterial host, the GC content of these phages varied, with phages Arawn and Idris having the highest GC content (46.9%), which does agree with the observation made previously that shorter phage genomes tend to have higher GC contents (Almpanis et al., 2018). There is, however, no linear relationship between GC content and genome length with these five phages (**Table 2**). Interestingly, phages Arawn and Idris were also predicted to be able to undergo the lysogenic cycle, yet the GC content of their genomes is higher than that of the bacterial host, which is 39.7%. These findings do not follow trends observed previously that phages either maintain a GC content similar to the host, or lower (AT rich), but may instead suggest that the phage genome integrates into an area of the host genome that is more GC rich than average (Almpanis et al., 2018).

With genome lengths of around 30–40 Kbp, this is similar to the 33.5–34.6 Kbp of other lytic rumen phage genomes belonging to *Siphoviridae* that were sequenced previously (Gilbert et al., 2017), and in the range of what has been observed from members of the *Siphoviridae* family generally (Hatfull, 2008). The number of ORFs in these Butyrivibrio phage genomes that share similarity with currently known nucleotide or amino acid sequences in the databases ranges from 40 to 52% of the total that were predicted, which is more than the ∼25–27% predicted for *Bacteroides* and *Ruminococcus* phages annotated previously, but not as many as the 54% of ORFs annotated for the *Streptococcus* phage (Gilbert et al., 2017). This lack of confidence in assigning functional annotation to all predicted ORFs stems from reliance on sequence similarity matching and poor protein characterization in phage genomes more generally. This lack of similarity to known sequences therefore is not particularly surprising, but it may suggest that the phages isolated in this study are considerably dissimilar to those isolated in other environments (Hatfull and Hendrix, 2011; Berg Miller et al., 2012). Additionally, phage genomes from the rumen are currently poorly represented in the public databases (Namonyo et al., 2018; Lawrence et al., 2019), limiting the conclusions that can be formed about the rumen phage population.

Butyrivibrio phage Bo-Finn, which was isolated from cow rumen fluid, was identified to be the same species as phage Arian, which was isolated from cow feces. This suggests that phage populations are present throughout the digestive tract and may be evidence for passage of phages that reside in the rumen through the digestive system and finally into the excretions of the ruminant animal. Whilst it is generally accepted that the fecal microbiome cannot be used to represent the rumen microbiome, but instead is more representative of the hindgut microbiota (Noel et al., 2019), in the instance of phages, sampling of feces for phages may reflect phage populations in the rumen, but this is something that requires further confirmation.

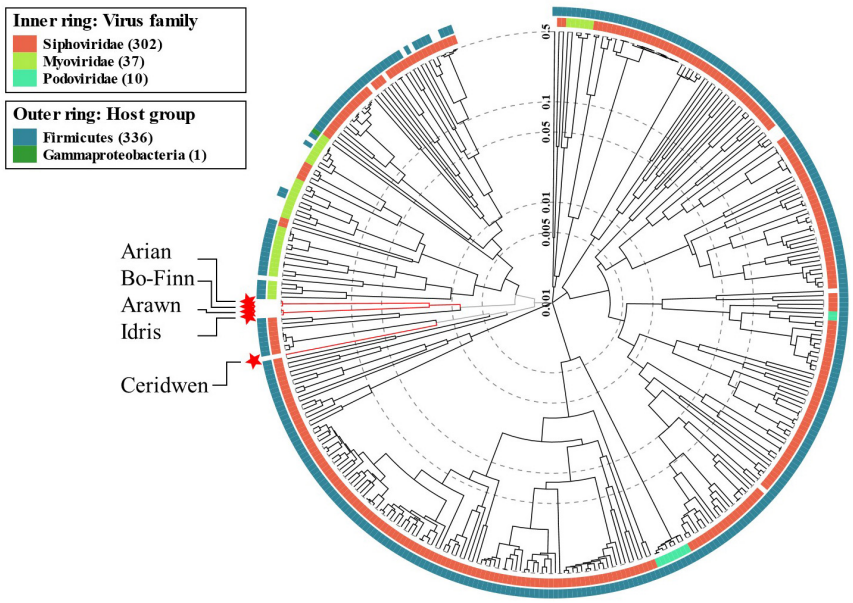

FIGURE 4 | Phage phylogenetic tree. The results of the ViPTree analysis (Nishimura et al., 2017) using a protein distance metric based on normalized tBLASTx scores plotted on a log scale. The tree includes 358 related taxa, with the five Butyrivibrio phages in this study highlighted with red stars and labeled with arrows. Taxa are also annotated with the virus family they belong to (if known) on the inner ring; red for *Siphoviridae,* lime green for *Myoviridae* and turquoise for *Podoviridae*. The phyla the bacterial host belongs to for each taxa are given in the outer ring; *Firmicutes* in blue and *Gammaproteobacteria* in green. A more detailed tree with labeled taxa can be seen in **Supplementary Figure S2**.

Phylogeny Analysis Reveals Wider Context of Butyrivibrio Phages

There are no significant alignments to any other phage genome in the NCBI viral reference database that exceeded 8% coverage of the query genome, something that is not unusual (Hatfull and Hendrix, 2011), suggesting that these five genomes are novel. However, some predicted genes in the Butyrivibrio phage genomes did show some level of similarity to other phage genes in the databases. Phylogenetic analysis of phage proteomes using ViPTree (Nishimura et al., 2017) revealed that four of the five phages are more closely related to each other than to any other phages (**Figure 4**). The next closest relatives of these four phages are Lactobacillus and Paenibacillus phages. Phage Ceridwen, on the other hand, is in a clade of Clostridium phages (**Supplementary Figure S2**). The long branches between the Butyrivibrio phages and its nearest relatives, however, suggests low protein sequence similarity. The closest relatives to all of the Butyrivibrio phages also belong to the *Siphoviridae* family, offering further evidence that the Butyrivibrio phages belong to this family as well (**Figure 4**). (The full phylogenetic tree with visible labels created by ViPTree can be seen in **Supplementary Figure S2**).

The genomes of the 13 closest relatives of the five Butyrivibrio phages were analyzed using VICTOR to determine whether the Butyrivibrio phages, based on genome BLAST distance, belonged to an existing species, genus, or VICTOR family. It was revealed that Butyrivibrio phage Arian and Bo-Finn belonged to the same species, whilst the other Butyrivibrio phages belonged to a unique species, which was not shared with any other phage genomes. Butyrivibrio phages Arawn and Idris belonged to the same genus, which was distinct from Arian and Bo-Finn, and from Ceridwen. The Butyrivibrio phages did, however, belong to the same VICTOR family, and none of the Butyrivibrio phages belonged to the same genus or family as any other phage genomes. Phylogenomic analysis, therefore, suggests four species and three genera that are novel and unique.

No Evidence of Phage Interactions Found in Host Genome

The reference genome for *Butyrivibrio fibrisolvens* DSM 3071 did not show an intact prophage but had two regions of incomplete prophages: one with seven predicted phage proteins of which six were highly similar to other phage proteins, and another with 10, of which six were also highly similar to other phage prophages. None of these predicted prophage proteins shared any similarity to proteins in the Butyrivibrio phage genomes from this study. Furthermore, none of the phage proteins from this study shared similarity above the thresholds to the bacterial host genome. The absence of a prophage similar to the Butyrivibrio phage genomes in this study suggests that these phages are more likely to undergo the lytic lifecycle.

Additionally, the host genome had one cas9 ortholog, and 14 CRISPR arrays, suggesting previous interaction with bacteriophages. However, none of these spacers were similar to any of the Butyrivibrio phages in this study, nor were any of the CRISPR spacers predicted in other microbial genomes in the Hungate1000 collection (Seshadri et al., 2018).

CONCLUSION

This study presents five novel phage genomes isolated from rumen-associated samples, and the first phages isolated and sequenced that target *Butyrivibrio fibrisolvens*. The phages were isolated from ruminant fecal and rumen fluid samples and their genomes sequenced, analyzed, and made publicly available, a contribution that doubles the number of cultured and sequenced phages from rumen-associated samples that are publicly available. These five phages represent four novel species from three separate genera and were shown based on genomic characteristics and phylogenetic analysis to likely belong to the *Siphoviridae* family. The closest relatives based on proteome analysis to Butyrivibrio phage Ceridwen were Clostridium phages, whereas Butyrivibrio phages Arian, Bo-Finn, Arawn and Idris were more closely related to each other than to any other phage genomes.

All of these Butyrivibrio phages were observed undergoing the lytic lifecycle, and further evidence in the genomes of Arian, Bo-Finn and Ceridwen suggest that these three are virulent phages. Phages Arawn and Idris, however, harbor a number of lysogeny-associated genes, suggesting the possibility of these phages to be temperate. Yet, the lack of evidence from sequence similarity analyses of integration of these phages into the bacterial genomes of *Butyrivibrio* species does cast doubt on their lysogenic capabilities.

The addition of these five rumen-specific phage genomes to the reference databases is a small, but important contribution and will help to annotate known cultured isolates in genomic and metagenomic datasets not just from the rumen (Gilbert and Ouwerkerk, 2020), but also from other environments. There is no reason to believe that *B. fibrisolvens* would be a more or less efficient or likely phage host than any other bacterium, and the identification of five phages belonging to three different genera active against this single host suggests that there is a lot more to be discovered. However, it is likely that an effort on the international scale is required, similar to the strategies of the Hungate collection (Seshadri et al., 2018), to achieve a more representative sample of this diversity.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the BioProject Accession PRJNA613207; Bo-Finn (MN882552), Arawn (MN882550), Idris (MN882554), Ceridwen (MN882553), and Arian (MN882551).

ETHICS STATEMENT

The animal study was reviewed and approved by Aberystwyth University Animal Welfare and Ethics Review Board.

AUTHOR CONTRIBUTIONS

JF, AK-S, and CC conceived the project. JF carried out experimental work and analysis with support from JP, AK-S, CC, DR, and AC. All authors contributed to manuscript writing and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01588/full#supplementary-material

REFERENCES

Adriaenssens, E. M., and Brister, J. R. (2017). How to name and classify your phage: an informal guide. *Viruses* 9, 1–9. doi: 10.3390/v9040070

Adriaenssens, E. M., and Cowan, D. A. (2014). Using signature genes as tools to assess environmental viral ecology and diversity. *Appl. Environ. Microbiol.* 80, 4470–4480. doi: 10.1128/AEM.00878-14

Almpanis, A., Swain, M., Gatherer, D., and McEwan, N. (2018). Correlation between bacterial G+C content, genome size and the G+C content of associated plasmids and bacteriophages. *Microb. Genomics* 4:e000168. doi: 10.1099/mgen.0.000168

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Anderson, C. L., Sullivan, M. B., and Fernando, S. C. (2017). Dietary energy drives the dynamic response of bovine rumen viral communities. *Microbiome* 5:155. doi: 10.1186/s40168-017-0374-3

Andrews, S. (2010). *FastQC, Simon Andrews, version 0.11.8*, Available online at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed August 13, 2019).

Antibody Design Laboratories (2015). *Small-Scale Preparation of Filamentous Bacteriophage by PEG Precipitation*. Available online at:

http://www.abdesignlabs.com/technical-resources/bacteriophage-preparation/ (accessed September 13, 2019)

Argov, T., Sapir, S. R., Pasechnek, A., Azulay, G., Stadnyuk, O., Rabinovich, L., et al. (2019). Coordination of cohabiting phage elements supports bacteria–phage cooperation. *Nat. Commun.* 10:5288. doi: 10.1038/s41467-019-13296-x

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387

Aziz, R. K., Ackermann, H. W., Petty, N. K., and Kropinski, A. M. (2018). "Essential steps in characterizing bacteriophages: biology, taxonomy, and genome analysis," in *Methods in Molecular Biology*, ed. J. M. Walker (Totowa, NJ: Humana Press Inc.), 197–215. doi: 10.1007/978-1-4939-7343-9_15

Bailly-Bechet, M., Vergassola, M., and Rocha, E. (2007). Causes for the intriguing presence of tRNAs in phages. *Genome Res.* 17, 1486–1495. doi: 10.1101/gr.6649807

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C., et al. (2000). Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl. Environ. Microbiol.* 66, 1654–1661. doi: 10.1128/AEM.66.4.1654-1661.2000

Berg Miller, M. E., Yeoman, C. J., Chia, N., Tringe, S. G., Angly, F. E., Edwards, R. A., et al. (2012). Phage-bacteria relationships and CRISPR elements revealed by a metagenomic survey of the rumen microbiome. *Environ. Microbiol.* 14, 207–227. doi: 10.1111/j.1462-2920.2011.02593.x

Bourdin, G., Schmitt, B., Guy, L. M., Germond, J. E., Zuber, S., Michot, L., et al. (2014). Amplification and purification of T4-Like *Escherichia coli* phages for phage therapy: from laboratory to pilot scale. *Appl. Environ. Microbiol.* 80, 1469–1476. doi: 10.1128/AEM.03357-13

Brandes, N., and Linial, M. (2016). Gene overlapping and size constraints in the viral world. *Biol. Direct* 11, 1–15. doi: 10.1186/s13062-016-0128-3

Brown, D. W., and Moore, W. E. C. (1960). Distribution of *Butyrivibrio fibrisolvens* in Nature. *J. Dairy Sci.* 43, 1570–1574. doi: 10.3168/jds.S0022-0302(60)90377-5

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 10:421. doi: 10.1186/1471-2105-10-421

Chan, P. P., and Lowe, T. M. (2019). tRNAscan-SE: searching for tRNA genes in genomic sequences. *Methods Mol. Biol.* 1962, 1–14. doi: 10.1007/978-1-4939-9173-0_1

Cotta, M. A., and Hespell, R. B. (1986). Proteolytic activity of the ruminal bacterium *Butyrivibrio fibrisolvens*. *Appl. Environ. Microbiol.* 52, 51–58. doi: 10.1073/pnas.85.12.4426

Couvin, D., Bernheim, A., Toffano-Nioche, C., Touchon, M., Michalik, J., Néron, B., et al. (2018). CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Res.* 46, W246–W251. doi: 10.1093/nar/gky425

Darling, A. C. E., Mau, B., Blattner, F. R., and Perna, N. T. (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 14, 1394–1403. doi: 10.1101/gr.2289704

Delcher, A. L., Bratke, K. A., Powers, E. C., and Salzberg, S. L. (2007). Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* 23, 673–679. doi: 10.1093/bioinformatics/btm009

Durante-Rodríguez, G., Mancheño, J. M., Díaz, E., and Carmona, M. (2016). Refactoring the λ phage lytic/lysogenic decision with a synthetic regulator. *Microbiologyopen* 5, 575–581. doi: 10.1002/mbo3.352

Eddy, S. R. (2011). Accelerated profile HMM searches. *PLoS Comput. Biol.* 7:e2195. doi: 10.1371/journal.pcbi.1002195

Gilbert, R., and Ouwerkerk, D. (2020). The genetics of rumen phage populations. *Proceedings* 36:165. doi: 10.3390/proceedings2019036165

Gilbert, R. A., Kelly, W. J., Altermann, E., Leahy, S. C., Minchin, C., Ouwerkerk, D., et al. (2017). Toward understanding phage: host interactions in the rumen; complete genome sequences of lytic phages infecting rumen bacteria. *Front. Microbiol.* 8:2340. doi: 10.3389/fmicb.2017.02340

Gilbert, R. A., Townsend, E. M., Crew, K. S., Hitch, T. C. A., Friedersdorff, J. C. A., Creevey, C. J., et al. (2020). Rumen virus populations: technological advances enhancing current understanding. *Front. Microbiol.* 11:450. doi: 10.3389/fmicb.2020.00450

Gutiérrez, D., Fernández, L., Rodríguez, A., García, P., Gutiérrez, D., Fernández, L., et al. (2018). Practical method for isolation of phage deletion mutants. *Methods Protoc.* 1:6. doi: 10.3390/mps1010006

Haft, D. H., Selengut, J. D., and White, O. (2003). The TIGRFAMs database of protein families. *Nucleic Acids Res.* 31, 371–373. doi: 10.1093/nar/gkg128

Hatfull, G. F. (2008). Bacteriophage genomics. *Curr. Opin. Microbiol.* 11, 447–453. doi: 10.1016/j.mib.2008.09.004

Hatfull, G. F., and Hendrix, R. W. (2011). Bacteriophages and their genomes. *Curr. Opin. Virol.* 1, 298–303. doi: 10.1016/j.coviro.2011.06.009

Hobson, P. N. (1969). "Rumen bacteria," in *Methods in Microbiology*, eds J. R. Norris and D. W. Ribbons (London: Academic Press), 131–149.

Hoogenraad, N. J., Hirk, F. J., Holmes, I., and Millis, N. F. (1967). Bacteriophages in rumen contents of sheep. *J. Gen. Virol.* 1, 575–576. doi: 10.1099/0022-1317-1-4-575

Hyatt, D., Chen, G.-L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinform.* 11:119. doi: 10.1186/1471-2105-11-119

Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T., and Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* 9:5114. doi: 10.1038/s41467-018-07641-9

Joshi, N. A., and Fass, J. N. (2011). *Sickle: A Sliding-Window, Adaptive, Quality-Based Trimming Tool for FastQ Files, version 1.33* Available online at: https://github.com/najoshi/sickle (accessed August 14, 2019).

Kepler, C. R., Hirons, K. P., McNeill, J. J., and Tove, S. B. (1966). Intermediates and products of the biohydrogenation of linoleic acid by *Butyrinvibrio fibrisolvens*. *J. Biol. Chem.* 241, 1350–1354.

Klieve, A. V. (2005). "Bacteriophages," in *Methods in Gut Microbial Ecology for Ruminants*, eds H. P. S. Makkar and C. S. McSweeney (Dordrecht: Springer), 39–46.

Klieve, A. V., Bain, P. A., Yokoyama, M. T., Ouwerkerk, D., Forster, R. J., and Turner, A. F. (2004). Bacteriophages that infect the cellulolytic ruminal bacterium *Ruminococcus albus* AR67. *Lett. Appl. Microbiol.* 38, 333–338. doi: 10.1111/j.1472-765X.2004.01493.x

Klieve, A. V., and Bauchop, T. (1988). Morphological diversity of ruminal bacteriophages from sheep and cattle. *Appl. Environ. Microbiol.* 54, 1637–1641. doi: 10.1128/aem.54.6.1637-1641.1988

Klieve, A. V., and Gilbert, R. A. (2005). "Bacteriophage populations," in *Methods in Gut Microbial Ecology for Ruminants*, eds H. P. S. Makkar and C. S. Mcsweeney (Dordrecht: Springer), 129–137. doi: 10.1007/1-4020-3791-0_10

Klieve, A. V., Hudman, J. F., and Bauchop, T. (1989). Inducible bacteriophages from ruminal bacteria. *Appl. Environ. Microbiol.* 55, 1630–1634. doi: 10.1128/aem.55.6.1630-1634.1989

Krogh, A., Larsson, B., Von Heijne, G., and Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580. doi: 10.1006/jmbi.2000.4315

Lawrence, D., Baldridge, M. T., and Handley, S. A. (2019). Phages and human health: more than idle hitchhikers. *Viruses* 11:587. doi: 10.3390/v11070587

Li, H. (2013). *Aligning Sequence Reads, Clone Sequences and Assembly Contigs with BWA-MEM*. Available online at: http://arxiv.org/abs/1303.3997 (accessed September 12, 2019)

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Lima, T., Auchincloss, A. H., Coudert, E., Keller, G., Michoud, K., Rivoire, C., et al. (2009). HAMAP: a database of completely sequenced microbial proteome sets and manually curated microbial protein families in UniProtKB/Swiss-Prot. *Nucleic Acids Res.* 37, D471–D478. doi: 10.1093/nar/gkn661

Lomsadze, A., Gemayel, K., Tang, S., and Borodovsky, M. (2018). Modeling leaderless transcription and atypical genes results in more accurate gene prediction in prokaryotes. *Genome Res.* 28, 1079–1089. doi: 10.1101/gr.230615.117

Mavrich, T. N., and Hatfull, G. F. (2017). Bacteriophage evolution differs by host, lifestyle and genome. *Nat. Microbiol.* 2:17112. doi: 10.1038/nmicrobiol.2017.112

McNair, K., Bailey, B. A., and Edwards, R. A. (2012). PHACTS, a computational approach to classifying the lifestyle of phages. *Bioinformatics* 28, 614–618. doi: 10.1093/bioinformatics/bts014

McNair, K., Zhou, C., Dinsdale, E. A., Souza, B., and Edwards, R. A. (2019). PHANOTATE: a novel approach to gene identification in phage genomes. *Bioinformatics* 35, 4537–4542. doi: 10.1093/bioinformatics/btz265

Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P., and Göker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinform.* 14:60. doi: 10.1186/1471-2105-14-60

Meier-Kolthoff, J. P., and Göker, M. (2017). VICTOR: genome-based phylogeny and classification of prokaryotic viruses. *Bioinformatics* doi: 10.1093/bioinformatics/btx440

Nale, J. Y., Spencer, J., Hargreaves, K. R., Trzepiński, P., Douce, G. R., and Clokie, M. R. J. (2016). Bacteriophage combinations significantly reduce *Clostridium difficile* growth in vitro and proliferation in vivo. *Antimicrob. Agents Chemother.* 60, 968–981. doi: 10.1128/AAC.01774-15

Namonyo, S., Wagacha, M., Maina, S., Wambua, L., and Agaba, M. (2018). A metagenomic study of the rumen virome in domestic caprids. *Arch. Virol.* 163, 3415–3419. doi: 10.1007/s00705-018-4022-4

Neph, S., Kuehn, M. S., Reynolds, A. P., Haugen, E., Thurman, R. E., Johnson, A. K., et al. (2012). BEDOPS: high-performance genomic feature operations. *Bioinformatics* 28, 1919–1920. doi: 10.1093/bioinformatics/bts277

Nishimura, Y., Yoshida, T., Kuronishi, M., Uehara, H., Ogata, H., and Goto, S. (2017). ViPTree: the viral proteomic tree server. *Bioinformatics* 33, 2379–2380. doi: 10.1093/bioinformatics/btx157

Noel, S. J., Olijhoek, D. W., Mclean, F., Løvendahl, P., Lund, P., and Højberg, O. (2019). Rumen and fecal microbial community structure of holstein and jersey dairy cows as affected by breed, diet, and residual feed intake. *Animals* 9:498. doi: 10.3390/ani9080498

Odegrip, R., Nilsson, A. S., and Haggård-Ljungquist, E. (2006). Identification of a gene encoding a functional reverse transcriptase within a highly variable locus in the P2-like coliphages. *J. Bacteriol.* 188, 1643–1647. doi: 10.1128/JB.188.4.1643-1647.2006

Orpin, C. G., and Munn, E. A. (1973). The occurrence of bacteriophages in the rumen and their influence on rumen bacterial populations. *Experentia* 30, 1018–1021.

Parmar, N. R., Jakhesara, S. J., Mohapatra, A., and Joshi, C. G. (2016). Rumen virome: an assessment of viral communities and their functions in the rumen of an Indian buffalo. *Curr. Sci.* 111, 919–925. doi: 10.18520/cs/v111/i5/919-925

Paynter, M. J., Ewert, D. L., and Chalupa, W. (1969). Some morphological types of bacteriophages in bovine rumen contents. *Appl. Microbiol.* 18, 942–943. doi: 10.1128/aem.18.5.942-943.1969

Quinlan, A. R., and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842. doi: 10.1093/bioinformatics/btq033

Ramsay, A. G., Scott, K. P., Martin, J. C., Rincon, M. T., and Flint, H. J. (2006). Cell-associated α-amylases of butyrate-producing Firmicute bacteria from the human colon. *Microbiology* 152, 3281–3290. doi: 10.1099/mic.0.29233-0

Rice, P., Longden, L., and Bleasby, A. (2000). EMBOSS: the European molecular biology open software suite. *Trends Genet.* 16, 276–277. doi: 10.1016/S0168-9525(00)02024-2

Rihtman, B., Meaden, S., Clokie, M. R. J., Koskella, B., and Millard, A. D. (2016). Assessing Illumina technology for the high-throughput sequencing of bacteriophage genomes. *PeerJ* 4:e2055. doi: 10.7717/peerj.2055

Rumney, C. J., Duncan, S. H., Henderson, C., and Stewart, C. S. (1995). Isolation and characteristics of a wheatbran—degrading *Butyrivibrio* from human faeces. *Lett. Appl. Microbiol.* 20, 232–236. doi: 10.1111/j.1472-765X.1995.tb00435.x

Russell, D. A. (2018). "Sequencing, assembling and finishing complete bacteriophage genomes," in *Bacteriophages. Methods in Molecular Biology*, Vol. 3, eds M. R. J. Clokie, A. M. Kropinski, and R. Lavigne (New York: Humana Press), 109–125. doi: 10.1007/978-1-4939-7343-9_9

Sales, M., Lucas, F., and Blanchart, G. (2000). Effects of ammonia and amino acids on the growth and proteolytic activity of three species of rumen bacteria: *Prevotella albensis, Butyrivibrio fibrisolvens,* and *Streptococcus bovis*. *Curr. Microbiol.* 40, 380–386. doi: 10.1007/s002840010074

Sampaio, M., Rocha, M., Oliveira, H., and Dias, O. (2019). Predicting promoters in phage genomes using PhagePromoter. *Bioinformatics* 35, 5301–5302. doi: 10.1093/bioinformatics/btz580

Sazinas, P., Redgwell, T., Rihtman, B., Grigonyte, A., Michniewski, S., Scanlan, D. J., et al. (2018). Comparative genomics of bacteriophage of the genus seuratvirus. *Genome Biol. Evol.* 10, 72–76. doi: 10.1093/gbe/evx275

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., et al. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotechnol.* 36, 359–367. doi: 10.1038/nbt.4110

Solovyev, V., and Salamov, A. (2011). "Automatic annotation of microbial genomes and metagenomic sequences," in *Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies*, ed. R. W. Li (Washington, DC: Agricultural Research Service).

Stark, W. M. (2017). Making serine integrases work for us. *Curr. Opin. Microbiol.* 38, 130–136. doi: 10.1016/j.mib.2017.04.006

Tang, F., Bossers, A., Harders, F., Lu, C., and Smith, H. (2013). Comparative genomic analysis of twelve *Streptococcus suis* (pro)phages. *Genomics* 101, 336–344. doi: 10.1016/j.ygeno.2013.04.005

Veesler, D., and Cambillau, C. (2011). A common evolutionary origin for tailed-bacteriophage functional modules and bacterial machineries. *Microbiol. Mol. Biol. Rev.* 75, 423–433. doi: 10.1128/mmbr.00014-11

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., et al. (2014). Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 9:e112963. doi: 10.1371/journal.pone.0112963

Wick, R. R., Schultz, M. B., Zobel, J., and Holt, K. E. (2015). Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* 31, 3350–3352. doi: 10.1093/bioinformatics/btv383

Zhang, Z., Schwartz, S., Wagner, L., and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* 7, 203–214. doi: 10.1089/10665270050081478

Zhou, C. L. E., Malfatti, S., Kimbrel, J., Philipson, C., McNair, K., Hamilton, T., et al. (2019). multiPhATE: bioinformatics pipeline for functional annotation of phage isolates. *Bioinformatics* 35, 4402,4404. doi: 10.1093/bioinformatics/btz258

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., and Wishart, D. S. (2011). PHAST: a fast phage search tool. *Nucleic Acids Res.* 39, 347–352. doi: 10.1093/nar/gkr485

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Anaerobic Fungi: Past, Present, and Future

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Anaerobic fungi (AF) play an essential role in feed conversion due to their potent fiber degrading enzymes and invasive growth. Much has been learned about this unusual fungal phylum since the paradigm shifting work of Colin Orpin in the 1970s, when he characterized the first AF. Molecular approaches targeting specific phylogenetic marker genes have facilitated taxonomic classification of AF, which had been previously been complicated by the complex life cycles and associated morphologies. Although we now have a much better understanding of their diversity, it is believed that there are still numerous genera of AF that remain to be described in gut ecosystems. Recent marker-gene based studies have shown that fungal diversity in the herbivore gut is much like the bacterial population, driven by host phylogeny, host genetics and diet. Since AF are major contributors to the degradation of plant material ingested by the host animal, it is understandable that there has been great interest in exploring the enzymatic repertoire of these microorganisms in order to establish a better understanding of how AF, and their enzymes, can be used to improve host health and performance, while simultaneously reducing the ecological footprint of the livestock industry. A detailed understanding of AF and their interaction with other gut microbes as well as the host animal is essential, especially when production of affordable high-quality protein and other animal-based products needs to meet the demands of an increasing human population. Such a mechanistic understanding, leading to more sustainable livestock practices, will be possible with recently developed -omics technologies that have already provided first insights into the different contributions of the fungal and bacterial population in the rumen during plant cell wall hydrolysis.

Keywords: anaerobic digestion, carbohydrate-active enzymes, food security, herbivores, methanogenesis, Neocallimastigomycota, rumen, sustainable agriculture

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INTRODUCTION

The ability of herbivorous animals to ferment recalcitrant plant fiber into utilizable forms of energy, most cases in the form of volatile fatty acids (VFAs), has been attributed to the trillions of microbial cells that inhabit their gastrointestinal tract (Dearing and Kohl, 2017). Importantly, energy stored within complex plant carbohydrates is made accessible to the host animal

only through the synergistic activity of its gut microbes (Morais and Mizrahi, 2019).

Independent of which type of mammalian herbivore digestive physiology is considered, all mammalian herbivores have evolved specialized gut compartments to give home to a complex microbial ecosystem of bacteria, anaerobic fungi (AF), protozoa, methanogenic archaea and bacteriophages (Morgavi et al., 2013). All three of the major types of herbivorous mammals depend on these microbiomes and their proper function to support their health and performance: ruminants (e.g., cattle, goats, and sheep), pseudoruminants (e.g., camelids and hippopotami) and hindgut herbivores (e.g., elephants, donkeys, horses and zebras). Furthermore, in foregut fermenting ruminants and pseudoruminants the forestomach microbes themselves also serve as a substantial source of protein and vitamins for the host unlike in hindgut herbivores (Mizrahi, 2013; Nagaraja, 2016). An excellent review of herbivore gastrointestinal physiology, including detailed drawings, and its role on microbial fermentation of plant biomass was summarized by Dehority (2002).

Foregut fermenters are capable of an enhanced degradation of plant biomass that is facilitated by a prolonged (60–90 h) retention time of the feed material in the rumen, the first and largest of three pre-gastric chambers of the foregut fermenter digestive tract. Gastric digestion in the ruminant digestive system occurs in the abomasum, which is the fourth chamber of the ruminant's foregut. Plant material in the cecum and colon of a hindgut fermenter, on the other hand, is retained on average only half as long (i.e., 30–40 h in equines) as it is in the ruminant's digestive tract and is consequently less completely digested (Trinci et al., 1994).

Despite the recent significant advances in our understanding of how bacteria and archaea influence the function, resilience, and the environmental footprint of herbivorous mammals such as ruminants (Kittelmann et al., 2014; Henderson et al., 2015; Liu et al., 2017; Huws et al., 2018; Wallace et al., 2019), our knowledge of AF and their influence on the host animal remains limited. This limited knowledge is perhaps not surprising considering that until the mid-20th century it was still believed that all fungi required oxygen (Trinci et al., 1994). It was only in 1975 that the ground-breaking work of Colin Orpin unequivocally confirmed the existence of AF, changing the accepted dogma of the time. Shortly after AF were first isolated and described in the rumen (Orpin, 1975, 1976, 1977a,b), they were also isolated from the horse cecum (Orpin, 1981). Since then, many more AF have been isolated from a wide range of domesticated and wild herbivores with eighteen different genera characterized to date (Hanafy et al., 2020).

To date, AF have been most extensively studied in ruminants, where they are recognized as an important microbe for good rumen function. This is primarily due to their role as highly efficient degraders of recalcitrant plant material (Li et al., 2017; Gilmore et al., 2019). Rumen AF are also syntrophic partners of the methanogenic archaea (Li et al., 2017; Gilmore et al., 2019). Additional insights into the currently understudied herbivore gut mycobiome has the potential to expand our scientific knowledge about life in the absence of oxygen, as well as open new avenues to improve the feed conversion efficiency of plant-based animal feeds. In this review, we discuss the current understanding of AF biology, ecology and their role in livestock production, along with future perspectives on how their true value can be realized.

TAXONOMY

Anaerobic fungi were first documented over 100 years ago, when their flagellated zoospores were mistakenly identified as flagellate protozoa (Liebetanz, 1910; Braune, 1913). After first being incorrectly classified as Protozoa (Liebetanz, 1910; Braune, 1913), they were reclassified, due to the significant evidence collected over many years by Orpin, as belonging to the fungal phylum Chytridiomycetes (Barr, 1980, 1988). In 2007, they were acknowledged as being a distinct phylum, the Neocallimastigomycota (Hibbett et al., 2007). Recently, Tedersoo et al. (2018) proposed a new fungal subkingdom, Chytridiomycota, grouping the Neocallimastigomycota with two additional phyla, the aerobic Chytridiomycota and Monoblepharomycota, thereby acknowledging the monophyletic origin of the zoosporic chitinous fungi (Ebersberger et al., 2012).

The Neocallimastigomycota contains only one order (*Neocallimastigales*) and one family (*Neocallimastigaceae*) comprising eighteen genera; namely the monocentric rhizoidal *Neocallimastix, Piromyces*, *Oontomyces*, *Buwchfawromyces*, *Pecoramyces*, *Liebetanzomyces*, *Feramyces*, *Agriosomyces*, *Aklioshbomyces*, *Capellomyces*, *Ghazallomyces*, *Joblinomyces*, *Khoyollomyces*, and *Tahromyces*; the polycentric rhizoidal *Anaeromyces* and *Orpinomyces*; and the bulbous *Caecomyces* and *Cyllamyces*. Key morphological features of AF taxa, such as the number of flagella on zoospores, type of thallus and rhizoids, steps of zoosporangial development, and the shape of sporangia, are listed in **Table 1**.

Although morphological features have been crucial for the classification of AF in the past, this approach is encumbered with difficulties due to the extensive morphological variations, pleomorphism in sporangial and rhizoidal structures, similarities in morphological features of monocentric/uniflagellate genera, failure to produce sporangia, and the absence of zoosporogenesis in some polycentric species. Hence, ribosomal RNA (rRNA) operon-based analysis is also needed to verify and support classification of AF. The topology of the ribosomal RNA (*rrn*) operon, indicating regions that have been used for taxonomic classification, is shown in **Figure 1**. This culture-independent approach, which in many cases is based on the nucleotide sequence of the internal transcribed spacer 1 (ITS1) region, suggests that the digestive tract of wild and domestic herbivores harbors several clades and genera-equivalent groups within the *Neocallimastigaceae* that have not yet been cultured (Koetschan et al., 2014; Paul et al., 2018).

Although the ITS1 region is currently the molecular marker of choice to assign taxonomy to AF, there are some limitations to this marker, including a high (up to 13%) variation of the ITS1 sequence of clones from a single culture (Callaghan et al., 2015). This variability makes classification of new isolates specifically challenging. Furthermore, the

TABLE 1 | Key morphological features of characterized genera of anaerobic fungi.

Genus	Morphology [zoospore (z), thallus (t), rhizomycelium (r)]	Miscellaneous features	References
Agriosomyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Endogenous and exogenous zoosporangial development, rhizoids are swollen below the sporangial tightly constricted neck, swollen sporangiophores	Hanafy et al. (2020)
Aklioshbomyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Bi or triflagellate zoospores, endogenous and exogenous zoosporangial development, papillated sporangia, pseudo-intercalary endogenous sporangia occasionally, unbranched sporangiophores	Hanafy et al. (2020)
Anaeromyces	Uniflagellate (z) Polycentric (t) Filamentous (r)	Sporangia with acuminate (mucronate) apex, can be located on erect, solitary, unbranched sporangiophore, hyphae are highly branched, often with numerous constrictions (sausage-like appearance), sometimes with root-like appearance	Breton et al. (1990)
Buwchfawromyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Extensive rhizoidal system with twisted rhizoids, sporangia with no apical projections, septum can be visible, nuclei located in sporangia, but not observed in sporangiophores or rhizoids	Callaghan et al. (2015)
Caecomyces	Uniflagellate (z) Monocentric (t) Bulbous (r)	Bi or quadriflagellate zoospores, vegetative stage is absent of developed branching rhizoidal system, consists of spherical or ovoid bodies (holdfast or haustoria), tubular sporangiophores and bulbous rhizoids, nuclei usually present both in sporangia and vegetative cells	Gold et al. (1988)
Capellomyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Endogenous and exogenous zoosporangial development, unbranched sporangiophores can exhibit subsporangial swelling, zoospores released through apical pore	Hanafy et al. (2020)
Cyllamyces	Uniflagellate (z) Polycentric (t) Bulbous (r)	Bi or triflagellate zoospores, bulbous holdfast without rhizoids with multiple sporangia, which can be born on a single elongate or branched sporangiophore, nuclei present in bulbous holdfast and sporangiophores	Ozkose et al. (2001)
Feramyces	Polyflagellate (z) Monocentric (t) Filamentous (r)	Extensive highly branched rhizoidal system with wide and narrow hyphae, wide hyphae with constrictions at irregular intervals, single terminal sporangium per thallus with the occasional formation of pseudo-intercalary sporangia, sporangiophores frequently coiled or wide and flattened, often forming an apophysis-like or eggcup-like swelling below the sporangium, both endogenous and exogenous zoosporangial development, zoospores are released through apical pore with the sporangial wall staying intact, or through detachment of the whole sporangium	Hanafy et al. (2018)
Ghazallomyces	Polyflagellate (z) Monocentric (t) Filamentous (r)	Endogenous and exogenous zoosporangial development, highly branched rhizoids, unbranched sporangiophores, pleomorphic sporangia with septum, sporangial necks constricted with narrow port, zoospores released through apical pore	Hanafy et al. (2020)
Joblinomyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Biflagellate zoospores, both endogenous and exogenous zoosporangial development, sporangiophores vary in length, zoospores released through wide apical pore resulting in empty cup-shaped sporangium	Hanafy et al. (2020)
Khoyollomyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Endogenous and exogenous zoosporangial development, highly branched rhizoids, intercalary swellings in broad hyphae, multisporangiate thallus, branched sporangiophores with two to four sporangia, zoospores released through wide apical pore	Hanafy et al. (2020)
Liebetanzomyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Endogenous and exogenous zoosporangial development, extensive anucleate rhizoidal system without constrictions, single terminal sporangium per thallus, sporangium with septum on sporangiophore of variable length, sometimes forming eggcup-like structure below the sporangium or showing cyst-like structure. Pleomorphism in sporangial and rhizoidal structures on different substrates is typical	Joshi et al. (2018)
Neocallimastix	Polyflagellate (z) Monocentric (t) Filamentous (r)	Rhizoid tubular or inflated below the neck of sporangia, sporangia located on unbranched or branched sporangiophores	Heath et al. (1983)
Oontomyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Intercalary rhizoidal swellings, sporangia never mucronated, formed terminally, long sporangiophores can be separated from the rhizomycelium by distinct constriction	Dagar et al. (2015)
Orpinomyces	Polyflagellate (z) Polycentric (t) Filamentous (r)	Polynucleate rhizomycelium of extensively branched hyphae, wider hyphae can have tightly constricted points at close intervals (bead-like or sausage-like appearance)	Barr et al. (1989)
Pecoramyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Biflagellate zoospores, both endogenous and exogenous zoosporangial development, single terminal sporangium formed per thallus, sporangiophores unbranched, often forming apophysis-like or eggcup-like swelling below sporangium. Extensive anucleate rhizoidal system lacks rhizoidal swellings or constrictions	Hanafy et al. (2017)
Piromyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Bi or quadriflagellate zoospores, both endogenous and exogenous zoosporangial development, rhizoids with or without subsporangial swelling, septum often in mature zoosporangia	Barr et al. (1989)
Tahromyces	Uniflagellate (z) Monocentric (t) Filamentous (r)	Bi or triflagellate zoospores, both endogenous and exogenous zoosporangial development, branched rhizoids, short swollen sporangiophores, sporangia with septum, sporangial necks constricted	Hanafy et al. (2020)

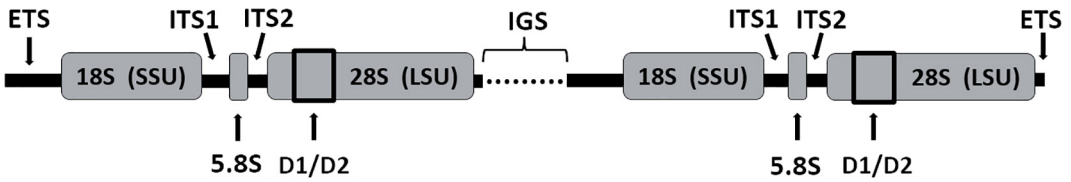

FIGURE 1 | Topology of the ribosomal RNA (*rrn*) operon. Genes of the small (SSU; 18*S*), large (LSU; 28*S*) and 5.8*S* subunit, internal transcribed spacer 1 (ITS1) and 2 (ITS2), flanked by the external transcribed spacer (ETS) regions and linked by the intergenic spacer (IGS).

ITS1 region itself ranges in size (Edwards et al., 2008) and recent work of Edwards et al. (2019) indicated preferential amplification of smaller sized ITS1 regions. PCR primer choice is also crucial, which was highlighted by the finding that the sequence amplified by a widely used primer (i.e., MN100F) is not conserved in all AF (Kittelmann et al., 2013; Callaghan et al., 2015).

These problematic aspects of using the ITS1 as phylogenetic marker gene, and the resulting instability of the ITS1 phylogeny, has led to recent efforts to explore the potential of using the sequence of the large 28S rRNA subunit (LSU) as a phylogenetic marker instead (as the 18S rRNA gene is too conserved in AF). It appears that the D1/D2 region of the LSU has a taxonomic resolution similar to the ITS1 region (Wang et al., 2017). However, the more conserved size of the D1/D2 marker and lower heterogeneity within individual cultures enables a more stable phylogenetic backbone for AF classification. Since it provides molecular support for all currently accepted genera of AF, and provides a higher resolution compared to the ITS1 region, utilization of the D1/D2 region of the LSU as a phylogenetic marker seems favorable (Dagar et al., 2011; Wang et al., 2017; Hanafy et al., 2020).

A number of AF specific primers targeting the LSU region have been published (Dollhofer et al., 2016; Nagler et al., 2018), and employed to study AF community composition of environmental samples (Dollhofer et al., 2017; Nagler et al., 2019). However, a sufficiently large reference database for these taxonomic marker genes from previously characterized taxa and their type specimens is still lacking (Wang et al., 2017). There is also a challenge of how to relate LSU sequences to the clades that until now only contain "unculturable" representatives and that were identified by environmentally derived ITS1 sequences (Edwards et al., 2019). Despite the advances of the LSU D1/D2 region as a phylogenetic marker, ITS1 still remains the most accepted phylogenetic marker for AF. As such, it is likely that ITS1 will continue to be used in the near future as the primary barcode to assess AF diversity and community structure in environmental samples. However, it is undisputable that to advance a more accurate and higher resolution classification of members belonging to the AF, a continuous expansion and curation of an AF LSU reference database is need. An alternative option could also be a more complex database that integrates the use of different reference taxonomic marker genes (Wurzbacher et al., 2019). A comprehensive ITS1 sequence database and associated taxonomy files (Koetschan et al., 2014), including the most recently described genera, are available from the AF Network website[1].

Latest DNA sequence technologies (i.e., PacBio and Nanopore sequencing) generating long reads appear to offer solutions to overcome some of these challenges, specifically the incompleteness, fragmentation and non-overlapping of extant ribosomal data. These new sequencing techniques enable the generation of full-length reference sequences (up to 10 kb in length) that span several regions suitable for taxonomic classification and intraspecies assignment, including the above mentioned ITS1 and LSU gene. Primers targeting the whole fungal ribosomal tandem repeat region, consisting of ETS, SSU, ITS1, 5.8S, ITS2, LSU, and IGS (**Figure 1**), have already been successfully applied to specimens of three fungal phyla, including early diverging fungi (Wurzbacher et al., 2019). A stable and reliable AF classification system would be enormously facilitated by the ability to widely utilize the complete ribosomal operon sequence as phylogenetic marker, assuming a corresponding well-curated reference database will be available in the future.

LIFE CYCLE

One of the additional and major challenges that contribute to the current lack of a consistent and standardized taxonomic classification system for AF is the morphological transformations AF undergo throughout their corresponding life cycles (**Table 1**). AF reproduce asexually and they alternate between a motile zoospore and a non-motile vegetative stage. Flagellate zoospores, which are released from mature sporangia, actively move toward freshly ingested plant tissues in the rumen; a chemotactic response triggered by soluble sugars (Orpin and Bountiff, 1978) and phenolic acids (Wubah and Kim, 1996). Zoospore liberation is influenced by diet, and in ruminants is induced by water-soluble haems and other porphyrins (Orpin and Greenwood, 1986; Orpin, 1994). Although zoospores are motile for several hours (Lowe et al., 1987a), they tend to attach to plant fragments within 30 min after being released from a sporangium (Heath et al., 1986; Edwards et al., 2008). After attachment, the zoospores shed their flagella and form a cyst. The encysted fungus then germinates to produce a fungal thallus that is composed of the sporangium and a filamentous rhizomycelium or a bulbous holdfast.

[1]https://www.anaerobicfungi.org

In most of the monocentric AF, thallus development is of the endogenous type, where on germination, the zoospore cyst develops a germ tube that branches and grows into a rhizoid system. The nucleus remains in the zoospore cyst, which develops into a new sporangium and the anucleate rhizoids. Since the zoospore cyst retains the nucleus and develops into a sporangium, the sporangial development type is referred to as endogenous, and since this type of development results in a single sporangium per thallus, it is said to be monocentric. In some of the monocentric AF, such as the *Piromyces* spp., thallus development is of the exogenous type, which involves a two-sided germination of the zoospore cyst. During this process, a germ tube develops into a rhizoid system during the endogenous sporangial development, but once a substantial rhizoid has developed, a tubular outgrowth (the sporangiophore or sporangial stalk) emerges on the side opposite of the main rhizoid and the sporangium develops at the apex of the outgrowth. As the original nucleus escapes the zoospore cyst and develops elsewhere, the sporangial development is said to be exogenous (Barr et al., 1989). Both types are strictly determinate. In other members of the monocentric AF, such as the *Capellomyces*, both exogenous and endogenous sporangial development takes place (Hanafy et al., 2017, 2018, 2020; Joshi et al., 2018).

Polycentric AF display an exogenous thallus development, during which a one-sided germination of the zoospore cyst occurs. During the germination process, the content of the zoospore cyst migrates into germ tube which then develops into a nucleated branched rhizomycelium capable of developing multiple sporangia. Currently it appears that the remaining cyst, which has been emptied, has no further function. Development of thalli of polycentric fungi is said to be non-determinate.

In the case of the bulbous genera, nuclei are observed in the vegetative parts of the thallus (holdfast/branched sporangiophores), consistent with exogenous development. Thallus development in the bulbous genera is of a limited polycentric type, where the encysted zoospore forms a bulbous holdfast without rhizoids. Bulbous holdfasts give rise to single or multiple sporangia including branched sporangiophores. Growth in these fungi is not non-determinate like the thalli of polycentric fungi, but not as strictly determinate as in the case of the monocentric filamentous AF (Ozkose et al., 2001).

Our current understanding of the AF life cycle is based on what has been learned from rumen AF, but it is likely that AF associated with pseudoruminants pass through similar, if not even identical, life cycles stages. However, there are numerous aspects of AF biology that remain to be reassessed for hindgut herbivores. The cause of zoospore release in the hindgut is unclear, as it is not known if the known triggering compounds in the rumen (i.e., water-soluble haems and other porphyrins) can survive passage through the gastric stomach and small intestine. Whereas zoospores that are released within the rumen locate freshly ingested plant material chemotactically using soluble sugars (Orpin and Bountiff, 1978) and phenolic acids (Wubah and Kim, 1996), it is unclear to what extent these chemotactic signals are available to (and used by) AF in the cecum and colon of hindgut herbivores.

Regardless of herbivore type, the life cycle of AF has been proposed to contain a resting phase. While resting structures are still not fully understood, they provide a compelling explanation for why some of the currently known AF can be cultured from fecal material after prolonged periods of desiccation and oxygen exposure (Milne et al., 1989; Davies et al., 1993; McGranaghan et al., 1999; Griffith et al., 2009). To date resting cysts (Orpin, 1981), melanized resistant sporangia (Wubah et al., 1991) and multi-chambered spore-like structures (Brookman et al., 2000) have been described in different AF taxa. Although it may well be the case that there is no resting structure common to all AF, with taxon-specific structures instead, resting structures are thought to play an important role in the inter-animal transfer of AF. For example, it has been suggested that the survival of AF in saliva is likely to be an important transfer mechanism in ruminants and pseudoruminants (Lowe et al., 1987b). In hindgut fermenters, feces may play a more important role as a transfer mechanism between animals than saliva, particularly as certain hindgut fermenters, like foals, exhibit coprophagic behavior (Marinier and Alexander, 1995).

As zoospores and fungal thalli represent different parts of the same AF life cycle, consistent and accurate enumeration of AF is challenging. Approaches used to count exclusively free zoospores (France et al., 1990), fungal colonies on agar strips (Ushida et al., 1989), or both morphologies in culture supernatants (Theodorou et al., 1990; Obispo and Dehority, 1992; Griffith et al., 2009), and chitin measurements (Gay, 1991; Rezaeian et al., 2004b) have, in recent years, been widely replaced by molecular quantification via real-time PCR. This method overcomes the contrast within the life cycle between low zoospore numbers yet high AF vegetative biomass, as well as the paucity or absence of zoosporogenesis observed in some polycentric axenic cultures (Ho and Bauchop, 1991). On the other hand, the real-time PCR approach to quantify fungal biomass possesses its own challenges, such as the varying amount of fungal biomass produced by monocentric, polycentric and bulbous genera relative to DNA content. This makes translating quantitative estimates derived from real-time PCR into fungal biomass very challenging (Denman and McSweeney, 2006; Edwards et al., 2008). Estimating AF abundance by quantifying the number of ITS1 spacer regions [(Mura et al., 2019) or rRNA genes, i.e., 5.8S rRNA gene (Edwards et al., 2008) or LSU (Nagler et al., 2018)] seems to be taxon independent. However, it still remains to be determined if all AF have the same copy number of the *rrn* operon.

INFLUENCE OF HOST ON ANAEROBIC FUNGI COMMUNITY

Although diet has a significant effect on the structure of the AF community, host animal phylogeny has been shown to be a more important factor (Liggenstoffer et al., 2010; Kittelmann et al., 2012). Kittelmann et al. (2012) provided strong evidence that both diet and ruminant species, as well as interactions between those two parameters, affect the diversity of the AF community by subjecting cattle, sheep and deer to three different diets (summer pasture, winter pasture, and silage). Recent studies

revealed that the genetic background of the host animal can influence the activity of the entire rumen microbiota, including the community of rumen AF (Roehe et al., 2016). In addition to the genetic background, several breed-associated phenotypes, such as eating frequency, dry matter intake, and rumen size potentially contribute to the variations in rumen microbiota that is observed among various breeds (Wallace et al., 2019; Zhang et al., 2020).

Anaerobic fungi were reported to be present in domesticated and non-domesticated equine species, with the AF community composition in horses and ponies being more similar to zebras than donkeys (Edwards et al., 2020b). In a separate study, AF diversity in donkeys was shown to be higher when compared to that of ponies and pony × donkey hybrids (Edwards et al., 2020a). Several studies have revealed that the genus *Khoyollomyces* (formerly known as AL1) is almost exclusively found in equines (Liggenstoffer et al., 2010; Mura et al., 2019; Edwards et al., 2020b; Hanafy et al., 2020). This may be due to the growth or metabolic characteristics of *Khoyollomyces,* making it more adapted to growth in the equine hindgut. Metabolic differences have previously been reported for equine and rumen strains of *Piromyces*, with equine strains possessing faster growth and higher fiber degradation capacity compared to rumen isolates (Julliand et al., 1998). This is perhaps not surprising considering the fundamental differences between ruminants (where freshly ingested feed directly enters the rumen), and hindgut herbivores (where feed first passes through the stomach and small intestine before reaching the hindgut) in terms of the main gut site where fiber degradation primarily occurs. There is also evidence indicating that the genus *Oontomyces* is specific to camelids (Dagar et al., 2015). Further research is needed to understand key factors that limit, or conversely broaden, the host distribution of certain AF taxa.

ROLE OF ANAEROBIC FUNGI IN METHANOGENESIS

Ruminants are a significant source of anthropogenic methane (CH_4) producing ∼90 Tg of CH_4 annually (Reay et al., 2018), with exact values differing based on the methodology employed to quantify emissions (Capper and Bauman, 2013; Lyu et al., 2018). Although methanogenesis occurs in the rumen, there appears to be no direct benefit of the microbial generated CH_4 (which is released into the environment) to the animal itself. The ruminant provides the anaerobic environment that is necessary for the archaea, first described as archaebacteria in the late 1970s by Woese and Fox (1977), that are capable of CH_4 production. Due to the complex nature of the rumen microbiome and the interactions between the individual community members and their biochemistry, the role of AF in methanogenesis can only be understood in light of knowledge about archaea and processes facilitated by them. There are three different biochemical routes by which archaea can produce CH_4: (1) acetoclastic methanogenesis, (2) hydrogenotrophic methanogenesis, and (3) methylotrophic methanogenesis (Borrel et al., 2013; Offre et al., 2013).

Although most gut methanogens are thought to be hydrogenotrophic (Ferry, 2010; Borrel et al., 2013; Lyu et al., 2018), the majority of the global microbial CH_4 is produced via acetoclastic methanogenesis (Ferry, 2010; Lyu et al., 2018). In the rumen ecosystem, both acetate and hydrogen are produced (Baldwin and Allison, 1983) and are available for methanogenesis. However, since their turnover rates in the rumen are high, the contribution of CH_4 produced via the acetoclastic pathway accounts only for a small fraction of the overall CH_4 produced in the rumen (Baldwin and Allison, 1983; Janssen, 2010). During hydrogenotrophic CH_4 synthesis H_2 and CO_2 are combined to yield CH_4 (Baldwin and Allison, 1983), with contributions from the newly described methylotrophic methanogens (Poulsen et al., 2013).

Under normal circumstances, H_2 seldom reaches high concentrations in the rumen and the dissolved H_2 concentration is usually about 0.1–50 μM, which is 0.014–6.8% of its maximum solubility at 39°C and 1 atm (Hegarty and Gerdes, 1999; Janssen, 2010). The scarcity and poor water solubility of H_2 limits the access of methanogens to molecular hydrogen, necessitating the development of close physical contact and intimate syntrophic partnership between H_2 producers and H_2 metabolizers. Extreme forms of these interspecies H_2-transfer are the methanogenic endo- and ecto-symbionts of rumen protozoa (Embley and Finlay, 1994). The close association seen between rumen ciliates and methanogens (Vogels et al., 1980) seems a more general feature of anaerobic ciliates, aimed at boosting their metabolic rate (Rotterová et al., 2020). Although AF are not known to have methanogenic endo- or ecto-symbionts, they do contain modified mitochondria known as hydrogenosomes (van der Giezen, 2009), and there is *in vitro* based evidence of cross-feeding (syntrophy) between hydrogenic AF and methanogenic archaea in the herbivore gut ecosystem (Yarlett et al., 1986).

Differential centrifugation of cellular fractions revealed that fungal hydrogenosomes convert malate or pyruvate under anaerobic conditions into H_2, CO_2, and acetate with the concomitant production of ATP (Yarlett et al., 1986; Marvin-Sikkema et al., 1993). This is similar to the process found in trichomonads, anaerobic urogenital parasites, where hydrogenosomes were first discovered (Müller, 1993). The H_2 produced is the result of the action of the oxygen-sensitive enzyme hydrogenase (Davidson et al., 2002), which is the terminal electron acceptor for the metabolism coming from pyruvate. The excess H_2 produced by AF can be used by methanogens to regenerate oxidized nucleotides (e.g., NAD, NADP) (Yarlett et al., 1986). Indeed, methanogenic archaea have even been found attached to the surface of fungal rhizoids and sporangia (Bauchop and Mountfort, 1981; Jin et al., 2011), which is likely to improve interspecies hydrogen transfer. Within the rumen microbiome, symbiotic ecto- and endosymbiotic partnership have been reported for rumen protozoa and methanogens (Finlay et al., 1994; Irbis and Ushida, 2004; Valle et al., 2015). Since other fungi have been shown to accommodate prokaryotic endosymbionts (Partida-Martinez et al., 2007), there is the possibility that methanogenic archaea can also exist intracellularly within AF, although such an intimate symbiotic relationship has not been reported until today.

There have been numerous co-culture studies, particularly with hydrogenotrophic *Methanobrevibacter* isolates and representatives of the genera *Piromyces*, *Neocallimastix*, *Orpinomyces*, *Caecomyces*, and *Anaeromyces* (Edwards et al., 2017). Methane, CO_2, formate and acetate are the main products when AF are grown in the presence of methanogens, whereas H_2, lactate, succinate and ethanol production is drastically decreased compared to the corresponding pure anaerobic fungal cultures (Bauchop and Mountfort, 1981; Marvin-Sikkema et al., 1990). This difference between co- and pure cultures is due to the inter-species hydrogen transfer in the methanogenic co-cultures influencing the efficiency of anaerobic fungal fermentation. This shifts AF product formation away from more oxidized end-products (i.e., lactate and ethanol) and toward the production of more reduced products (i.e., formate and acetate). Recently the life cycle stage has also been shown to shift metabolite production of AF grown in methanogenic co-culture (Li et al., 2019).

In exchange for the excess H_2 produced by AF, methanogens have a beneficial effect on AF growth and activity. This is evidenced in terms of increased cellulolytic enzyme activity and dry matter disappearance in methanogenic co-cultures compared to anaerobic fungal cultures alone (Bauchop and Mountfort, 1981; Jin et al., 2011). The ability of AF and methanogen co-cultures to rapidly convert lignocellulose containing plant material into CH_4 also has good potential for biotechnological applications (Cheng et al., 2009; Jin et al., 2011; Peng et al., 2016), particularly in terms of biogas production from lignocellulosic waste streams. Conversely, in ruminants CH_4 production is viewed as an unfavorable outcome of the fermentation. In ruminants, eructation of H_2 in the form of large amounts of CH_4 represents a loss of energy for the animal in addition to being a significant source of anthropogenic CH_4.

Several rodent hindgut fermenters and non-ruminant foregut fermenters also emit CH_4 of a magnitude as high as ruminants, but in contrast equids, macropods and rabbits produce much less (Clauss et al., 2020). There has been a move in recent years to understand why some domesticated ruminants produce lower CH_4 compared to others (Shi et al., 2014), and a subsequent push to utilize feed-based approaches to decrease ruminal methanogenesis (Jeyanathan et al., 2014). However, it remains to be determined to what extent the growth and activity of rumen AF is affected by reducing the number and/or activity of methanogenic archaea using these approaches. Being able to understand the interdependencies of these populations will be important for informing a holistic understanding of the microbiome as it pertains to ruminant function. Current knowledge regarding interactions of AF with bacteria and protozoa is more limited, and has been reviewed elsewhere (Gordon and Phillips, 1993; Edwards et al., 2017).

ROLE OF ANAEROBIC FUNGI IN PLANT CELL-WALL DEGRADATION

In vitro studies suggest that the contribution of AF to the ruminal degradation of plant material could be more significant than the contribution of cellulolytic rumen bacteria (Joblin et al., 1989; Lee et al., 2000b). This is likely due to the broad range of enzymes that are produced by the AF combined with their physical ability to break open fibrous materials through their penetrating hyphal tips. These tips have high concentrations of fibrolytic enzymes, whose enzymatic activity subsequently also increases nutrient access for other cellulolytic microbes (Ho et al., 1988; Ljungdahl, 2008; Haitjema et al., 2014; Dagar et al., 2015; Solomon et al., 2016). Whilst rumen zoospore numbers are low compared to counts of bacteria and archaea, AF have been shown to represent up to 20% of the rumen microbial biomass (Rezaeian et al., 2004a) and 10–16% of rRNA transcript abundance (Elekwachi et al., 2017). The observation that some AF species are capable of releasing up to 95% of the fermentable sugars from untreated plant leaves during a 4-day incubation period (Sijtsma and Tan, 1996) further highlights their critical role during the rumen digestion of fibrous plant biomass. These findings have led to the general belief that AF have been essential in the successful evolution of mammalian herbivores (Wang et al., 2019).

Understanding how AF and their carbohydrate-active enzymes (CAZymes) contribute to the degradation of indigested biomass in the herbivore gut is rather limited compared to the knowledge of the role of bacteria. This is partially due to the more extensive genomic resources that exist for rumen bacteria compared to AF (Hess et al., 2011; Seshadri et al., 2018; Stewart et al., 2019). AF genome information that is currently publicly available is summarized in **Table 2**, and includes the AF strain host isolation source and number of CAZymes identified.

One striking feature of AF besides their large repertoire and diversity of CAZymes (**Supplementary Table S1**), is the ability of their CAZymes to form cellulosomes. Cellulosomes, first identified in anaerobic bacteria, are extracellular multi-enzyme complexes that tether together an assortment of cellulases and related accessory enzymes (Lamed et al., 1983). The assembly of these AF multi-protein complexes, in some cases with individual building blocks from different species, is facilitated by fungal dockerins, which can directly bind to plant cell wall components without the need for a scaffoldin (Fanutti et al., 1995). This is in stark contrast to bacterial cellulosomes, which are highly species-specific (Bayer et al., 2004).

Cellulosomes have been linked to the improved fitness and biomass-degradation phenotype of both anaerobic bacteria and AF (Bayer et al., 2004; Henske et al., 2018) by enabling the synergistic activity of the individual biomass-degrading enzymes. Cellulosomes have been shown to increase cellulolytic activity over free enzymes by up to 12-fold (Krauss et al., 2012). Synthetic cellulosomes, inspired by bacterial cellulosomes, have shown promise for industrial applications due to outperforming free cellulolytic enzymes when produced in recombinant systems (Gilmore et al., 2020). Although evidence of AF cellulosomes emerged about 20 years ago (Wilson and Wood, 1992), it is only recent work that has provided strong support for the hypothesis that these multi-enzyme machineries might hold the secret to the superior biomass-degrading capability of AF. However, cellulosomes from AF still remain poorly understood (Haitjema et al., 2017).

Horizontal gene transfer (HGT) between the different populations of rumen microbes appears to have played a

TABLE 2 | Publicly available genomes of anaerobic fungi.

Organism	Strain	Host	Genome size[a] [base pairs]	Gene count[a]	CAZyme count[a]	References
Anaeromyces robustus	S4	Sheep	71,685,009	12,832	1,766	Haitjema et al. (2017)
Caecomyces churrovis	–	Sheep	165,495,782	15,009	ND[b]	Henske et al. (2017)
Neocallimastix californiae	G1	Goat	193,495,782	20,219	2,743	Haitjema et al. (2017)
Pecoramyces ruminantium (formerly *Orpinomyces* sp.)	C1A	Cow	100,954,185	18,936	2,029	Youssef et al. (2013)
Piromyces finnis	Pirfi3	Horse	56,455,805	10,992	1,463	Haitjema et al. (2017)
Piromyces sp.	E2	Elephant	71,019,055	14,648	3,819	Haitjema et al. (2017)

[a] *https://mycocosm.jgi.doe.gov (Grigoriev et al., 2013).*
[b] *Not determined.*

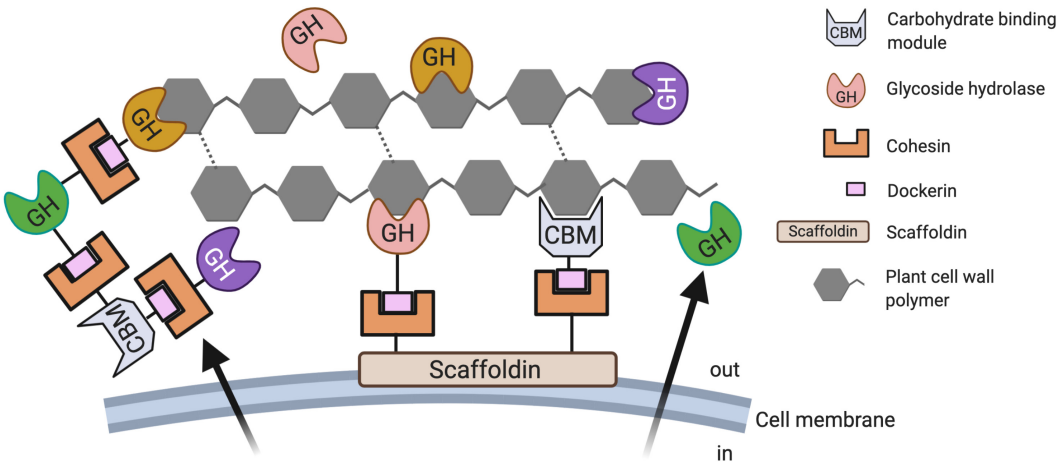

FIGURE 2 | Carbohydrate-active enzymes employed by anaerobic fungi during biomass conversion. Anaerobic fungi (AF) deploy various strategies for the degradation of plant biomass. It has been suggested that their ability to produce secreted free CAZymes, cell-bound multi-enzyme complexes (cellulosomes), as well as free cellulosomes might provide the AF with the competitive advantage over the CAZyme repertoire produced by anaerobic bacteria (Henske et al., 2017).

significant role in the evolution of the Neocallimastigomycota (Murphy et al., 2019). HGT between rumen bacteria and rumen AF was proposed by Garcia-Vallvé et al. (2000) as a major mechanism that allowed rumen AF to acquire many of the CAZymes that have now been identified in their genomes (**Supplementary Table S1**). Since then, numerous other authors have supported the hypothesis that the acquisition of CAZymes from bacterial donors enabled the rumen AF, as well as rumen protozoa (Ricard et al., 2006), to successfully compete for plant carbohydrates in the herbivore gut (Duarte and Huynen, 2019; Wang et al., 2019). Despite the numerous CAZyme families that AF acquired via HGT, the cellulosome of AF appears to have unique attributes that distinguish it from those that are found in gut bacteria.

Cellulosomes, regardless of their origin, are typically composed of several enzymes (i.e., cellulases and hemicellulases) that contain an active site, one or several carbohydrate binding modules and a dockerin that facilitates the "docking" of the enzymatic multi-modular complex to one of the multiple cohesins that are displayed by a scaffoldin. In anaerobic bacteria, cellulosomes are anchored to the bacterial cell wall via dockerin-scaffoldin modules (Fontes and Gilbert, 2010). In contrast, AF cellulosomes are not necessarily anchored to the fungal cell wall, but can be also found as free multi-enzyme complexes that are released into the extracellular matrix (Haitjema et al., 2014). These free cellulosomes enable an increased concentration of enzymes that possess catalytic and carbohydrate binding sites without the need to have them linked to and displayed on the AF cell surface. More importantly, it has been suggested that these free cellulosomes are non-species specific and theoretically enable the synthesis of cellulosomes using components of different donors (Nagy et al., 2007).

Another aspect that distinguishes AF cellulosomes from their bacterial counterparts is the presence of modules that have sequence signatures which are conserved within members of glycoside hydrolase (GH) families GH3, GH6, and GH45 (Haitjema et al., 2017). The ability to expand the substrate repertoire of AF cellulosomes by incorporating these GH signatures might provide additional enzymatic capabilities not conveyed by bacterial cellulosomes, such as the conversion of cellulose to monosaccharides by the β-glucosidase activity of GH3.

Besides cellulosomes, free CAZymes are also produced AF, which ultimately leaves the AF with several hydrolytic mechanisms to attack plant cell wall polymers from multiple directions (Haitjema et al., 2017) (**Figure 2**). The ability to

produce secreted free CAZymes, cell-bound cellulosomes, as well as free cellulosomes might provide the AF with the competitive advantage over the CAZyme repertoire produced by the cellulolytic anaerobic bacteria also resident in the herbivore gut (Henske et al., 2017). It has also been proposed that the superior efficiency in biomass degradation of AF might be caused to a great extent by the ability to simultaneously employ a diverse set of cellulosome-bound as well as free (not cellulosome associated) CAZymes that also display a significant functional overlap (Couger et al., 2015).

This hypothesis, as well as the ability of AF to contribute a complementary set of CAZymes to the ones provided by bacteria was recently supported by genome-centric metaproteome and metatranscriptome approaches (Hagen et al., 2020). Shotgun metaproteome and metatranscriptome data were mapped back on previously assembled rumen genomes from prokaryotes and cultured AF, as well as on Metagenome Assembled Genomes to determine the origin of the different CAZymes detected in the microbiome that colonized recalcitrant plant material during rumen-incubation (Hagen et al., 2020). Results from this study revealed that the bacterial population contributed CAZymes mostly associated with the degradation of more readily degradable carbohydrates such as hemicellulose, whereas fungi provided CAZymes (e.g., enzymes belonging to the GH family GH5, GH6, GH8, and GH48) that targeted the more recalcitrant plant cell wall components. These data also provided clear evidence for the involvement of AF cellulosomes in the biomass degradation process. It can be assumed that a more detailed understanding of AF and their enzymatic repertoire, in the context of a highly diverse and synergistically working microbial ecosystem, will emerge as more of these complex and large-scale meta-omics data sets are generated and analyzed.

ANAEROBIC FUNGI AS FEED ADDITIVES TO PROMOTE ANIMAL HEALTH AND PERFORMANCE

The capability of AF to colonize and degrade otherwise recalcitrant plant structures via a set of highly efficient enzymes has led to an increased interest in the application of AF, and their enzymes, to boost the digestibility of low-quality feed and to increase the overall feed efficiency of herbivorous animals. Enabling the utilization of low-quality feed will be essential to enable the production of high-quality animal proteins, especially during a time when terrestrial areas for the production of high-quality feed will become scarce due to a changing climate and the rapid urbanization of areas that are currently being used to grow feed crops.

Several *in vivo* studies indicated that continuous dosing of ruminants with live culture of AF results in the changes of numerous parameters indicative of enhanced feed digestibility and feed efficiency, with benefits of AF dosing appearing to be more pronounced in young ruminants (Sehgal et al., 2008). Measurements performed to determine the animal's ability to digest feedstuff in response to AF dosing, include dry matter (DM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility, animal growth, milk yield and VFA concentrations (all parameters increased when AF were added) (Dey et al., 2004; Paul et al., 2004, 2011; Tripathi et al., 2007; Saxena et al., 2010).

Probiotic capabilities of live AF cultures for ruminants were indicated in the 1990s when living AF cultures were added to the rumen of cattle and sheep from which fungi had been previously removed. Forage intake by such fungus-free early weaned calves was 35% higher in those that had been dosed with *Neocallimastix* sp. R1 (Theodorou et al., 1990), and dosing of fungus-free sheep with *Neocallimastix* sp. SLl resulted in a 40% increase in intake of a straw based diet (Gordon and Phillips, 1993).

Despite the work that has been conducted to investigate how dosing the rumen ecosystem with AF cultures affected the abundance of the native fungal, bacterial and even the ciliate rumen populations (Lee et al., 2000a; Paul et al., 2004, 2011), there has been no evidence that suggests that dosing with AF, and therefore the increase of AF concentration in the rumen, resulted in a decrease of bacteria or ciliate protozoa nor in a drop of feed digestibility. In contrast to this, a positive correlation between fungal and bacterial concentrations, most likely due to the fact that the hyphae of AF physically open the plant tissue thereby increasing surface area available for colonization and nutrient access for other fibrolytic rumen microbes, has been reported (Bauchop, 1979; Akin and Borneman, 1990). This increased accessibility would also explain why counts of the holotrichs, the starch degrading protozoal population, increased in response to dosing with AF, while the overall count of the ciliate rumen protozoa remained stable (Paul et al., 2004).

Besides the lack of evidence that an increase in AF by dosing reduces the abundance of other rumen populations, it appears to be noteworthy that none of the studies centering on AF dosing has looked at potential changes of fiber-colonization by AF after the dosing event. Considering that dosing with AF cultures improved fiber digestion, VFA production and animal performance related parameters, suggesting that the altered microbiome is more efficient in the digestion of fibrous feeds than the original community, a closer investigation of the fiber colonization and deconstruction process post-dosing seems warranted.

Whereas ruminal fiber digestion improved in response to live AF, no shift in fermentation pattern was observed when AF-derived enzymes were added to the diet (Lee et al., 2000a). This highlights the importance of using viable cultures of AF as ruminant feed additives (Paul et al., 2011). In contrast to this, spent media containing AF enzymes seems to be effective in improving monogastric livestock production (Theodorou et al., 1996). Cell walls of cereals, major components of swine and poultry feed, contain difficult to digest non-starch polysaccharides, such as β-glucans in barley and wheat and arabinoxylans in rye and oats (Sánchez-Rodríguez et al., 2012). These polymers can have anti-nutritional effects due to their low digestibility and tendency to form high-molecular-weight aggregates that reduce passage rate, decrease diffusion of digestive enzymes, promote endogenous losses, and stimulate unwanted bacterial proliferation (Bedford and Schulze, 1998). Previous studies suggest that recombinant GHs from AF expressed in

Lactobacillus reuteri maintain their fiber-degrading capability and their resistance to bile salt and acids, while *L. reuteri* itself still retained its high adhesion efficiency to mucin and mucus (Liu et al., 2005a,b, 2007; Cheng et al., 2014), which would explain the decomposition-promoting effect of these recombinant proteins in the monogastric animal. Despite these promising characteristics for recombinant AF-derived GHs, the ability to efficiently generate significant amounts of cheap, stable, and active recombinant enzymes will be essential for low-cost production and large-scale application of these enzymes as a feed additive for monogastrics.

Tannins present in many feeds and forages are inhibitory to rumen microbes, and are an anti-nutritional issue for ruminants. Likewise, upon anaerobic degradation of phenolic compounds present in fibrous feeds, different phenolic monomers (i.e., ferulic acid, *p*-coumaric acid, vanillic acid, vanillin, catechol etc.) are released into the rumen that are inhibitory to microbiota. Therefore, attempts were made to identify rumen microbes that had tannin or phenolic monomer tolerating or degrading capability, so that they could be utilized as direct fed microbials to mitigate adverse effects of these anti-nutritional factors.

The anaerobic rumen fungus *Piromyces* sp. FNG5, isolated from a wild herbivore, was found to be tolerant to phenolic monomers and its pure culture degraded p-coumaric acid (38.5–65.1%), 65.2–74.1% ferulic acid (65.2–74.1%) and vanillic acid (34.1–66.8%) after 14 days of incubation (Paul et al., 2003). McSweeney et al. (2001) reported that sheep fed with condensed tannins from *Calliandra calothyrsus* had reduced ruminal AF concentration, but the inhibitory effect was less prominent compared to rumen bacteria. Paul et al. (2006) reported that addition of *Piromyces* sp. FNG5 significantly increased *in vitro* degradation (12%) of condensed tannins and this AF isolate could tolerate tannic acid concentrations up to 20 g/L. This amount is higher than the theoretic tannic acid level expected in the rumen of animals fed a diet composed exclusively of high tannin content plants. Conversely, Kok et al. (2013) found that *L. leucocephala* hybrid-Bahru (containing condensed tannins) when fed to goats, significantly decreased ruminal AF concentration. Saminathan et al. (2019) reported that high MW fractions of condensed tannins had inhibitory effect on ruminal AF, but relative abundance of *Piromyces* 4 was increased indicating that this group of uncultured AF is likely to be tannin resistant. The mechanisms by which some AF species can overcome the growth inhibitory effects of condensed tannins or phenolic monomers is unknown. Whether AF produce tannase, or not, remains to be established; but many isolates of AF, especially those from wild ruminants adapted to tannin rich and fibrous diets, were shown to produce a variety of esterases capable of degrading phenolic compounds (Paul et al., 2003). It is possible that these esterases are directly linked the ability of these AF to overcome growth inhibition caused by phenolic compounds.

Although these studies highlight the potential of using AF as probiotics to enhance digestibility of highly fibrous or tanniferous feed, boosting ruminant livestock production, economic aspects such as the need to repeatedly administer oral-dosages of AF to maintain the desired response have to be considered when discussing AF as probiotics on a commercial scale (Ribeiro et al., 2016).

As well as positively impacting forage intake and feed digestibility, AF have the potential to contribute to the protein supply of the host animal. This is both indirectly through the production of proteolytic enzymes in the rumen and directly as a source of microbial protein. Unlike the cellulolytic rumen bacteria, many isolates of AF are protease positive and capable of penetrating the proteinaceous layer of feed particles (Wallace and Joblin, 1985; Asao et al., 1993; Michel et al., 1993; Yanke et al., 1993). *In vitro* studies with defined populations of both proteolytic and non-proteolytic rumen bacteria and a proteolytic *Neocallimastix frontalis* strain have further indicated that *N. frontalis* was able to contribute to rumen protein degradation, particularly when protein was associated with feed particles (Wallace and Munro, 1986).

Besides producing proteases, AF directly contribute to protein supply of the host in terms of being part of the microbial biomass that passes down to the intestines from rumen, for subsequent digestion and absorption. Gulati et al. (1989) showed that AF cells were composed of proteins with a well-balanced combination of amino acids that were highly available to the ruminant host. A high proportion of the protein components of three monocentric AF (i.e., *Neocallimastix* sp. LMI, *Piromyces* sp. SMI and *Caecomyces* sp. NMI) was digested and absorbed in the intestine of sheep, with digestibility factors of 0.91–0.98 (Gulati et al., 1988, 1989). These high *in vivo* digestibility values for AF protein compared favorably with a value of 0.77 for mixed rumen bacteria protein measured in a similar manner (Gulati et al., 1990). Although the amount of nutritional nitrogen derived directly from anaerobic rumen fungi might only amount to a small portion of the total nitrogen that is absorbed by the animal, its importance lies in its high quality and immediate availability.

DIETARY MANIPULATION OF COMMENSAL ANAEROBIC FUNGI

Rather than directly adding more AF to the ruminant animal, there have been numerous attempts to improve the concentration of AF in the rumen by providing animals with feed that increases their overall concentration. It was previously believed that increasing dietary recalcitrant fiber may increase fungal population in rumen, as some of the plant fiber breakdown products were shown to have a positive effect on zoosporogenesis and chemotactic effects on fungal zoospores. Few AF were seen in the rumen of animals that were fed lush pasture (i.e., legume or grass when green and leafy), and the number of AF increased when animals were fed the same pasture after it had matured and was more recalcitrant (Bauchop, 1989; Kostyukovsky et al., 1991). However, results later showed the opposite effect, with an increased AF count in ruminants fed a low-lignin diet reported compared to animals provided with more recalcitrant feed (Gordon and Phillips, 1998). To make matters even more complex, other studies suggested that there was no direct effect of the hay type (with different levels of lignin content) on AF populations (Sekine et al., 1995).

It has been suggested that an appropriate amount of starch or concentrate in diet may support ruminal AF growth and stimulate zoosporogenesis (Matsui et al., 1997), but *in vivo* study findings remain inconclusive (Ishaq et al., 2017). A possible explanation for these different responses to starch rich feed is that only some of the AF, namely species of the genera *Neocallimastix*, *Piromyces* and *Orpinomyces*, have been shown to produce amylases and, therefore, have the ability to ferment starch grains (Phillips and Gordon, 1988; McAllister et al., 1993; Yanke et al., 1993). More work in this area needs to be conducted *in vivo* before final conclusions can be made. Furthermore, feeding starch or concentrates tends to increase ruminal ciliate protozoal concentrations, and protozoa are known to predate on AF zoospores (Morgavi et al., 1994).

Beneficial effects of sulfur supplementation, specifically for low sulfur diets, on the number of AF in sheep and their relative contribution to fiber degradation was reported in the early 1980s (Akin et al., 1983). This beneficial effect was further confirmed in subsequent studies using alkali treated wheat straw (Gordon et al., 1983; Gulati et al., 1985; Weston et al., 1988) and other poor quality feeds (Morrison et al., 1990). Promkot and Wanapat (2009) suggested beneficial manipulation of AF concentrations was possible through provision of an appropriate dietary supplement containing sulfur. For a supplement of this type to be effective, it should ideally contain a single organic sulfur compound which is readily utilized by the rumen AF but not by other components of the rumen microbiota (i.e., bacteria, archaea, and protozoa).

Two organic sulfur nutrients, mercapto-1-propionic acid (MPA) and 3-mercapto-1-propanesulfonic acid, were tested in cattle trials and compared to an inorganic sulfur supplement. It was reported that the organic sulfur sources improved nitrogen utilization and microbial protein production, but surprisingly this was concluded to be due to a general improvement in the efficiency of microbial fermentation of lignocellulose and not from specific stimulation of ruminal AF (McSweeney and Denman, 2007). Conversely, in a patent (Gordon and Phillips, 2002) it was reported that administering an effective amount of a degradation resistant sulfur source (MPA or its functional equivalent) promoted the growth of AF in the rumen of animals fed low sulfur content diets. Within this patent, it was also demonstrated that ruminal MPA infusion increased AF zoospores concentrations, and had a strong, positive, response on the digestive performance of sheep. However, additional scientific literature in this field is scarce and a sulfur supplement specific for promoting anaerobic rumen fungi remains to be identified.

Influence of other dietary supplements on the rumen AF community has been less studied, but some interesting findings have been reported. Thiamine supplementation, used to attenuate rumen metabolic disorder caused by high concentrate diet through buffering the rumen pH, increased significantly the proportion of ruminal AF in dairy cows (Xue et al., 2018). Plant oils, which are attractive feed additives used to mitigate CH_4 emissions, seem to have a negative effect on AF. The addition of soya oil significantly reduced ruminal AF diversity in steers (Boots et al., 2013). In dairy cows, sunflower oil addition decreased the concentration and diversity of AF. However, responses at the genus level were dependent on concentrate/forage ratios (Tapio et al., 2017). The addition of rapeseed oil led to a considerable decrease in the ruminal AF population, but the mechanism was not further investigated (Fonty and Grenet, 1994). Previously, Elliott et al. (1987) found that feeding a supplement of sunflower meal to sheep consuming a barley straw diet resulted in decrease of ruminal AF concentration to below detectable levels. In another study, the feeding of calcium salts of medium chain fatty acids (C6–Cl2) to sheep resulted in reduced numbers of AF zoospores in the rumen, whereas the salts of long chain fatty acids ($C \geq 14$) had no effect on AF (Ushida et al., 1992). This indicates that the inhibitory effects of the long chain fatty acids common in oilseed meals can be alleviated, at least partly, by chemical pretreatment.

Anaerobic fungi are also sensitive to a shortage of nitrogen. A low protein diet decreased rumen AF concentration in dairy cows compared to a high protein diet (Belanche et al., 2012). However, the AF community composition was modified by the level of dietary protein only when cows consumed the starch-rich diet, but not the fiber-rich diet (Belanche et al., 2012). This highlights the potential for further complexities when trying to determine the effects of individual dietary components on ruminal AF.

FUTURE PERSPECTIVES AND OPPORTUNITIES FOR ANAEROBIC FUNGI BASED APPLICATIONS IN ANIMAL PRODUCTION AND HEALTH

With consumer demand for affordable high-quality animal products increasing and with a decline in natural resources, such as farmable land area, it will be essential to create new and refine existing strategies to improve the utilization of low-quality forages for animal feed. Such strategies will rely heavily on approaches that render the recalcitrant fraction of the plant material more accessible to the fermenting microorganisms that are indigenous to the herbivore gut. AF with their ability to break open recalcitrant plant structures will play a significant role in these new approaches.

Whilst AF are clearly beneficial for the ruminant host, the underlying mechanisms to boost indigenous AF populations through standard feed components such as fiber, starch, nitrogen and lipid are inherently complex and are still not well understood. As such, there still remains great interest in developing a reliable and reproducible feed-based strategy to increase AF in order to improve animal performance and health. However, whilst the benefits of AF for ruminants has been well established, their value for hindgut herbivores remains to be confirmed. The observed increase in feed intake with AF supplemented ruminants is thought to be due to the AF causing more rapid clearance of digesta from the rumen, due to their physical and enzymatic disruption of fibrous plant particles (Gordon and Phillips, 1998). If this is also the case in equines, development

of an equine AF probiotic may enable replacement of some of the energy dense concentrates used in horse feeds with more bulky fibrous feeds. This will contribute to reducing the risk of colic and dysbiosis of hindgut microbiota, which is commonly observed in working and/or performance equines fed high grain/concentrate diets in order to meet their higher energy requirements (Shirazi-Beechey, 2008; Durham, 2013; Julliand and Grimm, 2017).

Most of the studies focused on AF-based strategies for improving animal production and health have relied on the repeated oral-dosing of AF. This approach can only become economically feasible if the cost of industrial scale production of an AF probiotic is significantly less than the economic return gained by livestock producers. Considering the significant benefits that have been reported for live AF supplementation to date for ruminants, the probiotic use of AF is likely to have a significant return on investment for ruminant livestock producers. One possibility to produce an AF probiotic could be the use of encapsulated cultures. However, methods for large-scale production of encapsulated AF are currently not available, and would have to be developed before this approach could become commercially feasible. An alternative approach could be the use of AF resting structures, with their subsequent revival into active hydrolytic AF occurring within the host. Fundamental understanding of these structures is, however, currently too limited for this to be practically realized in the near future. Advances in understanding of the biology of the AF resting phase would not only facilitate the utilization of AF in livestock production, but also their application in other areas where lignocellulosic plant material is used to produce biofuels and platform chemicals. As such, characterization of the resting phase of AF should be a high priority research area for development.

The resulting increased efficiency of livestock production through the application of AF will undoubtedly have beneficial impacts in terms of the environmental footprint and sustainability of livestock production. The impact of AF based strategies on ruminant derived methane production remains to be determined, however, it is clear that increased efficiency of lignocellulosic plant material utilization will also decrease the need for arable land for animal feed production. Recent advances in molecular techniques enable a detailed understanding of role of the AF and how it affects the performance and health of its herbivorous host. Research based on state-of-the-art methods will allow the development of more advanced and holistic approaches to manipulate the composition and function of the gut microbiome and ultimately the health and performance of the host animal. Such progress will facilitate a more sustainable livestock industry to provide affordable high-quality animal products for a growing global population.

CONCLUSION

Although AF were first observed more than hundred years ago, it was not until Colin Orpin's ground-breaking work in the mid 1970s that AF were correctly identified, overturning the paradigm of the time that all fungi required oxygen. Considering AF have only been effectively studied for ∼50 years, advances made during this time have been significant. Whereas initial work required the refinement of isolation and cultivation techniques and was mostly driven by morphological observations and phenotypic characterization, more recent insights have been enabled by novel molecular approaches. These molecular techniques have already greatly advanced understanding of the complexity and diversity of the AF, however, our full understanding is still far from complete. With the ability to sequence long genomic regions such as the entire *rrn* operon, it is inevitable that a more accurate and complete understanding of the AF phylogeny will soon emerge. Understanding the phylogenetic relationship of individual AF will be essential to increasing understanding of their evolutionary history, and factors that drive their niche specialization within and between different types of host. However, to practically develop AF for application in livestock production, as well as other industries, functional systems microbiology approaches will be key. Technologies such as (meta)genomics, (meta)transcriptomics and proteomics will enable the pinpointing of specific genes, proteins and reactions that are employed by AF in response to extrinsic conditions (e.g., host genotype) and changes therein (e.g., host health status and dietary composition). Despite the significant progress made to date, the ecological role of AF and their quantitative contribution to host function and health still remains to be clarified in the full range of mammalian herbivores where they naturally reside. Furthermore, a key phase of the AF life cycle, the resting phase, is an area of very limited knowledge that urgently needs to be researched. Together, newly obtained knowledge in these areas will enable utilization of AF and their enzymes to transform the sustainability and environmental footprint of livestock agriculture, as well as revolutionizing biotechnological processes involving plant-based feedstocks. Whilst we increasingly understand more about the evolution, biology and ecology of AF, there still remains many key "why" questions to be answered: "Why" are AF sensitive to oxygen? "Why" do AF have the lowest GC-content among all known microorganisms? "Why" are AF the only fungi with polyflagellate zoospores, hydrogenosomes, and cellulosomes? Considering that the AF phylum is currently composed of just one family, are we only looking at the tip of an iceberg? Or have we missed something crucial when classifying these microorganisms? Whatever the answers are, we know for certain that many fundamental questions still remain to be answered before the true potential of this highly valuable and paradigm shifting phylum of microorganisms is fully understood and can be harnessed.

AUTHOR CONTRIBUTIONS

MH designed the manuscript, coordinated the co-author contributions, and wrote the plant cell-wall degradation section. KF wrote the habitat, life cycle, morphology and taxonomy section and contributed to other sections. SP and AP contributed to the feed additive section. MG contributed to the

methanogenesis section. CS co-authored the plant cell wall degradation section. JE wrote the section on hindgut herbivores and contributed to other sections. All authors reviewed the manuscript, offered critical feedback, and approved the final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.584893/full#supplementary-material

REFERENCES

Akin, D. E., and Borneman, W. S. (1990). Role of rumen fungi in fiber degradation. *J. Dairy Sci.* 73, 3023–3032. doi: 10.3168/jds.s0022-0302(90)78989-8

Akin, D. E., Gordon, G. L., and Hogan, J. P. (1983). Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. *Appl. Environ. Microbiol.* 46, 738–748. doi: 10.1128/aem.46.3.738-748.1983

Asao, N., Ushida, K., and Kojima, Y. (1993). Proteolytic activity of rumen fungi belonging to the genera *Neocallimastix* and *Piromyces*. *Lett. Appl. Microbiol.* 16, 247–250. doi: 10.1111/j.1472-765x.1993.tb01410.x

Baldwin, R. L., and Allison, M. J. (1983). Rumen metabolism. *J. Anim. Sci.* 57, 461–477.

Barr, D. J. S. (1980). An outline for the reclassification of the *Chytridiales*, and for a new order, the *Spizellomycetales*. *Can. J. Bot.* 58, 2380–2394. doi: 10.1139/b80-276

Barr, D. J. S. (1988). How modern systematics relates to the rumen fungi. *Biosystems* 21, 351–356. doi: 10.1016/0303-2647(88)90032-9

Barr, D. J. S., Kudo, H., Jakober, K. D., and Cheng, K. J. (1989). Morphology and development of rumen fungi: *Neocallimastix* sp., *Piromyces communis*, and *Orpinomyces bovis* gen.nov., sp.nov. *Can. J. Bot.* 67, 2815–2824. doi: 10.1139/b89-361

Bauchop, T. (1979). Rumen anaerobic fungi of cattle and sheep. *Appl. Environ. Microbiol.* 38, 148–158. doi: 10.1128/aem.38.1.148-158.1979

Bauchop, T. (1989). Biology of gut anaerobic fungi. *Biosystems* 23, 53–64. doi: 10.1016/0303-2647(89)90008-7

Bauchop, T., and Mountfort, D. O. (1981). Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl. Environ. Microbiol.* 42, 1103–1110. doi: 10.1128/aem.42.6.1103-1110.1981

Bayer, E. A., Belaich, J. P., Shoham, Y., and Lamed, R. (2004). The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annu. Rev. Microbiol.* 58, 521–554. doi: 10.1146/annurev.micro.57.030502.091022

Bedford, M. R., and Schulze, H. (1998). Exogenous enzymes for pigs and poultry. *Nutr. Res. Rev.* 11, 91–114. doi: 10.1079/nrr19980007

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M., Pinloche, E., and Newbold, C. J. (2012). Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J. Nutr.* 142, 1684–1692. doi: 10.3945/jn.112.159574

Boots, B., Lillis, L., Clipson, N., Petrie, K., Kenny, D. A., Boland, T. M., et al. (2013). Responses of anaerobic rumen fungal diversity (phylum *Neocallimastigomycota*) to changes in bovine diet. *J. Appl. Microbiol.* 114, 626–635. doi: 10.1111/jam.12067

Borrel, G., O'Toole, P. W., Harris, H. M. B., Peyret, P., Brugère, J.-F., and Gribaldo, S. (2013). Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis. *Genome Biol. Evol.* 5, 1769–1780. doi: 10.1093/gbe/evt128

Braune, R. A. (1913). Untersuchungen über die im wiederkäuermagen vorkommenden protozoen. *Arch. Protistenk.* 32, 111–170.

Breton, A., Bernalier, A., Dusser, M., Fonty, G., Gaillard-Martinie, B., and Guillot, J. (1990). *Anaeromyces mucronatus* nov. gen., nov. sp. A new strictly anaerobic rumen fungus with polycentric thallus. *FEMS Microbiol. Lett.* 58, 177–182. doi: 10.1111/j.1574-6968.1990.tb13974.x

Brookman, J. L., Ozkose, E., Rogers, S., Trinci, A. P. J., and Theodorou, M. K. (2000). Identification of spores in the polycentric anaerobic gut fungi which enhance their ability to survive. *FEMS Microbiol. Ecol.* 31, 261–267. doi: 10.1111/j.1574-6941.2000.tb00692.x

Callaghan, T. M., Podmirseg, S. M., Hohlweck, D., Edwards, J. E., Puniya, A. K., Dagar, S. S., et al. (2015). *Buwchfawromyces eastonii* gen. nov., sp. nov.: a new anaerobic fungus (*Neocallimastigomycota*) isolated from buffalo faeces. *Mycokeys* 9, 11–28. doi: 10.3897/mycokeys.9.9032

Capper, J. L., and Bauman, D. E. (2013). The role of productivity in improving the environmental sustainability of ruminant production systems. *Annu. Rev. Anim. Biosci.* 1, 469–489. doi: 10.1146/annurev-animal-031412-103727

Cheng, H. L., Hu, C. Y., Lin, S. H., Wang, J. Y., Liu, J. R., and Chen, Y. C. (2014). Characterization of two truncated forms of xylanase recombinantly expressed by *Lactobacillus reuteri* with an introduced rumen fungal xylanase gene. *Enzyme Microb. Technol.* 64–65, 6–10. doi: 10.1016/j.enzmictec.2014.06.004

Cheng, Y. F., Edwards, J. E., Allison, G. G., Zhu, W. Y., and Theodorou, M. K. (2009). Diversity and activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on lignocellulose in consecutive batch culture. *Bioresour. Technol.* 100, 4821–4828. doi: 10.1016/j.biortech.2009.04.031

Clauss, M., Dittmann, M. T., Vendl, C., Hagen, K. B., Frei, S., Ortmann, S., et al. (2020). Review: comparative methane production in mammalian herbivores. *Animal* 14, s113–s123.

Couger, M. B., Youssef, N. H., Struchtemeyer, C. G., Liggenstoffer, A. S., and Elshahed, M. S. (2015). Transcriptomic analysis of lignocellulosic biomass degradation by the anaerobic fungal isolate *Orpinomyces* sp. strain C1A. *Biotechnol. Biofuels* 8:208.

Dagar, S. S., Kumar, S., Griffith, G. W., Edwards, J. E., Callaghan, T. M., Singh, R., et al. (2015). A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*). *Fungal Biol.* 119, 731–737. doi: 10.1016/j.funbio.2015.04.005

Dagar, S. S., Kumar, S., Mudgil, P., Singh, R., and Puniya, A. K. (2011). D1/D2 domain of large-subunit ribosomal DNA for differentiation of *Orpinomyces* spp. *Appl. Environ. Microbiol.* 77, 6722–6725. doi: 10.1128/aem.05441-11

Davidson, E. A., van der Giezen, M., Horner, D. S., Embley, T. M., and Howe, C. J. (2002). An [Fe] hydrogenase from the anaerobic hydrogenosome-containing fungus *Neocallimastix frontalis* L2. *Gene* 296, 45–52. doi: 10.1016/s0378-1119(02)00873-9

Davies, D. R., Theodorou, M. K., Brooks, A. E., and Trinci, A. P. (1993). Influence of drying on the survival of anaerobic fungi in rumen digesta and faeces of cattle. *FEMS Microbiol. Lett.* 106, 59–63. doi: 10.1111/j.1574-6968.1993.tb05935.x

Dearing, M. D., and Kohl, K. D. (2017). Beyond fermentation: other important services provided to endothermic herbivores by their gut microbiota. *Integr. Comp. Biol.* 57, 723–731. doi: 10.1093/icb/icx020

Dehority, B. A. (2002). Gastrointestinal tracts of herbivores, particularly the ruminant: anatomy, physiology and microbial digestion of plants. *J. Appl. Anim. Res.* 21, 145–160. doi: 10.1080/09712119.2002.9706367

Denman, S. E., and McSweeney, C. S. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol.* 58, 572–582. doi: 10.1111/j.1574-6941.2006.00190.x

Dey, A., Sehgal, J. P., Puniya, A. K., and Singh, K. (2004). Influence of an anaerobic fungal culture (*Orpinomyces* sp.) administration on growth rate, ruminal fermentation and nutrient digestion in calves. *Asian Australas. J. Anim. Sci.* 17, 820–824. doi: 10.5713/ajas.2004.820

Dollhofer, V., Callaghan, T. M., Dorn-In, S., Bauer, J., and Lebuhn, M. (2016). Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi. *J. Microbiol. Methods* 127, 28–40. doi: 10.1016/j.mimet.2016.05.017

Dollhofer, V., Callaghan, T. M., Griffith, G. W., Lebuhn, M., and Bauer, J. (2017). Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants. *Bioresour. Technol.* 235, 131–139. doi: 10.1016/j.biortech.2017.03.116

Duarte, I., and Huynen, M. A. (2019). Contribution of lateral gene transfer to the evolution of the eukaryotic fungus *Piromyces* sp. E2: massive bacterial transfer

of genes involved in carbohydrate metabolism. *bioRxiv [Preprint]* doi: 10.1101/514042

Durham, A. E. (2013). "Intestinal disease," in *Equine Applied and Clinical Nutrition*, eds R. J. Geor, P. A. Harris, and M. Coenen (Philadelphia, PA: W.B. Saunders), 568–581.

Ebersberger, I., de Matos Simoes, R., Kupczok, A., Gube, M., Kothe, E., Voigt, K., et al. (2012). A consistent phylogenetic backbone for the fungi. *Mol. Biol. Evol.* 29, 1319–1334. doi: 10.1093/molbev/msr285

Edwards, J. E., Forster, R. J., Callaghan, T. M., Dollhofer, V., Dagar, S. S., Cheng, Y., et al. (2017). PCR and omics based techniques to study the diversity, ecology and biology of anaerobic fungi: insights, challenges and opportunities. *Front. Microbiol.* 8:1657. doi: 10.3389/fmicb.2017.01657

Edwards, J. E., Hermes, G. D. A., Kittelmann, S., Nijsse, B., and Smidt, H. (2019). Assessment of the accuracy of high-throughput sequencing of the ITS1 region of *Neocallimastigomycota* for community composition analysis. *Front. Microbiol.* 10:2370. doi: 10.3389/fmicb.2019.02370

Edwards, J. E., Kingston-Smith, A. H., Jimenez, H. R., Huws, S. A., Skot, K. P., Griffith, G. W., et al. (2008). Dynamics of initial colonization of nonconserved perennial ryegrass by anaerobic fungi in the bovine rumen. *FEMS Microbiol. Ecol.* 66, 537–545. doi: 10.1111/j.1574-6941.2008.00563.x

Edwards, J. E., Schennink, A., Burden, F., Long, S., van Doorn, D. A., Pellikaan, W. F., et al. (2020a). Domesticated equine species and their derived hybrids differ in their fecal microbiota. *Anim. Microbiome* 2:8.

Edwards, J. E., Shetty, S. A., van den Berg, P., Burden, F., van Doorn, D. A., Pellikaan, W. F., et al. (2020b). Multi-kingdom characterization of the core equine fecal microbiota based on multiple equine (sub)species. *Anim. Microbiome* 2:6. doi: 10.1111/eve.03_12792

Elekwachi, C. O., Wang, Z., Wu, X., Rabee, A., and Forster, R. J. (2017). Total rRNA-Seq analysis gives insight into bacterial, fungal, protozoal and archaeal communities in the rumen using an optimized RNA isolation method. *Front. Microbiol.* 8:1814. doi: 10.3389/fmicb.2017.01814

Elliott, R., Ash, A. J., Calderon-Cortes, F., Norton, B. W., and Bauchop, T. (1987). The influence of anaerobic fungi on rumen volatile fatty acid concentrations in vivo. *J. Agric. Sci.* 109, 13–17. doi: 10.1017/s0021859600080928

Embley, T. M., and Finlay, B. J. (1994). The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. *Microbiology* 140(Pt 2), 225–235. doi: 10.1099/13500872-140-2-225

Fanutti, C., Ponyi, T., Black, G. W., Hazlewood, G. P., and Gilbert, H. J. (1995). The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *J. Biol. Chem.* 270, 29314–29322. doi: 10.1074/jbc.270.49.29314

Ferry, J. G. (2010). How to make a living by exhaling methane. *Annu. Rev. Microbiol.* 64, 453–473. doi: 10.1146/annurev.micro.112408.134051

Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M., and Hirt, R. P. (1994). Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiol. Lett.* 117, 157–161. doi: 10.1111/j.1574-6968.1994.tb06758.x

Fontes, C. M., and Gilbert, H. J. (2010). Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. *Annu. Rev. Biochem.* 79, 655–681. doi: 10.1146/annurev-biochem-091208-085603

Fonty, G., and Grenet, E. (1994). "Effects of diet on the fungal population of the digestive tract of ruminants," in *Anaerobic Fungi: Biology, Ecology and Function*, eds D. O. Mountfort and C. G. Orpin (New York, NY: Marcel Dekker), 229–239. doi: 10.1201/9781003067085-8

France, J., Theodorou, M. K., and Davies, D. (1990). Use of zoospore concentrations and life cycle parameters in determining the population of anaerobic fungi in the rumen ecosystem. *J. Theor. Biol.* 147, 413–422. doi: 10.1016/s0022-5193(05)80496-5

Garcia-Vallvé, S., Romeu, A., and Palau, J. (2000). Horizontal gene transfer of glycosyl hydrolases of the rumen fungi. *Mol. Biol. Evol.* 17, 352–361. doi: 10.1093/oxfordjournals.molbev.a026315

Gay, L. (1991). Chitin content and chitin synthase activity as indicators of the growth of three different anaerobic rumen fungi. *FEMS Microbiol. Lett.* 64, 99–102. doi: 10.1111/j.1574-6968.1991.tb04643.x

Gilmore, S. P., Lankiewicz, T. S., Wilken, S. E., Brown, J. L., Sexton, J. A., Henske, J. K., et al. (2019). Top-down enrichment guides in formation of synthetic microbial consortia for biomass degradation. *ACS Synth. Biol.* 8, 2174–2185. doi: 10.1021/acssynbio.9b00271

Gilmore, S. P., Lillington, S. P., Haitjema, C. H., de Groot, R., and O'Malley, M. A. (2020). Designing chimeric enzymes inspired by fungal cellulosomes. *Synth. Syst. Biotechnol.* 5, 23–32. doi: 10.1016/j.synbio.2020.01.003

Gold, J. J., Heath, I. B., and Bauchop, T. (1988). Ultrastructural description of a new chytrid genus of caecum anaerobe, *Caecomyces equi* gen. nov., sp. nov., assigned to the Neocallimasticaceae. *Biosystems* 21, 403–415. doi: 10.1016/0303-2647(88)90039-1

Gordon, G. L., and Phillips, M. W. (1998). The role of anaerobic gut fungi in ruminants. *Nutr. Res. Rev.* 11, 133–168. doi: 10.1079/nrr19980009

Gordon, G. L., and Phillips, M. W. (2002). Fungal sulphur source and methods of using the same. U.S. Patent No 645,858,0B1. Washington, DC: U.S. Patent and Trademark Office.

Gordon, G. L. R., Gulati, S. K., and Ashes, J. R. (1983). Influence of low-sulphur straw on anaerobic fungal numbers in a sheep rumen. *Proc. Nutr. Soc. Aust.* 8:188.

Gordon, G. L. R., and Phillips, M. W. (1993). Removal of anaerobic fungi from the rumen of sheep by chemical treatment and the effect on feed consumption and in vivo fibre digestion. *Lett. Appl. Microbiol.* 17, 220–223. doi: 10.1111/j.1472-765x.1993.tb01451.x

Griffith, G. W., Ozkose, E., Theodorou, M. K., and Davies, D. R. (2009). Diversity of anaerobic fungal populations in cattle revealed by selective enrichment culture using different carbon sources. *Fungal Ecol.* 2, 87–97. doi: 10.1016/j.funeco.2009.01.005

Grigoriev, I. V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., et al. (2013). MycoCosm portal: gearing up for 1000 fungal genomes. *Nucleic Acids Res.* 42, D699–D704.

Gulati, S. K., Ashes, J. R., and Gordon, G. L. (1988). Digestibility of sulphur amino acids in rumen fungal species. *Proc. Nutr. Soc. Aust.* 13:133.

Gulati, S. K., Ashes, J. R., and Gordon, G. L. R. (1990). Comparative digestibility of sulphur amino acids in rumen bacteria and fungal proteins by sheep. *Proc. Nutr. Aust.* 15, 128–131.

Gulati, S. K., Ashes, J. R., Gordon, G. L. R., Connell, P. J., and Rogers, P. L. (1989). Nutritional availability of amino acids from the rumen anaerobic fungus *Neocallimastix* sp. LM1 in sheep. *J. Agric. Sci.* 113, 383–387. doi: 10.1017/s002185960007009x

Gulati, S. K., Ashes, J. R., Gordon, G. L. R., and Phillips, M. W. (1985). Possible contribution of rumen fungi to fibre digestion in sheep. *Proc. Nutr. Soc. Aust.* 10:96.

Hagen, L. H., Brooke, C. G., Shaw, C., Norbeck, A. D., Piao, H., Arntzen, M. Ø., et al. (2020). Proteome specialization of anaerobic fungi during ruminal degradation of recalcitrant plant fiber. *ISME J.* doi: 10.1038/s41396-020-00769-x [Epub ahead of print].

Haitjema, C. H., Gilmore, S. P., Henske, J. K., Solomon, K. V., de Groot, R., Kuo, A., et al. (2017). A parts list for fungal cellulosomes revealed by comparative genomics. *Nat. Microbiol.* 2:17087.

Haitjema, C. H., Solomon, K. V., Henske, J. K., Theodorou, M. K., and O'Malley, M. A. (2014). Anaerobic gut fungi: advances in isolation, culture, and cellulolytic enzyme discovery for biofuel production. *Biotechnol. Bioeng.* 111, 1471–1482. doi: 10.1002/bit.25264

Hanafy, R. A., Elshahed, M. S., Liggenstoffer, A. S., Griffith, G. W., and Youssef, N. H. (2017). *Pecoramyces ruminantium*, gen. nov., sp. nov., an anaerobic gut fungus from the feces of cattle and sheep. *Mycologia* 109, 231–243. doi: 10.1080/00275514.2017.1317190

Hanafy, R. A., Elshahed, M. S., and Youssef, N. H. (2018). *Feramyces austinii*, gen. nov., sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild Barbary sheep and fallow deer. *Mycologia* 110, 513–525. doi: 10.1080/00275514.2018.1466610

Hanafy, R. A., Lanjekar, V. B., Dhakephalkar, P. K., Callaghan, T. M., Dagar, S. S., Griffith, G. W., et al. (2020). Seven new *Neocallimastigomycota* genera from wild, zoo-housed, and domesticated herbivores greatly expand the taxonomic diversity of the phylum. *Mycologia* doi: 10.1080/00275514.2019.1696619 [Epub ahead of print].

Heath, I. B., Bauchop, T., and Skipp, R. A. (1983). Assignment of the rumen anaerobe *Neocallimastix frontalis* to the *Spizellomycetales* (Chytridiomycetes) on the basis of its polyflagellate zoospore ultrastructure. *Can. J. Bot.* 61, 295–307. doi: 10.1139/b83-033

Heath, I. B., Kaminskyj, S. G., and Bauchop, T. (1986). Basal body loss during fungal zoospore encystment: evidence against centriole autonomy. *J. Cell Sci.* 83, 135–140.

Hegarty, R., and Gerdes, R. (1999). Hydrogen production and transfer in the rumen. *Recent Adv. Anim. Nutr. Aust.* 12, 37–44.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census, C., et al. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567.

Henske, J. K., Gilmore, S. P., Knop, D., Cunningham, F. J., Sexton, J. A., Smallwood, C. R., et al. (2017). Transcriptomic characterization of *Caecomyces churrovis*: a novel, non-rhizoid-forming lignocellulolytic anaerobic fungus. *Biotechnol. Biofuels* 10:305.

Henske, J. K., Wilken, S. E., Solomon, K. V., Smallwood, C. R., Shutthanandan, V., Evans, J. E., et al. (2018). Metabolic characterization of anaerobic fungi provides a path forward for bioprocessing of crude lignocellulose. *Biotechnol. Bioeng.* 115, 874–884. doi: 10.1002/bit.26515

Hess, M., Sczyrba, A., Egan, R., Kim, T., Chokhawala, H., Schroth, G., et al. (2011). Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* 331, 463–467. doi: 10.1126/science.1200387

Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., et al. (2007). A higher-level phylogenetic classification of the Fungi. *Mycol. Res.* 111, 509–547.

Ho, Y. W., Abdullah, N., and Jalaludin, S. (1988). Penetrating structures of anaerobic rumen fungi in cattle and swamp buffalo. *Microbiology* 134, 177–181. doi: 10.1099/00221287-134-1-177

Ho, Y. W., and Bauchop, T. (1991). Morphology of three polycentric rumen fungi and description of a procedure for the induction of zoosporogenesis and release of zoospores in cultures. *J. Gen. Microbiol.* 137, 213–217. doi: 10.1099/00221287-137-1-213

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., et al. (2018). Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9:2161. doi: 10.3389/fmicb.2018.02161

Irbis, C., and Ushida, K. (2004). Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa. *J. Gen. Appl. Microbiol.* 50, 203–212. doi: 10.2323/jgam.50.203

Ishaq, S. L., AlZahal, O., Walker, N., and McBride, B. (2017). An investigation into rumen fungal and protozoal diversity in three rumen fractions, during high-fiber or grain-induced sub-acute ruminal acidosis conditions, with or without active dry yeast supplementation. *Front. Microbiol.* 8:1943. doi: 10.3389/fmicb.2017.01943

Janssen, P. H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* 160, 1–22. doi: 10.1016/j.anifeedsci.2010.07.002

Jeyanathan, J., Martin, C., and Morgavi, D. P. (2014). The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8, 250–261. doi: 10.1017/s1751731113002085

Jin, W., Cheng, Y. F., Mao, S. Y., and Zhu, W. Y. (2011). Isolation of natural cultures of anaerobic fungi and indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. *Bioresour. Technol.* 102, 7925–7931. doi: 10.1016/j.biortech.2011.06.026

Joblin, K. N., Campbell, G. P., Richardson, A. J., and Stewart, C. S. (1989). Fermentation of barley straw by anaerobic rumen bacteria and fungi in axenic culture and in co-culture with methanogens. *Lett. Appl. Microbiol.* 9, 195–197. doi: 10.1111/j.1472-765x.1989.tb00323.x

Joshi, A., Lanjekar, V. B., Dhakephalkar, P. K., Callaghan, T. M., Griffith, G. W., and Dagar, S. S. (2018). *Liebetanzomyces polymorphus* gen. et sp. nov., a new anaerobic fungus (*Neocallimastigomycota*) isolated from the rumen of a goat. *Mycokeys* 40, 89–110.

Julliand, V., and Grimm, P. (2017). The impact of diet on the hindgut microbiome. *J. Equine Vet. Sci.* 52, 23–28. doi: 10.1016/j.jevs.2017.03.002

Julliand, V., Riondet, C., de Vaux, A., Alcaraz, G., and Fonty, G. (1998). Comparison of metabolic activities between *Piromyces citronii*, an equine fungal species, and *Piromyces communis*, a ruminal species. *Anim. Feed Sci. Technol.* 70, 161–168. doi: 10.1016/s0377-8401(97)00043-6

Kittelmann, S., Naylor, G. E., Koolaard, J. P., and Janssen, P. H. (2012). A proposed taxonomy of anaerobic fungi (class neocallimastigomycetes) suitable for large-scale sequence-based community structure analysis. *PLoS One* 7:e36866. doi: 10.1371/journal.pone.0036866

Kittelmann, S., Pinares-Patino, C. S., Seedorf, H., Kirk, M. R., Ganesh, S., McEwan, J. C., et al. (2014). Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLoS One* 9:e103171. doi: 10.1371/journal.pone.0103171

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., et al. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One* 8:e47879. doi: 10.1371/journal.pone.0047879

Koetschan, C., Kittelmann, S., Lu, J., Al-Halbouni, D., Jarvis, G. N., Müller, T., et al. (2014). Internal transcribed spacer 1 secondary structure analysis reveals a common core throughout the anaerobic fungi (*Neocallimastigomycota*). *PLoS One* 9:e91928. doi: 10.1371/journal.pone.0091928

Kok, C. M., Sieo, C. C., Tan, H. Y., Saad, W. Z., Liang, J. B., and Ho, Y. W. (2013). Anaerobic cellulolytic rumen fungal populations in goats fed with and without *Leucaena leucocephala* hybrid, as determined by real-time PCR. *J. Microbiol.* 51, 700–703. doi: 10.1007/s12275-013-2540-z

Kostyukovsky, V. A., Okunev, O. N., and Tarakanov, B. V. (1991). Description of two anaerobic fungal strains from the bovine rumen and influence of diet on the fungal population in vivo. *J. Gen. Microbiol.* 137, 1759–1764. doi: 10.1099/00221287-137-7-1759

Krauss, J., Zverlov, V. V., and Schwarz, W. H. (2012). In vitro reconstitution of the complete *Clostridium thermocellum* cellulosome and synergistic activity on crystalline cellulose. *Appl. Environ. Microbiol.* 78, 4301–4307. doi: 10.1128/aem.07959-11

Lamed, R., Setter, E., and Bayer, E. A. (1983). Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. *J. Bacteriol.* 156, 828–836. doi: 10.1128/jb.156.2.828-836.1983

Lee, S. S., Ha, J. K., and Cheng, K. J. (2000a). Influence of an anaerobic fungal culture administration on in vivo ruminal fermentation and nutrient digestion. *Anim. Feed Sci. Technol.* 88, 201–217. doi: 10.1016/s0377-8401(00)00216-9

Lee, S. S., Ha, J. K., and Cheng, K. J. (2000b). Relative contributions of bacteria, protozoa, and fungi to in vitro degradation of orchard grass cell walls and their interactions. *Appl. Environ. Microbiol.* 66, 3807–3813. doi: 10.1128/aem.66.9.3807-3813.2000

Li, Y., Jin, W., Mu, C., Cheng, Y., and Zhu, W. (2017). Indigenously associated methanogens intensified the metabolism in hydrogenosomes of anaerobic fungi with xylose as substrate. *J. Basic Microbiol.* 57, 933–940. doi: 10.1002/jobm.201700132

Li, Y., Li, Y., Jin, W., Sharpton, T. J., Mackie, R. I., Cann, I., et al. (2019). Combined genomic, transcriptomic, proteomic, and physiological characterization of the growth of *Pecoramyces* sp. F1 in monoculture and co-culture with a syntrophic methanogen. *Front. Microbiol.* 10:435. doi: 10.3389/fmicb.2019.00435

Liebetanz, E. (1910). Die parasitischen protozoen des wiederkauermagens. *Arch. Protistenkunde* 19, 19–90.

Liggenstoffer, A. S., Youssef, N. H., Couger, M. B., and Elshahed, M. S. (2010). Phylogenetic diversity and community structure of anaerobic gut fungi (phylum *Neocallimastigomycota*) in ruminant and non-ruminant herbivores. *ISME J.* 4, 1225–1235. doi: 10.1038/ismej.2010.49

Liu, C., Meng, Q., Chen, Y., Xu, M., Shen, M., Gao, R., et al. (2017). Role of age-related shifts in rumen bacteria and methanogens in methane production in cattle. *Front. Microbiol.* 8:1563. doi: 10.3389/fmicb.2017.01563

Liu, J. R., Yu, B., Lin, S. H., Cheng, K. J., and Chen, Y. C. (2005a). Direct cloning of a xylanase gene from the mixed genomic DNA of rumen fungi and its expression in intestinal *Lactobacillus reuteri*. *FEMS Microbiol. Lett.* 251, 233–241. doi: 10.1016/j.femsle.2005.08.008

Liu, J. R., Yu, B., Liu, F. H., Cheng, K. J., and Zhao, X. (2005b). Expression of rumen microbial fibrolytic enzyme genes in probiotic *Lactobacillus reuteri*. *Appl. Environ. Microbiol.* 71, 6769–6775. doi: 10.1128/aem.71.11.6769-6775.2005

Liu, J. R., Yu, B., Zhao, X., and Cheng, K. J. (2007). Coexpression of rumen microbial beta-glucanase and xylanase genes in *Lactobacillus reuteri*. *Appl. Microbiol. Biotechnol.* 77, 117–124. doi: 10.1007/s00253-007-1123-5

Ljungdahl, L. G. (2008). The cellulase/hemicellulase system of the anaerobic fungus *Orpinomyces* PC-2 and aspects of its applied use. *Ann. N. Y. Acad. Sci.* 1125, 308–321. doi: 10.1196/annals.1419.030

Lowe, S. E., Grifith, G. G., Milne, A., Theodorou, M. K., and Trinci, A. P. J. (1987a). The life cycle and growth kinetics of an anaerobic rumen fungus. *J. Gen. Microbiol.* 133, 1815–1827. doi: 10.1099/00221287-133-7-1815

Lowe, S. E., Theodorou, M. K., and Trinci, A. P. J. (1987b). Isolation of anaerobic fungi from saliva and faeces of sheep. *Microbiology* 133, 1829–1834. doi: 10.1099/00221287-133-7-1829

Lyu, Z., Shao, N., Akinyemi, T., and Whitman, W. B. (2018). Methanogenesis. *Curr. Biol.* 28, R727–R732.

Marinier, S. L., and Alexander, A. J. (1995). Coprophagy as an avenue for foals of the domestic horse to learn food preferences from their dams. *J. Theor. Biol.* 173, 121–124. doi: 10.1006/jtbi.1995.0049

Marvin-Sikkema, F. D., Pedro Gomes, T. M., Grivet, J.-P., Gottschal, J. C., and Prins, R. A. (1993). Characterization of hydrogenosomes and their role in glucose metabolism of *Neocallimastix* sp. L2. *Arch. Microbiol.* 160, 388–396.

Marvin-Sikkema, F. D., Richardson, A. J., Stewart, C. S., Gottschal, J. C., and Prins, R. A. (1990). Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. *Appl. Environ. Microbiol.* 56, 3793–3797. doi: 10.1128/aem.56.12.3793-3797.1990

Matsui, H., Ushida, K., and Kojima, Y. (1997). Effect of dietary concentrate on fungal zoosporogenesis in sheep rumen. *Asian Australas. J. Anim. Sci.* 10, 599–602. doi: 10.5713/ajas.1997.599

McAllister, T. A., Dong, Y., Yanke, L. J., Bae, H. D., Cheng, K. J., and Costerton, J. W. (1993). Cereal grain digestion by selected strains of ruminal fungi. *Can. J. Microbiol.* 39, 367–376. doi: 10.1139/m93-054

McGranaghan, P., Davies, J. C., Griffith, G. W., Davies, D. R., and Theodorou, M. K. (1999). The survival of anaerobic fungi in cattle faeces. *FEMS Microbiol. Ecol.* 29, 293–300. doi: 10.1111/j.1574-6941.1999.tb00620.x

McSweeney, C. S., and Denman, S. E. (2007). Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. *J. Appl. Microbiol.* 103, 1757–1765. doi: 10.1111/j.1365-2672.2007.03408.x

McSweeney, C. S., Palmer, B., Bunch, R., and Krause, D. O. (2001). Effect of the tropical forage calliandra on microbial protein synthesis and ecology in the rumen. *J. Appl. Microbiol.* 90, 78–88. doi: 10.1046/j.1365-2672.2001.01220.x

Michel, V., Fonty, G., Millet, L., Bonnemoy, F., and Gouet, P. (1993). In vitro study of the proteolytic activity of rumen anaerobic fungi. *FEMS Microbiol. Lett.* 110, 5–9. doi: 10.1111/j.1574-6968.1993.tb06287.x

Milne, A., Theodorou, M. K., Jordan, M. G. C., Kingspooner, C., and Trinci, A. P. J. (1989). Survival of anaerobic fungi in feces, in saliva, and in pure culture. *Exp. Mycol.* 13, 27–37. doi: 10.1016/0147-5975(89)90005-4

Mizrahi, I. (2013). "Rumen symbioses," in *The Prokaryotes: Prokaryotic Biology and Symbiotic Associations*, eds E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson (Berlin: Springer), 533–544. doi: 10.1007/978-3-642-30194-0_1

Morais, S., and Mizrahi, I. (2019). Islands in the stream: from individual to communal fiber degradation in the rumen ecosystem. *FEMS Microbiol. Rev.* 43, 362–379. doi: 10.1093/femsre/fuz007

Morgavi, D. P., Kelly, W. J., Janssen, P. H., and Attwood, G. T. (2013). Rumen microbial (meta)genomics and its application to ruminant production. *Animal* 7(Suppl. 1), 184–201. doi: 10.1017/s1751731112000419

Morgavi, D. P., Sakurada, M., Mizokami, M., Tomita, Y., and Onodera, R. (1994). Effects of ruminal protozoa on cellulose degradation and the growth of an anaerobic ruminal fungus, *Piromyces* sp. strain OTS1, in vitro. *Appl. Environ. Microbiol.* 60, 3718–3723. doi: 10.1128/aem.60.10.3718-3723.1994

Morrison, M., Murray, R. M., and Boniface, A. N. (1990). Nutrient metabolism and rumen micro-organisms in sheep fed a poor-quality tropical grass hay supplemented with sulphate. *J. Agric. Sci.* 115, 269–275. doi: 10.1017/s0021859600075237

Müller, M. (1993). The hydrogenosome. *J. Gen. Microbiol.* 139, 2879–2889.

Mura, E., Edwards, J., Kittelmann, S., Kaerger, K., Voigt, K., Mrazek, J., et al. (2019). Anaerobic fungal communities differ along the horse digestive tract. *Fungal Biol.* 123, 240–246. doi: 10.1016/j.funbio.2018.12.004

Murphy, C. L., Youssef, N. H., Hanafy, R. A., Couger, M. B., Stajich, J. E., Wang, Y., et al. (2019). Horizontal gene transfer as an indispensable driver for evolution of *Neocallimastigomycota* into a distinct gut-dwelling fungal lineage. *Appl. Environ. Microbiol.* 85:e00988-19.

Nagaraja, T. G. (2016). "Microbiology of the rumen," in *Rumenology*, eds D. D. Millen, M. De Beni Arrigoni, and R. D. Lauritano Pacheco (Cham: Springer International Publishing), 39–61.

Nagler, M., Kozjek, K., Etemadi, M., Insam, H., and Podmirseg, S. M. (2019). Simple yet effective: microbial and biotechnological benefits of rumen liquid addition to lignocellulose-degrading biogas plants. *J. Biotechnol.* 300, 1–10. doi: 10.1016/j.jbiotec.2019.05.004

Nagler, M., Podmirseg, S. M., Griffith, G. W., Insam, H., and Ascher-Jenull, J. (2018). The use of extracellular DNA as a proxy for specific microbial activity. *Appl. Microbiol. Biotechnol.* 102, 2885–2898. doi: 10.1007/s00253-018-8786-y

Nagy, T., Tunnicliffe, R. B., Higgins, L. D., Walters, C., Gilbert, H. J., and Williamson, M. P. (2007). Characterization of a double dockerin from the cellulosome of the anaerobic fungus *Piromyces equi*. *J. Mol. Biol.* 373, 612–622. doi: 10.1016/j.jmb.2007.08.007

Obispo, N. E., and Dehority, B. A. (1992). A most probable number method for enumeration of rumen fungi with studies on factors affecting their concentration in the rumen. *J. Microbiol. Methods* 16, 259–270. doi: 10.1016/0167-7012(92)90016-w

Offre, P., Spang, A., and Schleper, C. (2013). Archaea in biogeochemical cycles. *Annu. Rev. Microbiol.* 67, 437–457. doi: 10.1146/annurev-micro-092412-155614

Orpin, C. G. (1975). Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* 91, 249–262. doi: 10.1099/00221287-91-2-249

Orpin, C. G. (1976). Studies on the rumen flagellate *Sphaeromonas communis*. *J. Gen. Microbiol.* 94, 270–280. doi: 10.1099/00221287-94-2-270

Orpin, C. G. (1977a). The occurrence of chitin in the cell walls of the rumen organisms *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*. *J. Gen. Microbiol.* 99, 215–218. doi: 10.1099/00221287-99-1-215

Orpin, C. G. (1977b). The rumen flagellate *Piromonas communis*: its life-history and invasion of plant material in the rumen. *J. Gen. Microbiol.* 99, 107–117. doi: 10.1099/00221287-99-1-107

Orpin, C. G. (1981). Isolation of cellulolytic phycomycete fungi from the caecum of the horse. *J. Gen. Microbiol.* 123, 287–296. doi: 10.1099/00221287-123-2-287

Orpin, C. G. (1994). "Anaerobic fungi: taxonomy, biology and distribution in nature," in *Anaerobic Fungi*, Vol. 12, eds D. O. Mountfort and C. G. Orpin (New York, NY: Dekker), 1–45. doi: 10.1201/9781003067085-1

Orpin, C. G., and Bountiff, L. (1978). Zoospore chemotaxis in the rumen phycomycete *Neocallimaslix frontalis*. *J. Gen. Microbiol.* 104, 113–122. doi: 10.1099/00221287-104-1-113

Orpin, C. G., and Greenwood, Y. (1986). The role of haems and related compounds in the nutrition and zoosporogenesis of the rumen chytridiomycete *Neocallimastix frontalis* H8. *Microbiology* 132, 2179–2185. doi: 10.1099/00221287-132-8-2179

Ozkose, E., Thomas, B. J., Davies, D. R., Griffith, G. W., and Theodorou, M. K. (2001). *Cyllamyces aberensis* gen.nov. sp.nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. *Can. J. Bot.* 79, 666–673. doi: 10.1139/cjb-79-6-666

Partida-Martinez, L. P., Monajembashi, S., Greulich, K. O., and Hertweck, C. (2007). Endosymbiont-dependent host reproduction maintains bacterial-fungal mutualism. *Curr. Biol.* 17, 773–777. doi: 10.1016/j.cub.2007.03.039

Paul, S. S., Bu, D., Xu, J., Hyde, K. D., and Yu, Z. (2018). A phylogenetic census of global diversity of gut anaerobic fungi and a new taxonomic framework. *Fungal Divers.* 89, 253–266. doi: 10.1007/s13225-018-0396-6

Paul, S. S., Deb, S. M., Punia, B. S., Das, K. S., Singh, G., Ashar, M. N., et al. (2011). Effect of feeding isolates of anaerobic fungus *Neocallimastix* sp. CF 17 on growth rate and fibre digestion in buffalo calves. *Arch. Anim. Nutr.* 65, 215–228. doi: 10.1080/1745039x.2011.559722

Paul, S. S., Kamra, D. N., Sastry, V. R., and Sahu, N. P. (2006). Effect of adding an anaerobic fungal culture isolated from a wild blue bull (*Boselophus tragocamelus*) to rumen fluid from buffaloes on in vitro fibrolytic enzyme activity, fermentation and degradation of tannins and tannin-containing Kachnar tree (*Bauhinia variegata*) leaves and wheat straw. *J. Sci. Food Agric.* 86, 258–270. doi: 10.1002/jsfa.2303

Paul, S. S., Kamra, D. N., Sastry, V. R., Sahu, N. P., and Kumar, A. (2003). Effect of phenolic monomers on biomass and hydrolytic enzyme activities of an

anaerobic fungus isolated from wild nil gai (*Baselophus tragocamelus*). *Lett. Appl. Microbiol.* 36, 377–381. doi: 10.1046/j.1472-765x.2003.01331.x

Paul, S. S., Kamra, D. N., Sastry, V. R. B., Sahu, N. P., and Agarwal, N. (2004). Effect of administration of an anaerobic gut fungus isolated from wild blue bull (*Boselaphus tragocamelus*) to buffaloes (*Bubalus bubalis*) on in vivo ruminal fermentation and digestion of nutrients. *Anim. Feed Sci. Technol.* 115, 143–157. doi: 10.1016/j.anifeedsci.2004.01.010

Peng, X. N., Gilmore, S. P., and O'Malley, M. A. (2016). Microbial communities for bioprocessing: lessons learned from nature. *Curr. Opin. Chem. Eng.* 14, 103–109. doi: 10.1016/j.coche.2016.09.003

Phillips, M. W., and Gordon, G. L. R. (1988). Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. *Biosystems* 21, 377–383. doi: 10.1016/0303-2647(88)90036-6

Poulsen, M., Schwab, C., Borg Jensen, B., Engberg, R. M., Spang, A., Canibe, N., et al. (2013). Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. *Nat. Commun.* 4:1428.

Promkot, C., and Wanapat, M. (2009). Effect of elemental sulfur supplementation on rumen environment parameters and utilization efficiency of fresh cassava foliage and cassava hay in dairy cattle. *Asian Australas. J. Anim. Sci.* 22, 1366–1376. doi: 10.5713/ajas.2009.90141

Reay, D. S., Smith, P., Christensen, T. R., James, R. H., and Clark, H. (2018). Methane and global environmental change. *Annu. Rev. Environ. Resour.* 43, 165–192.

Rezaeian, M., Beakes, G. W., and Parker, D. S. (2004a). Distribution and estimation of anaerobic zoosporic fungi along the digestive tracts of sheep. *Mycol. Res.* 108(Pt 10), 1227–1233. doi: 10.1017/s0953756204000929

Rezaeian, M., Beakes, G. W., and Parker, D. S. (2004b). Methods for the isolation, culture and assessment of the status of anaerobic rumen chytrids in both in vitro and in vivo systems. *Mycol. Res.* 108, 1215–1226. doi: 10.1017/s0953756204000917

Ribeiro, G. O., Gruninger, R. J., Badhan, A., and McAllister, T. A. (2016). Mining the rumen for fibrolytic feed enzymes. *Anim. Front.* 6, 20–26. doi: 10.2527/af.2016-0019

Ricard, G., McEwan, N. R., Dutilh, B. E., Jouany, J.-P., Macheboeuf, D., Mitsumori, M., et al. (2006). Horizontal gene transfer from Bacteria to rumen Ciliates indicates adaptation to their anaerobic, carbohydrates-rich environment. *BMC Genomics* 7:22. doi: 10.1186/1471-2164-7-22

Roehe, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., McKain, N., Ross, D. W., et al. (2016). Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. *PLoS Genet.* 12:e1005846. doi: 10.1371/journal.pgen.1005846

Rotterová, J., Salomaki, E., Pánek, T., Bourland, W., Žihala, D., Táborskı, P., et al. (2020). Genomics of new ciliate lineages provides insight into the evolution of obligate anaerobiosis. *Curr. Biol.* 30, 2037–2050.e6.

Saminathan, M., Kumari Ramiah, S., Gan, H. M., Abdullah, N., Wong, C. M. V. L., Ho, Y. W., et al. (2019). In vitro study on the effects of condensed tannins of different molecular weights on bovine rumen fungal population and diversity. *Ital. J. Anim. Sci.* 18, 1451–1462. doi: 10.1080/1828051x.2019.1681304

Sánchez-Rodríguez, C., Bauer, S., Hématy, K., Saxe, F., Ibáñez, A. B., Vodermaier, V., et al. (2012). Chitinase-like1/pom-pom1 and its homolog CTL2 are glucan-interacting proteins important for cellulose biosynthesis in *Arabidopsis*. *Plant Cell* 24, 589–607. doi: 10.1105/tpc.111.094672

Saxena, S., Sehgal, J., Puniya, A., and Singh, K. (2010). Effect of administration of rumen fungi on production performance of lactating buffaloes. *Benef. Microbes* 1, 183–188. doi: 10.3920/bm2009.0018

Sehgal, J. P., Jit, D., Puniya, A. K., and Singh, K. (2008). Influence of anaerobic fungal administration on growth, rumen fermentation and nutrient digestion in female buffalo calves. *J. Anim. Feed Sci.* 17, 510–518. doi: 10.22358/jafs/66678/2008

Sekine, J., Shinoda, M., Kamel, H., and Oura, R. (1995). Effect of kinds of bay on population densities of rumen anaerobic fungi. *Indian J. Anim. Sci.* 65, 1352–1355.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., et al. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 collection. *Nat. Biotechnol.* 36, 359–367. doi: 10.1038/nbt.4110

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., et al. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Res.* 24, 1517–1525. doi: 10.1101/gr.168245.113

Shirazi-Beechey, S. P. (2008). Molecular insights into dietary induced colic in the horse. *Equine Vet. J.* 40, 414–421. doi: 10.2746/042516408x314075

Sijtsma, L., and Tan, B. (1996). Degradation of perennial ryegrass leaf and stem cell walls by the anaerobic fungus *Neocallimastix* sp. strain CS3b. *Appl. Environ. Microbiol.* 62, 1437–1440. doi: 10.1128/aem.62.4.1437-1440.1996

Solomon, K. V., Haitjema, C. H., Henske, J. K., Gilmore, S. P., Borges-Rivera, D., Lipzen, A., et al. (2016). Early-branching gut fungi possess a large, comprehensive array of biomass-degrading enzymes. *Science* 351, 1192–1195. doi: 10.1126/science.aad1431

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R., and Watson, M. (2019). Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nat. Biotechnol.* 37, 953–961. doi: 10.1038/s41587-019-0202-3

Tapio, I., Fischer, D., Blasco, L., Tapio, M., Wallace, R. J., Bayat, A. R., et al. (2017). Taxon abundance, diversity, co-occurrence and network analysis of the ruminal microbiota in response to dietary changes in dairy cows. *PLoS One* 12:e0180260. doi: 10.1371/journal.pone.0180260

Tedersoo, L., Sánchez-Ramírez, S., Kõljalg, U., Bahram, M., Döring, M., Schigel, D., et al. (2018). High-level classification of the Fungi and a tool for evolutionary ecological analyses. *Fungal Divers.* 90, 135–159. doi: 10.1007/s13225-018-0401-0

Theodorou, M. K., Gill, M., King-Spooner, C., and Beever, D. E. (1990). Enumeration of anaerobic chytridiomycetes as thallus-forming units: novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. *Appl. Environ. Microbiol.* 56, 1073–1078. doi: 10.1128/aem.56.4.1073-1078.1990

Theodorou, M. K., Zhu, W. Y., Rickers, A., Nielsen, B. B., Gull, K., and Trinci, A. P. J. (1996). "Biochemistry and ecology of anaerobic fungi," in *Human and Animal Relationships*, eds D. H. Howard and J. D. Miller (Berlin: Springer), 265–295.

Trinci, A. P. J., Davies, D. R., Gull, K., Lawrence, M. I., Nielsen, B. B., Rickers, A., et al. (1994). Anaerobic fungi in herbivorous animals. *Mycol. Res.* 98, 129–152. doi: 10.1016/s0953-7562(09)80178-0

Tripathi, V. K., Sehgal, J. P., Puniya, A. K., and Singh, K. (2007). Effect of administration of anaerobic fungi isolated from cattle and wild blue bull (*Boselaphus tragocamelus*) on growth rate and fibre utilization in buffalo calves. *Arch. Anim. Nutr.* 61, 416–423. doi: 10.1080/17450390701556759

Ushida, K., Tanaka, H., and Kojima, Y. (1989). A simple in situ method for estimating fungal population size in the rumen. *Lett. Appl. Microbiol.* 9, 109–111. doi: 10.1111/j.1472-765x.1989.tb00302.x

Ushida, K., Umeda, M., Kishigami, N., and Kojima, Y. (1992). Effect of medium-chain and long-chain fatty acid calcium salts on rumen microorganisms and fiber digestion in sheep. *Nihon Chikusan Gakkaiho* 63, 591–597. doi: 10.2508/chikusan.63.591

Valle, E. R., Henderson, G., Janssen, P. H., Cox, F., Alexander, T. W., and McAllister, T. A. (2015). Considerations in the use of fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy to characterize rumen methanogens and define their spatial distributions. *Can. J. Microbiol.* 61, 417–428. doi: 10.1139/cjm-2014-0873

van der Giezen, M. (2009). Hydrogenosomes and mitosomes: conservation and evolution of functions. *J. Eukaryot. Microbiol.* 56, 221–231. doi: 10.1111/j.1550-7408.2009.00407.x

Vogels, G. D., Hoppe, W. F., and Stumm, C. K. (1980). Association of methanogenic bacteria with rumen ciliates. *Appl. Environ. Microbiol.* 40, 608–612. doi: 10.1128/aem.40.3.608-612.1980

Wallace, R. J., and Joblin, K. N. (1985). Proteolytic activity of a rumen anaerobic fungus. *FEMS Microbiol. Lett.* 29, 19–25. doi: 10.1111/j.1574-6968.1985.tb00828.x

Wallace, R. J., and Munro, C. A. (1986). Influence of the rumen anaerobic fungus *Neocallimastix frontalis* on the proteolytic activity of a defined mixture of rumen bacteria growing on a solid substrate. *Lett. Appl. Microbiol.* 3, 23–26. doi: 10.1111/j.1472-765x.1986.tb01539.x

Wallace, R. J., Sasson, G., Garnsworthy, P. C., Tapio, I., Gregson, E., Bani, P., et al. (2019). A heritable subset of the core rumen microbiome dictates dairy

cow productivity and emissions. *Sci. Adv.* 5:eaav8391. doi: 10.1126/sciadv.aav8391

Wang, X., Liu, X., and Groenewald, J. Z. (2017). Phylogeny of anaerobic fungi (phylum *Neocallimastigomycota*), with contributions from yak in China. *Antonie Van Leeuwenhoek* 110, 87–103. doi: 10.1007/s10482-016-0779-1

Wang, Y., Youssef, N. H., Couger, M. B., Hanafy, R. A., Elshahed, M. S., and Stajich, J. E. (2019). Molecular dating of the emergence of anaerobic rumen fungi and the impact of laterally acquired genes. *mSystems* 4:e00247-19.

Weston, R. H., Lindsay, J. R., Purser, D. B., Gordon, G. L. R., and Davis, P. (1988). Feed intake and digestion responses in sheep to the addition of inorganic sulfur to a herbage diet of low sulfur content. *Aust. J. Agric. Res.* 39, 1107–1119. doi: 10.1071/ar9881107

Wilson, C. A., and Wood, T. M. (1992). The anaerobic fungus *Neocallimastix frontalis*: isolation and properties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen-bond-ordered cellulose. *Appl. Microbiol. Biotechnol.* 37, 125–129.

Woese, C. R., and Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5088–5090. doi: 10.1073/pnas.74.11.5088

Wubah, D. A., Fuller, M. S., and Akin, D. E. (1991). Resistant body formation in *Neocallimastix* sp., an anaerobic fungus from the rumen of cow. *Mycologia* 83, 40–47. doi: 10.2307/3759831

Wubah, D. A., and Kim, D. S. H. (1996). Chemoattraction of anaerobic ruminai fungi zoospores to selected phenolic acids. *Microbiol. Res.* 151, 257–262. doi: 10.1016/s0944-5013(96)80022-x

Wurzbacher, C., Larsson, E., Bengtsson-Palme, J., Van den Wyngaert, S., Svantesson, S., Kristiansson, E., et al. (2019). Introducing ribosomal tandem repeat barcoding for fungi. *Mol. Ecol. Resour.* 19, 118–127. doi: 10.1111/1755-0998.12944

Xue, F., Nan, X., Sun, F., Pan, X., Guo, Y., Jiang, L., et al. (2018). Metagenome sequencing to analyze the impacts of thiamine supplementation on ruminal fungi in dairy cows fed high-concentrate diets. *AMB Express* 8:159.

Yanke, L. J., Dong, Y., McAllister, T. A., Bae, H. D., and Cheng, K. J. (1993). Comparison of amylolytic and proteolytic activities of ruminal fungi grown on cereal grains. *Can. J. Microbiol.* 39, 817–820. doi: 10.1139/m93-121

Yarlett, N., Orpin, C. G., Munn, E. A., Yarlett, N. C., and Greenwood, C. A. (1986). Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. *Biochem. J.* 236, 729–739. doi: 10.1042/bj2360729

Youssef, N. H., Couger, M. B., Struchtemeyer, C. G., Liggenstoffer, A. S., Prade, R. A., Najar, F. Z., et al. (2013). The genome of the anaerobic fungus *Orpinomyces* sp. strain C1A reveals the unique evolutionary history of a remarkable plant biomass degrader. *Appl. Environ. Microbiol.* 79, 4620–4634. doi: 10.1128/aem.00821-13

Zhang, Q., Difford, G., Sahana, G., Løvendahl, P., Lassen, J., Lund, M. S., et al. (2020). Bayesian modeling reveals host genetics associated with rumen microbiota jointly influence methane emission in dairy cows. *ISME J.* 14, 2019–2033. doi: 10.1038/s41396-020-0663-x

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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