frontiers | Research Topics

Rumen microbiome dynamics and their implications in health and environment

Edited by
Robert W. Li, Christina D. Moon and Diego P. Morgavi

Published in
Frontiers in Microbiology

ISSN 1664-8714
ISBN 978-2-8325-2753-5
DOI 10.3389/978-2-8325-2753-5

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Rumen microbiome dynamics and their implications in health and environment

Topic editors

Robert W. Li — Agricultural Research Service, United States Department of Agriculture, United States

Christina D. Moon — AgResearch Ltd, New Zealand

Diego P. Morgavi — INRAE Clermont-Auvergne-Rhône-Alpes, France

Citation

Li, R. W., Moon, C.D., Morgavi, D. P., eds. (2023). *Rumen microbiome dynamics and their implications in health and environment*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-2753-5

Table of contents

frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 06 June 2023
DOI 10.3389/fmicb.2023.1223885

OPEN ACCESS

EDITED AND REVIEWED BY
Matthias Hess,
University of California, Davis, United States

*CORRESPONDENCE
Robert W. Li
robert.li@usda.gov

RECEIVED 16 May 2023
ACCEPTED 18 May 2023
PUBLISHED 06 June 2023

CITATION
Li RW, Moon CD and Morgavi DP (2023) Editorial: Rumen microbiome dynamics and their implications in health and environment. *Front. Microbiol.* 14:1223885. doi: 10.3389/fmicb.2023.1223885

Editorial: Rumen microbiome dynamics and their implications in health and environment

Robert W. Li[1]*, Christina D. Moon[2] and Diego P. Morgavi[3]

[1]United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Beltsville, MD, United States, [2]AgResearch Ltd., Grassland Research Centre, Palmerston North, New Zealand, [3]INRAE, Clermont-Auvergne-Rhône-Alpes, Saint-Genès-Champanelle, France

KEYWORDS

rumen, microbial ecosystem, resilience, methane, ruminant (source: MeSH), inhibitor, early life

Editorial on the Research Topic
Rumen microbiome dynamics and their implications in health and environment

As part of the unique digestive system of ruminants, the rumen harbors a dazzling array of microbial diversity. As members of a complex and dynamic microbial ecosystem, bacteria, protozoa, fungi, archaea and viruses in the rumen interact with each other and contribute, individually or in concert, to rumen function. Ruminal microorganisms convert plant fiber into short-chain fatty acids (SCFA) and other metabolites for the production of meat and milk, a key determinant for feed use efficiency. Rumen microbes are also capable of detoxification and metabolizing xenobiotics, particularly aromatic compounds, and secondary plant metabolites. As a result, the rumen as an efficient bioremediation system can be used to clean up environmental toxins. Ruminal biohydrogenation, a process by which bacteria convert dietary unsaturated fatty acids to saturated fatty acids, affects fatty acid profiles of ruminant products. On the other hand, rumen methanogens are a major contributor to methane emissions; ruminant farming accounts for up to 25% of global anthropogenic methane emissions. For example, a cow can release up to 500 L of methane each day; globally, cattle production releases up to 100 million tons of methane annually. Moreover, methane is 28 times more potent than CO_2 in contributing to global warming. Dozens of methane inhibitors have been tested in the past decades. Although some of these inhibitors reduce methane production by up to 90%, obstacles prevent their widespread use. Inhibition may be transient, lasting only a few weeks and dissipates when inhibitors are withdrawn. When used at doses required to achieve strong effects, many methane inhibitors interfere with feed intake, digestion, and rumen fermentation, harming production traits such as live weight gain. Interventions that incur costs and losses to producers hamper sustainable technology transfer.

The collective efforts of a total of 97 authors from 16 countries, from Austria to the United States of America, resulted in the publication of 15 papers in the Research Topic entitled "Rumen microbiome dynamics and their implications in health and environment." This special collection covers a broad range of research subjects related to the rumen microbiome and its manipulation. The host species examined include multiple breeds of major ruminant species, sheep, goats, and cattle, under various production systems.

First, Lobo and Faciola conducted a comprehensive review of the recent advances in ruminal viruses that infect not only bacteria (bacteriophages or phages), but also archaea (archaeaphages) and the eukaryotes fungi (mycophages) and protozoa as well as their role in modulating the interactions among other ruminal microorganisms. These viruses, resident or transient in the rumen environment, are concentrated in a handful of viral families and associated with the dominant ruminal bacterial phyla. The authors also discussed the potential application of ruminal phage therapy in livestock production, including in controlling pathogens and microbes harboring antibiotic resistant genes, reducing methane production, and regulating gut homeostasis, such as reverting rumen dysbiosis and alleviating ruminal acidosis.

Five publications in this collection investigated the establishment and development of the rumen microbiota during early life in three major ruminant species, sheep, goats and cattle. Mao et al. compared two weaning schemes, 30-day weaning and 45-day weaning, as well as their respective 5-days post-weaning controls in male Hu lambs, on the rumen bacterial and archaeal colonization and development. While the weaning schemes have little effect on the rumen archaeal population, the rumen bacterial communities became more stable and experienced less fluctuation at the later weaning age, suggesting that later weaning may benefit the rumen microbial establishment. Further, several taxa, such as *Fibrobacter*, had a significant and positive correlation with rumen papillae length. In another study (Yin et al.), the rumen bacterial establishment in the same sheep breed was monitored from birth to 120 days of age. The authors concluded that the rumen microbial community and function of rumen, as judged by its lack of resilience and resistance to disturbances, are not well-established before lambs reach 20 days of age, providing a window of opportunities for ruminal intervention for desired growth performance at later stages. Artiles-Ortega et al. attempted to understand if prenatal exposure to a protein-rich legume *Leucaena leucocephala* with toxic secondary metabolites would affect post-weaning performance and immune status in goat kids. Their findings suggest that the prenatal adaptation increases dry matter intake after weaning, resulting in higher daily bodyweight gain. Furthermore, postnatal supplementation of live yeast favors the maturation of the rumen bacterial community and protozoa colonization; and early-life dietary interventions can have persistent effects on production traits. In calves, Huuki et al. demonstrated that rumen fluid from an adult cow administered fresh to calves for 6 weeks prior to weaning enhanced the maturation of rumen bacterial and archaeal communities and resulted in improved feed intake and significant weight gain compared to untreated monozygotic twin calves used as controls. Cristobal-Carballo et al. investigated the effect of divergent feeding regimes during the first 41 weeks of life of Hereford–Friesian-cross female calves on long-term rumen fermentation performance and found that the rumen microbiota and associated fermentation end-products are largely driven by the diet consumed at the time of sampling and that early dietary interventions have little detectable long-term microbial imprint potential on rumen function.

Beef production relying on the use of resilient (rustic) breeds may alleviate some of the challenges posed by climate change, due to their ability for better adaptation and efficient utilization of low-quality fibers. Daghio et al. compared the rumen microbial composition and metabolites as well as growth traits of two rustic breeds under two production systems, feedlot and pasture grazing. Their findings suggest that while the production system shapes the rumen microbiome structure, the cattle breed is the main factor that influences bacterial communities, supporting the notion that host genetics may play an important role in determining rumen microbial composition. Furthermore, steer performance is likely affected by the rumen capacity for SCFA production and the presence of hydrogen sinks that divert hydrogen to processes alternative to methanogenesis. In dairy cattle, progressive mechanisms of adaptation in the rumen and hindgut of cows fed increasing amounts of starch with or without a phytogenic feed additive have been investigated by Ricci et al.. The gradual inclusion of starch in the diet and the phytogenic additive altered microbiota composition and metabolic activity in all gut compartments surveyed. Further, the microbiota has capacity to adjust differentially, yet rapidly, to changing dietary conditions. Last, Li et al. examined the effect of herbal tea residue on the growth performance of Simmental crossbred finishing steers. Their findings demonstrate that while herbal tea residue does not appear to promote cattle growth, its supplementation tend to improve rumen fermentation by increasing the propionic acid concentration and the propionate-to-acetate ratio, indirectly leading to the improvement of muscle quality, such as tenderness and oleic acid and linoleic acid concentrations in the longissimus dorsi. Together, this study provides evidence that herbal tea residues can be used as a functional roughage to modulate rumen fatty acid composition and muscle glucolipid metabolism.

Six papers in this collection aimed to develop effective methane mitigation strategies in ruminants. Brede et al. used the rumen simulation technique (RUSITEC) in a 38-day trial to investigate the effect of a feed additive consisting of garlic powder and bitter orange extracts on rumen fermentation and methane production. This feed additive does not appear to affect the microbial or prokaryote population but does alter ruminal SCFA concentration profiles. The intervention led to a transient reduction in the abundance of *Methanomicrobia*, the most abundant archaeal group in the system, and a concomitant reduction in the copy number of a key gene in methanogenesis that encodes methyl coenzyme-M reductase, resulting in a transient inhibition in methane production. However, the practical impact of the feed additive on production settings remained to be documented. Künzel et al. used a similar RUSITEC system to understand how two brown seaweeds from Iceland affect methanogenesis and nutrient conversion. Their findings demonstrated that while the inclusion of two dose levels of both seaweeds tested shows a small yet significant reduction in methane production, the intervention also resulted in a concomitant reduction on overall rumen fermentation, which raised serious questions of their utilization by farmers. Bharanidharan et al. also evaluated the potential of seeds of *Pharbitis nil*, a plant species widely distributed in East Asia, as a possible dietary strategy for ruminal methane mitigation using *in vitro* and *in sacco* methods. The plant indeed inhibited methanogenesis by up to 50%, depending on the dose tested. Furthermore, this plant can also modulate the rumen

microbiota, including decreasing the Bacteroidetes-to-Firmicutes ratio. The additional benefit of this plant as feed additive lies in its antiprotozoal potential. In addition to the *in vitro* studies, Thirumalaisamy et al. examined the effect of a long-term supplementation of silkworm pupae oil on methane emissions and production traits in adult sheep. A daily silkworm pupae oil supplementation for 180 days reduced methane emissions up to 25% while maintaining higher bodyweight gains. However, the study also confirmed the transient nature of silkworm pupae oil on methane inhibition where daily methane emissions reverted to pre-supplementation levels shortly after the supplementation ceased. Similarly, methane emissions can be decreased by up to 90% in calves receiving a combination of two known methane inhibitors, chloroform and 9,10-anthraquinone, in the first 12 weeks of life (Cristobal-Carballo et al.), compared to control calves. Moreover, no negative impacts of the treatment on dry matter intake and growth were noticed. Similar to the *in vitro* data, these methanogen inhibitors have limited effect on rumen bacterial communities while reducing the abundance of methanogens, such as *Methanobrevibacter* and *Methanosphaera*. However, once methane inhibition ceased, the methanogen community, rumen metabolites and hydrogen emissions reversed to the baseline, providing further evidence that imprinting a rumen microbiota with lower methane yield during early life is still difficult.

To overcome the difficulties of transient methanogenesis inhibition of known methane inhibitors and natural products for on-farm pragmatic applications, Smith et al. took a different approach, aiming to develop strategies for genetic selection to achieve permanent and accumulative reductions to the methane emission related to livestock farming. Using residual methane emissions (RME) as an optimal phenotype for assessing the methanogenic potential of ruminants, they measured RME for 282 crossbred finishing beef cattle and then ranked the animals with high or low RME, differing in ∼30% difference in RME. The rumen in the low-RME cattle tends to have higher abundances of lactic-acid-producing bacteria (LAB) as well as significantly higher abundances of methanogens, such as *Methanosphaera*, the latter of which is likely under the control of host genetics and negatively correlated with RME and positively correlated with rumen propionate levels. These taxa can be used as potential biomarkers of the methanogenic potential in beef cattle. The findings from this study likely generated microbial targets for future environmentally focused breeding programs.

The valuable contribution of this special collection to the rumen microbiology literature is vividly evident by its initial impacts, a total of 6,367 downloads and 30,255 views during the first few months of its inception. The collection was ranked one of Outstanding Research Topics of 2022 by Frontiers. The findings from these studies provide novel insights into host-rumen microbe interactions, which should facilitate the development and optimization of ruminal manipulation and enteric methane control strategies. We wish to express our gratitude to all contributing authors for their outstanding research efforts. Without their hard work, this collection would not have come into existence. Finally, we want to thank the editorial board members and staff of Frontiers in Microbiology, particularly the section editors of Systems Microbiology, for their support to this Research Topic.

Author contributions

RL: conceptualization, data curation, and writing—original draft preparation. CM and DM: writing—revision. All authors contributed to the article and approved the submitted version.

Conflict of interest

CM was employed by AgResearch Ltd.

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.

Publisher's note

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 March 2021
doi: 10.3389/fmicb.2021.636865

Weaning Age Affects the Development of the Ruminal Bacterial and Archaeal Community in Hu Lambs During Early Life

Huiling Mao[1,2], Yanfang Zhang[1], Yan Yun[1], Wenwen Ji[1], Zhao Jin[1], Chong Wang[1]* and Zhongtang Yu[2]*

[1] College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A & F University, Hangzhou, China,
[2] Department of Animal Sciences, The Ohio State University, Columbus, OH, United States

OPEN ACCESS

Edited by:
Diego P. Morgavi, INRAE Clermont-Auvergne-Rhône-Alpes, France

Reviewed by:
Charles James Newbold, Scotland's Rural College, United Kingdom
Yue-Qin Tang, Sichuan University, China

***Correspondence:**
Huiling Mao
maohl@zafu.edu.cn
Chong Wang
wangcong992@163.com

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *02 December 2020*
Accepted: *25 February 2021*
Published: *23 March 2021*

Citation:
Mao H, Zhang Y, Yun Y, Ji W, Jin Z, Wang C and Yu Z (2021) Weaning Age Affects the Development of the Ruminal Bacterial and Archaeal Community in Hu Lambs During Early Life. Front. Microbiol. 12:636865. doi: 10.3389/fmicb.2021.636865

Weaning plays an important role in many animal processes, including the development of the rumen microbiota in ruminants. Attaining a better understanding of the development of the rumen microbial community at different weaning stages can aid the identification of the optimal weaning age. We investigated the effects of weaning age on ruminal bacterial and archaeal communities in Hu lambs. Thirty male Hu lambs were randomly assigned to two weaning-age groups: a group weaned at 30 days of age (W30) and a group weaned at 45 days of age (W45), with each group having five replicate pens. On the weaning day (day 30 for W30 and day 45 for W45) and at 5 days postweaning [day 35 for W30 (PW30) and day 50 for W45 (PW45)], one lamb from each replicate was randomly selected and sacrificed. Rumen contents were collected to examine the ruminal microbiota. Compared to W30, PW30 had a decreased relative abundance of *Bacteroidetes*. At genus level, the extended milk replacer feeding (W45 vs. W30) increased the relative abundance of *Ruminococcus* while decreased that of *Prevotella* and *Dialister*. Compared to W30, PW30 exhibited decreased relative abundances of *Prevotella*, *Dialister* and *Bacteroides* but an increased unclassified *Coriobacteriaceae*. No significant difference was noted in the detected archaeal taxa among the animals. The function "biosynthesis of secondary metabolites" was less predominant in PW30 than in W30, whereas the opposite held true for "metabolism of cofactors and vitamins." Some bacterial genera were significantly correlated with rumen volatile fatty acid (VFA) concentration or other animal measures, including negative correlations between ruminal VFA concentration and unclassified *Mogibacteriaceae* and unclassified *Veillonellaceae*; positive correlations of ruminal papillae length with *Fibrobacter* and *unclassified Lachnospiraceae,* but negative correlations with *Mitsuokella* and *Succiniclasticum*; and negative correlations between plasma D-lactate concentration and *Prevotella*, unclassified *Paraprevotellaceae*, and *Desulfovibrio*. Our results revealed that the ruminal bacterial community underwent

larger changes over time in lambs weaned at 30 days of age than in lambs weaned half a month later. Thus, extending milk replacer feeding to 45 days weaning was recommended from the perspective of the rumen microbial community in the Hu lamb industry.

Keywords: weaning age, postweaning, lamb, rumen, bacteria, archaea, weaning stress

INTRODUCTION

Early weaning of production animals has many merits; for example, it enhances growth, feedlot performance (Myers et al., 1999a) and carcass quality (Myers et al., 1999b) and shortens reproduction cycles in dams (Thu and Trach, 2012). However, early weaning is a potent stressor that increases morbidity and mortality in young animals, including lambs (Li et al., 2018). The extreme dietary shift imposed by weaning can influence the severity of production losses through the weaning transition (Meale et al., 2016; Mao et al., 2019a). To reduce weaning stress, a weaning strategy is required in which young animals are weaned when they reach solid food and milk or milk replacer allowances are gradually decreased.

During and immediately after birth, the digestive tract of lambs is rapidly colonized by microbiota. Many factors influence microbial colonization, such as the dam's vaginal microbiome and the types of microbes in the surrounding environment (Meale et al., 2016). Some researchers have found that the rumen microbiome shifts with host age (Jami et al., 2013; Rey et al., 2013). Some studies have investigated the transition of the rumen microbiome induced by weaning (Meale et al., 2017; Li C. et al., 2020). Wang et al. (2016) reported that the influence of weaning on ruminal microflora probably came from the stress of physiology and psychology. Li Y. et al. (2020) have made a research on the dynamics of young forest musk deer intestinal microbiota during the weaning transition, and they believed that weaning stress may affect the composition of the intestinal microbiota. However, the stress of weaning at different ages on the rumen microbiome remains poorly understood. In this study, the effect of weaning age (two ages) on the ruminal microbial communities of Hu lambs was investigated, and differences between postweaning intervals at each weaning age were evaluated. The aim was to identify an appropriate weaning age for lambs from the perspective of the rumen microbial community.

MATERIALS AND METHODS

Study Animals and Sample Collection

As part of a previous study (Mao et al., 2019b), rumen samples were collected from Hu lambs. Briefly, 30 healthy male Hu lambs (3.60 ± 0.37 kg) at 5 days of age were selected, maintained at a breeding farm and fed milk replacer (MR). The lambs were randomly assigned to two weaning-age groups: one group weaned at 30 days of age (W30) and one group weaned at 45 days of age (W45). Each group has 15 lambs, and three lambs with similar body weight within the same group were placed in one pen. Individual pens were considered as experimental replicates (n = 5). On the weaning day (day 30 for the W30 group and day 45 for the W45 group) and at five days postweaning [day 35 for the W30 group (PW30) and day 50 for the W45 group (PW45)], one lamb of each replicate per group was randomly selected and sacrificed (n = 5). All the lambs were also offered, *ad libitum*, starter pellets and hay of Chinese wild rye from day 10 (**Supplementary Table 1**). Body weight were recorded for 2 consecutive days every 10 days before morning feeding and average daily gain was calculated. Before slaughter, blood samples were collected, and plasma was prepared to determine the concentration of D-lactate. Immediately after slaughter, rumen content samples were collected and stored at −80°C for subsequent microbial DNA extraction, and rumen tissues were sampled to measure ruminal papillae length. Additional procedures are as described in Mao et al. (2019b), with the exception that in the present study, data collected on the day of animal sacrifice were used for bacterial and archaeal analysis.

DNA Extraction and Data Processing

The cetyltrimethyl ammonium bromide method as described by Gagen et al. (2010) was used to extract the total DNA of the rumen content samples. The hypervariable V3-V4 region of the bacterial and archaeal 16S rRNA gene was amplified using primers 341F (5′-ACTCCTACGGGRSGCAGCAG-3′) and 806F (5′-GGACTACVVGGGTATCTAATC-3′) (Wang and Qian, 2009). Each primer had six base barcodes to identify samples. The amplicon libraries were pooled at an equimolar ratio and sequenced using the 2 × 250 paired-end protocol on an Illumina HiSeq platform (Realbio Genomics Institute, Shanghai, China).

Microbiome bioinformatics were performed with QIIME 2 (Bolyen et al., 2018). The paired-end sequencing reads were assembled using the PANDAseq assembler (Masella et al., 2012). Operational taxonomic units (OTUs) were clustered at a 97% identity threshold, and taxa were assigned using the core set in the Greengenes 16S reference database (13_8 version) (McDonald et al., 2012). Alpha diversity measurements of the ruminal bacterial and archaeal communities including the observed_OTUs, Shannon index (Shannon and Weaver, 1949), Faith's phylogenetic diversity (Faith, 1992), and evenness calculated by QIIME 2. The overall dissimilarity of the microbial community between weaning ages was evaluated by principal coordinates analysis (PCoA) based on unweighted

Abbreviations: ANOSIM, analysis of similarity; ANOVA, analysis of variance; DNA, deoxyribonucleic acid; KO, KEGG orthology; LEfSe, linear discriminate analysis effect size; MR, milk replacer; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; VFA, volatile fatty acid.

UniFrac distances (Lozupone and Knight, 2005). The significance of differences between groups was tested by analysis of similarity (ANOSIM). A significant difference was declared at $0.5 < R < 0.75$ with $P < 0.05$, whereas a trend was declared at $0.3 < R < 0.5$ with $P < 0.05$, and no difference was declared at $R < 0.3$. Data on plasma D-lactate concentration, rumen papillae length and width, and ruminal total VFA concentrations were obtained from our previous study (Mao et al., 2019b) and analyzed for correlations with bacterial taxa. Based on comparison of the 16S rRNA gene sequences against a Green genes reference taxonomy (Green genes 13.8), the functional capabilities of the rumen microbiota were predicted by using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013). Briefly, after the abundance of each OTU was normalized to marker gene copy number, the KEGG database was used to predict the functions.

Statistical Analysis

The alpha diversity indices (observed numbers of OTUs, Shannon index, Faith's phylogenetic diversity, and evenness) of bacteria and archaea, and phenotypic data (plasma D-lactate concentration, ruminal papillae length and width, and ruminal VFA concentration) were analyzed by one-way ANOVA using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC, United States). Statistical significance was set at $P \leq 0.05$. For the relative abundances of rumen bacteria, all the taxa analyzed in the present study were identified in at least three lambs at each group. It meant that only the bacteria that were observed in at least 60% of samples were considered in the relative abundance analysis. To show the changes in bacterial relative abundances, bacterial data were subjected to LEfSe analysis (Segata et al., 2011). A significant change was observed with a LDA (Linear Discriminant Analysis) score > 2.0 calculated by LEfSe. Spearman's rank correlations between the relative abundances of major (ruminal bacterial and archaeal taxa (i.e., taxa with relative abundances > 0.5%) and plasma D-lactate, ruminal papillae length and width, and ruminal VFA concentration were analyzed using the PROC CORR procedure of SAS. The correlation coefficients were plotted using GraphPad Prism 7.

RESULTS

Bacterial Community Composition

At least 43,063 sequences were obtained from each sample, and 575 operational taxonomic units (OTUs, 144 ± 28 OTUs per sample) defined based on 97% similarity were detected. Good's coverage for each sample was greater than 99%, indicating sufficient sequencing depth to detect most of the rumen bacteria of the Hu lambs in this study. As shown in **Table 1**, extending MR feeding for 15 days increased the number of observed OTUs and Faith's phylogenetic diversity ($P < 0.01$), but there was no significant difference in any of the alpha diversity indices between weaning and postweaning ($P > 0.05$).

The PCoA showed that extending the period of MR feeding significantly altered the rumen bacterial composition (**Figure 1A**). ANOSIM revealed significant differences in bacterial community composition between treatments W30 and PW30 ($R = 0.594$, $P = 0.032$), W30 and W45 ($R = 0.521$, $P = 0.032$), and PW30 and PW45 ($R = 0.646$, $P = 0.027$). No significant difference was found between treatments W45 and PW45 ($R = 0$, $P = 0.493$).

TABLE 1 | Alpha diversity index values of ruminal bacteria and archaea in different groups.

Domain	Indices	Treatments				SEM	*P*-value
		W30	PW30	W45	PW45		
Bacteria	Observed OTUs	123^{b}	121^{b}	164^{a}	167^{a}	10.0	<0.01
	Shannon	5.00^{ab}	4.38^{b}	4.92^{ab}	5.30^{a}	0.260	0.15
	PD index[1]	9.81^{b}	9.35^{b}	11.9^{a}	12.3^{a}	0.51	<0.01
	Evenness	0.72	0.63	0.67	0.72	0.034	0.25
Archaea	Observed OTUs	3^{b}	6^{a}	6^{a}	8^{a}	0.74	<0.01
	Shannon	0.64^{b}	1.22^{b}	1.40^{ab}	2.19^{a}	0.263	0.01
	PD index[1]	0.11^{b}	0.20^{a}	0.21^{a}	0.23^{a}	0.017	<0.01
	Evenness	0.33^{b}	0.49^{ab}	0.54^{ab}	0.73^{a}	0.087	0.05

[1]*Faith's phylogenetic diversity.*

[a,b]*Means within a row with different superscripts differ significantly ($P < 0.05$).*

Ten bacterial phyla were detected. *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria* were the four predominant phyla, representing 55.7, 25.7, 16.3, and 1.91% of the total sequences, respectively (**Figure 2A**). The relative abundance of *Bacteroidetes and Proteobacteria* were increased in W30 compared with PW30 (**Figure 3A**). However, there were no significant differences in the relative abundances of any of the phyla between W45 and PW45. A total of 108 bacterial genera were detected, of which 29 had relative abundances greater than 0.5% (**Figure 2B**). With the extension of MR feeding (W45 vs. W30; **Figure 3B**), the relative abundances of *Prevotella* and *Dialister* decreased, whereas the abundance of *Ruminococcus* increased. Among the lambs weaned at 30 days (**Figure 3C**), the abundances of *Prevotella*, *Dialister* and *Bacteroides* decreased from weaning to 5 days postweaning, whereas *UN_Coriobacteriaceae* abundance increased. However, there was no differentially abundant features were found between weaning and postweaning among the lambs weaned at 45 days.

Archaeal Community Composition

Following denoising and chimera checking, an average of 4,686 archaeal sequences per rumen sample were obtained, with an average of six OTUs per sample. Analysis of the archaeal 16S rRNA gene sequences revealed that weaning age had a significant effect ($P < 0.01$) on the number of observed OTUs and Faith's phylogenetic diversity (**Table 1**) and that both of these alpha diversity indexes increased from weaning to postweaning ($P < 0.01$) among the lambs weaned at 30 days but not among those weaned at 45 days (**Table 1**). A PCoA of overall archaeal community diversity was performed to examine differences among the four treatments (**Figure 1B**). ANOSIM revealed significant differences in archaeal community composition between treatments W30 and PW30 ($R = 0.625$, $P = 0.036$) and treatments W30 and W45 ($R = 0.562$, $P = 0.038$).

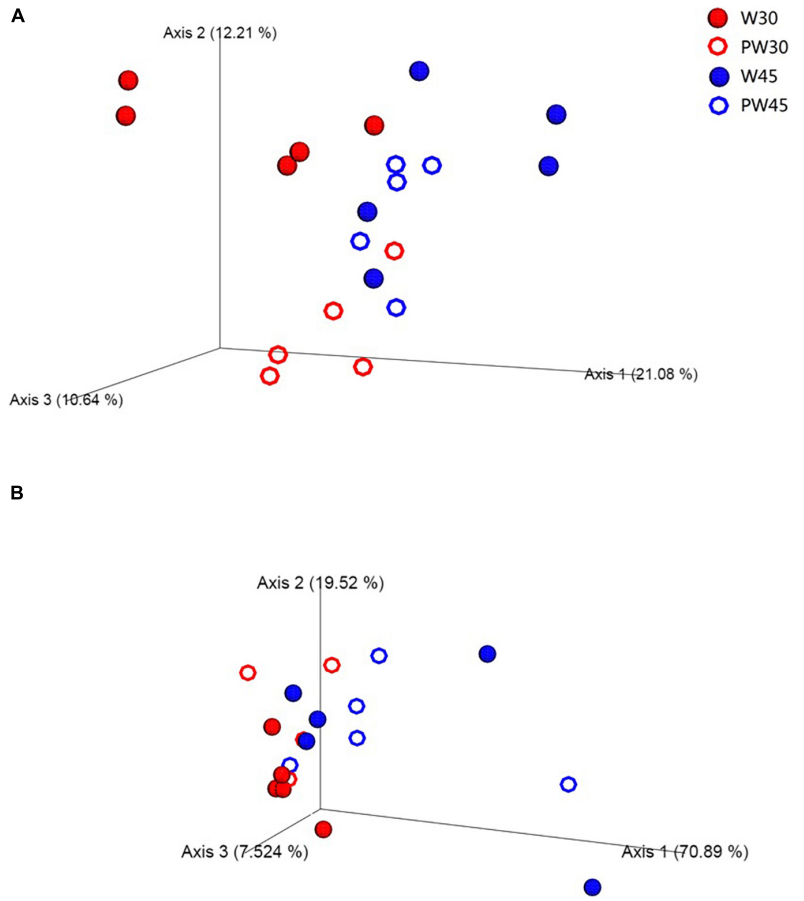


FIGURE 1 | Principal coordinates analysis (PCoA) of bacterial **(A)** and archaeal **(B)** communities in the rumen based on unweighted UniFrac distances.

However, there was no significant difference between treatments in any other comparisons ($P > 0.05$).

Only one phylum, *Euryarchaeota*, was identified in all the rumen samples. We detected two genera of *Euryarchaeota* in all of the treatments, with *Methanobrevibacter* being much more predominant (92.3–97%) than *Methanosphaera* (3–7.7%). There was no significant difference in the relative abundance of either of these two genera among the four treatment groups ($P > 0.05$).

Predicted Rumen Microbial Functions

The functional features predicted from the 16S rRNA sequences did not differ ($P > 0.05$) in relative abundance between the two weaning ages (W30 vs. W45) or between weaning and 5 days postweaning among the lambs weaned at 45 days (W45 vs. PW45). However, the relative abundance of sequences associated with the function "biosynthesis of secondary metabolites" significantly increased ($P < 0.05$) from weaning to postweaning among the lambs weaned at 30 days (W30 vs. PW30) (**Figure 4**).

Relationships Between Bacterial Taxa and Phenotypic Variables

The plasma D-lactate, VFA, ruminal papillae length and width of W30 and W45 were obtained from our previous study (Mao et al., 2019b), and re-analyzed with data of 20 lambs for bacteria analysis (**Table 2**). Correlation analysis showed that plasma D-lactate was negatively correlated (**Figure 5**) with the relative abundances of *Prevotella* ($P < 0.05$), *unclassified Paraprevotellaceae* ($P < 0.01$) and *Desulfovibrio* ($P < 0.05$). Ruminal papillae length was positively correlated with the relative abundances of *Fibrobacter* ($P < 0.05$) and unclassified *Lachnospiraceae* ($P < 0.05$) but negatively correlated with the relative abundance of *Mitsuokella* ($P < 0.05$). Ruminal papillae width was negatively correlated with only the relative abundance of *Succiniclasticum* ($P < 0.05$). Ruminal total VFA concentration was negatively correlated with the relative abundances of unclassified genera within *Mogibacteriaceae* ($P < 0.05$) and *Veillonellaceae* ($P < 0.05$).

DISCUSSION

Understanding the impacts of weaning age on animal growth, rumen tissue structure, and the structure and functionality of the rumen microbiome during early life and the changes that occur postweaning can aid the identification of an appropriate weaning age for achieving optimal health and growth performance

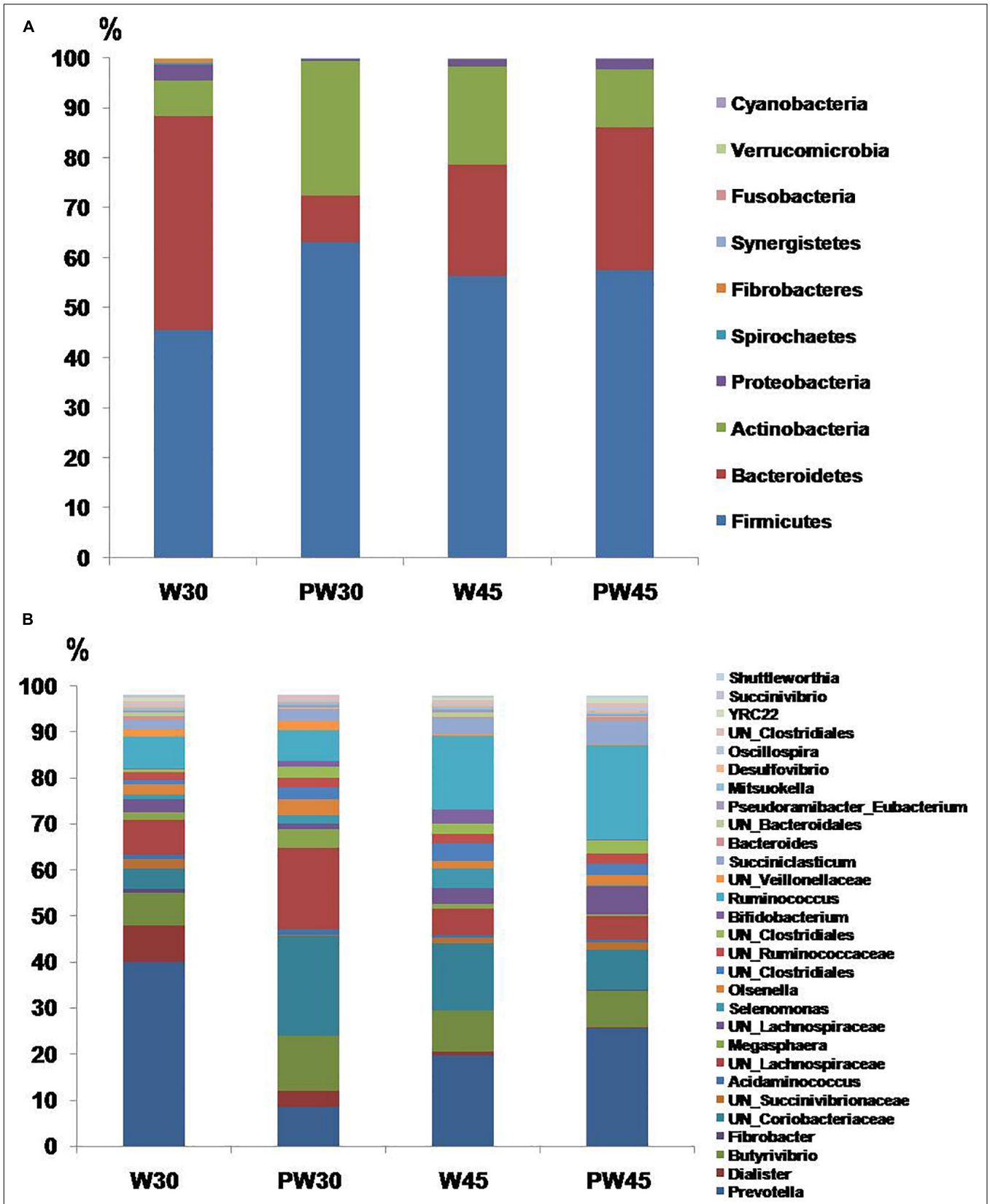


FIGURE 2 | Ruminal bacterial phyla **(A)** and genera **(B)** in the four groups. Only those genera with relative abundances of >0.5% are shown.

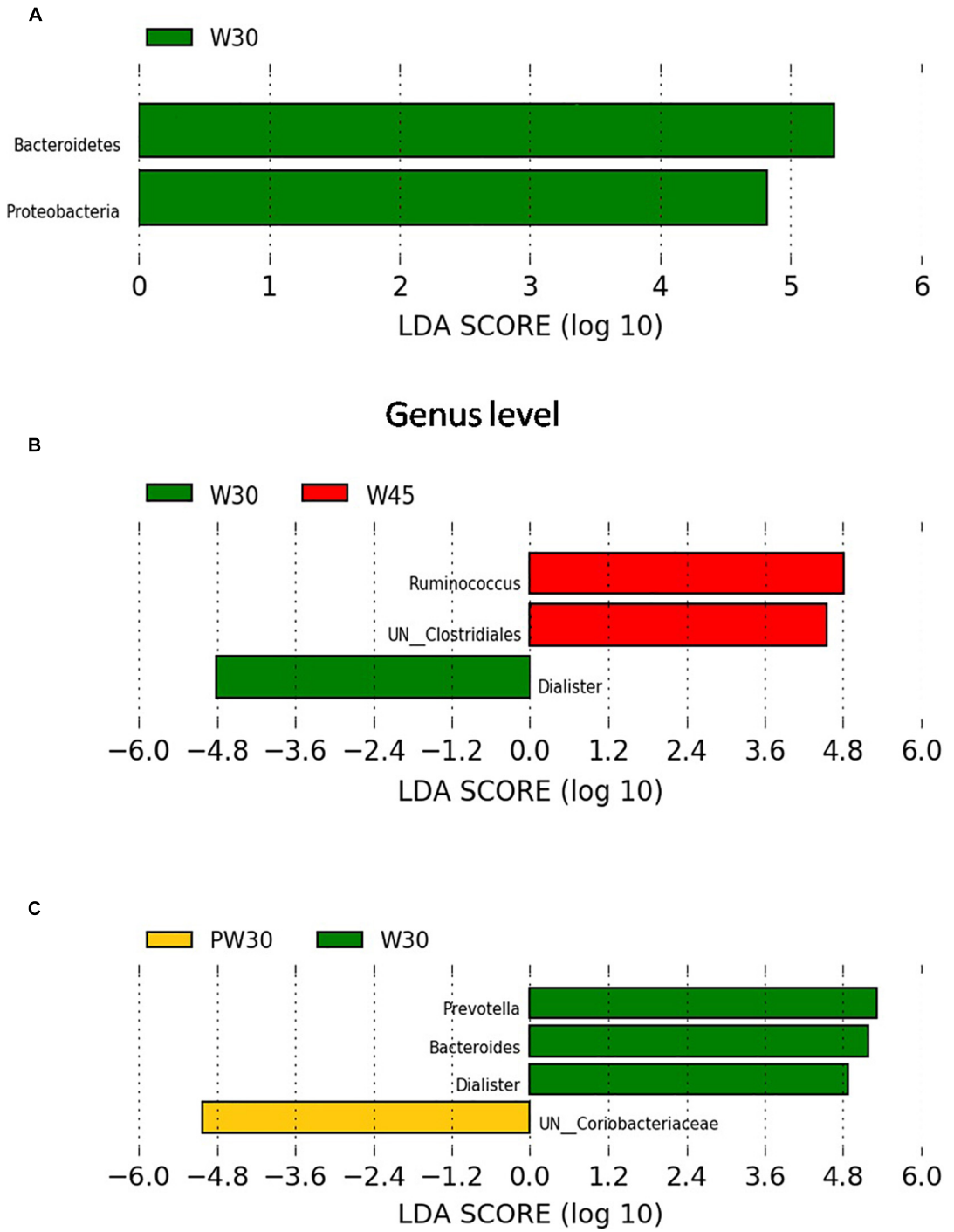


FIGURE 3 | Phylum level changes between W30 and PW30 **(A)**, no differentially abundant features were found neither between W30 and W45 nor between W45 and PW45. Genus level changes between W30 and W45 **(B)**, and between W30 and PW30 **(C)**, no differentially abundant features were found between W45 and PW45.

throughout adulthood. In this study, we characterized the rumen microbiota at two different weaning ages (30 vs. 45 days) and the changes that occur postweaning (5 d) in the diversity, composition and predicted functional features of the rumen bacterial and archaeal communities. The lambs in the two weaning-age groups received the same diet and exhibited similar

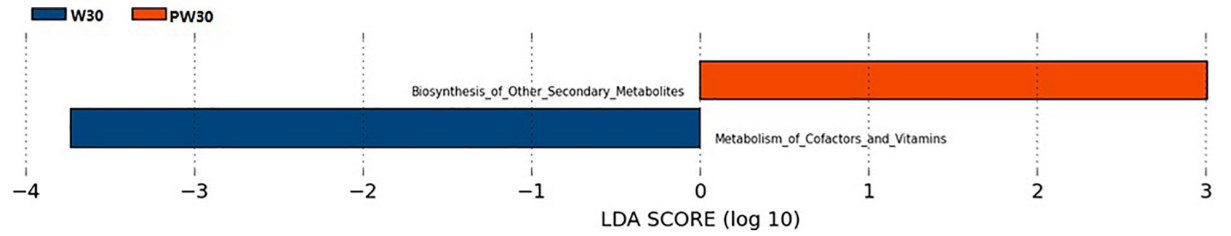


FIGURE 4 | Differences in predicted metagenomic functions of rumen bacteria between the W30 and PW30 groups of lambs. No functional feature was found to differ between W45 and PW45 lambs by LEfSe.

levels of feed intake (Mao et al., 2019b), allowing us to determine the effects of weaning age on the aforementioned measures. Additionally, we investigated whether weaning stress influenced microbial colonization in the rumen, information that may aid the selection of an appropriate weaning age.

The gastrointestinal tract of most newborn animals is thought to be sterile, but at birth, microbes from the surrounding environment and the dam rapidly colonize the rumen (Yáñez-Ruiz et al., 2015). Jami et al. (2013) reported that some rumen bacteria essential for mature rumen function were present as early as 1 day after birth, and Li et al. (2012) found that all major types of rumen bacteria could be detected in the rumen of 14-day-old calves. Guzman et al. (2015) suggested that inoculation occurs even before birth, noting that members of typical functional bacteria have been found in the rumen of calves less than 20 min after birth. However, there was no significant difference in abundance of any of the top four bacterial phyla between the two weaning ages (W30 and W45) in this study. This finding suggests that the rumen microbiota might have largely achieved stability by the age of 30 days while lambs were consuming the same diet. This interpretation is in general agreement with Yáñez-Ruiz et al. (2015) finding that the rumen microbiota no longer exhibited clear temporal changes at the phylum level beyond 15 days of age. Furthermore, Meale et al. (2016) suggested that the developing gut (preweaning) contains the same dominant phyla as the more mature gut, although the relative abundances vary with developmental stage. In the present study, the relative abundance of *Bacteroidetes* decreased from 0 to 5 days postweaning among the lambs weaned early (at day 30). Interestingly, none of the major bacterial phyla exhibited significant changes in relative abundance from 0 to 5 days postweaning among the lambs weaned at 45 days of age. This finding suggests that early weaning stress could have a negative effect on rumen development. *Bacteroidetes* plays a role in the normal development of the digestive tract, affecting traits such as the growth and volume increase of the rumen (Thomas et al., 2011; Lin et al., 2018).

Seven genera exhibited significant differences in relative abundance among treatments. While it did not mean these seven microbial genera have the most important functions in the complex rumen environment. Many microbes share the same metabolic pathways in the rumen; thus, a significant change in microbial community composition may not entail a shift in function (Yang et al., 2018). Consistent with this observation, Li et al. (2012) found that all of the functional classes in the rumen were similar between 14-day-old and 42-day-oldcalves. In this study, extension of the MR feeding period decreased the abundances of *Prevotella* and *Dialister* and increased the abundance of *Ruminococcus*. *Prevotella* strains are highly amylolytic and proteolytic (Matsui et al., 2000; Xu and Gordon, 2003). Members of *Dialister* have been reported to play roles in altering the buffering capacity of the rumen and fluid turnover (Myer et al., 2015). The members of *Ruminococcus* are cellulolytic, fiber-degrading bacteria (Ezaki, 2015) and can produce all organic acids. Zhong et al. (2019) found that increasing the relative abundance of *Ruminococcaceae* increased butyrate production during the transition from weaning to postweaning, which stimulating jejunal adaption toward gut health in piglets. The results of the present study suggested that extending the duration of MR feeding induces microbial changes and potentially affects rumen digestion. From weaning to postweaning, the abundances of *Prevotella*, *Dialister*, *and Bacteroides* decreased, and the abundance of unclassified Coriobacteriaceae increased among lambs weaned at 30 days of age, whereas no abundance differences between weaning and postweaning were observed among lambs weaned at day 45. These findings suggest that early weaning stress could change the microbial composition, and Bailey et al. (2011) also believed that the relative abundance of bacteria in the genus *Bacteroides* decrease under the influence of stressors. Weaning stress may be an reason for the decrease in *Bacteroides* after weaning (Li Y. et al., 2020). Additionally, extending the period of MR feeding seems to increase rumen microbial diversity. A high level of diversity could provide "functional redundancy" that makes the ecosystem more resilient to environmental stressors and more

TABLE 2 | Phenotypic variables of Hu lambs of different groups.

Items	Treatments				SEM	*P*-value
	W30	PW30	W45	PW45		
D-lactate, ng/mL	1024^{ab}	1081^{a}	950^{c}	976^{bc}	20.4	<0.01
Rumen papillae						
Length, μm	581^{b}	814^{ab}	985^{ab}	1307^{a}	153	0.08
Width, μm	323	362	369	383	36.6	0.26
Total VFA, mg/ml	1.58^{ab}	2.38^{a}	2.50^{a}	1.08^{b}	0.314	0.05

^{a,b,c}*Means within a row with different superscripts differ significantly ($P < 0.05$).*

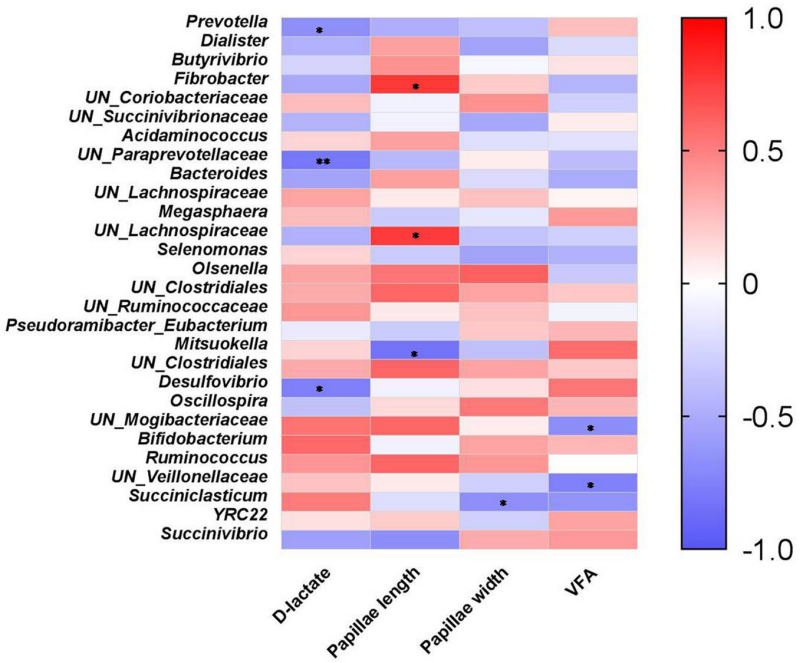


FIGURE 5 | Spearman's correlation coefficients for individual major rumen bacterial genera (each with >0.5% relative abundance) and phenotypic variables. Color represents the correlation coefficient, with red representing a positive correlation and blue denoting a negative correlation. $^{*}P < 0.05$ and $^{**}P < 0.01$.

stable (Konopka, 2009). In our previous study (Mao et al., 2017), rumen microbial changes in response to weaning were observed; however, only some known cellulolytic bacteria were quantified, and postweaning changes were not investigated. Therefore, the present study provides a more comprehensive assessment of how weaning affects rumen microbial colonization, taking weaning stress into consideration.

In the present study, only *Euryarchaeota* phylum was identified in all the rumen samples. This finding was similar to Kumar et al. (2015), who reported *Euryarchaeota* was the dominant archaea phylum in the rumen microbiome. Moreover, archaeal communities were concluded to less diverse (3–4% of the rumen microbiome) and less variable than other microbial domains (Jeyanathan et al., 2011), which might be due to the establishment of stable methanogenic communities in the rumen at a very early age (Su et al., 2014).

Based on the PICRUSt analysis of the rumen microbiome, we speculate that weaning stress of W45 lambs was lower than that of W30 lambs. However, despite a lower abundance of the genus *Prevotella* in the lambs weaned at 30 days than in those weaned at 45 days, no significant differences in amino acid metabolism were observed between these groups. This finding may have been due to the establishment of the functional maturity of the rumen prior to microbial maturity. Meale et al. (2016) found that among the 10.75% of OTUs assigned to a KO category, none were associated with significant changes in carbohydrate metabolism. However, the predictions of PICRUSt are based on known microbial functions, and many of the functions of unclassified bacteria may be over- or underestimated (Yang et al., 2018). Wilkinson et al. (2018) indicated that the functional profiles predicted by CowPI better match estimates for both the meta-genomic and transcriptomic datasets than PICRUSt. On the other hand, metagenomic and metabolomic analyses might be used to analyze the functions of the rumen microbiome to better understand the effects of changes of bacterial taxa on rumen function, functional inferences from 16S data should not replace metagenomic and metatranscriptomic approaches. Therefore, we could improve the function prediction in the future study.

The VFAs in the rumen stimulate rumen epithelial metabolism (Baldwin and McLeod, 2000), and butyrate has been shown to be the most stimulatory (Górka et al., 2011). Butyrate promotes cell proliferation and inhibits cell apoptosis, leading to the functional maturation of intestinal epithelial cells (Hamer et al., 2008; Zhong et al., 2019). The genus *Butyrivibrio* represents a major group of butyrate producers in the rumen (Bryant, 1986). In the present study, the abundance of this genus was positively correlated with papillae length in the rumen. This result is consistent with that of Yang et al. (2018). We observed a positive correlation between *Succinivibrio* abundance and rumen VFA concentration, which corroborates the finding of Rey et al. (2013), who found a similar correlation in the rumen of 3- to 12-day-old calves.

CONCLUSION

In conclusion, this study provides new insights into the colonization of the weaning-lamb rumen and associations within the rumen microbiota. The results suggest that extending the period of MR feeding could influence the microbial composition

and potentially benefit rumen development and mitigate the negative effect of weaning stress. Weaning at the age of 45 days may be recommended from the perspective of the rumen microbial community.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI sequence read archive under BioProject PRJNA683734.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Use and Care Committee, Zhejiang A & F University (Hangzhou, China).

AUTHOR CONTRIBUTIONS

HM and CW: conceptualization and methodology. HM and YZ: investigation and data collection. YY, WJ, and ZJ: sample analysis. CW: formal statistical analysis. HM: writing-original draft preparation, project administration, and funding acquisition. ZY: writing-review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This experiment was funded by the National Natural Science Foundation of China (31902179), National College Students Innovation and Entrepreneurship Training Program (202010341039), and Zhejiang Xinmiao Talents Program (2020R412046).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.636865/full#supplementary-material

REFERENCES

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G., and Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav. Immun.* 25, 397–407. doi: 10.1016/j.bbi.2010.10.023

Baldwin, R. L., and McLeod, K. R. (2000). Effects of diet forage:concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. *J. Anim. Sci.* 78, 771–783. doi: 10.2527/2000.783771x

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., et al. (2018). QIIME 2: reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ* 6:e27295v2.

Bryant, M. P. (1986). "Genus IV. Butyrivibrio Bryant and small 1956, 18, emend. Moore, Johnson and Holdeman 1976, 241," in *Bergey's Manual of Systematic Bacteriology*, Vol. 2, eds P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (Baltimore, MD: Williams &Wilkins), 1376–1379.

Ezaki, T. (2015). "Ruminococcus," in *Bergey's Manual of Systematics of Archaea and Bacteria*, ed. W. B. Whitman (Hoboken, NJ: JohnWiley & Sons). doi: 10.1002/9781118960608.gbm00678

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Cons.* 61, 1–10. doi: 10.1016/0006-3207(92)91201-3

Gagen, E. J., Denman, S. E., Padmanabha, J., Zadbuke, S., Jassim, R., Morrison, M., et al. (2010). Functional gene analysis suggests different acetogen population in the bovine rumen and tammar wallaby forestomach. *Appl. Environ. Microbiol.* 76, 7785–7795. doi: 10.1128/AEM.01679-10

Górka, P., Kowalski, Z. M., Pietrzak, P., Kotunia, A., Jagusiak, W., Holst, J. J., et al. (2011). Effect of method of delivery of sodium butyrate on rumen development in newborn calves. *J. Dairy Sci.* 94, 5578–5588. doi: 10.3168/jds.2011-4166

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B., and Franks, A. E. (2015). Presence of selected methanogens, fibrolytic bacteria, and *Proteobacteria* in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS One* 10:e0133048. doi: 10.1371/journal.pone.0133048

Hamer, H. M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F., and Brummer, R. J. (2008). Review article: the role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* 27, 104–119. doi: 10.1111/j.1365-2036.2007.03562.x

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Jeyanathan, J., Kirs, M., Ronimus, R. S., Hoskin, S. O., and Janssen, P. H. (2011). Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets. *FEMS Microbiol.Ecol.* 76, 311–326. doi: 10.1111/j.1574-6941.2011.01056.x

Konopka, A. (2009). What is microbial community ecology? *ISME J.* 3, 1223–1230. doi: 10.1038/ismej.2009.88

Kumar, S., Indugu, N., Vecchiarelli, B., and Pitta, D. W. (2015). Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows. *Front. Microbiol.* 6:781. doi: 10.3389/fmicb.2015.00781

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814-821. doi: 10.1038/nbt.2676

Li, C., Wang, W., Liu, T., Zhang, Q., Wang, G., Li, F., et al. (2018). Effect of early weaning on the intestinal microbiota and expression of genes related to barrier function in lambs. *Front. Microbiol.* 9:1431. doi: 10.3389/fmicb.2018.01431

Li, C., Zhang, Q., Wang, G., Niu, X., Wang, W., Li, F., et al. (2020). The functional development of the rumen is influenced by weaning and associated with ruminal microbiota in lambs. *Anim. Biotechnol.* 1–17. doi: 10.1080/10495398.2020.1812618

Li, R. W., Connor, E. E., Li, C., Baldwin, V. I., Ransom, L., and Sparks, M. E. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. *Environ. Microbiol.* 14, 129–139. doi: 10.1111/j.1462-2920.2011.02543.x

Li, Y., Shi, M., Zhang, T., Hu, X., Zhang, B., Xu, S., et al. (2020). Dynamic changes in intestinal microbiota in young forest musk deer during weaning. *PeerJ* 8:e8923. doi: 10.7717/peerj.8923

Lin, X., Wang, J., Hou, Q. L., Wang, Y., Hu, Z. Y., Shi, K. R., et al. (2018). Effect of hay supplementation timing on rumen microbiota in suckling calves. *MicrobiologyOpen* 7:e00430. doi: 10.1002/mbo3.430

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Mao, H., Wang, C., and Yu, Z. (2019a). Dietary leucine supplementation enhances the health of early weaned Hu lambs. *Anim. Feed Sci. Thechnol.* 247, 248–254. doi: 10.1016/j.anifeedsci.2018.11.020

Mao, H., Wang, C., and Yu, Z. (2019b). Weaning ages do not affect the overall growth or carcass traits of Hu sheep. *Animals* 9:356. doi: 10.3390/ani9060356

Mao, H., Xia, Y., Tu, Y., Wang, C., and Diao, Q. (2017). Effects of various weaning times on growth performance, rumen fermentation and microbial diversity and

microbial population of yellow cattle calves. *Asian Australas. J. Anim. Sci.* 30, 1557–1562. doi: 10.5713/ajas.16.0981

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., and Neufeld, J. D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13:31. doi: 10.1186/1471-2105-13-31

Matsui, H., Ogata, K., Tajima, K., Nakamura, M., Nagamine, T., Aminov, R. I., et al. (2000). Phenotypic characterization of polysaccharidases produced by four Prevotella type strains. *Curr. Microbiol.* 41, 45–49. doi: 10.1007/s002840010089

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., et al. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618. doi: 10.1038/ismej.2011.139

Meale, S. J., Li, S., Azevedo, P., Derakhshani, H., DeVries, T. J., Plaizier, J. C., et al. (2017). Weaning influences the severity of gastrointertinal microbiome shifts in dairy calves. *Sci. Rep.* 7:189. doi: 10.1038/s41598-017-00223-7

Meale, S. J., Li, S., Azevedo, P., Derakhshani, H., Plaizier, J. C., Khafipour, E., et al. (2016). Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front. Microbiol.* 7:582. doi: 10.3389/fmicb.2016.00582

Myer, P. R., Smith, T. P., Wells, J. E., Kuehn, L. A., and Freetly, H. C. (2015). Rumen microbiome from steers differing in feed efficiency. *PLoS One* 10:e0129174. doi: 10.1371/journal.pone.0129174

Myers, S. E., Faulkner, D. B., Ireland, F. A., Berger, L. L., and Parrett, D. F. (1999a). Production systems comparing early weaning to normal weaning with or without creep feeding for beef steers. *J. Anim. Sci.* 77, 300–310. doi: 10.2527/1999.772300x

Myers, S. E., Faulkner, D. B., Ireland, F. A., and Parrett, D. F. (1999b). Comparison of three weaning ages on cow-calf performance and steer carcass traits. *J. Anim. Sci.* 77, 323–329. doi: 10.2527/1999.772323x

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., and Monteil, V. (2013). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116, 245–257. doi: 10.1111/jam.12405

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

Shannon, C., and Weaver, W. (1949). *The Mathematical Theory of Communication*. Urbana: University of Illinois Press.

Su, Y., Bian, G., Zhu, Z., Smidt, H., and Zhu, W. (2014). Early methanogenic colonisation in the faeces of Meishan and Yorkshire piglets as determined by pyrosequencing analysis. *Archaea* 2014:547908. doi: 10.1155/2014/547908

Thomas, F., Hehemann, J. H., Rebuffet, E., Czjzek, M., and Michel, G. (2011). Environmental and gut bacteroidetes: the food connection. *Front. Microbiol.* 2:93. doi: 10.3389/fmicb.2011.00093

Thu, C. T. T., and Trach, N. X. (2012). Effects of early weaning on postpartum resumption of reproduction in mother buffaloes and growth of their calves. *Livest. Res. Rural. Dev.* 24:19.

Wang, L., Xu, Q., Kong, F., Yang, Y., Wu, D., Mishra, S., et al. (2016). Exploring the goat rumen microbiome from seven days to two year. *PLoS One* 11:e0154354. doi: 10.1371/journal.pone.0154354

Wang, Y., and Qian, P. Y. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One* 4:e7401. doi: 10.1371/journal.pone.0007401

Wilkinson, T. J., Huws, S. A., Edwards, J. E., Kingston-Smith, A. H., Siu-Ting, K., Hughes, M., et al. (2018). CowPI: a rumen microbiome focussed version of the PICRUSt functional inference software. *Front. Microbiol.* 9:1095. doi: 10.3389/fmicb.2018.01095

Xu, J., and Gordon, J. I. (2003). Honor thy symbionts. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10452–10459. doi: 10.1073/pnas.1734063100

Yang, B., Le, J., Wu, P., Liu, J., Guan, L., and Wang, J. (2018). Alfalfa intervention alters rumen microbial community development in Hu lambs during early life. *Front. Microbiol.* 9:574. doi: 10.3389/fmicb.2018.00574

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

Zhong, X., Zhang, Z., Wang, S., Cao, L., Zhou, L., Sun, A., et al. (2019). Microbial-Driven butyrate regulates jejunal homeostasis in piglets during the weaning stage. *Front. Microbiol.* 9:3335. doi: 10.3389/fmicb.2018.03335

Conflict of Interest: The 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 April 2021
doi: 10.3389/fmicb.2021.652031

Correlation of Breed, Growth Performance, and Rumen Microbiota in Two Rustic Cattle Breeds Reared Under Different Conditions

Matteo Daghio[1], Francesca Ciucci[2], Arianna Buccioni[1], Alice Cappucci[2,3], Laura Casarosa[2], Andrea Serra[2,3], Giuseppe Conte[2,3], Carlo Viti[1], Breanne M. McAmmond[4], Jonathan D. Van Hamme[4] and Marcello Mele[2,3]*

[1] Dipartimento di Scienze e Tecnologie Agrarie Alimentari Ambientali e Forestali, University of Florence, Florence, Italy, [2] Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, University of Pisa, Pisa, Italy, [3] Centro di Ricerche Agro-ambientali "E. Avanzi", University of Pisa, Pisa, Italy, [4] Department of Biological Sciences, Thompson Rivers University, Kamloops, BC, Canada

OPEN ACCESS

Edited by:
Robert W. Li,
United States Department of Agriculture, United States

Reviewed by:
Devin Holman,
Agriculture and Agri-Food Canada, Canada
Waleed Abu Al-Soud,
Al Jouf University, Saudi Arabia

***Correspondence:**
Carlo Viti
carlo.viti@unifi.it

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: 11 January 2021
Accepted: 19 March 2021
Published: 29 April 2021

Citation:
Daghio M, Ciucci F, Buccioni A, Cappucci A, Casarosa L, Serra A, Conte G, Viti C, McAmmond BM, Van Hamme JD and Mele M (2021) Correlation of Breed, Growth Performance, and Rumen Microbiota in Two Rustic Cattle Breeds Reared Under Different Conditions. Front. Microbiol. 12:652031. doi: 10.3389/fmicb.2021.652031

The use of rustic cattle is desirable to face challenges brought on by climate change. Maremmana (MA) and Aubrac (AU) are rustic cattle breeds that can be successfully used for sustainable production. In this study, correlations between two rearing systems (feedlot and grazing) and the rumen microbiota, the lipid composition of rumen liquor (RL), and the growth performance of MA and AU steers were investigated. Bacterial community composition was characterized by high-throughput sequencing of 16S rRNA gene amplicons, and the RL lipid composition was determined by measuring fatty acid (FA) and the dimethyl acetal profiles. The main factor influencing bacterial community composition was the cattle breed. Some bacterial groups were positively correlated to average daily weight gain for the two breeds (i.e., Rikenellaceae RC9 gut group, *Fibrobacter* and *Succiniclasticum* in the rumen of MA steers, and Succinivibrionaceae UCG-002 in the rumen of AU steers); despite this, animal performance appeared to be influenced by short chain FAs production pathways and by the presence of H_2 sinks that divert the H_2 to processes alternative to the methanogenesis.

Keywords: growth performance, high-throughput sequencing, microbiota, rumen, rustic cattle

INTRODUCTION

Maremmana (MA) and Aubrac (AU) cattle are rustic breeds well suited for diverse farming conditions, due to their ability to efficiently use low-quality dietary fiber (Renand et al., 2002; Sargentini, 2011; Gallo et al., 2014; Bongiorni et al., 2016; Conte et al., 2019). AU is a breed that originated on the Massif Central in southern France and currently is mainly raised for beef production. The MA breed is typically raised in the Maremma region in Central Italy. It was formerly selected as a work animal, but is now raised only for beef production.

It has been suggested that the use of more resilient breeds (rustic breeds) for beef production is desirable to face challenges brought on by climate change (Horizon, 2020). Rustic breeds may better adapt to challenging and diverse livestock systems due to specific genetic traits.

Furthermore, the rumen microbiota plays an important role in the adaptive response of animals to environmental challenges (Zhong et al., 2019). Several authors have hypothesized that host genetics can be fundamental in selecting rumen microorganisms and suggested that breeding strategies could exploit the abundance of rumen microbial genes to select animals with desirable traits (Roehe et al., 2016).

Molecular analysis of rumen liquor (RL) can provide detailed information on the composition of rumen microbiota, and associations between rumen microbial community structure and feeding regimens, diet composition, and host genetics have been made (Li et al., 2019b; Vasta et al., 2019). There is increasing interest in understanding associations between rumen microbiota, rumen metabolism, and animal performance in order to evaluate how changes in rumen microbial community composition may affect the efficiency and quality of beef production (Vasta et al., 2019). Analysis of fatty acids (FAs) and dimethyl acetals (DMAs) originating from rumen microorganisms could be used to understand the effects of a feeding regimen and its influence on rumen microbial metabolism [lipolysis, biohydrogenation (BH) of dietary lipids, synthesis of membrane lipids] (Alves et al., 2013). Previous studies have suggested that the composition of FAs and DMAs in RL may be associated with changes in the rumen microbial community composition resulting from changes in animal diet or feeding regimen (Cappucci et al., 2018; Mannelli et al., 2018) (e.g., grazing vs. feedlot). However, no data are available allowing for comparisons of rumen lipid composition between different rustic cattle breeds maintained on the same farming systems. Moreover, to date, no data are available describing rumen microbial community composition of rustic breeds as affected by different feeding regimens.

The aim of this study was to investigate the patterns of rumen microbial community composition of steers from two cattle breeds (AU and MA steers) as affected by two different feeding regimens (feedlot vs. grazing) and to explore associations with the growth performance of the steers.

MATERIALS AND METHODS

Experimental Design

Forty 4.5-months-old MA and AU (n = 20 each) steers with an average body weight of 250 kg were allotted into 2 experimental groups as follows: 10 AU and 10 MA were fed in a feedlot (2,500 m^2), whereas 10 AU and 10 MA were fed in a grazing (10 ha) system. Each feedlot or pasture area was equally subdivided into 3 lots. Within each rearing system, 10 subjects *per* breed were randomly allotted into 2 subgroups of 3 steers and 1 group of 4 animals, in order to obtain 3 replicates for each treatment and each breed.

The steers in the feedlot were fed grass hay *ad libitum* and concentrated feed (1 kg/100 kg of live weight *per* head and *per* day), whereas grazing animals received grass hay *ad libitum* in addition to the fresh forage available on the pasture that consisted of 62% grass, 17.5% legumes (mainly white clover), and 20.5% other species (**Supplementary Table 1**). The same kind of concentrate feed used for steers in the feedlot was administered to grazing steers when grass availability was limited [the amount of concentrate was decided monthly on the basis of the individual average daily weight gain (ADG) and was not greater than 1 kg/100 kg of live weight *per* head]. All the animals had free access to water.

Crude protein (CP) ether extract and ash were determined in feeds according to the AOAC (2000) methods. Fiber fractions were analyzed according to Van Soest et al. (1991). Net energy (NE) content of feeds was estimated according to Cornell Net Carbohydrates and Protein System for cattle (Fox et al., 1992). Productivity was evaluated by weighing animals monthly to assess ADG. All steers were slaughtered at 600 kg (between 20 and 22 months old).

All experiments in this study were performed in accordance with the approved guidelines from the European directive 2010/63/UE and DL 4/03/2014 no. 26.

Sampling of RL and Determination of FAs and DMAs

RL samples were collected from freshly slaughtered steers. The whole rumen content was collected, mixed, and filtered on a sterile cheese cloth (Ramos-Morales et al., 2014) to obtain 200 ml of RL for chemical and microbial analyses. This procedure (i.e., mixing the whole rumen content) ensured the collection of a representative sample of each rumen. The liquid-associated bacteria, and the solid-adherent bacteria associated to the food particles that were not removed by filtering on cheese cloth, were collected. The reproducibility of the results was ensured by sampling 20 animals for each breed. Approximately 2 ml of RL was immediately stored at –80°C for microbial DNA extraction. The rest of the samples were stored at –20°C and then lyophilized (ScanVAC CoolSafe 55-4 lyophilizer, LaboGene ApS DK-3450, Allerød, Denmark) for the analysis of lipid composition.

Lipid Extraction and Identification of Fatty Acid Methyl Esters and DMAs

Lipids were derivatized by a direct acid/basic double transesterification of freeze-dried RL (Alves et al., 2013). Identification of fatty acid methyl esters (FAMEs) and DMAs was performed by thin-layer chromatography purification of the esterified fraction (Alves et al., 2013). The identification of DMAs was obtained by gas chromatography–mass spectrometry (GC–MS), according to Alves et al. (2013).

The composition of FAs was characterized using a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD, United States) as previously reported (Alves et al., 2013; Cappucci et al., 2018). Every single FAME was identified through comparison to a standard FAME mixture containing 52 standards (Nu-Chek-Prep Inc., Elysian, MN, United States). Nonanoic acid and nonadecanoic acid were used as internal standards. The identification of the 18:1 isomers was based on a mixture of commercial standards (Supelco, Bellefonte PA, United States) and on the basis of the isomeric profiles (Kramer et al., 2004). Individual FA and DMA profiles were expressed in g/100 g of total FAs and DMAs, respectively.

DNA Extraction, Sequencing, and Bioinformatics

DNA was extracted from 185 μl of RL by using the Fast DNA Spin for soil kit (MP Biomedicals, Solon, OH, United States) following the manufacturer's protocol modified as previously reported (Mannelli et al., 2018).

Bacterial 16S rRNA gene amplicons were generated with a double step PCR protocol. The 341F and 806R primers (Bergmann et al., 2011; Zeng et al., 2013) were used for the first round. The same primers with the addition of adaptors and Ion Xpress barcodes were used in the second round. All reactions contained 10 μl of 2× GoTaq Green Master Mix (Promega Corporation, Madison, WI, United States), 2 μl forward primer (1 μM final concentration), 2 μl reverse primer (1 μM final concentration), 1 μl of template DNA, and water for a total volume of 20 μl. The thermocycler program for first PCR consisted of: 95°C for 4 min followed by 25 cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 2 min, with a final extension at 72°C for 5 min. The second PCR used the same conditions except that an annealing temperature of 65°C and 20 cycles was used. After each round of PCR, amplicons were cleaned in a 96-well plate using Agencourt AMPure XP beads (Beckman Coulter, Inc.). Amplicons were sequenced using an Ion S5^{TM} XL sequencer (Thermo Fisher Scientific) on an Ion 530 chip using 400 bp chemistry.

The UPARSE pipeline (USEARCH 8.1) was used to process the obtained sequences (Edgar, 2010, 2013). The forward primer was removed, the sequences shorter than 350 bp were eliminated, and the remaining sequences were truncated at 350 bp. Low-quality sequences (i.e., sequences with a total expected error > 2) were eliminated. The sequences present only once in the entire dataset (singletons) were removed, and the sequences were grouped into operational taxonomic units (OTUs) at 97% similarity. A representative sequence was selected for each OTU. Representative sequences were classified against SILVA database v138 (Pruesse et al., 2007) using the function assignTaxonomy (confidence 80%) in the DADA2 package, version 1.14.0 (Callahan et al., 2016) in R 3.6.1 (R Core Team, 2020). OTUs with a relative abundance lower than 0.005% in all the samples were removed from the whole dataset. A total of 1,249,694 high-quality sequences were obtained with an average of 31,242 ± 14,662 sequences *per* sample (average ± standard deviation). A randomly rarefied dataset (5,456 sequences per sample—i.e., sequences in the sample with the lowest number of sequences) was generated. The Chao1 index, the ACE index, the Simpson index, and the Shannon diversity index were calculated using the vegan package, version 2.5-6 (Oksanen et al., 2019) in R 3.6.1 (R Core Team, 2020).

Statistical Analysis

Statistical analysis of RL, FAs, and DMAs was performed by the following general linear model (SAS Institute, Charlotte, NC, United States) (SAS Institute, 2008):

$$Y_{ijz} = \mu + B_i + R_j + B_i \times R_j + \varepsilon_{ijz}$$

Where:

y = observation

μ = overall mean

B_i = fixed effect of the i-th breed: AU and MA (i: from 1 to 2)

R_j = fixed effect of the j-th rearing system: pasture and feedlot (j: from 1 to 2)

$B_i \times R_j$ = interaction effect of the i-th breed and j-th rearing system

ε_{ijz} = random error.

In cases of a significant effect for the $B_i \times R_j$ interaction, a *post-hoc* HSD analysis of Tukey was performed. Probability of significant effect due to experimental factors was fixed at $p < 0.05$.

Data from the characterization of the microbial communities were processed using the vegan package, version 2.5-6 (Oksanen et al., 2019) in R 3.6.1 (R Core Team, 2020). A non-metric multidimensional scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) based on Hellinger transformed OTU abundance data were performed using the metaMDS and the adonis2 functions, respectively. Both the NMDS and the PERMANOVA were performed on the Bray–Curtis dissimilarity index. The taxa with different relative abundances between the conditions (i.e., breed, rearing system, and interaction of breed × rearing system) were identified by a Kruskal–Wallis test and by a *post-hoc* Dunn test with the Benjamini–Hochberg correction for multiple comparison. The Kruskal–Wallis test and the Dunn test were performed to detect significant differences between the conditions for the calculated diversity indexes. Differences were considered significant for $p < 0.05$. The Spearman correlations were performed to identify the bacterial genera correlated to the ADG. Correlations were considered significant for $p < 0.1$.

Nucleotide Sequence Accession Number

The sequences are available at the National Centre for Biotechnology Information (NCBI), BioProject number PRJNA682716, under the following BioSample accession numbers: SAMN17005974–SAMN17006013.

RESULTS

FA and DMA Profiles

The total percentage of saturated fatty acids (SFAs) was higher in the RL from steers reared in the feedlot system (**Table 1**), with stearic acid (SA, C18:0) being the most relatively abundant, followed by palmitic acid (PA, C16:0) (**Table 2**). The RL from MA steers had a higher content of PA and a lower content of SA than the RL from steers of the same breed reared in the feedlot, and to the AU steers, regardless of the rearing system (**Table 2**). The overall content of odd and branched chain FAs (OBCFAs) was higher in the RL from grazing steers of both breeds (**Table 1**); however, some differences were significant between breeds for specific OBCFA. The RL from AU steers contained a higher percentage of C15:0 *ante* and C16:0 *ante*. In contrast, the RL from MA steers had a higher percentage of C16:0 *iso* and C17:0 *ante* (**Table 2**). A significant breed × rearing system interaction was found for C17:0 *iso* that was found at a higher percentage in the RL of grazing MA steers. The RL from AU steers contained a

TABLE 1 | Relative percentage (g/100 g of total FAs + DMAs) of the main classes of fatty acids and dimethyl acetals in the rumen liquor from Aubrac or Maremmana steers maintained in feedlot or on pasture.

	AU		MA		SE	*p*-value		
	Grazing	Feedlot	Grazing	Feedlot		B	R	B × R
SFAs	72.667	76.167	67.674	75.215	1.363	0.045	0.001	0.167
UFAs	21.390	18.683	20.534	17.761	1.309	0.522	0.054	0.981
PUFAs	4.934^{b}	4.559^{b}	8.254^{a}	5.271^{b}	0.529	0.001	0.005	0.025
MUFAs	16.456	14.124	12.280	12.490	0.974	0.007	0.307	0.223
PUFAs *n*-6	3.539	3.558	5.452	4.514	0.426	0.003	0.312	0.293
PUFAs *n*-3	1.351^{b}	0.961^{b}	2.721^{a}	0.727^{b}	0.220	0.018	<0.001	0.001
n-6/*n*-3	2.981^{c}	5.168^{b}	2.436^{c}	8.467^{a}	0.765	0.091	<0.001	0.024
trans 18:1 tot	10.126	8.197	5.244	5.548	0.755	<0.001	0.312	0.167
VA/*trans* 18:1 tot	0.462	0.401	0.603	0.414	0.034	0.034	0.001	0.081
OCFAs tot	4.719	4.294	4.243	3.236	0.213	0.002	0.003	0.203
OBCFAs tot	6.983	6.131	7.379	5.363	0.370	0.635	<0.001	0.144
OCDMAs tot	2.509	2.071	1.530	0.929	0.142	<0.001	0.001	0.590
DMAs tot	5.221	4.491	5.785	4.213	0.287	0.637	<0.001	0.172
BCFAs tot	4.486^{b}	3.907^{b}	6.621^{a}	4.289^{b}	0.394	0.004	0.001	0.041
BCFAs *iso*	1.723	1.533	2.615	1.813	0.144	<0.001	0.003	0.061
BCFAs *ante*	2.766	2.380	3.961	2.454	0.152	0.004	0.003	0.054

AU, Aubrac; MA, Maremmana; SE, Standard Error; B, breed; R, rearing system; SFAs, saturated fatty acids; UFAs, unsaturated fatty acids; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; VA, vaccenic acid; OCFAs, odd chain fatty acids; OBCFAs, odd and branched chain fatty acids; OCDMAs, odd chain dimethyl acetals; DMAs, dimethyl acetals; BCFAs, branched chain fatty acids. a, b, c are the probability of significant effect due to experimental treatment; means within a row with different letters differ ($p < 0.05$).

higher percentage of odd chain FAs (OCFAs) C13:0, C15:0, C17:0, and C23:0 than the RL from MA steers.

The overall percentage of polyunsaturated fatty acids (PUFAs) was significantly higher in RL from grazing MA steers, as a consequence of the significant breed × rearing system interaction (**Table 1**). Considering the PUFA *n*–3 FA, the interaction was significant for C22:5 *n*–3 ($p < 0.001$) and for C18:4 *n*–3 ($p = 0.034$). Linoleic acid (C18:2 *n*–6) was the most relatively abundant PUFA, and it was found at higher percentage in RL from MA steers, irrespective of the rearing system, together with *n*–6 (C20:3 *n*–6, C22:4 *n*–6) PUFAs. The *cis* and *trans* C18:1 isomers were differentially distributed in RL according to the breed and feeding strategy. Vaccenic acid (VA, C18:1 *trans*11) was the C18:1 *trans* isomer with the highest concentration in the RL, regardless of the breed and the rearing system. The highest concentration of VA was detected in the RL from AU steers, whereas the C18:1 *cis*9, the most abundant among the C18:1 *cis* isomers and monounsaturated fatty acids (MUFAs), was contained at the greatest concentration in the RL from MA steers. The content of C18:1 *trans*6–8, C18:1 *trans*9, C18:1 *trans*10, C18:1 *trans*12, C18:1 *trans15*, C18:1 *trans*16, C18:1 *cis*11, and C18:1 *cis*12 was higher in the RL from AU steers. The interaction of breed × rearing system was significant in a few cases: C18:1 *trans*12, C18:1 *trans*15, C18:1 *cis*12, and C18:1 *trans*16, being the FAs found at the lowest percentage in the RL from MA grazing steers. Grazing activity resulted in a significant increase of α-linolenic acid (α-LNA, C18:3 *n*–3) in the RL, especially for the MA steers that had the highest amount of α-LNA in their RL ($p = 0.148$). A decrease of the *n*–6/*n*–3 ratio in the RL from grazing steers was also observed, regardless of the breed. The concentration of conjugated linoleic acid (CLA) did not vary across treatments (**Table 2**).

The total content of DMA in RL did not vary between breeds but was higher in the RL from grazing steers (**Table 1**). Nineteen different DMAs were identified in the RL (**Table 3**). The most abundant DMA was DMA C16:0, followed by DMA C15:0 *iso* for AU steers and DMA C14:0 for MA steers. Overall, 16 DMAs changed significantly in their content according to the breed factor, and only DMA C15:0 *ante*, DMA C17:0, and DMA C18:1 *trans*11 did not significantly differ between the two breeds. DMA C15:0 *iso*, DMA C16:1, and DMA C18:1 *cis*9 were significantly more abundant in the RL from AU steers, whereas the relative percentages of the other DMAs were higher in the RL from MA steers. In four cases (DMA C14:0, DMA C15:0, DMA C18:1 *cis*11, and DMA C18:1 *cis*12), the interaction breed × rearing system was significant. The rearing system showed a significant effect only for DMA C18:1 *cis*11. Overall, the content of odd chain DMAs and of branched chain DMAs was higher in the RL from AU steers, mainly due to the higher content of DMA C15:0 *iso* (**Table 3**).

Taxonomic Composition of the Bacterial Communities

Bacterial communities in the rumen of AU and MA steers reared in two different systems were characterized by high-throughput sequencing of 16S rRNA gene amplicons. Rarefaction curves (**Supplementary Figure 1**), obtained by plotting the number of OTUs vs. the number of sampled sequences, indicated that the depth of the sampling was enough to describe the biodiversity within the dataset.

TABLE 2 | Relative percentage of selected fatty acids (g/100 g of total FAs) in the rumen liquor from Aubrac or Maremmana steers maintained in feedlot or on pasture.

	AU		MA		SE	*p*-value		
	Grazing	Feedlot	Grazing	Feedlot		B	R	B × R
C12:0	0.340	0.254	0.197	0.195	0.046	0.045	0.370	0.391
C13:0 *iso*	0.084	0.073	0.067	0.061	0.009	0.144	0.380	0.800
C13:0 *ante*	0.013	0.015	0.017	0.012	0.002	0.853	0.308	0.085
C13:0	0.113	0.124	0.045	0.045	0.013	<0.001	0.691	0.696
C14:0 *iso*	0.326	0.247	0.316	0.245	0.029	0.825	0.018	0.894
C14:0	1.495	1.701	1.050	0.964	0.113	<0.001	0.616	0.224
C15:0 *iso*	0.641	0.609	0.771	0.549	0.048	0.492	0.016	0.068
C15:0 *ante*	1.964	1.786	1.419	1.156	0.097	<0.001	0.037	0.678
C15:0	1.466	1.323	1.336	1.003	0.089	0.021	0.015	0.313
C16:0 *iso*	0.361	0.273	0.956	0.647	0.057	<0.001	0.002	0.074
C16:0 *ante*	0.463	0.284	0.297	0.104	0.066	0.017	0.011	0.921
C16:0	17.719^{b}	16.485^{b}	23.766^{a}	18.795^{b}	0.722	<0.001	<0.001	0.019
C17:0 *iso*	0.335^{b}	0.294^{b}	0.624^{a}	0.352^{b}	0.047	0.001	0.003	0.026
C16:1 *cis*7	0.116	0.107	0.056	0.059	0.009	<0.001	0.714	0.498
C16:1 *cis*9	0.150	0.138	0.206	0.090	0.060	0.946	0.310	0.415
C17:0 *ante*	0.478	0.405	1.089	0.653	0.093	<0.001	0.013	0.072
C17:0	0.764	0.717	0.678	0.575	0.038	0.007	0.070	0.489
C18:0 *iso*	0.072	0.109	0.047	0.051	0.023	0.092	0.403	0.487
C18:0	48.192^{b}	53.365^{a}	37.282^{c}	51.652^{a}	1.994	0.005	<0.001	0.035
C18:1 *trans*6–8	0.694	0.841	0.264	0.371	0.135	0.003	0.375	0.890
C18:1 *trans*9	0.378	0.451	0.197	0.247	0.066	0.009	0.375	0.870
C18:1 *trans*10	2.407	2.027	0.597	0.993	0.400	0.002	0.985	0.363
C18:1 *trans*11	4.889	3.385	3.375	2.424	0.459	0.015	0.015	0.570
C18:1 *trans*12	0.786^{a}	0.686^{a}	0.437^{b}	0.622^{a}	0.044	<0.001	0.367	0.004
C18:1 *cis*9	4.836	4.312	6.290	5.968	0.310	<0.001	0.202	0.758
C18:1 *trans*15	0.684^{a}	0.538^{b}	0.291^{c}	0.491^{b}	0.040	<0.001	0.531	< 0.001
C18:1 *cis*11	0.984	1.098	0.540	0.658	0.095	<0.001	0.253	0.983
C18:1 *cis*12	0.353^{a}	0.337^{a}	0.216^{b}	0.323^{a}	0.021	0.001	0.044	0.007
C18:1 *cis*13	0.038	0.028	0.031	0.028	0.003	0.260	0.076	0.265
C18:1 *trans*16	0.834^{a}	0.642^{b}	0.400^{c}	0.643^{b}	0.045	<0.001	0.598	< 0.001
C19:1 *trans*7	0.096	0.075	0.062	0.059	0.009	0.012	0.188	0.338
C18:2 *n*-6	3.632	3.635	5.673	4.632	0.454	0.003	0.284	0.281
C20:0	0.806^{a}	0.824^{a}	0.896^{a}	0.717^{b}	0.036	0.824	0.040	0.013
C20:1 *cis*11	0.102	0.109	0.073	0.063	0.014	0.013	0.902	0.547
C18:3 *n*-3	0.959	0.517	1.340	0.369	0.170	0.518	<0.001	0.148
C18:2 *cis*9*trans*11	0.046	0.042	0.085	0.032	0.013	0.283	0.047	0.081
C21:0	0.046	0.044	0.057	0.044	0.008	0.514	0.357	0.456
C18:4 *n*-3	0.151^{b}	0.173^{b}	0.255^{a}	0.078^{c}	0.043	0.929	0.094	0.034
C22:0	0.513^{b}	0.465^{b}	0.678^{a}	0.416^{b}	0.037	0.142	<0.001	0.009
C20:3 *n*-6	0.020	0.021	0.045	0.030	0.005	0.001	0.161	0.095
C23:0	0.221	0.155	0.111	0.101	0.026	0.005	0.179	0.312
C24:0	0.606	0.509	0.724	0.525	0.040	0.123	0.001	0.236
C22:4 *n*-6	0.082	0.066	0.086	0.057	0.007	0.702	0.005	0.403
C22:5 *n*-3	0.315^{b}	0.317^{b}	1.300^{a}	0.316^{b}	0.111	<0.001	<0.001	<0.001
Unknown	0.405^{c}	0.377^{c}	5.419^{a}	2.473^{b}	0.332	<0.001	<0.001	<0.001

AU, Aubrac; MA, Maremmana; SE, Standard Error; B, breed; R, rearing system; Unknown, sum of three unknown peaks. a, b, c are the probability of significant effect due to experimental treatment; means within a row with different letters differ ($p < 0.05$).

Chao1, ACE, Shannon, and Simpson diversity indexes (**Figure 1**) were calculated and clearly showed a difference (in terms of both richness and evenness) between the rumen bacterial communities in the two breeds. Conversely, no differences were observed in the diversity for the two rearing systems.

An NMDS plot showed that the composition of the microbial communities was affected by the breed (**Figure 2**),

TABLE 3 | Relative percentage of dimethyl acetals (g/100 g of total DMAs) in the rumen liquor from Aubrac or Maremmana steers maintained in feedlot or on pasture.

	AU		MA		SE	*p*-value		
	Grazing	Feedlot	Grazing	Feedlot		B	R	B × R
DMA C13:0	0.785	0.995	1.104	1.610	0.141	<0.001	0.142	0.366
DMA C13:0 *iso*	0.548	0.540	1.171	0.599	0.171	0.025	0.113	0.113
DMA C14:0	5.454^{c}	7.845^{b}	10.114^{a}	9.735^{a}	0.536	<0.001	0.097	0.012
DMA C14:0 *iso*	6.837	6.331	7.865	8.017	0.919	0.017	0.794	0.754
DMA C15:0	5.180^{c}	5.720^{c}	7.021^{a}	6.046^{b}	0.238	<0.001	0.321	0.003
DMA C15:0 *ante*	2.998	2.788	3.697	3.180	0.644	0.503	0.568	0.781
DMA C15:0 *iso*	24.628	23.026	6.261	5.389	1.008	<0.001	0.245	0.782
DMA C16:0	30.499	30.682	40.405	38.091	1.274	<0.001	0.370	0.267
DMA C16:1	1.920	1.765	0.796	0.964	0.185	<0.001	0.967	0.423
DMA C16:0 *iso*	1.649	1.538	4.127	4.444	0.299	<0.001	0.814	0.547
DMA C17:0	0.426	0.458	0.320	0.466	0.075	0.455	0.286	0.517
DMA C17:1	1.439	1.140	1.867	1.460	0.258	0.044	0.179	0.805
DMA C17:0 *ante*	0.615	0.755	1.393	1.449	0.171	<0.001	0.625	0.772
DMA C17:0 *iso*	0.613	0.699	1.014	0.793	0.102	0.003	0.537	0.163
DMA C18:0	2.853	3.214	3.348	3.382	0.159	<0.001	0.290	0.269
DMA C18:1 *cis*11	2.584^{b}	2.338^{b}	1.954^{b}	4.299^{a}	0.368	0.012	0.012	0.002
DMA C18:1 *cis*12	0.870^{c}	0.464^{d}	1.055^{b}	1.752^{a}	0.222	<0.001	0.568	0.024
DMA C18:1 *cis*9	8.452	7.921	4.793	6.333	0.673	<0.001	0.508	0.162
DMA C18:1 *trans*11	1.650	1.779	1.694	1.991	0.192	0.109	0.321	0.717

DMA, dimethyl acetal; AU, Aubrac; MA, Maremmana; SE, Standard Error; B, breed; R, rearing system. a, b, c are the probability of significant effect due to experimental treatment; means within a row with different letters differ ($p < 0.05$).

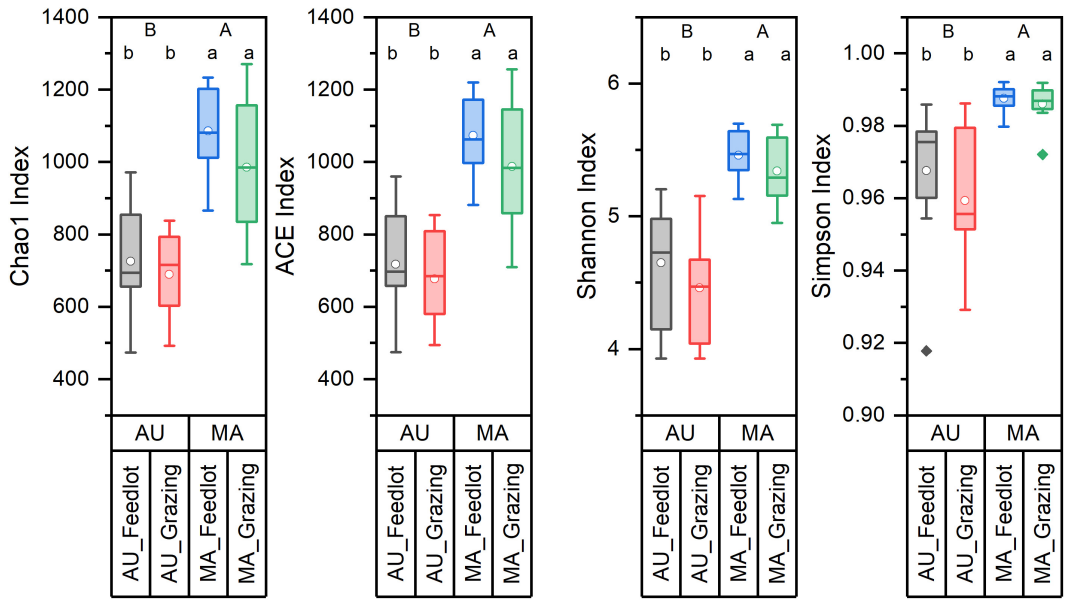


FIGURE 1 | Diversity indices of the rumen microbiota of Aubrac (AU) and Maremmana (MA) breeds in the two rearing systems (feedlot and grazing). Uppercase letters (A and B) show significant differences ($p < 0.05$) between breeds. Lowercase letters (a and b) show significant differences ($p < 0.05$) due to the interaction breed × rearing system. The biodiversity is lower in the bacterial communities in the rumen of AU steers.

an observation confirmed by PERMANOVA ($R^2 = 0.28$, $p < 0.001$). Furthermore, despite the diversity within the microbial communities in the two rearing systems not being different, a significant difference in the composition (i.e., in the structure) of the bacterial communities was observed for the rearing system ($R^2 = 0.04$, $p = 0.021$) and for the interaction breed × rearing system ($R^2 = 0.04$, $p = 0.029$).

Considering the whole dataset, the family Prevotellaceae (phylum *Bacteroidetes*) was the most relatively abundant (average relative abundance ∼38%, with a range between ∼10

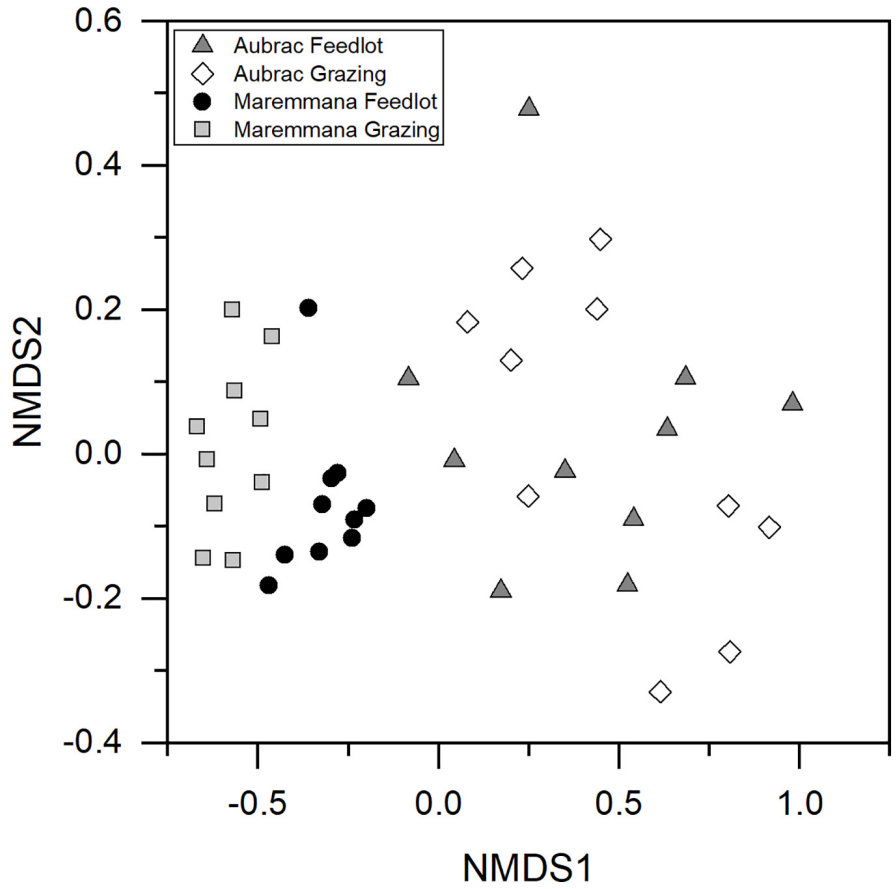


FIGURE 2 | Non-metric multidimensional scaling plot based on Hellinger transformed OTU abundance data. The bacterial communities selected in the rumen of the two breeds reared in different conditions are different.

and ∼58%), followed by the families Ruminococcaceae and Lachnospiraceae (phylum *Firmicutes*), with an average relative abundance of ∼11% (range between ∼6 and ∼28%) and ∼8% (range between ∼4 and ∼18%), respectively (**Figure 3A**). At the genus level (**Figure 3B**), the most relatively abundant groups were *Prevotella* 1 (phylum *Bacteroidetes*, average ∼27%, with a range between ∼6 and ∼57%) and Rikenellaceae RC9 gut group (phylum *Bacteroidetes*, average ∼7%, with a range between ∼1 and ∼18%), followed by the genus *Succiniclasticum* (phylum *Firmicutes*, average ∼4%, with a range between < 1 and ∼9%).

Only 16 families showed an average relative abundance of 1%, or higher, in at least one group (i.e., AU grazing, AU feedlot, MA grazing, MA feedlot— **Supplementary Table 2**). Twelve families showed significantly different relative abundances between the two breeds. In particular, the families Prevotellaceae, Fibrobacteraceae, Acidaminococcaceae, Veillonellaceae, and Succinivibrionaceae were more relatively abundant in the AU breed, whereas the families F082, p-251-o5, Christensenellaceae, Family XIII, and Ruminococcaceae were more abundant in the MA breed. Furthermore, the relative abundance of the family Rikenellaceae was higher (∼12%) in the MA steers reared in grazing conditions than in the AU steers reared in both pasture and feedlot, whereas the relative abundance of this family in MA steers reared in feedlot was intermediate (**Supplementary Table 2**). Similarly, the family Saccharimonadaceae showed a higher relative abundance in the rumen of MA steers reared in the feedlot than of AU steers, regardless of the rearing conditions, and the relative abundance in the RL from MA steers reared in pasture was intermediate (**Supplementary Table 2**).

Twenty genera with an average relative abundance of 1%, or higher, in at least one group were observed (**Supplementary Table 3**). The genera *Prevotella* (*Prevotella* 1 and *Prevotella* 7), *Fibrobacter*, *Oribacterium*, and *Succiniclasticum* were more relatively abundant in the rumen of AU steers, whereas the genera *Acetitomaculum*, *Saccharofermentans*, and some members of the families Christensenellaceae and Ruminococcaceae (Christensenellaceae R-7 group, Ruminococcaceae NK4A214 group, Ruminococcaceae UCG-010, *Ruminococcus* 2) were more relatively abundant in the rumen of MA steers, regardless of the rearing system. Four genera had a different relative abundance not only considering the breed but also considering the interaction breed × rearing system: the Lachnospiraceae NK3A20 group and *Candidatus Saccharimonas* showed a significantly higher relative abundance

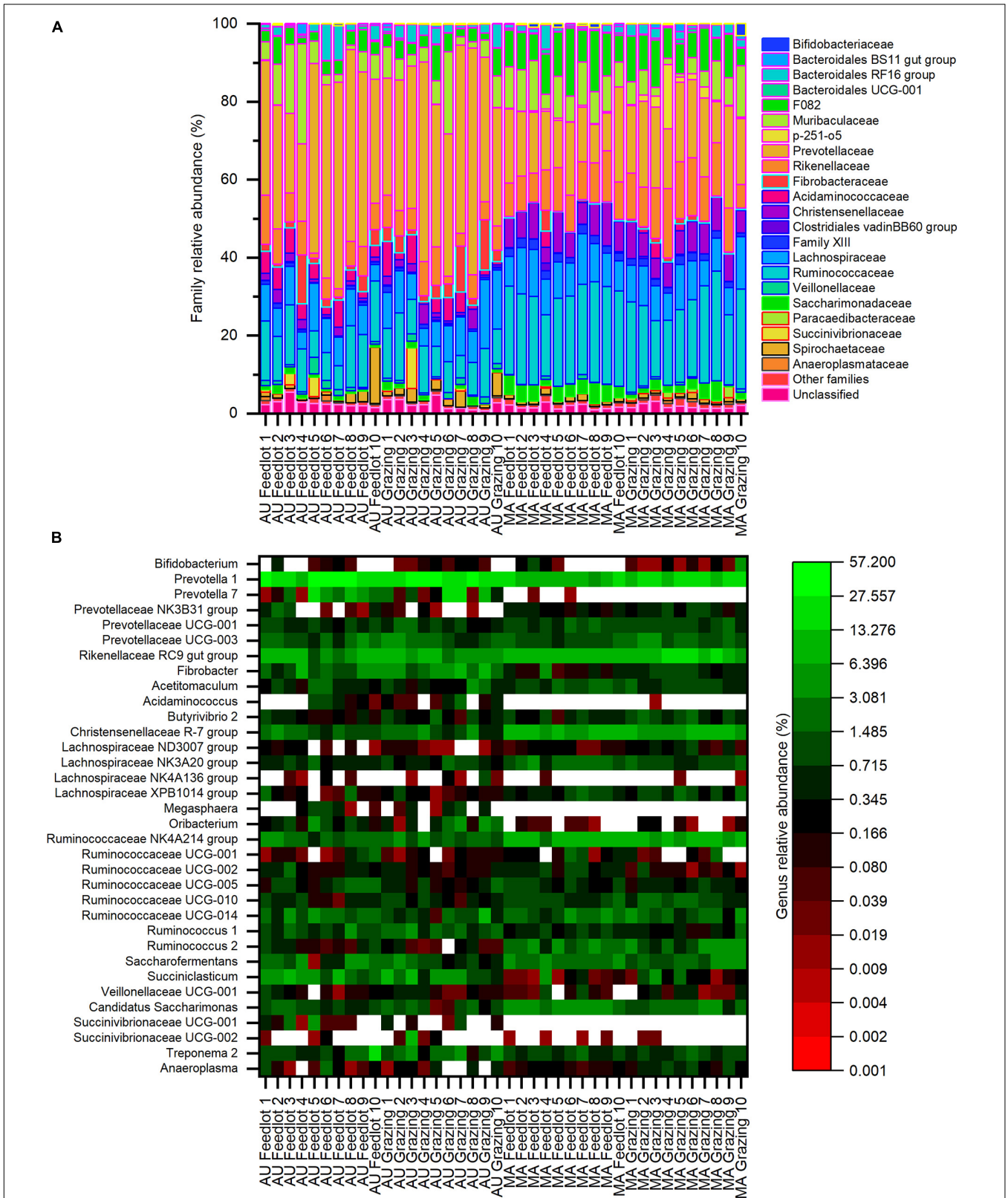


FIGURE 3 | Composition of bacterial communities at the **(A)** family and **(B)** genus levels for the Aubrac (AU) and Maremmana (MA) breeds in the two rearing systems (feedlot and grazing). Only the genera with a relative abundance of at least 1% in at least one sample are reported. Within the whole dataset, the most relatively abundant families were Prevotellaceae, Ruminococcaceae, and Lachnospiraceae, whereas the most relatively abundant genus was *Prevotella*.

in the rumen microbial communities in MA steers reared in the feedlot than in AU steers reared in both grazing and feedlot systems (**Supplementary Table 3**). Similarly, the Rikenellaceae RC9 gut group had a higher relative abundance in MA steers in pasture conditions than in AU steers in both rearing systems, whereas the genus *Ruminococcus* 1 showed the opposite and had a higher relative abundance in the rumen of AU grazing steers than of MA grazing steers (**Supplementary Table 3**).

Correlation Between Bacterial Taxa and Growth Performance

The ADG values were less than 1 kg *per* head and *per* day and were higher for the AU steers than for the MA steers (**Supplementary Table 4**). To correlate the bacterial groups to growth performance, Spearman correlations were obtained independently for each breed. Only genera with a relative abundance of at least 1% in at least one sample were considered for each breed. At the genus level, different bacterial genera showed a significant correlation with growth performance ($p < 0.1$) in the two breeds (**Figure 4**). The groups Ruminococcaceae UCG-01, *Treponema* 2 (AU), Lachnospiraceae NK3A20 group, and *Saccharofermentans* (MA) were negatively correlated to growth performance. Conversely, the relative abundance of Succinivibrionaceae UCG-002 (AU), Rikenellaceae RC9 gut group, *Fibrobacter*, and *Succiniclasticum* (MA) increased in accordance to growth performance.

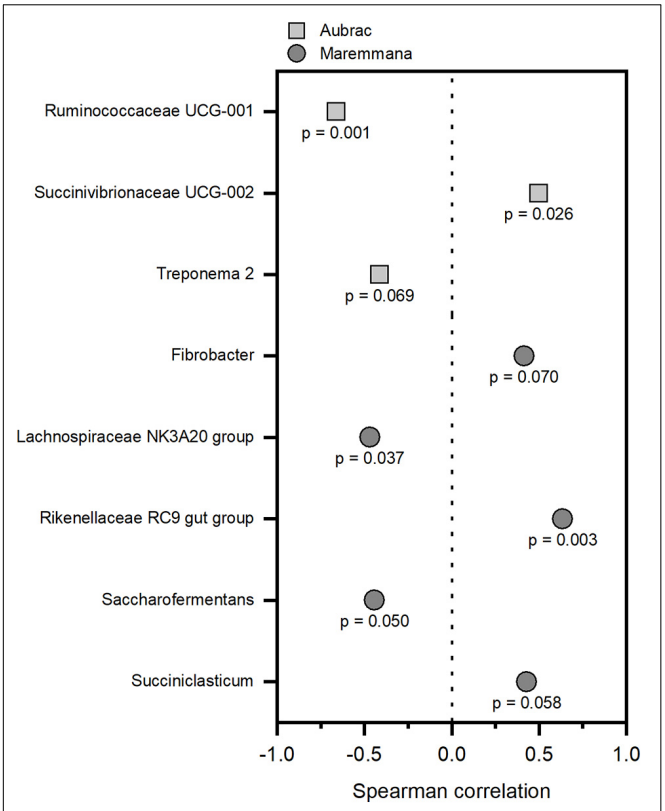


FIGURE 4 | Correlations between the rumen bacteria and the average daily weight gain of the Aubrac cattle and the Maremmana cattle. Only significant correlations ($p < 0.1$) were reported. Correlations were calculated independently for each breed, and only the genera with a relative abundance of 1%, or higher, in at least one sample were considered.

DISCUSSION

AU and MA steers were reared under two different conditions (i.e., grazing and feedlot). The ADG was recorded, and at the end of the rearing period, samples of RL were collected to analyze the FA and DMA profiles and to characterize the bacterial communities. The diet is a key factor that modulates the microbial community and, consequently, the BH of FAs in the rumen (Vasta et al., 2019). However, the breed can also influence the rumen microbiota, methane emissions, and feed efficiency (Paz et al., 2016; Roehe et al., 2016; Li et al., 2019a,b). Hence, the presence of functional FAs in animal products can be influenced both by host genetics and by the feeding strategy (Vasta et al., 2019).

In our study, SA and PA were the most relatively abundant FAs in all the samples, which are consistent with the findings from several authors (Cersosimo et al., 2016; Buccioni et al., 2017; Cappucci et al., 2018; Mannelli et al., 2018). FA profiles, however, showed several differences in the RL collected from the two breeds. The SA content was lower in the RL from grazing animals than in the RL from animals fed in feedlot, with the lowest percentage recorded in grazing MA steers. The feeding strategy based on pasture increased the percentage of C18:3 *n*–3. This observation can be explained since this FA is a structural FA whose presence can be observed in fresh herbs. This finding (i.e., higher percentage of C18:3 *n*–3 in RL of grazing steers, linked to lower concentration of SA in RL of MA steers reared in pasture) suggests less intensive BH in the RL of grazing MA steers. A different modulation of the BH pathway in the two breeds, regardless of the feeding strategy, is confirmed also by the amount of C18:1 *trans* isomers that is higher in the RL of AU steers than of MA steers. These FAs are important markers of rumen microbial metabolism and are produced not only by hydrogenation of PUFAs but also by isomerization of MUFAs (Mosley et al., 2002). A feeding strategy based on grazing is able to modify the FAs profile in the rumen (Collomb et al., 2002). Grazing animals can select plant species, or specific components of forage plants, with different secondary metabolites or C18:3 *n*3 contents. This can lead to a different intake of such molecules. MA and AU could have a different feeding behavior and could be characterized by a different rate of passage of forage in the rumen due to their physiology. These factors could have induced changes in the profile of the microbial community, thus affecting the rate of BH (Lee et al., 2003; Cabiddu et al., 2010; Vasta et al., 2019). The effect of polyphenols on the bacterial communities involved in BH in the rumen of animals fed fresh grass has been described in the literature and can explain the reduced disappearance of PUFAs (Vasta et al., 2019). The accumulation of PUFAs can further influence the microbial activity due to their antibacterial properties, leading to additional changes in the BH pathway

(Jenkins, 1994; Chilliard et al., 2001). In this trial, the animals grazed on a pasture composed of grass and legumes and a previous study showed that the proportion of C18 PUFAs by-passing the rumen and entering the duodenum was higher in steers fed legume silage than in steers fed grass silage (Lee et al., 2003). Furthermore, a negative relationship between the content of polyphenols in several legumes and the trend of BH of C18:3 *n*3 during *in vitro* fermentation has been demonstrated (Cabiddu et al., 2010). Moreover, differences in the amount of C18:1 *cis*9 and C18:3 *cis*9 *cis*12 *cis*15 (higher in MA steers) and VA (higher in AU steers) could be related to a selective action in protecting *cis*9 double bound from hydrogenation in MA steers and in lowering *trans*11 hydrogenation in AU steers, which can be influenced by the different compositions of microbial communities between the two breeds.

OBCFAs are largely present in bacterial membranes (Kaneda, 1991) and are therefore considered microbial markers in the rumen ecosystem (Vlaeminck et al., 2006; Bessa et al., 2009). The results of the present study provide evidence that the total concentration of OBCFA was higher in the RL of grazing steers and, in particular, in MA grazing steers. Other authors observed that the proportion of even-chain saturated FA decreased, and that of BCFA increased with increasing forage content (Bas et al., 2003). In our study, grazing regimen increased the concentration of *iso* branched chain FAs, in particular 14:0 *iso*,15:0 *iso*, 16:0 *iso*, and 17:0 *iso*. An increase of amylolytic bacteria can increase *ante iso* and linear OCFAs, whereas an increase in cellulolytic bacteria can result in a higher amount of *iso* FAs (Vlaeminck et al., 2006). Our data suggest that that the feeding regimen of MA grazing steers was probably richer in forage than that of AU grazing steers, possibly due to more intense grazing activity by MA steers. Although the individual consumption of forage and concentrate was not recorded during the experiment, the total amount of concentrate consumed by the grazing MA steers as a group was lower than that recorded by the grazing AU steers (data not shown), providing indirect confirmation of this hypothesis.

Microbial plasmalogens (detected as DMAs) have been used to compare microbial communities in RL when different diets were provided (Mannelli et al., 2019). Several authors observed that specific DMAs are associated with specific bacterial taxa under certain conditions, indicating that plasmalogen lipid profiles can be considered as a tool for the identification of the effect that a change in environmental conditions can induce on a microbial community (Miyagawa, 1982; Minato et al., 1988). In this study, DMA C16:0 was the most abundant in all groups, which was in agreement with previous results (Alves et al., 2013). The main factor affecting DMA profile in this study was the breed, and the results were confirmed by the 16S rRNA gene amplicon sequencing.

Differences in the composition of the bacterial communities in RL between the two breeds were observed by high-throughput sequencing of 16S rRNA gene amplicons. Bacterial diversity was higher in the rumen communities of MA steers than of AU steers. AU steers, however, showed higher ADG. This observation is in agreement with previous findings in which the more efficient animals were characterized by a lower biodiversity of the microbial taxa and of the metabolic pathways active in the rumen (Shabat et al., 2016). The most relatively abundant groups were the same in the two breeds (i.e., Prevotellaceae, Ruminococcaceae, and Lachnospiraceae at the family level and *Prevotella* 1, Rikenellaceae RC9 gut group, and *Succiniclasticum* at the genus level), regardless of the rearing system. The genus *Prevotella* is a dominant member of the bacterial community in the rumen (Stevenson and Weimer, 2007; Henderson et al., 2015; Mannelli et al., 2019) and is among the bacterial groups of the so-called rumen core microbiota (Henderson et al., 2015), along with members of the families Ruminococcaceae and Lachnospiraceae (e.g., microorganisms of the genera *Ruminococcus* and *Butyrivibrio*) (Henderson et al., 2015; Mannelli et al., 2019). The importance of these bacteria in the ecology of rumen communities and their key role in the degradation of cellulose, pectin, and hemicellulose has been also well documented (Kabel et al., 2011; Seshadri et al., 2018).

The presence of bacteria belonging to the genus *Succiniclasticum* is widely described in rumen communities (Holman and Gzyl, 2019; Auffret et al., 2020). Members of this genus are involved in the conversion of succinate to propionate (van Gylswyk, 1995). Furthermore, the abundance of propionate-producing bacteria (i.e., *Succiniclasticum* spp.) is positively correlated to animal feed efficiency (Auffret et al., 2020; Clemmons et al., 2020), since propionate is the main precursor of glucogenesis (Shabat et al., 2016). This is also confirmed by our study, in which a positive correlation was observed between the genus *Succiniclasticum* and the growth performance in the MA steers, indicating that the relative abundance of this genus in the rumen increased according to the ADG. Other than the genus *Succiniclasticum*, the genus *Fibrobacter* was also correlated with the growth of MA steers in our study. Members of the genus *Fibrobacter* are indeed involved in the conversion of cellulose to succinate that can be used as a substrate for the production of propionate by the genus *Succiniclasticum* (Holman and Gzyl, 2019). Furthermore, these two genera (i.e., *Fibrobacter* and *Succiniclasticum*) were more abundant in the rumen of AU steers (which showed higher ADG) than of MA steers, despite the fact that their presence in AU steers was not correlated to the growth performance, suggesting the occurrence of different fermentation patterns in the two breeds. The presence of the Rikenellaceae RC9 gut group (family Rikenellaceae) has been widely observed in the gastrointestinal tract of several ruminants (Holman and Gzyl, 2019; Tong et al., 2020). The Rikenellaceae RC9 gut group was positively correlated with growth performance in MA steers and was found to be more relatively abundant in the MA steers (in particular when this breed is reared under grazing conditions) than in the AU steers. These microorganisms can be involved in the production of short chain FAs (Holman and Gzyl, 2019) and in the scavenging of H_2, lowering methanogenesis rates (Tong et al., 2020). This reinforces the hypothesis that an increase in growth performance can be correlated to the production of propionate, which is not only a glucogenic precursor but also a sink of H_2 leading to more efficient energy recovery.

Two bacterial taxa (i.e., Lachnospiraceae NK3A20 group—family Lachnospiraceae and *Saccharofermentans*—family Ruminococcaceae) were negatively correlated with growth performance in MA steers. This result is consistent with the observation of another study in which microorganisms assigned to the family Lachnospiraceae and to the genus *Saccharofermentans* were dominant in the rumen of dairy cows with low milk yields (Sofyan et al., 2019). Despite the fact that *Saccharofermentans* and Lachnospiraceae spp. are involved in the production of short chain FAs, the negative correlation of their relative abundance with growth performance of MA steers could suggest that the pathways that involve these groups are less efficient in this breed (i.e., MA), than the pathways that involve the genus *Succiniclasticum* and the Rikenellaceae RC9 gut group.

Correlation with ADG in the AU steers differed from those in the MA steers, in accordance with our previous observations, which clearly showed that the main driving factor that influenced the microbial communities in this work was the breed. A significant effect of breed on the rumen microbiota was reported in Holstein and Jersey cows (Paz et al., 2016) and in Angus, Charolais, and Kinsella composite hybrid cattle (Li et al., 2019a). Our study extends this observation to MA and AU cattle, which are rustic breeds, that are well-adapted to extensive farming conditions, with potential advantages in terms of sustainability of animal production. The only similarity that was observed in the two breeds is in regard to members of the family Ruminococcaceae, since in the rumen of AU steers, an uncultured member of this family (i.e., Ruminococcaceae UCG-001) had a negative correlation with performance, in analogy to the genus *Saccharofermentans* in the rumen of MA steers. Further, the genus *Treponema* had a negative correlation with growth performance in AU steers. In the literature, the role of the genus *Treponema* in the rumen is still controversial, with both negative and positive correlations to animal performance reported. Negative correlations are usually explained by the potential pathogenic role of this microorganism (Mao et al., 2015), whereas positive correlations are explained by the fact that some species within this genus exhibit pectinolytic activity (Auffret et al., 2020). The only microbial group with a positive correlation with growth performance in the rumen of AU steers comprised an uncultured member of the family Succinivibrionaceae (i.e., Succinivibrionaceae UCG-002). These microorganisms are involved in the production of succinate and acetate, and their presence has been linked to low methane emissions from cows (Holman and Gzyl, 2019). Furthermore, a higher abundance of the genus *Succinivibrio* was observed in the rumen of cattle with higher feed efficiency (Auffret et al., 2020). Our observations suggest that different microbial groups can be correlated with animal performance in the two breeds, but that the microbial pathways that influence the performance could be the same (i.e., short chain FAs production and the presence of H_2 sinks as an alternative to methanogenesis). This evidence reinforces the hypothesis that different bacterial groups can have similar functions in the rumen ecosystem. Therefore, future studies should be focused on the impact that specific metabolic functions and pathways have on the rumen microbiota and on animal traits.

In this study, the microbial communities and the FA profiles in the rumens of two breeds of rustic cattle reared under different conditions were characterized. While the rearing system shaped the rumen microbiome structure, the main factor that influenced the bacterial communities was the cattle breed, indicating for the first time that host genetics plays an important role also in rustic cattle. This aspect is of particular interest since rustic cattle, such as AU and MA, are well suited to extensive farming in harsh environments, such as the Mediterranean area. Furthermore, while microorganisms involved in the production of propionate seem to have an influence on growth performance, different taxa had a positive influence on the weight gain in the two breeds, reinforcing once again the hypothesis that host genetics can influence the rumen microbiome.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, BioProject number PRJNA682716, BioSample accession numbers SAMN17005974-SAMN17006013.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because all experiments in this study were performed in accordance with the approved guidelines from the European directive 2010/63/UE and DL 4/03/2014 n 26.

AUTHOR CONTRIBUTIONS

MD, FC, AB, AS, CV, and MM conceived and designed the research. MD and FC wrote the manuscript. MD performed the bioinformatic analysis. FC, AC, and AS performed the field sampling. MD, GC, and MM performed the statistical analysis. LC and FC performed the chemical analyses. BM and JV performed the amplicons sequencing. AB, CV, and MM reviewed the manuscript. All authors read and approved the submitted version.

ACKNOWLEDGMENTS

This work was performed in the framework of the PROSMARTBEEF project, Rural Development Programme, Tuscany region, 2014–2020, 16.2 measure.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.652031/full#supplementary-material

REFERENCES

Alves, S. P., Santos-Silva, J., Cabrita, A. R. J., Fonseca, A. J. M., and Bessa, R. J. B. (2013). Detailed dimethylacetal and fatty acid composition of rumen content from lambs fed lucerne or concentrate supplemented with soybean oil. *PLoS One* 8:e58386. doi: 10.1371/journal.pone.0058386

AOAC (2000). *Official Methods of Analysis*, 17th Edn. Arlington, VA: AOAC.

Auffret, M. D., Stewart, R. D., Dewhurst, R. J., Duthie, C.-A., Watson, M., and Roehe, R. (2020). Identification of microbial genetic capacities and potential mechanisms within the rumen microbiome explaining differences in beef cattle feed efficiency. *Front. Microbiol.* 11:1229. doi: 10.3389/fmicb.2020.01229

Bas, P., Archimède, H., Rouzeau, A., and Sauvant, D. (2003). Fatty acid composition of mixed-rumen bacteria: effect of concentration and type of forage. *J. Dairy Sci.* 86, 2940–2948. doi: 10.3168/jds.S0022-0302(03)73891-0

Bergmann, G. T., Bates, S. T., Eilers, K. G., Lauber, C. L., Caporaso, J. G., Walters, W. A., et al. (2011). The under-recognized dominance of Verrucomicrobia in soil bacterial communities. *Soil Biol. Biochem.* 43, 1450–1455. doi: 10.1016/j.soilbio.2011.03.012

Bessa, R. J. B., Maia, M. R. G., Jerónimo, E., Belo, A. T., Cabrita, A. R. J., Dewhurst, R. J., et al. (2009). Using microbial fatty acids to improve understanding of the contribution of solid associated bacteria to microbial mass in the rumen. *Anim. Feed Sci. Technol.* 150, 197–206. doi: 10.1016/j.anifeedsci.2008.09.005

Bongiorni, S., Gruber, C. E. M., Chillemi, G., Bueno, S., Failla, S., Moioli, B., et al. (2016). Skeletal muscle transcriptional profiles in two Italian beef breeds, Chianina and Maremmana, reveal breed specific variation. *Mol. Biol. Rep.* 43, 253–268. doi: 10.1007/s11033-016-3957-3

Buccioni, A., Pallara, G., Pastorelli, R., Bellini, L., Cappucci, A., Mannelli, F., et al. (2017). Effect of dietary chestnut or quebracho tannin supplementation on microbial community and fatty acid profile in the rumen of dairy ewes. *Biomed. Res. Int.* 2017:4969076. doi: 10.1155/2017/4969076

Cabiddu, A., Salis, L., Tweed, J. K. S., Molle, G., Decandia, M., and Lee, M. R. F. (2010). The influence of plant polyphenols on lipolysis and biohydrogenation in dried forages at different phenological stages: in vitro study. *J. Sci. Food Agric.* 90, 829–835. doi: 10.1002/jsfa.3892

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

Cappucci, A., Alves, S. P., Bessa, R. J. B., Buccioni, A., Mannelli, F., Pauselli, M., et al. (2018). Effect of increasing amounts of olive crude phenolic concentrate in the diet of dairy ewes on rumen liquor and milk fatty acid composition. *J. Dairy Sci.* 101, 4992–5005. doi: 10.3168/jds.2017-13757

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

Chilliard, Y., Ferlay, A., and Doreau, M. (2001). Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livest. Prod. Sci.* 70, 31–48.

Clemmons, B. A., Powers, J. B., Campagna, S. R., Seay, T. B., Embree, M. M., and Myer, P. R. (2020). Rumen fluid metabolomics of beef steers differing in feed efficiency. *Metabolomics* 16:23. doi: 10.1007/s11306-020-1643-x

Collomb, M., Ueli, B., Sieber, R., Jeangros, B., and Bosset, J.-O. (2002). Correlation between fatty acids in cows' milk fat produced in the lowlands, mountains and highlands of Switzerland and botanical composition of the fodder. *Int. Dairy J.* 12, 661–666.

Conte, G., Serra, A., Casarosa, L., Ciucci, F., Cappucci, A., Bulleri, E., et al. (2019). Effect of linseed supplementation on total longissimus muscle lipid composition and shelf-life of beef from young Maremmana bulls. *Front. Vet. Sci.* 5:326. doi: 10.3389/fvets.2018.00326

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Fox, D. G., Sniffen, C. J., O'Connor, J. D., Russell, J. B., and Van Soest, P. J. (1992). A net carbohydrate and protein system for evaluating cattle diets: III. Cattle requirements and diet adequacy. *J. Anim. Sci.* 70, 3578–3596. doi: 10.2527/1992.70113578x

Gallo, L., De Marchi, M., and Bittante, G. (2014). A survey on feedlot performance of purebred and crossbred european young bulls and heifers managed under intensive conditions in Veneto, northeast Italy. *Ital. J. Anim. Sci.* 13:3285. doi: 10.4081/ijas.2014.3285

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Collaborators, G. R. 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. doi: 10.1038/srep14567

Holman, D. B., and Gzyl, K. E. (2019). A meta-analysis of the bovine gastrointestinal tract microbiota. *FEMS Microbiol. Ecol.* 95:fiz072. doi: 10.1093/femsec/fiz072

Horizon. (2020). *Work Programme 2016 - 2017. Food Security, Sustainable Agriculture and Forestry, Marine and MAritime and Inland Water Research and the Bioeconomy*. Vancouver, BC: Horizon.

SAS Institute (2008). *User's Guide: Statistics*. Cary, NC: SAS Institute.

Jenkins, T. C. (1994). Regulation of lipid metabolism in the rumen. *J. Nutr.* 124, 1372S–1376S.

Kabel, M. A., Yeoman, C. J., Han, Y., Dodd, D., Abbas, C. A., de Bont, J. A. M., et al. (2011). Biochemical characterization and relative expression levels of multiple carbohydrate esterases of the xylanolytic rumen bacterium *Prevotella ruminicola* 23 grown on an ester-enriched substrate. *Appl. Environ. Microbiol.* 77, 5671–5681. doi: 10.1128/AEM.05321-11

Kaneda, T. (1991). Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* 55, 288–302.

Kramer, J. K. G., Cruz-Hernandez, C., Deng, Z., Zhou, J., Jahreis, G., and Dugan, M. E. R. (2004). Analysis of conjugated linoleic acid and trans 18:1 isomers in synthetic and animal products. *Am. J. Clin. Nutr.* 79, 1137S–1145S. doi: 10.1093/ajcn/79.6.1137S

Lee, M. R. F., Harris, L. J., Dewhurst, R. J., Merry, R. J., and Scollan, N. D. (2003). The effect of clover silages on long chain fatty acid rumen transformations and digestion in beef steers. *Anim. Sci.* 76, 491–501. doi: 10.1017/S1357729800058719

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

Mannelli, F., Cappucci, A., Pini, F., Pastorelli, R., Decorosi, F., Giovannetti, L., et al. (2018). Effect of different types of olive oil pomace dietary supplementation on the rumen microbial community profile in Comisana ewes. *Sci. Rep.* 8:8455. doi: 10.1038/s41598-018-26713-w

Mannelli, F., Daghio, M., Alves, S. P., Bessa, R. J. B., Minieri, S., Giovannetti, L., et al. (2019). Effects of chestnut tannin extract, vescalagin and gallic acid on the dimethyl acetals profile and microbial community composition in rumen liquor: an in vitro study. *Microorganisms* 7: 202. doi: 10.3390/microorganisms7070202

Mao, S., Zhang, M., Liu, J., and Zhu, W. (2015). Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Sci. Rep.* 5:16116. doi: 10.1038/srep16116

Minato, H., Ishibashi, S., and Hamaoka, T. (1988). Cellular fatty acid and sugar composition of representative strains of rumen bacteria. *J. Gen. Appl. Microbiol.* 34, 303–319.

Miyagawa, E. (1982). Cellular fatty acid and fatty aldehyde composition of rumen bacteria. *J. Gen. Appl. Microbiol.* 28, 389–408.

Mosley, E. E., Powell, G. L., Riley, M. B., and Jenkins, T. C. (2002). Microbial biohydrogenation of oleic acid to trans isomers in vitro. *J. Lipid Res.* 43, 290–296.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *vegan: Community Ecology Package. R package version 2.5-6*.

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.* 7:1206. doi: 10.3389/fmicb.2016.01206

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned

ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–7196. doi: 10.1093/nar/gkm864

R Core Team (2020). *R: A language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Ramos-Morales, E., Arco-Pérez, A., Martín-García, A. I., Yáñez-Ruiz, D. R., Frutos, P., and Hervás, G. (2014). Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. *Anim. Feed Sci. Technol.* 198, 57–66. doi: 10.1016/j.anifeedsci.2014.09.016

Renand, G., Havy, A., and Turin, F. (2002). Caractérisation des aptitudes bouchères et qualités de la viande de trois systèmes de production de viande bovine à partir des races rustiques françaises Salers, Aubrac et Gasconne. *INRAE Prod. Anim.* 15, 171–183. doi: 10.20870/productions-animales.2002.15.3.3699

Roehe, R., Dewhurst, R. J., Duthie, C., 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

Sargentini, C. (2011). "La razza bovina maremmana come produttrice di carne, mediante allevamento in purezza o in incrocio," in *La razza bovina Maremmana* Editrice Innocenti, (Florence: Quaderni dei Georgofili), 71–84.

Seshadri, R., Leahy, S. C., Attwood, G. T., Hoong Teh, K., 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

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Miller, M. E. B., 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

Sofyan, A., Uyeno, Y., Shinkai, T., Hirako, M., Kushibiki, S., Kanamori, H., et al. (2019). Metagenomic profiles of the rumen microbiota during the transition period in low-yield and high-yield dairy cows. *Anim. Sci. J.* 90, 1362–1376. doi: 10.1111/asj.13277

Stevenson, D. M., and Weimer, P. J. (2007). Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75, 165–174. doi: 10.1007/s00253-006-0802-y

Tong, J., Zhang, H., Wang, J., Liu, Y., Mao, S., Xiong, B., et al. (2020). Effects of different molecular weights of chitosan on methane production and bacterial community structure in vitro. *J. Integr. Agric.* 19, 1644–1655. doi: 10.1016/S2095-3119(20)63174-4

van Gylswyk, N. O. (1995). Succiniclasticum ruminis gen. nov., sp . nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int. J. Syst. Bacteriol.* 45, 297–300.

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Vasta, V., Daghio, M., Cappucci, A., Buccioni, A., Serra, A., Viti, C., et al. (2019). Invited review: plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: experimental evidence and methodological approaches. *J. Dairy Sci.* 102, 3781–3804. doi: 10.3168/jds.2018-14985

Vlaeminck, B., Fievez, V., Cabrita, A. R. J., Fonseca, A. J. M., and Dewhurst, R. J. (2006). Factors affecting odd- and branched-chain fatty acids in milk: a review. *Anim. Feed Sci. Technol.* 131, 389–417. doi: 10.1016/j.anifeedsci.2006.06.017

Zeng, B., Li, G., Yuan, J., Li, W., Tang, H., and Wei, H. (2013). Effects of age and strain on the microbiota colonization in an infant human flora-associated mouse model. *Curr. Microbiol.* 67, 313–321. doi: 10.1007/s00284-013-0360-3

Zhong, S., Ding, Y., Wang, Y., Zhou, G., Guo, H., Chen, Y., et al. (2019). Temperature and humidity index (THI)-induced rumen bacterial community changes in goats. *Appl. Microbiol. Biotechnol.* 103, 3193–3203. doi: 10.1007/s00253-019-09673-7

Conflict of Interest: The 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 September 2021
doi: 10.3389/fmicb.2021.710914

Effect of Methane Inhibitors on Ruminal Microbiota During Early Life and Its Relationship With Ruminal Metabolism and Growth in Calves

*Omar Cristobal-Carballo[1,2], Susan A. McCoard[1], Adrian L. Cookson[2,3], Siva Ganesh[4], Katherine Lowe[1], Richard A. Laven[2] and Stefan Muetzel[1]**

[1] Ruminant Nutrition and Physiology Team, AgResearch Grasslands, Palmerston North, New Zealand, [2] School of Veterinary Science, Massey University, Palmerston North, New Zealand, [3] Food System Integrity, AgResearch Grasslands, Palmerston North, New Zealand, [4] Biostatistics Team, AgResearch Grasslands, Palmerston North, New Zealand

OPEN ACCESS

Edited by:
Diego P. Morgavi,
INRAE
Clermont-Auvergne-Rhône-Alpes,
France

Reviewed by:
Wenli Li,
U.S. Dairy Forage Research Center,
Agricultural Research Service,
United States Department
of Agriculture, United States
Sarah Jade Meale,
The University of Queensland,
Australia

***Correspondence:**
Stefan Muetzel
stefan.muetzel@agresearch.co.nz

Specialty section:
This article was submitted to
Systems Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 17 May 2021
Accepted: 17 August 2021
Published: 16 September 2021

Citation:
Cristobal-Carballo O,
McCoard SA, Cookson AL,
Ganesh S, Lowe K, Laven RA and
Muetzel S (2021) Effect of Methane
Inhibitors on Ruminal Microbiota
During Early Life and Its Relationship
With Ruminal Metabolism and Growth
in Calves.
Front. Microbiol. 12:710914.
doi: 10.3389/fmicb.2021.710914

The present study aimed to determine whether dietary supplementation with methanogen inhibitors during early life may lead to an imprint on the rumen microbial community and change the rumen function and performance of calves to 49-weeks of rearing. Twenty-four 4-day-old Friesian x Jersey cross calves were randomly assigned into a control and a treatment group. Treated calves were fed a combination of chloroform (CF) and 9,10-anthraquinone (AQ) in the solid diets during the first 12 weeks of rearing. Afterward, calves were grouped by treatments until week 14, and then managed as a single group on pasture. Solid diets and water were offered *ad libitum*. Methane measurements, and sample collections for rumen metabolite and microbial community composition were carried out at the end of weeks 2, 4, 6, 8, 10, 14, 24 and 49. Animal growth and dry matter intake (DMI) were regularly monitored over the duration of the experiment. Methane emissions decreased up to 90% whilst hydrogen emissions increased in treated compared to control calves, but only for up to 2 weeks after treatment cessation. The near complete methane inhibition did not affect calves' DMI and growth. The acetate:propionate ratio decreased in treated compared to control calves during the first 14 weeks but was similar at weeks 24 and 49. The proportions of *Methanobrevibacter* and *Methanosphaera* decreased in treated compared to control calves during the first 14 weeks; however, at week 24 and 49 the archaea community was similar between groups. Bacterial proportions at the phylum level and the abundant bacterial genera were similar between treatment groups. In summary, methane inhibition increased hydrogen emissions, altered the methanogen community and changed the rumen metabolite profile without major effects on the bacterial community composition. This indicated that the main response of the bacterial community was not a change in composition but rather a change in metabolic pathways. Furthermore, once methane inhibition ceased the methanogen community, rumen metabolites and hydrogen emissions became similar between treatment groups, indicating that perhaps using the treatments tested in this study, it is not possible to imprint a low methane microbiota into the rumen in the solid feed of pre-weaned calves.

Keywords: methane inhibitors, early life, rumen, metataxonomics, microbiota, imprinting, animal phenotype, fermentation profiles

INTRODUCTION

The rumen harbors a symbiotic community of microorganisms that degrade ingested plant components (Hobson and Stewart, 1997). Complex carbohydrates are hydrolyzed and fermented into short chain fatty acids (SCFA), mostly absorbed across the rumen wall and utilized as energy sources for the host ruminant (Van Soest, 1994). A by-product of acid formation in the rumen is hydrogen (H_2) that is converted to methane (CH_4), a potent greenhouse gas (Ellis et al., 2007). For decades, manipulations of the rumen microbiota have been attempted with the aim to improve animal performance or reduce CH_4 emissions (Haque, 2018). In adult ruminants, microbial manipulation, e.g., dietary interventions, microbial inhibitors, plant extracts, among others, have shown either no or only short-term post-treatment effects because of the well-established rumen microbiota (Weimer et al., 2010; Weimer, 2015). In young ruminants, it has been observed that microbial establishment progresses as solid feed intake increases and the rumen develops (Fonty et al., 1987; Jami et al., 2013; Rey et al., 2014; Dill-McFarland et al., 2017). Therefore, manipulations of the rumen microbiota during early life could be a feasible mechanism to promote changes in the community structure that will persist in later life (Yáñez-Ruiz et al., 2010; Abecia et al., 2014a,b; Belanche et al., 2019; Meale et al., 2021).

Dietary and chemical interventions during early life have been shown to alter rumen microbial composition and influence CH_4 emissions and SCFA production during and for up to 3 months after treatment cessation in small ruminants (Abecia et al., 2013, 2014b; Saro et al., 2018; Wang et al., 2019). In calves, dietary manipulations during early life have revealed that shifts in the ruminal bacterial community correlate to changes in fermentation patterns and the colonization by archaeal microorganisms (Dias et al., 2017). Such studies have also indicated that changes in the rumen microbial composition can persist to adulthood (Dill-McFarland et al., 2019). The intake of methane inhibitors, targeting the methyl-coenzyme M reductase, from birth until 3 weeks post-weaning have shown long-lasting changes for up to at least 1 year of life on the ruminal microbial ecosystem and CH_4 emissions, without differences in live weight (LW), Average daily gain (ADG) and SCFA production between treatments (Meale et al., 2021). Overall, findings in young ruminants suggest that alterations in the early establishment of rumen microbiota may influence the microbial succession process. Additionally, early life intervention studies have reported decreased methanogenesis in the short- and long-term post-treatment, with promising mitigation results in calves maintained throughout the first year of the animals' life. However, there is a need for more studies assessing the impact of microbial manipulation during early life on the long-term microbial establishment (bacteria and archaea), rumen function and performance in calves.

In a normal functioning rumen, H_2 released during rumen microbial fermentation is used by methanogens to reduce carbon dioxide (CO_2) to CH_4 (Ungerfeld and Kohn, 2006; Janssen, 2010). Methanogenic archaea make up only 3–4% of the rumen microbial population (Yanagita et al., 2000; Ziemer et al., 2000), but they play an important role in H_2 removal (Wolin et al., 1997; Joblin, 1999). The use of methanogen inhibitors in ruminants has shown to increase H_2 concentrations in the rumen (Bauchop, 1967; Kung et al., 2003), change the feed fermentation pathways toward production of less acetate and more propionate and butyrate, and change the composition of the methanogen community away from the dominant *Methanobrevibacter* species (Knight et al., 2011; Martinez-Fernandez et al., 2016). However, it is not clear if alterations of the methanogen community during the first weeks of life through methane inhibitor treatment affects subsequent H_2 emissions and succession of methanogen microbes following discontinuation of treatment.

Studies *in vitro* and *in vivo* have shown that chloroform (CF) and 9,10-anthraquinone (AQ) are potent methanogen inhibitors (Bauchop, 1967; Garcia-Lopez et al., 1996; Kung et al., 2003; Knight et al., 2011). The mechanism of action of CF on methanogens has not been confirmed. Available data indicates that CF interferes at the cobamide-dependent methyl transferase step of the methanogenesis pathway, but there could also be collateral inhibition of methyl transferases in other bacteria (Gunsalus and Wolfe, 1978; Graham and White, 2002). In contrast, AQ seems to interfere with the methyl-coenzyme M of methanogens by disrupting electron transfer during CH_4 formation (Garcia-Lopez et al., 1996; Kung et al., 1998). The use of these methane inhibitors can have adverse effects on feed intake, digestion, rumen fermentation and LW gain when added at high concentrations (Kung et al., 2003; Martinez-Fernandez et al., 2016) with these studies examining changes in methanogen populations, ruminal fermentation and CH_4/H_2 production in mature ruminants. To our knowledge, there is no evidence of the effects of feeding two methanogen inhibitors (CF and AQ) during early and adult life on rumen microbial establishment, rumen function and performance in calves. The use of CF and AQ was to provide a methane inhibition effect that would last over the treatment period even if the rumen community adapts to one of the inhibitors. The objective of the present study was to determine whether feeding CF and AQ in the solid feed diet during early life may lead to an imprint on the rumen microbial community, change the fermentation pathways and alter growth performance of calves to 49 weeks of age.

MATERIALS AND METHODS

Experimental Design

Twenty-four female dairy calves (Friesian x Jersey cross) were randomly allocated, following a simple randomization procedure, to a control and a treatment group. The treatment group had the methane inhibitors CF and AQ mixed into their starter concentrate and partial mixed ration (PMR) diets. The control diet did not contain methane inhibitors. The treatment period lasted for 12 weeks followed by a 37-week period in which both groups were fed similar diets. Animal manipulations were reviewed and approved (AE13132) by the Grasslands Animal Ethics Committee and complied with the institutional Codes of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the New Zealand Animal Welfare Act of 1999 and its amendments.

Animal Management

Calves were sourced from a single commercial farm at 4 days of age and an average weight of 33 ± 3.7 kg (mean ± S.D.). On arrival to the animal facility, calves were weighed and assigned to one of the experimental groups. Calves were housed in individual pens (1.5 m × 3 m) bedded with wood shavings. Experimental groups were housed in separate temperature-controlled rooms to avoid cross contamination of ruminal microbes between treatments. At week 10, after weaning off milk, calves were moved from individual to a single group pen within each treatment group. At week 14, calves from both groups were transferred outdoors and managed as one mob on pasture. LW was determined weekly during the first 10 weeks of life, fortnightly until week 24 of age, and monthly thereafter. ADG was calculated at the end of the experiment.

Feeding Management and Diet Composition

All calves were fed 4 L/d (2 meals of 2 L per day) of colostrum during the first 4 days of life prior to enrollment into the trial. From day 5 onward, calves were fed 4 L of reconstituted milk replacer (MR; 125 g/kg dry matter: 20.6% fat, 22.7% crude protein (CP) and 49.5% lactose; Milligans, Oamaru, New Zealand) split into two equal feeds of 2 L fed at 0800 and 1600 h using individual feeders. At week 4, calves were transitioned to once per day milk feeding, where 4 L was offered in the morning only. At week 10, calves were fully weaned from MR over a 10-day period reducing individual milk intake by 10% per day. Starter concentrate (Denver Stock Feeds, Palmerston North, New Zealand) was offered *ad libitum* from day 8. From week 4, calves were offered *ad libitum* access to starter concentrate plus a PMR. Calves were weaned off starter concentrate from weeks 12 to 14. At week 14, calves were moved outdoors and managed as a single mob on a ryegrass and red clover mixed sward, with continue free access to the PMR. Calves were weaned off PMR diet from week 15 to 17, reducing intake by 10% per day. Fresh water was available *ad libitum* throughout the study.

The ingredients used in the starter concentrate and PMR diets are given in **Table 1**. The chemical composition of the milk replacer, starter concentrate, PMR diet and pasture diets (**Table 2**) was determined by wet chemistry at the Nutrition Lab at Massey University (Palmerston North, New Zealand). Compositional analyses were carried out according to the methods of the Association of Official Analytical Chemists (AOAC, 1990, 2010, 2012). When animals were grazed, pasture samples were taken during methane measurements and scanned by near-infrared reflectance spectroscopy (NIRS; FeedTECH, AgResearch Ltd., Palmerston North, New Zealand) for ash, ether extract, CP, neutral detergent fiber (NDF) and water soluble carbohydrates (WSC) contents (Corson et al., 1999). Dry matter intake (DMI) of starter concentrate and PMR diets were measured on a daily basis only during the individual housing period. Additionally, DMI of starter concentrate, PMR and pasture were also determined when animals were brought into the respiration chambers to measure methane emission. The feed intake was calculated as the difference between feed offered and refused by the animal.

TABLE 1 | Ingredients of the starter concentrate and the partial mixed ration (PMR) diet.

Starter concentrate	g/kg	PMR diet	g/kg
Maize	108	Chopped hay	500
Barley	432	Barley	290
Peas	173	Soya	100
Soya	205	Molasses	100
Molasses	54	Di-calcium-phosphate	5.5
Sodium bicarbonate	20	Salt	3.0
Salt	5.0	Mineral/vitamin mix	1.5
Calf pre-mix	1.0		
Bovatec	0.6		
Rumasweet palatant	0.2		

TABLE 2 | Chemical composition (g/kg) of the milk replacer, starter concentrate, partial mixed ration (PMR) diet and pastures.

Diet	Milk replacer[1]	Starter concentrate	PMR diet	Pasture Wk24[a]	Pasture Wk49[a]
Dry matter[2]	97.0	88.7	82.8	17.6	20.2
Crude protein[3]	22.7	20.8	13.3	21.8	18.9
WSC[4]	49.5*	56.1	5.1	11.6	8.7
NDF[5]	0.0	15.5	43.8	43.3	49.3
Ether extract[6]	20.6	1.7	1.1	3.5	2.1
Ash[7]	6.2	5.9	5.6	9.5	9.2

[a]Chemical composition of pastures was scanned using the scanned by near-infrared reflectance spectroscopy (NIRS; Corson et al., 1999).
[1]Manufacturers data.
[2]Method 945.15; AOAC, 2010.
[3]Method 992.15; AOAC, 2010.
[4]Water soluble carbohydrates (WSC); (Paul and Southgate, 1978).
[5]Neutral detergent fiber (NDF); method 7.074; AOAC, 1990.
[6]Method 954.02; AOAC, 1990.
[7]Method 942.05; AOAC, 2012.
**Lactose.*

Dry matter content was determined by drying the feed offer and refusal for 48 h at 105°C.

Methane Inhibitors

The inhibitors used in this study were 9,10-AQ (A90004, Sigma-Aldrich, St Louis, MO, United States) and CF (C2432, Sigma-Aldrich, St Louis, MO, United States). The CF was complexed with β-cyclodextrin (β-CD; Trappsol®, TBCDF-F, Cyclodextrin Technologies Development Ltd., Gainesville, FL, United States) to stop evaporation from the feed and to render it odorless. 50 ml of CF were added to β-CD dissolved at 10% (w/v) in 10 L of water. The mix was kept at 4°C and shaken every hour during an 8 h period, after which it settled over night. The supernatant liquid was decanted and the sediment filtered using Whatman no. 54 filter paper, dried in the fridge at 4°C and transferred to a jar. Concentrations of 9,10-AQ and CF fed to the calves in the starter concentrate and PMR diets were 500 and 50 mg/kg of feed, respectively. The choice and dose of the CF/AQ mix was based on a dose response test run *in vitro* (Muetzel, personal communication). Both AQ and CF were pre-mixed into approximately 5 kg of starter concentrate using a food processor

and then mixed into the total batch amount using a concrete mixer. The same process was used for the PMR diet, where the inhibitors were first mixed into 5 kg of soybean meal, then into the total soybean meal using a concrete mixer and, finally, the rest of the ingredients were incorporated in a large mixer. The final mixes were prepared twice a week and stored at 4°C until used.

Measurements of Gas Emissions and Dry Matter Intake

Emissions of CH_4 and H_2 were measured at weeks 2, 4, 6, 8, 10, 14, 24 and 49. Measurements were carried out in open circuit respiratory chambers (Pinares-Patiño et al., 2012) for 24 h. The air flow through the chambers was adjusted to 600 L/min to account for the low CH_4 emissions of a young animal. For the last two measurements, the airflow was increased to 1,000 and 1,500 L/min, respectively. Calves entered the chambers in the morning (0800 h), when solid diets, i.e., the starter concentrate and/or PMR diets (week 2–14), or fresh grass (week 24 and 29), were offered. During the milk feeding period, MR was offered before entering to the chambers in the morning (0800 h) and on weeks 2 and 4 also before the afternoon feed allocation (1600 h). For the last two measurements, the same type of pasture that the animals were consuming in their allocated paddocks was cut daily (Aorangi Farm, AgResearch, New Zealand) and transported to the Animal Facility at Grasslands. For measurements at week 24 and 49, calves were moved to indoor yards and adapted to eat fresh cut pasture in pens for five to 7 days prior to entering to the chambers. Animals in the chambers were offered solid diets *ad libitum* and refusals were collected to determine DMI from the difference between feed allowance and refusals.

Rumen Fluid Sampling

Rumen samples were taken via stomach tubing after removing the calves from the respiration chambers (weeks 2, 4, 6, 8, 10, 14, 24 and 49). Each sample was subsampled for SCFA analysis (1.8 ml) and DNA extraction (0.9 ml). Samples for SCFA analysis were centrifuged (20,000 × *g*, 10 min, 4°C) and an aliquot of 0.9 ml of the supernatant was collected into 0.1 ml of internal standard (19.8 mM ethylbutyrate in 20% v/v phosphoric acid) and stored at −20°C until analysis. Rumen samples for subsequent DNA extraction and microbial community analysis were snap-frozen and stored at −20°C.

Short Chain Fatty Acid Analysis

Samples for SCFA were thawed and centrifuged (20,000 × *g*, 10 min, 4°C) and 0.8 ml of the supernatant was collected into a crimp cap glass vial. Gas chromatography was used to analyze SCFA composition (Attwood et al., 1998) in a HP 6,890 gas chromatograph equipped with a flame ionization detector using a Zebron ZB-FFAP 30.0 m × 0.53 mm I.D × 1 μm film column (Tavendale et al., 2005).

DNA Extractions, Amplification of Target Genes and Amplicon Pooling

Nucleic acids were extracted from 200 μl of the rumen fluid using the phenol-CF, bead beating, with filtration kit for purification II (PCQI) method (Henderson et al., 2013; Kittelmann et al., 2013). A total of 120 ng of DNA contained in 6 μl of water were divided into 3 aliquots of 20 μl each (Kittelmann et al., 2013). DNA extracts were quantified on a FlexStation 3 (Molecular Devices, LLC. San Jose, CA, United States) and run on a 1% agarose gel with a lambda-*Hin*dIII marker to determine sizing and integrity. DNA primers are presented in **Supplementary Table 1**. PCR amplicon reactions (30 and 35 cycles for bacteria and archaea, respectively), targeting the region of the 16S rRNA genes in the microbial groups bacteria and archaea, were prepared as described by Kittelmann et al. (2013), with the following modifications. Triplicate PCR products were pooled, and the correct sizes of PCR amplicons and the absence of signal from negative controls were verified by agarose gel electrophoresis and quantified by fluorescence using the Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, United States). For each amplicon, 150 ng from the same target gene and region (i.e., all bacteria and archaea amplicons) were pooled, concentrated and quantified (Quant-iT dsDNA HS assay kit; Invitrogen, Carlsbad, CA, United States). Each pool was then purified using the NucleoMag NGS kit (Macherey-Nagel, Dueren, Germany), with a final purification of the amplicons performed with the QIAquick PCR Purification kit (Qiagen, Valencia, CA, United States). The resulting DNA concentration was quantified using Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA, United States). Both pools were then diluted to 6.0×10^9 copies per μl and combined at a bacteria to archaea ratio of 5:1 (Kittelmann et al., 2013).

Before sequencing, pooled libraries were checked for quality control (QC) with the Labchip GX Touch HT Instrument (PerkinElmer, Waltham, MA, United States) using the DNA High Sensitivity assay. Amplicons were sequenced at the Massey Genome Service/New Zealand Genomics Limited using Illumina MiSeq system (Massey University, Palmerston North, New Zealand). The pooled library was run on one Illumina MiSeq; 2 × 250 base PE run version 2 chemistry (Reagent Kit v2, 500 cycles; Invitrogen, Carlsbad, CA, United States). An Illumina prepared PhiX control library for the run was loaded onto the Illumina MiSeq run at 20% volume. Sequence reads were provided in fastq format. Raw sequence reads were deposited in the European Nucleotide Archive under the accession number PRJEB37781.

Phylogenetic Analysis

Sequencing reads were quality-filtered using the DynamicTrim function of SolexaQA (Cox et al., 2010). Reads were then processed and analyzed using QIIME version 1.8 (Caporaso et al., 2010). Sequencing reads were grouped, using the UCLUST algorithm, into operational taxonomic units (OTUs) sharing similarities over 97% for bacteria and 99% for archaea (Edgar, 2010). Sequences were then assigned to phylogenetic groups using the BLAST (version 2.4.0) algorithm (Altschul et al., 2012). Bacterial 16S rRNA genes were assigned using SILVA 123 (Henderson et al., 2019) and archaea 16S rRNA genes using RIM-DB (Seedorf et al., 2014). OTU-tables generated by QIIME were used for downstream statistical analysis.

Statistical Analysis

The effects of including CF and AQ (methane inhibitors) in the solid feed diet of calves from 1 to 12 weeks of age on DMI, ADG and LW, rumen function (SCFA and enteric emissions) and microbial community composition (bacteria and archaea) were evaluated during and after treatment.

The effects of treatment on rumen function, and DMI, ADG and LW were analyzed by fitting a linear mixed effect (LME) model via the restricted maximum likelihood (REML) framework as implemented in the *NLME* package in R (Pinheiro et al., 2015; R Core Team, 2016). DMI, LW, ADG, CH_4, and H_2 emissions, total concentrations and individual proportions of SCFA were fitted in an LME model and included treatment and time as fixed effects and animal as random effect. LW was adjusted to initial LW at trial entry. The equation used is as follows:

$$y_{ijk} = \mu + \beta_k + \alpha_i + \gamma_j + (\alpha\gamma)_{ij} + u_{ik} + e_{ijk}$$

where y_{ijk} is response at time j for the kth animal in the ith treatment, μ is the general mean, β_k is the covariate for the animals within treatments (only used for analysis of LW), α_i is the effect of the ith treatment, γ_j is the effect of the jth time, $(\alpha\gamma)_{ij}$ is the interaction between treatment and time, u_{ik} is the normally distributed random experimental error for the experimental units (the animals within treatments) with constant variance $\sigma_u{}^2$, and e_{ijk} is the normally distributed random experimental error on repeated measures with variance $\sigma_e{}^2$. The resulting models were analyzed by repeated-measures ANOVA and Tukey's post-test to determine the longitudinal effects of the intake of methanogen inhibitors during early life. Predicted means from the model, together with estimates of the standard error of the mean (SEM), permutation *F*-test of the model (1,000 permutations), and pairwise comparisons (Tukey test) were obtained using the *PREDICTMEANS* package in R (Luo et al., 2014). Significance was declared when $P \leq 0.050$ (Ganesh and Cave, 2018).

The OTU-tables of the rumen microbial community generated by QIIME were analyzed to determine changes in the alpha and beta diversity of the bacteria and archaea communities produced by treatment interventions. The alpha diversity of the rumen bacterial and archaeal microbiota were analyzed using the Shannon index in the *VEGAN* package of R (Oksanen et al., 2017). The effects of methanogen inhibitors on the Shannon index of the microbial (bacterial and archaeal) community were analyzed using an LME model and repeated measurements ANOVA as described for rumen function, and DMI and ADG data. Predicted means from the models, together with estimates of the SEM were obtained, and pairwise comparisons were done using Tukey's test. The beta diversity of the entire community and abundant community of bacteria and archaea (abundant microbes are described below) were analyzed using the partial least squares discriminant analysis (PLSDA) in the *MixOmics* package of R (Lê Cao et al., 2016).

Univariate analyses were done to identify the effects of methane inhibitors in the abundant bacteria and archaea taxa. Abundant bacterial phyla, bacterial genera and archaea species were defined as organisms with an average proportion $\geq$0.5, $\geq$0.5 and $\geq$1.0%, respectively, across the complete dataset. The effect of treatment on the abundant bacterial and archaeal communities was analyzed using an LME model and repeated measurements ANOVA as indicated for rumen function and animal DMI and ADG. After checking for normality, the data of the most abundant bacterial and archaeal taxa were natural logarithm transformed. Predicted means from the model, together with the 95% confidence interval (CI) of the geometric mean, permutation *F*-test of the model, and pairwise comparisons were obtained, and back transformed using the *PREDICTMEANS* package of R. Pairwise *P*-values were calculated using the Benjamini–Hochberg test (BH; Benjamini and Hochberg, 1995). Transformed mean and 95% CI were reported as indicated by Bland and Altman (1996). Significance was declared when $P \leq 0.050$.

RESULTS

Animal Performance

The effect of methane inhibitors inclusion in the solid feed diet on DMI for treated and control calves is shown in **Table 3**. DMI of starter concentrate, PMR, pasture and total DMI of all solid feed diets increased over time ($P < 0.001$) in both treatment groups. Total mixed ratio intake was 11% lower ($P = 0.022$) in treated compared to control calves. Concentrate intake had a treatment by time interaction effect tendency ($P > 0.090$), but the *post hoc* (Tukey test) analysis did not show differences ($P \geq 0.468$) between treated and control calves. No other treatment by time effects were observed for PMR, pasture and total DMI of all solid feed diets. LW and ADG of treated and control calves is presented in **Figure 1**. There was a treatment by time interaction ($P < 0.001$) for overall LW gain throughout the study, nevertheless this was primarily influenced by a 5.6% difference in LW between treated and control calves at week 44 (231 kg vs. 245 kg, $P < 0.001$; **Figure 1A**). However, there was no evidence ($P = 0.087$) of an effect of treatment on ADG (Trt = 0.613 kg/d [C.I. = 0.590 – 0.637] vs. Ctrl = 0.642 kg/d [C.I. = 0.619 – 0.666]). ADG declined from week 14 to 18 following removal of the starter concentrate with recovery of growth rates observed by week 20 (**Figure 1B**).

Rumen Fermentation

The intake of methane inhibitors on the rumen function is shown in **Table 4**. Treatment by time interaction effects ($P \leq 0.001$) were evident during the first 14 weeks of rearing for yield and production of CH_4 and H_2, and the percentage of acetate, propionate, caproate, valerate and isovalerate. Treated calves had average CH_4 yield decreases ($P < 0.001$) of 7.9 $\pm$ 1.90-fold (mean $\pm$ SEM), and H_2 yield ($P < 0.001$) increases of 88.7 $\pm$ 48.78-fold compared to control calves, but similar values for CH_4 yield and H_2 yield in both groups at 24 and 49 weeks. The average proportion of acetate decreased 1.2 $\pm$ 0.02-fold ($P < 0.001$) and those of propionate increased 1.3 $\pm$ 0.05-fold ($P = 0.001$) in treated compared to control calves, but the proportions of butyrate were similar ($P = 0.246$) in both groups. When CH_4 was inhibited, treated calves had valerate 2.1 $\pm$ 0.17-fold greater ($P < 0.001$), caproate 2.8 $\pm$ 0.08-fold greater ($P < 0.001$)

TABLE 3 | Effect of methane inhibitors[1] on dry matter intake (DMI).

		Rearing period (weeks)														P-val		
	Treatment	1	2	3	4*	5	6	7	8	9	10	14	24	49	SED	P-Tx	P-Tm	P-Int
Concentrate (kg)	Ctrl	0.11	0.24	0.37	0.54	0.67	0.68	0.75	0.74	0.81	0.82	–	–	–	0.046	0.117	<0.001	0.090
	Trt	0.09	0.21	0.34	0.46	0.53	0.57	0.61	0.66	0.75	0.83	–	–	–				
PMR (kg)	Ctrl	–	–	–	0.17	0.21	0.37	0.54	0.73	1.04	1.21	2.38	–	–	0.062	0.022	<0.001	0.150
	Trt	–	–	–	0.15	0.21	0.30	0.43	0.57	0.83	1.09	2.33	–	–				
Pasture (kg)	Ctrl	–	–	–	–	–	–	-	–	–	–	–	3.05	6.84	0.466	0.524	<0.001	0.360
	Trt	–	–	–	–	–	–	-	–	–	–	–	2.87	7.28				
Total DMI (kg)	Ctrl	0.11	0.24	0.37	0.65	0.88	1.05	1.29	1.47	1.85	2.03	2.38	3.05	6.84	0.193	0.396	<0.001	0.572
	Trt	0.09	0.21	0.34	0.59	0.75	0.87	1.04	1.23	1.58	1.93	2.33	2.87	7.28				

DMI was calculated for starter concentrate, partial mixed rations (PMR), pasture and total DMI in dairy calves. Results[2] are the means and standard error of the differences (SED), P-value for treatment (P-Tx), time (P-Tm) and interaction (P-Int). Significance of pairwise comparisons (Tukey post hoc analysis) between treatments are shown in bold at each sampling time.

[1] The treatment involved dosing a mix of 9,10-anthraquinone (AQ) and chloroform (CF) at 500 and 50 mg/kg of feed, respectively, from arrival until week 12 of the rearing period, after which both groups were on the same control diet.

[2] Repeated measurements were used to analyze the long-term effects of the methane inhibitors in the rumen function of calves.

**Calves were introduced to PMR diet during week 4, where 4 control and 3 treated calves did not consumed PMR; therefore, the mean of the total DMI does not correspond to the sum of the means of concentrate and PMR.*

and isovalerate ($P < 0.001$) 2.4 ± 0.19-fold greater average proportions than control calves. No interaction effects were observed for isobutyrate ($P = 0.462$) or total SCFA concentrations ($P = 0.723$). Similar SCFA profiles were observed in both groups at weeks 24 and 49.

Rumen Microbiota

A total of 9,527,448 reads were obtained from 190 rumen samples, using the Illumina MiSeq platform, with an average of 42,445 bacteria and 7,699 archaea sequences per sample. The number of OTUs was 1,500 and 40 for bacteria and archaea, respectively. A total of 245 bacteria and 17 archaea taxa were analyzed after using a minimal sample cut-off of 200 reads.

Figure 2 shows the normalized Shannon index for bacteria and archaea of treated and control calves across sampling periods. The bacterial Shannon index (**Figure 2A**) increased ($P < 0.001$) over time, but it was similar for the effects of treatment ($P = 0.713$) and treatment by time interaction ($P = 0.907$). The archaeal Shannon index (**Figure 2B**) showed an effect of treatment by time interaction effect ($P < 0.001$), where treated calves had a greater diversity at weeks 2 ($P < 0.001$) and 4 ($P = 0.002$) compared to control calves. In treated calves (0.48 ± 0.024), the archaea community showed a 24.5% greater ($P = 0.002$) Shannon index than in control calves (0.36 ± 0.024). Time effects ($P < 0.001$) were evident in calves at weeks 6 (0.33 ± 0.038) and 14 (0.30 ± 0.037) and had a lower ($P < 0.001$) archaeal diversity than calves at week 49 (0.56 ± 0.038).

The beta diversity of the bacteria dataset is shown in **Figure 3**. The PLSDA of the whole dataset (245 bacterial genera) in **Figure 3A** shows no clustering for the treatments, but a continuum across the two dimensions according to animal age or diet. **Figure 3B** shows the analysis for the abundant bacteria that occurred on average at a level higher than 0.5%, where there was no clustering treatments and less clear continuum of the timeline was observed. The beta diversity of the archaeal community is shown in **Figure 4**. The whole archaea community and the abundant subset in treated calves clustered apart from control calves during the first 14 weeks (**Figures 4A,B**). While at week 24 and 49, both groups clustered together within two defined clusters in the whole archaea community (17 archaea species; **Figure 4A**), but in the abundant archaea (7 archaea species) only the community at week 49 of both treatments clustered separately (**Figure 4B**).

Bacterial Community Composition

The bacterial community, from the selected cut offs of OTUs, showed 18 different phyla. These phyla corresponded to 32 classes, 46 orders, 81 families and 244 genera (**Supplementary Tables 2–6**). Phyla with a relative abundance ≥0.5% across samples showed that *Firmicutes* (43.1 ± 6.89%; mean ± S.D.) and *Bacteroidetes* (43.1 ± 7.22%) were the most abundant phyla. Whilst *Proteobacteria*, *Spirochaetae*, *Tenericutes*, *Fibrobacteres*, *Actinobacteria*, and *Cyanobacteria* together represented only 12.8 ± 4.71% of the bacteria phyla in the rumen. The effect of methane inhibitors on the abundant bacterial phyla at different sampling times is shown in **Table 5**. A treatment by time interaction effect was observed in *Fibrobacteres* ($P = 0.048$) and *Cyanobacteria* ($P = 0.003$). Pairwise analysis ($P = 0.137$) did not show differences for *Fibrobacteres* at any of the sampling times between treatments. The relative abundance of *Cyanobacteria* decreased in treated calves at weeks 10 and 14 (5.6- and 10.7-fold change, respectively; $P < 0.01$) with respect to control calves. Time effects were observed ($P < 0.001$) in the abundant bacterial phyla composition, for all except the *Actinobacteria* ($P = 0.125$). The proportions of *Bacteroidetes* and *Firmicutes* were constant until week 49, when *Bacteroidetes* dominated the ruminal community. Other changes over time were the general decline in *Proteobacteria* and at the same time an increase in *Spirochetes* until weaning (week 10) that decreased thereafter.

At the genus level, 41 bacteria genera had a relative abundance ≥0.50% across sampling times. These 41 genera accounted for 85.7 ± 5.38% (mean ± S.D.) of the community across sampling

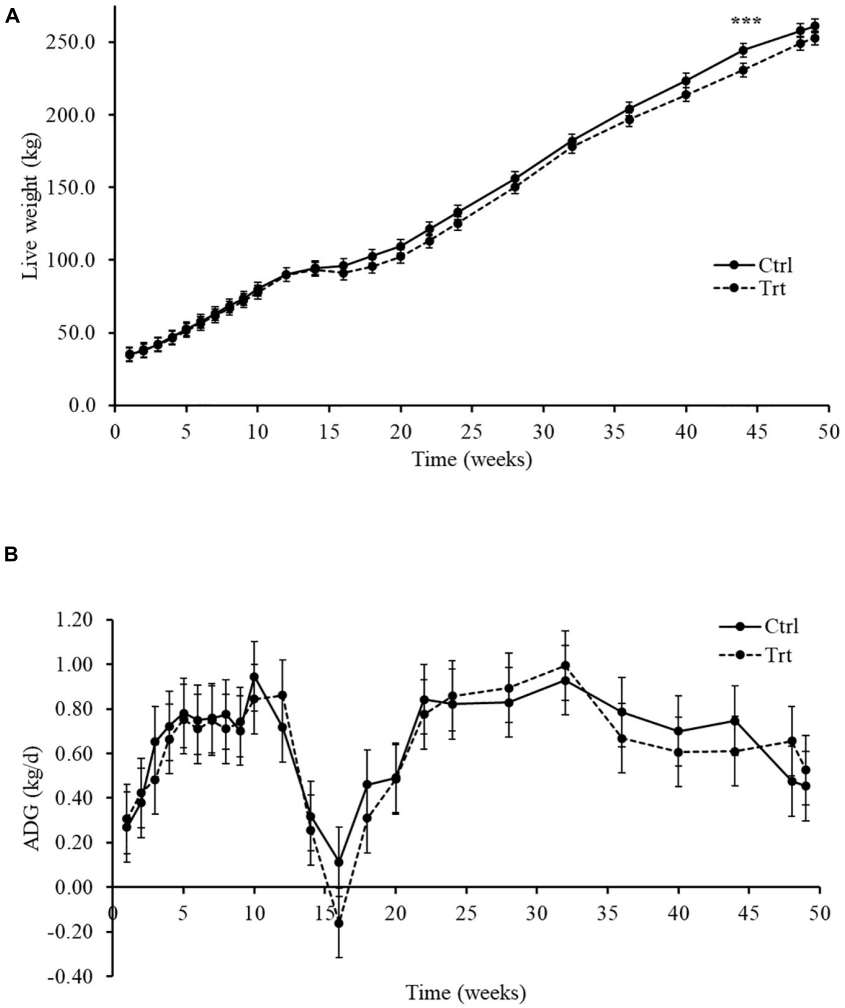


FIGURE 1 | Growth performance from week 1 to 49 in control group (Ctrl) and treated (Trt) calves. Growth performance was measure as: **(A)** Live weight (kg) and **(B)** average daily gain (ADG; kg/d). Calves during the rearing time were fed as follows: milk twice a day and *ad libitum* control and treatment concentrates at weeks 2 and 4; milk once a day and *ad libitum* control and treatment concentrates and partial mixed ration (PMR) diet at weeks 6, 8, and 10; concentrates step-down weaned and PMR diets fed *ad libitum* until week 14; and grazing a mixed sward of ryegrass/clover as one mob at weeks 24 and 49. Predicted means and their least square of the difference (LSD) are presented. Significance of pairwise comparisons (Tukey post-hoc analysis) between treatments are indicated by asterisks as: $^{***}P < 0.001$.

times. On average, *Prevotella* 1 was the most abundant genus (24.7 ± 11.23%), followed by *Christensenellaceae* R-7 group (5.2 ± 5.54%), *Rikenellaceae* RC9 gut group (4.3 ± 3.87%), *Ruminococcus* 2 (4.1 ± 5.61%) and *Sharpea* (3.2 ± 6.54%). The abundant bacteria genera for treated and control calves during different sampling times are shown in **Table 6**. Treatment by time interaction effects ($P \leq 0.048$) were observed in 15 abundant bacteria genera; however, pairwise comparison adjusted to Benjamini–Hochberg (BH) showed significance ($P \leq 0.040$) in only 11 abundant bacteria genera for treated when compared to control calves at random weeks during and 2 weeks after treatment. Treatment effects ($P < 0.031$) were observed in 9 of the 41 abundant bacteria genera. Samples from inhibitor-treated calves indicated proportional increases of *Rikenellaceae* RC9 gut group, *Succiniclasticum*, *Lachnospiraceae* NK3A20 group, *Ruminococcaceae* UCG 002 and p-2534-18B5 gut group, whilst decreases of *Ruminococcus* 1, *Ruminococcaceae* NK4A214 group, *Ruminoclostridium* 5 and *Ruminococcaceae* UCG 005 when compared to control calves. Most of the abundant bacterial genera were affected by time ($P \leq 0.029$), except for *Succinivibrionaceae* UCG 002, *Ruminiclostridium* 5 and *Eubacterium ventriosum* group ($P \geq 0.261$).

Archaeal Community

The archaeal community was represented by 17 species at a cut-off of ≥200 amplicons across sampling times. The archaeal community of treated calves had a reduced number of amplicons during and 2 weeks after administration of methane inhibitors compared to control calves (**Supplementary Figure 1**). The effect of methanogen inhibitors on the abundant archaea species

TABLE 4 | Effect of methane inhibitors[1] on rumen function[2].

		Rearing period (weeks)									*P*-value		
	Treatment	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Methane production (g/d)	Ctrl	1.53	**7.44**	**19.04**	**25.80**	**44.04**	**50.81**	63.80	145.40	3.037	<0.001	<0.001	<0.001
	Trt	0.69	**1.04**	**1.50**	**3.81**	**2.70**	**12.28**	58.76	143.13				
Methane yield (g/kg DMI)	Ctrl	5.16	**15.72**	**25.80**	**23.99**	**20.20**	**21.08**	22.33	21.86	2.161	<0.001	<0.001	<0.001
	Trt	4.58	**4.38**	**2.27**	**3.85**	**1.41**	**5.39**	21.08	20.26				
Hydrogen production (g/d)	Ctrl	0.17	0.14	**0.42**	**0.07**	**0.02**	**0.04**	0.08	0.29	0.388	<0.001	<0.001	<0.001
	Trt	0.35	0.82	**2.48**	**3.29**	**5.93**	**4.35**	0.09	0.44				
Hydrogen yield (g/kg DMI)	Ctrl	0.98	**0.38**	**0.89**	**0.07**	**0.01**	**0.02**	0.03	0.07	0.437	<0.001	<0.001	<0.001
	Trt	1.68	**2.64**	**3.41**	**2.93**	**2.97**	**1.88**	0.02	0.06				
SCFA (mM)	Ctrl	81.60	77.36	76.26	80.46	85.48	80.17	75.47	74.39	7.190	0.313	0.667	0.723
	Trt	72.57	84.39	78.00	73.13	79.50	71.69	74.47	72.57				
Acetate (%)	Ctrl	**53.67**	**54.50**	**62.92**	**61.86**	**62.66**	**67.21**	67.56	69.61	2.131	<0.001	<0.001	<0.001
	Trt	**48.71**	**44.82**	**47.77**	**47.71**	**49.11**	**52.46**	67.73	69.22				
Propionate (%)	Ctrl	**26.01**	**26.53**	**22.23**	**23.22**	**22.36**	**17.73**	18.63	17.68	2.115	<0.001	<0.001	0.001
	Trt	**32.90**	**35.45**	**27.78**	**28.76**	**33.73**	**25.54**	18.47	18.15				
Butyrate (%)	Ctrl	15.18	13.46	10.09	**10.06**	10.67	10.55	9.80	8.42	2.198	0.154	0.011	0.246
	Trt	12.83	12.82	14.05	**14.89**	11.12	13.82	9.77	8.48				
Caproate (%)	Ctrl	1.17	0.64	**0.68**	**0.67**	0.78	0.58	0.38	0.25	0.267	0.027	<0.001	<0.001
	Trt	0.93	0.76	**1.81**	**1.94**	1.14	0.74	0.33	0.25				
Valerate (%)	Ctrl	2.72	**2.55**	**1.89**	**1.62**	**1.69**	**1.47**	1.13	1.06	0.366	<0.001	<0.001	<0.001
	Trt	3.05	**3.87**	**4.80**	**3.94**	**3.48**	**2.79**	1.13	1.03				
Isobutyrate (%)	Ctrl	0.64	1.06	0.97	1.11	0.75	1.07	1.09	1.36	0.120	0.914	<0.001	0.462
	Trt	0.71	0.92	1.13	1.20	0.61	0.95	1.13	1.33				
Isovalerate (%)	Ctrl	0.62	1.27	**1.23**	1.46	1.09	**1.39**	1.41	1.61	0.432	0.075	<0.001	<0.001
	Trt	0.87	1.36	**2.62**	1.56	0.81	**3.69**	1.45	1.54				

Results[3] are the means and standard error of the differences (SED), P-value for treatment (P-Tx), time (P-Tm) and interaction (P-Int). Significance of pairwise comparisons (Tukey post hoc analysis) between treatments are shown in bold at each sampling time.

[1]The treatment involved dosing a mix of 9,10-anthraquinone (AQ) and chloroform (CF) at 500 and 50 mg/kg of feed, respectively, from arrival until week 12 of the rearing period, after which both groups were on the same control diet.

[2]Rumen Function was defined as: methane and hydrogen production (g/d) and yield (g/kg DMI), and total concentrations (mM) and individual proportions (%) of short chain fatty acids (SCFA) in dairy calves.

[3]Repeated measurements were used to analyze the long-term effects of the methane inhibitors in the rumen function of calves.

is shown in **Table 7**. Treatment by time interaction effects ($P \leq 0.011$) were observed during and 2 weeks after the intake of methane inhibitors. *Post hoc* analyses of *Methanomassiliicoccales* (*Mcc.*) Group 12 sp. ISO4-H5 showed greater ($P < 0.001$; between 6- and 68-fold increases) relative abundance from 2 to 10 weeks, and *Methanosphaera* (*Msp.*) sp. ISO3-F5 showed lower ($P < 0.001$) abundance at weeks 6 and 14 (12 and 31-fold decrease, respectively), in treated when compared to control calves. *Methanobrevibacter (Mbb.) ruminantium* and *Mmc.* Group 4 sp. MpT1 had greater ($P < 0.001$) proportions in treated calves in week 2 (10- and 8-fold change, respectively); however, *Mmc.* Group 4 sp. MpT1 showed significant decreases ($P < 0.001$) in treated calves at weeks 6, 8 and 14 (between 9 and 57-fold reductions) when compared to control calves. Treatment effects showed greater proportions ($P < 0.001$) of *Mmc.* Group 12 sp. ISO4-H5 (2.9% [C.I. = 1.71 – 4.87] vs. 0.4% [C.I. = 0.25 – 0.72]), but lower proportions of *Mbb. gottschalkii* ($P = 0.028$; 34.0% [C.I. = 26.66 – 43.29] vs. 50.8% [C.I. = 40.01 - 64.49]) and *Msp.* sp. ISO3-F5 ($P = 0.004$; 1.0% [C.I. = 0.59 – 1.75] vs. 3.5% [C.I. = 2.12 – 5.80]) in treated when compared to control calves. At weeks 24 and 49, the archaeal groups did not differ between treatments, and *Mmc.* Group 12 sp. ISO4-H5 was almost imperceptible in the rumen of both groups of calves. Time effects ($P \leq 0.016$) were observed across all the abundant archaea species, except for *Mbb. gottschalkii* whose relative abundance did not change ($P = 0.061$) with time.

DISCUSSION

This study utilized known CH_4 inhibitors that were incorporated into the solid diet of calves up to 12 weeks of age to investigate the impact on the rumen microbial composition, fermentation profiles, enteric emissions and animal feed intake and growth. In the present study, the inclusion of a CF/AQ mix in the PMR reduced the intake of this diet, but it did not affect the total DMI of solid feeds (concentrate and PMR) of treated calves, resulting in similar growth to control calves during the treatment interventions. Observed results from the current study supported those reported in adult ruminants that showed no effects on feed intake and animal production when receiving comparable

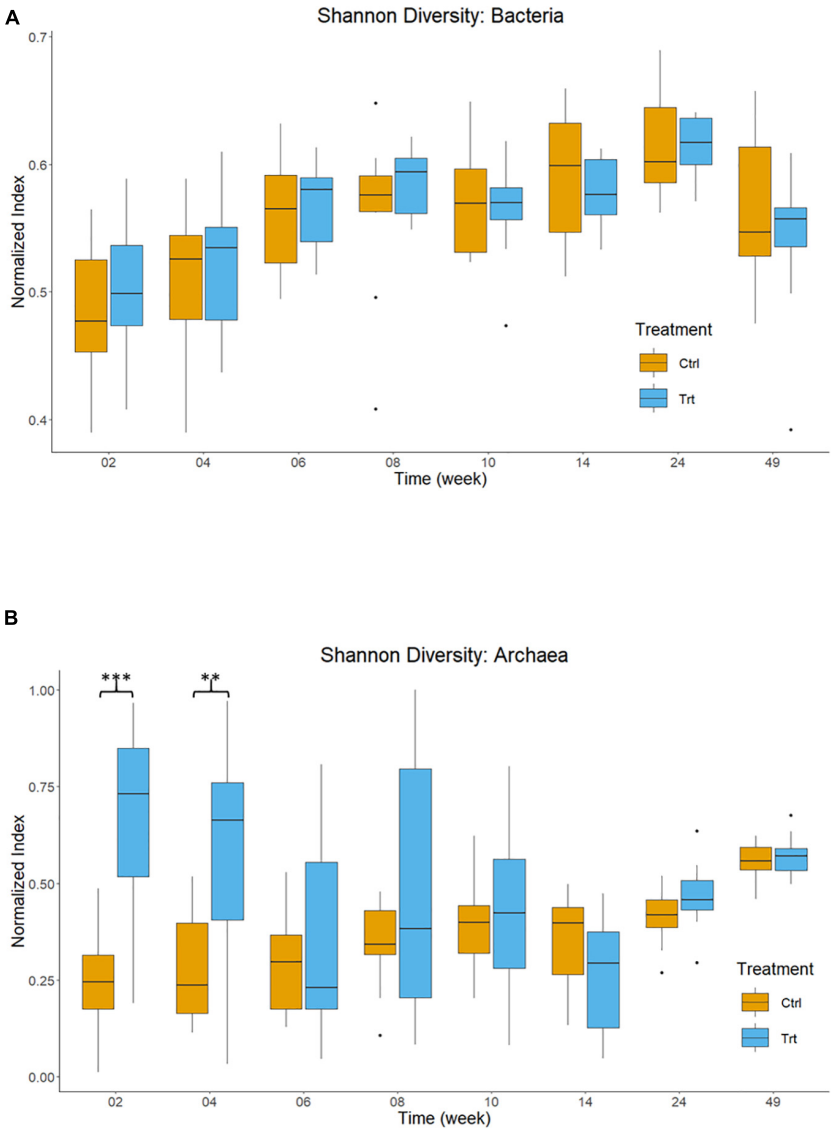


FIGURE 2 | Shannon diversity indices of the rumen microbiota in control (orange) and treatment (blue) calves across different ages. Calves during the rearing time were fed as follows: milk twice a day and *ad libitum* control and treatment concentrates at weeks 2 and 4; milk once a day and *ad libitum* control and treatment concentrates and partial mixed diet (PMR) diet at weeks 6, 8, and 10; concentrates step-down weaned and PMR diets fed *ad libitum* until week 14; and grazing a mixed sward of ryegrass/clover as one mob at weeks 24 and 49. Sampling times were from 2 to 49 weeks. Shannon index for **(A)** bacteria and **(B)** archaea. Boxplots represent the 25th and 75th percentiles, lines within boxes are the medians, the whiskers extend to the most extreme data points, and dots represent the outliers. Significance of pairwise comparisons (Tukey *post hoc* analysis) between treatments are indicated by asterisks as: $^{**}P < 0.010$; $^{***}P < 0.001$.

doses of halogenated or synthetic compounds to reduce CH_4 emissions (Sawyer et al., 1974; Martínez-Fernández et al., 2014; Hristov et al., 2015; Martinez-Fernandez et al., 2016, 2018). However, negative dose effects have been indicated in sheep fed up to 66 mg/kg of AQ in the diet for 8 weeks, showing reductions of 19% in DMI and numerical decreases of up to 31% in ADG when compared to controls (Kung et al., 2003). Calves' LW, ADG, and DMI during the intake of CH_4 inhibitors in this study were in accordance with those observed in goat kids drenched with bromochloromethane (BCM; Abecia et al., 2013) and calves receiving 3-nitrooxypropanol (3-NOP; Meale et al., 2021) in which DMI and growth were not affected; however, treated goat kids had greater weight gains than their control counterparts, whilst no such effect was observed in the present calf study. Post-weaning, the growth check observed following removal of the starter concentrate over a 2 week period from week 14 to 16 is consistent with prior observations in artificially reared lambs (Jensen et al., 2017). These results suggest that a 2 week transition period to remove starter concentrates provided *ad libitum* to young ruminants is insufficient to enable the rumen and metabolic system of the animal to adapt to the diet change. Therefore, the use of CF/AQ mix in the starter concentrate

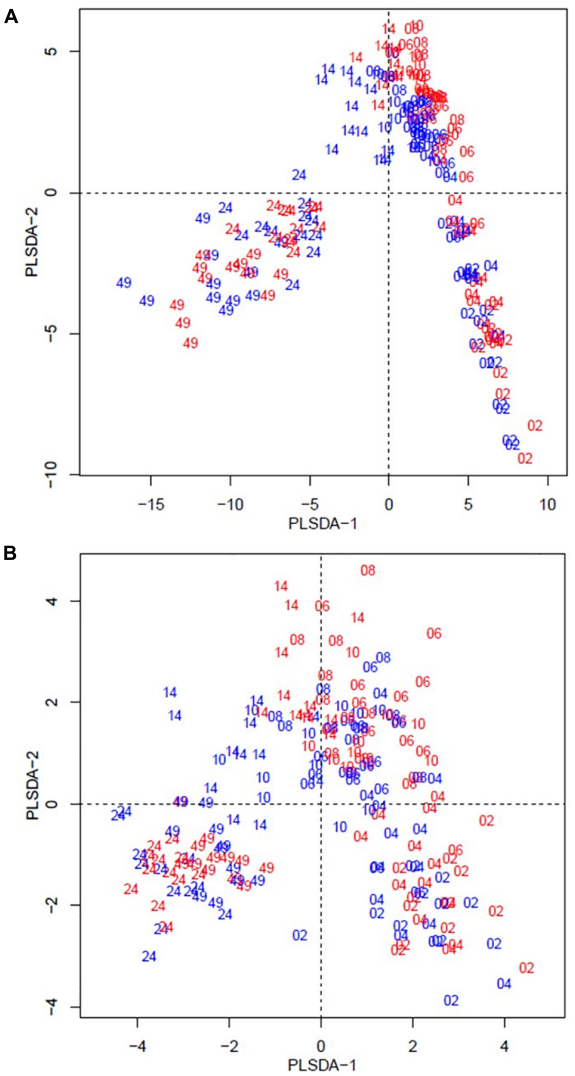


FIGURE 3 | Partial least square discriminant analysis (PLSDA) of the bacteria community at the genus level in treatment (red) and control (blue) dietary treatment across sampling periods. Calves during the rearing time were fed as follows: milk twice a day and *ad libitum* control and treatment concentrates at weeks 2 and 4; milk once a day and *ad libitum* control and treatment concentrates and partial mixed diet (PMR) diet at weeks 6, 8, and 10; concentrates step-down weaned and PMR diets fed *ad libitum* until week 14; and grazing a mixed sward of ryegrass/clover as one mob at weeks 24 and 49. Sampling times were from 2 to 49 weeks. **(A)** PLSDA of the bacteria community – 245 bacterial genera. **(B)** PLSDA of the abundant (=0.5 %) bacteria – 41 abundant bacterial genera. Calves from the treated and control groups are represented in red and blue, respectively. The numbers correspond to the sampling time in weeks.

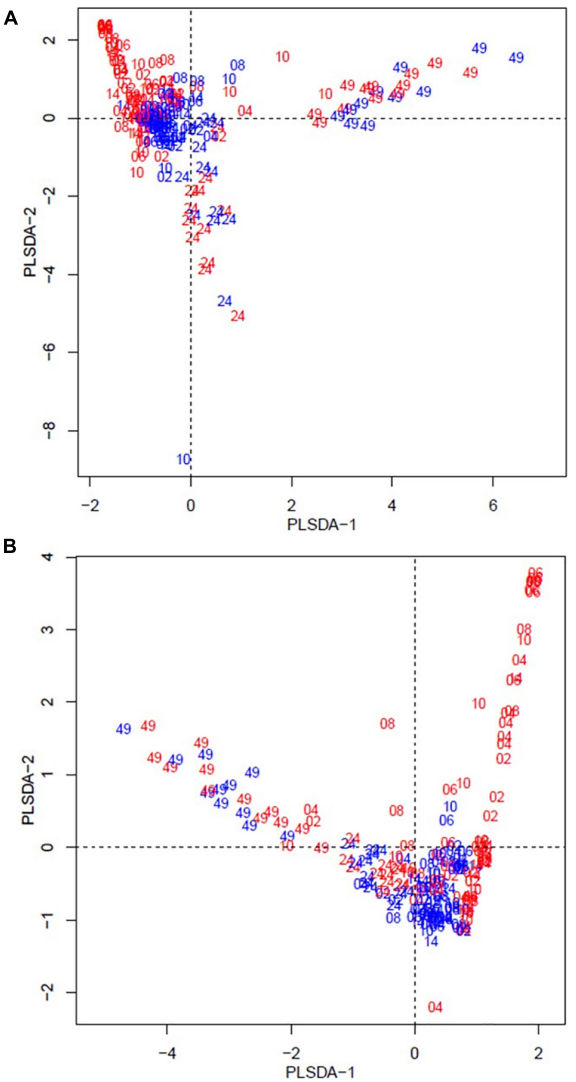


FIGURE 4 | Partial least square discriminant analysis (PLSDA) of the archaea community at the species level for treated (red) and control (blue) calves across sampling times. Calves during the rearing time were fed as follows: milk twice a day and *ad libitum* control and treatment concentrates at weeks 2 and 4; milk once a day and *ad libitum* control and treatment concentrates and partial mixed diet (PMR) diet at weeks 6, 8, and 10; concentrates step-down weaned and PMR diets fed *ad libitum* until week 14; and grazing a mixed sward of ryegrass/clover as one mob at weeks 24 and 49. Sampling times were from 2 to 49 weeks. **(A)** PLSDA of the archaea community – 17 archaeal species. **(B)** PLSDA of the abundant (=0.5 %) archaea community – 7 archaeal species. Calves from the treated and control groups are represented in red and blue, respectively. The numbers correspond to the sampling time in weeks.

diets of calves during rumen development can be used without detrimental effects on DMI and growth in the animal; however, further digestibility studies are required to assess the effect that a CF/AQ mix has when used in PMR diet and the post-treatment growth effects on calves of similar breed.

Feeding a CF/AQ mix in the solid feed to calves in the present study showed similar pattern on enteric emissions as reported using CF in dairy cows and steers (Knight et al., 2011; Martinez-Fernandez et al., 2016), and using AQ in sheep (Kung et al., 2003), which resulted in decreasing CH_4 and increasing H_2 emissions during treatment but with no differences detected 7 and 14 days post-treatment. Hydrogen disposal, via molecular H_2, is an important part of the adaptive changes in fermentation pathways in treated calves. In total, molecular H_2 accounted

TABLE 5 | Effect of methane inhibitors[1] on the relative abundance of bacterial phyla (%)[2] at different sampling times[3].

		Time (week)									*P*-value		
Taxa- Phylum	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Firmicutes	Ctrl	44.03	37.35	41.97	36.52	46.37	48.82	53.30	30.99	5.066	0.301	<0.001	0.100
	Trt	36.61	29.24	45.48	47.67	42.04	44.23	50.59	30.10				
Bacteroidetes	Ctrl	36.17	41.46	38.54	39.92	36.51	38.57	40.22	58.75	5.133	0.264	<0.001	0.540
	Trt	37.00	46.13	37.59	35.73	43.25	42.96	41.73	59.61				
Proteobacteria	Ctrl	2.53	4.71	1.10	5.11	4.59	2.85	0.68	2.30	0.417	0.482	<0.001	0.052
	Trt	8.09	9.57	3.36	2.27	2.59	2.31	0.56	2.09				
Spirochaetae	Ctrl	0.15	0.51	2.90	4.21	1.70	0.68	0.77	0.89	0.172	0.535	<0.001	0.264
	Trt	0.35	0.77	1.80	2.14	2.63	1.28	0.91	0.93				
Tenericutes	Ctrl	0.12	0.24	0.34	0.37	0.62	1.44	1.42	1.34	0.096	0.625	<0.001	0.771
	Trt	0.06	0.23	0.34	0.60	0.81	1.84	1.49	1.38				
Fibrobacteres	Ctrl	0.01	0.04	0.22	0.27	0.76	0.65	0.39	1.03	0.053	0.751	<0.001	0.048
	Trt	0.03	0.09	0.04	0.17	0.86	0.21	0.58	1.63				
Actinobacteria	Ctrl	0.78	0.62	0.42	0.49	0.49	0.59	0.48	0.50	0.065	0.574	0.125	0.126
	Trt	0.33	0.76	0.24	0.60	0.90	0.44	0.55	0.36				
Cyanobacteria*	Ctrl	0.01	0.01	0.06	0.11	**0.28**	**0.75**	0.36	0.91	0.030	0.001	<0.001	0.003
	Trt	0.01	0.01	0.02	0.04	**0.05**	**0.07**	0.27	0.95				

Calves were arranged in a control (Ctrl) and treatment (Trt) group. Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment (P-Tx), time (P-Tm) and interaction (P-Int). Significance of pairwise comparisons (Benjamini–Hochberg test) between treatments are shown in bold at each sampling time.

[1]Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to the concentrate and partial mixed ration diet (PMR) until week 12.

[2]Abundant bacterial phyla were defined as organisms with an average proportion ≥0.05%.

[3]Sampling times were from 2 to 49 weeks. Calves during the rearing time were fed as follows: milk twice a day and ad libitum control and treatment concentrates at weeks 2 and 4; milk once a day and ad libitum control and treatment concentrates and PMR diet at weeks 6, 8 and 10; concentrates step-down weaned and PMR diets fed ad libitum until week 14; and grazing a mixed sward of ryegrass/clover as one mob at weeks 24 and 49.

[4]A repeated measurement analysis was carried out to determine the effect of methane inhibitors on the bacteria community structure at the phylum level and their carry-over effects.

**Environmental bacterial phylum.*

for 26% of the H_2 that was not captured in methane. This proportion of molecular H_2 is in accordance with the 15 to 30% value observed by Martinez-Fernandez et al. (2016) in adult cattle. In response to CH_4 inhibition, the proportion of propionate was increased in the current trial. The propionate fermentation pathway consumes H_2 and is in direct competition with methanogenesis (Iannotti et al., 1973; Wolin, 1976; Morvan et al., 1996). However, because only rumen concentrations in this experiment were measured, no quantitative estimate of the H_2 captured in propionate could be made. Following methane inhibition, the observed increases in ruminal H_2 in treated calves additionally enhanced the fermentation pathways that consume H_2 such as valerate and caproate, as also indicated in a meta-analysis study by Ungerfeld (2015). It has been indicated that H_2 is redirected toward the production of SCFA that require a net incorporation of H_2 when produced from glucose, i.e., production of valerate and caproate via propionyl-CoA, or SCFA whose production results in less release of H_2 per unit of glucose compared to acetate, i.e., caproate via acetyl-CoA/butyryl-CoA (Ungerfeld, 2020). Acetogenesis is another potential pathway of H_2 disposal in the rumen, but can be excluded here since CF inhibits the acetyl-CoA cleavage pathway of acetogens (Scholten et al., 2000) although no measurements of the pathway were made in this experiment.

In ruminants, methanogens have been found in neonatal animals (Guzman et al., 2015), indicating that microbial colonization of the gastrointestinal tract (GIT) occurred during or directly after birth (Malmuthuge and Griebel, 2018). In the current study, calves entered the experiment at approximately 4 days of age, therefore these animals had already been exposed to maternal and environmental microbial communities. Exposing the community to increasing amounts of CF/AQ, during the first 12 weeks of rearing, affected the diversity of the archaea community acquired during the first 4 days of the calves' life. However, the observed changes of the archaeal community diversity were not maintained, returning to control levels 12 weeks after treatment cessation. Similar observations have been previously reported by Abecia et al. (2013) in goat kids ingesting BCM that showed reductions in the archaeal diversity while ingesting the inhibitors, but 12 weeks after treatment cessation all groups had similar diversities. Our results indicate that the application of CF/AQ in the solid feed during the first 12 weeks of rearing does not lead to a permanent change in the diversity of the archaeal community in growing ruminants.

The composition of the methanogen community in the rumen has been suggested to change from birth to adulthood (Guzman et al., 2015; Friedman et al., 2017). In the present study, changes in the archaeal community corresponded to those reported in calves receiving 3-NOP, in which the archaea diversity increased as the animal aged (Meale et al., 2021). In adult ruminants, the family *Methanobacteriaceae*, which includes *Methanobrevibacter* spp. and *Methanosphaera* spp., represents up to 90% of the rumen

TABLE 6 | Effect of methane inhibitors[1] on the relative abundance of bacterial genus (%)[2] at different sampling times[3].

		Time (week)									*P*-value		
Taxa- Genus	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Prevotella 1	Ctrl	18.36	19.61	20.21	19.24	18.14	18.93	28.10	39.13	4.280	0.945	<0.0001	0.007
	Trt	21.82	27.44	13.03	11.71	23.91	19.84	29.09	39.91				
Christensenellaceae R-7 group	Ctrl	0.34	1.00	4.44	4.63	5.38	4.56	3.79	5.43	0.780	0.608	<0.0001	0.544
	Trt	0.33	0.57	3.85	8.38	6.37	8.41	4.46	5.26				
Rikenellaceae RC9 gut group	Ctrl	**0.13**	0.99	2.04	3.14	2.75	4.13	2.04	4.64	0.637	0.007	<0.0001	0.515
	Trt	**0.55**	1.93	5.32	6.12	5.02	9.31	2.24	4.53				
Ruminococcus 2	Ctrl	2.62	1.19	1.91	2.85	4.22	3.87	0.43	0.27	0.354	0.074	<0.0001	0.288
	Trt	0.52	0.41	4.25	1.99	3.24	2.03	0.44	0.25				
Sharpea	Ctrl	3.55	1.41	0.61	0.53	1.79	0.07	0.01	0.01	0.231	0.644	<0.0001	0.855
	Trt	5.46	2.18	0.93	0.92	2.14	0.26	0.00	0.01				
Bacteroidales BS11 gut group	Ctrl	0.13	1.59	2.95	2.25	1.69	3.05	1.62	2.49	0.324	0.392	<0.0001	0.846
	Trt	0.09	1.08	1.23	2.16	1.49	1.31	1.76	3.02				
Bacteroidales S24-7 group	Ctrl	0.09	0.62	2.80	4.44	2.08	1.05	1.96	1.81	0.333	0.876	<0.0001	0.281
	Trt	0.11	0.29	2.05	1.90	2.52	2.43	2.11	2.39				
Ruminobacter	Ctrl	0.02	0.04	**0.02**	1.18	2.43	0.42	0.02	0.03	0.079	0.363	<0.0001	0.016
	Trt	0.03	0.13	**0.85**	0.62	0.66	0.32	0.02	0.03				
Ruminiclostridium 9	Ctrl	0.03	0.05	0.17	0.20	0.35	2.42	5.86	0.32	0.204	0.508	<0.0001	0.662
	Trt	0.04	0.06	0.20	0.45	1.03	1.59	4.54	0.28				
Lachnospiraceae UCG 005	Ctrl	0.39	0.37	**0.02**	0.02	0.01	0.01	0.01	0.03	0.018	0.484	<0.0001	0.017
	Trt	0.24	0.09	**0.21**	0.07	0.01	0.01	0.01	0.02				
Treponema 2	Ctrl	0.02	0.09	0.88	2.00	0.96	0.43	0.65	0.72	0.153	0.284	<0.0001	0.867
	Trt	0.06	0.21	1.42	1.59	1.76	0.81	0.76	0.77				
Roseburia	Ctrl	2.05	0.82	**0.23**	0.06	0.07	0.24	1.48	0.46	0.171	0.081	<0.0001	0.001
	Trt	4.08	2.35	**1.25**	0.15	0.14	0.11	0.85	0.41				
p-2534-18B5 gut group	Ctrl	0.01	0.01	**0.02**	0.19	0.23	**0.07**	0.10	0.02	0.100	0.000	<0.0001	<0.0001
	Trt	0.01	0.04	**5.13**	1.41	0.53	**0.74**	0.11	0.02				
Lachnospiraceae NK3A20 group	Ctrl	0.01	**0.02**	**0.08**	**0.36**	1.23	1.70	1.22	1.14	0.186	0.001	<0.0001	0.010
	Trt	0.02	**0.18**	**1.09**	**1.76**	1.61	3.28	1.23	1.13				
Succinivibrio	Ctrl	0.68	0.77	0.03	0.05	0.08	0.02	0.00	0.02	0.050	0.604	<0.0001	0.526
	Trt	1.96	0.55	0.03	0.01	0.06	0.01	0.00	0.02				
Succiniclasticum	Ctrl	0.21	1.09	0.76	1.51	0.81	0.65	0.98	1.56	0.237	0.004	<0.0001	0.151
	Trt	0.70	2.47	2.24	1.82	1.99	1.07	1.10	1.47				
Ruminococcus 1	Ctrl	0.45	0.60	1.18	1.44	1.74	1.85	3.17	1.65	0.247	0.009	<0.0001	0.666
	Trt	0.18	0.37	0.70	1.01	1.36	1.42	2.44	1.71				
Ruminococcaceae UCG 014	Ctrl	0.35	0.43	0.85	1.07	1.53	1.77	1.23	0.97	0.171	0.167	<0.0001	0.827
	Trt	0.14	0.29	0.80	1.30	0.97	0.95	1.11	0.93				
Succinivibrionaceae UCG 002	Ctrl	0.06	**0.03**	0.03	0.10	0.06	0.09	0.05	0.07	0.018	0.615	0.261	0.011
	Trt	0.02	**0.64**	0.11	0.06	0.09	0.04	0.02	0.07				
Ruminococcaceae NK4A214 group	Ctrl	**0.36**	0.27	0.82	0.86	0.91	0.76	2.75	2.38	0.197	0.031	<0.0001	0.044
	Trt	**0.09**	0.31	0.58	0.63	0.57	0.63	2.52	2.48				
Ruminiclostridium 5	Ctrl	0.31	**0.93**	**1.35**	**0.77**	0.39	**0.99**	0.28	0.24	0.076	<0.0001	0.273	0.001
	Trt	0.08	**0.04**	**0.08**	**0.11**	0.18	**0.22**	0.32	0.24				
Prevotellaceae UCG 001	Ctrl	0.06	0.48	0.65	0.45	0.51	0.95	0.89	1.67	0.125	0.630	<0.0001	0.034
	Trt	0.23	0.51	0.24	0.28	0.39	0.33	1.05	2.12				
Prevotellaceae UCG 003	Ctrl	0.07	0.05	0.39	0.49	1.49	1.14	0.87	2.60	0.150	0.676	<0.0001	0.105
	Trt	0.07	0.16	0.34	0.46	0.49	0.60	1.26	2.48				
Selenomonas 1	Ctrl	0.27	0.23	0.16	0.10	0.16	0.29	3.41	0.91	0.130	0.059	<0.0001	0.294
	Trt	0.50	0.35	0.12	0.23	0.39	0.35	2.87	0.87				
Fibrobacter	Ctrl	0.01	0.04	0.22	0.27	0.76	0.65	0.39	1.03	0.081	0.751	<0.0001	0.048
	Trt	0.03	0.09	0.04	0.17	0.86	0.21	0.58	1.63				
Pseudobutyrivibrio	Ctrl	0.13	0.16	0.15	0.19	0.16	0.75	1.93	1.42	0.115	0.559	<0.0001	0.869
	Trt	0.19	0.14	0.15	0.29	0.27	0.53	2.07	1.41				

(Continued)

TABLE 6 | (Continued)

		Time (week)									*P*-value		
Taxa- Genus	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Mollicutes RF9	Ctrl	0.04	0.17	0.26	0.32	0.60	1.22	1.12	0.88	0.119	0.106	<0.0001	0.985
	Trt	0.05	0.25	0.28	0.55	0.76	1.79	1.13	0.84				
Prevotella 7	Ctrl	0.68	0.10	0.01	0.01	0.01	0.01	0.44	0.03	0.027	0.771	<0.0001	0.755
	Trt	0.54	0.21	0.03	0.01	0.01	0.01	0.23	0.03				
Kandleria	Ctrl	0.00	0.00	0.01	0.02	0.07	0.02	1.93	0.03	0.047	0.985	<0.0001	0.759
	Trt	0.00	0.00	0.00	0.01	0.02	0.03	1.83	0.03				
Succinimonas	Ctrl	0.02	0.02	0.02	0.02	0.02	0.01	1.00	0.01	0.027	0.436	<0.0001	0.824
	Trt	0.09	0.02	0.02	0.03	0.04	0.02	1.00	0.01				
Ruminococcaceae UCG 005	Ctrl	0.15	0.19	**0.57**	0.70	0.43	0.62	0.53	0.58	0.075	0.015	<0.0001	0.291
	Trt	0.06	0.09	**0.16**	0.54	0.52	0.35	0.57	0.45				
Eubacterium coprostanoligenes group	Ctrl	0.03	0.12	0.52	0.70	0.71	1.03	0.96	0.91	0.100	0.136	<0.0001	0.417
	Trt	0.03	0.10	0.28	0.38	0.37	0.53	1.04	0.91				
Erysipelotrichaceae UCG 002	Ctrl	0.00	0.01	0.03	0.01	0.05	0.01	0.05	0.00	0.004	0.884	0.002	0.116
	Trt	0.00	0.00	0.01	0.01	0.01	0.09	0.02	0.00				
Atopobium	Ctrl	0.41	0.19	0.28	0.35	0.39	0.51	0.30	0.32	0.060	0.254	0.015	0.036
	Trt	0.13	0.30	0.09	0.40	0.67	0.29	0.31	0.21				
Lachnospiraceae uncultured	Ctrl	0.15	0.14	0.57	0.20	0.15	0.17	0.21	0.25	0.043	0.482	0.029	0.172
	Trt	0.18	0.15	0.23	0.33	0.22	0.30	0.21	0.26				
Prevotellaceae NK3B31 group	Ctrl	**0.01**	0.05	0.04	0.02	0.08	0.09	0.20	0.27	0.024	0.275	0.001	0.002
	Trt	**0.17**	0.43	0.19	0.05	0.04	0.02	0.19	0.23				
Sphaerochaeta	Ctrl	0.10	0.23	0.34	0.66	0.29	0.18	0.09	0.04	0.042	0.764	<0.0001	0.572
	Trt	0.19	0.29	0.21	0.30	0.44	0.21	0.06	0.03				
Lachnospiraceae NK4A136 group	Ctrl	0.05	0.18	0.14	0.20	0.29	0.51	0.60	0.38	0.059	0.338	<0.0001	0.584
	Trt	0.10	0.14	0.40	0.29	0.33	0.67	0.46	0.36				
Ruminococcaceae UCG 002	Ctrl	**0.04**	0.19	0.36	0.27	0.25	0.33	0.49	0.37	0.073	0.001	<0.0001	0.033
	Trt	**0.25**	0.37	0.68	0.66	0.52	0.76	0.38	0.34				
Bacteroidales RF16 group	Ctrl	0.01	0.01	0.03	0.25	**0.47**	**0.85**	0.57	1.28	0.070	0.073	<0.0001	0.000
	Trt	0.01	0.02	0.13	0.18	**0.10**	**0.11**	0.66	1.33				
Eubacterium ventriosum group	Ctrl	0.02	0.04	0.06	0.05	0.04	0.05	0.04	0.06	0.007	0.092	0.274	0.714
	Trt	0.02	0.01	0.01	0.02	0.02	0.04	0.04	0.05				

Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment (P-Tx), time (P-Tm) and interaction (P-Int). Significance of pairwise comparisons (Benjamini–Hochberg test) between treatments are shown in bold at each sampling time.

[1]Calves were arranged in a control (Ctrl) and treatment (Trt) group. Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to treated partial mixed ration diet (PMR) until week 12.

[2]Abundant bacterial genera were defined as organisms with an average proportion ≥0.5%.

[3]Sampling times were from 2 to 49 weeks. Calves during the rearing time were fed as follows: milk twice a day and ad libitum control and treatment concentrates at weeks 2 and 4; milk once a day and ad libitum control and treatment concentrates and PMR diet at weeks 6, 8 and 10; concentrates step-down weaned and PMR diets fed ad libitum until week 14; and grazing a mixed sward of ryegrass/clover as one mob at weeks 24 and 49.

[4]A repeated measurement analysis was carried out to determine the effect of methane inhibitors on the bacteria community structure at the genus level and their carry-over effects.

archaea (Henderson et al., 2015; Seedorf et al., 2015; Friedman et al., 2017). The relative abundance of this family in control calves showed adult-like proportions, representing an average of 93.8 ± 3.89% of the archaea community between 2 and 49 weeks. Methanogen inhibitors like CF and 3-NOP in steers have been shown to decrease the relative abundance of *Methanobacteriaceae* (Martinez-Fernandez et al., 2018). These results were consistent with the reduction of *Mbb. gottschalkii* and *Msp.* ISO3-F5 found in calves fed CF/AQ. Little treatment by time interaction effects observed between treatment calves for *Methanobacteriaceae* in the present study agreed with those reported in calves receiving 3-NOP from birth until 3 weeks post-weaning by Meale et al. (2021). Quantitative PCR analysis confirmed that 3-NOP and CF reduces *Methanobrevibacter*; however, only 3-NOP decreased the abundance of Methanomassiliicoccaceae family (Martinez-Fernandez et al., 2018). The mix of inhibitors in our experiment did not affect Methanomassiliicoccaceae confirming the results from Martinez-Fernandez et al. (2018) and also indicating that AQ does not appear to have a specific effect against this archaea family. The most abundant species of *Methanobrevibacter* grow from reducing H_2 and CO_2 (Miller et al., 1986), whilst *Methanosphaera* is a methanogen that reduces methanol with H_2 and is dependent on acetate as a carbon source (Fricke et al., 2006). However, *Methanomassiliicoccales (Mmc.)* spp. are obligatory hydrogen-dependent methylotrophic methanogens and require compounds

TABLE 7 | Effect of methane inhibitors[1] on the relative abundance of archaeal species (%)[2] at different sampling times[3].

Relative abundance		Time (week)									*P*-value		
Taxa- Genus	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Methanobrevibacter gottschalkii clade	Ctrl	46.52	49.71	**52.03**	45.77	46.54	59.39	59.89	48.63	15.849	0.028	0.061	0.183
	Trt	31.59	45.54	**14.89**	21.23	36.53	40.96	56.46	46.19				
Methanobrevibacter ruminantium clade	Ctrl	**2.75**	4.55	4.80	13.07	16.97	10.05	15.56	11.72	3.943	0.843	0.016	0.011
	Trt	**26.58**	7.74	2.74	7.06	7.37	3.81	23.07	16.78				
MethanomassiliicoccalesGroup12 sp. ISO4-H5	Ctrl	**0.11**	**0.33**	**1.63**	**0.96**	**1.36**	1.51	0.07	0.13	1.460	<0.001	<0.001	<0.001
	Trt	**7.48**	**7.52**	**15.37**	**15.02**	**8.22**	4.72	0.06	0.17				
Methanosphaera sp. ISO3-F5	Ctrl	2.03	2.99	**2.73**	1.73	3.04	**2.18**	7.01	16.97	1.531	0.004	<0.001	<0.001
	Trt	1.03	0.98	**0.22**	0.68	0.67	**0.07**	8.91	16.51				
MethanomassiliicoccalesGroup10 sp.	Ctrl	0.05	0.10	0.02	0.07	0.12	0.15	1.48	9.08	0.470	0.397	<0.001	0.137
	Trt	0.65	0.09	0.06	0.07	0.15	0.03	0.72	7.96				
Methanosphaera sp. Group5	Ctrl	0.13	0.21	0.11	0.06	0.11	0.07	2.24	2.46	0.281	0.202	<0.001	0.569
	Trt	0.54	1.66	0.05	0.05	0.19	0.02	2.80	1.73				
MethanomassiliicoccalesGroup4 sp. MpT1	Ctrl	**0.19**	0.39	**0.38**	**0.51**	0.81	**1.14**	0.09	0.81	0.199	0.051	<0.001	<0.001
	Trt	**1.53**	0.53	**0.01**	**0.06**	1.24	**0.02**	0.12	0.96				

Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment (P-Tx), time (P-Tm) and interaction (P-Int). Significance of pairwise comparisons (Benjamini–Hochberg test) between treatments are shown in bold at each sampling time.

[1]Calves were arranged in a control (Ctrl) and treatment (Trt) group. Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to treated partial mixed ration diet (PMR) until week 12.

[2]Abundant archaeal species were defined as organisms with an average proportion ≥1.0%.

[3]Sampling times were from 2 to 49 weeks. Calves during the rearing time were fed as follows: milk twice a day and ad libitum control and treatment concentrates at weeks 2 and 4; milk once a day and ad libitum control and treatment concentrates and PMR diet at weeks 6, 8 and 10; concentrates step-down weaned and PMR diets fed ad libitum until week 14; and grazing a mixed sward of ryegrass/clover as one mob at weeks 24 and 49.

[4]A repeated measurement analysis was carried out to determine the effect of methane inhibitors on the archaeal community structure at the species level and their carry-over effect.

like methanol, methylamine, dimethylamine, and trimethylamine as their major energy and carbon source (Lang et al., 2015; Li et al., 2016). In the present study, it is not clear how the use of two different methanogen inhibitors affects the growth and abundance of the different archaea species (Borrel et al., 2014). Additionally, the observed changes in the archaeal community were driven by the diet offered at the time as observed in control calves. In addition, care has to be taken when evaluating the relative abundances, as an increase in abundance of one group can be due to either an increase in the target groups' numbers or a major decrease in other groups (**Supplementary Figure 1**).

The use of methanogen inhibitors did not affect the diversity of the bacterial community in the present study. This is a novel observation in young calves and an unexpected result since the rumen metabolite profiles (gases and SCFA) were altered by the CF/AQ mix fed to the calves. The results in the present study agree with those reported in lambs and calves also receiving methane inhibitors from birth until weaning off milk and 3 weeks after weaning off milk, respectively, in which alterations of the fermentation pathways did not affect the bacterial diversity in the rumen when compared to controls (Abecia et al., 2018; Meale et al., 2021). Bacterial community diversity increased over time in the present study. Similar results have been shown for growing calves in previous studies (Jami et al., 2013; Dill-McFarland et al., 2017; Meale et al., 2021). In addition to animal age, bacterial community diversity can be affected by the diet (Kim et al., 2016; Martinez-Fernandez et al., 2016). In our study, the effects of time on bacteria community diversity reported here are a combination of animal age and changing diet.

Despite the differences in intra-ruminal H_2 concentrations observed between treatment groups in the present study, the bacterial community at the phylum level was similar between studied groups. Our results differed from those in cannulated steers where increases in relative abundance of *Bacteroidetes* and decreases in *Firmicutes* in response to CF were described (Martinez-Fernandez et al., 2016). Changes in the abundant bacterial genera were few notwithstanding the fermentation shifts in the concentration of the different SCFA observed in the rumens of treated compared to control calves. The increase of *Lachnospiraceae* NK3A20 observed in treated calves was in accordance with data from CH_4 inhibition studies in goat kids supplemented with rhubarb root containing emodin, a derivative of AQ (Wang et al., 2017). This family possess a large and diverse repertoire of glycoside hydrolases and polysaccharide lyases (Seshadri et al., 2018), having the capacity to ferment polysaccharides or fumarate to acetate, succinate and CO_2, while no H_2 is formed (Janssen and Hugenholtz, 2003). In the present study, CH_4 inhibition appeared to increase *Rikenellaceae* RC9 gut group, *Roseburia* and p-2534-18B5 gut group to different extents. This is similar to the observation where these bacteria groups were increased by the ingestion of concentrate diets (Henderson et al., 2015), in the sense that diets rich in grains are known to lead to lower CH_4 emissions, because starch utilizing bacteria tend to produce less H_2 (Stewart et al., 1997; Janssen, 2010). Therefore, the metabolism of these

bacteria is not likely to be affected by the partial pressure of H_2 in the rumen.

Cellulolytic microbes such as *Fibrobacter* were not affected by CH_4 inhibition in the present study, which is in accordance with previous observations inhibiting CH_4 production in steers dosed with CF (16 and 26 mg/kg of LW) (Martinez-Fernandez et al., 2016) and in steers receiving CF (16 mg/kg of LW) or 3-NOP (2.5 g/animal) (Martinez-Fernandez et al., 2018). These results confirm that *Fibrobacter* can tolerate H_2 accumulation (Wolin et al., 1997) because its major end product is succinate (Abdul Rahman et al., 2016). In contrast, calves treated with CF/AQ mixed in the diet showed that the proportion of cellulolytic *Ruminiclostridium* and *Ruminococcus* was reduced with increased H_2 concentrations, similar to observations with BCM (50 mg/kg LW) in the diet (Mitsumori et al., 2012). Inhibition of these cellulolytic bacteria genera has been observed previously during *in vitro* experiments, in which inhibition of CH_4 production with haloforms reduced *Ruminococcus* populations, whilst *Fibrobacter* numbers increased (Goel et al., 2009). The class *Clostridia* is predominated by H_2-producing cellulolytic bacteria, and the H_2 accumulation significantly inhibited their H_2-producing activity (Lay, 2001). The increased partial pressure of H_2 influences the metabolism of these fiber-degrading genera by inhibiting NADH oxidation, whilst H_2 is diverted to form other end products such as succinate and ethanol (Wolin et al., 1997). Therefore, the degradation of cellulose by these *Ruminiclostridium* and *Ruminococcus* (Ruminococcaceae family) may be impaired by the increased H_2 pressure in the rumen.

Methane inhibition did not have any effect on the most abundant genus *Prevotella* capable of degrading a broad spectrum of polysaccharides and peptides in the diet. They also have the ability to use different pathways in response to H_2 pressure which makes them very flexible under high H_2 conditions (Marounek and Dušková, 1999; Seshadri et al., 2018) as induced in the treated calves. Given the minor changes observed in the rumen community composition, this indicates that methane inhibition, other than dietary changes, result predominantly in a shift of metabolic pathways of an existing community rather than a change in the microbial communities. Further investigations are necessary to elucidate the mechanisms that the rumen bacterial population uses to adapt to high intra-ruminal H_2 pressure, produced by the inhibition of methanogen microbes with CF/AQ in the diet, and to explore whether imprinting of the rumen microbiota is possible.

CONCLUSION

Collectively, this study showed that CF/AQ mix inclusion in the starter diets during the first 12 weeks of rearing did not affect the DMI and growth of dairy calves. Methane inhibition changed the composition of the target community, the methanogens, but had only minor effects on the bacterial community indicating the importance of the metabolic flexibility of many rumen microorganisms. This metabolic flexibility, however, may account for the observation that there was no lasting effect of the microbial community as it returns to the energetically more favorable pathways once CH_4 inhibition/H_2 pressure has been removed. Metabolic flexibility of the rumen microbiota, however, may be overcome if the treatments begin earlier in the rumen and more work is needed to establish the start and the duration of such early life interventions.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the European Nucleotide Archive, accession number PRJEB37781.

ETHICS STATEMENT

The animal study was reviewed and approved by Grasslands Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

SM designed the study, secured funding, and generated the respiration chamber data. OC-C generated the microbial community data and wrote the initial draft manuscript. SG and OC-C undertook the statistical analysis. All authors contributed to the data interpretation and manuscript editing.

FUNDING

This study was funded by Sustainable Land use and Climate Change Fund Project "Hydrogen management in the rumen" (SLMACC-AGR30783) the Ministry for Primary Industries, New Zealand, AgResearch Strategic Science Investment Fund, New Zealand, and the New Zealand AID Program (MFAT), and CONACYT and INIFAP in Mexico for Ph.D. stipend support.

ACKNOWLEDGMENTS

The authors acknowledge our animal facility team for taking care of the animals and sample collected with special thanks to Sarah Maclean and Holly Kjestrup. The authors also want to thank Sarah Lewis for her work in aiding in the community and chromatography samples and Peter Janssen and Linley Schofield for critically reviewing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.710914/full#supplementary-material

REFERENCES

Abdul Rahman, N., Parks, D. H., Vanwonterghem, I., Morrison, M., Tyson, G. W., and Hugenholtz, P. (2016). A phylogenomic analysis of the bacterial phylum Fibrobacteres. *Front. Microbiol.* 6:1469. doi: 10.3389/fmicb.2015.01469

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., et al. (2018). Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Front. Microbiol.* 9:2227. doi: 10.3389/fmicb.2018.02227

Abecia, L., Martín-García, A. I., Martinez, G., Newbold, C. J., and Yáñez-Ruiz, D. R. (2013). Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. *J. Anim. Sci.* 91, 4832–4840. doi: 10.2527/jas.2012-6142

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J., et al. (2014a). Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Anim. Prod. Sci.* 54, 1449–1454. doi: 10.1071/an14337

Abecia, L., Waddams, K. E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Newbold, C. J., et al. (2014b). An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by Archaea. *Archaea* 2014:12.

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.

AOAC (1990). *Official Methods of Analysis*. Washington, DC: *AOAC*.

AOAC (2010). *Official Methods of Analysis of AOAC International*. Washington, DC: *AOAC*.

AOAC (2012). *Official Methods of Analysis of AOAC International*. Washington, DC: *AOAC*.

Attwood, G. T., Klieve, A. V., Ouwerkerk, D., and Patel, B. K. (1998). Ammonia-hyperproducing bacteria from New Zealand ruminants. *Appl. Environ. Microbiol.* 64, 1796–1804. doi: 10.1128/aem.64.5.1796-1804.1998

Bauchop, T. (1967). Inhibition of rumen methanogenesis by methane analogues. *J. Bacteriol.* 94, 171–175. doi: 10.1128/jb.94.1.171-175.1967

Belanche, A., Yáñez-Ruiz, D. R., Detheridge, A. P., Griffith, G. W., Kingston-Smith, A. H., and Newbold, C. J. (2019). Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. *Environ. Microbiol.* 21, 4360–4377. doi: 10.1111/1462-2920.14801

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Bland, J. M., and Altman, D. G. (1996). Transformations, means, and confidence intervals. *Br. Med. J.* 312:1079. doi: 10.1136/bmj.312.7038.1079

Borrel, G., Parisot, N., Harris, H. M., Peyretaillade, E., Gaci, N., Tottey, W., et al. (2014). Comparative genomics highlights the unique biology of Methanomassiliicoccales, a Thermoplasmatales-related seventh order of methanogenic archaea that encodes pyrrolysine. *BMC Genomics* 15:679. doi: 10.1186/1471-2164-15-679

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336.

Corson, D., Waghorn, G., Ulyatt, M., and Lee, J. (1999). "NIRS: forage analysis and livestock feeding," in *Proceedings of the Conference New Zealand Grassland Association*, Hawkes Bay, 127–132. doi: 10.33584/jnzg.1999.61.2340

Cox, M. P., Peterson, D. A., and Biggs, P. J. (2010). SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics* 11:485. doi: 10.1186/1471-2105-11-485

Dias, J., Marcondes, M. I., Noronha, M. F., Resende, R. T., Machado, F. S., Mantovani, H. C., et al. (2017). Effect of pre-weaning diet on the ruminal archaeal, bacterial, and fungal communities of dairy calves. *Front. Microbiol.* 8:1553. doi: 10.3389/fmicb.2017.01553

Dill-McFarland, K. A., Breaker, J. D., and Suen, G. (2017). Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci. Rep.* 7:40864.

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D., and Suen, G. (2019). Diet influences early microbiota development in dairy calves without long-term impacts on milk production. *Appl. Environ. Microbiol.* 85:e02141-18.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461.

Ellis, J., Kebreab, E., Odongo, N., Mcbride, B., Okine, E., and France, J. (2007). Prediction of methane production from dairy and beef cattle. *J. Dairy Sci.* 90, 3456–3466. doi: 10.3168/jds.2006-675

Fonty, G., Gouet, P., Jouany, J.-P., and Senaud, J. (1987). Establishment of the microflora and anaerobic fungi in the rumen of lambs. *J. Gen. Microbiol.* 133, 1835–1843. doi: 10.1099/00221287-133-7-1835

Fricke, W. F., Seedorf, H., Henne, A., Krüer, M., Liesegang, H., Hedderich, R., et al. (2006). The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H2 for methane formation and ATP synthesis. *J. Bacteriol.* 188, 642–658. doi: 10.1128/jb.188.2.642-658.2006

Friedman, N., Jami, E., and Mizrahi, I. (2017). Compositional and functional dynamics of the bovine rumen methanogenic community across different developmental stages. *Environ. Microbiol.* 19, 3365–3373. doi: 10.1111/1462-2920.13846

Ganesh, S., and Cave, V. (2018). P-values, p-values everywhere! *N. Z. Vet. J.* 66, 55–56.

Garcia-Lopez, P., Kung, L., and Odom, J. (1996). In vitro inhibition of microbial methane production by 9, 10-anthraquinone. *J. Anim. Sci.* 74, 2276–2284. doi: 10.2527/1996.7492276x

Goel, G., Makkar, H. P., and Becker, K. (2009). Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. *Br. J. Nutr.* 101, 1484–1492. doi: 10.1017/s0007114508076198

Graham, D. E., and White, R. H. (2002). Elucidation of methanogenic coenzyme biosyntheses: from spectroscopy to genomics. *Nat. Prod. Rep.* 19, 133–147. doi: 10.1039/b103714p

Gunsalus, R., and Wolfe, R. (1978). ATP activation and properties of the methyl coenzyme M reductase system in *Methanobacterium* thermoautotrophicum. *J. Bacteriol.* 135, 851–857. doi: 10.1128/jb.135.3.851-857.1978

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B., and Franks, A. E. (2015). Presence of selected methanogens, fibrolytic bacteria, and proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS One* 10:e0133048. doi: 10.1371/journal.pone.0133048

Haque, M. N. (2018). Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *J. Anim. Sci. Technol.* 60:15.

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.

Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., et al. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One* 8:e74787. doi: 10.1371/journal.pone.0074787

Henderson, G., Yilmaz, P., Kumar, S., Forster, R. J., Kelly, W. J., Leahy, S. C., et al. (2019). Improved taxonomic assignment of rumen bacterial 16S rRNA sequences using a revised SILVA taxonomic framework. *PeerJ.* 7:e6496.

Hobson, P. N., and Stewart, C. S. (1997). *The Rumen Microbial Ecosystem*. Dordrecht: Springer.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., et al. (2015). An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10663–10668. doi: 10.1073/pnas.1504124112

Iannotti, E., Kafkewitz, D., Wolin, M., and Bryant, M. (1973). Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of H2. *J. Bacteriol.* 114, 1231–1240. doi: 10.1128/jb.114.3.1231-1240.1973

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

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

Janssen, P. H., and Hugenholtz, P. (2003). Fermentation of glycolate by a pure culture of a strictly anaerobic gram-positive bacterium belonging to the family Lachnospiraceae. *Arch. Microbiol.* 179, 321–328.

Jensen, A., Khan, M., Knol, F., Peterson, S., Morel, P., Mckenzie, C., et al. (2017). "How does feeding meal affect growth of artificially reared East Friesian-cross

dairy lambs?" in *Proceedings of the New Zealand Society of Animal Production: New Zealand Society of Animal Production*, 13–17.

Joblin, K. N. (1999). Ruminal acetogens and their potential to lower ruminant methane emissions. *Aust. J. Agric. Res.* 50, 1307–1314. doi: 10.1071/ar99004

Kim, Y.-H., Nagata, R., Ohtani, N., Ichijo, T., Ikuta, K., and Sato, S. (2016). Effects of dietary forage and calf starter diet on ruminal pH and bacteria in holstein calves during weaning transition. *Front. Microbiol.* 7:1575. doi: 10.3389/fmicb.2016.01575

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

Knight, T., Ronimus, R., Dey, D., Tootill, C., Naylor, G., Evans, P., et al. (2011). Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. *Anim. Feed Sci. Technol.* 166, 101–112. doi: 10.1016/j.anifeedsci.2011.04.059

Kung, L. Jr., Hession, A. O., and Bracht, J. P. (1998). Inhibition of sulfate reduction to sulfide by 9, 10-anthraquinone in in vitro ruminal fermentations. *J. Dairy Sci.* 81, 2251–2256.

Kung, L., Smith, K., Smagala, A., Endres, K., Bessett, C., Ranjit, N., et al. (2003). Effects of 9, 10 anthraquinone on ruminal fermentation, total-tract digestion, and blood metabolite concentrations in sheep. *J. Anim. Sci.* 81, 323–328. doi: 10.2527/2003.811323x

Lang, K., Schuldes, J., Klingl, A., Poehlein, A., Daniel, R., and Brune, A. (2015). New mode of energy metabolism in the seventh order of methanogens as revealed by comparative genome analysis of "Candidatus Methanoplasma termitum." *Appl. Environ. Microbiol.* 81, 1338–1352. doi: 10.1128/aem.03389-14

Lay, J. J. (2001). Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. *Biotechnol. Bioeng.* 74, 280–287. doi: 10.1002/bit.1118

Lê Cao, K., Rohart, F., Gonzalez, I., and Dejean, S. (2016). *mixOmics: Omics Data Integration Project. R package Version 6.1. 1*.

Li, Y., Leahy, S. C., Jeyanathan, J., Henderson, G., Cox, F., Altermann, E., et al. (2016). The complete genome sequence of the methanogenic archaeon ISO4-H5 provides insights into the methylotrophic lifestyle of a ruminal representative of the Methanomassiliicoccales. *Stand. Genomic Sci.* 11:59.

Luo, D., Ganesh, S., and Koolaard, J. (2014). *Calculate Predicted Means for Linear Models. 0.99 ed*.

Malmuthuge, N., and Griebel, P. J. (2018). Fetal environment and fetal intestine are sterile during the third trimester of pregnancy. *Vet. Immunol. Immunopathol.* 204, 59–64. doi: 10.1016/j.vetimm.2018.09.005

Marounek, M., and Dušková, D. (1999). Metabolism of pectin in rumen bacteria *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*. *Lett. Appl. Microbiol.* 29, 429–433. doi: 10.1046/j.1472-765x.1999.00671.x

Martínez-Fernández, G., Abecia, L., Arco, A., Cantalapiedra-Hijar, G., Martín-García, A., Molina-Alcaide, E., et al. (2014). Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. *J. Dairy Sci.* 97, 3790–3799. doi: 10.3168/jds.2013-7398

Martinez-Fernandez, G., Denman, S. E., Yang, C., Cheung, J., Mitsumori, M., and Mcsweeney, C. S. (2016). Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. *Front. Microbiol.* 7:1122. doi: 10.3389/fmicb.2016.01122

Martinez-Fernandez, G., Duval, S., Kindermann, M., Schirra, H. J., Denman, S. E., and Mcsweeney, C. S. (2018). 3-NOP vs. halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Front. Microbiol.* 9:1582. doi: 10.3389/fmicb.2018.01582

Meale, S., Popova, M., Saro, C., Martin, C., Bernard, A., Lagree, M., et al. (2021). Early life dietary intervention in dairy calves results in a long-term reduction in methane emissions. *Sci. Rep.* 11, 1–13.

Miller, T. L., Wolin, M., and Kusel, E. (1986). Isolation and characterization of methanogens from animal feces. *Syst. Appl. Microbiol.* 8, 234–238. doi: 10.1016/s0723-2020(86)80084-4

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., et al. (2012). Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. *Br. J. Nutr.* 108, 482–491. doi: 10.1017/s0007114511005794

Morvan, B., Rieu-Lesme, F., Fonty, G., and Gouet, P. (1996). In vitroInteractions between rumen H2-producing cellulolytic microorganisms and H2-utilizing acetogenic and sulfate-reducing bacteria. *Anaerobe* 2, 175–180. doi: 10.1006/anae.1996.0023

Oksanen, F., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al. (2017). *vegan: Community Ecology Package. R Package Version 2.4-4*.

Paul, A., and Southgate, D. (1978). *McCance and Widdowson'sthe composition of foods*. London: HM Stationery Office.

Pinares-Patiño, C., Hunt, C., Martin, R., West, J., Lovejoy, P., and Waghorn, G. (2012). "New Zealand ruminant methane measurement centre," in *Technical Manual on Respiration Chamber Design*, eds C. S. Pinares-Patiño and G. C. Waghorn (Wellington: Ministry of Agriculture and Forestry), 11–30.

Pinheiro, J., Bates, D., Debroy, S., Sarkar, D., and Team, R. C. (2015). *nlme: Linear and nonlinear Mixed Efects Models. 3.1–121 ed*.

R Core Team (2016). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., and Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116, 245–257. doi: 10.1111/jam.12405

Saro, C., Hohenester, U. M., Bernard, M., Lagrée, M., Martin, C., Doreau, M., et al. (2018). Effectiveness of interventions to modulate the rumen microbiota composition and function in pre-ruminant and ruminant lambs. *Front. Microbiol.* 9:1273. doi: 10.3389/fmicb.2018.01273

Sawyer, M. S., Hoover, W., and Sniffen, C. J. (1974). Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. *J. Anim. Sci.* 38, 908–914. doi: 10.2527/jas1974.384908x

Scholten, J. C., Conrad, R., and Stams, A. J. (2000). Effect of 2-bromo-ethane sulfonate, molybdate and chloroform on acetate consumption by methanogenic and sulfate-reducing populations in freshwater sediment. *FEMS Microbiol. Ecol.* 32, 35–42. doi: 10.1111/j.1574-6941.2000.tb00696.x

Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P. H. (2014). RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *PeerJ* 2:e494. doi: 10.7717/peerj.494

Seedorf, H., Kittelmann, S., and Janssen, P. H. (2015). Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle. *Appl. Environ. Microbiol.* 81, 986–995. doi: 10.1128/aem.03018-14

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. doi: 10.1038/nbt.4110

Stewart, C., Flint, H., and Bryant, M. (1997). "The rumen bacteria," in *The Rumen Microbial Ecosystem*, eds P. N. Hobson and C. S. Stewart (Dordrecht: Springer), 10–72.

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., and Sivakumaran, S. (2005). Methane production from in vitro rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Anim. Feed Sci. Technol.* 123–124, Part 1, 403–419. doi: 10.1016/j.anifeedsci.2005.04.037

Ungerfeld, E., and Kohn, R. (2006). "The role of thermodynamics in the control of ruminal fermentation," 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), 55–85.

Ungerfeld, E. M. (2015). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Front. Microbiol.* 6:37. doi: 10.3389/fmicb.2015.00037

Ungerfeld, E. M. (2020). Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Front. Microbiol.* 11:589. doi: 10.3389/fmicb.2020.00589

Van Soest, P. J. (1994). *Nutritional Ecology of the Ruminant*. Ithaca, NY: Comstock Pub.

Wang, L., Zhang, K., Zhang, C., Feng, Y., Zhang, X., Wang, X., et al. (2019). Dynamics and stabilization of the rumen microbiome in yearling Tibetan sheep. *Sci. Rep.* 9, 1–9.

Wang, Z., Elekwachi, C., Jiao, J., Wang, M., Tang, S., Zhou, C., et al. (2017). Changes in metabolically active bacterial community during rumen

development, and their alteration by rhubarb root powder revealed by 16S rRNA amplicon sequencing. *Front. Microbiol.* 8:159. doi: 10.3389/fmicb.2017.00159

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

Weimer, P. J., Stevenson, D. M., Mantovani, H. C., and Man, S. L. C. (2010). Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents1. *J. Dairy Sci.* 93, 5902–5912. doi: 10.3168/jds.2010-3500

Wolin, M., Miller, T., and Stewart, C. (1997). "Microbe-microbe interactions," in *The Rumen Microbial Ecosystem*, eds P. N. Hobson and C. S. Stewart (Dordrecht: Springer), 467–491.

Wolin, M. (1976). "Interactions between H2-producing and methane-producing species," in *Microbial Production and Utilization of Gases (H2, CH4, CO)*, eds H. G. Schlegel, G. Gottschalk, and N. Pfennig (Göttingen: Akademie der Wissenchaften zur Göttingen).

Yanagita, K., Kamagata, Y., Kawaharasaki, M., Suzuki, T., Nakamura, Y., and Minato, H. (2000). Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of methanomicrobium mobile by fluorescence in situ hybridization. *Biosci. Biotechnol. Biochem.* 64, 1737–1742. doi: 10.1271/bbb.64.1737

Yáñez-Ruiz, D. R., Macías, B., Pinloche, E., and Newbold, C. J. (2010). The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. *FEMS Microbiol. Ecol.* 72, 272–278. doi: 10.1111/j.1574-6941.2010.00852.x

Ziemer, C. J., Sharp, R., Stern, M. D., Cotta, M. A., Whitehead, T. R., and Stahl, D. A. (2000). Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. *Environ. Microbiol.* 2, 632–643. doi: 10.1046/j.1462-2920.2000.00146.x

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 September 2021
doi: 10.3389/fmicb.2021.679135

Age-Related Changes in the Ruminal Microbiota and Their Relationship With Rumen Fermentation in Lambs

Xuejiao Yin†, Shoukun Ji†, Chunhui Duan, Peizhi Tian, Sisi Ju, Hui Yan, Yingjie Zhang and Yueqin Liu**

College of Animal Science and Technology, Hebei Agricultural University, Baoding, China

OPEN ACCESS

Edited by:
Diego P. Morgavi, INRAE Clermont-Auvergne-Rhône-Alpes, France

Reviewed by:
Beatrix Stessl, University of Veterinary Medicine Vienna, Austria
Robert W. Li, Agricultural Research Service, United States Department of Agriculture, United States

***Correspondence:**
Yingjie Zhang
zhangyingjie66@126.com
orcid.org/0000-0001-8470-9700
Yueqin Liu
liuyueqin66@126.com
orcid.org/0000-0002-5765-8060

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *11 March 2021*
Accepted: *31 August 2021*
Published: *20 September 2021*

Citation:
Yin X, Ji S, Duan C, Tian P, Ju S, Yan H, Zhang Y and Liu Y (2021) Age-Related Changes in the Ruminal Microbiota and Their Relationship With Rumen Fermentation in Lambs. Front. Microbiol. 12:679135. doi: 10.3389/fmicb.2021.679135

The rumen microbiota is vital for the health and growth performance of the host animal, mainly due to its role in the fermentation of ingested feed within the rumen. Attaining a better understanding of the development of the bacterial community and fermentation in the rumen can provide the theoretical basis for regulating feed utilization. This study analyzed the development of rumen bacteria in lambs from birth to 4 months of age using 16S-rRNA amplicon sequencing data and studied its relationship with ruminal fermentation. Serum levels of metabolites were monitored at 30, 60, 90, and 120 days of age, and the RandomForest approach was used to determine age-related changes in rumen bacteria. Levels of blood metabolites, ruminal fermentation, the rumen bacterial community and its functions were all affected by the age of the lambs ($P < 0.05$). Based on the Bray-Curtis distance within the age groups of the rumen microbiota, the similarity increased sharply after the lambs were weaned at 60 days of age ($P < 0.05$). The similarity between the samples collected from birth to 90 days of age and those collected at 120 days of age, increased after 20 days of age, reaching a maximum at 90 days vs. 120 days ($P < 0.05$). Some age-associated changes in the microbial genera were correlated with changes in the concentrations of volatile fatty acids and the levels of microbial crude protein in the rumen, including positive correlations between main volatile fatty acids and the genera of *Prevotella 1*, *Lachnospiraceae NK3A20 group*, *Ruminococcus gauvreauii group*, *Ruminococcaceae UCG-014*, and *Ruminococcus 2* ($P < 0.05$). These results indicated that the microbial community and the function of rumen was not well-established before 20 days of age, so there is a degree of plasticity in the rumen bacterial community during the first 20 days of post-natal development in lambs, and this might provide an opportunity for interventions to improve rumen fermentation and, thus, increase their growth performance.

Keywords: sheep, rumen microbiota, volatile fatty acid, microbiota crude protein, blood metabolites

INTRODUCTION

The rumen is vital for the metabolism, immunity, and health of the ruminant, which hosts a complex and dynamic ecosystem containing a great diversity of microbiota. The ruminant is completely dependent on the microbial community to ferment the ingested material (Huws et al., 2018; Morais and Mizrahi, 2019). The fermentation process provides most of the energy and

protein to meet the needs of the host, mainly in the form of short-chain fatty acids (SCFAs) and microbial proteins (Weimer, 2015). Therefore, to increase feed utilization efficiency and improve animal health and production, we need to thoroughly understand the development of the nutrient organ-rumen.

The colonization of the gastrointestinal tract of newborns starts at birth, and the succession process continues until the microbiota reaches a stable state later in life. Young animals such as lambs can be considered to be non-ruminants from a nutritional standpoint (Drackley, 2008). Various factors, such as diet and the surrounding environment, may influence microbial colonization (Zhu et al., 2018). Many events or disturbances may affect the rumen microbiota composition. These modifications may be more significant in successive colonization processes when the microbiota is less stable and relatively simple (Saro et al., 2018). Compared to less mature communities, a diverse and well-established microbial community within the rumen has higher resilience and is more resistant to disturbances. The composition and function of the microbiota will tend to return to their pre-treatment status once the disturbance ceases (Shade et al., 2012; Kittelmann et al., 2014). However, in young ruminants, the influence of alterations may persist for some time after the end of the modification. Saro et al. (2018) showed that a long-term early life intervention can affect the composition of the rumen microbial community and persist weeks after the intervention. Abecia et al. (2018) showed that the treatment effect persisted after the end of the modification in the early life of kid goats. Currently, many ways to change the rumen fermentation through early life microbiota have been studied, including feed styles (Zhang et al., 2019), weaning method (Mao et al., 2021), rumen fluid inoculation (Yu et al., 2020), and different additives (Lin et al., 2019; Wang et al., 2021). Thus, the rudimentary status of the ruminants' microbiota in the early period provides an opportunity for human intervention in the rumen colonization process. However, further study is essential to gain a better understanding of the rumen colonization process and the crucial period when it can be easily moderated. There is a dearth of study on the development of the rumen as a nutrient organ and the changing process of ruminal fermentation.

In order to determine an appropriate developmental stage for the timing of the interventions, we hypothesized that the rumen bacterial communities and ruminal fermentation would change significantly over the growing period of lambs and therein persisted the key microbiota required in this process. The present study was conducted to examine the changes of ruminal fermentation and the process of microbial colonization in the rumen. This information will improve our understanding of the microbial ecology in the rumen and will strengthen the methodology for modulating the microbial community to improve the productivity and health of ruminants.

MATERIALS AND METHODS

This study was conducted under the guidance of the Animal Care and Use Committee of Hebei Agricultural University (approval number: YJ201825).

Animals, Diets, and Experimental Design

The study was conducted between January and August 2019 at an experimental sheep farm facility in Hengshui, China. All sheep used in the study were of the Hu sheep breed. Twenty pregnant ewes (44.7 ± 1.7 kg BW, mean ± SD) carrying a singleton lamb were identified by ultrasonic examination (TTY2018, Liaochen, China). They were kept in individual pens (3.0 × 0.8 m) with free access to feed and water.

Ten newborn female lambs were included in this study (2.87 ± 0.28 kg BW). These lambs were raised according to conventional housing and growth practices used in our experimental facility. Briefly after birth, the lambs remained with their dams in individual pens and had free access to a starter commercial compound from day 15. On day 60, the lambs were weaned and offered a mixed ration at 0730 and 1,500 h each day with approximately 5% feed refusal. The ingredients and nutrient composition of the diets are provided in **Supplementary Table 1**. Fresh water was freely available throughout the experimental period. Unhealthy individuals ($n = 3$) were removed at 120 days of age.

Sample Collection

Blood samples (5 ml) were obtained from the lambs by jugular venipuncture on days 30, 60, 90, and 120 prior to the morning feeding (**Figure 1A**). They were allowed to coagulate, and the serum obtained by centrifugation at 3,000 × *g* for 15 min at 4°C was stored at −20°C prior to analysis.

The ruminal contents from each lamb were sampled at nine time points as follows: the first within 24 h after birth, then at the ages of 3, 10, 20, 30, 45, and 60 days before weaning, then at the ages of 90 and 120 days after weaning (**Figure 1A**). The rumen fluid samples were obtained 2 h after the morning feeding by use of an oral stomach tube, which was thoroughly cleaned using fresh warm water between sample collections (Ahmad et al., 2020). The rumen contents were collected into 20 mL cryopreservation tubes. All samples were immediately snap-frozen in liquid nitrogen and then stored at −80°C for further analysis.

Sample Measurements

The analyses of the serum concentrations of total protein (TP), albumin (ALB), glucose (GLU), non-esterified fatty acids (NEFA), and beta-hydroxybutyric acid (BHBA) were performed by an automatic biochemical analyzer using standard commercial kits supplied from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China).

The cryopreserved rumen fluid samples were thawed at 4°C and thoroughly mixed by vortexing. Later, 5 ml of the rumen fluid was taken and centrifuged at 3,000 × *g* for 10 min at 4°C. Then 1 ml of the supernatant was transferred to a 1.5-ml centrifuge tube containing 0.2 ml of metaphosphoric acid solution (25% w/v) with the internal standard 2-ethylbutyric acid. The mixture was placed in an ice-water bath for 30 min and centrifuged at 10,000 × *g* at 4°C. The resulting supernatant was transferred to a 1.5-ml centrifuge tube and stored at 4°C until analysis of the concentration of VFA.

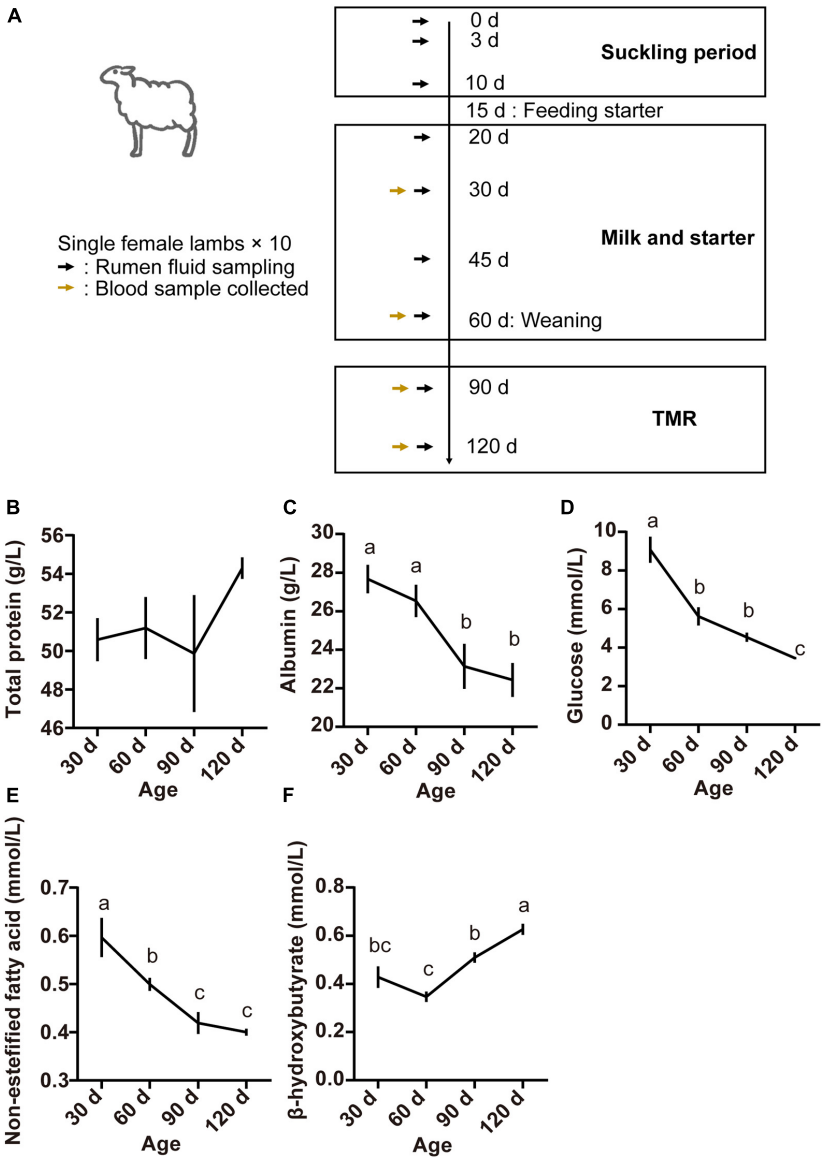


FIGURE 1 | Experimental design and sampling schedule of lambs **(A)**, serum concentrations of total protein **(B)**, albumin **(C)**, glucose **(D)**, non-esterified fatty acids **(E)**, and beta-hydroxybutyric acid **(F)** at different lamb ages. Data are expressed as mean ± SEM. ^{a,b}Letters denote significant differences between groups; groups that do not share the same letter are significantly different from each other ($P < 0.05$).

The concentration of VFA was determined by gas chromatography (Varian 450, Agilent Technologies China, Co., Ltd., China) using the conditions and subsequent test procedures described earlier (Ahmad et al., 2020). Ammonia N concentration was determined spectrophotometrically using a colorimetric method (Weatherburn, 1967). The concentration of microbial crude protein (MCP) was determined by the Coomassie brilliant blue method (Bradford, 1976).

DNA Extraction and Sequencing

The DNA of the rumen bacteria was extracted using the DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's protocol. The quality of the obtained DNA was checked using 1% agarose gel electrophoresis and spectrophotometer (optical density at 260/280 nm ratio). We used universal primers to amplify the V3-V4 hypervariable regions of bacterial 16S rRNA gene. The primers were (338F: 5′- ACTCCTACGGGAGGCAGCAG −3′, 806R: 5′-GGACTACHVGGGTWTCTAAT −3′) (Munyaka et al., 2015). For each sample, a 10-digit barcode sequence was added to the 5′ end of the forward and reverse primers (provided by Allwegene Company, Beijing). The PCR was carried out on a Mastercycler Gradient (Eppendorf, Germany) using 25 μl reaction volumes, containing 12.5 μl KAPA 2G Robust Hot Start Ready Mix, 1 μl Forward Primer (5 μM), 1 μl Reverse Primer (5 μM), 5 μl DNA (total template quantity was 30 ng), and 5.5 μl H_2O.

Cycling parameters were 95°C for 5 min, followed by 32 cycles of 95°C for 45 s, 55°C for 50 s, and 72°C for 45 s with a final extension at 72°C for 10 min. Three PCR products per sample were pooled to mitigate reaction-level PCR biases. The PCR products were extracted from 2% agarose gels and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany) following the manufacturer's instructions. Then they were quantified using QuantiFluor^{TM}-ST (Promega, Madison, WI, United States). After that, the amplicons were sequenced at the Allwegene Company, Beijing. Deep sequencing of DNA extracts was performed on an Illumina Miseq PE300 sequencing platform (Caporaso et al., 2012) at the Allwegene Company (Beijing, China). After the run, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline Version 2.6.

Bioinformatics Analysis

The raw data were first screened, and the sequences that were shorter than 300 bp or had a low quality score (≤20), contained ambiguous bases, or did not exactly match primer sequences and barcode tags were removed from consideration. Only clean sequences with an overlap longer than 10 bp were assembled using FLASH-1.2.11 (Magoc and Salzberg, 2011). Reads that could not be assembled were discarded. Chimera sequences were detected using usearch6.1 (Edgar, 2010). Qualified reads were separated using sample-specific barcode sequences and trimmed with Illumina Analysis Pipeline Version 2.6. The resulting dataset was analyzed using QIIME1 pipeline (version 1.5.0). The sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97% (Edgar, 2013) to calculate the richness and diversity indices. The Ribosomal Database Project (RDP) Classifier tool was used to classify all the sequences into different taxonomic groups (Cole et al., 2009). The microbiota Bray- Curtis similarity was calculated based on OTUs. Chao 1 index and Shannon index, which reflect alpha diversity, were calculated by QIIME. Non-metric multidimensional scaling (NMDS) plots of the Bray-Curtis metric calculated with square root transformed data in R (vegan package). Heat maps were generated using the package "pheatmap" of R (v4.0.0) software. The functional profiles of bacterial communities were predicted by the method of Tax4Fun2 (Wemheuer et al., 2020). The 16S rRNA function prediction was categorized into the Kyoto Encyclopedia of Genes and Genomes (KEGG).

RandomForest Analysis

We regressed (Subramanian et al., 2014) the relative abundances of bacterial taxa at the genus level along with the KEGG pathways against the ages of lambs using default parameters in the "RandomForest" package in R (Breiman, 2001) (ntree = 1,000, mtry = p/3, where p is the number of genera or the number of pathways) (Liaw and Wiener, 2002) in order to obtain the best discriminant performance of taxa and pathways across lambs' ages. Lists of taxa and pathways ranked by RandomForest were performed in order of feature importance. Ten-fold cross-validation [the rfcv() function in the R package "RandomForest," 10 repeats] was used to identify the number of marker taxa and pathways. After the minimum cross-validation error was obtained, we chose the number that stabilized against the cross-validation error curve as marker taxa and pathways correlating with the ages of lambs (Zhang et al., 2018).

Data Availability Statement

The datasets generated from the current study are available in the Genome Sequence Archive repository[1] under accession numbers PRJCA004193.

Statistical Analysis

All statistical analyses were carried out using R (v4.0.0) software. A completely randomized design was used to analyze the results. An individual lamb was considered as an experimental unit for every analysis. The ruminal fermentation parameters and serum parameters were processed by ANOVA after checking independency, normality, and homogeneity:

$$Yi = \mu + Xi + ei$$

where Y_i is the dependent parameter, μ is the overall mean, X_i is the age effect, and e_i is the residual error. Duncan's multiple range test was used when significant effects were detected between ages. Spearman correlation between parameters was performed with CORR procedures. ANOSIM was performed to compare the similarity of bacterial communities in the rumen of different ages of lambs (**Supplementary Data**). Comparisons between groups were performed using a Wilcoxon test or Kruskal-Wallis test with R software. All data are presented as mean ± SEM. P-value < 0.05 was considered to indicate statistical significance.

RESULTS

Blood Metabolites

Serum parameters for the different age groups are presented in **Figure 1**. The TP concentration was not affected by age ($P > 0.05$; **Figure 1B**). However, serum concentrations of ALB, GLU, and NEFA were higher at 30 days and declined thereafter ($P < 0.05$; **Figures 1C–E**). The concentration of BHBA increased from 60 to 120 days, finally reaching the highest at 120 days of age ($P < 0.05$; **Figure 1F**).

Ruminal Fermentation Parameters

Ruminal fermentation parameters were affected by the age of the lambs (**Figure 2**). Rumen ammonia N concentration was highest at 30 days of age ($P < 0.05$; **Figure 2A**). The concentration of MCP increased from birth to 10 days of age and became relatively stable thereafter ($P < 0.05$; **Figure 2B**). Except for valerate which appeared at 10 days of age (**Figure 2G**), other volatile fatty acids (total VFA, acetate, propionate, butyrate, isobutyrate, and isovalerate) appeared at 3 days of age, and the concentration of all volatile fatty acids reached the highest at 60 days of age ($P < 0.05$; **Figure 2**).

[1] http://gsa.big.ac.cn

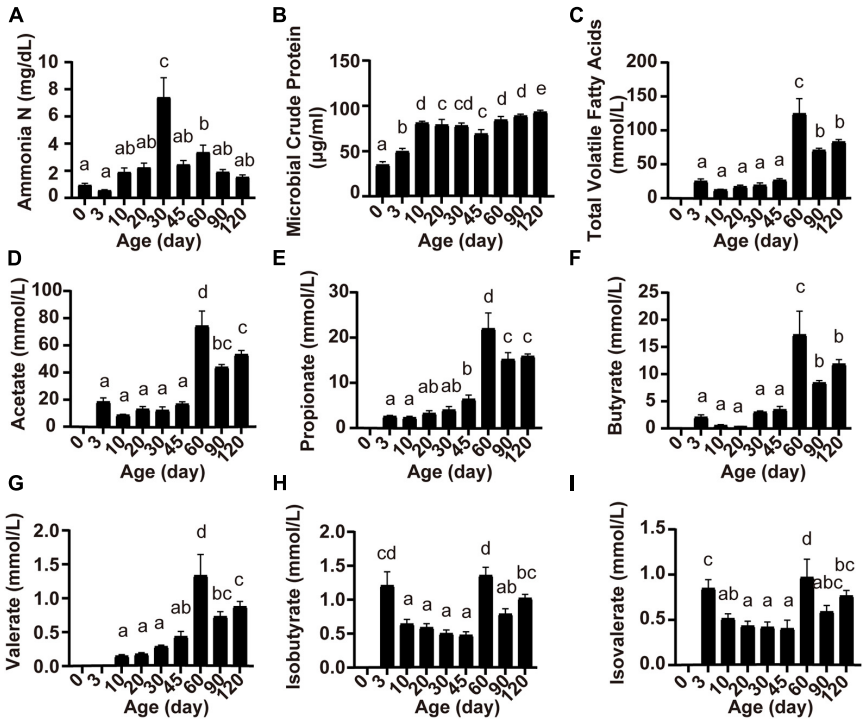


FIGURE 2 | Serum concentrations of ammonia N **(A)**, microbial crude protein **(B)**, total volatile fatty acids **(C)**, acetate **(D)**, propionate **(E)**, butyrate **(F)**, valerate **(G)**, isobutyrate **(H)**, and isovalerate **(I)** at different lamb ages. Data are expressed as mean ± SEM. ^{a,b}Letters denote significant differences between groups; groups that do not share the same letter are significantly different from each other ($P < 0.05$).

Changes in Rumen Microbiota and Taxonomic Composition

To investigate the development of the ruminal microbiota in these Hu sheep, we amplicon-sequenced the rumen fluid samples of the lambs from birth to 4 months of age (**Figure 1A**). The average number of high-quality rumen bacterial sequences generated per sample was 70,600. The Chao1 index, which reflects the species richness, increased after day 0 (**Figure 3A**), whereas the Shannon index, which indicates bacterial diversity, was observed lower at 3 days compared to the age groups of 45, 60, 90, and 120 days (**Figure 3B**, $P < 0.05$). The NMDS analysis, which compared the OTU community of each age group, revealed a high within-group similarity from 30 to 120 days of age (**Figure 3C**). The discrimination and high variance of samples collected at the 0, 3, and 10 age groups compared to later age groups confirmed the unique development of the bacterial community of each lamb before 10 days old. The samples collected at 20 days of age were also compositionally distinct to later samples but were similar to 30 days of age with less variance between ruminal samples. The significance of differences between groups was tested by analysis of similarity (ANOSIM, **Supplementary Data**). To further investigate the changes in specific rumen bacterial taxa during the early life of lambs, we compared the relative abundance of rumen bacteria at the phylum level (**Figure 3D**). The ruminal communities were dominated by Firmicutes (46.11%), Bacteroidetes (42.97%), and Proteobacteria (5.20%), accounting for 94.28% of all reads. From birth to 4 months of age, the relative abundances of Firmicutes and Bacteroidetes increased, whereas those of Proteobacteria and Fusobacteria decreased dramatically. The results showed that each age hosts its own particular bacterial community and the ruminal communities became more similar at the later time points.

The Stability of Ruminal Microbiota Over Time

The within-group similarity comparison based on the Bray-Curtis distance of the bacterial communities among age groups declared a significant change in an age-dependent way (**Figure 4A**). The within-group similarity between different individuals increased significantly after birth with the continued increase of the alpha diversity (Chao1 and Shannon index), while no difference was observed in the inter-individual variation at 3 days compared to the age groups of 10, 20, 30, 45, and 60 days (**Figure 4A**, $P < 0.05$). The results of the within-group similarity demonstrated that the rumen bacterial ecosystem of lambs was exposed to a very high rate of inter-individual diversity and low stability before weaning.

Besides, ruminal samples of lambs apparently clustered together in the NMDS graph after 20 days, suggesting that the entire ruminal microbiota underwent relatively minor changes after 20 days of age and the community become relatively stable (**Figure 3C**). To highlight the process of progressive

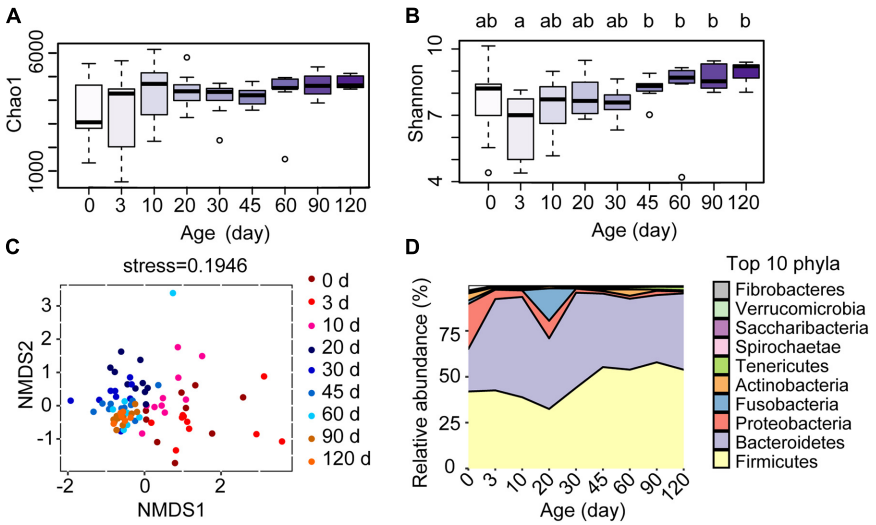


FIGURE 3 | Effect of the age of lambs on rumen bacterial communities. **(A)** Species richness estimates (Chao1 index) and **(B)** diversity (Shannon index) for the nine sampling time points. **(C)** Scatterplot from non-metric multidimensional scaling (NMSD) of bacterial Bray-Curtis distance in each sample. **(D)** Relative abundance of bacterial phyla at different ages of the lambs. ^{a,b}Letters denote significant differences between groups; groups that do not share the same letter are significantly different from each other ($P < 0.05$).

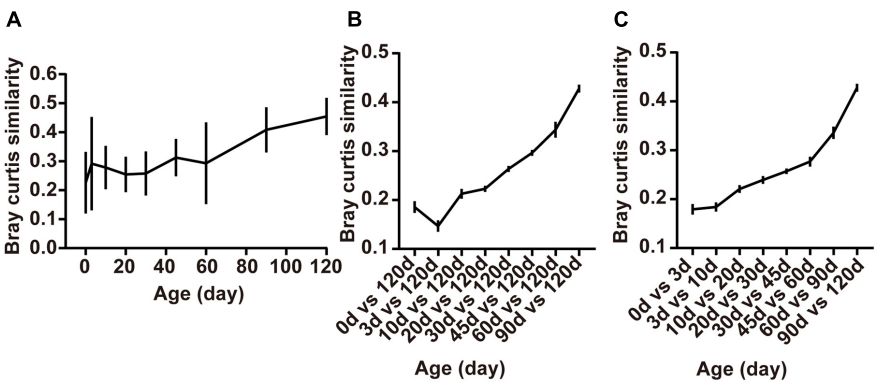


FIGURE 4 | Changes in rumen bacterial diversity as the lambs age. **(A)** The Bray-Curtis similarity within groups, **(B)** between samples collected from birth to 90 days of age and those collected at 120 days of age, and **(C)** the similarity between adjacent time points. Data are expressed as mean ± SEM.

approximation to the community structure of the mature rumen microbiota in lambs, we determined the similarity between the rumen bacteria of samples collected from birth to 90 days of age and those collected at 120 days of age (**Figure 4B**). The results demonstrated that the composition of the lambs' rumen bacterial ecosystem changed closer to the mature one, with a dramatic increase in the similarity after 20 days of age, reaching a maximum at 90 days vs. 120 days ($P < 0.05$, **Figure 4B**).

Furthermore, we determined the average Bray-Curtis similarity between adjacent age groups to describe the stability of an individual's bacteria. The similarity illustrated that the bacterial composition of each individual's rumen bacteria increased in an age-dependent manner. We found that rumen bacteria similarity between 0 and 3 days and 3 and 10 days was lower than the other groups. Meanwhile, the similarity between 90 and 120 days was the highest among the groups ($P < 0.05$, **Figure 4C**).

These results showed that large differences in bacterial community composition appear within the first few days after birth (days 0–3) and in the period shortly after providing starter feed (days 10–20). The bacterial communities changes fast during these periods, whereas a stable rumen bacterial community gradually becomes established after weaning (60 days of age).

Age-Discriminatory Bacteria in Rumen Fluid of Lambs

With the application of the RandomForest algorithm to correlate rumen microbiota composition with ages, we found that the cross-validation error curve stabilized when 27 genera were used. Thus, these 27 genera are defined as biomarker taxa in

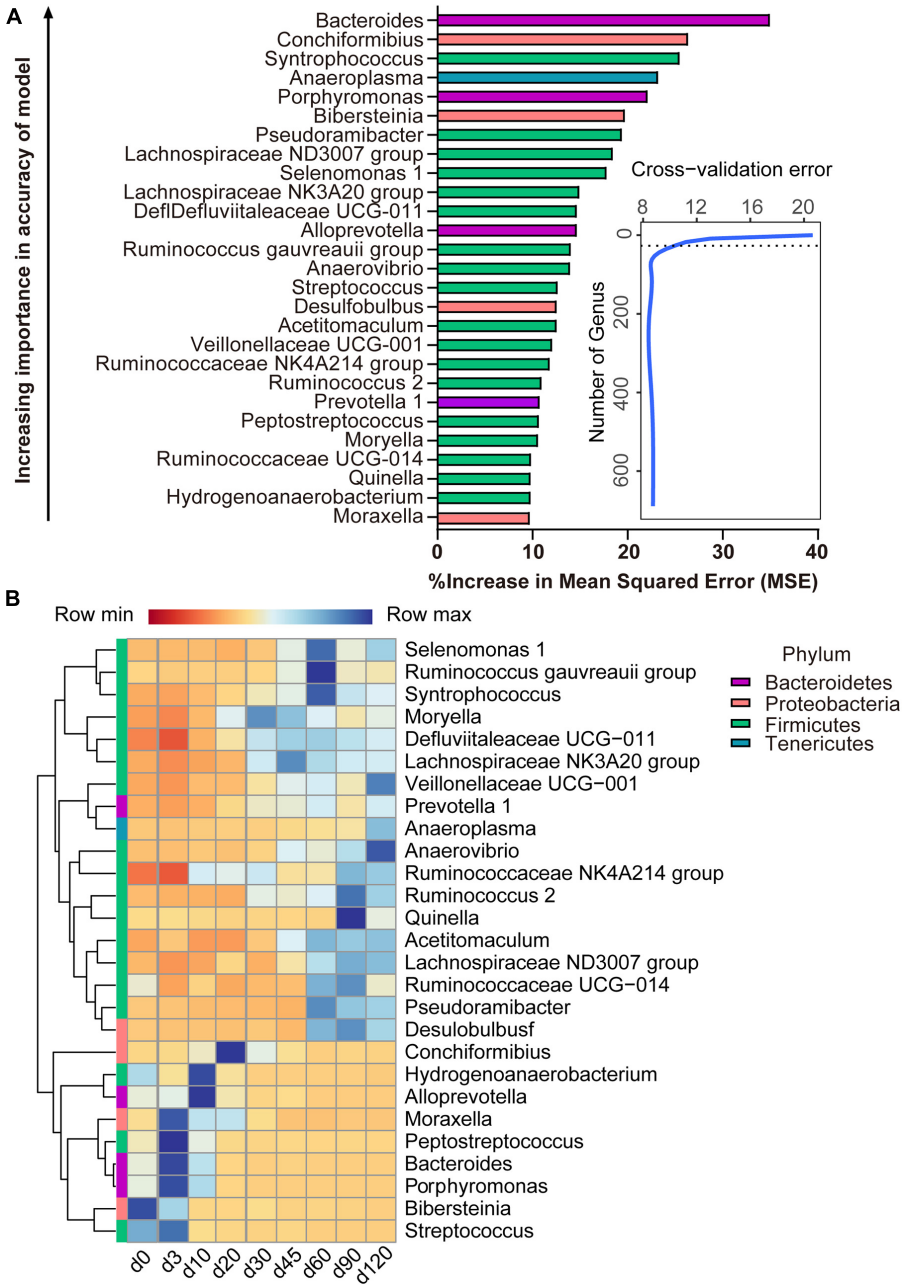


FIGURE 5 | Bacterial taxonomic biomarkers of lamb age and the rumen microbiota gradually stabilize. **(A)** The top 27 biomarkers of bacterial genera were identified by applying RandomForest regression of their relative abundances in lambs against chronological age. Ranking in descending order of importance to the accuracy of the model was to determine biomarker taxa. The insert illustrates the result of the 10-fold cross-validation error. **(B)** Heatmap of changes over ages showing the relative abundances of the top 27 age-predictive biomarkers for bacterial genera.

the model and are shown in **Figure 5A**. All bacterial taxa were ranked in a descending order of importance. The age-discriminatory genera mostly belonged to Firmicutes (18/27), Proteobacteria (4/27), and Bacteroidetes (4/27) phyla. Of these age-discriminatory genera, 12 taxa (out of 27) increased abundance with the age of the lambs (*Veillonellaceae UCG-001*, *Prevotella 1*, *Anaeroplasma*, *Anaerovibrio*, *Ruminococcaceae NK4A214 group*, *Ruminococcus 2*, *Quinella*, *Acetitomaculum*, *Lachnospiraceae ND3007 group*, *Ruminococcaceae UCG-014*, *Pseudoramibacter*, and *Desulfobulbus*), 6 taxa obtained the highest relative abundance at 60 days of age (*Selenomonas 1*, *Ruminococcus gauvreauii group*, *Syntrophococcus*, *Moryella*, *Defluviitaleaceae UCG-011*, and *Lachnospiracea NK3A20 group*), the abundance of 6 taxa increased within the first 3 days after birth and decreased thereafter (*Moraxella*, *Peptostreptococcus*, *Bacteroides*, *Porphyromonas*, *Bibersteinia*, and *Streptococcus*), and

3 taxa increased in abundance up to 20 days of age and then decreased (*Conchiformibius*, *Hydrogenoanaerobacterium*, and *Alloprevotella*) (**Figure 5B**).

Function Prediction of Rumen Microbiota

The list of 21 pathways, in the order of time-discriminatory importance, which are defined as biomarker pathways in the model, is presented in **Figure 6A** (citrate cycle; glycine, serine, and threonine metabolism; penicillin and cephalosporin biosynthesis; taurine and hypotaurine metabolism; pyruvate metabolism; biotin metabolism; lipoic acid metabolism; folate biosynthesis; porphyrin and chlorophyll metabolism; flavonoid biosynthesis; isoflavonoid biosynthesis; stilbenoid, diarylheptanoid, and gingerol biosynthesis; carbon metabolism; phosphatidylinositol signaling system; phospholipase D signaling pathway; peroxisome; amoebiasis; human papillomavirus infection; viral carcinogenesis; renal cell carcinoma; choline metabolism in cancer). Most (13/21) of the important pathways are metabolic (including processes such as the citrate cycle and the metabolism of glycine, serine and threonine, taurine and hypotaurine, pyruvate, biotin, lipoic acid, porphyrin, and chlorophyll, and carbon), as well as biosynthetic processes (for penicillin, cephalosporin, folate, flavonoid, isoflavonoid, stilbenoid, diarylheptanoid, and gingerol). All of these pathways had a high relative abundance from birth to 10 days of age, which decreased thereafter (**Figure 6B**).

Correlation Analyses of Age-Related Bacterial Genera and Functions With Rumen Fermentation and Serum Metabolite Levels

To investigate how the age-related genera and functions in rumen impact host metabolism, a Spearman correlation matrix was generated to explore these relationships. As shown in **Figure 7A**, the relative abundances of *Bacteroides*, *Moraxella*, *Peptostreptococcus*, *Streptococcus*, *Hydrogenoanaerobacterium*, *Conchiformibius*, *Porphyromonas*, *Alloprevotella*, and *Bibersteinia*

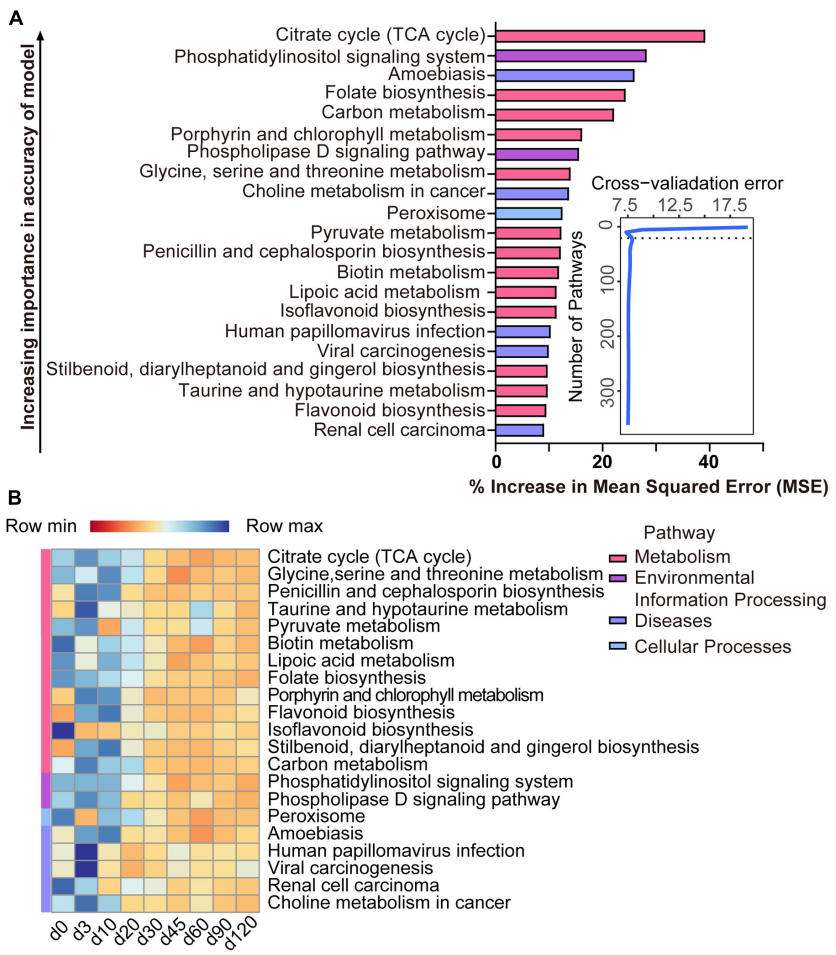


FIGURE 6 | Bacterial pathway biomarkers of lamb age and the rumen microbiota gradually stabilize. **(A)** The top 21 biomarkers of bacterial pathways were identified by applying RandomForest regression of their relative abundances in lambs against chronological age. Ranking in descending order of importance to the accuracy of the model was to determine biomarker taxa. The insert illustrates the result of the 10-fold cross-validation error. **(B)** Heatmap of changes over ages showing the relative abundances of the top 21 age-predictive biomarkers for bacterial pathways.

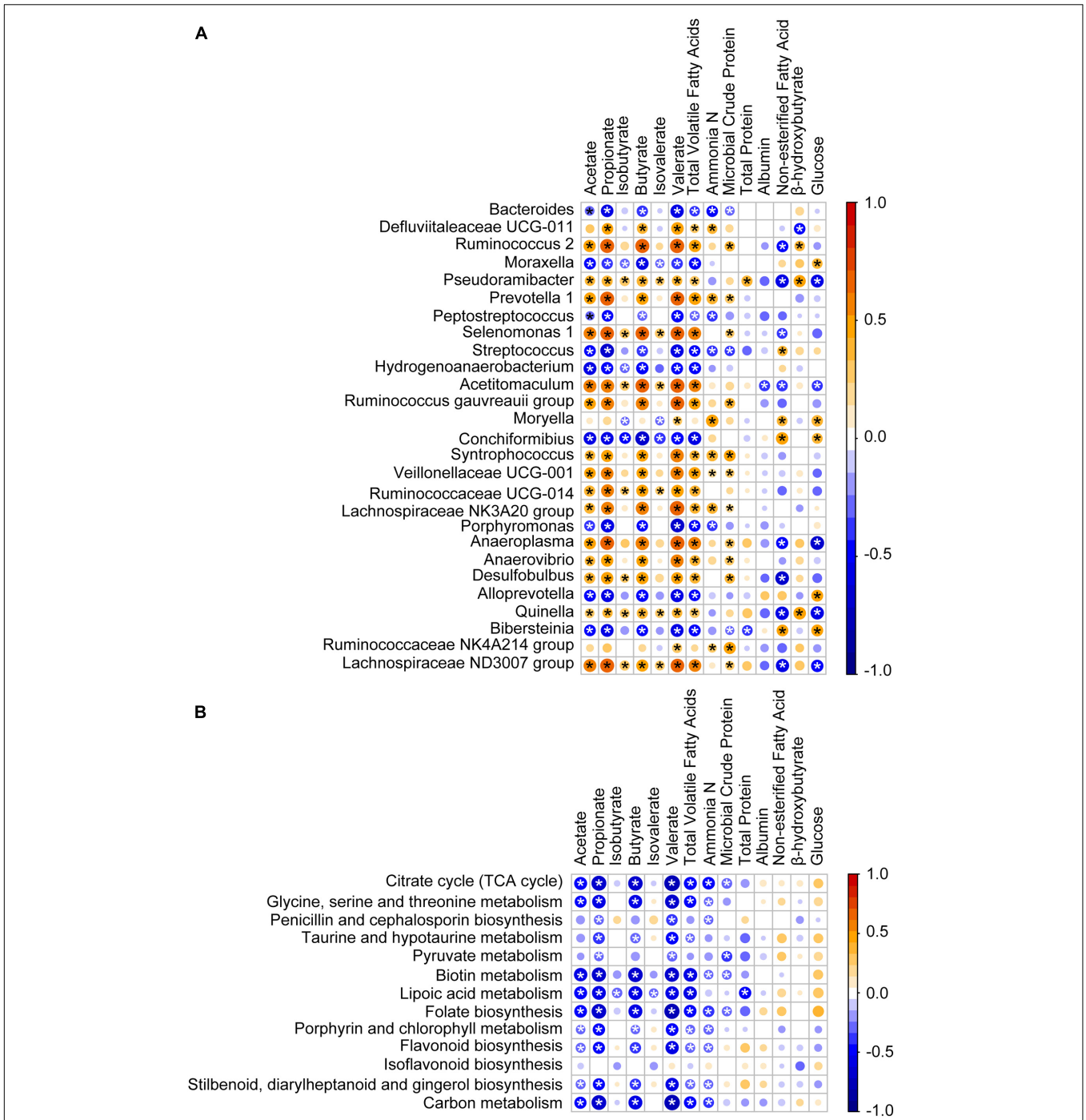


FIGURE 7 | Association of the age-associated genera and pathways with lamb ruminal fermentation and blood serum parameters. **(A)** Spearman correlation of ruminal fermentation, serum parameters, and age-associated genera identified by RandomForest. **(B)** Spearman correlation of ruminal fermentation, serum parameters, and age-associated pathway identified by RandomForest. Bubble size represents the absolute value of the correlation coefficient (r), and bubble color represents negative (blue) or positive (red) correlations, $^{*}P < 0.05$.

had negative ($P < 0.05$) Spearman correlations with acetate, propionate, butyrate, valerate, and total VFA. In contrast relative abundances of *Ruminococcus 2*, *Pseudoramibacter*, *Prevotella 1*, *Selenomonas 1*, *Acetitomaculum*, *Ruminococcus gauvreauii group*, *Syntrophococcus*, *Veillonellaceae UCG-001*, *Ruminococcaceae UCG-014*, *Lachnospiraceae NK3A20 group*, *Anaeroplasma*, *Anaerovibrio*, *Desulfobulbus*, *Quinella*, and *Lachnospiraceae ND3007 group* were positively ($P < 0.05$) correlated with acetate, propionate, butyrate, valerate, and total VFA. Relative abundances of *Pseudoramibacter*, *Acetitomaculum*,

Anaeroplasma, *Quinella*, and *Lachnospiraceae ND3007 group* were negatively ($P < 0.05$) correlated with NEFA and GLU and those of *Moryella*, *Conchiformibius,* and *Bibersteinia* were positively correlated with NEFA and GLU ($P < 0.05$). The relative abundances of the citrate cycle, the metabolism of glycine, serine and threonine, biotin, lipoic acid, the biosynthesis of folate, porphyrin, and chlorophyll, and carbon as well as of flavonoid, stilbenoid, diarylheptanoid, and gingerol were negatively correlated with acetate, propionate, butyrate, valerate, and total VFA ($P < 0.05$, **Figure 7B**).

DISCUSSION

In order to improve the efficiency of metabolic pathways, efforts have been made to investigate compounds that may modify key populations of microorganisms (Ahmad et al., 2020; Chen et al., 2020). However, because the rumen microbiota is generally well-established and hard to change by the time of such treatments, this approach has tended to be inconsistent or short-lived. Previous works have observed that the developing rumen in the newborn provides a unique opportunity to alter such a complex microbial ecosystem (Yá?ez-Ruiz et al., 2015; Abecia et al., 2018; Saro et al., 2018), but at this stage the most sensitive period of interventions on ruminal microbiota community and fermentation processes are not well known. The current study was designed to determine the effect of age on the rumen bacterial community and the ruminal fermentation of lambs. Both the composition and the fermentation of the microbial community were monitored from birth until lambs had access to adult diets. This study demonstrated that ruminal fermentation changed in an age-dependent manner that was correlated with some age-related alterations in rumen microbial taxa. It is important to know when modulating the composition and function of microbiota will be more effective. Our results indicated that it may be possible to manage these changes to improve rumen function and enhance the health and productivity of the host animals. In particular, these results suggest that the first 20 days from birth may be the most suitable period for the interventions.

The specific characteristic of ruminants is their ability to utilize the cellulose and hemicelluloses present in plant-based diets (Newbold and Ramos-Morales, 2020). In our study, as the lambs' ruminal microbiota developed, we recorded changes in the relative abundances of Proteobacteria, Bacteroidetes, and Firmicutes that were consistent with previous studies (Jami et al., 2013; Zhu et al., 2018). The relative abundances of Firmicutes and Bacteroidetes increased, while that of Proteobacteria decreased dramatically. From 10 days on, there were no further dramatic changes of bacterial phyla indicating that, despite the fact that no solid plant material was ingested by the lambs, the bacteria responsible for its digestion were already present. Although the major types of rumen bacteria were present within 20 days, our findings on similarity suggested that the bacterial community was still unstable at this stage and thus was not fully matured. This supports the proposal that the period from birth to 20 days of age provides a unique opportunity to manipulate the composition of ruminal microbiota in lambs.

After 20 days of age, there were no clear age-related changes at the bacterial phyla level; however, there was variation in the relative abundance of some genera, which corresponds with changes of the substrate in the rumen (Yá?ez-Ruiz et al., 2015). Genera that were abundant from birth to 20 days of age in this and other studies (Dill-McFarland et al., 2017; Li et al., 2018; Yousif et al., 2018) included *Bacteroides*, *Moraxella*, *Peptostreptococcus*, *Streptococcus*, *Hydrogenoanaerobacterium*, *Conchiformibius*, *Porphyromonas*, *Alloprevotella*, and *Bibersteinia*, and these had a negative correlation with the main VFAs. These early rumen colonizers may have been spread from the birth canal or other environmental sources and are likely to be important for the newborn. For instance, the abundance of *Bacteroides* in newborn lambs enables them to utilize some components of milk (Mach et al., 2015). For the ruminal fermentation, the total volatile fatty acids appeared at 3 days of age, indicating that at the beginning of birth, though the microbiota already persists in the rumen, it was almost impossible to produce the SCFAs. Our results proved that the lambs can be considered a non-ruminant from a functional point of view in the early period (Jiao et al., 2016). Later, the microbiota present in the rumen will help the host to ingest feed. One of the important events in the development of microbiota is starter feeding, which can effectively facilitate the growth and development of the rumen. In our study, the similarity between the samples collected from birth to 90 days of age and those collected at 120 days of age increased after starter feeding (15 days of age). And after feeding solid food, the relative abundance of *Bacteroides*, which was important to digest milk (Mach et al., 2015), decreased. Our findings demonstrated that, in some way, the starter feeding affected the composition of ruminal bacteria which stabilized the bacterial ecosystem, consistent with a previous study in lambs (Lin et al., 2019). The presence of dietary fiber leads to a high abundance of *Prevotella* species (Kolodziejczyk et al., 2019). The butyrate-producing bacteria, Lachnospiraceae (*Lachnospiraceae NK3A20 group*) and Ruminococcaceae (*Ruminococcus gauvreauii group*, *Ruminococcaceae UCG-014*, and *Ruminococcus 2*) are critical for rumen fermentation (Ma et al., 2020). In our study, the age-related genera, *Prevotella 1*, *Lachnospiraceae NK3A20 group*, *Ruminococcus gauvreauii group*, *Ruminococcaceae UCG-014,* and *Ruminococcus 2*, all had a higher relative abundance after 30 days of age especially after weaning (60 days of age) along with a positive correlation with acetate, propionate, butyrate, valerate, and total VFA. Meanwhile, the concentrations of SCFAs significantly increased from 60 days of age. The changes of relative abundance in lambs showed that weaning will significantly increase the proportion of the bacteria which can produce the SCFAs, thus increasing the concentrations of SCFAs. The changes in the gene involved in the KEGG pathway also showed that the fermentation way in the rumen was changed after 20 days of age. The relatively oxidized rumen environment of newborn lambs enables rumen microbes to exploit the citrate cycle for energy production (Backhed et al., 2015) as shown by the enhanced expression of genes related to citrate cycle enzymes at this age. Our results illustrated that the microbiota which helps the neonate to utilize breast milk decreased, and

then the microbiota which can ferment the solid food increased. Finally, a fully matured microbial ecosystem was established to digest plant material.

The feeding pattern will influence the bacterial communities in lambs. In our study, the lambs had free access to the starter from 15 days compared to a previous study in goats (free access to the starter from 25 days). The richness and diversity calculated by Chao and Shannon were higher in our study, and the relative abundance of Firmicutes and Bacteroidetes were different (Li et al., 2019). Compared to the Hu lambs that were weaned at 90 days, richness and diversity were higher in lambs 60, 90, and 120 days of age (Wang et al., 2016) in our study. The comparison of different feeding patterns indicated that both the time of feeding the starter and the time of weaning will affect the composition of the rumen bacterial community. Early intake of the starter and timely weaning will increase microbial diversity and make the microbial community mature earlier.

CONCLUSION

This study of the ruminal microbiota of lambs shows that the bacterial diversity increases with the age of lambs and the age-related abundances of particular genera are correlated with concentrations of volatile fatty acids and microbial crude protein. The genera of *Prevotella 1*, *Lachnospiraceae NK3A20 group*, *Ruminococcus gauvreauii group*, *Ruminococcaceae UCG-014*, and *Ruminococcus 2* are important in maturation of both the rumen bacterial community and rumen fermentation. Although the composition and function of the ruminal microbiota are not well-established by 20 days of age of lambs, the major types of rumen bacteria are already present at this stage. This period provides a unique opportunity for the potential manipulation of the ruminal microbial ecosystems. The findings of the present study may be instructive for improving the ruminal fermentation processes and reprogramming the efficiency of rumen microbiota.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://gsa.big.ac.cn, PRJCA004193.

ETHICS STATEMENT

This study was conducted under the guidance of the Animal Care and Use Committee of Hebei Agricultural University (approval number: YJ201825).

AUTHOR CONTRIBUTIONS

XY: software, formal prioritized as targets in strength-based analysis, and writing—original draft. SJ: methodology, data curation, and term. CD: writing—review and editing. SJ and PT: investigation. HY: validation and visualization. YZ: conceptualization, project administration, and funding acquisition. YL: supervision and resources. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the China Agriculture Research System (grant no. CARS-38) provided by YZ, the National Key R&D Program of China (grant no. 2018YFD0502100) provided by YZ, Precision Animal Husbandry Discipline Group Construction Project of Hebei Agricultural University (grant no. 1090064) provided by YL, and the Scientific Research Foundation of Hebei Agricultural University (grant no. YJ201825) provided by SJ.

ACKNOWLEDGMENTS

We thank the Sheep Industry Test Station for the provision of resources. We thank Wenxin Cao and Kang Wang from the College of Animal Science and Technology, Hebei Agricultural University for technical assistance with the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.679135/full#supplementary-material

REFERENCES

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., et al. (2018). Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Front. Microbiol.* 9:2227. doi: 10.3389/fmicb.2018.02227

Ahmad, A. A., Yang, C., Zhang, J., Kalwar, Q., Liang, Z., Li, C., et al. (2020). Effects of dietary energy levels on rumen fermentation, microbial diversity, and feed efficiency of Yaks (Bos grunniens). *Front. Microbiol.* 11:625. doi: 10.3389/fmicb.2020.00625

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17:852. doi: 10.1016/j.chom.2015.05.012

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Breiman, L. (2001). Random Forests. *Mach. Learn.* 45, 5–32. doi: 10.1023/A:1010933404324

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Chen, D., Yan, J., Shen, W., Song, Y., Lan, X., Yi, K., et al. (2020). Effect of inclusion of HMBi in the ration of goats on feed intake, nutrient digestibility, rumen bacteria community and blood serum parameters. *J. Anim. Physiol. Anim. Nutr.* 104, 987–997. doi: 10.1111/jpn.13270

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., et al. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* 37, D141–D145. doi: 10.1093/nar/gkn879

Dill-McFarland, K. A., Breaker, J. D., and Suen, G. (2017). Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci. Rep.* 7:40864. doi: 10.1038/srep40864

Drackley, J. K. (2008). Calf nutrition from birth to breeding. *Vet. Clin. North Am. Food Anim. Pract.* 24, 55–86. doi: 10.1016/j.cvfa.2008.01.001

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

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

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Jiao, J., Wu, J., Zhou, C., Tang, S., Wang, M., and Tan, Z. (2016). Composition of Ileal Bacterial Community in Grazing Goats Varies across Non-rumination, Transition and Rumination Stages of Life. *Front. Microbiol.* 7:1364. doi: 10.3389/fmicb.2016.01364

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

Kolodziejczyk, A. A., Zheng, D., and Elinav, E. (2019). Diet-microbiota interactions and personalized nutrition. *Nat. Rev. Microbiol.* 17, 742–753. doi: 10.1038/s41579-019-0256-8

Li, B., Zhang, K., Li, C., Wang, X., Chen, Y., and Yang, Y. (2019). Characterization and comparison of microbiota in the gastrointestinal tracts of the goat (Capra hircus) during preweaning development. *Front. Microbiol.* 10:2125. doi: 10.3389/fmicb.2019.02125

Li, Z., Wang, X., Zhang, T., Si, H., Nan, W., Xu, C., et al. (2018). The development of microbiota and metabolome in small intestine of Sika Deer (Cervus nippon) from birth to weaning. *Front. Microbiol.* 9:4. doi: 10.3389/fmicb.2018.00004

Liaw, A., and Wiener, M. (2002). Classification and Regression by randomForest. *R News* 2, 18–22.

Lin, L., Xie, F., Sun, D., Liu, J., Zhu, W., and Mao, S. (2019). Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. *Microbiome* 7:83. doi: 10.1186/s40168-019-0701-y

Ma, T., Villot, C., Renaud, D., Skidmore, A., Chevaux, E., Steele, M., et al. (2020). Linking perturbations to temporal changes in diversity, stability, and compositions of neonatal calf gut microbiota: prediction of diarrhea. *ISME J.* 14, 2223–2235. doi: 10.1038/s41396-020-0678-3

Mach, N., Berri, M., Estelle, J., Levenez, F., Lemonnier, G., Denis, C., et al. (2015). Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environ. Microbiol. Rep.* 7, 554–569. doi: 10.1111/1758-2229.12285

Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mao, H., Zhang, Y., Yun, Y., Ji, W., Jin, Z., Wang, C., et al. (2021). Weaning age affects the development of the ruminal bacterial and archaeal community in Hu lambs during early life. *Front. Microbiol.* 12:636865. doi: 10.3389/fmicb.2021.636865

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

Munyaka, P. M., Eissa, N., Bernstein, C. N., Khafipour, E., and Ghia, J. E. (2015). Antepartum antibiotic treatment increases offspring susceptibility to experimental colitis: a role of the gut microbiota. *PLoS One* 10:e0142536. doi: 10.1371/journal.pone.0142536

Newbold, C. J., and Ramos-Morales, E. (2020). Review: ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal* 14, s78–s86. doi: 10.1017/S1751731119003252

Saro, C., Hohenester, U. M., Bernard, M., Lagree, M., Martin, C., Doreau, M., et al. (2018). Effectiveness of interventions to modulate the rumen microbiota composition and function in pre-ruminant and ruminant lambs. *Front. Microbiol.* 9:1273. doi: 10.3389/fmicb.2018.01273

Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., Burgmann, H., et al. (2012). Fundamentals of microbial community resistance and resilience. *Front. Microbiol.* 3:417. doi: 10.3389/fmicb.2012.00417

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M. A., et al. (2014). Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* 510, 417–421. doi: 10.1038/nature13421

Wang, L., Xu, Q., Kong, F., Yang, Y., Wu, D., Mishra, S., et al. (2016). Exploring the goat rumen microbiome from seven days to two years. *PLoS One* 11:e0154354. doi: 10.1371/journal.pone.0154354

Wang, T., Jiao, J. X., Wang, H. C., Degen, A. A., Gou, N. N., Li, S. S., et al. (2021). The effects of supplementing sweet sorghum with grapeseeds on dry matter intake, average daily gain, feed digestibility and rumen parameters and microbiota in lambs. *Anim. Feed Sci. Tech.* 272:114750. doi: 10.1016/j.anifeedsci.2020.114750

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* 39, 971–974. doi: 10.1021/ac60252a045

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

Wemheuer, F., Taylor, J. A., Daniel, R., Johnston, E., Meinicke, P., Thomas, T., et al. (2020). Tax4Fun2: prediction of habitat-specific functional profiles and functional redundancy based on 16S rRNA gene sequences. *Environ. Microbiome* 15, 1034–1039. doi: 10.1186/s40793-020-00358-7

Yá?ez-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

Yousif, M. H., Li, J. H., Li, Z. Q., Maswayi Alugongo, G., Ji, S. K., Li, Y. X., et al. (2018). Low Concentration of antibiotics modulates gut microbiota at different levels in pre-weaning dairy calves. *Microorganisms* 6:118. doi: 10.3390/microorganisms6040118

Yu, S. B., Zhang, G. Y., Liu, Z. B., Wu, P., Yu, Z. T., and Wang, J. K. (2020). Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. *BMC Microbiol.* 20:29. doi: 10.1186/s12866-020-1716-z

Zhang, C., Li, M. M., Al-Marashdeh, O., Gan, L. P., Zhang, C. Y., and Zhang, G. G. (2019). Performance, rumen fermentation, and gastrointestinal microflora of lambs fed pelleted or unpelleted total mixed ration. *Anim. Feed Sci. Tech.* 253, 22–31. doi: 10.1016/j.anifeedsci.2019.05.003

Zhang, J., Zhang, N., Liu, Y. X., Zhang, X., Hu, B., Qin, Y., et al. (2018). Root microbiota shift in rice correlates with resident time in the field and developmental stage. *Sci. China Life Sci.* 61, 613–621. doi: 10.1007/s11427-018-9284-4

Zhu, Z., Kristensen, L., Difford, G. F., Poulsen, M., Noel, S. J., Abu Al-Soud, W., et al. (2018). Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows. *J. Dairy Sci.* 101, 9847–9862. doi: 10.3168/jds.2017-14366

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 October 2021
doi: 10.3389/fmicb.2021.691502

Long-Term Mootral Application Impacts Methane Production and the Microbial Community in the Rumen Simulation Technique System

*Johanna Brede[1], Manuela Peukert[2], Björn Egert[3], Gerhard Breves[1] and Melanie Brede[1]**

[1] Institute for Physiology and Cell Biology, University of Veterinary Medicine Hannover, Hanover, Germany, [2] Department of Safety and Quality of Meat, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Kulmbach, Germany, [3] Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany

OPEN ACCESS

Edited by:
Christina Diane Moon, AgResearch Ltd., New Zealand

Reviewed by:
Eva Ramos-Morales, Scotland's Rural College, United Kingdom
Diego P. Morgavi, INRAE Clermont-Auvergne-Rhône-Alpes, France

***Correspondence:**
Melanie Brede
melanie.brede@tiho-hannover.de

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *06 April 2021*
Accepted: *21 September 2021*
Published: *08 October 2021*

Citation:
Brede J, Peukert M, Egert B, Breves G and Brede M (2021) Long-Term Mootral Application Impacts Methane Production and the Microbial Community in the Rumen Simulation Technique System. Front. Microbiol. 12:691502. doi: 10.3389/fmicb.2021.691502

Methane emissions by ruminants contribute to global warming and result in a loss of dietary energy for the animals. One possibility of reducing methane emissions is by dietary strategies. In the present trial, we investigated the long-term effects of Mootral, a feed additive consisting of garlic powder (*Allium sativum*) and bitter orange extracts (*Citrus aurantium*), on fermentation parameters and the microbial community in the rumen simulation technique (RUSITEC) system. The experiment lasted 38 days and was divided into three phases: an equilibration period of 7 days, a baseline period (BL) of 3 days, and experimental period (EP) of 28 days. Twelve fermentation vessels were divided into three groups (n = 4): control (CON), short-term (ST), and long-term (LT) application. From day 11 to day 27, 1.7 g of Mootral was added to the ST vessels; LT vessels received 1.7 g of Mootral daily for the entire EP. With the onset of Mootral application, methane production was significantly reduced in both groups until day 18. Thereafter, the production rate returned to the initial quantity. Furthermore, the short chain fatty acid fermentation profile was significantly altered by Mootral application; the molar proportion of acetate decreased, while the proportions of propionate and butyrate increased. Metabolomic analysis revealed further changes in metabolite concentrations associated with the Mootral supplementation period. The methyl coenzyme-M reductase gene copy number was reduced in the liquid and solid phase, whereas the treatment did not affect the abundance of bacteria. At the end of the BL, Methanomicrobia was the most abundant archaeal class. Mootral supplementation induced an increase in the relative abundance of *Methanomassiliicoccales* and a reduction in the relative abundance of Methanomicrobia, however, this effect was transient. Abundances of bacterial families were only marginally altered by the treatment. In conclusion, Mootral has the transient ability to reduce methane production significantly due to a selective effect on archaea numbers and archaeal community composition with little effect on the bacterial community.

Keywords: methane, cattle, garlic, microbial community, RUSITEC

INTRODUCTION

In the rumen, methanogenic archaea produce methane mainly by reducing CO_2 with hydrogen (Morgavi et al., 2010). Additionally, formate, methanol, and methylamines serve as substrates for methanogenesis (Hungate et al., 1970; Patterson and Hespell, 1979). Methane production depends on various factors such as carbohydrate intake and composition, rumen retention time, rate of fermentation, and methanogenesis (Beauchemin, 2009). Reducing enteric methane emissions is desirable to reduce the contribution of livestock to greenhouse gas emissions.

Many strategies to reduce methane emissions have been evaluated, which can be differentiated into dietary and non-dietary approaches. The latter are, for example, defaunation (Machmuller et al., 2003), anti-methanogenic vaccination (Wedlock et al., 2010), and breeding of ruminants with low methane emissions (Attwood et al., 2011). Moreover, various dietary strategies have been examined regarding their anti-methanogenic effect. Firstly, there have been attempts to alter the diet in grain content, forage type, and quality (Haque, 2018). Secondly, the supplementation of lipids (Johnson and Johnson, 1995), and secondary plant compounds such as saponins (Lila et al., 2003) and tannins (Tavendale et al., 2005) have been tested. Furthermore, organic acids for example fumaric acid have been investigated (Riede et al., 2013). Moreover, the supplementation of chemicals such as 3-nitrooxypropanol (Martinez-Fernandez et al., 2018) and ionophores (Odongo et al., 2007) have been studied. These strategies either target the methanogens or alter metabolic processes so that less substrate is available for methanogenesis (Haque, 2018). Many of the described approaches have the potential to inhibit methane production, but their effectiveness is limited by practicability, decreased palatability or a negative impact on productivity. According to Beauchemin (2009), the ideal strategy is highly dependent on the particular farm, geographic region, feed, and the type of animal.

Among the dietary approaches, application of garlic and flavonoids has been the focus of many studies. Garlic contains allicin and is known for its antimicrobial properties (Ankri and Mirelman, 1999). The potential of garlic to reduce methane production has been widely studied *in vitro* (Busquet et al., 2005; Soliva et al., 2011; Mbiriri et al., 2017) and *in vivo* (Patra et al., 2011; Ma et al., 2016). Flavonoids from citrus fruits are a group of polyphenols with antioxidative, antimicrobial und anti-inflammatory properties (Kumar and Pandey, 2013). Hence, these plant compounds have also been investigated regarding their effect on methane production (Oskoueian et al., 2013).

Mootral (Mootral SA, Rolle, Switzerland) is a feed additive, which consists of garlic powder (*Allium sativum*) and bitter orange extracts (*Citrus aurantium*). The strong potential of Mootral to mitigate methane production has already been demonstrated *in vitro* by Eger et al. (2018). In this study, a 1 and 2 g/days application of Mootral were evaluated during 1 week of supplementation in the rumen simulation technique (RUSITEC)-system. Both doses substantially reduced the percentage of methane in the fermentation gas (by 95 and 99%, respectively). In a recently published study by Ahmed et al. (2021) application of Mootral at a level of 20% of the substrate led to a reduction of methane proportion up to 54% in a batch culture. In an *in vivo* study by Vrancken et al. (2019) a pellet with 3% Mootral powder reduced methane emissions in Jersey cows by 38% and in Holstein Friesian cows by 21%. In another study by Roque et al. (2019), Angus × Hereford cross steers were supplemented with 15 g of Mootral per day for a duration of 12 weeks. A reduction in methane production by 23% was reported.

However, although the effectiveness of Mootral in reducing methane production *in vitro* and *in vivo* has already been demonstrated, the mechanisms which lead to methane reduction still remain unclear. In the present study, we aimed to investigate the impact of a long-term Mootral application on fermentation parameters, methane production and its effects on the microbial community within the RUSITEC-system. Therefore, we intensively studied both, liquid- and solid-associated archaea and bacteria and identified specific metabolite alterations using an untargeted metabolomics approach.

MATERIALS AND METHODS

Ethics Statement

The fistulation of the donor cows was permitted by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany) under AZ 33.19-42502-05-13A373. In this project, all activities including animals were carried out under the requirements of the German Animal Welfare Act.

Experimental Setup

In 1977, Czerkawski and Breckenridge introduced the RUSITEC (Czerkawski and Breckenridge, 1977). For this experiment, two systems with six fermentation vessels each were applied, and all fermenters were fed with the same basal diet of 7 g of hay (70.1% of diet dry matter) and 3 g of concentrate (29.9% of dry matter, deuka Schaffutter, Deutsche Tiernahrung Cremer GmbH & Co. KG, Bramsche, Germany, **Supplementary Table 1**) per day. The same hay and concentrate was fed to the donor cows. The hay was cut into pieces of approximately 1 cm length. The trial lasted 38 days and was designed to compare the short- and long-term effects of Mootral (Mootral SA, Rolle, Switzerland, crude nutrients are presented in **Supplementary Table 1**). The product was produced as described by Eger et al. (2018). The powder used in this trial contained 1% allicin with a stability of 6 months. The experiment started 4 weeks after delivery of the product. The experiment consisted of three phases: equilibration (days 0–7), baseline period (BL, days 8–10), and experimental period (EP, days 11–38). The equilibration period was performed to ensure a stable microbial community composition in the system, and the BL aimed to verify that there were not differences in fermentation among the treatment groups before supplementation. The 12 vessels were divided into three treatment groups (each $n = 4$): control (CON), short-term (ST), and long-term (LT) application. The CON group remained untreated during the entire experiment. In the LT group, the diet was supplemented with 1.7 g Mootral (inclusion

rate: 17.7% of the diet dry matter content) for the entire EP, in the ST group 1.7 g was supplied from the beginning of the EP (day 11) until day 27, by adding Mootral into the feed bags.

At the start of the experiment, rumen fluid, and solid contents of two rumen-fistulated non-lactating German Black Pied cattle were collected as an inoculum 3 h after the morning feeding. The cows were fed a basal diet of 6.5 kg hay, 600 g concentrate, and 100 g minerals (VitaMineral Trockensteher, Agravis, Münster, Germany) once per day. The rumen fluid was removed from each cow's rumen and filled in a bottle *via* a pump. Solid rumen contents were collected from the solid phase in the dorsal rumen sac. Rumen contents were transported from the stable to the laboratory in closed well-filled containers to avoid extensive exposure to oxygen. Liquid contents from each cow were filtered through a gauze (Lohmann & Rauscher International GmbH & Co. KG, Rengsdorf, Germany) to remove larger feed particles and mixed in another bucket. Afterward, 660 mL of mixed rumen fluid was filled into each of the fermentation vessels. During the whole procedure, rumen contents were kept warm using a water bath (39°C). The solid rumen content was squeezed out through gauze and a total of 70 g was weighed into each of the 12 nylon bags (R712 Forage Bags *in situ*, ANKOM Technology, 6.75 cm × 12 cm, Gesellschaft für Analysetechnik HLS, Salzwedel, Germany). One of these inoculum bags was placed into the inner vessel of each of the fermentation vessel together with another nylon bag containing the formerly described basal diet (feed bag). The whole procedure was finished about 45 min after collection of rumen contents. A motor ensured a permanent up and down movement (6 times/min) of the inner vessel. A modified McDougall's buffer (Wetzels et al., 2018) was infused into the fermentation vessel using a pump (Typ B1, Ole Dich, Hvidore, Denmark) with a dilution rate of 0.77 L/days. At day 1, the inoculum bag was replaced after 24 h of incubation by a feed bag. At day 2, the feed bag from day 0 was exchanged for a new one. Thereafter, the feed bags were changed alternately, so that each feed bag remained in the fermentation vessel for 48 h. After removing the feed or inoculum bag from the fermentation vessel, it was transferred to a plastic bag and 40 mL of the modified McDougall's buffer was added. The bag was washed by squeezing for 60 s to detach the microorganisms loosely associated with the feed particles. Subsequently, the washing liquid was added back to the vessels. The daily overflow was collected in a glass flask *via* a butyl tube (8.0 mm × 12.0 mm, YMC Europe GmbH, Dinslaken, Germany). Glass flasks were placed in a styrofoam box filled with ice, to stop further fermentation processes. Daily, nitrogen (Widmann Gase GmbH, Elchingen, Germany) was infused into the overflow flasks after removing the fluid to maintain anaerobic conditions. To seal the overflow flask, a plug with a further butyl tube was placed into the aperture. The butyl tube was linked to a gas bag (Plastigas®, Linde AG, Pullach, Germany) to collect the fermentation gas for further evaluation of the gas volume. Gas samples for analysis of the gas composition were collected in a glass bulb (Pfeuffer, Hannover, Germany) installed between flask and gas bag.

Sampling and Measurements

Measurements of pH (Polyplast pH Sensor, Hamilton Bonaduz AG, CH-7402, Switzerland), redox potential (Polyplast ORP, Hamilton Bonaduz AG, connected with a Digital-pH-Meter 646, Knick GmbH & Co. KG, Berlin, Germany), and overflow volume were carried out daily for the entire duration of the experiment. Overflow samples for determining short chain fatty acid (SCFA) and ammonia-N concentrations were collected daily in BL and every fourth day during EP. The samples were stored at −18°C until further processing. The SCFA concentrations were analyzed by gas chromatography (Gas Chromatograph 5890 Series II, Hewlett Packard Enterprise GmbH, Böblingen, Germany) as previously described by Koch et al. (2006). To determine the ammonia-N-concentration, a photometric measurement was performed as described before (Riede et al., 2013). Gas volume was measured daily using a gas drum type meter (Dr.-Ing. RITTER Apparatebau GmbH & Co. KG, Bochum, Germany). Samples for measuring of CO_2 and CH_4 were collected with a glass bulb at days 10, 14, 18, 22, 26, 30, 34, and 38. The analysis of the gas samples was performed at the ISAH (Institute for Sanitary Engineering and Waste Management, Leibniz University Hannover, Germany) by gas chromatography as described by Eger et al. (2018). The total production rate was calculated by multiplying the percentages of CO_2 and CH_4 with the gas volume corrected for standard conditions (1,013 hPa, 0°C). Samples for qPCR of the bacterial 16S gene in solid- and liquid-associated microorganisms (SAM and LAM, respectively) were collected at days 9, 16, 23, 30, and 37 by freezing feed bags or 10 mL of fermenter fluid, respectively, in liquid nitrogen. Samples were stored at −80°C until further treatment. Prior to DNA extraction, the samples were freeze-dried and the solid samples were ground mechanically using a coffee grinder. For sequencing and qPCR of the methyl coenzyme-M reductase gene (*mcrA*) gene, samples were taken weekly during BL and EP (days 10, 17, 24, 31, and 38). For the LAM, 30 mL of the liquid was taken from the vessel and treated as described by Eger et al. (2018). Feed bags were treated as described by Brede et al. (2020). Additionally, samples for metabolome analysis were obtained from the effluent on a weekly basis (days 10, 17, 24, 31, and 38) and stored at −18°C.

DNA Extraction and Quantitative PCR

The DNeasy PowerSoil Kit (QIAGEN GmbH, Hilden, Germany) was used for DNA extraction in SAM and LAM samples as well as in qPCR samples to obtain the DNA for sequencing and qPCR analysis. Two modifications of the manufacture's protocol were applied: 250 μL or 250 mg of samples collected for sequencing and *mcrA* PCR and 100 mg of the freeze-dried samples were used, and at the final step, the membrane was washed with 50 μL of 70°C pre-warmed water instead of solution C6. The concentration and purity of genomic DNA was determined using the NanoDrop ONE (Thermo Fisher Scientific Inc., Madison, WI, United States). A quantitative PCR was performed by Microsynth AG (Balgach, Switzerland) to determine the absolute abundance of bacteria as described by Liu et al. (2012). The quantification of methanogenic archaea based on the abundance of the *mcrA* gene was conducted at the Institute for Physiology

and Cell Biology, University of Veterinary Medicine Hannover, Germany, by using primers against the *mcrA* gene as described by Denman et al. (2007). Reaction mixtures (20 μL) contained SensiFAST™ SYBR® No-ROX Kit (BioCat GmbH, Heidelberg, Germany), 500 nM of each primer and 10 ng template. The PCR product amplification was performed on a real-time PCR cycler (CFX96TM; Bio-Rad Laboratories Inc., Hercules, CA, United States) in accordance with the following protocol: 5 min at 95°C; 40 cycles of 15 s at 95°C; 30 s at 60°C; and 30 s at 72°C. In order to define the melt curve a thermal profile with a gradual increase in temperature (0.5°C/10 s) from 72 to 98°C was performed. Water was used as no-template control in each assay. Additionally, pool samples were calibrated with the NanoDrop ONE (Thermo Fisher Scientific Inc.) and were used to determine a standard curve (10^8–10^2 serial dilution). Each series of experiments was carried out twice. The efficiency of the PCR runs ranged between 88.6 and 90.3%.

Next Generation Sequencing

Sequencing of the hypervariable regions V3 and V4 of the 16S RNA gene was performed by Microsynth AG on the Illumina MiSeq using a v2 500 cycles kit using Arch349F and Arch806R as primer pair for archaea (Takai and Horikoshi, 2000), and 341F_ill (5′-CCT ACG GGN GGC WGC AG-3′) and 802R_ill (5′-GAC TAC HVG GGT ATC TAA TCC-3′) for bacteria. Paired-ends reads were filtered through Illumina's chastity filter, de-multiplexed and trimmed of Illumina adaptor residuals by Illumina real time analysis software. A quality check was performed with the software FastQC version 0.11.8. By using the software cutadapt v2.3, the locus specific V3 and V4 primers were cut from the sequencing reads. If trimming was not possible, the paired-end reads were discarded. The software USEARCH version 11.0.667 was used to unite the trimmed forward and reverse reads of each paired-end read. The merged sequences were filtered with the maximum of one "expected error" allowed. Reads containing ambiguous bases were discarded. Reads were denoised and OTUs (100% similarity) were compared to the SILVA v123 database as previously described (Eger et al., 2018). Alpha and beta diversity and the rarefaction were calculated by using the R package phyloseq v1.26. PERMANOVA was performed using the adonis2 function of vegan 2.5-7. The assessment of alpha diversity was conducted by using the Richness (Observed), Simpson and Shannon indices. The beta diversity calculation was performed with the unifrac distance measure. With the package DESeq2 v1.22.2 a differential OTU analysis based on normalized abundance counts was carried out and was considered significant at a log2fold change of at least ±2 and $P < 0.05$. In order to achieve a more specific classification, archaeal OTUs were compared to the JGI Integrated Microbial Genomes and Microbiomes (IGM/M) database[1].

Metabolome Analysis

The samples were analyzed at the Max Rubner-Institut (Department of Safety and Quality of Meat, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Kulmbach, Germany) by two-dimensional gas chromatography coupled with a quadrupole mass spectrometer (GC × GC qMS). The sample set consisted of biological study samples, quality check samples (QCs), and blank samples. The QC samples were prepared by combining an aliquot of 1 mL from each sample. Blank samples were matrix free. For sample preparation an aliquot of 1.5 mL of each sample was added to 20 μL of an internal standard mixture (**Supplementary Table 2**). Samples were centrifuged, filtered (0.2 μm), and freeze-dried. For the subsequent extraction, 200 μL of methanol (hypergrade LC/MS, Merck KGaA, Darmstadt, Germany) was added and shaken using the Bead Ruptor 24 Elite (Omni International Inc., Kennesaw GA, United States) for 30 s at 4 m/s. After a second centrifugation step (15 min at 4°C, 15,000 rpm) 150 μL of the supernatant was transferred into 2 mL glass vials containing a 200 μL glass insert, dried in a vacuum centrifuge (Christ Speedvac RVC 2-18 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and finally stored under protective argon atmosphere at −80°C until analysis. Before measurement, samples were derivatized by methoximation and silylation. Methoximation was performed using a 20 mg/mL solution of *O*-methoxylamine hydrochloride (Sigma-Aldrich, Darmstadt, Germany) in pyridine at 50°C for 1 h. In a second step, 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (Thermo Fisher Scientific GmbH, Dreieich, Germany) was added and samples were shaken at 70°C for 1 h. Derivatized samples were additionally centrifuged and the clear supernatant was transferred to fresh glass vials. The measurements were performed on a Shimadzu GCMS QP2010 instrument (Shimadzu Deutschland GmbH, Duisburg, Germany). Instrumentation and parameter details are provided in **Supplementary Table 3**. Full scan data were acquired in a mass range of 60–550 m/z.

Peaks of the acquired raw data were integrated using the GCMS Postrun Analysis Module within the instrument software GCMSsolution (Version 4.45, Shimadzu Deutschland GmbH). Subsequently, peak quality filtering, peak alignment, signal intensity drift correction, and quality assessment were performed as described by Egert et al. (2015) and Weinert et al. (2015). For visualization of chromatograms and annotation of compounds, we used the NIST 14 library database implemented in GC Image Software (Version 2.7, GC Image, LLC, Lincoln, NE, United States). A series of *n*-alkanes (C7–C30, Sulpelco, Merck KGaA) was used as a retention time standard.

Statistical Analysis

GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, United States) was applied for statistical analysis of biochemical data, qPCR results, and comparisons of relative abundances of microbial taxa. Residuals were tested for normal distribution by the Kolmogorov–Smirnov test. To determine time, treatment, and interaction (time × treatment) effects, a two-way ANOVA for repeated measurements and a *post hoc* Tukey test were performed. Due to missing values a mixed-effects analysis was carried out for pH value, α-diversity of solid-associated archaea and qPCR data of solid-associated bacteria. For qPCR data Tukey's multiple comparison test was subsequently applied. Relative abundances of bacteria and archaea families were tested

[1]https://img.jgi.doe.gov/

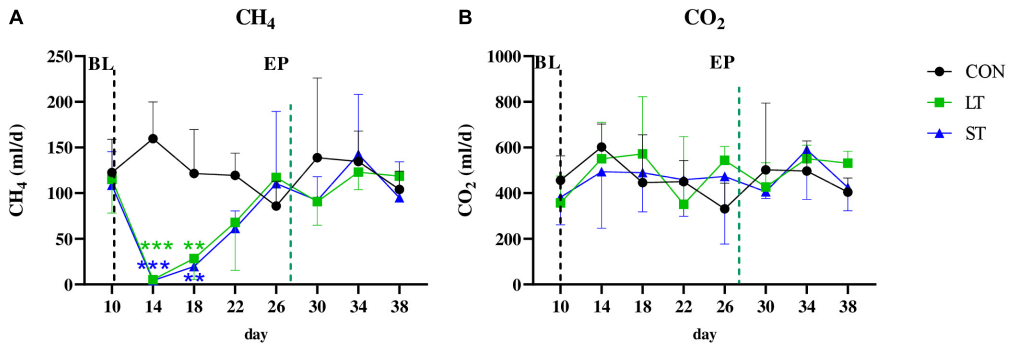


FIGURE 1 | Daily production rates of methane **(A)** and carbon dioxide **(B)** during baseline period (BL, day 10) and experimental period (EP). The black line indicates the beginning of the EP. The green line indicates termination of Mootral supplementation in the short-term (ST) group. CON, control group; LT, long-term group. Significant differences between treatments and the control group in the Tukey *post hoc* test are indicated by ***$P < 0.001$, **$P < 0.01$. Data are presented as means ± SD.

for normal distribution and compared with multiple *t*-tests corrected for multiple comparisons using Benjamini–Hochberg correction with an FDR of 1%. For all statistical test, the levels of significance were defined at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are presented as mean ± SD. As no time-dependent alterations were observed during BL, only data for the last day (day 10) are presented.

For multivariate data analyses of metabolomics data unbiased principle component analysis (PCA) and supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) were applied using the SIMCA-P+ software (version 13, Umetrics, Umeå, Sweden). For univariate statistical testing the Tukey-HSD, which includes a correction for family-wise error rates by multiple testing, was applied using JMP software (13.1.0, SAS Institute Inc., Cary, NC, United States). The heatmap illustration of metabolite data was created in MetaboAnalyst[2] (Chong et al., 2019).

Data Availability

The sequence datasets are available at the NCBI SRA database under the accession number PRJNA716515.

RESULTS

Production of Fermentation Gas

Mootral treatment significantly affected methane production ($P < 0.001$, two-way ANOVA for repeated measurements, **Figure 1A**). The ANOVA also revealed a significant effect of time ($P < 0.001$) and an interaction of time × treatment ($P < 0.01$). In the post-test, a significant reduction in daily methane production compared to the CON group was observed at day 14 ($P < 0.001$, −97%) and day 18 ($P < 0.01$, −83% for ST, −77% for LT) for both groups treated with the feed additive. Subsequently, the production rate increased again and both groups did not differ from the CON group, indicating that this effect was not persistent. In contrast, CO_2 production ranged from 331.43 ± 97.51 to 602.32 ± 87.20 mL in all three groups for the entire duration of the trial and did not differ among the three groups ($P > 0.05$, **Figure 1B**).

Fermentation Parameters

Several changes in fermentation parameters were detected due to the treatment with Mootral. In the LT and ST group, the pH value was marginally lower compared to the CON group at some timepoints (**Table 1**, time: $P < 0.001$, treatment: $P < 0.01$, interaction: $P < 0.01$); however, all measured values were within the physiological range. An increase in NH_3-N was observed in the LT group for the entire EP duration and in the ST within the supplementation time until day 27 (**Table 1**, time: $P < 0.001$, treatment: $P < 0.001$). This was accompanied by a slight increase in total SCFA production that was significant for the ST treatment at day 14 ($P < 0.01$) and for the LT treatment at days 22 and 26 ($P < 0.01$), as compared with the control (**Table 1**). Furthermore, Mootral treatment affected the SCFA composition. It led to a significant reduction in the molar proportion of acetate, in the ST group from day 14 to day 26 ($P < 0.001$) and at day 34 ($P < 0.01$), and in the LT group from day 14 to day 38 ($P < 0.05$) compared to CON group (**Table 2**). Propionate and butyrate proportions were significantly changed over time ($P < 0.001$, **Table 2**) with a slight increase in propionate and a transient elevation of butyrate. Moreover, a significant interaction of time and treatment ($P < 0.01$) was revealed for propionate and butyrate. Butyrate proportion was elevated in the LT group compared to the CON group at days 14, 18, and 26 ($P < 0.05$), and in the ST group at day 18 ($P < 0.01$). There were no significant differences among groups from day 26 onward. Additionally, the application of Mootral increased the valerate ($P < 0.001$) and isovalerate ($P < 0.01$) proportion (**Table 2**). Significant effects were also revealed for the factors time (both $P < 0.001$) and interaction ($P < 0.01$). The molar proportion of valerate was significantly higher in the LT group compared to the CON group ($P < 0.05$) from day 18 to day 38. In the ST group, the valerate proportion was increased compared to CON at days 22, 26, and 34 ($P < 0.05$). Regarding the isovalerate proportion the post-test

[2]https://www.metaboanalyst.ca/home.xhtml

TABLE 1 | Fermentation parameters at day 10 (baseline period) and every fourth day of experimental period (days 14–38).

Parameter	Day	Treatment			Pooled SD	*P*-value		
		CON	LT	ST		Treatment	Time	Interaction
pH	10	6.77	6.80	6.81	0.02	0.007	<0.001	0.009
	14	6.77^{a}	6.66^{b}	6.66^{ab}	0.03			
	18	6.76^{a}	6.70^{a}	6.69^{b}	0.02			
	22	6.81	6.76	6.76	0.01			
	26	6.77	6.72	6.73	0.01			
	30	6.79	6.95	6.8	0.03			
	34	6.79	6.74	6.79	0.02			
	38	6.82	6.78	6.81	0.02			
NH_3-N (mM)	10	6.77	7.07	6.55	0.17	<0.001	<0.001	<0.001
	14	7.12^{a}	9.05^{b}	9.77^{b}	0.42			
	18	6.71^{a}	9.75^{b}	9.97^{b}	0.26			
	22	6.28^{a}	9.17^{b}	9.56^{b}	0.29			
	26	6.66^{a}	9.42^{b}	9.87^{b}	0.40			
	30	6.82^{a}	9.61^{b}	7.52^{a}	0.12			
	34	6.74^{a}	8.62^{b}	7.65^{a}	0.31			
	38	6.63^{a}	8.70^{b}	7.26^{a}	0.57			
SCFA total (mmol/day)	10	27.62	26.8	24.83	1.39	0.185	<0.001	0.005
	14	29.11^{a}	33.59^{ab}	37.05^{b}	3.07			
	18	27.31	33.4	32.98	1.67			
	22	23.76^{a}	30.73^{b}	28.07^{ab}	1.77			
	26	24.87^{a}	31.91^{b}	30.65^{ab}	2.23			
	30	26.13	26.70	23.13	1.65			
	34	26.29	29.37	27.08	1.49			
	38	22.42	27.75	21.60	1.65			

ST, short-term group; LT, long-term group; CON, control group. Data are presented as means and pooled SD. Statistical analysis was performed by repeated measurements two-way ANOVA (NH_3-N and SCFA) or mixed-effects analysis (pH), followed by Tukey post hoc test. Significant differences within a row are indicated by different superscripts.

revealed a significant increase at days 14–22 ($P < 0.001$) in the LT and ST group compared to the CON group.

Effects of Mootral on Abundance of 16S rRNA Genes and Bacterial Community Composition

The abundance of the bacterial 16S rRNA gene was not altered by Mootral treatment (data not shown). By sequencing the bacterial 16S gene V3 and V4 regions, 1,031 OTUs were detected in LAM samples, 1,026 of these were bacterial OTUs. Of the 749 observed solid-associated OTUs, 744 were bacterial OTUs. Alpha-diversity measures did not differ among treatment groups (**Supplementary Figure 1**). In total 18 phyla were detected, the most abundant being Bacteroidetes (LAM: 40.24 ± 6%; SAM: 25.2 ± 3.5%) and Firmicutes (LAM: 31.55 ± 3.35%; SAM: 57.03 ± 6.45%). A total of 43 bacterial families were detected in the LAM samples and 39 families in the SAM samples. Only the 20 most abundant thereof will be discussed below (**Supplementary Figure 2**). Few differences among treatment groups were detected by multiple comparisons (**Table 3**). A lower abundance of the families Christensenellaceae and Succinivibrionaceae was observed in the LAM samples at day 17 in the LT group compared to the CON group (CON vs. LT, $P < 0.001$). At days 31 and 38 of the experiment, the relative abundance of Prevotellaceae was higher in the LT group compared to the CON group (CON vs. LT, $P < 0.001$). In the SAM samples, only the family Victivallaceae was less abundant in the LT group at day 17 (CON vs. LT, $P < 0.001$). Lactobacillaceae were numerically more abundant in the ST group at days 17 and 24, and in the LT group at days 31 and 38, however, due to a high variance within the treatments this effect was not significant.

Effects of Mootral on *mcrA* Copy Numbers and on the Archaeal Community

In contrast, *mcrA* copy numbers (**Figure 2**) were affected by time (SAM: $P < 0.001$; LAM: $P < 0.01$), treatment (both $P < 0.001$) and an interaction of both factors (LAM: $P < 0.001$; SAM: $P < 0.01$). Mootral treatment led to a significant decrease in *mcrA* gene copy number at day 17 ($P < 0.01$) in both LAM and SAM (**Figures 2A,B**). In LAM (**Figure 2A**), a reduced copy number was also observed at days 24 and 31 ($P < 0.01$). In SAM, *mcrA* gene copy numbers were significantly lower in LT group compared to both the ST and CON groups, at day 38 ($P < 0.05$, **Figure 2B**).

TABLE 2 | Molar proportions of acetate, propionate, butyrate, valerate, isovalerate at day 10 (baseline period), and every fourth day of experimental period (days 14–38).

Parameter	Day	Treatment			Pooled SD	*P*-value		
		CON	LT	ST		Treatment	Time	Interaction
Acetate (%)	10	58.23	57.69	59.77	0.52	<0.001	<0.001	<0.001
	14	58.62^{a}	48.66^{b}	47.98^{b}	0.79			
	18	57.59^{a}	48.48^{b}	49.40^{b}	0.72			
	22	59.87^{a}	51.21^{b}	50.51^{b}	0.88			
	26	59.74^{a}	51.94^{b}	54.50^{b}	1.18			
	30	57.84^{a}	54.89^{b}	56.98^{ab}	0.42			
	34	59.34^{a}	54.5^{b}	56.08^{b}	0.80			
	38	58.32^{a}	53.48^{b}	57.15^{a}	1.33			
Propionate (%)	10	31.43	31.76	31.33	0.47	0.095	<0.001	<0.001
	14	32.19^{a}	32.85^{a}	35.63^{b}	0.91			
	18	34.09	34.02	33.92	0.71			
	22	32.12^{a}	33.41^{ab}	34.97^{b}	1.14			
	26	33.03	35.43	35.14	1.03			
	30	34.35	34.94	34.35	0.85			
	34	32.05^{a}	36.27^{b}	34.55^{ab}	0.65			
	38	32.9^{a}	37.64^{b}	34.24^{a}	0.76			
Butyrate (%)	10	7.96	7.12	6.26	0.74	0.091	<0.001	0.005
	14	7.84^{a}	10.88^{b}	9.59^{ab}	0.86			
	18	7.99^{a}	11.14^{b}	11.20^{b}	0.64			
	22	7.80^{a}	9.89^{ab}	10.19^{b}	0.61			
	26	7.02^{a}	9.59^{b}	8.59^{ab}	0.68			
	30	7.43	8.99	7.70	0.63			
	34	7.76	8.59	7.76	0.47			
	38	8.15	8.18	7.06	0.78			
Valerate (%)	10	0	0	0	0	<0.001	<0.001	0.004
	14	0	0.15	0	0.08			
	18	0^{a}	0.80^{b}	0.21^{a}	0.12			
	22	0^{a}	0.83^{b}	0.77^{b}	0.26			
	26	0^{a}	1.32^{b}	0.78^{b}	0.26			
	30	0^{a}	0.96^{b}	0.22^{a}	0.15			
	34	0^{a}	0.65^{b}	0.71^{b}	0.20			
	38	0^{a}	0.71^{b}	0.54^{ab}	0.24			
Isovalerate (%)	10	2.37	3.43	2.65	0.45	0.004	<0.001	<0.001
	14	1.35^{a}	7.46^{b}	6.8^{b}	0.45			
	18	0.32^{a}	5.56^{b}	5.27^{b}	0.82			
	22	0.21^{a}	4.66^{b}	3.56^{b}	0.54			
	26	0.20	1.72	0.98	0.32			
	30	0.38	0.22	0.74	0.60			
	34	0.85	0	0.91	0.52			
	38	0.62	0	1.02	0.45			

ST, short-term group; LT, long-term group; CON, control group. Data are presented as means and pooled SD. Significant differences within a row are indicated by different superscripts.

In the liquid samples, archaea sequencing resulted in 2,630,324 total reads, ranging from 270 to 810,506 with a mean of 37,046. Within these, 71 OTUs were identified, 42 of them belonged to the domain *Archaea* and accounted for 92.9% of the reads. In the solid samples, a total of 2,279,860 reads were obtained, the range was from 2,147 to 105,637 with a mean of 38,642 among all samples. In solid samples, 97.0% of the sequencing reads belonged to *Archaea* and 40 of the detected 54 OTUs were archaeal OTUs. All archaeal OTUs belonged to the phylum Euryarchaeota. The three classes Methanomicrobia, Thermoplasmata, and Methanobacteria were identified.

Mootral application also changed archaeal alpha-diversity measures. In both, LAM and SAM samples, the number of observed OTUs decreased up until day 31 in ST group and up until day 38 in the LT group ($P < 0.05$, **Figures 3A,B**). Moreover the Simpson Diversity index decreased for the LT and ST groups at days 17 and 24 in SAM ($P < 0.001$, **Figure 3B**), for LT at days 17, 24, and 38 in LAM ($P < 0.05$, **Figure 3A**), as well as for ST

TABLE 3 | Differentially abundant bacterial families by treatment group in liquid- and solid-associated microbiota (LAM and SAM, respectively).

	Day	Treatment			Pooled SD
		CON	LT	ST	
LAM					
Christensenellaceae	10	0.37	0.30	0.43	0.14
	17	0.64[a]	0.30[b]	0.31[b]	0.06
	24	0.73	0.57	0.50	0.10
	31	1.05	0.62	0.70	0.29
	38	1.10	0.82	0.75	0.29
Prevotellaceae	10	16.4	15.1	15.9	3.47
	17	13.5	17.4	20.5	3.01
	24	13.8	17.2	17.8	2.44
	31	20.3[a]	31.7[b]	18.2[ab]	1.76
	38	15.1[a]	24.2[b]	15.0[a]	1.76
Succinivibrionaceae	10	13.71	11.44	12.34	4.31
	17	12.32[a]	4.01[ab]	3.02[b]	2.30
	24	11.53	8.45	8.76	5.37
	31	8.69	1.64	4.50	2.35
	38	8.69	1.64	4.50	2.35
SAM					
Victivallaceae	10	0.59	0.59	0.69	0.14
	17	0.58[a]	0.16[b]	0.17[ab]	0.09
	24	0.84	0.39	0.33	0.16
	31	0.86	0.17	0.53	0.25
	38	0.86	0.23	0.82	0.15

ST, short-term group; LT, long-term group; CON, control group. Data are mean relative abundances (%) and pooled SD. Families that differed significantly by multiple t-test ($P < 0.05$, Benjamini–Hochberg correction, FDR = 1%). Different superscript indicate significant differences within a row.

at day 24 in LAM ($P < 0.05$). The Shannon index was affected at days 14, 24, and 38 in SAM ($P < 0.01$, **Figure 3B**), but only at days 17 and 38 in LAM ($P < 0.05$, **Figure 3A**). In the beta-diversity plots of archaea, all 12 communities clustered close together in both liquid and solid samples at day 10 (**Figure 4**). However, at day 17 the LT and ST communities were clearly separated from the day 17 CON samples as well as from the day 10 samples. At day 24 and day 31, the LT and ST samples were widespread in the plot, whereas the two groups treated with Mootral moved closer to CON group at day 38 but remained more variable than the samples of the CON group. PERMANOVA revealed a clear effect of time and interaction for both phases ($P < 0.001$), as well as a treatment effect for LAM ($P < 0.05$) and SAM ($P < 0.01$).

At day 10, the Methanomicrobiaceae was the family with the highest abundance in all three treatment groups (LAM: 61.7 ± 5.1%; SAM: 90.9 ± 1.9%, **Table 4**). Additionally, the families Thermoplasmatales incertae sedis (LAM: 35.9 ± 4.8%; SAM: 8.7 ± 1.7%), Methanobacteriaceae (LAM: 2.3 ± 0.5%; SAM: 0.6 ± 0.3%), and Methanosarcinaceae (LAM: 0.008 ± 0.08%; SAM: 0.001 ± 0.001%) were detected. After treatment with Mootral Thermoplasmatales incertae sedis increased in the LT group and ST group. In contrast, a significant reduction in the proportion of Methanomicrobiaceae was observed (**Table 4**).

The analysis of differentially abundant OTUs revealed significant alterations in liquid (**Figure 5A**) and solid (**Figure 5B**) archaeal communities among treatment groups and at different time points. None of the OTUs differed among the groups at day 10, before treatments were applied. Mootral application diminished the abundance of members of the genera *Methanomicrobium* (*Methanomicrobium mobile* DSM 1539), *Methanobrevibacter* (*Methanobrevibacter thaueri* DSM 11995, *Methanobrevibacter* sp. YE 315), and the family Thermoplasmatales incertae sedis (Unclassified archaeon ISO4-G1, *Candidatus Methanoplasma termitum* MpT1 and *Methanomassiliicoccales* archaeon RumEn M2) at days 17 and 24 in SAM and LAM (**Figure 5**), while it increased the relative abundance of several OTUs from Thermoplasmatales incertae sedis. In the LT group, *Methanobrevibacter olleyae* YLM1 was enriched at several time-points in both phases. While LT and ST samples were similar at day 24 for both phases, at day 31, *M. thaueri* DSM 11995 and nine OTUs from Thermoplasmatales incertae sedis increased again in ST SAM samples. In LAM, 12 OTUs displayed consistent differences among the CON and LT groups throughout the entire treatment period. Five of these 12

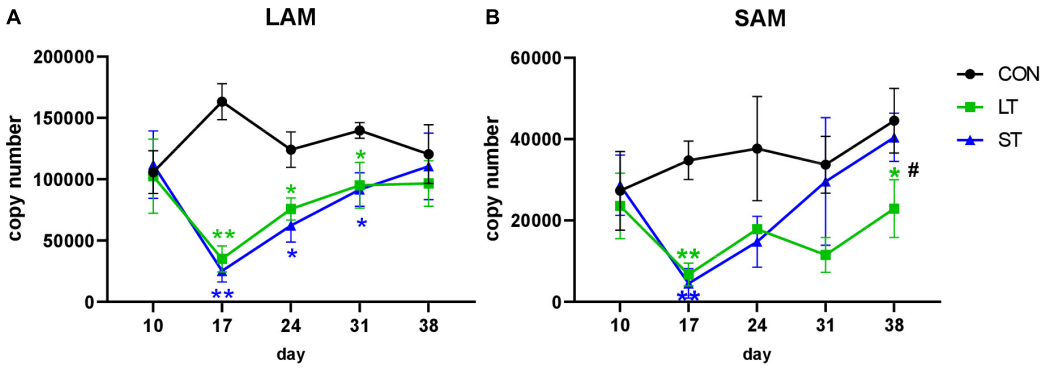


FIGURE 2 | Copy numbers of the *mcrA* gene in LAM **(A)** and SAM **(B)** samples. ST, short-term group; LT, long-term group; CON, control group. Significant treatment effects of LT or ST, compared to CON in Tukey *post hoc* test are indicated by $^{**}P < 0.01$, $^{*}P < 0.05$. Significant treatment effects between ST and LT are indicated by $^{\#}P < 0.05$. Data are presented as means ± SD.

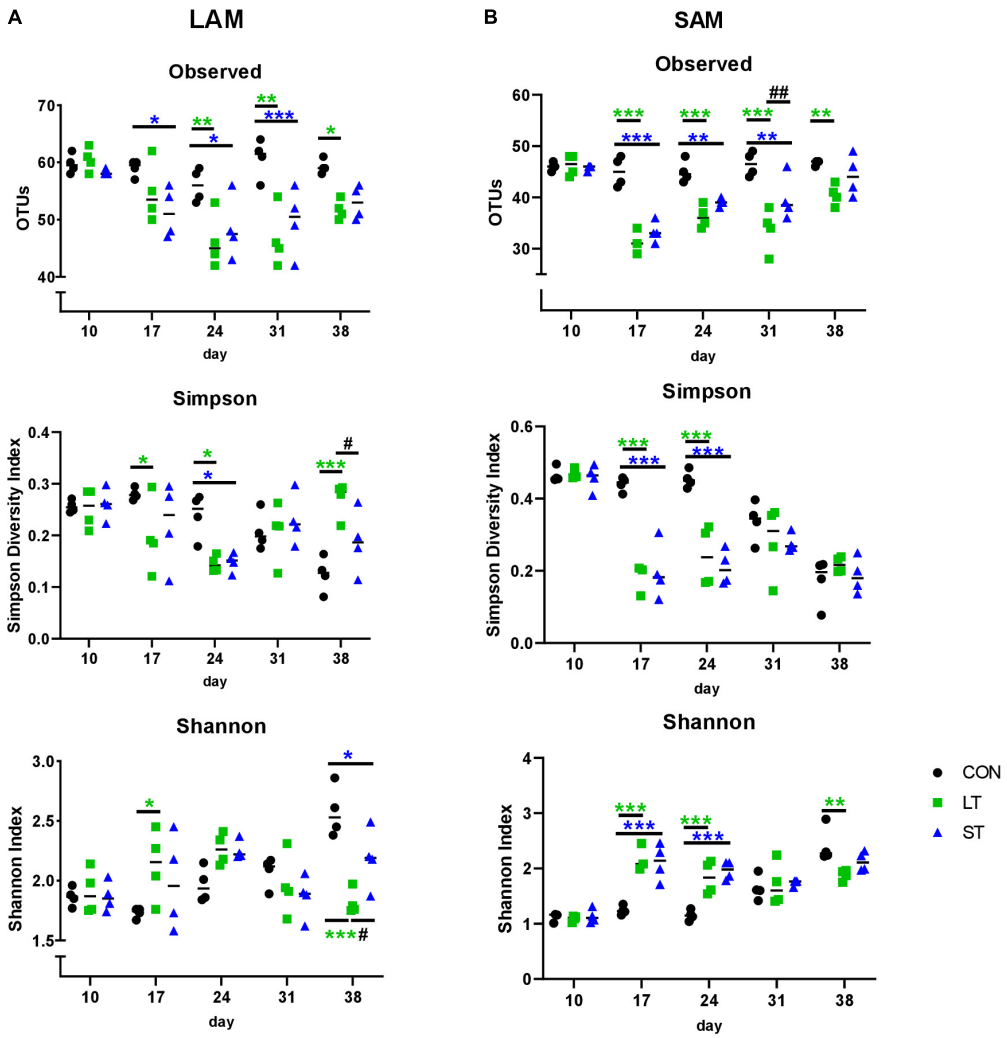


FIGURE 3 | Number of observed operational taxonomic units (OTUs), Simpson's Diversity Index, and Shannon's Index in LAM **(A)** and SAM **(B)** of *Archaea* by treatment groups (n = 4). Individual data and medians are presented. ST, short-term group; LT, long-term group; CON, control group. The treatment effects of the Tukey *post hoc* test are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (CON vs. LT or CON vs. ST); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (LT vs. ST). Time effects are not shown.

OTUs also differed between the ST and CON group up until day 31, but not at day 38.

Effects of Mootral on the Rumen Metabolome

Metabolite profiles of the samples were compared with focus being placed on differences among treatments (comparison among BL, and CON, ST, and LT groups at day 17, and time-dependent shifts in the ST and LT groups). Addition of Mootral lead to a clear separation of ST and LT samples at day 17, from BL samples at day 10 in PCA and OPLS-DA (**Supplementary Figure 3A**, **Figure 6A**, R^2 = 0.97 and Q^2 = 0.88). For the LT group, a clear separation of day 10 CON and LT samples vs. LT samples from day 17 to day 38 (Mootral addition) was confirmed (R^2 = 0.98, Q^2 = 0.94, **Figure 6B**). In contrast, in the ST group, a model with three groups was applied, comparing "before treatment" samples from day 10 (BL, CON10, and ST10), with Mootral treatment (ST17 and ST24) and "after treatment" samples (ST31 and ST38). Both PCA and OPLS-DA (R^2 = 0.91, Q^2 = 0.77, **Figure 6C** and **Supplementary Figure 3C**) indicated clear differentiation between pre-treated and treated samples. Samples collected after treatment were not clearly separated from day 10 samples in PCA (**Supplementary Figure 3C**) and in the OPLS-DA they formed a third cluster (**Figure 6C**). To determine compounds which were responsible for the observed clustering in the OPLS-DA model, variable importance in projection (VIP) score was calculated. A threshold of VIP >1 was chosen, as this indicated an above average contribution of this compound for the model. For the LT group, 194 compounds were significantly different ($P < 0.05$) and exhibited a VIP score >1; for ST group, 222 compounds differed. About 38% (106) of these compounds could be identified. The 20 identified compounds with the highest VIP scores for both comparisons (ST

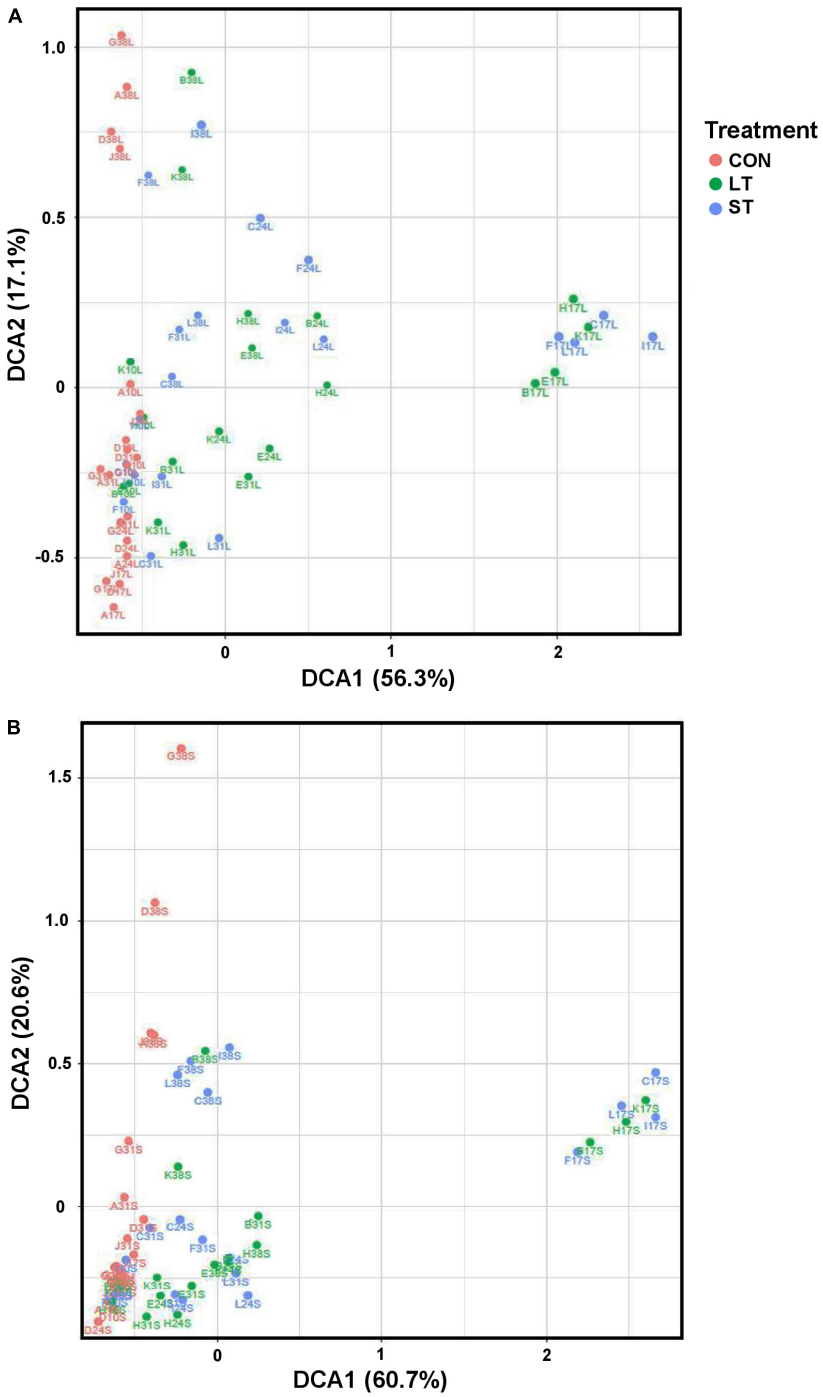


FIGURE 4 | The beta-diversity of *Archaea* is indicated by Detrending Correspondence Analysis (DCA) based on the UniFrac distances. Treatment groups are distinguished by color: CON, red; LT, green; ST, blue. The labels represent the day (10, 17, 24, 31, and 38) and the name of the fermentation vessel (A–L). Panel **(A)** shows the LAM, and **(B)** shows the SAM archaeal communities.

and LT) were selected (**Supplementary Table 4**). Four clusters were identified (**Supplementary Figure 4**). The first cluster was more highly concentrated when the samples were treated with Mootral compared to BL (day 10), ST31 and ST38 samples and could therefore contain compounds derived from Mootral itself or degradation products of Mootral. The second cluster was enriched in BL samples and was persistently reduced by Mootral application. It consisted of propylene glycol, two branched-chain SCFA, 2-hydroxycaproic acid, and two monosaccharides. A third group consisting mainly of plant-derived compounds showed a

TABLE 4 | Differentially abundant archaeal families by treatment group in liquid- and solid-associated microbiota (LAM and SAM, respectively).

	Day	Treatment			Pooled SD
		CON	LT	ST	
LAM					
Methanobacteriaceae	10	2.18	2.42	2.36	0.52
	17	1.51	3.25	1.63	1.32
	24	1.43[a]	8.80[ab]	14.29[b]	4.02
	31	1.87	3.39	1.05	1.77
	38	7.99	7.00	2.74	3.00
Methanomicrobiaceae	10	60.42	61.04	63.74	5.60
	17	66.68[a]	8.24[b]	4.94[b]	3.22
	24	61.36[a]	34.54[ab]	16.36[b]	9.88
	31	54.57	48.16	55.82	15.44
	38	16.86	14.60	27.87	9.80
Methanosarcinaceae	10	0.01	0.01	0.01	0.01
	17	0.01[a]	0.07[b]	0.10[ab]	0.07
	24	0.16	0.03	3.97E−03	0.10
	31	0.35	2.20E−03	2.46E−03	0.19
	38	1.39	1.66E−03	0.06	0.59
Thermoplasmatales incertae sedis	10	37.39	36.53	33.89	5.24
	17	31.80[a]	88.44[b]	93.33[b]	3.99
	24	37.06[a]	56.63[ab]	69.35[b]	11.79
	31	43.21	48.44	43.13	15.08
	38	73.76	78.40	69.33	9.63
SAM					
Methanobacteriaceae	10	0.74	0.52	0.63	0.28
	17	0.70[a]	2.24[b]	3.22	1.78
	24	1.64	7.55	10.30	4.06
	31	1.28	6.24	1.75	2.73
	38	6.07	9.21	2.03	4.65
Methanomicrobiaceae	10	90.99	91.34	90.26	2.61
	17	90.02[a]	8.42[b]	10.86[b]	4.43
	24	90.24[a]	62.86[ab]	54.98[b]	11.93
	31	77.91	73.19	72.83	9.59
	38	37.78	38.83	41.96	15.51
Methanosarcinaceae	10	2.13E−03	2.79E−04	1.52E−03	8.45E−04
	17	2.35E−03	0.06	0.05	0.09
	24	0.01	0	2.10E−03	0.01
	31	0.06	0	3.42E−03	0.04
	38	0.25	4.69E−03	0.01	0.09
Thermoplasmatales incertae sedis	10	8.27	8.14	9.11	1.92
	17	9.28[a]	89.28[b]	85.87[b]	6.13
	24	8.11[a]	29.59[ab]	34.72[b]	9.59
	31	20.76	20.57	25.42	7.17
	38	55.90	51.96	56.00	12.49

ST, short-term group; LT, long-term group; CON, control group. Data are mean relative abundances (%) and pooled SD. Families that differed significantly by multiple t-test ($P < 0.05$, Benjamini–Hochberg correction, FDR = 1%). Different superscript indicate significant differences within a row.

similar pattern; however, a slight recovery of these compounds appeared in ST31 and ST38. The fourth cluster exhibited a suppression during Mootral treatment, but clearly recovered after terminating the supplementation in ST group (ST31 and ST38). This cluster consisted of several fatty acids, phenoxyethanol, 5-hydroxytryptophan, 4-isopropylphenol, and an indole derivate.

DISCUSSION

The strong potential of Mootral, a product consisting of garlic and citrus extracts, to reduce methane emissions has been previously revealed *in vitro* and *in vivo* (Eger et al., 2018; Roque et al., 2019; Vrancken et al., 2019). The aim of the present study was to examine the long-term effects of Mootral on ruminal fermentation parameters, archaeal, and bacterial community structure as well as on the rumen metabolome in a long-term *in vitro* trial.

Mootral Dosage and Potential Effects of Mootral Degradation

Due to the use of natural compounds in this product, the concentrations of active substances varies. As a reference, the concentration of allicin was determined. The dose applied here resulted in an allicin concentration of approximately 20 mg/L in the fermentation vessels, which is comparable to doses applied in previous *in vitro* experiments (Busquet et al., 2005; Chaves et al., 2008; Eger et al., 2018) and far lower than the likely toxicity for rumen bacteria (300–3,000 mg/L) (Busquet et al., 2005). In general, the dosage applied in the RUSITEC system needs to be higher compared to *in vivo* dosing due to the higher liquid to solid ratio (Carro et al., 2009), however, also *in vivo* quite high dosages (2 g allicin/65 kg body weight) have been used (Ma et al., 2016).

Due to a low allicin concentration in the Mootral powder used in this study, a relatively large amount had to be used. As Mootral is a natural product with various active compounds, an inactive placebo for the control group was not available. Therefore, it cannot be excluded, that some of the effects observed in this study are due to nutrient utilization of the product. Metabolites, whose concentrations were increased at the same timepoints when Mootral was applied are probably derived from the product itself. As the crude protein content of the powder is about 22% (**Supplementary Table 1**), elevated NH_3-N levels are presumably related to the higher protein supply. Mootral application also increased the concentration of benzeneprenoic acid (hydrocinnamic acid) and other aromatic compounds. Hydrocinnamic acid is produced by rumen bacteria from cinnamic acid (Martin, 1982) which can be found in various plants. Bitter oranges not only contain isoflavonoids such as naringin, but, e.g., also phenolic acids, hydroxybenzoic acid, and cinnamic-acid (Jabri Karoui and Marzouk, 2013), therefore it is most likely that the increase in the concentrations of aromatic compounds is based on supply by Mootral application. The concentration of pyridoxine, a vitamin B6, was also increased during Mootral application. In contrast to other B vitamins its rumen balance is often negative (Castagnino et al., 2016; Beaudet et al., 2020) and an increase in its concentration may be beneficial for the animal.

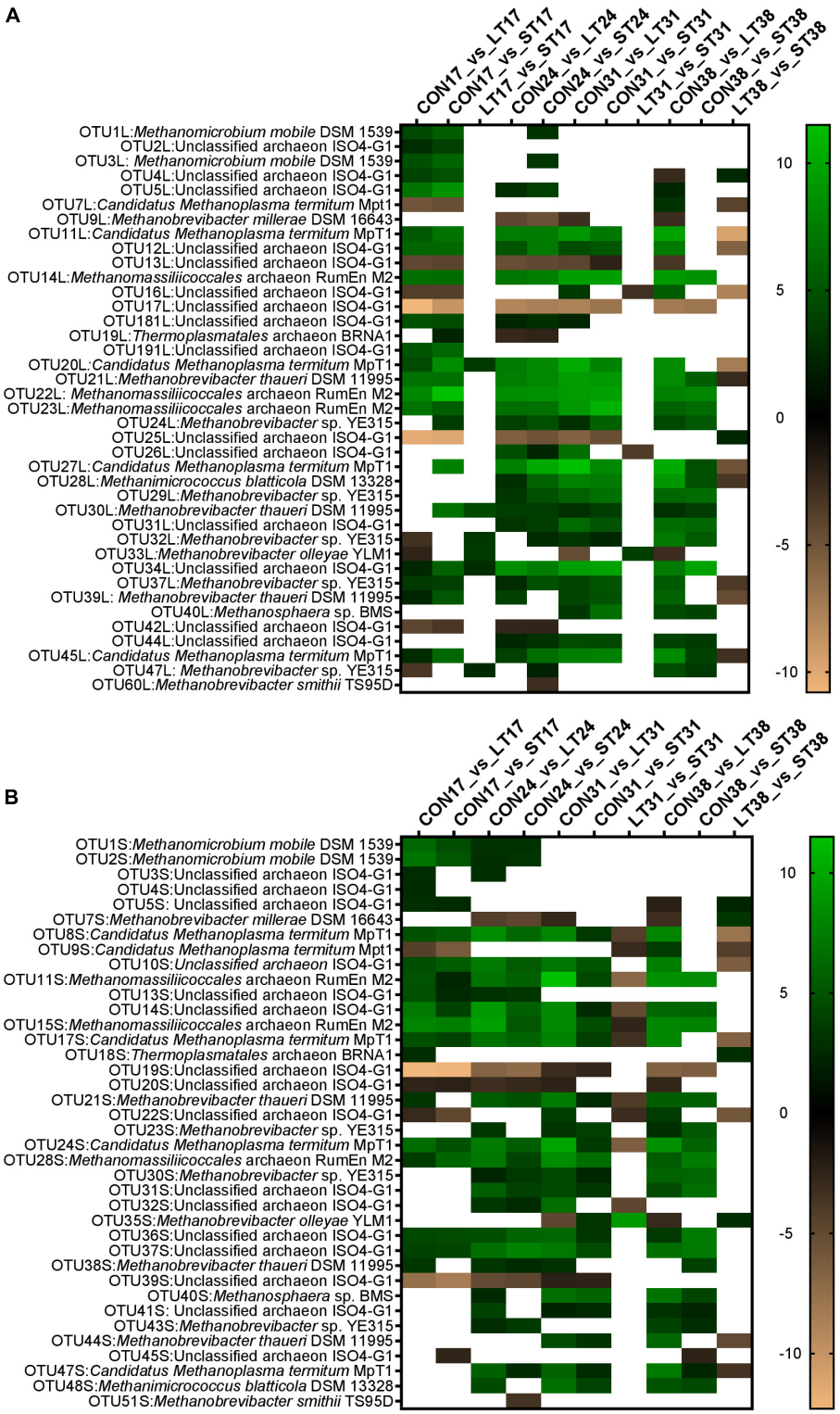


FIGURE 5 | Heatmaps of significantly differentially abundant ($P < 0.05$, differential OTU analysis, DESeq2) archaeal operational taxonomic units (OTUs) in liquid-associated **(A)** and solid-associated **(B)** microbiota based on log2fold changes, which is indicated by the brown to green color scale. A threshold of ±2 was applied, and white indicate no significant differences. The heatmap displays the significant differences at days 17, 24, 31, and 38 between the three treatment groups (CON, LT, and ST). No significant differences were detected at day 10 and at day 24 between LT and ST group.

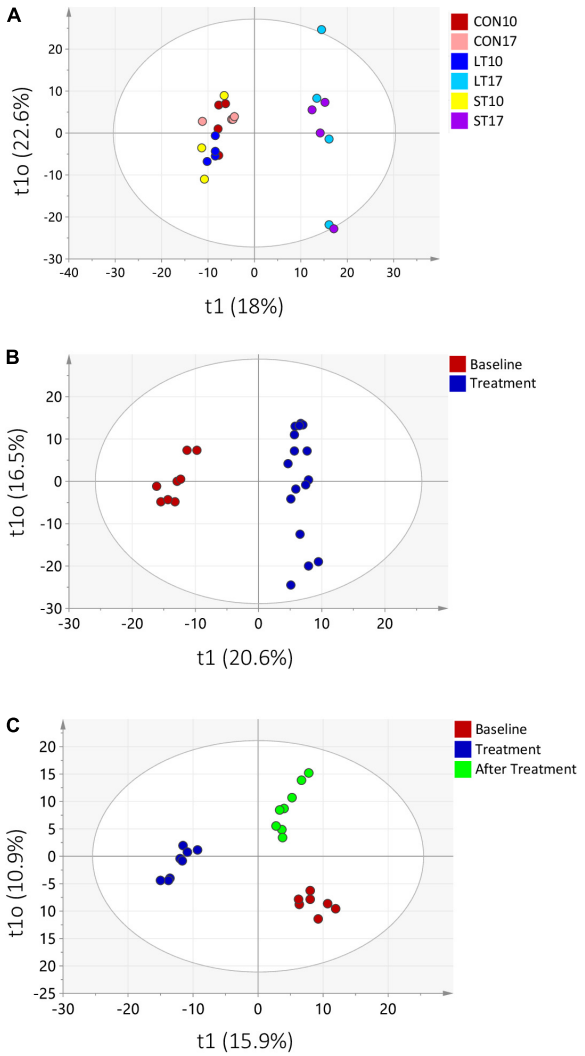


FIGURE 6 | Orthogonal projections to latent structures discriminant analysis plot of the metabolomics analysis **(A)** for all three treatment groups at days 10 and 17, **(B)** the long term (LT) group at days 17–38 (Treatment) vs. LT and CON at day 10 (BL) and **(C)** the short term (ST) group at days 17 and 24 (Treatment), at days 31 and 38 (After Treatment) vs. CON and ST at day 10 (BL).

Effects of Mootral on the Fermentation Pattern, the Bacterial Community Composition and Metabolites in the Rumen Simulation Technique System

In the present trial, the supplementation of Mootral induced an increase in the total SCFA production in the RUSITEC-system. This was due to an increased production of propionate, butyrate, valerate, and isovalerate. However, this effect was transient for total SCFA, butyrate, and isovalerate even in the LT group. Effects on pH, SCFA production and SCFA molar proportions resembled those observed by Eger et al. (2018) and Ahmed et al. (2021). Previous studies with garlic compounds and flavonoids are variable, but they often also report an increase in total SCFA accompanied by a lower acetate, and higher propionate or butyrate proportions (Busquet et al., 2005; Balcells et al., 2012; Patra and Yu, 2015; Ma et al., 2016). Metabolomics revealed that Mootral supplementation suppressed the production of several fatty acids (short, medium, and long chained), phenol derivates, and plant-derived compounds. Changes in carbohydrate metabolism were indicated by accumulation of a disaccharide and suppression of several monosaccharides, levoglucosan, and tyrosol.

The abundance of bacteria was not affected by Mootral application, which is in agreement with previous reports where garlic oil (Patra and Yu, 2015) or the flavonoid naringin (Oskoueian et al., 2013) were investigated *in vitro* using rumen fluid from dairy cattle (roughage: concentrate 43:57) or male cattle (roughage: concentrate 60:40), respectively. In contrast, *in vitro* treatment with garlic powder resulted in a dose-dependent reduction in the number of bacteria with a straw and concentrate diet (Wanapat et al., 2008). Treatment with garlic oil led to a reduction in the total number of the cellulolytic bacteria *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus* (Patra and Yu, 2012). Cellulolytic bacteria mainly produce acetate (Dijkstra et al., 2012), which was lower in proportions in Mootral-treated groups in the present study and is a source of hydrogen for methane production. However, only the families Succinivibrionaceae, Prevotellaceae, Christensenellaceae, and Victivallaceae were affected at single time-points by Mootral treatment in our study. Ahmed et al. (2021) also observed a treatment related effect on the family Prevotellaceae, as the relative abundance was significantly increased with a treatment of 20% Mootral. Nevertheless, they only observed additional effects for Veillonellaceae. Altogether, the impact of Mootral on the bacterial community appears to be small and was only visible at single time-points. However, most rumen bacteria have a certain metabolic flexibility to degrade various substrates (Comtet-Marre et al., 2017) and therefore may result in differing fermentation profiles. Thus, transient shifts in fermentation products may occur without major alterations in the bacterial community composition. As the shifts in methane production were not in parallel to shifts in fermentation products, the effects of Mootral on methane production are not simply linked to the changing bacterial fermentation pattern.

Effects of Mootral on Methane Production and the Archaeal Community

Regarding their ability to reduce methane production, both garlic and flavonoids have been described as very effective (Busquet et al., 2005; Patra et al., 2011; Soliva et al., 2011; Ramos-Morales et al., 2018). Compared to this study, a similar effect on methane production was observed in the previous *in vitro* study by Eger et al. (2018), in which a 95% reduction in methane production was achieved during an 8 day supplementation period. The reduction in *in vivo* methane production in beef cattle or dairy cattle by Mootral was considerably less, ranging from 21 to 38% (Roque et al., 2019; Vrancken et al., 2019). In the *in vitro* study by Ahmed et al. (2021), a reduction in the percentage of methane in the fermentation gas by 54% could be achieved in a batch

culture system when 20% Mootral was administered using rumen fluid from sheep fed grass and concentrate (50:50). However, the total production rate in the supplemented group did not differ from the control group (Ahmed et al., 2021). A discrepancy between the decrease in methane production achieved *in vivo* compared to *in vitro* was also observed by Fievez et al. (2003) when comparing the effects of different fish oil types in a batch culture incubation with an *in vivo* application. An inhibition of rumen methanogenesis was observed in both approaches, but the effect was one-fifth *in vivo*. It appears that the methane-reducing effect of Mootral is reproducible in different animals and with different diets and *in vitro* systems. However, as our long-term study indicates, this effect appears to be transient, at least *in vitro*, as methane production was not significantly reduced after 12 days of supplementation. Long-term responses to different methane reducing compounds differ. Long-term application may result in a better and more persistent decrease in methane production (Belanche et al., 2020) or reveal a transient effect (Guan et al., 2006). Patra et al. (2011) did not observe an effect of garlic bulb on methane emissions of sheep after 21 days of feeding. Schilde et al. (2021) revealed that 3-NOP combined with a high concentrate ration *in vivo* persistently reduced methane production, while when a high forage ration was fed, methane production recovered after 3–4 months. In contrast to the short suppression of methane production in our study, the two *in vivo* studies using Mootral (Roque et al., 2019; Vrancken et al., 2019) detected effects over a 12-week supplementation period; however, these two studies used higher concentrate proportions compared to our *in vitro* study. Therefore, further studies on the time-course of methane emissions and ruminal parameters in response to Mootral supplementation in combination with different feeding strategies *in vivo* and *in vitro* are needed to reveal whether the transient effects observed here are linked to the *in vitro* system or to the dietary strategy and whether they transfer to the *in vivo* situation.

Busquet et al. (2005) discussed that the mechanism of methane inhibition by organosulfur components in garlic could be based on an inhibition of the 3-hydroxy-3-methyl-glutaryl coenzyme A, which is needed to produce membrane lipids only found in archaea, however, they could not clearly prove or reject this hypothesis. However, this agrees with previous reports on allicin inhibiting enzymes by reacting with their thiol group (Ankri and Mirelman, 1999). Alterations to the liquid-associated archaeal communities have already been reported by Eger et al. (2018) where a similar diet was used in the RUSITEC system. In the present study solid-associated archaea were additionally investigated. The qPCR targeting the *mcrA* gene revealed a significant reduction in methanogenic archaea, which is in line with previous studies on garlic or flavonoids (Oskoueian et al., 2013; Patra and Yu, 2015; Ma et al., 2016). However, this effect was not consistent over time indicating an adaption of these microorganisms to the treatment. In addition, the application of Mootral resulted in a reduction in the relative abundance of Methanomicrobiaceae and an increase in *Methanomassiliicoccales*. This is in line with the finding of Ahmed et al. (2021), who observed a dose-dependent increase in the *Methanomassiliicoccaceae*. Interestingly, in the previous studies by Eger et al. (2018), *Methanobrevibacteriaceae* dominated before treatment and were reduced by Mootral application; however, this resulted in a comparable decrease in methane reduction. Nonetheless, as with methane production, the Mootral-induced structural changes in the microbial community composition were transient, indicating an adaptation to the product. The ability of the microbial ecosystem to adapt to dietary strategies to reduce methanogenesis is well known (Beauchemin, 2009). This effect might not have occurred in the *in vitro* study by Ahmed et al. (2021), as a short-term batch culture was used, whereas in our trial, the supplementation lasted for up to 28 days. Saro et al. (2018) reported that feeding growing lambs with a combination of garlic and linseed oil for 4 weeks did not result in persisting methane reduction or changes of the archaeal population.

Although the composition of the ST and LT archaeal communities was similar to the CON group by the end of the experiment, the log2fold change revealed an increased abundance of some OTUs in the treated groups. This was particularly applicable to OTUs belonging to *Methanomassiliicoccales*. When this analysis was performed these OTUs were still classified as Thermoplasmatales incertae sedis in the database, however, nowadays their classification has to be updated. Most *Methanomassiliicoccales* OTUs were identified as ISO4-G1 or RumEn M2, which are now classified to belong to the host-associated clade *Methanomethylophilaceae* (Söllinger et al., 2016; Cozannet et al., 2020). It has been demonstrated that they produce methane in a H_2-dependent methylotrophic way and that their reduction is accompanied by a reduced methane production (Borrel et al., 2013; Poulsen et al., 2013). In contrast, in the present study, an increase in the proportion of *Methanomassiliicoccales* and decrease in the abundance of Methanomicrobiaceae accompanied the reduction in methane production. However, differing results for several OTUs that appeared to be closely related indicate that *Methanomassiliicoccales* have a certain metabolic flexibility. Methyl-reducing archaea such as *Methanomassiliicoccus luminyensis* have been demonstrated to be able to use lower H_2 pressures compared to "classic hydrogenotrophic" (growing on $CO_2 + H_2$) such as *Methanobrevibacter* strains (Feldewert et al., 2020). One potential mode of action of Mootral could therefore be to provide an alternative sink for H_2, thereby favoring obligate methylotrophic archaea. Calculated based on SCFA, hydrogen production was not reduced by Mootral addition, raising the question of the fate of the hydrogen (Eger et al., 2018). Moreover, a higher availability of methyl-compounds through Mootral addition may favor these archaea. The results of Eger et al. (2018) implied that the effect of Mootral is primarily mediated *via* a reduction in *Methanobrevibacter* spp. of the SGMT clade. In the present study, we also revealed a suppression of *M. thaueri* and an additional suppression of *Methanomicrobium mobile*, while an enrichment of *M. olleyae* which belong to the lower-methane production RO-clade (Danielsson et al., 2012) was observed in the LT group. Some of these shifts persisted, while the total archaeal family composition resembled the CON group at the end of the experiment. To our knowledge, there are currently no data on microbial community composition available *in vivo*, and

it has to be investigated whether these shifts are mirrored and persistent *in vivo*.

CONCLUSION

By using a long-term *in vitro*-system, we demonstrated that the methane-reducing effect of Mootral is mainly based on direct effects on the liquid and solid-associated archaea. Although some changes in fermentation products were observed, the bacterial community was barely affected. As most of the effects were transient even with continued supplementation, it remains to be studied, whether this effect also occurs *in vivo* and under different feeding strategies or if it is related to the *in vitro* system in order to find a practicable application scheme for the on-farm use of Mootral.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA716515.

ETHICS STATEMENT

The animal study was reviewed and approved by the Lower Saxony State Office for Consumer Protection and Food Safety Oldenburg, Germany AZ 33.19-42502-05-13A373.

AUTHOR CONTRIBUTIONS

GB and MB designed the experiments. JB, MB, and MP were responsible for data acquisition and statistical analysis. BE processed the metabolomics data. JB and MB wrote the manuscript. All authors approved the final version of the manuscript.

FUNDING

This study was funded by Mootral SA.

ACKNOWLEDGMENTS

The authors would like to thank Marion Burmester and Kerstin Kiri for their help with the biochemical analysis. Additionally, the authors would like to thank Nadine Schnepel for conducting the qPCR and Corinna Lorey for analyzing the gas samples. This study was included in the doctoral thesis of JB (Brede, 2020). The authors thank Frances Sherwood-Brock for proofreading the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.691502/full#supplementary-material

REFERENCES

Ahmed, E., Yano, R., Fujimori, M., Kand, D., Hanada, M., Nishida, T., et al. (2021). Impacts of Mootral on methane production, rumen fermentation, and microbial community in an *in vitro* Study. *Front. Vet. Sci.* 7:623817. doi: 10.3389/fvets.2020.623817

Ankri, S., and Mirelman, D. (1999). Antimicrobial properties of allicin from garlic. *Microbes Infect.* 1, 125–129. doi: 10.1016/s1286-4579(99)80003-3

Attwood, G. T., Altermann, E., Kelly, W. J., Leahy, S. C., Zhang, L., and Morrison, M. (2011). Exploring rumen methanogen genomes to identify targets for methane mitigation strategies. *Anim. Feed Sci. Technol.* 16, 65–75. doi: 10.1016/j.anifeedsci.2011.04.004

Balcells, J., Aris, A., Serrano, A., Seradj, A. R., Crespo, J., and Devant, M. (2012). Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. *J. Anim. Sci.* 90, 4975–4984. doi: 10.2527/jas.2011-4955

Beauchemin, K. (2009). Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture, Veterinary Science. *Nut. Nat. Res.* 4:35. doi: 10.1079/pavsnnr20094035

Beaudet, V., Gervais, R., Chouinard, P. Y., Graulet, B., Martin, C., Doreau, M., et al. (2020). Effects of increasing amounts of extruded linseed in the diet on apparent ruminal synthesis of some B vitamins in dairy cows. *Animal* 14, 1885–1891. doi: 10.1017/s1751731120000671

Belanche, A., Newbold, C. J., Morgavi, D. P., Bach, A., Zweifel, B., and Yáñez-Ruiz, D. R. (2020). A meta-analysis describing the effects of the essential oils blend Agolin Ruminant on performance, rumen fermentation and methane emissions in dairy cows. *Animals* 10:620. doi: 10.3390/ani10040620

Borrel, G., O'Toole, P. W., Harris, H. M., 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

Brede, J. (2020). *Einfluss von pflanzlichen Substanzen auf die ruminale Methanproduktion*. Hanover: Tierärztliche Hochschule Hanover.

Brede, M., Orton, T., Pinior, B., Roch, F.-F., Dzieciol, M., Zwirzitz, B., et al. (2020). PacBio and Illumina MiSeq amplicon sequencing confirm full recovery of the bacterial community after subacute ruminal acidosis challenge in the RUSITEC system. *Front. Microbiol.* 11:1813. doi: 10.3389/fmicb.2020.01813

Busquet, M., Calsamiglia, S., Ferret, A., Carro, M. D., and Kamel, C. (2005). Effect of garlic oil and four of its compounds on rumen microbial fermentation. *J. Dairy Sci.* 88, 4393–4404. doi: 10.3168/jds.S0022-0302(05)73126-X

Carro, M. D., Ranilla, M. J., Martin-Garcia, A. I., and Molina-Alcaide, E. (2009). Comparison of microbial fermentation of high- and low-forage diets in Rusitec, single-flow continuous-culture fermenters and sheep rumen. *Animal* 3, 527–534. doi: 10.1017/s1751731108003844

Castagnino, D. S., Kammes, K. L., Allen, M. S., Gervais, R., Chouinard, P. Y., and Girard, C. L. (2016). Particle length of silages affects apparent ruminal synthesis of B vitamins in lactating dairy cows. *J. Dairy Sci.* 99, 6229–6236. doi: 10.3168/jds.2016-11274

Chaves, A. V., He, M. L., Yang, W. Z., Hristov, A. N., McAllister, T. A., and Benchaar, C. (2008). Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria. *Can. J. Animal Sci.* 88, 117–122. doi: 10.4141/cjas07061

Chong, J., Wishart, D. S., and Xia, J. (2019). Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr. Protoc. Bioinform.* 68:e86. doi: 10.1002/cpbi.86

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

Cozannet, M., Borrel, G., Roussel, E., Moalic, Y., Allioux, M., Sanvoisin, A., et al. (2020). New insights into the ecology and physiology of *Methanomassiliicoccales* from terrestrial and aquatic environments. *Microorganisms* 9:30. doi: 10.3390/microorganisms9010030

Czerkawski, J. W., and Breckenridge, G. (1977). Design and development of a long-term rumen simulation technique (Rusitec). *Br. J. Nutr.* 38, 371–384. doi: 10.1079/bjn19770102

Danielsson, R., Schnürer, A., Arthurson, V., and Bertilsson, J. (2012). Methanogenic population and CH_4 production in swedish dairy cows fed different levels of forage. *Appl. Environm. Microbiol.* 78, 6172–6179. doi: 10.1128/AEM.00675-12

Denman, S. E., Tomkins, N. W., and McSweeney, C. S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol. Ecol.* 62, 313–322. doi: 10.1111/j.1574-6941.2007.00394.x

Dijkstra, J., Ellis, J., Kebreab, E., Strathe, A., López, S., France, J., et al. (2012). Ruminal pH regulation and nutritional consequences of low pH. *Animal Feed Sci. Technol.* 172, 22–33.

Eger, M., Graz, M., Riede, S., and Breves, G. (2018). Application of Mootral^{TM} reduces methane production by altering the archaea community in the rumen simulation technique. *Front. Microbiol.* 9:2094. doi: 10.3389/fmicb.2018.02094

Egert, B., Weinert, C. H., and Kulling, S. E. (2015). A peaklet-based generic strategy for the untargeted analysis of comprehensive two-dimensional gas chromatography mass spectrometry data sets. *J. Chromatogr. A* 1405, 168–177. doi: 10.1016/j.chroma.2015.05.056

Feldewert, C., Lang, K., and Brune, A. (2020). The hydrogen threshold of obligately methyl-reducing methanogens. *FEMS Microbiol. Lett.* 367:137. doi: 10.1093/femsle/fnaa137

Fievez, V., Dohme, F., Danneels, M., Raes, K., and Demeyer, D. (2003). Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation *in vitro* and *in vivo*. *Animal Feed Sci. Technol.* 104, 41–58. doi: 10.1016/S0377-8401(02)00330-9

Guan, H., Wittenberg, K. M., Ominski, K. H., and Krause, D. O. (2006). Efficacy of ionophores in cattle diets for mitigation of enteric methane. *J. Anim. Sci.* 84, 1896–1906. doi: 10.2527/jas.2005-652

Haque, M. N. (2018). Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *J. Anim. Sci. Technol.* 60:15. doi: 10.1186/s40781-018-0175-7

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.

Jabri Karoui, I., and Marzouk, B. (2013). Characterization of bioactive compounds in Tunisian bitter orange (*Citrus aurantium* L.) peel and juice and determination of their antioxidant activities. *Biomed. Res. Int.* 2013:345415. doi: 10.1155/2013/345415

Johnson, K. A., and Johnson, D. E. (1995). Methane emissions from cattle. *J. Animal Sci.* 73, 2483–2492. doi: 10.2527/1995.7382483

Koch, M., Strobel, E., Tebbe, C. C., Heritage, J., Breves, G., and Huber, K. (2006). Transgenic maize in the presence of ampicillin modifies the metabolic profile and microbial population structure of bovine rumen fluid *in vitro*. *Br. J. Nutr.* 96, 820–829. doi: 10.1017/BJN20061889

Kumar, S., and Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. *Sci. World J.* 2013:162750. doi: 10.1155/2013/162750

Lila, Z. A., Mohammed, N., Kanda, S., Kamada, T., and Itabashi, H. (2003). Effect of sarsaponin on ruminal fermentation with particular reference to methane production *in vitro*. *J. Dairy Sci.* 86, 3330–3336. doi: 10.3168/jds.S0022-0302(03)73935-6

Liu, C. M., Aziz, M., Kachur, S., Hsueh, P. R., Huang, Y. T., Keim, P., et al. (2012). BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. *BMC Microbiol.* 12:56. doi: 10.1186/1471-2180-12-56

Ma, T., Chen, D., Tu, Y., Zhang, N., Si, B., Deng, K., et al. (2016). Effect of supplementation of allicin on methanogenesis and ruminal microbial flora in Dorper crossbred ewes. *J. Anim. Sci. Biotechnol.* 7:57. doi: 10.1186/s40104-015-0057-5

Machmuller, A., Soliva, C. R., and Kreuzer, M. (2003). Effect of coconut oil and defaunation treatment on methanogenesis in sheep. *Reprod. Nutr. Dev.* 43, 41–55. doi: 10.1051/rnd:2003005

Martin, A. K. (1982). The origin of urinary aromatic compounds excreted by ruminants 1. The metabolism of quinic, cyclohexanecarboxylic and non-phenolic aromatic acids to benzoic acid. *Br. J. Nutr.* 47, 139–154. doi: 10.1079/BJN19820019

Martinez-Fernandez, G., Duval, S., Kindermann, M., Schirra, H. J., Denman, S. E., and McSweeney, C. S. (2018). 3-NOP vs. halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Front. Microbiol.* 9:1582. doi: 10.3389/fmicb.2018.01582

Mbiriri, D. T., Cho, S., Mamvura, C. I., and Choi, N. J. (2017). Effects of a blend of garlic oil, nitrate and fumarate on *in vitro* ruminal fermentation and microbial population. *J. Anim. Physiol. Anim. Nutr.* 101, 713–722. doi: 10.1111/jpn.12508

Morgavi, D. P., Forano, E., Martin, C., and Newbold, C. J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal* 4, 1024–1036. doi: 10.1017/s1751731110000546

Odongo, N. E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M. M., Hook, S. E., et al. (2007). Long-term effects of feeding monensin on methane production in lactating dairy cows. *J. Dairy Sci.* 90, 1781–1788. doi: 10.3168/jds.2006-708

Oskoueian, E., Abdullah, N., and Oskoueian, A. (2013). Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *Biomed. Res. Int.* 2013:349129. doi: 10.1155/2013/349129

Patra, A. K., Kamra, D. N., Bhar, R., Kumar, R., and Agarwal, N. (2011). Effect of Terminalia chebula and Allium sativum on *in vivo* methane emission by sheep. *J. Anim. Physiol. Anim. Nutr.* 95, 187–191. doi: 10.1111/j.1439-0396.2010.01039.x

Patra, A. K., and Yu, Z. (2012). Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. *Appl. Environm. Microbiol.* 78, 4271–4280. doi: 10.1128/aem.00309-12

Patra, A. K., and Yu, Z. (2015). Effects of adaptation of *in vitro* rumen culture to garlic oil, nitrate, and saponin and their combinations on methanogenesis, fermentation, and abundances and diversity of microbial populations. *Front. Microbiol.* 6:1434. doi: 10.3389/fmicb.2015.01434

Patterson, J. A., and Hespell, R. B. (1979). Trimethylamine and methylamine as growth substrates for rumen bacteria and *Methanosarcina barkeri*. *Curr. Microbiol.* 3, 79–83. doi: 10.1007/bf02602436

Poulsen, M., Schwab, C., Jensen, B. 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. doi: 10.1038/ncomms2432

Ramos-Morales, E., Rossi, G., Cattin, M., Jones, E., Braganca, R., and Newbold, C. J. (2018). The effect of an isoflavonid-rich liquorice extract on fermentation, methanogenesis and the microbiome in the rumen simulation technique. *FEMS Microbiol. Ecol.* 94:9. doi: 10.1093/femsec/fiy009

Riede, S., Boguhn, J., and Breves, G. (2013). Studies on potential effects of fumaric acid on rumen microbial fermentation, methane production and microbial community. *Arch. Anim. Nutr.* 67, 368–380. doi: 10.1080/1745039x.2013.830518

Roque, B. M., Van Lingen, H. J., Vrancken, H., and Kebreab, E. (2019). Effect of Mootral—a garlic- and citrus-extract-based feed additive—on enteric methane emissions in feedlot cattle. *Transl. Anim. Sci.* 3, 1383–1388. doi: 10.1093/tas/txz133

Saro, C., Hohenester, U. M., Bernard, M., Lagrée, M., Martin, C., Doreau, M., et al. (2018). Effectiveness of interventions to modulate the rumen microbiota composition and function in pre-ruminant and ruminant lambs. *Front. Microbiol.* 9:1273. doi: 10.3389/fmicb.2018.01273

Schilde, M., Zeyner, A., and Dänicke, S. (2021). Effects of 3-nitrooxypropanol and varying concentrate feed proportions in the ration on methane emission, rumen fermentation and performance of periparturient dairy cows. *Archiv. Anim. Nutr.* 75:1877986. doi: 10.1080/1745039X.2021.1877986

Soliva, C. R., Amelchanka, S. L., Duval, S. M., and Kreuzer, M. (2011). Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). *Br. J. Nutr.* 106, 114–122. doi: 10.1017/s0007114510005684

Söllinger, A., Schwab, C., Weinmaier, T., Loy, A., Tveit, A. T., Schleper, C., et al. (2016). Phylogenetic and genomic analysis of *Methanomassiliicoccales* in

wetlands and animal intestinal tracts reveals clade-specific habitat preferences. *FEMS Microbiol. Ecol.* 92:149. doi: 10.1093/femsec/fiv149

Takai, K., and Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl. Environ. Microbiol.* 66, 5066–5072. doi: 10.1128/aem.66.11.5066-5072.2000

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., and Sivakumaran, S. (2005). Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Animal Feed Sci. Technol.* 12, 403–419. doi: 10.1016/j.anifeedsci.2005.04.037

Vrancken, H., Suenkel, M., Hargreaves, P. R., Chew, L., and Towers, E. (2019). Reduction of enteric methane emission in a commercial dairy farm by a novel feed supplement. *Open J. Animal Sci.* 3:24. doi: 10.4236/ojas.2019.93024

Wanapat, M., Khejornsart, P., Pakdee, P., and Wanapat, S. (2008). Effect of supplementation of garlic powder on rumen ecology and digestibility of nutrients in ruminants. *J. Sci. Food Agri.* 88, 2231–2237. doi: 10.1002/jsfa.3333

Wedlock, D. N., Pedersen, G., Denis, M., Dey, D., Janssen, P. H., and Buddle, B. M. (2010). Development of a vaccine to mitigate greenhouse gas emissions in agriculture: vaccination of sheep with methanogen fractions induces antibodies that block methane production *in vitro*. *N. Z. Vet. J.* 58, 29–36. doi: 10.1080/00480169.2010.65058

Weinert, C. H., Egert, B., and Kulling, S. E. (2015). On the applicability of comprehensive two-dimensional gas chromatography combined with a fast-scanning quadrupole mass spectrometer for untargeted large-scale metabolomics. *J. Chromatogr. A* 1405, 156–167. doi: 10.1016/j.chroma.2015.04.011

Wetzels, S. U., Eger, M., Burmester, M., Kreienbrock, L., Abdulmawjood, A., Pinior, B., et al. (2018). The application of rumen simulation technique (RUSITEC) for studying dynamics of the bacterial community and metabolome in rumen fluid and the effects of a challenge with *Clostridium perfringens*. *PLoS One* 13:e0192256. doi: 10.1371/journal.pone.0192256

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 October 2021
doi: 10.3389/fmicb.2021.711040

Effect of Divergent Feeding Regimes During Early Life on the Rumen Microbiota in Calves

*Omar Cristobal-Carballo[1,2], Sue A. McCoard[1], Adrian L. Cookson[3,2], Richard A. Laven[2], Siva Ganesh[4], Sarah J. Lewis[1] and Stefan Muetzel[1]**

[1] Ruminant Nutrition and Physiology Team, AgResearch Grasslands, Palmerston North, New Zealand, [2] School of Veterinary Medicine, Massey University, Palmerston North, New Zealand, [3] Food System Integrity Team, AgResearch Grasslands, Palmerston North, New Zealand, [4] Biostatistics Team, AgResearch Grasslands, Palmerston North, New Zealand

The objective of this study was to determine whether divergent feeding regimes during the first 41 weeks of the life of a calf are associated with long-term changes in the rumen microbiota and the associated fermentation end-products. Twenty-four calves (9 ± 5 days of age) were arranged in a 2 × 2 factorial design with two divergent treatments across three dietary phases. In phase 1 (P01), calves were offered a low-milk volume/concentrate starter diet with early weaning (CO) or high-milk volume/pasture diet and late weaning (FO). In phase 2 (P02), calves from both groups were randomly allocated to either high-quality (HQ) or low-quality (LQ) pasture grazing groups. In phase 3 (P03), calves were randomly allocated to one of two grazing groups and offered the same pasture-only diet. During each dietary phase, methane (CH_4) and hydrogen (H_2) emissions and dry matter intake (DMI) were measured in respiration chambers, and rumen samples for the evaluation of microbiota and short-chain fatty acid (SCFA) characterizations were collected. In P01, CO calves had a higher solid feed intake but a lower CH_4 yield (yCH_4) and acetate:propionate ratio (A:P) compared with FO calves. The ruminal bacterial community had lower proportions of cellulolytic bacteria in CO than FO calves. The archaeal community was dominated by *Methanobrevibacter boviskoreani* in CO calves and by *Mbb. gottschalkii* in FO calves. These differences, however, did not persist into P02. Calves offered HQ pastures had greater DMI and lower A:P ratio than calves offered LQ pastures, but yCH_4 was similar between groups. The cellulolytic bacteria had lower proportions in HQ than LQ calves. In all groups, the archaeal community was dominated by *Mbb. gottschalkii*. No treatment interactions were observed in P02. In P03, all calves had similar DMI, CH_4 and H_2 emissions, SCFA proportions, and microbial compositions, and no interactions with previous treatments were observed. These results indicate that the rumen microbiota and associated fermentation end-products are driven by the diet consumed at the time of sampling and that previous dietary interventions do not lead to a detectable long-term microbial imprint or changes in rumen function.

Keywords: calves, early-life, microbial community, fermentation, dietary transitions, enteric emissions

OPEN ACCESS

Edited by:
Diego P. Morgavi, INRAE Clermont-Auvergne-Rhône-Alpes, France

Reviewed by:
Jeyamalar Jeyanathan, Ghent University, Belgium
Paul E. Smith, Teagasc Grange Animal and Bioscience Research Department (ABRD), Ireland

****Correspondence:***
Stefan Muetzel
stefan.muetzel@agresearch.co.nz

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *17 May 2021*
Accepted: *31 August 2021*
Published: *20 October 2021*

Citation:
Cristobal-Carballo O, McCoard SA, Cookson AL, Laven RA, Ganesh S, Lewis SJ and Muetzel S (2021) Effect of Divergent Feeding Regimes During Early Life on the Rumen Microbiota in Calves. Front. Microbiol. 12:711040. doi: 10.3389/fmicb.2021.711040

INTRODUCTION

The rumen is a fermentation chamber occupied by a diverse, interactive, and dynamic microbiota comprised of many species of bacteria, archaea, protozoa, and fungi (Hobson and Stewart, 1997). These microorganisms convert ingested feed into short-chain fatty acids (SCFA) and microbial biomass, which are the main source of energy and amino acids for ruminants (Puniya et al., 2015; Huws et al., 2018; Gruninger et al., 2019). Other fermentation end-products, including hydrogen (H_2) and carbon dioxide (CO_2), formic acid, and methyl groups, are utilized by methanogens to produce methane (CH_4) (Moss et al., 2000; Liu and Whitman, 2008; Janssen, 2010). Methane production is both a loss of dietary gross energy (Bergman, 1990) and a greenhouse gas (Johnson and Johnson, 1995). Thus, manipulation of the ruminal ecosystem has been used in attempts to improve the efficiency of feed conversion and decrease environmental impacts (Mizrahi et al., 2021). However, manipulations in adult ruminants have shown limited and only short-term effects after treatment cessation (Weimer, 1998). The microbiota in the rumen of mature ruminants is characterized by a high degree of redundancy and resilience, which provides stability to the rumen environment and maintains the digestive function of the host across a range of feeding and management conditions (Weimer, 2015). These properties thus represent a barrier to manipulating rumen fermentation by selectively targeting groups of microorganisms. However, some studies in small ruminants suggest that in early life the rumen microbial community may be more plastic and, therefore, easier to manipulate (Yáñez-Ruiz et al., 2010; Abecia et al., 2014; De Barbieri et al., 2015).

During early postnatal life, the rumen microbiota of the young ruminant is very responsive to dietary interventions (Yáñez-Ruiz et al., 2010; Abecia et al., 2014; O'Hara et al., 2020). While sterile *in utero* (Malmuthuge and Griebel, 2018; Husso et al., 2021), the rumen of newborn animals undergoes a rapid microbial colonization during and after birth from maternal (Taschuk and Griebel, 2012; Yeoman et al., 2018) and environmental sources (Dehority and Orpin, 1997; Curtis and Sloan, 2004). After the initial colonization, microbial groups critical to the degradation of feed have been observed in the undeveloped rumen, as early as the third day of age (Fonty et al., 1987; Minato et al., 1992; Guzman et al., 2015; Wang et al., 2017). The rumen microbiota rapidly shifts toward obligate anaerobic microbes as young ruminants start to transition from milk to solid diets (Walters et al., 2011; Rey et al., 2014). In post-weaned calves, the consumption of solid diets is associated with a progressive shift in ruminal microbial composition toward a more diverse microbiota (Rey et al., 2014; Dias et al., 2017; Dill-McFarland et al., 2017). The ruminal microbiota in young ruminants acquires an adult-like composition as the solid feed intake increases between the weaning transition and 1 year of age (Dill-McFarland et al., 2019), with recent studies indicating that an increased solid feed intake can result in adult-like fermentation profiles (Cristobal-Carballo et al., 2019). As a result, dietary interventions, aimed at altering ruminal microbial composition and fermentation profiles, may be most effective during the weaning transition of young ruminants. However, there is little information available on the effect of early life nutrition of calves during the transition to weaning and immediately afterward on the rumen prokaryotic community and fermentation profiles. The aim of this study was to determine whether contrasting feeding regimes pre- and post-weaning could imprint the rumen microbial community and produce associated changes in rumen fermentation.

MATERIALS AND METHODS

Animal procedures were reviewed and approved by the Grasslands Animal Ethics Committee (AE 13297) and complied with the institutional Codes of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the New Zealand Animal Welfare Act of 1999 and its amendments.

Experimental Design

Twenty-four calves were randomly selected and balanced across dietary treatments from a parent production study using 200 Hereford–Friesian-cross female calves (Burggraaf et al., 2020). Treatments in the large production study were balanced for live weight and arrival date of the calves. The study was carried out in a 2 × 2 factorial design with different dietary treatments across three dietary phases. In phase 1 (P01, 0–14 weeks), calves were reared using either a low-milk volume and concentrate starter diet with early weaning (CO) or high-milk volume and pasture diet with later weaning (FO). In phase 2, post-weaning (P02, 14–19 weeks), each group of calves was evenly divided and randomly allocated to either a high-quality (HQ) or low-quality pasture (LQ) diet. The outcome was the generation of four groups in P02, where the main effects of pre-weaning rearing system and post-weaning diet quality and the interactions were compared. In phase 3 (P03, 30 to 41 weeks), all calves were randomly allocated to two groups that equally represented all four treatment groups and managed under commercial grazing conditions on the same farm. For this study, measurements and sampling were undertaken in week 9 (P01), week 19 (P02), and week 41 (P03).

Animal and Feeding Management

Calves from P01 were managed in two pre-weaning rearing systems: FO calves were housed during week 1 and then moved to paddocks of ryegrass/white clover pasture from weeks 2 to 12. These calves were fed whole milk powder (WMP; **Table 1**; NZ Agbiz, Auckland, New Zealand) at 8.0 L/calf/day (1,000 g of WMP; 125 g/L of water), divided in two feeds for 5 weeks. In this group, the intake of WMP was increased from 5.0 to 8.0 L/calf/day during the first 2 weeks. Calves were fed 8.0 L once per day from weeks 5 to 9, then 4 L once a day for 2 weeks, then were gradually weaned over the following week. Calves from the CO group were housed on arrival and fed WMP at 4.0 L/calf/day (500 g WMP/day; mixed as per the FO group), divided in two feeds for 5 weeks and then once a day for 2 weeks, before abruptly weaning off milk at the end of week 7. This group was offered *ad libitum* starter concentrate (**Table 1**; Denver Stock Feeds, Palmerston North, New Zealand) from weeks 1 to 7, then calves

TABLE 1 | Chemical composition (% of dry matter) of the whole milk powder (WMP), concentrate and pastures[a] fed to calves in phase 1 (P01), pastures of high[b] (HQ) and low quality[c] (LQ) in phase 2 (P02) and pastures[d] fed to all calves in phase 3 (P03).

Feed	P01			P02		P03
	WMP	Pasture[a]	Concentrate	HQ Pasture[b]	LQ Pasture[c]	Pasture[d]
Dry matter (%)[e]	95.2	18.7	93.8	21.5	37.7	14.9
Crude protein[f]	24.1	14.8	19.8	19.8	7.3	19.2
ME (MJ/kg DM)[g]		10.5	13.8	10.9	7.6	11.2
NDF[h]	–	49.5	16.2	47.9	64.2	53
ADF[i]	–	25.4	5.9	25.2	35.7	27
Lignin[j]	–	1.8	–	3.8	4.2	2.9
Lipids[k]	28.4	1.1	2.3	2.3	1.1	2.8
Ash[l]	5.5	6.4	6.2	8.8	5.5	10.3
Soluble sugars[m]	41.5[n]	19.3	8	9.3	9	11.4
Starch[o]	–	–	36.8	–	–	–

[a] *Pasture was composed of ryegrass/white clover mixed sward. Chemical composition of pastures was scanned using near-infrared reflectance spectroscopy (NIRS; Corson et al., 1999).*
[b] *Calves were grazed in irrigated pastures. Chemical composition of pastures was scanned using near-infrared reflectance spectroscopy (NIRS; Corson et al., 1999).*
[c] *Calves were grazed in unirrigated pastures. Chemical composition of pastures was scanned using near-infrared reflectance spectroscopy (NIRS; Corson et al., 1999).*
[d] *Pasture was composed of ryegrass/white clover mixed sward. Chemical composition of pastures was scanned using near-infrared reflectance spectroscopy (NIRS; Corson et al., 1999).*
[e] *Method 945.15; AOAC, 2010.*
[f] *Method 992.15; AOAC, 2010.*
[g] *Metabolizable energy content of pellets was calculated using the equation ME = DOMD% × 0.16 (Alderman and Cottrill, 1996).*
[h] *Neutral detergent fiber; Method 7.074; AOAC, 1990.*
[i] *Acid detergent fiber; method 7.074; AOAC, 1990.*
[j] *Lignin; method 7.074; AOAC, 1990.*
[k] *Method 954.02; AOAC, 1990.*
[l] *Method 942.05; AOAC, 2012.*
[m] *Paul, A.A and Southgate, D.A. The Composition of Foods. 4th Edition, 1978.*
[n] *Lactose.*
[o] *Method 996.11; AOAC, 2010.*

were transferred to paddocks of ryegrass/white clover pasture with starter concentrate reduced to 1.5 kg/calf/day for 2 weeks and finally to 1 kg/calf/day for two more weeks until weaning off concentrate starter at week 12.

In P02, calves in the HQ and LQ groups were grazed on pastures of either high quality (HQ; irrigated pasture) or low quality (LQ; non-irrigated pasture). Calves grouped in LQ were also fed grass silage to meet dry matter intake (DMI) requirements. The botanical composition of high-quality pastures was 31.0% of ryegrass, 35.5% of white clover, 7.3% of herbs, 3.4% other grasses, and 22.4% of dead material (DM basis), while the botanical composition of non-irrigated low-quality pastures was of 16.3% of ryegrass, 4.0% of white clover, 1.3% of herbs, 16.0% other grasses, and 61.4% of dead material (DM basis) (Burggraaf et al., 2020). In P03, from approximately 7 months of age, the calves were randomly allocated to one of two groups which were balanced for all four previous treatments and grazed commercially on ryegrass/white clover pasture. Fresh water was available *ad libitum* at all times.

Enteric Emissions and Animal Performance Measurements

Enteric emissions [methane (CH_4) and hydrogen (H_2)] and animal performance [DMI and live weight (LW)] measurements were performed from the 24 selected calves, at weeks 9 (P01), 19 (P02), and 41 (P03). Before enteric emission measurements, calves were adapted to confinement conditions in covered yards as follows: in a group pen all together for the first 5 days and then in individual crates from days 5 to 7. Enteric emission measurements were carried out in open circuit respiratory chambers (Pinares-Patiño et al., 2012) over a 48-h period. The air flow through the chambers was set at 700, 1,000, and 1,200 L/min during the three measurement phases, respectively, to account for the increasing gas emissions as the solid feed intake of the calves increased. Calves entered the chambers in the morning (0900 h) when feed was offered: WMP (for FO calves in P01) and fresh solid feed (starter concentrate and/or pasture, depending on the phase and group). For LQ calves in P02, these animals received only low-quality grass (**Table 1**) during confinement and in respiration chambers; no grass silage was offered to these animals. Enteric emission measurements were paused for ∼45 min every morning to offer fresh feed and clean the chambers. During the adaptation and enteric emission measurement phases, pasture was cut daily, transported to the animal facility, and offered *ad libitum*. Water was available *ad libitum*.

Samples of concentrate and WMP were analyzed for chemical composition by wet chemistry (Hill Laboratories Ltd., Hamilton, New Zealand). Pasture samples of ryegrass/white clover-mixed sward were analyzed by near-infrared reflectance spectroscopy (NIRS; FeedTECH, Palmerston North, New Zealand). The chemical composition of the offered diets during the different rearing phases is shown in **Table 1**. During the gas measurement phases, DMI was calculated from the difference between the allowance and the residual feed. Milk DMI was not included in the calculation of the total DMI to estimate methane or hydrogen yield because most of the milk DM bypasses the rumen (Wise and Anderson, 1939) and, therefore, has little effect on rumen fermentation (Lane et al., 2000). Live weights were recorded a day before methane emission measurements during the morning and before feeding. Total daily production (g/day) of CH_4 and H_2 (pCH_4 and pH_2, respectively) were calculated from the enteric emission measurements (Pinares and Waghorn, 2014). Daily enteric emissions and animal performance parameters were used to calculate CH_4 and H_2 yield (yCH_4 and yH_2, respectively; g per kg DMI).

Sampling and Fermentation Analysis of Rumen Contents

Rumen samples were collected in the morning *via* an oral stomach tube (Henderson et al., 2013) after enteric emission

measurements and before feeding new milk and/or solid feed. Oral stomach tubing was performed using a stainless steel pipe (25 mm outside diameter, wall thickness 1.2 mm) measuring 520 mm in length with a "T" handle 350 mm from one end. The stainless steel pipe was used to guide the lavage tube over the back of the tongue to ensure it entered the rumen. The lavage tube (19 mm outside diameter) enabled contents to be aspirated using a 400-ml syringe from the center of the dorsal rumen. A modification of the technique was used at 9 and 19 weeks, where the stainless steel pipe was not used due to the size of the animals. Each sample of 10 to 30 ml of rumen fluid was subsampled for SCFA analysis and DNA extraction. For DNA analyses, 900 μl of each rumen fluid sample was snap frozen in a cool rack and immediately stored at −20°C until analysis. For SCFA analysis, 1.8 ml of each rumen sample was prepared as per Guyader et al. (2016). Gas chromatography was then used to analyze SCFA composition, as per Attwood et al. (1998), using a gas chromatograph (Model 6869, Hewlett-Packard, Montreal, QC, Canada) equipped with an auto-sampler, fitted with a Zebron ZB-FFAP 30.0 m × 0.53 mm I.D. × 1 μm film column (Phenomenex, Torrance, CA, United States) and a flame ionization detector set at 265°C.

Microbial DNA Extraction, Library Preparation, and Sequencing

DNA was extracted from 200 μl of thawed and vortexed rumen fluid samples using the phenol–chloroform, bead beating with filtration kit for purification II (PCQI) (Rius et al., 2012; Henderson et al., 2013). Primers used for PCR amplification of bacterial and archaeal 16S rRNA genes are listed in **Supplementary Table S1**. Amplification reactions used for PCR targeting the regions of bacterial (30 cycles) and archaeal (35 cycles) 16S rRNA genes were prepared in triplicate as described by Kittelmann et al. (2013). PCR products were pooled, and the correct size products (∼500 bp) were verified by agarose gel electrophoresis and quantified by fluorescence using the Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, United States). Bacteria and archaea PCR reactions included a negative control for each separate amplification run. Negative control reactions containing no template DNA were performed alongside each PCR amplification and were included in subsequent analyses to confirm no amplification of product. Agarose gel electrophoresis was performed using 2 μl of PCR product on a 1% (w/v) agarose gel containing SYBR Safe. Each amplicon (150 ng) from the same target gene and region (i.e., all bacteria and archaea amplicons) was pooled. Pooled samples were concentrated (vacuum dried) and the final PCR product concentration was determined using Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA, United States). Pools were purified using the NucleoMag NGS kit (Macherey-Nagel, Dueren, Germany). The final purification of amplicons was done using the QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) and the DNA concentration quantified using Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA, United States). Both pools were diluted to 6.0×10^9 copies per μl and combined at a "bacteria to archaea" ratio of 5:1 (Kittelmann et al., 2013). Pooled libraries were checked for quality control (QC) using Labchip GX Touch HT instrument (PerkinElmer, Waltham, MA, United States). Amplicons were sequenced using the Illumina MiSeq system according to the protocol of the manufacturer (Illumina, San Diego, CA, United States) at Massey Genome Service, Massey University, Palmerston North, New Zealand. The pooled library was run on one Illumina MiSeq (500 cycle V 2 kit). A control library for the run, Illumina-prepared PhiX, was loaded onto the Illumina MiSeq run at 20% volumes. Sequence reads were provided in fastq format. The sequences obtained were deposited in the European Nucleotide Archive under the accession number PRJEB37783.

Phylogenetic Analysis of Sequencing Data

Sequencing reads were quality-filtered using the DynamicTrim function of SolexaQA (Cox et al., 2010). Reads were then processed and analyzed using the QIIME software package 1.8 (Caporaso et al., 2010). Sequencing reads were grouped into operational taxonomic units (OTUs) sharing over 97% and 99% similarity for bacteria and archaea, respectively, by using the UCLUST algorithm (Edgar, 2010). Sequences were assigned to phylogenetic kingdoms using the BLAST (version 2.4.0) algorithm (Altschul et al., 1990). Bacterial 16S rRNA genes were assigned using SILVA 123 (Henderson et al., 2019) and archaeal 16S rRNA genes using RIM-DB (Seedorf et al., 2014). QIIME-generated OTU tables were used for downstream statistical analysis.

Statistical Analysis

Data were checked for normality using *Q*–*Q* plots alongside the Shapiro–Wilk's *W* test. After normality assessment, univariate analyses were performed using a linear mixed effect (LME) model *via* the restricted maximum likelihood (REML) framework as implemented in the *NLME* package in R (Pinheiro et al., 2015; R Core Team, 2016). The resulting LME models were analyzed using analysis of variance (ANOVA). Predicted means from the model, together with estimates of the standard error of the mean and pairwise comparisons (Tukey's or Benjamini–Hochberg test), were obtained and back transformed (where applicable) using the *PREDICTMEANS* package of R (Luo et al., 2014). Statistical significance was declared at a P-value ≤ 0.05.

Dietary effects were evaluated on animal performance, enteric emissions, and rumen fermentation data. In P01, dietary treatment (FO or CO) was used as a fixed effect and animal as a random effect. Data from P02 and P03 were analyzed using dietary treatments from P01 (FO and CO) and P02 (HQ and LQ) as fixed effects and animal as random effect. Live weight analysis for each feeding phase was adjusted using the initial LW as covariate in the model. The resulting LME models were analyzed using one-way ANOVA for P01 and a 2 × 2 factorial ANOVA for P02 and P03. Treatment effects were assessed and predicted means from the model, together with estimates of the standard errors of the means, were obtained and compared using Tukey's test.

A total of 364 bacterial OTUs and 17 archaea OTUs (**Supplementary Tables S2, S3**) were analyzed after using a minimum average cutoff of 70 reads per sample. The alpha

diversity of the bacterial and archaeal community of calves under contrasting dietary management conditions was analyzed separately using Shannon index in the *Vegan* package of R (Oksanen et al., 2017). Dietary treatment effects for the Shannon index of the microbial (bacterial and archaeal) community during P01, P02, and P03 were fitted in an LME model and analyzed using ANOVA as described for DMI, rumen fermentation, and gas emissions data. Predicted means from the models, together with estimates of the standard error of the mean (SEM), were obtained, and pairwise comparisons were done using Tukey's test. Beta diversity of the bacteria and archaea community in each group of calves was analyzed using a partial least squares discriminant analysis (PLSDA) using the *mixOmics* package of R (Lê Cao et al., 2016). Groups of calves in the PLSDA analysis were assigned combining phase and treatments as follows: phase 1 (P01) corresponded to groups FO and CO, and phases 2 (P02) and 3 (P03) were the groups formed by the combination of dietary treatments from P01 and P02, resulting in FOHQ, FOLQ, COHQ, and COLQ. Additionally, a PLSDA was conducted for abundant microbes. Abundant microbes were defined as bacteria genera and archaea species with a relative abundance $\geq$0.70% and $\geq$1.00%, respectively. The aim was to identify whether the abundant microbiota showed a similar cluster separation pattern to that observed in the whole microbiota. Association scores for bacteria and archaea were visualized using clustered image maps (CIM) representing the first two dimensions (Henderson et al., 2015).

Univariate analyses were used to determine the effect of dietary treatments on the abundant microbial community. The abundant microbial community was defined from the OTU data table as those taxa with an overall relative abundance across phases $\geq$1.0 and $\geq$0.7% at bacteria phylum and genus level, respectively, and $\geq$1.0% at species level for archaea. After checking for normality, bacteria (phyla and genera) and archaea (species) community data were transformed using natural logarithm. The analysis of the abundant microbial community in each feeding phase was assessed as described for animal performance, enteric emissions, and rumen fermentation data. Predicted means from the models, together with estimates of the confident intervals (CI) with upper limit (UL) and lower limit (LL), were obtained and back transformed, and pairwise comparisons were done using the Benjamini–Hochberg test.

RESULTS

Animal Performance, Enteric Emissions, and Rumen Fermentation

The effects of dietary treatments on animal performance, enteric emissions, and rumen fermentation are presented in **Table 2**. In P01 (week 9), CO calves had a 136% greater ($P < 0.01$) DMI than FO calves (total DMI, including milk intake, CO = 2.10 kg vs. FO = 1.84 kg; $P < 0.01$). Live weight was 8% lower ($P = 0.02$) in CO than in FO calves. Daily pCH_4 was 25% higher in CO than FO calves but this was not significant ($P = 0.06$). Calves in the CO group had 47% lower ($P < 0.01$) yCH_4 than FO calves. Hydrogen production and yield were not affected ($P \geq 0.33$) by the dietary regime. Total SCFA concentrations in the rumen were 45% higher ($P < 0.01$) in CO than FO calves. Compared with FO calves, the proportion of acetate in CO calves was lower ($P < 0.01$), while the proportions of propionate and valerate were greater ($P < 0.01$). The proportions of butyrate and caproate were similar ($P \geq 0.11$) in both groups, while both isobutyrate and isovalerate were lower ($P < 0.01$) in CO than FO calves.

In P02 (week 19), CO calves were 10% lighter ($P < 0.01$) than FO calves. Isovalerate proportions were higher in CO than FO calves (1.00 vs. 0.87; $P = 0.03$). No other effects from P01 dietary regimes (i.e., CO and FO) and no interactions between the dietary regimes in P01 and P02 (i.e., HQ and LQ) were observed ($P \geq 0.13$). Dry matter intake was 41% greater in HQ than LQ calves. Live weight in HQ calves was 8% lower ($P = 0.03$) than in LQ calves. The pCH_4 and pH_2 were 31% ($P < 0.01$) and 133% ($P = 0.03$) greater in HQ than LQ calves, respectively. No differences between yCH_4 and yH_2 were observed between these groups ($P \geq 0.15$). Total SCFA concentrations in HQ calves were 36% greater ($P < 0.01$) than in LQ calves. In HQ calves, acetate proportions were lower ($P < 0.01$), while propionate and valerate proportions were greater ($P < 0.01$) than in LQ calves. The proportions of butyrate and caproate were 20% lower and 8% greater in HQ than in LQ calves, but these were not significant ($P = 0.07$). Both isobutyrate and isovalerate proportions were greater ($P < 0.01$) in HQ than LQ calves.

In P03 (week 41), CO calves were 6% lighter than FO calves, but this was not significant ($P = 0.08$), while HQ calves were 23% heavier ($P < 0.01$) than LQ calves. No direct effect of previous dietary treatments ($P \geq 0.12$) or their interactions ($P \geq 0.14$) was observed on animal performance, enteric emissions, and rumen fermentation.

Rumen Microbial Diversity

Negative control reactions containing no template for bacteria and archaea resulted in no 16S rRNA amplicons after PCR; therefore, no subsequent analysis was undertaken. After merging, filtering, and trimming, Illumina sequencing generated a total of 8,087,270 bacterial and archaeal 16S rRNA sequences from the 72 samples. The average number of sequences of bacteria and archaea was 97,286 $\pm$ 29,785 SD and 15,037 $\pm$ 2,875 SD as per sample, respectively, while the number of OTUs was 1,509 and 41 for bacteria and archaea, respectively.

Figure 1 shows the Shannon index of the bacterial and archaeal community in each of the dietary treatment groups of calves during each sampling phase. In P01, the bacteria diversity in CO calves was lower ($P = 0.01$) than in FO calves. However, the Shannon index for bacteria in P02 and P03 did not show dietary effects from P01 and P02 ($P \geq 0.69$) or dietary interaction effects ($P \geq 0.70$; **Figure 1A**). The archaea diversity did not show dietary treatment effect ($P \geq 0.29$) in P01, or carryover effects ($P \geq 0.17$) from P01, dietary treatment effects during P02, or their interaction effects during P02 and P03 (**Figure 1B**).

Figure 2 shows the beta diversity analysis of the bacteria and archaea for the community and abundant microbes, respectively, during each feeding phase. The PLSDA for the bacteria community (364 bacteria genus; **Figure 2A**) and abundant bacteria (25 genus; **Figure 2B**) in CO calves differed from pasture-fed calves in P01–P03. Within pasture-fed calves, the beta diversity for the bacteria community differed between calves in

TABLE 2 | Effect of dietary treatments[a] on dry matter intake (DMI)[b], live weight (LW)[c], enteric emissions[d], and fermentation profiles[e] in calves during three measurement phases[f].

	P01				P02									P03								
	FO	CO	SEM	*P*-T1	FO	CO	SEM	HQ	LQ	SEM	*P*-T1	*P*-T2	*P*-int	FO	CO	SEM	HQ	LQ	SEM	*P*-T1	*P*-T2	*P*-int
DMI (kg/day)	0.89	2.1	0.039	< 0.01	3.50	3.31	0.202	3.98	2.83	0.202	0.52	< 0.01	0.81	4.11	3.91	0.168	4.20	3.81	0.168	0.4	0.12	0.87
LW (kg)	82.0	75.6	1.751	0.02	123.1	111.3	2.859	121.7	112.8	2.931	< 0.01	0.03	0.78	192.7	182.5	3.974	206.9	168.3	4.073	0.08	< 0.01	0.59
pCH_4 (g/day)[g]	14.59	18.20	1.265	0.06	67.84	62.90	2.321	74.78	56.97	2.321	0.15	< 0.01	0.41	121.6	117.8	4.428	124.6	114.7	4.428	0.55	0.13	0.46
yCH_4 (g/kg of DMI)[h]	16.21	8.66	0.690	< 0.01	20.60	19.42	1.412	18.87	21.16	1.412	0.56	0.27	0.91	29.81	30.41	0.901	29.91	30.32	0.901	0.65	0.75	0.68
pH_2 (g/day)[i]	0.123	0.211	0.063	0.33	0.101	0.062	0.020	0.114	0.049	0.020	0.18	0.03	0.76	0.033	0.036	0.022	0.034	0.035	0.022	0.93	0.97	0.67
yH_2 (g/kg)[j]	0.143	0.101	0.035	0.41	0.027	0.019	0.005	0.029	0.017	0.005	0.26	0.11	0.15	0.008	0.009	0.005	0.007	0.009	0.005	0.91	0.82	0.44
SCFA (mM)[k]	70.1	101.6	6.150	< 0.01	63.5	64.7	3.831	73.8	54.4	3.831	0.82	< 0.01	0.28	74.5	71.0	5.894	76.2	69.4	5.894	0.68	0.43	0.18
Acetate (%)	62.48	45.05	1.080	< 0.01	70.31	70.6	0.386	68.55	72.36	0.386	0.6	< 0.01	0.8	67.96	67.89	0.548	68.02	67.84	0.548	0.93	0.82	0.26
Propionate (%)	21.83	39.14	1.061	< 0.01	17.51	17.19	0.295	17.98	16.72	0.295	0.45	< 0.01	0.75	16.53	16.63	0.427	16.41	16.75	0.427	0.87	0.58	0.92
Butyrate (%)	12.04	10.99	0.801	0.37	9.19	8.95	0.255	9.41	8.73	0.255	0.52	0.07	0.71	11.71	11.64	0.276	11.77	11.58	0.276	0.87	0.65	0.14
Valerate (%)	1.35	3.53	0.179	< 0.01	0.94	1.00	0.039	1.20	0.74	0.039	0.32	< 0.01	0.32	0.99	1.02	0.038	0.99	1.02	0.038	0.49	0.67	0.43
Caproate (%)	0.45	0.68	0.097	0.11	0.29	0.34	0.027	0.28	0.35	0.027	0.17	0.07	0.13	0.12	0.13	0.008	0.13	0.11	0.008	0.43	0.17	0.72
Isobutyrate (%)	0.92	0.28	0.042	< 0.01	0.89	0.92	0.023	1.22	0.58	0.023	0.45	< 0.01	0.86	1.30	1.27	0.054	1.28	1.29	0.054	0.74	0.84	0.25
Isovalerate (%)	0.94	0.33	0.082	< 0.01	0.87	1.00	0.039	1.35	0.53	0.039	0.03	< 0.01	0.88	1.40	1.42	0.065	1.41	1.41	0.065	0.88	0.96	0.28

Results are the means and standard error of the means (SEM), P-value for treatment effect for FO vs. CO (P01), treatment effect for HQ vs. LQ (P02) and their interactions (P-int).

[a] *Dietary treatments corresponded to phase 1 (P01) concentrate (CO) vs. pasture (FO) diets and phase 2 (P02) high-quality (HQ) vs. low-quality (LQ) pastures, with measurements in P01 (9 weeks), P02 (19 weeks), and phase 3 (P03; 41 weeks) when all calves were offered a common pasture diet.*

[b] *DMI (kg/day) was measured in two consecutive days during gas emission measurements.*

[c] *LW (kg) was analyzed adjusting LW to initial LW.*

[d] *Methane (CH_4) and hydrogen (H_2) production in two consecutive days (g/day) and yield per kilogram of DMI (y; g/kg DMI) measured.*

[e] *Total concentrations (mM) and individual proportions (%) of short-chain fatty acids (SCFA).*

[f] *Dietary treatments in each phase were evaluated as follows: a one-way ANOVA in P01 (9 weeks) to analyze FO vs. CO diets and a 2 × 2 factorial ANOVA in P02 and P03 to evaluate FO vs. CO and HQ vs. LQ dietary treatment effects and their interactions.*

[g] *Methane production per animal (g of CH_4/day).*

[h] *Methane yield (g of CH_4/kg of DMI).*

[i] *Hydrogen production per animal (g of H_2/day).*

[j] *Hydrogen yield (g of H_2/kg of DMI).*

[k] *Short-chain fatty acids.*

P01–P02 and those in P03; however, for the abundant bacteria, differences were only observed between pasture-fed calves in P01 and those in P03. The PLSDA of the archaea community (17 species; **Figure 2C**) and abundant archaea (7 species; **Figure 2D**) showed that concentrate-fed calves had different archaea diversity than those pasture-fed calves. Beta diversity within pasture-fed calves, for the archaea community and for the abundant archaea, showed that calves from the HQ groups (FOHQ and COHQ) in P02 differed from the other groups of calves in P01–P03.

Bacterial Community

The most prominent difference in P01 was a decrease ($P < 0.01$) in the proportion of *Fibrobacteres* and *Tenericutes* in CO compared with FO calves. No other differences were observed for bacteria phyla composition between treatments (**Table 3**). At the genus level, members of the *Firmicutes* phylum had greater ($P < 0.01$) *Lachnospiraceae* NK3A20 group, *Roseburia*, *Erysipelotrichaceae* UCG-002, and *Succiniclasticum* proportions, but lower ($P < 0.01$) *Ruminiclostridium* 9, *Ruminococcaceae* NK4A214 group, *Ruminococcus* 1, and *Kandleria* proportions in CO compared with FO calves. On the other hand, members of the *Bacteroidetes* phylum showed greater ($P < 0.01$) *Prevotella* 7 proportions but lower ($P < 0.01$) *Prevotella* 1; *Bacteroidales* BS11, RF16, and S24-7; *Prevotellaceae* UCG-003; and *Rikenellaceae* RC9 gut group proportions in CO compared with FO calves (**Table 4**).

During P02, the bacterial community in HQ when compared with LQ calves had greater *Firmicutes* proportions ($P < 0.01$) but lower *Fibrobacteres* proportions ($P < 0.01$). At the genus level, members of the *Firmicutes* phylum in HQ calves had greater ($P \leq 0.02$) proportions of *Butyrivibrio* 2, *Pseudobutyrivibrio*, *Roseburia*, *Ruminiclostridium* 9, and *Ruminococcaceae* NK4A214, but lower ($P \leq 0.02$) *Christensenellaceae* R-7, *Ruminococcus* 1,

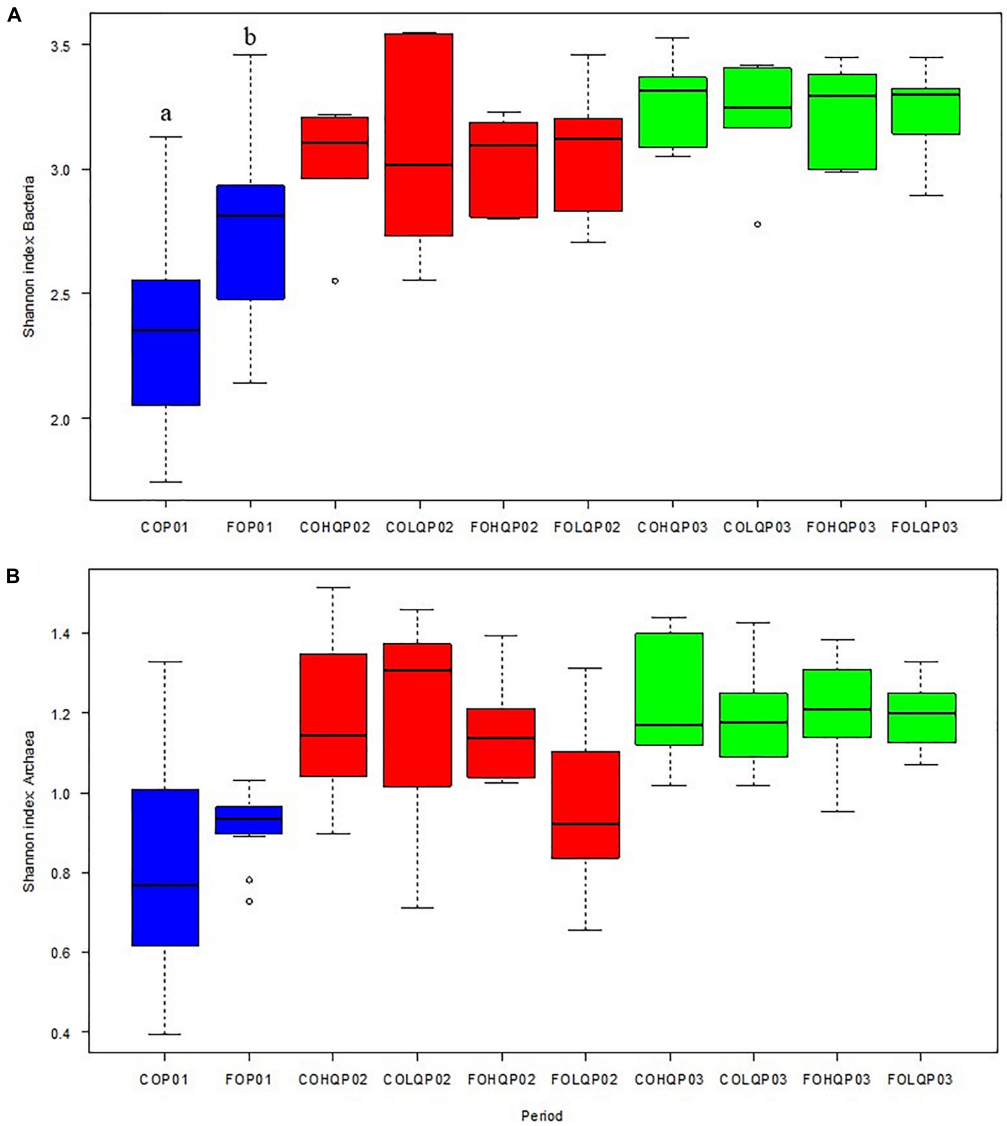


FIGURE 1 | Effects of dietary treatments in the Shannon diversity indices of the microbial communities in the rumen of calves during the three measurement phases. The alpha diversity of dietary treatment concentrate (CO) vs. pasture (FO) during phase 1 (P01; blue) and the combination of dietary treatments from P01 and high-quality (HQ) vs. low-quality (LQ) pastures from phase 2 (P02), resulting in FOHQ, FOLQ, COHQ, and COLQ, evaluated during P02 (red) and phase 3 (P03; green) are shown for **(A)** bacteria Shannon index and **(B)** archaea Shannon index. Boxplots represent the 25th and 75th percentiles, the whiskers extend to the most extreme data points, lines within boxes are the medians, and dots represent outliers.

Saccharofermentans, and *Succiniclasticum* compared with LQ calves. Conversely, members of the *Bacteroidetes* phylum in HQ calves had lower ($P \leq 0.03$) *Bacteroidales* BS11 and S24-7, *Prevotellaceae* UCG-003, and *Rikenellaceae* RC9 gut proportions than LQ calves. No effects ($P > 0.10$) from dietary treatments in P01 or the interaction ($P = 0.07$) between P01 and P02 were observed in P02 for the most abundant bacteria phyla and genera (**Tables 3**, **4**).

In P03, no direct effect of previous dietary treatments or their interactions was observed on the abundant bacteria at the phylum and genus levels (**Tables 3**, **4**). Low abundant bacterial genera showed similar patterns to abundant bacteria (**Supplementary Table S2**).

Archaeal Community

Table 5 shows the effects that dietary treatments during the three feeding phases have on the main archaea species in calves. During P01, the methanogenic community in CO calves was dominated by *Methanobrevibacter* (*Mbb.*) *boviskoreani*, while in FO calves, this was dominated by *Mbb. gottschalkii*. The abundant archaeal community in CO calves had greater ($P < 0.01$) proportions of *Mbb. boviskoreanii*, *Methanosphaera* (*Mph.*) A4, and *Mph.* Group 5, respectively, but lower ($P < 0.01$) proportions of *Mbb. gottschalkii*, *Mbb. ruminantium*, and *Mph. ISO3_F5* when compared with FO calves (**Supplementary Figure S1**).

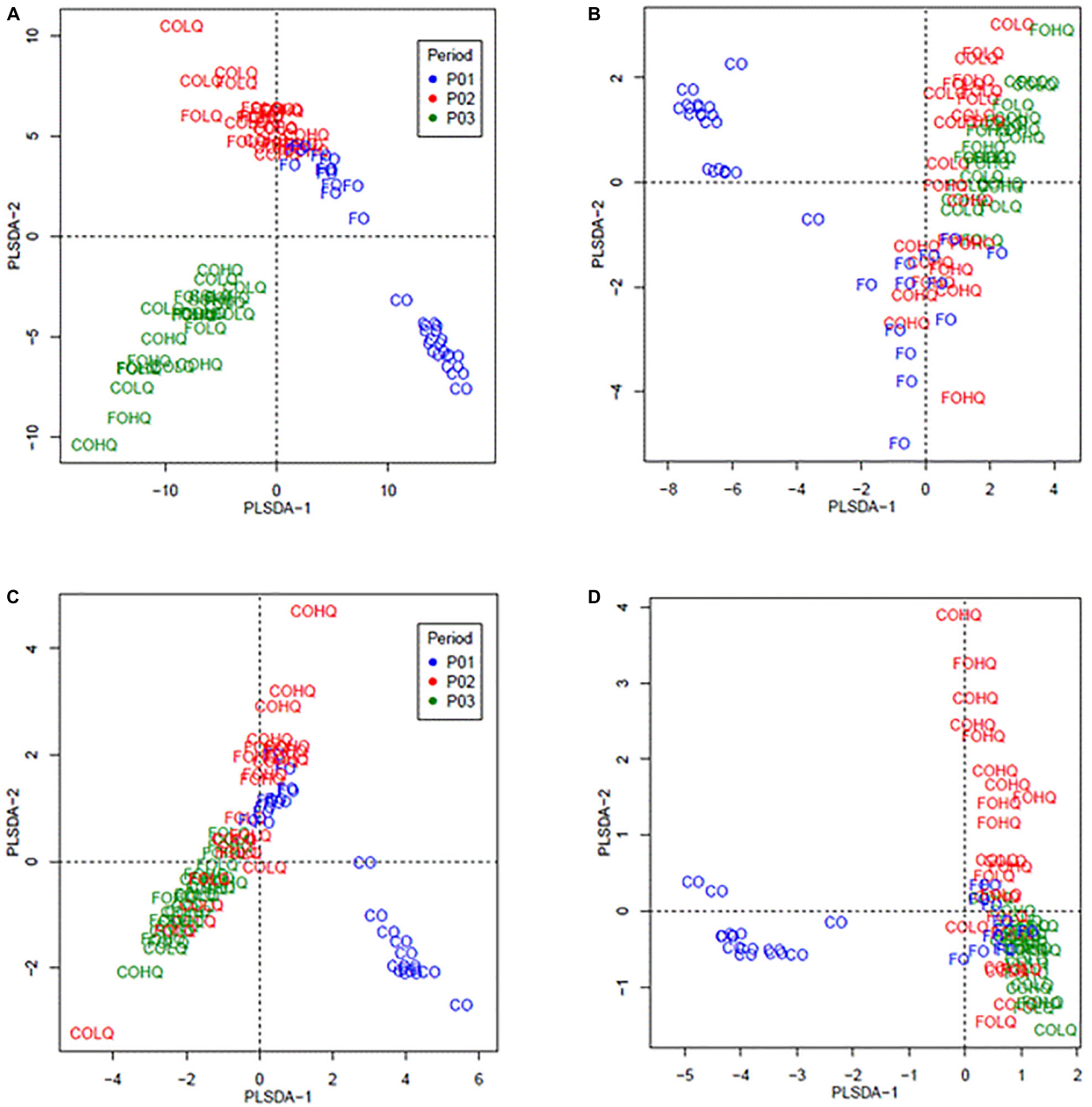


FIGURE 2 | Partial least square discriminant analysis (PLSDA) of the bacteria community at the genus level and archaea community at the species level from calves fed different treatments and treatment combinations in three sampling phases. Dietary treatments corresponded to: phase 1 (P01), concentrate (CO) vs. pasture (FO); and phase 2 (P02) high-quality (HQ) vs. low-quality (LQ) pastures. The treatment groups analyzed by phase were as follows: phase 1 (P01; blue) corresponded to groups from FO and CO; phase 2 (P02; red) and phase 3 (P03; green) were the groups formed by the combination of dietary treatments from P01 and P02, resulting in FOHQ, FOLQ, COHQ, and COLQ. **(A)** PLSDA of the bacteria community—364 bacterial genera, **(B)** PLSDA of the abundant (>0.7%) bacteria—25 abundant genera, **(C)** PLSDA of the archaea community—17 archaeal species, and **(D)** PLSDA of the abundant (>1.0%) archaea—7 abundant species.

In P02, the archaea community was dominated by *Mbb. gottschalkii* in both treatment groups. Compared with LQ calves, the abundant archaea community composition in HQ calves had greater ($P \leq 0.04$) proportions of *Mph.* sp. A4, *Mph.* Group 5, and *Mph. ISO3_F5*, but lower ($P < 0.01$) proportions of *Methanomassiliicoccales* (*Mmc.*) Group 10 sp. During P02, the archaea community did not show carryover effects from P01 treatments ($P \geq 0.24$) or interactions between P01 and P02 treatments ($P \geq 0.32$) in the abundant archaeal species.

In P03, animals that previously grazed the HQ swards showed greater ($P < 0.01$) *Mbb. ruminantium* proportions when compared with LQ calves. No main effects of P01 diets ($P \geq 0.08$) on the relative proportions of the abundant archaea were observed in P03.

TABLE 3 | Effect of dietary treatments[a] on the abundant bacteria phylum[b] during the measurement phases: 1 (P01), 2 (P02), and 3 (P03)[c].

	P01				
	CO	**95% CI (UL–LL)**	**FO**	**95% CI (UL–LL)**	***P*-val**
Actinobacteria	1.01	(0.635–1.623)	0.67	(0.420–1.073)	0.226
Bacteroidetes	40.45	(33.032–49.542)	52.93	(43.219–64.821)	0.074
Cyanobacteria	0.13	(0.068–0.244)	0.31	(0.164–0.591)	0.064
Fibrobacteres	0.01	(0.005–0.034)	0.40	(0.158–1.017)	< 0.0001
Firmicutes	48.73	(39.936–59.466)	40.45	(33.149–49.361)	0.199
Proteobacteria	0.49	(0.307–0.781)	0.43	(0.267–0.679)	0.676
Tenericutes	0.29	(0.176–0.486)	0.89	(0.536–1.485)	0.005
F:B[d]	1.20	(0.786–1.846)	0.76	(0.499–1.171)	0.131

	P02										
	CO	**95% CI (UL–LL)**	**FO**	**95% CI (UL–LL)**	**HQ**	**95% CI (UL–LL)**	**LQ**	**95% CI (UL–LL)**	***P*-T1**	***P*-T2**	***P*-int**
Actinobacteria	0.73	(0.547–0.986)	1.03	(0.771–1.389)	1.04	(0.774–1.395)	0.73	(0.545–0.982)	0.102	0.094	0.820
Bacteroidetes	55.23	(51.252–59.508)	55.46	(51.467–59.759)	53.59	(49.732–57.744)	57.15	(53.040–61.585)	0.935	0.218	0.627
Cyanobacteria	0.76	(0.552–1.039)	0.57	(0.412–0.776)	0.34	(0.245–0.462)	1.27	(0.927–1.746)	0.188	< 0.0001	0.576
Fibrobacteres	0.57	(0.377–0.851)	0.53	(0.351–0.792)	0.22	(0.144–0.324)	1.38	(0.921–2.080)	0.798	< 0.0001	0.512
Firmicutes	35.67	(32.431–39.233)	35.80	(32.551–39.378)	39.02	(35.480–42.921)	32.73	(29.754–35.994)	0.955	0.013	0.437
Proteobacteria	1.09	(0.797–1.499)	1.24	(0.901–1.695)	1.02	(0.741–1.394)	1.33	(0.969–1.822)	0.572	0.226	0.072
Tenericutes	1.79	(1.454–2.209)	1.49	(1.211–1.840)	1.56	(1.265–1.922)	1.72	(1.392–2.115)	0.212	0.508	0.728
F:B[d]	0.65	(0.547–0.763)	0.65	(0.546–0.763)	0.73	(0.616–0.861)	0.57	(0.485–0.677)	1.000	0.047	0.509

	P03										
	CO	**95% CI (UL–LL)**	**FO**	**95% CI (UL–LL)**	**HQ**	**95% CI (UL–LL)**	**LQ**	**95% CI (UL–LL)**	***P*-T1**	***P*-T2**	***P*-int**
Actinobacteria	0.6	(0.460–0.776)	0.62	(0.475–0.801)	0.71	(0.550–0.926)	0.52	(0.398–0.670)	0.858	0.082	0.338
Bacteroidetes	54.27	(51.198–57.522)	53.1	(50.092–56.279)	53.19	(50.178–56.376)	54.17	(51.110–57.423)	0.586	0.646	0.289
Cyanobacteria	0.36	(0.266–0.495)	0.32	(0.235–0.438)	0.35	(0.260–0.484)	0.33	(0.241–0.449)	0.571	0.727	0.571
Fibrobacteres	1.01	(0.732–1.398)	1.1	(0.795–1.518)	1.04	(0.749–1.431)	1.07	(0.776–1.483)	0.712	0.873	0.686
Firmicutes	36.88	(34.191–39.787)	38.57	(35.756–41.608)	37.8	(35.042–40.778)	37.63	(34.887–40.597)	0.394	0.932	0.243
Proteobacteria	1.19	(0.978–1.458)	1.12	(0.916–1.365)	1.31	(1.076–1.605)	1.02	(0.832–1.241)	0.631	0.072	0.914
Tenericutes	2.27	(1.975–2.613)	2.02	(1.756–2.324)	2.19	(1.902–2.516)	2.1	(1.824–2.413)	0.23	0.663	0.882
F:B[d]	0.68	(0.595–0.776)	0.73	(0.636–0.830)	0.72	(0.622–0.812)	0.69	(0.608–0.794)	0.469	0.803	0.259

[a] *Dietary treatments corresponded to phase 1 (P01) concentrate (CO) vs. pasture (FO) diets and phase 2 (P02) high-quality (HQ) vs. low-quality (LQ) pastures, with measurements in P01 (9 weeks), P02 (19 weeks), and phase 3 (P03; 41 weeks) when all calves were offered a common pasture diet.*
[b] *Measured effect corresponded to the seven most abundant ruminal bacterial phyla.*
[c] *Dietary treatments in each phase were evaluated as follows: a one-way ANOVA in P01 to analyze FO vs. CO diets and a 2 × 2 factorial ANOVA in P02 and P03 to evaluate FO vs. CO and HQ vs. LQ dietary treatment effects and their interactions.*
[d] *Firmicutes: Bacteroidetes ratio.*

DISCUSSION

Ruminal microorganisms are required for the degradation of plant components (Puniya et al., 2015; Huws et al., 2018; Gruninger et al., 2019). The establishment of these microbes in the rumen has been shown to be a dynamic progression from birth to adulthood (Jami et al., 2013; Rey et al., 2014). Recent studies have suggested that early interventions in life might imprint the microbial community, with such interventions having a persistent effect throughout the adult life of the animal (Yáñez-Ruiz et al., 2015). In this study, we have shown that feeding contrasting diets in early life (1 to 30 weeks) affects rumen fermentation patterns and rumen microbiota composition at the time of sampling; however, a permanent microbial or rumen fermentation imprint was not achieved.

Animal Performance, Rumen Enteric Emissions, and Fermentation Profiles

In pre-weaned ruminants, the intake of solid feed is affected by milk management (e.g., amount of milk, age at weaning, and weaning method) and both access to and the type of solid feed offered (Khan et al., 2011; Abbas et al., 2017). The above was observed in the present study in P01, where pre-weaning milk management and type of solid diet access resulted in differences in solid DMI. Despite the increased DMI in CO calves, the greater daily milk allowance and duration of milk feeding in FO calves resulted in heavier pre-weaning LW, as reported in the wider cohort of animals from the parent study (Burggraaf et al., 2020) and prior studies (Muir et al., 2002; Khan et al., 2011). Differences in solid feed intake between groups corresponded to differences in CH_4 production between CO

TABLE 4 | Effect of dietary treatments[a] on the abundant bacteria genus[b] during the three measurement phases[c].

Genus	P01					P02						
	CO	95% CI (UL–LL)	FO	95% CI (UL–LL)	*P*-val	CO	95% CI (UL–LL)	FO	95% CI (UL–LL)	HQ	95% CI (UL–LL)	LQ	95% CI (UL–LL)	*P*-T1	*P*-T2	*P*-int
Bacteroidales BS11 gut group	0.02	(0.014–0.041)	1.51	(0.875–2.592)	<0.0001	2.59	(1.858–3.613)	3.28	(2.351–4.572)	1.72	(1.232–2.395)	4.9	(3.546–6.896)	0.309	0.000	0.692
Bacteroidales RF16 group	0.00	(0.002–0.004)	0.86	(0.584–1.277)	<0.0001	0.88	(0.526–1.488)	0.90	(0.538–1.522)	0.63	(0.376–1.064)	1.3	(0.752–2.129)	0.950	0.063	0.981
Bacteroidales S24-7 group	0.25	(0.161–0.392)	1.48	(0.945–2.308)	<0.0001	1.07	(0.716–1.597)	1.33	(0.892–1.989)	0.87	(0.582–1.298)	1.6	(1.098–2.447)	0.429	0.030	0.651
Prevotella 1	0.78	(0.321–1.893)	39.31	(16.199–95.412)	<0.0001	35.27	(30.002–41.453)	35.13	(29.884–41.291)	37.82	(32.174–44.455)	32.8	(27.867–38.504)	0.972	0.205	0.620
Prevotella 7	18.38	(7.415–45.541)	0.35	(0.142–0.874)	<0.0001	0.06	(0.034–0.093)	0.06	(0.039–0.106)	0.11	(0.069–0.189)	0.0	(0.019–0.053)	0.706	0.001	0.990
Prevotellaceae UCG-001	0.87	(0.443–1.701)	0.86	(0.440–1.686)	0.9852	2.85	(1.997–4.065)	2.16	(1.512–3.078)	2.92	(2.045–4.162)	2.1	(1.477–3.007)	0.262	0.192	0.224
Prevotellaceae UCG-003	0.00	(0.003–0.006)	1.17	(0.758–1.808)	<0.0001	1.82	(1.312–2.511)	2.60	(1.883–3.602)	1.46	(1.053–2.014)	3.2	(2.347–4.491)	0.116	0.002	0.305
Rikenellaceae RC9 gut group	0.23	(0.162–0.319)	1.66	(1.185–2.331)	<0.0001	4.27	(3.432–5.324)	4.62	(3.707–5.751)	3.62	(2.904–4.505)	5.5	(4.381–6.796)	0.610	0.012	0.254
Fibrobacter	0.01	(0.005–0.036)	0.40	(0.152–1.053)	<0.0001	0.57	(0.377–0.851)	0.53	(0.351–0.792)	0.22	(0.144–0.324)	1.4	(0.921–2.080)	0.798	<0.0001	0.512
Christensenellaceae R-7 group	0.09	(0.056–0.142)	2.94	(1.836–4.697)	<0.0001	6.55	(5.607–7.656)	7.67	(6.562–8.961)	5.74	(4.913–6.709)	8.8	(7.489–10.225)	0.152	0.001	0.090
Butyrivibrio 2	0.01	(0.006–0.012)	0.41	(0.277–0.595)	<0.0001	0.92	(0.624–1.357)	0.93	(0.634–1.377)	1.41	(0.955–2.077)	0.6	(0.414–0.900)	0.955	0.005	0.636
Lachnospiraceae NK3A20 group	5.95	(3.206–11.04)	1.96	(1.054–3.630)	0.0150	1.86	(1.449–2.382)	2.17	(1.696–2.789)	2.26	(1.763–2.897)	1.8	(1.395–2.293)	0.361	0.180	0.341
Pseudobutyrivibrio	0.19	(0.114–0.302)	0.73	(0.451–1.196)	0.0004	1.13	(0.835–1.530)	1.05	(0.778–1.425)	1.61	(1.187–2.173)	0.7	(0.548–1.003)	0.734	0.001	0.454
Roseburia	6.59	(3.899–11.127)	0.67	(0.398–1.136)	<0.0001	0.34	(0.225–0.502)	0.27	(0.180–0.403)	0.72	(0.479–1.069)	0.1	(0.085–0.189)	0.431	<0.0001	0.561
[Eubacterium] coprostanoligenes group	0.30	(0.220–0.408)	0.94	(0.690–1.281)	<0.0001	1.07	(0.897–1.284)	1.16	(0.973–1.393)	1.00	(0.839–1.201)	1.2	(1.040–1.490)	0.511	0.093	0.036
Ruminiclostridium 9	0.00	(0.003–0.008)	2.71	(1.555–4.712)	<0.0001	0.59	(0.336–1.022)	0.62	(0.356–1.083)	1.34	(0.767–2.333)	0.3	(0.156–0.474)	0.881	0.000	0.672
Ruminococcaceae NK4A214 group	0.07	(0.043–0.107)	1.89	(1.204–2.970)	<0.0001	1.93	(1.559–2.382)	2.09	(1.691–2.585)	2.41	(1.949–2.979)	1.7	(1.352–2.067)	0.577	0.019	0.946
Ruminococcaceae UCG-014	1.27	(0.813–1.981)	0.97	(0.624–1.520)	0.3932	1.35	(1.048–1.727)	1.15	(0.896–1.476)	1.21	(0.939–1.547)	1.3	(1.000–1.647)	0.364	0.715	0.925
Ruminococcus 1	0.55	(0.308–0.985)	2.10	(1.176–3.767)	0.0027	1.14	(0.943–1.371)	0.99	(0.822–1.194)	0.90	(0.750–1.090)	1.2	(1.033–1.502)	0.290	0.020	0.448
Saccharofermentans	0.00	(0.002–0.006)	0.74	(0.410–1.321)	<0.0001	0.78	(0.635–0.962)	0.68	(0.548–0.831)	0.59	(0.478–0.723)	0.9	(0.730–1.105)	0.309	0.007	0.136
Erysipelotrichaceae UCG-002	3.78	(0.824–17.337)	0.09	(0.020–0.421)	0.0017	0.01	(0.005–0.018)	0.01	(0.007–0.024)	0.02	(0.012–0.040)	0.0	(0.003–0.011)	0.565	0.006	0.377
Kandleria	0.00	(0.001–0.008)	1.82	(0.785–4.195)	<0.0001	0.09	(0.039–0.196)	0.04	(0.017–0.082)	0.42	(0.189–0.943)	0.0	(0.003–0.017)	0.127	<0.0001	0.079
Succiniclasticum	2.21	(1.634–2.981)	0.95	(0.705–1.286)	0.0005											
Selenomonas 1	0.01	(0.007–0.020)	0.89	(0.537–1.470)	<0.0001											
Mollicutes RF9	0.28	(0.160–0.490)	0.80	(0.459–1.411)	0.0114											

(Continued)

TABLE 4 | (Continued)

Genus	P01										
	CO	95% CI (UL–LL)	FO	95% CI (UL–LL)	*P*-val						
Succiniclasticum	1.27	(0.959–1.682)	1.54	(1.159–2.034)	1.09	(0.824–1.445)	1.8	(1.350–2.367)	0.331	0.017	0.165
Selenomonas 1	0.00	(0.003–0.006)	0.01	(0.004–0.008)	0.01	(0.007–0.012)	0.0	(0.002–0.004)	0.269	<0.0001	0.228
Mollicutes RF9	0.01	(0.012–0.019)	0.01	(0.010–0.015)	0.01	(0.010–0.016)	0.0	(0.011–0.018)	0.213	0.607	0.782
	P03										
	CO	95% CI (UL–LL)	FO	95% CI (UL–LL)	HQ	95% CI (UL–LL)	LQ	95% CI (UL–LL)	*P*-T1	*P*-T2	*P*-int
Bacteroidales BS11 gut group	3.66	(3.085–4.331)	3.77	(3.180–4.465)	3.71	(3.130–4.395)	3.71	(3.133–4.400)	0.795	0.993	0.198
Bacteroidales RF16 group	0.89	(0.644–1.218)	0.73	(0.532–1.006)	0.82	(0.594–1.123)	0.79	(0.577–1.091)	0.385	0.894	0.026
Bacteroidales S24-7 group	3.70	(3.034–4.510)	3.40	(2.789–4.146)	3.58	(2.937–4.366)	3.51	(2.881–4.282)	0.538	0.888	0.173
Prevotella 1	33.59	(29.770–37.902)	32.74	(29.019–36.945)	32.68	(28.966–36.879)	33.65	(29.824–37.971)	0.758	0.725	0.839
Prevotella 7	0.06	(0.040–0.093)	0.08	(0.052–0.120)	0.08	(0.050–0.116)	0.06	(0.042–0.097)	0.383	0.526	0.823
Prevotellaceae UCG-001	2.60	(2.148–3.141)	2.56	(2.116–3.093)	2.45	(2.028–2.965	2.71	(2.241–3.276)	0.907	0.447	0.154
Prevotellaceae UCG-003	2.64	(2.146–3.252)	2.42	(1.965–2.978)	2.63	(2.132–3.232)	2.43	(1.978–2.997)	0.539	0.599	0.598
Rikenellaceae RC9 gut group	3.71	(3.260–4.225)	3.69	(3.240–4.195)	3.64	(3.197–4.140)	3.76	(3.306–4.281)	0.937	0.706	0.754
Fibrobacter	1.01	(0.732–1.398)	1.10	(0.795–1.518)	1.04	(0.749–1.431)	1.07	(0.776–1.483)	0.712	0.873	0.686
Christensenellaceae R-7 group	7.26	(6.358–8.301)	9.17	(8.025–10.479)	8.74	(7.647–9.985)	7.62	(6.672–8.712)	0.018	0.147	0.404
Butyrivibrio 2	0.61	(0.466–0.796)	0.62	(0.471–0.804)	0.67	(0.509–0.869)	0.56	(0.431–0.736)	0.645	0.370	0.645
Lachnospiraceae NK3A20 group	0.69	(0.572–0.836)	0.86	(0.710–1.037)	0.73	(0.608–0.888)	0.81	(0.668–0.976)	0.109	0.472	0.607
Pseudobutyrivibrio	1.02	(0.826–1.271)	0.92	(0.738–1.136)	1.06	(0.852–1.311)	0.89	(0.715–1.101)	0.450	0.247	0.034
Roseburia	0.34	(0.283–0.418)	0.27	(0.222–0.327)	0.29	(0.237–0.349)	0.32	(0.266–0.392)	0.078	0.385	0.059
[Eubacterium] coprostanoligenes group	1.23	(1.009–1.494)	1.23	(1.013–1.501)	1.15	(0.943–1.397)	1.32	(1.084–1.605)	0.972	0.310	0.386
Ruminiclostridium 9	0.12	(0.066–0.204)	0.13	(0.071–0.220)	0.13	(0.075–0.234)	0.11	(0.062–0.192)	0.840	0.608	0.133
Ruminococcaceae NK4A214 group	2.87	(2.502–3.298)	2.98	(2.594–3.418)	3.07	(2.671–3.520)	2.79	(2.430–3.203)	0.705	0.325	0.810
Ruminococcaceae UCG-014	0.67	(0.531–0.843)	0.61	(0.481–0.763)	0.62	(0.492–0.781)	0.65	(0.519–0.824)	0.530	0.738	0.901
Ruminococcus 1	1.97	(1.519–2.552)	1.75	(1.352–2.271)	1.64	(1.263–2.121)	2.11	(1.626–2.731)	0.515	0.166	0.053
Saccharofermentans	1.02	(0.887–1.167)	1.00	(0.871–1.147)	0.97	(0.846–1.113)	1.05	(0.914–1.202)	0.851	0.419	0.117
Erysipelotrichaceae UCG-002	0.00	(0.002–0.007)	0.00	(0.001–0.003)	0.01	(0.002–0.005)	0.00	(0.001–0.005)	0.061	0.794	0.336
Kandleria	0.12	(0.044–0.345)	0.10	(0.035–0.277)	0.11	(0.040–0.311)	0.11	(0.039–0.307)	0.757	0.986	0.122
Succiniclasticum	2.66	(2.152–3.297)	2.18	(1.761–2.698)	2.20	(1.776–2.721)	2.64	(2.133–3.269)	0.181	0.219	0.053
Selenomonas 1	0.85	(0.545–1.335)	0.71	(0.451–1.106)	0.71	(0.456–1.118)	0.84	(0.539–1.321)	0.543	0.590	0.133
Mollicutes RF9	1.46	(1.182–1.793)	1.33	(1.077–1.633)	1.36	(1.106–1.677)	1.42	(1.152–1.746)	0.517	0.776	0.870

[a] *Dietary treatments corresponded to phase 1 (P01) concentrate (CO) vs. pasture (FO) diets and phase 2 (P02) high-quality (HQ) vs. low-quality (LQ) pastures, with measurements in P01 (9 weeks), P02 (19 weeks), and phase 3 (P03; 41 weeks) when all calves were offered a common pasture diet.*

[b] *Measured effect corresponded to the 35 bacterial genera with a relative abundance > 0.50% across rumen samples.*

[c] *Dietary treatments in each phase were evaluated as follows: a one-way ANOVA in P01 to analyze FO vs. CO diets and a 2 × 2 factorial ANOVA in P02 and P03 to evaluate FO vs. CO and HQ vs. LQ dietary treatment effects and their interactions.*

and FO calves, where greater DMI was associated with greater CH_4 production (Jonker et al., 2016; Bird-Gardiner et al., 2017). However, CH_4 production per kilogram of DMI (yCH_4) was lower in CO calves with higher energy content in grain-based diets than in FO calves with a forage diets (**Table 1**), as previously stated by Johnson and Johnson (1995). Differences in dietary nutrient composition and its digestibility include changes in ruminal pH and in cellulolytic activity and fiber degradation, level of starch by-pass to the intestine, and percentage of SCFA which all may influence methanogenesis (Benchaar et al., 2001; Jentsch et al., 2007). Beauchemin and McGinn (2005) indicated that feeding high-concentrate diets (47–58% of starch) decreases methane yield and lowers the acetate:propionate ratio. In the rumen, the fermentation of diets rich in structural carbohydrates produces greater proportions of acetate with the release of hydrogen, whereas the intake of diets rich in starch contents results in greater propionate proportions without hydrogen production (Ungerfeld, 2020). The propionate pathway competes for hydrogen with hydrogenotrophic methanogens (Janssen, 2010). Therefore, the high availability of starch in the diet of CO calves resulted in a reduction of methane yield in part due to an increased propionate production (Sauvant et al., 2011; Williams et al., 2019). Moreover, the rumen pH of grass-fed ruminants ranges between 6.0 and 7.0 under normal physiological conditions (Grünberg and Constable, 2009). However, the consumption of diets rich in grains results in greater concentration of SCFA and production of lactic acid that can build up in the rumen and reduce the ruminal

TABLE 5 | Effect of dietary treatments[a] on the abundant archaea species[b] during the three measurement phases[c].

Species	P01				
	CO	95% CI (UL–LL)	FO	95% CI (UL–LL)	*P*-val
Methanomassiliicoccales Group 10 sp.	0.15	(0.053–0.451)	0.61	(0.210–1.789)	0.073
Methanobrevibacter.boviskoreani.clade	64.03	(47.111–87.013)	0.05	(0.036–0.067)	<0.001
Methanobrevibacter.gottschalkii.clade	2.53	(1.332–4.812)	57.40	(30.195–109.124)	<0.001
Methanobrevibacter.ruminantium.clade	0.81	(0.272–2.416)	24.36	(8.177–72.556)	<0.001
Methanosphaera sp. A4	8.58	(4.322–17.029)	0.05	(0.027–0.106)	<0.001
Methanosphaera sp. Group 5	4.35	(2.719–6.954)	1.73	(1.084–2.772)	0.009
Methanosphaera sp. ISO3_F5	0.07	(0.032–0.144)	4.35	(2.062–9.194)	<0.001

	P02										
Species	CO	95% CI (UL–LL)	FO	95% CI (UL–LL)	HQ	95% CI (UL–LL)	LQ	95% CI (UL–LL)	*P*-T1	*P*-T2	*P*-int
Methanomassiliicoccales Group 10 sp.	1.69	(0.889–3.201)	1.48	(0.777–2.799)	0.56	(0.297–1.070)	4.41	(2.325–8.372)	0.761	<0.001	0.966
Methanobrevibacter boviskoreani.clade	0.06	(0.023–0.149)	0.03	(0.012–0.075)	0.04	(0.014–0.088)	0.05	(0.020–0.128)	0.294	0.564	0.586
Methanobrevibacter gottschalkii.clade	58.91	(51.408–67.507)	65.36	(57.035–74.896)	58.16	(50.750–66.643)	66.21	(57.775–75.867)	0.274	0.176	0.787
Methanobrevibacter.ruminantium.clade	10.25	(5.966–17.622)	10.35	(6.020–17.782)	10.61	(6.172–18.229)	10.00	(5.820–17.191)	0.981	0.875	0.847
Methanosphaera sp. A4	0.08	(0.034–0.184)	0.06	(0.024–0.133)	0.13	(0.055–0.299)	0.04	(0.015–0.082)	0.577	0.036	0.327
Methanosphaera sp. Group 5	9.03	(5.219–15.626)	5.76	(3.327–9.960)	14.94	(8.636–25.853)	3.48	(2.011–6.020)	0.240	<0.001	0.373
Methanosphaera sp. ISO3_F5	3.34	(1.885–5.9226)	3.36	(1.894–5.948)	6.13	(3.460–10.870)	1.83	(1.032–3.241)	0.991	0.005	0.323

	P03										
Species	CO	95% CI (UL–LL)	FO	95% CI (UL–LL)	HQ	95% CI (UL–LL)	LQ	95% CI (UL–LL)	*P*-T1	*P*-T2	*P*-int
Methanomassiliicoccales Group 10 sp.	4.65	(3.189–6.787)	4.60	(3.154–6.712)	3.55	(2.433–5.179)	6.03	(4.133–8.797)	0.966	0.052	0.882
Methanobrevibacter.boviskoreani.clade	0.04	(0.025–0.066)	0.03	(0.021–0.056)	0.05	(0.030–0.080)	0.03	(0.018–0.046)	0.628	0.113	0.016
Methanobrevibacter.gottschalkii.clade	64.56	(60.961–68.375)	64.22	(60.635–68.008)	62.89	(59.380–66.602)	65.93	(62.249–69.819)	0.892	0.239	0.937
Methanobrevibacter.ruminantium.clade	11.94	(9.892–14.405)	11.99	(9.933–14.464)	15.35	(12.719–18.521)	9.32	(7.725–11.250)	0.975	<0.001	0.833
Methanosphaera sp. A4	0.04	(0.009–0.133)	0.04	(0.009–0.130)	0.04	(0.010–0.143)	0.03	(0.009–0.121)	0.982	0.860	0.674
Methanosphaera sp. Group 5	3.01	(2.116–4.283)	2.44	(1.717–3.476)	2.40	(1.689–3.420)	3.06	(2.151–4.354)	0.393	0.324	0.200
Methanosphaera sp. ISO3_F5	6.92	(5.061–9.453)	10.27	(7.517–14.042)	8.93	(6.531–12.200)	7.96	(5.825–10.881)	0.077	0.595	0.668

[a] *Dietary treatments corresponded to phase 1 (P01) concentrate (CO) vs. pasture (FO) diets and phase 2 (P02) high-quality (HQ) vs. low-quality (LQ) pastures, with measurements in P01 (9 weeks), P02 (19 weeks), and phase 3 (P03; 41 weeks) when all calves were offered a common pasture diet.*

[b] *Measured effect corresponded to the seven archaeal species with a relative abundance > 1.00% across rumen samples.*

[c] *Dietary treatments in each phase were evaluated as follows: a one-way ANOVA in P01 to analyze FO vs. CO diets and a 2 × 2 factorial ANOVA in P02 and P03 to evaluate FO vs. CO and HQ vs. LQ dietary treatment effects and their interactions.*

pH below 6.0 (Dijkstra et al., 2012). Ruminal pH was not measured in the current study, but it can be speculated that CO calves with higher SCFA concentrations and less fiber contents in the diet had a lower ruminal pH than the grazing groups (Hook et al., 2011). Reduction in ruminal pH may affect methanogenic microbes and further decrease CH_4 yield (Van Kessel and Russell, 1996).

During P02, results from the parent production trial (Burggraaf et al., 2020) showed no compensatory growth in CO calves reared on restricted milk, which correspond to observations in previous studies (Wardrop, 1966). Conversely, no growth checks were observed in FO calves, which indicated that an adequate rumen development was achieved, despite the high volume of milk fed, consistent with prior studies (Khan et al., 2011). Forage quality in this feeding phase was critical for lifetime performance of post-weaned calves, where calves fed HQ forages resulted in heavier LW when compared with calves fed LQ forages. This agrees with de Clifford et al. (2014), who showed that improved growth of calves is achieved when fed forages with higher metabolizable energy, metabolizable protein, and digestibility. The differences in methane production during P01 did not persist when these group of calves were allocated into different forage treatment diets in P02. During this P02, the intake of low-quality pastures with high fiber contents lowered DMI, resulting in low CH_4 production (g/day). These observations corresponded to those reported in growing heifers, where DMI was reduced in forage diets with low-quality and high NDF content (Pino et al., 2018). The lack of difference in methane yield (g/kg of DM) between calves grazed in high- or low-quality pasture is likely a result of the effects that the diet had on daily methane output and DMI; this lack of a response of methane yield to pasture quality has been also shown in adult cattle (Jonker et al., 2016) and sheep (Muetzel and Clark, 2015). Conversely, the intake of grasses with high protein and low fiber content increased the total concentration of SCFA and decreased the acetate to propionate ratio, i.e.,

HQ calves, characteristic of the intake of grasses with high organic matter digestibility (Owens and Basalan, 2016; Pino et al., 2018). On the other hand, reduced proportions of protein degradation products, i.e., isobutyrate and isovalerate, in LQ calves reflected the low crude protein content in the pasture (Brinkhaus et al., 2017).

When the animals were on the same diet in P03, treatment differences in LW were sustained due to the lack of compensatory growth following the dietary intervention in P01 and P02. Results from the present study and the parent production study (Burggraaf et al., 2020) showed that extended nutritional restrictions imposed during the first 7 months of age limited the capacity of cattle to exhibit compensatory growth. This agrees with similar studies where severe pre- and post-weaning nutritional restrictions limit the capacity of cattle to exhibit compensatory growth and achieve equivalent weight for age in later life (Ryan, 1990; Hearnshaw, 1997; Shamay et al., 2005). No differences of DMI, CH_4 emissions, and SCFA profiles were observed between the two groups. Similar results were observed in lambs fed high and low fiber diets in early life with no effect on rumen metabolites after 16 to 20 weeks of treatment cessation (Yáñez-Ruiz et al., 2010). Our results indicate that dietary composition at the time of measurement is the major driver for DMI, rumen fermentation pathways, and methane production independently of the previous feeding regimes, thus showing that at the metabolic level no imprint of pre- and post-weaning treatments had occurred.

Bacterial Composition in the Rumen

The bacterial diversity in the rumen is host specific; however, variations in the composition of the ingested diet result in diversity changes of the prokaryotic domains harbored in the rumen (Henderson et al., 2015). In the present study, differences in bacterial diversity observed between treatments in the different dietary phases were consistent with those reported in young and adult ruminants, suggesting that the degradation of more structural diets is a complex process which requires a more diverse consortium of microbes working together (Kim et al., 2016; Belanche et al., 2019).

In young ruminants, the bacteria present in the rumen is largely represented by the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, whose changes in relative abundance have been associated with animal growth and diet (Li et al., 2012; Jami et al., 2013; Rey et al., 2014). Previously, reports in calves showed that the fiber content of the diet led to an increased *Firmicutes*:*Bacteroidetes* ratio (F:B ratio) (Kim et al., 2016), but no such difference was observed in P01 of the present experiment despite the differences in structural contents between diets. It cannot be elucidated whether restriction in forage intake by the allowance of high-milk volumes might affect the F:B ratio as observed in FO calves from the present study. However, in P02, the intake of low-quality pastures increased F:B ratio as previously stated. The intake of diets rich in fiber, e.g., FO in P01 and LQ in P02, resulted in increased proportion of cellulose-degrading microorganism such as *Fibrobacteres* (Ransom-Jones et al., 2012). The lack of any differences in P03 confirms that diet at the time is the major driver of the microbial community at the phylum level and that changes observed by differences in diet composition pre- and post-weaning do not lead to a permanent change of the rumen microbiota.

Prevotella is one of the most abundant ruminal bacterial groups and plays a key role in the degradation and utilization of a large variety of carbohydrates and proteins entering the rumen (Cotta, 1992; Kim et al., 2016; Solden et al., 2016). In the present study, *Prevotella* was the dominant genus in the rumen of calves independent of age and diet, as observed previously in young and adult ruminants (Rey et al., 2014; Henderson et al., 2015). However, our results indicate that the differences between *Prevotella* 7 and *Prevotella* 1 are driven by dietary composition. The dominance of *Prevotella* 7 was only observed in CO calves in accordance to sheep fed 95% concentrates (McLoughlin et al., 2020), while *Prevotella* 1 prevailed in FO and all the other groups of grazing calves similar to reports in sheep fed high forage diets (Xie et al., 2019). *Prevotella* 1 group includes the species *P. ruminicola*, *P. brevis*, and *P. bryantii* (Henderson et al., 2019) that produce mainly acetate and succinate (Avguštin et al., 1997), rather than propionate (Avguštin et al., 1997; Seshadri et al., 2018). *Prevotella* 1 species possess extensive repertoires of polysaccharide utilization loci and carbohydrate active enzymes targeting various plant polysaccharides (Accetto and Avguštin, 2019). *Prevotella* 7 includes species like *P. albensis* (Henderson et al., 2019) that mostly produce acetate, succinate, and propionate (Avguštin et al., 1997; Seshadri et al., 2018). Annotation of *de novo* assembled contigs from metagenomic data not only identified sequences encoding for α-amylase enzymes in uncharacterized strains of *P. albensis* but also revealed the potential to metabolize xylan as an alternative substrate (Bandarupalli and St-Pierre, 2020). The higher proportion of propionate in the rumen of concentrate-fed calves was at least partially due to the differences in these two dominant rumen bacterial genera.

The intake of concentrates in CO calves increased the relative abundance of bacteria from the genera *Roseburia*, *Lachnospiraceae* NK3A20 group, and *Erysipelotrichaceae* UCG-002, which have a high affinity for utilizing highly degradable mono- and polysaccharides (Stanton et al., 2009; Huo et al., 2014). Increases of these soluble carbohydrate-utilizing genera have been observed in the rumen contents of cattle and sheep fed greater ratios of dietary concentrates (McLoughlin et al., 2020). *Roseburia* and *Lachnospiraceae* NK3A20 are butyrate-producing microorganisms (Duncan et al., 2002). However, even though the principal fermentation product of these organisms is butyrate, no effect on the proportion or concentrations (CO = 11.3 mM vs. FO = 8.4 mM, SED = 1.62; $P = 0.09$) of this SCFA between the two groups was observed. This may be because *Roseburia* and *Lachnospiraceae* NK3A20 made up only 6.6% and 6.0% of the community, respectively. Likewise, the family *Erysipelotrichaceae* ferments a wide range of sugars to produce mainly lactic acid (Deusch et al., 2017). Studies in low methane-emitting sheep have shown that high proportions of *Erysipelotrichaceae* are associated with increases in lactic acid production, resulting in less hydrogen and methane formation (Kamke et al., 2016). In the present study, the relative abundance of *Erysipelotrichaceae*

UCG-002 in the CO group may have favored the production of propionate by lactate-utilizing bacteria, i.e., *Megasphaera* (CO = 0.33% vs. FO = 0.00%, SED = 0.086; $P < 0.01$) (Kamke et al., 2016). Reductions in methane formation are attributed to the H_2 utilization for propionate formation, which competes with the most common hydrogenotrophic methanogens (Liu and Whitman, 2008; Ungerfeld, 2020). Therefore, increases in the intake of starch favored the proportions of amylolytic microorganisms, whose metabolism increased the proportions of propionate during ruminal fermentation but reduced the production of hydrogen and ultimately its availability to be used by methanogens. However, although the amylolytic bacterial microbiota dominated during the pre-weaning rearing phase in concentrate-fed calves, it did not persist into the post-weaning phases of grazing calves.

Interestingly, *Kandleria*, which degrades different sugars, including D-galactose and lactose (lactate producer) (Kumar et al., 2018), was found in high proportions in calves from the FO group. The genus *Kandleria* has been isolated from the rumen of young calves fed on only milk diets (Salvetti et al., 2011). Therefore, the observed relative abundance of this genus in FO calves may be associated with the degradation of milk sugars leaking into the rumen. This can be confirmed by the low relative abundance of *Kandleria* in FO calves when transitioning into P02 and P03 despite the soluble sugars from fresh mixed sward of ryegrass and white clover.

Bacteria from the genus *Ruminococcus* and *Fibrobacter* are considered major cellulolytic degraders (Koike and Kobayashi, 2001; Ransom-Jones et al., 2012; Abdul Rahman et al., 2016). The proportion of *Fibrobacter* in P01 and P02 was higher in calves consuming diets with the highest fiber contents within each dietary phase. Such association of high relative abundance of *Fibrobacter* has been shown in heifers (Petri et al., 2013) and sheep (Belanche et al., 2019) fed forage diets. Similar effects have also been observed for the various genera in the *Ruminococcaceae*, but their proportional increases with the increase in fiber content of the diets was not as pronounced as for *Fibrobacter*, which may be due to the fact that *Ruminococcaceae* have a much wider spectrum of metabolizable substrates compared with *Fibrobacter* (Thurston et al., 1994).

Besides the known fiber degraders, some unclassified *Bacteroidetes* genera such as *Rikenellaceae* RC9, *Bacteroidales*, and *Prevotellaceae* UCG-003 and the *Firmicutes* genus *Christensenellaceae* R-7 were increased in calves fed diets rich in fiber, i.e., LQ calves. The genus *Rikenellaceae* RC9, with no as yet defined metabolic function, is one of the most prevalent microbes in the rumen microbiota (Henderson et al., 2015; De Mulder et al., 2017) and abundant in rich fibrous diets (Petri et al., 2013; Schären et al., 2017). We found similar results with increased proportions of *Rikenellaceae* in the high fiber treatments in P01 and P02. Correspondingly, hemicellulose and monomeric sugar (xylose, fucose, mannose, and rhamnose) degraders (Ormerod et al., 2016; Solden et al., 2017) from *Bacteroidales*, such as the genera BS11 gut group and S24-7 group, were increased in calves with high fiber intakes. In the present study, *Christensenellaceae* R-7 was the second most abundant bacteria genus in the rumen of grazing calves. These also appear to be related to fiber degradation as their levels were increased in the high fiber treatments in the first two phases. These findings agreed with reports in dairy cows, where increases of fiber in the diet resulted in an increase of this genus (Lima et al., 2015). Our data suggest that not only are the well-described families like *Fibrobacteracea* and *Ruminococcaceae* involved in plant fiber degradation but also members of the *Rikenellaceae* RC9, *Bacteroidales* BS11, *Bacteroidales* S24-7, and *Christensenellaceae* R-7. However, further studies of these genera are required to investigate their growth, ecology, and metabolism when ruminants are fed diets rich in fiber.

The ingestion of high-quality forage diets, i.e., HQ calves, with less NDF and ADF content favored the growth of bacteria from the *Firmicutes* genera such as *Ruminococcaceae* NK4A214, *Butyrivibrio* 2, *Ruminiclostridium* 9, and *Pseudobutyrivibrio* (Rainey, 1996; Ravachol et al., 2016). In post-weaned calves, *Butyrivibrio* and *Pseudobutyrivibrio* were found in greater proportion when transitioning into forages with lower contents of hemicellulose. Species belonging to the genus *Butyrivibrio* and *Pseudobutyrivibrio* are important degraders of plant polysaccharides, i.e., hemicelluloses (arabinoxylans) and pectin (Palevich et al., 2019b). However, some species of *Butyrivibrio* are unable to grow on structural plant components, and their role in the rumen appears to be as a utilizer of monosaccharides, disaccharides, and oligosaccharides made available by the degradative activities of other bacterial species (Palevich et al., 2017; Palevich et al., 2019a). Correspondingly, bacterial species from the genus *Pseudobutyrivibrio* are metabolically versatile and capable of growing on a range of simple mono- or oligosaccharides derived from complex plant polysaccharides such as pectins, mannans, starch, and hemicelluloses (Palevich et al., 2020). These findings may explain the increased relative abundance of *Butyrivibrio* and *Pseudobutyrivibrio* in high-quality forages.

Archaea Composition in the Rumen

Rumen archaea are much less diverse than rumen bacteria, which likely reflects the narrow range of substrates they use (Janssen, 2010; Seedorf et al., 2014). Variations in the dietary composition have been shown to alter the archaeal community (Henderson et al., 2015) due to changes in fermentation patterns that affect the proportion of their substrates and metabolic activity (Lana et al., 1998; Ungerfeld, 2020). In the current study, the archaeal microbiota of grazing calves, across all treatments, was dominated by *Mbb. gottschalkii* and *Mbb. ruminantium*, which was in agreement with observations from adult ruminants fed diets with high fiber contents (Henderson et al., 2015; Seedorf et al., 2015). Conversely, the group of calves fed high proportions of concentrate in the diet showed increases of *Mbb. boviskoreani*, an organism that has been previously found and isolated from cattle fed diets rich in concentrates (Lee et al., 2013; Snelling et al., 2019). Therefore, these results indicate that the fermentation of diets with high contents in structural carbohydrates favored the prevalence of hydrogenotrophic archaea such as *Mbb. gottschalkii* and *Mbb. ruminantium*. Additionally, as previously discussed, rumen pH was likely to be lower in CO calves, which may

decrease the relative abundance of abundant archaea species such as *Mbb. gottschalkii* and *Mbb. Ruminantium* in these calves. The metabolic activity of these methanogen species, however, begins to be inhibited when the pH drops below their optimum pH of 7.0–7.2 for growing (Miller and Lin, 2002; Janssen, 2010). In contrast, *Mbb. boviskoreani* growth is supported at a ruminal pH of 5.5, whereas its optimum pH is between 6.0 and 7.0 (Lee et al., 2013). Another observation is the low relative abundance of *Methanomassiliicoccus* (*Mmc.*) Group 10 sp. and *Methanosphaera* (*Mph.*) *ISO3_F5* in the CO group. Our results agreed with the low proportions of *Mmc.* reported in heifers fed diets rich in concentrate (Zhang et al., 2017). However, it is not clear whether the low abundance of *Mmc.* observed in concentrate-fed calves is due to a reduction in ruminal pH or a competition for substrates, e.g., methanol, with methylotrophic archaea such as *Mph*, as further discussed.

The intake of highly digestible diets, i.e., CO and HQ treatments, showed high proportions of the genera *Methanosphaera* (*Mph*). These methanogens reduce methanol (Fricke et al., 2006; Kelly et al., 2019). Methanol in the rumen is derived from the demethoxylation of dietary pectins and other methylated plant polysaccharides *via* pectin methylesterase activity (Dehority, 1969; Kelly et al., 2019). Additionally, methanol production is negatively affected by pasture maturity that has lower pectin degradation (Dehority et al., 1962). Clover and other non-grass pasture species usually contain higher proportion of pectins than grasses (Thomson, 1984; Hammond et al., 2011). In cows, the intake of diets rich in clover can favor the relative abundance of *Mph. sp. Group 5* and *Mph. sp. ISO3_F5* in the rumen (Bowen et al., 2018; Smith et al., 2020). In the present study, calves consuming high-quality sward with 35.5% of white clover (DM basis) may have produced more methanol, favoring the increase of *Mph. sp. Group 5* and *Mph. sp. ISO3_F5* when compared with the intake of non-irrigated low-quality pastures with 4.0% of white clover and more fibrous contents (Burggraaf et al., 2020). Conversely, *Mph. sp. A4* was found in high proportions in CO diets similar to that observed in pre-weaned calves by Dias et al. (2017), who indicated that the pectins present in the starter concentrate may contribute to the formation of methanol and increases in the relative abundance of this archaea species. However, further studies are required to elucidate that the production of methanol, and a hypothetical reduction in ruminal pH, in concentrate-fed calves may correspond to the increasing proportion of *Mph. sp. A4* in the rumen.

In the present study, calves consuming diets rich in fiber showed increases in *Mmc*. This order is a methylotrophic methanogen that utilizes compounds like methanol, methylamines, dimethylamine, and trimethylamine (Borrel et al., 2012; Poulsen et al., 2013). Plant-derived glycine, betaine, and choline are rapidly metabolized by ruminal bacteria using choline trimethylamine lyase (Kelly et al., 2019). Fiber-rich diets, where fermentation results in high ratios of acetate to propionate, are associated with a greater concentration of methylamine, dimethylamine, and trimethylamine compared with highly digestible diets such as corn silage (Deusch et al., 2017). In our study, the fiber content in the diets and the acetate to propionate ratios were higher in calves consuming greater fiber contents in the diet, which might have resulted in greater production of methylamines. Morgavi et al. (2015) showed that *Mph.* and *Mmc.* occupy similar trophic niches; however, the more versatile use of substrates by *Mmc.* explained their higher relative abundance in the rumen of lambs after receiving an inoculum of rumen fluid obtained from wethers fed a hay diet. Therefore, the intake of swards rich in fiber with low white clover content may produce high concentrations of methylamines in the rumen, offering a competitive advantage to low abundant methylotrophic methanogens from the order *Methanomassiliicoccales* over the genus *Methanosphaera*, whose growth is limited by the availability of methanol in the rumen (Kelly et al., 2019). Our results indicate that the apparent methanogen structure community, specifically the low abundant archaea, is affected by changes in the chemical composition of the diet consumed.

CONCLUSION

In conclusion, our results showed that the rumen microbial community in the growing calf is diet dependent, with early life differences having only negligible effects on the microbiota of the growing ruminant. Different dietary regimes, pre- and post-weaning, were unable to leave a microbial imprint in the rumen of calves when the animals were fed a common diet. These findings showed that interventions after feeding colostrum to calves did not leave a permanent effect in the early microbial colonization and function in the rumen. Further studies should target earlier microbial interventions, during microbial colonization of the rumen milieu, in an attempt to imprint the ruminal microbiota.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the European Nucleotide Archive, accession number PRJEB37783.

ETHICS STATEMENT

The animal study was reviewed and approved by Grasslands Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

SMu and SMc designed the study and secured funding. OC-C, SMu, and SL generated the data. SG and OC-C completed the statistical analysis and all authors contributed to the interpretation. OC-C wrote the initial manuscript and all authors contributed to editing.

FUNDING

This study was funded by the Ministry of Primary Industries (FACCE # A21059 JPI Program), AgResearch Strategic Science Investment Fund, and New Zealand AID Program (MFAT), and CONACYT and INIFAP in Mexico for Ph.D. stipend support.

ACKNOWLEDGMENTS

The authors thank Vicki Burggraaf and Muhammad A. Khan (AgResearch), and Paul Muir and Beverly Thomson (On-Farm Research) for their support with coordinating the use of the subset of animals from the parent study for this research. The authors thank Sarah MacLean, Frederik Knol, Daniel Robinson, and Greg Skelton for their technical support for animal husbandry, sampling, and measurement procedures and Steve Lees, Richard Templeton, and the Aorangi farm staff for management of the subset of calves used in this study. The authors also gratefully acknowledge Sinead Leahy for the phylogenetic analysis of sequencing data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.711040/full#supplementary-material

REFERENCES

Abbas, W., Bhatti, S. A., Khan, M. S., Saeed, N., Warriach, H. M., Wynn, P., et al. (2017). Effect of weaning age and milk feeding volume on growth performance of Nili-Ravi buffalo calves. *Ital. J. Anim. Sci.* 16, 490–499. doi: 10.1080/1828051X.2017.1291282

Abdul Rahman, N., Parks, D. H., Vanwonterghem, I., Morrison, M., Tyson, G. W., and Hugenholtz, P. (2016). A phylogenomic analysis of the bacterial phylum Fibrobacteres. *Front. Microbiol.* 6:1469. doi: 10.3389/fmicb.2015.01469

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J., et al. (2014). Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Anim. Produc. Sci.* 54, 1449–1454. doi: 10.1071/AN14337

Accetto, T., and Avguštin, G. (2019). The diverse and extensive plant polysaccharide degradative apparatuses of the rumen and hindgut Prevotella species: a factor in their ubiquity? *Syst. Appl. Microbiol.* 42, 107–116. doi: 10.1016/j.syapm.2018.10.001

Alderman, G., and Cottrill, B. (1996). *Energy and protein requirements of ruminants*. Acribia, SA: CABI Publishing.

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

AOAC (1990). *Official Methods of Analysis.* Washington, DC: AOAC.

AOAC (2010). *Official Methods of Analysis of AOAC International.* Washington, DC: AOAC.

AOAC (2012). *Official Methods of Analysis of AOAC International.* Washington, DC: AOAC.

Attwood, G. T., Klieve, A. V., Ouwerkerk, D., and Patel, B. K. C. (1998). Ammonia-hyperproducing bacteria from New Zealand ruminants. *Appl. Environ. Microbiol.* 64, 1796–1804. doi: 10.1128/AEM.64.5.1796-1804.1998

Avguštin, G., Wallace, R. J., and Flint, H. J. (1997). Phenotypic diversity among ruminal isolates of *Prevotella* ruminicola: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella* ruminicola. *Int. J. Syst. Evol. Microbiol.* 47, 284–288. doi: 10.1099/00207713-47-2-284

Bandarupalli, V. V. K., and St-Pierre, B. (2020). Identification of a candidate starch utilizing strain of prevotella albensis from bovine rumen. *Microorganisms* 8:2005. doi: 10.3390/microorganisms8122005

Beauchemin, K., and McGinn, S. (2005). Methane emissions from feedlot cattle fed barley or corn diets. *J. Anim. Sci.* 83, 653–661. doi: 10.2527/2005.833653x

Belanche, A., Kingston-Smith, A. H., Griffith, G. W., and Newbold, C. J. (2019). A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. *Front. Microbiol.* 10:122. doi: 10.3389/fmicb.2019.00122

Benchaar, C., Pomar, C., and Chiquette, J. (2001). Evaluation of dietary strategies to reduce methane production in ruminants: a modelling approach. *Can. J. Anim. Sci.* 81, 563–574. doi: 10.4141/A00-119

Bergman, E. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590. doi: 10.1152/physrev.1990.70.2.567

Bird-Gardiner, T., Arthur, P., Barchia, I., Donoghue, K., and Herd, R. (2017). Phenotypic relationships among methane production traits assessed under ad libitum feeding of beef cattle. *J. Anim. Sci.* 95, 4391–4398. doi: 10.2527/jas2017.1477

Borrel, G., Harris, H. M., Tottey, W., Mihajlovski, A., Parisot, N., Peyretaillade, E., et al. (2012). Genome sequence of "Candidatus Methanomethylophilus alvus" Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. *J. Bacteriol.* 194, 6944–6945. doi: 10.1128/JB.01867-12

Bowen, J. M., Mccabe, M. S., Lister, S. J., Cormican, P., and Dewhurst, R. J. (2018). Evaluation of microbial communities associated with the liquid and solid phases of the rumen of cattle offered a diet of perennial ryegrass or white clover. *Front. Microbiol.* 9:2389. doi: 10.3389/fmicb.2018.02389

Brinkhaus, A. G., Wyss, U., Arrigo, Y., Girard, M., Bee, G., Zeitz, J., et al. (2017). In vitro ruminal fermentation characteristics and utilisable CP supply of sainfoin and birdsfoot trefoil silages and their mixtures with other legumes. *Animal* 11, 580–590. doi: 10.1017/S1751731116001816

Burggraaf, V. T., Craigie, C. R., Muir, P. D., Khan, M. A., Thomson, B. C., Knol, F. W., et al. (2020). Effect of rearing diet and early post-weaning pasture quality on the life-time growth, meat quality, carcass traits and environmental impact of dairy-beef cattle. *Livestock Sci.* 2020:104031. doi: 10.1016/j.livsci.2020.104031

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Corson, D., Waghorn, G., Ulyatt, M., and Lee, J. (1999). "NIRS: forage analysis and livestock feeding," in *Proceedings of the Conference New Zealand Grassland Association*, Hawkes Bay, 127–132. doi: 10.33584/jnzg.1999.61.2340

Cotta, M. A. (1992). Interaction of ruminal bacteria in the production and utilization of maltooligosaccharides from starch. *Appl. Environ. Microbiol.* 58, 48–54. doi: 10.1128/aem.58.1.48-54.1992

Cox, M. P., Peterson, D. A., and Biggs, P. J. (2010). SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinform.* 11:485. doi: 10.1186/1471-2105-11-485

Cristobal-Carballo, O., Khan, M. A., Knol, F. W., Lewis, S. J., Stevens, D. R., Laven, R. A., et al. (2019). Impact of weaning age on rumen development in artificially reared lambs. *J. Anim. Sci.* 97, 3498–3510. doi: 10.1093/jas/skz148

Curtis, T. P., and Sloan, W. T. (2004). Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. *Curr. Opin. Microbiol.* 7, 221–226. doi: 10.1016/j.mib.2004.04.010

De Barbieri, I., Hegarty, R. S., Silveira, C., Gulino, L. M., Oddy, V. H., Gilbert, R. A., et al. (2015). Programming rumen bacterial communities in newborn Merino lambs. *Small Ruminant Res.* 129, 48–59. doi: 10.1016/j.smallrumres.2015.05.015

de Clifford, R., Hickson, R., Martin, N., and Back, P. (2014). Growth rates of dairy heifers fed alternative feeds. *Proc. NZ Soc. Anim. Produc.* 74, 29–34.

De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Haegeman, A., De Tender, C., et al. (2017). Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiol. Ecol.* 93:fiw251. doi: 10.1093/femsec/fiw251

Dehority, B. (1969). Pectin-fermenting bacteria isolated from the bovine rumen. *J. Bacteriol.* 99, 189–196. doi: 10.1128/jb.99.1.189-196.1969

Dehority, B., and Orpin, C. (1997). "Development of, and natural fluctuations in, rumen microbial populations," in *The rumen microbial ecosystem*, eds P. N. Hobson and C. S. Stewart (Dordrecht: Springer), 196–245. doi: 10.1007/978-94-009-1453-7_5

Dehority, B. A., Johnson, R. R., and Conrad, H. R. (1962). Digestibility of forage hemicellulose and pectin by rumen bacteria in vitro and the effect of lignification thereon. *J. Dairy Sci.* 45, 508–512. doi: 10.3168/jds.S0022-0302(62)89436-3

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

Dias, J., Marcondes, M. I., Noronha, M. F., Resende, R. T., Machado, F. S., Mantovani, H. C., et al. (2017). Effect of pre-weaning diet on the ruminal archaeal, bacterial, and fungal communities of dairy calves. *Front. Microbiol.* 8:1553. doi: 10.3389/fmicb.2017.01553

Dijkstra, J., Ellis, J., Kebreab, E., Strathe, A., López, S., France, J., et al. (2012). Ruminal pH regulation and nutritional consequences of low pH. *Anim. Feed Sci. Technol.* 172, 22–33. doi: 10.1016/j.anifeedsci.2011.12.005

Dill-McFarland, K. A., Breaker, J. D., and Suen, G. (2017). Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci. Rep.* 7:40864. doi: 10.1038/srep40864

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D., and Suen, G. (2019). Diet influences early microbiota development in dairy calves without long-term impacts on milk production. *Appl. Environ. Microbiol.* 85:e2141–18. doi: 10.1128/AEM.02141-18

Duncan, S. H., Hold, G. L., Barcenilla, A., Stewart, C. S., and Flint, H. J. (2002). Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *Int. J. Syst. Evol. Microbiol.* 52, 1615–1620. doi: 10.1099/00207713-52-5-1615

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fonty, G., Gouet, P., Jouany, J.-P., and Senaud, J. (1987). Establishment of the microflora and anaerobic fungi in the rumen of lambs. *Microbiology* 133, 1835–1843. doi: 10.1099/00221287-133-7-1835

Fricke, W. F., Seedorf, H., Henne, A., Krüer, M., Liesegang, H., Hedderich, R., et al. (2006). The genome sequence of Methanosphaera stadtmanae reveals why this human intestinal archaeon is restricted to methanol and H2 for methane formation and ATP synthesis. *J. Bacteriol.* 188, 642–658. doi: 10.1128/JB.188.2.642-658.2006

Grünberg, W., and Constable, P. D. (2009). "CHAPTER 6 - Function and Dysfunction of the Ruminant Forestomach," in *Food Animal Practice*, Fifth Edn, eds D. E. Anderson and D. M. Rings (Saint Louis: W.B. Saunders), 12–19. doi: 10.1016/B978-141603591-6.10006-5

Gruninger, R., Ribeiro, G., Cameron, A., and Mcallister, T. (2019). Invited review: Application of meta-omics to understand the dynamic nature of the rumen microbiome and how it responds to diet in ruminants. *Animal* 13, 1843–1854. doi: 10.1017/S1751731119000752

Guyader, J., Tavendale, M., Martin, C., and Muetzel, S. (2016). Dose-response effect of nitrate on hydrogen distribution between rumen fermentation end products: An in vitro approach. *Anim. Produc. Sci.* 56, 224–230. doi: 10.1071/AN15526

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B., and Franks, A. E. (2015). Presence of selected methanogens, fibrolytic bacteria, and *proteobacteria* in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PloS One* 10:e0133048. doi: 10.1371/journal.pone.0133048

Hammond, K., Hoskin, S., Burke, J., Waghorn, G., Koolaard, J., and Muetzel, S. (2011). Effects of feeding fresh white clover (Trifolium repens) or perennial ryegrass (Lolium perenne) on enteric methane emissions from sheep. *Anim. Feed Sci. Technol.* 166, 398–404. doi: 10.1016/j.anifeedsci.2011.04.028

Hearnshaw, H. (1997). "Effect of pre-weaning nutrition on post-weaning growth carcass and meat quality traits," in *Proceedings of the Growth and Development Workshop*, eds D. W. Hennessy, S. R. McLennan, and V. H. Oddy (Armidale, NSW: The Cattle and Beef CRC for Meat Quality, University of New England), 59–67.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Collaborators, G. R. 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. doi: 10.1038/srep14567

Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., et al. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One* 8:e74787. doi: 10.1371/journal.pone.0074787

Henderson, G., Yilmaz, P., Kumar, S., Forster, R. J., Kelly, W. J., Leahy, S. C., et al. (2019). Improved taxonomic assignment of rumen bacterial 16S rRNA sequences using a revised SILVA taxonomic framework. *PeerJ* 7:e6496. doi: 10.7717/peerj.6496

Hobson, P. N., and Stewart, C. S. (1997). *The Rumen Microbial Ecosystem*. Springer: Netherlands. doi: 10.1007/978-94-009-1453-7

Hook, S. E., Steele, M. A., Northwood, K. S., Wright, A.-D. G., and Mcbride, B. W. (2011). Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. *Microb. Ecol.* 62, 94–105. doi: 10.1007/s00248-011-9881-0

Huo, W., Zhu, W., and Mao, S. (2014). Impact of subacute ruminal acidosis on the diversity of liquid and solid-associated bacteria in the rumen of goats. *World J. Microbiol. Biotechnol.* 30, 669–680. doi: 10.1007/s11274-013-1489-8

Husso, A., Lietaer, L., Pessa-Morikawa, T., Grönthal, T., Govaere, J., Van Soom, A., et al. (2021). The composition of the microbiota in the full-term fetal gut and amniotic fluid: A bovine cesarean section study. *Front. Microbiol.* 12:626421. doi: 10.3389/fmicb.2021.626421

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

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

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

Jentsch, W., Schweigel, M., Weissbach, F., Scholze, H., Pitroff, W., and Derno, M. (2007). Methane production in cattle calculated by the nutrient composition of the diet. *Arch. Anim. Nutr.* 61, 10–19. doi: 10.1080/17450390601106580

Johnson, K. A., and Johnson, D. E. (1995). Methane emissions from cattle. *J. Anim. Sci.* 73, 2483–2492. doi: 10.2527/1995.7382483x

Jonker, A., Muetzel, S., Molano, G., and Pacheco, D. (2016). Effect of fresh pasture forage quality, feeding level and supplementation on methane emissions from growing beef cattle. *Anim. Produc. Sci.* 56, 1714–1721. doi: 10.1071/AN15022

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., et al. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4, 1–16. doi: 10.1186/s40168-016-0201-2

Kelly, W. J., Leahy, S. C., Kamke, J., Soni, P., Koike, S., Mackie, R., et al. (2019). Occurrence and expression of genes encoding methyl-compound production in rumen bacteria. *Anim. Microb.* 1:15. doi: 10.1186/s42523-019-0016-0

Khan, M., Weary, D., and Von Keyserlingk, M. (2011). Invited review: Effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. *J. Dairy Sci.* 94, 1071–1081. doi: 10.3168/jds.2010-3733

Kim, Y.-H., Nagata, R., Ohtani, N., Ichijo, T., Ikuta, K., and Sato, S. (2016). Effects of dietary forage and calf starter diet on ruminal pH and bacteria in holstein calves during weaning transition. *Front. Microbiol.* 7:1575. doi: 10.3389/fmicb.2016.01575

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

Koike, S., and Kobayashi, Y. (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: Fibrobacter succinogenes, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.* 204, 361–366. doi: 10.1111/j.1574-6968.2001.tb10911.x

Kumar, S., Treloar, B. P., Teh, K. H., Mckenzie, C. M., Henderson, G., Attwood, G. T., et al. (2018). Sharpea and Kandleria are lactic acid producing rumen bacteria that do not change their fermentation products when co-cultured with a methanogen. *Anaerobe* 54, 31–38. doi: 10.1016/j.anaerobe.2018.07.008

Lana, R. P., Russell, J. B., and Van Amburgh, M. E. (1998). The role of pH in regulating ruminal methane and ammonia production. *J. Anim. Sci.* 76, 2190–2196. doi: 10.2527/1998.7682190x

Lane, M. A., Baldwin, R. L., and Jesse, B. W. (2000). Sheep rumen metabolic development in response to age and dietary treatments. *J. Anim. Sci.* 78, 1990–1996. doi: 10.2527/2000.7871990x

Lê Cao, K., Rohart, F., Gonzalez, I., and Dejean, S. (2016). *mixOmics: Omics Data Integration Project. R package Version 6.1. 1.*

Lee, J.-H., Kumar, S., Lee, G.-H., Chang, D.-H., Rhee, M.-S., Yoon, M.-H., et al. (2013). *Methanobrevibacterboviskoreani* sp. nov., isolated from the rumen of Korean native cattle. *Int. J. Syst. Evol. Microbiol.* 63, 4196–4201. doi: 10.1099/ijs.0.054056-0

Li, R. W., Connor, E. E., Li, C., Baldwin, V. I. R. L., and Sparks, M. E. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. *Environ. Microbiol.* 14, 129–139. doi: 10.1111/j.1462-2920.2011.02543.x

Lima, F. S., Oikonomou, G., Lima, S. F., Bicalho, M. L., Ganda, E. K., De Oliveira Filho, J. C., et al. (2015). Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows. *Appl. Environ. Microbiol.* 81, 1327–1337. doi: 10.1128/AEM.03138-14

Liu, Y., and Whitman, W. B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. N.Y. Acad. Sci.* 1125, 171–189. doi: 10.1196/annals.1419.019

Luo, D., Ganesh, S., and Koolaard, J. (2014). *Calculate Predicted Means for Linear Models. 0.99.*

Malmuthuge, N., and Griebel, P. J. (2018). Fetal environment and fetal intestine are sterile during the third trimester of pregnancy. *Vet. Immunol. Immunopathol.* 204, 59–64. doi: 10.1016/j.vetimm.2018.09.005

McLoughlin, S., Spillane, C., Claffey, N., Smith, P. E., O'rourke, T., Diskin, M. G., et al. (2020). Rumen microbiome composition is altered in sheep divergent in feed efficiency. *Front. Microbiol.* 11:1981. doi: 10.3389/fmicb.2020.01981

Miller, T. L., and Lin, C. (2002). Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. *Int. J. Syst. Evol. Microbiol.* 52, 819–822.

Minato, H., Otsuka, M., Shirasaka, S., Itabashi, H., and Mitsumori, M. (1992). Colonization of microorganisms in the rumen of young calves. *J. General Appl. Microbiol.* 38, 447–456. doi: 10.2323/jgam.38.447

Mizrahi, I., Wallace, R. J., and Moraïs, S. (2021). The rumen microbiome: balancing food security and environmental impacts. *Nat. Rev. Microbiol.* 19, 553–566. doi: 10.1038/s41579-021-00543-6

Morgavi, D. P., Rathahao-Paris, E., Popova, M., Boccard, J., Nielsen, K. F., and Boudra, H. (2015). Rumen microbial communities influence metabolic phenotypes in lambs. *Front. Microbiol.* 6:1060. doi: 10.3389/fmicb.2015.01060

Moss, A. R., Jouany, J. P., and Newbold, J. (2000). Methane production by ruminants: its contribution to global warming. *Ann. Zootech.* 49, 231–253. doi: 10.1051/animres:2000119

Muetzel, S., and Clark, H. (2015). Methane emissions from sheep fed fresh pasture. *NZ J. Agric. Res.* 58, 472–489. doi: 10.1080/00288233.2015.1090460

Muir, P., Fugle, C., and Ormond, A. (2002). Calf rearing using a once-a-day milk feeding system: Current best practice. *Proc. NZ Grassland Assoc.* 64, 21–24. doi: 10.33584/jnzg.2002.64.2469

O'Hara, E., Kenny, D., Mcgovern, E., Byrne, C. J., Mccabe, M. S., Guan, L., et al. (2020). Investigating temporal microbial dynamics in the rumen of beef calves raised on two farms during early life. *FEMS Microbiol. Ecol.* 96:203.

Oksanen, F., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al. (2017). *vegan: Community Ecology Package. R package version 2.4-4.* Available Online at: https. *CRAN. R-pro j ect. org/pack age= vega n.*

Ormerod, K. L., Wood, D. L. A., Lachner, N., Gellatly, S. L., Daly, J. N., Parsons, J. D., et al. (2016). Genomic characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of homeothermic animals. *Microbiome* 4:36. doi: 10.1186/s40168-016-0181-2

Owens, F. N., and Basalan, M. (2016). *Ruminal fermentation," in Rumenology*. Cham: Springer, 63–102. doi: 10.1007/978-3-319-30533-2_3

Palevich, N., Kelly, W. J., Ganesh, S., Rakonjac, J., and Attwood, G. T. (2019a). Butyrivibrio hungatei MB2003 competes effectively for soluble sugars released by Butyrivibrio proteoclasticus B316T during growth on xylan or pectin. *Appl. Environ. Microbiol.* 85:e2056–18. doi: 10.1128/AEM.02056-18

Palevich, N., Kelly, W. J., Leahy, S. C., Denman, S., Altermann, E., Rakonjac, J., et al. (2019b). Comparative genomics of rumen *Butyrivibrio spp.* uncovers a continuum of polysaccharide-degrading capabilities. *Appl. Environ. Microbiol.* 86:e1993–19. doi: 10.1128/AEM.01993-19

Palevich, N., Kelly, W. J., Leahy, S. C., Altermann, E., Rakonjac, J., and Attwood, G. T. (2017). The complete genome sequence of the rumen bacterium Butyrivibrio hungatei MB2003. *Stand. Genom. Sci.* 12, 1–14. doi: /10.1186/s40793-017-0285-8

Palevich, N., Maclean, P. H., Kelly, W. J., Leahy, S. C., Rakonjac, J., and Attwood, G. T. (2020). Complete genome sequence of the polysaccharide-degrading rumen bacterium pseudobutyrivibrio xylanivorans MA3014 reveals an incomplete glycolytic pathway. *Genome Biol. Evol.* 12, 1566–1572. doi: 10.1093/gbe/evaa165

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

Pinares, C., and Waghorn, G. (2014). *Technical manual on respiration chamber designs*. Wellington, New Zealand: Ministry of Agriculture and Forestry.

Pinares-Patiño, C., Hunt, C., Martin, R., West, J., Lovejoy, P., and Waghorn, G. (2012). "New Zealand ruminant methane measurement centre, AgResearch, Palmerston North," in *Technical manual on respiration chamber design*, eds C. S. Pinares-Patiño and G. C. Waghorn (Wellington, New Zealand: Ministry of Agriculture and Forestry), 11–30.

Pinheiro, J., Bates, D., Debroy, S., Sarkar, D., and Team, R. C. (2015). *nlme: Linear and Nonlinear Mixed Efects Models. 3.1-121.*

Pino, F., Mitchell, L. K., Jones, C. M., and Heinrichs, A. J. (2018). Comparison of diet digestibility, rumen fermentation, rumen rate of passage, and feed efficiency in dairy heifers fed ad-libitum versus precision diets with low and high quality forages. *J. Appl. Anim. Res.* 46, 1296–1306. doi: 10.1080/09712119.2018.1498788

Poulsen, M., Schwab, C., Jensen, B. B., Engberg, R., Spang, A., Canibe, N., et al. (2013). Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. *Nat. Commun.* 4:1428.

Puniya, A. K., Singh, R., and Kamra, D. N. (2015). *Rumen microbiology: from evolution to revolution*. Cham: Springer. doi: 10.1007/978-81-322-2401-3

Rainey, F. (1996). *Genus XV. Pseudobutyrivibrio van Gylswyk, Hippe and Rainey, 1996, 561", in: Bergey's manual of systematic bacteriology*. New York: Springer.

Ransom-Jones, E., Jones, D. L., Mccarthy, A. J., and Mcdonald, J. E. (2012). The fibrobacteres: An important phylum of cellulose-degrading bacteria. *Microb. Ecol.* 63, 267–281. doi: 10.1007/s00248-011-9998-1

Ravachol, J., De Philip, P., Borne, R., Mansuelle, P., Maté, M. J., Perret, S., et al. (2016). Mechanisms involved in xyloglucan catabolism by the cellulosome-producing bacterium Ruminiclostridium cellulolyticum. *Sci. Rep.* 6:22770. doi: 10.1038/srep22770

R Core Team (2016). *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation for Statistical Computing.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., and Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116, 245–257.

Rius, A. G., Kittelmann, S., Macdonald, K. A., Waghorn, G. C., Janssen, P. H., and Sikkema, E. (2012). Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture. *J. Dairy Sci.* 95, 5024–5034. doi: 10.3168/jds.2012-5392

Ryan, W. (1990). Compensatory growth in cattle and sheep. *Nutr. Abst. Rev. Ser. Livestock Feeds Feed.* 60, 653–664.

Salvetti, E., Felis, G. E., Dellaglio, F., Castioni, A., Torriani, S., and Lawson, P. A. (2011). Reclassification of *Lactobacillus* catenaformis (Eggerth 1935) Moore and Holdeman 1970 and *Lactobacillus* vitulinus Sharpe et al. 1973 as Eggerthia

catenaformis gen. nov., comb. nov. and *Kandleria vitulina* gen. nov., comb. nov., respectively. *Int. J. Syst. Evol. Microbiol.* 61, 2520–2524. doi: 10.1099/ijs.0.029231-0

Sauvant, D., Giger-Reverdin, S., Serment, A., and Broudiscou, L. (2011). Influences of diet and rumen fermentation on methane production by ruminants. *INRA Productions Anim.* 24, 433–446. doi: 10.20870/productions-animales.2011.24.5.3276

Schären, M., Drong, C., Kiri, K., Riede, S., Gardener, M., Meyer, U., et al. (2017). Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows. *J. Dairy Sci.* 100, 2765–2783. doi: 10.3168/jds.2016-11994

Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P. H. (2014). RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *PeerJ* 2:e494. doi: 10.7717/peerj.494

Seedorf, H., Kittelmann, S., and Janssen, P. H. (2015). Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle. *Appl. Environ. Microbiol.* 81, 986–995. doi: 10.1128/AEM.03018-14

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. doi: 10.1038/nbt.4110

Shamay, A., Werner, D., Moallem, U., Barash, H., and Bruckental, I. (2005). Effect of nursing management and skeletal size at weaning on puberty, skeletal growth rate, and milk production during first lactation of dairy heifers. *J. Dairy Sci.* 88, 1460–1469. doi: 10.3168/jds.S0022-0302(05)72814-9

Smith, P. E., Enriquez-Hidalgo, D., Hennessy, D., Mccabe, M. S., Kenny, D. A., Kelly, A. K., et al. (2020). Sward type alters the relative abundance of members of the rumen microbial ecosystem in dairy cows. *Sci. Rep.* 10, 1–10. doi: 10.1038/s41598-020-66028-3

Snelling, T. J., Auffret, M. D., Duthie, C.-A., Stewart, R. D., Watson, M., Dewhurst, R. J., et al. (2019). Temporal stability of the rumen microbiota in beef cattle, and response to diet and supplements. *Anim.Microbiome* 1:16. doi: 10.1186/s42523-019-0018-y

Solden, L. M., Hoyt, D. W., Collins, W. B., Plank, J. E., Daly, R. A., Hildebrand, E., et al. (2016). New roles in hemicellulosic sugar fermentation for the uncultivated Bacteroidetes family BS11. *ISME J.* 11:691. doi: 10.1038/ismej.2016.150

Solden, L. M., Hoyt, D. W., Collins, W. B., Plank, J. E., Daly, R. A., Hildebrand, E., et al. (2017). New roles in hemicellulosic sugar fermentation for the uncultivated Bacteroidetes family BS11. *ISME J.* 11:691.

Stanton, T., Duncan, S., and Flint, H. (2009). *Genus XVI. Roseburia Stanton and Savage 1983a, 626", in: Bergey's manual of systematic bacteriology*. New York: Springer.

Taschuk, R., and Griebel, P. J. (2012). Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract. *Anim. Health res. Rev.* 13, 129–141. doi: 10.1017/S1466252312000096

Thomson, D. (1984). "The nutritive value of white clover," in *Forage legumes*, ed. D. J. Thomson (Dunston, UK: British Grassland Society Occasional Symposium), 78–92.

Thurston, V., Dawson, K. A., and Strobel, H. J. (1994). Pentose utilization by the ruminal bacterium *Ruminococcus* albus. *Appl. Environ. Microbiol.* 60, 1087–1092. doi: 10.1128/aem.60.4.1087-1092.1994

Ungerfeld, E. M. (2020). Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Front. Microbiol.* 11:589. doi: 10.3389/fmicb.2020.00589

Van Kessel, J. A. S., and Russell, J. B. (1996). The effect of pH on ruminal methanogenesis. *FEMS Microbiol. Ecol.* 20, 205–210. doi: 10.1111/j.1574-6941.1996.tb00319.x

Walters, W. A., Caporaso, J. G., Lauber, C. L., Berg-Lyons, D., Fierer, N., and Knight, R. (2011). PrimerProspector: de novo design and taxonomic analysis of barcoded polymerase chain reaction primers. *Bioinformatics* 27, 1159–1161. doi: 10.1093/bioinformatics/btr087

Wang, Z., Elekwachi, C., Jiao, J., Wang, M., Tang, S., Zhou, C., et al. (2017). Changes in metabolically active bacterial community during rumen development, and their alteration by rhubarb root powder revealed by 16S rRNA amplicon sequencing. *Front. Microbiol.* 8:159. doi: 10.3389/fmicb.2017.00159

Wardrop, I. (1966). The effects of the plane of nutrition in early post-natal life on the subsequent growth and development of cattle. *Aus. J. Agric. Res.* 17, 375–385. doi: 10.1071/AR9660375

Weimer, P. J. (1998). Manipulating ruminal fermentation: a microbial ecological perspective. *J. Anim. Sci.* 76, 3114–3122. doi: 10.2527/1998.76123114x

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

Williams, S. R. O., Hannah, M., Jacobs, J. L., Wales, W. J., and Moate, P. J. (2019). Volatile fatty acids in ruminal fluid can be used to predict methane yield of dairy cows. *Animals* 9:1006. doi: 10.3390/ani9121006

Wise, G. H., and Anderson, G. (1939). Factors affecting the passage of liquids into the rumen of the dairy Calf. I. method of administering liquids: drinking from open pail versus sucking through a rubber Nipple1. *J. Dairy Sci.* 22, 697–705. doi: 10.3168/jds.S0022-0302(39)92926-7

Xie, F., Zhang, L., Jin, W., Meng, Z., Cheng, Y., Wang, J., et al. (2019). Methane emission, rumen fermentation, and microbial community response to a nitrooxy compound in low-quality forage fed hu sheep. *Curr. Microbiol.* 76, 435–441. doi: 10.1007/s00284-019-01644-5

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

Yáñez-Ruiz, D. R., Macías, B., Pinloche, E., and Newbold, C. J. (2010). The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. *FEMS Microbiol. Ecol.* 72, 272–278. doi: 10.1111/j.1574-6941.2010.00852.x

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J., and Aldridge, B. M. (2018). Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. *Sci. Rep.* 8, 1–14. doi: 10.1038/s41598-018-21440-8

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

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

REVIEW
published: 08 December 2021
doi: 10.3389/fmicb.2021.763416

Ruminal Phages – A Review

*Richard R. Lobo and Antonio P. Faciola**

Department of Animal Sciences, University of Florida, Gainesville, FL, United States

The rumen ecosystem is a complex and dynamic environment, which hosts microorganisms including archaea, bacteria, protozoa, fungi, and viruses. These microorganisms interact with each other, altering the ruminal environment and substrates that will be available for the host digestion and metabolism. Viruses can infect the host and other microorganisms, which can drive changes in microorganisms' lysis rate, substrate availability, nutrient recycling, and population structure. The lysis of ruminal microorganisms' cells by viruses can release enzymes that enhance feedstuff fermentation, which may increase dietary nutrient utilization and feed efficiency. However, negative effects associated to viruses in the gastrointestinal tract have also been reported, in some cases, disrupting the dynamic stability of the ruminal microbiome, which can result in gastrointestinal dysfunctions. Therefore, the objective of this review is to summarize the current knowledge on ruminal virome, their interaction with other components of the microbiome and the effects on animal nutrition.

Keywords: microbiome, phage, ruminal ecology, ruminant nutrition, virome

OPEN ACCESS

Edited by:
Diego P. Morgavi, INRAE Clermont-Auvergne-Rhône-Alpes, France

Reviewed by:
Jessica Charlotte Abigail Friedersdorff, Aberystwyth University, United Kingdom
T. G. Nagaraja, Kansas State University, United States

***Correspondence:**
Antonio P. Faciola
afaciola@ufl.edu

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

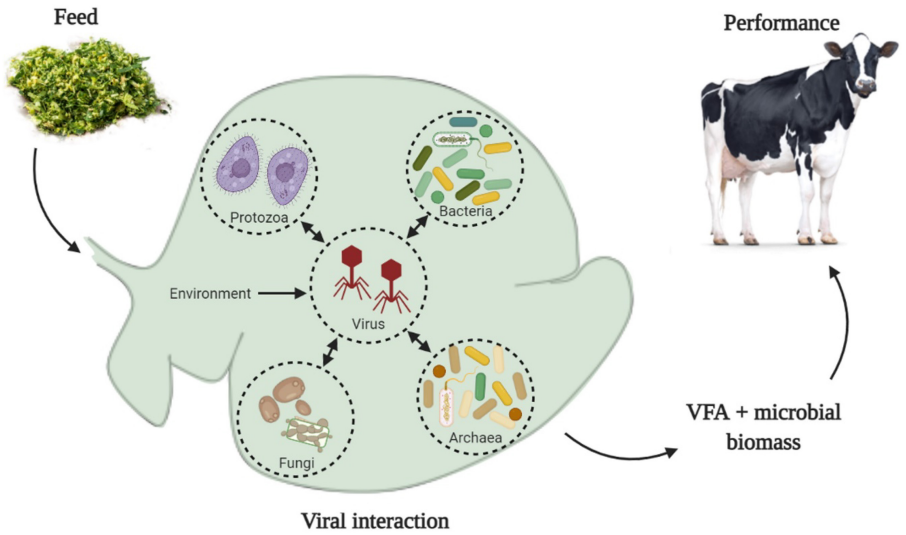
Received: *23 August 2021*
Accepted: *18 November 2021*
Published: *08 December 2021*

Citation:
Lobo RR and Faciola AP (2021) Ruminal Phages – A Review. Front. Microbiol. 12:763416. doi: 10.3389/fmicb.2021.763416

INTRODUCTION

The rumen is a complex and dynamic ecosystem inhabited by anaerobic bacteria, protozoa, fungi, archaea and viruses (Huws et al., 2018), all living in the same environment and interacting with each other. This microbial community uses a wide range of feed components as substrates for its own growth, including cellulose- and starch-rich substrates. In a healthy ruminant this process generates volatile fatty acids which are absorbed by the ruminal epithelium and used as the major energy source (approximately 70%) for the host animal (Bergman, 1990). Microbial growth also supplies microbial biomass which, given its protein concentration, amino acid profile, and digestibility, represent an important source of metabolizable protein for the host (Schwab and Broderick, 2017).

Ruminal bacteria, archaea, protozoa, and, to a less extent, fungi have been vastly studied in the past decades; however, ruminal viruses have mostly been under investigated but have been gaining more attention due to their biotechnological potential and possible effects on animal health and production. The most known description of viruses is that they are simple infectious particles composed of genetic material (DNA or RNA in a single or double strand) and an outer shell composed of protein, this simple structure is called a virion or free-virus (Lodish et al., 2000), also some viruses have an outer membrane and are called enveloped viruses (Tsai, 2007). The viruses population in the environment is referred to as the virome (Ross et al., 2013). Those viruses that specifically target and infect bacteria are called bacteriophages (phages) (Clokie et al., 2011). There are also viruses that infect other prokaryotes in the rumen such as archaea which may also be called archaeaphages or archaeal viruses, and eukaryotes such as fungi (called mycophages or mycoviruses) and protozoa (protozoan viruses) (Gilbert et al., 2020). Phages, particularly infecting



GRAPHICAL ABSTRACT | Ruminal viruses can interact with all groups of microorganisms in the ruminal environment, which include bacteria, archaea, fungi, and protozoa. Physical-chemical parameters of the ruminal environment, such as pH, concentration of volatile fatty acids (VFA), ammonia, and other chemical components, can be modulated by the animal's diet. We hypothesized that such changes in the physical-chemical parameters of the ruminal environment can modify the infectivity of viral particles and change the lysis rate of microbial cells, consequently, changes on VFA production and microbial biomass could affect animals' performance.

bacteria, have been reported to be the most numerous virus population in the rumen, as well as the most studied, and will therefore be the focal point of this review.

The first description of these submicroscopic agents in the ruminal environment occurred in the 1960s when it was demonstrated that viruses were inhabitants of the rumen and not just transient of the gastro-intestinal tract (GIT), from feed and water ingested (Adams et al., 1966; Hoogenraad et al., 1967). These resident viruses use ruminal microorganisms to proliferate themselves (Gilbert and Klieve, 2015) and represent a significant part of the ruminal microbiome. The viral particle count reported in the literature ranges from 5×10^7 to 1.4×10^{10} (**Table 1**).

Aside from resident viruses, transient viruses from feed and water sources can also be found in the rumen, which would likely make up a small part of the ruminal virome; however, they are not well understood and very little is known about their interaction with the ruminal microbiome. According to Klieve et al. (1996), the majority of the resident phages in the rumen have a symbiotic relationship with other ruminal microorganisms, and are often found integrated in host genomes as prophages. This interaction can create a long term and symbiotic association with the host cells, where the genome of the phage is inserted into the chromosome of the host organism and this process ends up forming the prophage. However, a predation interaction can also take place, where infection of the host cell by phages occurs with a short association, rapid death of the host cell, and release of new virions. These interactions between phages and microorganisms can drive changes in the microbial ecology of the environment (Koskella and Brockhurst, 2014). Those changes can modify ruminal fermentation and affect health of the animals, and consequently, animal performance (Namonyo et al., 2018) and will be covered in more details later on in the article.

Because of the limited knowledge about the ruminal virome and its interaction with other ruminal microorganisms, the objective of this review is to summarize the knowledge available in the literature in ruminal phages and create a comprehensive review, including the viral biology, their interaction with other microorganisms in complex environments, and their implication in the ruminant nutrition and health.

REPLICATION CYCLE

The lifecycle of the phage is used to classify them into virulent (or strict lytic phages) and temperate phages (**Figure 1**). Virulent ones use the lytic cycle, which inject their own genetic material into the host cell and quickly reprogram the cell machinery to replicate the viral particles and transcribe viral proteins, leading to a fast death of the host and consequently release of new virions in the environment (Payet and Suttle, 2013).

On the other hand, temperate phages use the lysogenic cycle, which will inject their genetic material into the host cell, where the viral genome is incorporated into the host cell genome, creating the prophage (Payet and Suttle, 2013) (**Figure 1**). The host cell (lysogen) then replicates itself, and the resulting daughter cells both harbor the prophage integrated in their genomes. This prophage can stay in a dormancy stage for several generations and can be activated or induced by physical or chemical environmental perturbations, which will start to produce viral particles and eventually cell lysis (Paul, 2008).

Viral infection is highly specific and host cell receptors may be composed of lipopolysaccharide, sugars, proteins, and fimbriae

TABLE 1 | Summary of published electron microscopy (EM) and pulse-field gel electrophoresis (PFGE) studies characterizing the phage population in the ruminal environment.

Phage particle count[a]	Method used	Morphology types	Host species	Number of animals	Feed used	Source
NA	EM	NA	Sheep	NA	Lucerne chaff	Hoogenraad et al. (1967)
5×10^7	EM	6	Cattle	1	Alfalfa hay	Paynter et al. (1969)
NA	EM	NA	Reindeer	NA	NA	Tarakanov (1972)
2×10^7 to 1×10^8	EM	26	Cattle/sheep	9	Chaffed rice straw and oaten chaff	Klieve and Bauchop (1988)
1.4×10^{10}	PFGE	NA	Sheep	1	Oaten chaff and lucerne chaff	Klieve and Swain (1993)

[a]*Estimation of free phages total count.*
NA – Non-available.

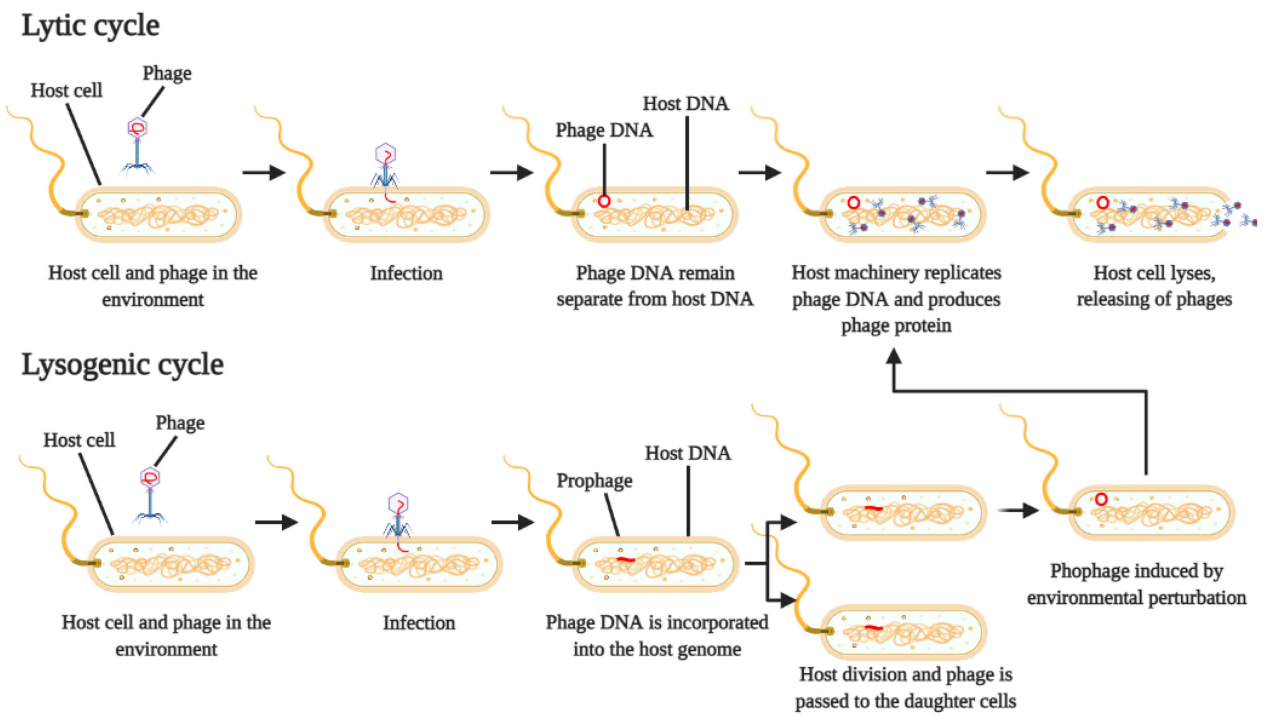


FIGURE 1 | Schematic representation of phage reproduction by lytic and lysogenic cycle. Adapted from Payet and Suttle (2013).

structures, which will determine the range of organisms that can host the virus (Matsuzaki et al., 2005). Some phages only target and infect one host species or even strain, and may be classes as monovalent, whilst others can infect multiple strains and be classed as polyvalent (Parasion et al., 2014).

Phages are commonly associated with their destructive effects on the host cell; however, injection of genetic material into the host cell can actually guide it through a process of evolution and diversification via horizontal gene transfer (HGT) (Berg Miller et al., 2012; Koskella and Brockhurst, 2014; Gilbert et al., 2020). Horizontal gene transfer can occur in both prokaryotic and eukaryotic cells by a process called transduction, where a newly formed virus incorporate host genes and transfers it to another cell (Touchon et al., 2017). However, these genes usually have to improve the adaptation chances of the recipient cell or it will no longer survive in the new lineage formed (Soucy et al., 2015).

Prokaryotic cells can use a strategy of their adaptive immune elements to incorporate the foreign genetic material into their own genome via clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) protein (Barrangou et al., 2007; Berg Miller et al., 2012; Barrangou and Marraffini, 2014). The CRISPR/Cas process occurs in a large number of species of archaea and almost all species of bacteria in the rumen and horizontal gene transfer is a common and important evolutionary practice of the living organisms in the ruminal environment (Berg Miller et al., 2012). The presence of CRISPR/Cas genes can provide historic information of phage-bacteria interaction and metagenomic analysis of the rumen microbiome (Berg Miller et al., 2012) and genomic analysis of bacterial host have detected CRISPR/Cas genes (Gilbert et al., 2017; Friedersdorff et al., 2020) suggesting previous interactions between phages and bacterial host.

KEY GROUPS OF THE RUMEN VIROME

Viruses that infect bacteria and those that infect archaea are often presented together due to its similarity (Gilbert and Klieve, 2015). A pioneer study of the characterization of ruminal phages using electron microscopy (EM) observed six different morphological viral types in the bovine ruminal content (Paynter et al., 1969), which is a small number when compared to the 26-40 morphological types observed by Ritchie et al. (1970) and Klieve and Bauchop (1988). Using molecular approaches (metagenomic) to identify phages and prophages in the ruminal environment, Berg Miller et al. (2012) and Anderson et al. (2017) identified a large number of viral DNA fragments in a large number of viral species (1500 and 2243, respectively), those DNA sequences were assessed for homology to the viral genome database, and the majority of them could not be identified, meaning that the majority of the phages and prophages in the ruminal environment were unknown.

Both, Berg Miller et al. (2012) and Anderson et al. (2017), observed that viruses (phages and prophages) from the families *Myoviridae*, *Siphoviridae*, *Mimiviridae*, and *Podoviridae* were the most abundant in the ruminal environment, their results corroborates the findings on morphological studies by Ritchie et al. (1970). According to Klieve et al. (1996), the majority of the phages in the ruminal environment are in a lysogenic state (prophages), which represents a symbiotic coexistence between phages and other microorganisms. Corroborating these results, Berg Miller et al. (2012) reported that prophages outnumbered lytic phages approximately 2:1 and the majority of the viruses (both prophages and phages) are proportionally associated with the dominant ruminal bacterial phyla (*Firmicutes* and *Proteobacteria*).

Culture-based studies have been used to isolate and culture new phages. Gilbert et al. (2017) reported the genomic study of five isolated phages of the families *Siphoviridae* and *Podoviridae*. Isolated phages were described as predators of ruminal bacteria of the genera *Bacteroides*, *Ruminococcus*, and *Streptococcus* and a co-examination of bacterial genomes suggests that these microorganisms have genes responsible for modulating phage:host interactions, such as CRISPR/Cas elements and restriction-modification phage defense. However, the authors suggested that even bacterial strains within the same genus could have different receptivity to phage infection and replication than others.

Another recent study reported five more phage genomes isolated from the ruminal environment, which double the currently available phage genomes (Friedersdorff et al., 2020). These phages have been observed in a lytic lifestyle, as a free phage; however, genomic analysis indicates that some have a potential to undergo temperate lifecycle. Also, the authors reported that the five phages identified were isolated from a single host (*Butyrivibrio fibrisolvens*) and they belong to three different genera, which indicate that there is much to be discovered and an international effort is required to achieve a greater understanding of the viral diversity and interaction.

Only a few studies on ruminal viruses that interact with prokaryotic organisms are available in the literature; however, fewer report viruses with eukaryotic (i.e., anaerobic fungi and protozoa) interactions. Anaerobic fungi is an important component of the microbial community in the ruminal environment, playing a key role in the degradation of plant cell wall material (Rabee et al., 2019; Azad et al., 2020). While the importance of anaerobic fungi in ruminant nutrition is known, very little is known about the mycoviruses that infect them. To our knowledge, only one study reporting mycoviruses in the rumen is available (Hitch et al., 2019). The authors reported the presence of 30 mycoviruses in the ruminal environment, which the majority were classified in the *Partitiviridae*, *Alphaflexiviridae*, and *Betaflexiviridae* families; however, similar to the bacteriophage analysis, the majority of the DNA sequences obtained could not be classified (only 0.025% of the contigs were related to known mycoviruses), indicating that more studies are needed to access a better identification of mycoviruses.

Anderson et al. (2017), in their metagenomic analysis of viruses, observed ten DNA fragments that belong to viruses that have eukaryotic cells as target hosts (fungi and protozoa) were identified, based on the homology with known viruses; however, no further explanations were provided. To the best of our knowledge, this is the only study evaluating ruminal protozoa viruses. However, it has been documented that viruses can infect protozoa in other environments (Barrow et al., 2020), suggesting that ruminal protozoa can also be infected by viruses, leading to speculations that protozoal population changes can be driven by viral activity, with possible implications in the fiber and protein utilization.

VIRAL INFLUENCE IN COMPLEX ENVIRONMENTS

As mentioned previously, phages are predators of other organisms. In a complex environment, such as the marine environment, viruses can be considered drivers of nutrient and energy cycles (Suttle, 2007). Phages can modulate bacterial populations through host cell lysis, which could in turn effect ruminant nutrition. Although this hypothesis has not been fully proven, we know from other microbiomes that virus can be considered drivers of nutrient and energy cycles (Suttle, 2007).

According to Mojica and Brussaard (2014), after the release of viral progeny, free virions are exposed to the environmental physicochemical conditions of the water (such as pH, temperature, and metabolites presented in the water) which can affect the interaction of the viruses with other organisms. For example, environmental conditions can change infectivity, modify the viruses' structure, and adsorption of it by the host organism. Anthropogenic activities in the marine environment can change physicochemical conditions and metabolites composition of the water, these changes have impacts in the virus-host interaction and consequently on rate of host lysis (Mojica and Brussaard, 2014; Johannessen et al., 2017); however, the exact way that environmental properties affect the viruses are still unknown.

In addition to marine environments, the gut virome in humans has been well studied (Minot et al., 2011; Beller and Matthijnssens, 2019). Published studies reported that the human gut virome is driven by diet (Minot et al., 2011; Oh et al., 2019; Rasmussen et al., 2019). However, there is little explanation of how viral population structure is driven by diet and how it can affect other organisms within the GIT.

A couple of studies are available evaluating the effects of the diet on the ruminal virome (Swain et al., 1996; Anderson et al., 2017). Swain et al. (1996) using pulse-field gel electrophoresis and two groups of sheep, one fed oaten and chaff-lucerne at a ratio of 70:30 and the other fed pasture, reported differences in the viral population between groups. They hypothesized that differences on viral population between dietary groups are due to the specific dietary regime and suggested that it is possible to manipulate the rate of bacterial lysis (Swain et al., 1996). Also, the authors observed a diurnal fluctuation in the viral population in response to feeding.

In a more recent study, Anderson et al. (2017) used a metagenomic approach, to evaluate the effect of diets varying in energy content, nutritional composition, and fiber source in beef steers. They demonstrated that virus population can change according to the diet and viruses can modulate the microbiome, impacting microbial metabolism.

Based on knowledge from other fields, such as the marine environment, it can be speculated that when new free virions are produced in the rumen, physicochemical properties (such as pH, ammonia, volatile fatty acids, and other metabolites) can modulate viral activity, increasing or reducing their infectivity to the next host cell (**Figure 2**); however, to our knowledge this has not yet been shown in the rumen.

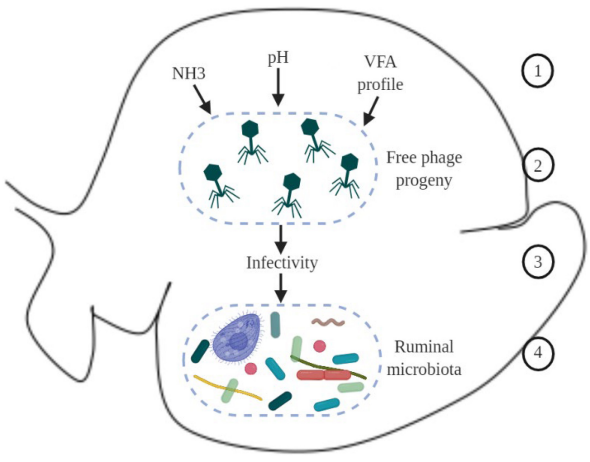


FIGURE 2 | Schematic overview of hypothetical environmental factors in the ruminal environment that could affect virus dynamics and phage-host interaction. 1 – physicochemical parameters in the ruminal environment, such as ammonia (NH_3), pH, and VFA (volatile fatty acids); 2 – new phages produced; 3 – physicochemical parameters in the environment can modulate infectivity of new phages; 4 – changes in the infectivity of phages can modulate the ruminal microbiome.

As discussed previously, viruses can infect ruminal microorganisms and many of these interactions between the viruses and microorganisms in the ruminal environment can generate a fragmentation of the host cell, which releases particles of the host cell, including proteins, nucleic acids, and cell wall fragments, which can be used by other organisms from the microbiome as source of nitrogen and energy, in a process referred as intra-ruminal recycling (Firkins et al., 1992; Hartinger et al., 2018) (**Figure 3A**). The microorganisms involved in the recycling process utilize substrates that are not primarily absorbed by the animal, using it as a source of nutrient to produce microbial biomass, and later on the microbial biomass produced can be digested, absorbed, and metabolized by the animal (Silva et al., 2019; Gnetegha Ayemele et al., 2020).

In addition, a recent study suggested that the bacterial host cell lysis can release enzymes as well, including those involved in carbohydrate fermentation (Solden et al., 2018). These enzymes can help in the feed degradation and enhance ruminal fermentation. These findings support the idea that phages in the rumen can influence microbial lysis and nutrient recycling.

Phages can drive the environment ecology by bloom of lytic activity, which is illustrated in **Figure 3B**, this process occurs in the rumen and was first presented by Swain et al. (1996). These lytic activities affect the dominant microbial population in the environment in a process named top-down or "kill the winner," which is a theoretical model, in which a specific organism starts to dominate the ecosystem, this specific organism will likely be preyed upon by its natural viral predator, which will control the organism population, creating the opportunity for other organisms to emerge. However, this has not been experimentally demonstrated in the ruminal environment (Gilbert et al., 2020).

THE PROSPECTUS OF PHAGE THERAPY IN RUMINANTS

After the discovery of phages infecting bacterial cells and their potential to kill the host, Felix d'Herelle was the first to use phages as a treatment to reduce human infections (d'Herelle, 1917). This technology has continued to be developed since then and studies of biological control of several human infections have been carried out, bacterial diseases such as pneumonia (Dufour et al., 2019; Anand et al., 2020) and skin infections (Jault et al., 2019) are some examples of its application. In addition, studies using phage therapy to control antibiotic resistant organisms have been successfully used (Schooley et al., 2017).

According to Gilbert and Klieve (2015), in agriculture, phage therapy approaches for biological control have caught the attention of researchers. This shift from antibiotic use to the phage therapy is advantageous due to the fact that the isolation of a phage of interest is relatively simple, fast, and inexpensive, also, due to its high specificity of target, which avoids disturbances of local microbiome (Parasion et al., 2014).

Phage therapy has been studied as a strategy to reduce methane (Leahy et al., 2010; Morkhade et al., 2020). As ruminal fermentation leads to the eventual production of methane, it is thought that perturbations of microbial populations that

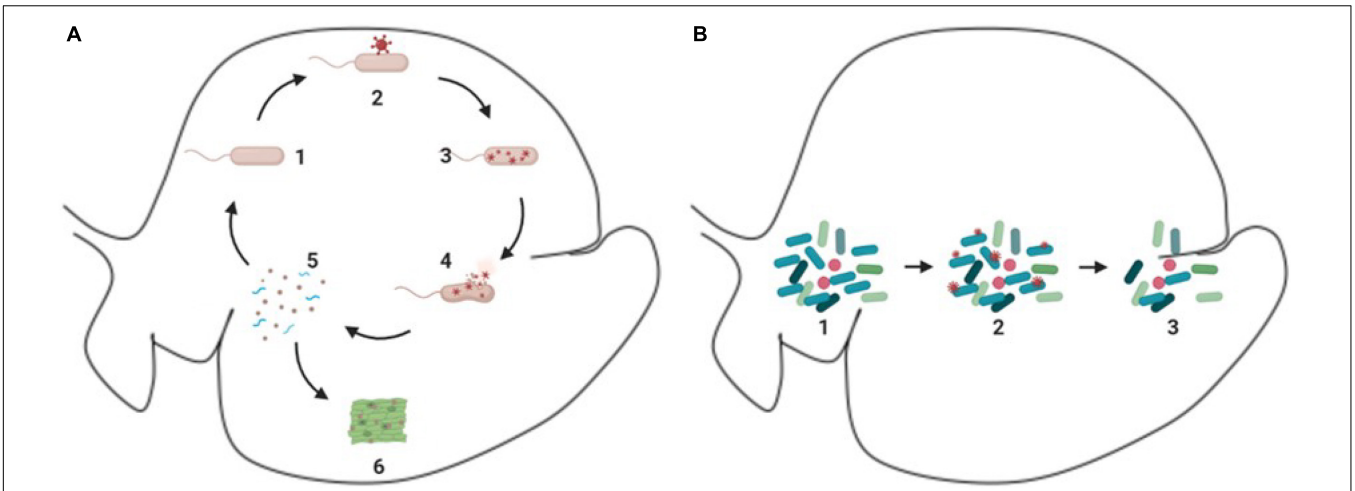


FIGURE 3 | Schematic demonstration of phages and ruminal microorganisms. **(A)** Process of intra-ruminal recycling and enzymatic release. 1 – microorganism in the environment (e.g., rod like microorganism); 2 – attachment of phages (red circular structure) to microorganism; 3 – reproduction of phages (red circular structure) inside of the host cell; 4 – lysis of the host cell and release of virions (red circular structure) and cellular particles; 5 – cellular particles released (blue spiral like particles), which can be used as substrate to other microorganisms; 6 – enzymes released can act in the degradation of feedstuff. **(B)** Bloom of lytic cycle. 1 – microbiome with predominant species (dark blue rod like microorganisms); 2 – phages (small red circular particles) attachment to individuals of the predominant species (dark blue rod like microorganisms); 3-reduction of the predominant species (dark blue rod like microorganisms).

contribute to this process would result in a reduction of methane. Methanogens act as a hydrogen sink, combining hydrogen with carbon dioxide to produce methane (Ungerfeld, 2020), and therefore targeting methanogens with phage activity to control their population and activity would lead to a reduction in methane production. Indeed, this was shown to be the case when archaea population is reduced, the activity of methane pathway is decreased and the hydrogen is redirected toward other metabolic pathways available in the rumen that are more beneficial, such as propionate production (Martin and Macy, 1985). According to Leahy et al. (2010), the discovery of a prophage with 69 phage-related proteins have been described from *Methanobrevibacter ruminantium*, including lytic enzymes which have a potential to be applied as a biocontrol agent for ruminal methanogens, such as Peir from the Methanobrevibacterium prophage φmru. These enzymes may potentially be used to reduce the archaea population in the rumen which could lead to reductions in methane emission and consequently less energy loss from feed.

Altermann et al. (2018) studied the viability of the production of phage-derived lytic enzymes, they used the viral enzyme PeiR from methanogen virus that has the capability to infect *Methanobrevibacter ruminantium*. They fused the gene of PeiR to polyhydroxyalkanoate synthase gene and added it in the genome of *Escherichia coli*. Translation of this gene produced a polyhydroxyakanoate with an active PeiR enzyme at the surface of this nanoparticle. Altermann et al. (2018) also reported that these nanoparticles were able to kill not only the original methanogen host strain cell but a wide range of other ruminal methanogen strains in an *in vitro* pure culture, reducing methane emission by up to 97%.

Another application of phage therapy is to revert cases of dysbiosis. According to Belizário and Napolitano (2015) and Gutiérrez and Domingo-Calap (2020), phage therapy can be used to reestablish the homeostasis of the host microbiome. Under dysbiosis, the homeostasis of the host microbiome is altered, which leads to an increase in pathogenic organisms and a reduction on symbiotic organisms (Palmela et al., 2018), using phage therapy, the organisms that are causing the dysbiosis can be targeted, leading to a reestablishment of homeostasis of the host microbiome.

Recently, a study was published testing an endolysin (LyJH307) to reduce *Streptococcus bovis*, which is a lactic acid-producing bacteria that is highly correlated to development of subacute ruminal acidosis (Kim et al., 2020). According to the authors, the viral molecule LyJH307 presented a potent lytic activity in a wide range of pH and temperature. Also, it was effective in the control of growth not only *S. bovis* isolated from rumen, but in in different groups of *S. bovis*, suggesting that this molecule have the potential to be used in the control of *S. bovis* which is one of the biggest contributors to the development of subacute ruminal acidosis.

USE OF TECHNOLOGY

For the scope of this article, only a brief summary of the technologies used in phages studies was included; however, a comprehensive publication on advances in technology for studying rumen virome has been recently published by Gilbert et al. (2020). The infection of bacteria by phages was first noted in the early 1900's (Twort, 1915; D'Herelle, 1918), a few years later, EM was developed (Haguenau et al., 2003). Electron microscopy is a technique that enable researchers to visualize particles at a nanometer scale, providing a direct image of the subject of study (Richert-Pöggeler et al., 2019), in this case the viral particle.

Electron microscopy enabled pioneer studies with focus on visualization and enumeration of ruminal phages (Hoogenraad et al., 1967; Paynter et al., 1969).

Early work using EM to study the ruminal environment, used ruminal fluid filtered through muslin (which is a type of cotton cloth) and stained for negative contrast using potassium phosphotungstate (Hoogenraad et al., 1967). The results of this study showed a large number of viral particles, either free or attached to different types of cells, especially icosahedral particles and tailed phages (Hoogenraad et al., 1967), although it was observed a large number of phages in the rumen, a quantitative estimation of phages was not made.

Following this pioneering study, a few other studies were published using similar methodologies to investigate ruminal phages from different species of ruminants, such as cattle (Paynter et al., 1969; Klieve and Bauchop, 1988), reindeer (Tarakanov, 1972), and sheep (Klieve and Bauchop, 1988). The morphology of the phages was studied in all of these studies, which reported up to 26 different types of phages and an estimation of viral particle counts ranging from 5×10^7 to 1.4×10^8 viral particles per mL of ruminal content of sheep and cattle (**Table 1**).

Isolation of phages from the ruminal environment was also carried out. Adams et al. (1966) successfully isolated phages from ruminal fluid and were able to demonstrate the concept of phage specificity. They reported that phages that were able to infect *Serratia* host strains from the rumen were unable to infect *Serratia* strains from other environments, such as soil, water, and sewage (Adams et al., 1966). Also, Hoogenraad et al. (1967) demonstrated with EM that phages in the rumen were different from phages from other environments. The isolation technique commonly used was culture-based, in which double-layer agar plates were used for detection of clearing zones within bacterial monolayers (Klieve, 2005). However, those culture-based methods tend to favor the isolation of phages undergoing lytic cycle (Gilbert et al., 2020), which were the most studies forms of phages.

This technology has been used to this date; however, morphological study of phages occasionally create issues such as misclassification due to similar morphological types (Ackermann, 2013) or even identify a false viral particle, such as in cases where cytoplasmic structures were identified as viral particles (Bullock et al., 2021). Also, compared to modern DNA techniques, EM underestimates the richness of viral species (Ross et al., 2013). Nevertheless, EM is still being used as a powerful tool to study the morphology of new isolated phages (Lima et al., 2019; Qin et al., 2019; Park et al., 2020).

The fast development of molecular biology methodologies has allowed rumen microbiologists to use those techniques in the study of ruminal phages (Orpin et al., 1988; Flint, 1997). Techniques based on genome length (such as electrophoresis and blotting techniques) and restriction enzyme mapping have been more commonly utilized (Gilbert et al., 2020). Klieve and Swain (1993), used intact genome lengths and pulsed field gel electrophoresis techniques to build a population profile and estimate phages count, which ranged from 3×10^9 to 1.6×10^{10} viral particle per mL of ruminal content. The pattern observed was mainly of DNA length ranging from 30 to 200 kilo bases, which comprises many types of phages, including temperate ones.

With the advance of molecular biology techniques, total DNA sequencing (commonly known as shotgun metagenomics) (Ross et al., 2013; Anderson et al., 2017; Namonyo et al., 2018) has been used and reported as a good tool for the study of ruminal virome; however, metaproteomics (Solden et al., 2018) and metatranscriptomics (Hitch et al., 2019) have also been used. In shotgun sequencing reports, high throughput sequencing tool is used to sequence the total DNA of a sample, providing millions of reads which are assembled by bioinformatics tools allowing the researchers to identify DNA viruses and annotate the genome. However, the shotgun metagenomic does not include population of RNA viruses and metatranscriptomic is needed to capture this population.

Also, sequencing of DNA from isolated viruses is a powerful strategy to obtain the genomic information, functional prediction, and a better understanding of phages and its interaction with bacteria. Together, Gilbert et al. (2017) and Friedersdorff et al. (2020), reported the entire genome of 10 ruminal phages. According to Friedersdorff et al. (2020), sequenced phages were in a lytic life cycle; however, the functional genomic analysis enabled to infer that some of the phages had lysogeny-associated genes, which suggest that some of these phages could have a temperate life cycle as well. Gilbert et al. (2017), provided insights on how lytic phages interact with the host bacteria in the rumen environment.

TABLE 2 | Summary of published studies characterizing the ruminal virome using high throughput sequencing tools.

Host species	Richness	Method used	Number of animals	Feed used	Source
Cattle	17,993	N of unique viral contigs[a]	3	NA	Berg Miller et al. (2012)
Cattle	435,304	CatchAll (Allen et al., 2013)	13	6 kg of concentrate + *ad libitum* lucerne hay	Ross et al. (2013)
Buffalo	3,239	N of unique viral contigs[a]	1	Pasture	Parmar et al. (2016)
Cattle	∼1000	Chao1	5	Total mixed ration	Anderson et al. (2017)
Sheep and goat	179 and 1,456	N of unique viral contigs[a]	8 and 8	NA	Namonyo et al. (2018)
Moose	810	N of unique viral contigs[a]	1	Wild pasture	Solden et al. (2018)
Sheep	2,466	N of unique viral contigs[a]	20	Pelleted lucerne	Hitch et al. (2019)

[a]*Number of assembled contigs and identified as virus by homology to other known viruses.*

The use of "*omics*" approaches, enable researchers to identify a large number species, Ross et al. (2013) reported an estimation of diversity of up to 27,000 species in the cow's ruminal virome; however, the majority of these species still unknown. Namonyo et al. (2018) reported that more than 90% of the viral reads could not be identified, due to the limited availability of viral genomes in current databases, suggesting that a great amount of genomes are still unknown, creating opportunities to more identification studies. Also, *in silico* analysis of the viral genome, suggested that ruminal phages have glycosidic hydrolases, which could potentially increase the degradation of carbohydrates and consequently increase dietary energy efficiency (Anderson et al., 2017). Indeed "*omic*" approaches can help in the development of the understanding of the virome and its interactions; however, a great amount of variability of the estimated richness and small number of samples were observed (**Table 2**).

CONCLUSION

Viruses are an important component of the ruminal environment and likely play roles in the ecology of the rumen; however, their activation mechanisms in the rumen remain unclear and their interactions with other components of the microbiome, diet, and physicochemical properties of the ruminal environment and subsequent effects on the health and production of livestock animals. The limited number of viral studies, lack of rumen-representative data, limited number of rumen isolates of phages, and very little information on mycophages and protozoal viruses creates a variety of opportunities for future studies. Also, very little is known about the effects of transient viruses in the gastrointestinal tract. An international effort to investigate phages should be developed, similar to the global effort employed to understand the bacterial population and their interactions with health of humans and animals.

Moreover, phages can be used as a powerful biotechnological tool in livestock production, they may have applications in areas such as pathogen control, gastrointestinal tract homeostasis regulators, methane emission reducers, and thus improve energy efficiency in the rumen. However, phages' current applications in livestock production systems are still below their potential due to the lack of knowledge and research in the basic and applied ruminal virome.

AUTHOR CONTRIBUTIONS

RL and AF conceived the idea. RL collected literature data, created the tables and figures, and wrote the first draft of the manuscript. AF reviewed and edited the manuscript. Both authors approved the final version of the manuscript.

FUNDING

RL was sponsored by the National Science Foundation (NSF) awarded to AF. Award number CBET – 1856009.

ACKNOWLEDGMENTS

We acknowledge our colleagues at the University of Florida for the great discussions and feedback. Also, Rosalind A. Gilbert from University of Queensland for the thoughtful advices.

REFERENCES

Ackermann, H. W. (2013). Sad state of phage electron microscopy. Please shoot the messenger. *Microorganisms* 2, 1–10. doi: 10.3390/microorganisms2010001

Adams, J. C., Gazaway, J. A., Brailsford, M. D., Hartman, P. A., and Jacobson, N. L. (1966). Isolation of bacteriophages from the bovine rumen. *Experientia* 22, 717–718. doi: 10.1007/BF01901335

Allen, H. K., Bunge, J., Foster, J. A., Bayles, D. O., and Stanton, T. B. (2013). Estimation of viral richness from shotgun metagenomes using a frequency count approach. *Microbiome* 11, 1–7. doi: 10.1186/2049-2618-1-5

Altermann, E., Schofield, L. R., Ronimus, R. S., Beattie, A. K., and Reilly, K. (2018). Inhibition of rumen methanogens by a novel archaeal lytic enzyme displayed on tailored bionanoparticles. *Front. Microbiol.* 9:2378. doi: 10.3389/fmicb.2018.02378

Anand, T., Virmani, N., Kumar, S., Mohanty, A. K., Pavulraj, S., Bera, B. C., et al. (2020). Phage therapy for treatment of virulent *Klebsiella pneumoniae* infection in a mouse model. *J. Glob. Antimicrob. Resist.* 21, 34–41. doi: 10.1016/j.jgar.2019.09.018

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

Azad, E., Fehr, K. B., Derakhshani, H., Forster, R., Acharya, S., Khafipour, E., et al. (2020). Interrelationships of fiber-associated anaerobic fungi and bacterial communities in the rumen of bloated cattle grazing alfalfa. *Microorganisms* 8, 1–17. doi: 10.3390/microorganisms8101543

Barrangou, R., and Marraffini, L. A. (2014). CRISPR-cas systems: prokaryotes upgrade to adaptive immunity. *Mol. Cell* 54, 234–244. doi: 10.1016/j.molcel.2014.03.011

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

Barrow, P., Dujardin, J. C., Fasel, N., Greenwood, A. D., Osterrieder, K., Lomonossoff, G., et al. (2020). Viruses of protozoan parasites and viral therapy: is the time now right? *Virol. J.* 17:142. doi: 10.1186/s12985-020-01410-1

Belizário, J. E., and Napolitano, M. (2015). Human microbiomes and their roles in dysbiosis, common diseases, and novel therapeutic approaches. *Front. Microbiol.* 6:1050. doi: 10.3389/fmicb.2015.01050

Beller, L., and Matthijnssens, J. (2019). What is (not) known about the dynamics of the human gut virome in health and disease. *Curr. Opin. Virol.* 37, 52–57. doi: 10.1016/j.coviro.2019.05.013

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

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590. doi: 10.1152/physrev.1990.70.2.567

Bullock, H. A., Goldsmith, C. S., Zaki, S. R., Martines, R. B., and Miller, S. E. (2021). Difficulties in differentiating coronaviruses from subcellular structures in human tissues by electron microscopy. *Emerg. Infect. Dis.* 27, 1023–1031. doi: 10.3201/eid2704.204337

Clokie, M. R. J., Millard, A. D., Letarov, A. V., and Heaphy, S. (2011). Phages in nature. *Bacteriophages* 1, 31–45. doi: 10.4161/BACT.1.1.14942

d'Herelle, F. (1917). On an invisible microbe antagonistic toward dysenteric bacilli. *C. R. Acad. Sci.* 165, 373–375. doi: 10.1016/j.resmic.2007.07.005

D'Herelle, F. (1918). Technique de la recherche du microbe filtrant bacteriophage (*Bacteriophagum intestinale*). *C. R. Seances Soc. Biol. Fil.* 81, 1160–1162.

Dufour, N., Delattre, R., Chevallereau, A., Ricard, J. D., and Debarbieux, L. (2019). Phage therapy of pneumonia is not associated with an overstimulation of the inflammatory response compared to antibiotic treatment in mice. *Antimicrob. Agents Chemother.* 63: e00379–19. doi: 10.1128/AAC.00379-19

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

Friedersdorff, J. C. A., Kingston-Smith, A. H., Pachebat, J. A., Cookson, A. R., Rooke, D., and Creevey, C. J. (2020). The isolation and genome sequencing of five novel bacteriophages from the rumen active against *Butyrivibrio fibrisolvens*. *Front. Microbiol.* 11:1588. doi: 10.3389/FMICB.2020.01588

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_9

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

Gnetegha Ayemele, A., Ma, L., Park, T., Xu, J., Yu, Z., and Bu, D. (2020). Giant milkweed (*Calotropis gigantea*): a new plant resource to inhibit protozoa and decrease ammoniagenesis of rumen microbiota in vitro without impairing fermentation. *Sci. Total Environ.* 743:140665. doi: 10.1016/j.scitotenv.2020.140665

Gutiérrez, B., and Domingo-Calap, P. (2020). Phage therapy in gastrointestinal diseases. *Microorganisms* 8:1420. doi: 10.3390/microorganisms8091420

Haguenau, F., Hawkes, P. W., Hutchison, J. L., Satiat-Jeunemaître, 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

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, 1–21. doi: 10.1186/s40104-018-0249-x

Hitch, T. C. A., Edwards, J. E., and Gilbert, R. A. (2019). Metatranscriptomics reveals mycoviral populations in the ovine rumen. *FEMS Microbiol. Lett.* 366:161. doi: 10.1093/femsle/fnz161

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

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

Jault, P., Leclerc, T., Jennes, S., Pirnay, J. P., Que, Y. A., Resch, G., et al. (2019). Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect. Dis.* 19, 35–45. doi: 10.1016/S1473-3099(18)30482-1

Johannessen, T. V., Larsen, A., Bratbak, G., Pagarete, A., Edvardsen, B., Egge, E. D., et al. (2017). Seasonal dynamics of haptophytes and dsDNA algal viruses suggest complex virus-host relationship. *Viruses* 9:84. doi: 10.3390/v9040084

Kim, H., Lee, H. G., Kwon, I., and Seo, J. (2020). Characterization of endolysin LyJH307 with antimicrobial activity against *Streptococcus bovis*. *Animals* 10:963. doi: 10.3390/ANI10060963

Klieve, A. V. (2005). *Methods in Gut Microbial Ecology for Ruminants*, 1st Edn, eds H. P. S. Makkar and C. S. McSweeney (Berlin: Springer-Verlag), doi: 10.1007/1-4020-3791-0

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 Swain, R. A. (1993). Estimation of ruminal bacteriophage numbers by pulsed-field gel etectrophoresis and laser densitometry. *Appl. Environ. Microbiol.* 59, 2299–2303.

Klieve, A. V., Swain, R. A., and Nolan, J. V. (1996). Bacteriophages in the rumen: types presents, population size, and implications for the efficiency of feed utilization. *Aust. Soc. Anim. Prod.* 2, 92–94.

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

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., 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

Lima, J. A., Neto, J. P. N., Castro, K. S., da Rosa, A. P., de, A. T., Tesh, R., et al. (2019). Characterization of triniti virus supports its reclassification in the family peribunyaviridae. *J. Gen. Virol.* 100, 137–144. doi: 10.1099/jgv.0.001196

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., and Darnell, J. (2000). "Viruses: structure, function, and uses," in *Molecular Cell Biology* ed. San Francisco (New York, NY: W. H. Freeman), 4.

Martin, S. A., and Macy, J. M. (1985). Effects of monensin, pyromellitic diimide and 2-bromoethanesulfonic acid on rumen fermentation in vitro. *J. Anim. Sci.* 60, 544–550. doi: 10.2527/JAS1985.602544X

Matsuzaki, S., Rashel Jumpei Uchiyama, M., Sakurai, S., Ujihara Masayuki Kuroda, T., Ikeuchi, M., Tani Mikiya Fujieda, T., et al. (2005). Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. *J. Infect. Chemother.* 11, 211–219. doi: 10.1007/s10156-005-0408-9

Minot, S., Sinha, R., Chen, J., Li, H., 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

Mojica, K. D. A., and Brussaard, C. P. D. (2014). Factors affecting virus dynamics and microbial host-virus interactions in marine environments. *FEMS Microbiol. Ecol.* 89, 495–515. doi: 10.1111/1574-6941.12343

Morkhade, S. J., Bansod, A. P., Kolaskar, A. G., and Thakare, S. O. (2020). A complete review on: methanogens methane producers of rumen and abatement strategies-biotechnology and microbiological strategies review. *Int. J. Vet. Sci. Anim. Husb.* 5, 11–17.

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

Oh, J. H., Alexander, L. M., Pan, M., Schueler, K. L., Keller, M. P., Attie, A. D., et al. (2019). Dietary fructose and microbiota-derived short-chain fatty acids promote bacteriophage production in the gut symbiont *Lactobacillus reuteri*. *Cell Host Microbe* 25, 273–284.e6. doi: 10.1016/j.chom.2018.11.016

Orpin, C. G., Hazlewood, G. P., and Mann, S. P. (1988). Possibilities of the use of recombinant-DNA techniques with rumen micro-organisms. *Anim. Feed Sci. Technol.* 21, 161–174. doi: 10.1016/0377-8401(88)90097-1

Palmela, C., Chevarin, C., Xu, Z., Torres, J., Sevrin, G., Hirten, R., et al. (2018). Adherent-invasive *Escherichia coli* in inflammatory bowel disease. *Gut* 67, 574–587. doi: 10.1136/gutjnl-2017-314903

Parasion, S., Kwiatek, M., Gryko, R., Mizak, L., and Malm, A. (2014). Bacteriophages as an alternative strategy for fighting biofilm development. *Polish J. Microbiol.* 63, 137–145.

Park, M., Cho, Y. J., Kim, D., Yang, C. S., Lee, S. M., Dawson, T. L., et al. (2020). A novel virus alters gene expression and vacuolar morphology in *Malassezia* cells and induces a TLR3-mediated inflammatory immune response. *Host Microbe Biol.* 11, 1–19. doi: 10.1128/mBio.01521-20

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. doi: 10.18520/cs/v111/i5/919-925

Paul, J. H. (2008). Prophages in marine bacteria: dangerous molecular time bombs or the key to survival in the seas? *ISME J.* 2, 579–589. doi: 10.1038/ismej.2008.35

Payet, J. P., and Suttle, C. A. (2013). To kill or not to kill: the balance between lytic and lysogenic viral infection is driven by trophic status. *Limnol. Oceanogr.* 58, 465–474. doi: 10.4319/lo.2013.58.2.0465

Paynter, M. J. B., Ewert, D. L., and Chalupa, W. (1969). Some morphological types of bacteriophages in bovine rumen contents. *Appl. Environ. Microbiol.* 18, 942–943.

Qin, P., Du, E.-Z., Luo, W.-T., Yang, Y.-L., Zhang, Y.-Q., Wang, B., et al. (2019). Characteristics of the life cycle of porcine deltacoronavirus (PDCoV) in vitro: replication kinetics, cellular ultrastructure and virion morphology, and evidence of inducing autophagy. *Viruses* 11:455. doi: 10.3390/v11050455

Rabee, A. E., Forster, R. J., Elekwachi, C. O., Kewan, K. Z., Sabra, E. A., Shawket, S. M., et al. (2019). Community structure and fibrolytic activities of anaerobic rumen fungi in dromedary camels. *J. Basic Microbiol.* 59, 101–110. doi: 10.1002/jobm.201800323

Rasmussen, T. S., de Vries, L., Kot, W., Hansen, L. H., Castro-Mejía, J. L., Vogensen, F. K., et al. (2019). Mouse vendor influence on the bacterial and viral gut composition exceeds the effect of diet. *Viruses* 11:435. doi: 10.3390/v11050435

Richert-Pöggeler, K. R., Franzke, K., Hipp, K., and Kleespies, R. G. (2019). Electron microscopy methods for virus diagnosis and high resolution analysis of viruses. *Front. Microbiol.* 10:3255. doi: 10.3389/fmicb.2018.03255

Ritchie, A. E., Robinson, I. M., and Allisin, M. J. (1970). "Rumen bacteriophage: survey of morphological types," in *Microscopie Electronique, Societe Francaise de Microscopie Electronique*, Vol. 3, ed. P. Favard (Paris: Societe Francaise de Microscopie Electronique), 333–334.

Ross, E. M., Petrovski, S., Moate, P. J., and Hayes, B. J. (2013). Metagenomics of rumen bacteriophage from thirteen lactating dairy cattle. *BMC Microbiol.* 13:242. doi: 10.1186/1471-2180-13-242

Schooley, R. T., Biswas, B., Gill, J. J., Hernandez-Morales, A., Lancaster, J., Lessor, L., et al. (2017). Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrob. Agents Chemother.* 61:e00954. doi: 10.1128/AAC.00954-17

Schwab, C. G., and Broderick, G. A. (2017). A 100-year review: protein and amino acid nutrition in dairy cows. *J. Dairy Sci.* 100, 10094–10112. doi: 10.3168/jds.2017-13320

Silva, L. F. P., Dixon, R. M., and Costa, D. F. A. (2019). Nitrogen recycling and feed efficiency of cattle fed protein-restricted diets. *Anim. Prod. Sci.* 59:2093. doi: 10.1071/AN19234

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

Soucy, S. M., Huang, J., and Gogarten, J. P. (2015). Horizontal gene transfer: building the web of life. *Nat. Rev. Genet.* 16, 472–482. doi: 10.1038/nrg3962

Suttle, C. A. (2007). Marine viruses - Major players in the global ecosystem. *Nat. Rev. Microbiol.* 5, 801–812. doi: 10.1038/nrmicro1750

Swain, R. A., Nolan, J. V., and Klieve, A. V. (1996). Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. *Appl. Environ. Microbiol.* 62, 994–997.

Tarakanov, B. V. (1972). The electron-microscopy examination of the microflora of reindeer rumen. *Mikrobiologia* 41, 862–870.

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

Tsai, B. (2007). Penetration of nonenveloped viruses into the cytoplasm. *Annu. Rev. Cell Dev. Biol.* 23, 23–43.

Twort, F. W. (1915). An investigation on the nature of ultra-microscopic viruses. *Lancet* 186, 1241–1243. doi: 10.1016/S0140-6736(01)20383-3

Ungerfeld, E. M. (2020). Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Front. Microbiol.* 11:589. doi: 10.3389/FMICB.2020.00589

Conflict of Interest: The 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 January 2022
doi: 10.3389/fmicb.2021.758395

Fresh Rumen Liquid Inoculant Enhances the Rumen Microbial Community Establishment in Pre-weaned Dairy Calves

*Hanna Huuki[1,2], Seppo Ahvenjärvi[3], Paula Lidauer[4], Milka Popova[5], Johanna Vilkki[2], Aila Vanhatalo[1] and Ilma Tapio[2]**

[1] Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland, [2] Production Systems, Genomics and Breeding, Natural Resources Institute Finland (Luke), Jokioinen, Finland, [3] Production Systems, Animal Nutrition, Natural Resources Institute Finland (Luke), Jokioinen, Finland, [4] Production Systems, Welfare of Farmed Animals, Natural Resources Institute Finland (Luke), Jokioinen, Finland, [5] Institute National de la Recherche Agronomique, UMR 1213 Herbivores, Clermont Université, VetAgro Sup, UMR Herbivores, Clermont-Ferrand, France

OPEN ACCESS

Edited by:
Christina Diane Moon, AgResearch Ltd., New Zealand

Reviewed by:
Alejandro Belanche, University of Zaragoza, Spain
Robert W. Li, Agricultural Research Service, United States Department of Agriculture, United States

***Correspondence:**
Ilma Tapio
ilma.tapio@luke.fi

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *13 August 2021*
Accepted: *30 November 2021*
Published: *12 January 2022*

Citation:
Huuki H, Ahvenjärvi S, Lidauer P, Popova M, Vilkki J, Vanhatalo A and Tapio I (2022) Fresh Rumen Liquid Inoculant Enhances the Rumen Microbial Community Establishment in Pre-weaned Dairy Calves. Front. Microbiol. 12:758395. doi: 10.3389/fmicb.2021.758395

The development of the functional rumen in calves involves a complex interplay between the host and host-related microbiome. Attempts to modulate rumen microbial community establishment may therefore have an impact on weaning success, calf health, and animal performance later in life. In this experiment, we aimed to elucidate how rumen liquid inoculum from an adult cow, provided to calves during the pre-weaning period, influences the establishment of rumen bacterial, archaeal, fungal, and ciliate protozoan communities in monozygotic twin calves (n = 6 pairs). The calves were divided into treatment (T-group) and control (C-group) groups, where the T-group received fresh rumen liquid as an oral inoculum during a 2–8-week period. The C-group was not inoculated. The rumen microbial community composition was determined using bacterial and archaeal 16S ribosomal RNA (rRNA) gene, protozoal 18S rRNA gene, and fungal ITS1 region amplicon sequencing. Animal weight gain and feed intake were monitored throughout the experiment. The T-group tended to have a higher concentrate intake (Treatment: $p < 0.08$) and had a significantly higher weekly weight gain (Treatment: $p < 0.05$), but no significant difference in volatile fatty acid concentrations between the groups was observed. In the T-group, the inoculum stimulated the earlier establishment of mature rumen-related bacterial taxa, affecting significant differences between the groups until 6 weeks of age. The inoculum also increased the archaeal operational taxonomic unit (OTU) diversity (Treatment: $p < 0.05$) but did not affect the archaeal quantity. Archaeal communities differed significantly between groups until week 4 ($p = 0.02$). Due to the inoculum, ciliate protozoa were detected in the T-group in week 2, while the C-group remained defaunated until 6 weeks of age. In week 8, *Eremoplastron dilobum* was the dominant ciliate protozoa in the C-group and *Isotricha* sp. in the T-group, respectively. The Shannon diversity of rumen anaerobic fungi reduced with age (Week: $p < 0.01$), and community establishment was influenced by a change

of diet and potential interaction with other rumen microorganisms. Our results indicate that an adult cow rumen liquid inoculum enhanced the maturation of bacterial and archaeal communities in pre-weaning calves' rumen, whereas its effect on eukaryotic communities was less clear and requires further investigation.

Keywords: microbiome manipulation, microbiome establishment, dairy calf, ciliate protozoa, anaerobic fungi, bacteria, archaea, rumen function

INTRODUCTION

The microbial ecosystem inhabiting the rumen enables ruminants to utilize plant materials as feed. However, immediately after birth, ruminant feed digestion more resembles that of monogastrics (Comline and Titchen, 1951; Baldwin et al., 2004; Meale et al., 2017). It takes several weeks of morphological, physiological, and metabolical changes, induced by the ontogenic regulation, nutrition, and establishment of rumen microorganisms, for the rumen to become fully functional to provide for most of the animal's energy needs (Baldwin et al., 2004; Khan et al., 2016; Meale et al., 2017; Malmuthuge et al., 2019). The successful development of functional rumen during the pre-weaning period may affect animal performance later in life (Yáñez-Ruiz et al., 2015). Understanding how this system can be influenced is therefore one of the current research focal points worldwide.

Active gut colonization of newborns starts at birth through exposure to the microbiomes of the dam's vagina and skin, feces, mouth, colostrum, and milk (Yeoman et al., 2018; Klein-Jöbstl et al., 2019), as well as through the housing environment, with other conspecifics (Bird et al., 2010). During the pre-ruminant phase (0–14 days), when calves are sustained on a liquid diet, the rumen function is still very low, as most of the liquid is digested by the enzymes in the abomasum and small intestine (Comline and Titchen, 1951; Ørskov et al., 1970; Baldwin et al., 2004). Nevertheless, during this period, the rumen starts being populated by the aerobic and facultative anaerobic bacteria, gradually changing to obligate anaerobes (Jami et al., 2013; Rey et al., 2014). Cellulolytic and proteolytic bacteria, necessary for a functional rumen, have been detected in the rumen as early as 20 min to 3 days after birth (Li et al., 2012; Rey et al., 2012, 2014; Jami et al., 2013; Guzman et al., 2015). Methanogenic archaea also start populating the rumen during the first days of life (Guzman et al., 2015; Friedman et al., 2017), while anaerobic fungi have been observed in the rumen at around 1 week after birth (Fonty et al., 1987).

In the transition phase (from 2 weeks to weaning), solid feed particles and liquid start entering the rumen, fueling the fermentation process and causing physical stress that promotes the expansion of the volume and muscular development of the rumen and rumen motility (Khan et al., 2016). During this period, the papillae's growth increases the rumen epithelium surface area needed for more efficient volatile fatty acid (VFA) absorption and metabolism (Baldwin et al., 2004; Khan et al., 2016; Meale et al., 2017). Rumination starts at around 2 weeks of age, and after 6 weeks, it gradually reaches a level similar to that of adult cows (Swanson and Harris, 1958). The transition phase is related to further rearrangements of microbial community composition (Dias et al., 2017), with ciliate protozoa being the last microorganisms to colonize the rumen. They have been detected in animals after 2 weeks of age, once stable bacterial community and rumen conditions have been established (Eadie, 1962b; Fonty et al., 1988).

Attempts to affect early life rumen microbial colonization and development have explored various strategies. Natural rearing with the mother affects the microbiota development (Abecia et al., 2017; Belanche et al., 2019b) and can have a positive impact on young ruminants' performance (Belanche et al., 2019a). Dietary intervention studies have demonstrated that supplementing the liquid diet with concentrate (Malmuthuge et al., 2013; Jiao et al., 2015; Dias et al., 2017) or forage (Jiao et al., 2015; Carballo et al., 2019; Dill-McFarland et al., 2019) can promote rumen maturation and alter the rumen microbial community. The use of antimicrobials has been found to alter microbial communities and reduce methane emissions (Abecia et al., 2013; Meale et al., 2021). Similarly, the inoculation of calves with rumen liquid influences their rumen bacterial community composition by developing into more adult-like, improved papillae growth and increases dry matter intake (Cox et al., 2019). In lambs and goats, a rumen inoculum has improved growth performance (Zhong et al., 2014; Belanche et al., 2020), dry matter intake (De Barbieri et al., 2015; Belanche et al., 2020), and microbial colonization during the pre-weaning period (Belanche et al., 2020; Palma-Hidalgo et al., 2021), while no effect on calf growth and microbial composition was observed when bacteria- or protozoa-enriched inoculum was administered (Cersosimo et al., 2019).

Despite research efforts, the mechanisms of how rumen microbial community development can be modulated remain elusive. The genetics of an individual plays a role, as monozygotic or dizygotic twins have more similar microbiota than non-related individuals in humans (Turnbaugh et al., 2009; Goodrich et al., 2014, 2016; Koo et al., 2019) and calves (Mayer et al., 2012). In this study, we therefore used monozygotic twin calves to reduce the potential variation in microbial community establishment caused by the differences in animals' genetic background. Our goal was to modulate rumen microbial community colonization in calves during the pre-weaning period by inoculating them with fresh rumen liquid obtained from a cow with a low residual energy intake value (Mäntysaari et al., 2012). We assessed the effect of inoculation on the establishment of rumen general and core microbiome and rumen fermentation characteristics. We hypothesized that the microbial community structure of treated

calves would more closely resemble that of the adult cow and that the inoculum might promote fermentation processes in the rumen and potentially stimulate the growth of calves.

MATERIALS AND METHODS

Animals and Experimental Design

Fifteen Nordic Red dairy cows were impregnated with twin embryos produced by embryo splitting (Herr and Reed, 1991) at the Natural Resources Institute Finland (Luke). Six pregnancies resulted in the calving of twins (July 23–October 28, 2017). After birth, the calves were left with their dam for an hour and then separated into individual pens (147 cm × 172 cm) that prevented physical contact and microbial exchange between the calves or contact with other animals throughout the experiment. Pens were furnished with straw bedding, a heat lamp, a basket for hay, and buckets for water and concentrate. The calves were weighed at birth and within each pair were randomly assigned to either an inoculation treatment (T-group) or a control group (C-group).

The calves from both groups were managed and fed in the same way. Within 2 h of birth, all the calves received 2 L of the same high-quality (Brix value 24) colostrum mix as the first meal to ensure uniformity in the passive transfer of immunity *via* the colostrum. Colostrum from 3 cows was collected in advance, aliquoted into 0.5-L portions, and stored at −20°C until use. Before use, portions were warmed to 40°C, pooled, and bottle-fed to the calves. As the second meal, the twins received their own dam's colostrum from the first milking and thereafter were fed bulk colostrum. From day (d)4 onward, they received 2 L of milk four times a day. From d8, the milk was gradually replaced with the milk replacer (Startti Maito Instant, Valio, Finland). Weaning was carried out gradually, starting on d29. The calves were completely weaned from the milk replacer after the experiment by d57. The detailed schedule of the weaning process is presented in **Supplementary Table 1**. Hay and calf concentrate (Pikku-mullin-herkku, Raisio Agro, Finland) were offered from d1 and topped up according to consumption. Starting from week 7, grass silage was offered up to a maximum of 2 kg/day. The dietary ingredients and chemical composition of feeds are presented in **Supplementary Tables 2**, **3**.

Starting from week 2, the calves in the T-group received rumen liquid obtained from a fistulated multiparous cow (176–269 days in milk) fed a concentrate:forage diet (ratio 48:52) and previously identified as a feed-efficient animal based on a low residual energy intake (Mäntysaari et al., 2012). Freshly collected rumen liquid was given to the calves orally through a silicone tube attached to a syringe. During weeks 2 and 3, the oral dose was 5 ml/day, given 3 times a week (Monday, Wednesday, and Friday). Later, in week 4, the dose was increased to 10 ml/day. The inoculation treatment ended when the calves were 8 weeks old.

The calves' health was monitored and recorded daily by the farm veterinarian. During the treatment period, two calves from both groups were offered electrolyte (Benfital Plus nutrient supplement, Boehringer Ingelheim Danmark A/S) for diarrhea, and two calves in the T-group and one in the C-group received a dose of activated carbon paste (Lehmän HIILI-pasta, FinnCow, Finland). Animal weight was measured at birth and once a week thereafter. The weekly weight gain was calculated by subtracting the previous week's weight from the current weight.

Feed refusals were recorded after each meal to measure the intake of liquid diet, concentrate, and grass silage. Daily feed intake was calculated by subtracting the weight of leftovers from that of the offered feed. Hay was only monitored by recording the offered amount of hay per day. Real consumption was impossible to measure due to the hay falling onto the straw bedding. The solid feed intake was reported as g of dry matter based on the feed analysis.

Sample Collection

Blood samples were collected from the jugular vein in K2E EDTA tubes (Greiner Bio-one, Austria) once a week during the 8-week period. Analysis of blood immunoglobulin G (IgG) was performed at Movet oy (Kuopio, Finland) to confirm the proper transfer of passive immunity.

Rumen fluid was collected *via* the esophageal 1-m polyvinyl chloride (PVC) tube (9/13 mm inner/outer diameter) into a glass jar attached to a manual vacuum pump (Ruminator[1], Germany). The samples were collected 3 h after morning feeding in weeks 2, 4, 6, and 8 at least 24 h after the previous inoculation. Immediately after collection, 500-μl aliquots were snap-frozen in dry ice and stored at −80°C until DNA extraction.

The rumen sample collection for VFA and ammonia-N determination was carried out as described by Ahvenjärvi and Huhtanen (2018). Briefly, fresh rumen liquid was filtered through two layers of cheesecloth, a 5-ml aliquot was mixed with 0.5 ml of saturated mercuric (II) chloride solution and 2 ml of 1 M sodium hydroxide solution, and stored at −20°C for later VFA analysis with gas chromatography (Huhtanen et al., 1998). To determine the ammonia-N concentrations, 15 ml of rumen liquid was mixed with 0.3 ml of 50% sulfuric acid and stored at −20°C for later analysis based on the direct colorimetric method (McCullough, 1967).

To assess the rumen inoculum microbial community composition, 500-μl samples from each inoculum batch were collected, snap-frozen in dry ice, and stored at −80°C until DNA extraction. Before DNA extraction, samples from each week were pooled into 16 samples, so that they represented the different weeks throughout the treatment period.

DNA Extraction, Library Preparation, and Sequencing

The total DNA was extracted from 500 μl of rumen liquid samples, as described by Rius et al. (2012), and stored at −20°C for further analysis. Libraries of the bacterial and archaeal 16S ribosomal RNA (rRNA) V4 region were prepared following the "16S metagenomics sequencing library preparation" protocol (Illumina) using 515F and 806R primers (Caporaso et al., 2011) with Illumina adapters. The same protocol was adapted for the library preparation of the ciliate protozoa 18S rRNA V3 region using 316F and 539R primers (Sylvester et al., 2004) and for the internal transcribed spacer 1 (ITS1) region of rumen

[1]profs-products.com

TABLE 1 | Alpha-diversity estimates for bacterial, archaeal, ciliate protozoan, and fungal communities in treatment (T-group) and control (C-group) calves during the 8-week experimental period.

Study week	Group	Bacteria			Archaea			Ciliate protozoa			Anaerobic fungi		
		N	Observed OTUs	Shannon diversity	Simpson	*N*	Observed OTUs	Shannon diversity	Simpson	*N*	Observed OTUs	Shannon diversity	Simpson	*N*	Observed OTUs	Shannon diversity	Simpson
Week 2	**T-group**	6	482 ± 133	5.20 ± 0.50	0.92 ± 0.03	6	8 ± 4	1.70 ± 0.60	0.59 ± 0.12	5	73 ± 9	2.20 ± 0.30	0.69 ± 0.06	5	438 ± 107	4.00 ± 0.50	0.88 ± 0.03
	C-group	6	411 ± 41	5.30 ± 0.40	0.94 ± 0.02	5	2 ± 1	0.50 ± 0.40	0.18 ± 0.17	0	–	–	–	6	467 ± 87	4.40 ± 0.40	0.91 ± 0.02
Week 4	**T-group**	6	963 ± 315	7.00 ± 0.90	0.97 ± 0.02	6	15 ± 4	2.30 ± 0.50	0.69 ± 0.10	6	84 ± 11	2.50 ± 0.60	0.74 ± 0.10	3	440 ± 41	4.30 ± 0.00	0.90 ± 0.01
	C-group	6	597 ± 88	6.11 ± 0.30	0.97 ± 0.01	6	8 ± 2	1.50 ± 0.40	0.48 ± 0.12	0	–	–	–	6	392 ± 111	3.60 ± 1.00	0.81 ± 0.14
Week 6	**T-group**	6	1,063 ± 516	7.20 ± 1.30	0.97 ± 0.02	6	16 ± 8	2.10 ± 0.70	0.61 ± 0.17	6	89 ± 11	2.40 ± 0.90	0.67 ± 0.25	3	431 ± 117	4.10 ± 0.50	0.88 ± 0.04
	C-group	6	732 ± 93	6.60 ± 0.50	0.97 ± 0.01	6	9 ± 3	1.60 ± 0.60	0.52 ± 0.21	3	82 ± 24	2.20 ± 1.10	0.60 ± 0.33	4	313 ± 115	3.00 ± 1.10	0.71 ± 0.24
Week 8	**T-group**	6	1,015 ± 601	6.80 ± 1.60	0.96 ± 0.03	6	13 ± 6	2.00 ± 0.40	0.62 ± 0.07	6	74 ± 18	1.90 ± 1.20	0.50 ± 0.30	2	328 ± 33	2.80 ± 0.10	0.64 ± 0.08
	C-group	6	837 ± 200	6.60 ± 0.70	0.96 ± 0.03	6	13 ± 6	1.70 ± 0.20	0.53 ± 0.13	4	76 ± 19	1.60 ± 1.20	0.42 ± 0.32	4	252 ± 158	2.40 ± 0.80	0.61 ± 0.14
***p*-value**	**Wk**		<0.001	<0.001	0.002		0.005	0.072	0.207		0.131	0.369	0.414		0.048	0.007	0.003
	Trt		0.228	0.421	0.757		0.015	0.003	0.001		0.642	0.499	0.554		0.543	0.238	0.320
	Trt × Wk		<0.001	0.002	0.019		0.002	0.009	0.012		0.437	0.737	0.796		0.212	0.012	0.011

The number of samples for each group and week is indicated as N. Data are presented as averages ± standard deviation. The statistical significance of Treatment (Trt), Week (Wk), and Treatment by Week interaction (Trt × Wk) was tested with the Kruskal–Wallis rank sum test. The C-group remained defaunated until week 6. The statistical analyses for ciliate protozoa between the groups were performed for week 6 and week 8 data only. OTU, operational taxonomic unit.

anaerobic fungi using Neo18SF and Neo5.8SR primers (Edwards et al., 2008). A detailed description of primers is provided in **Supplementary Table 4**, and protocols for library preparation are in **Supplementary Material 1**. Libraries were sequenced on Illumina MiSeq (Finnish Functional Genomics Centre, Turku) using the Paired-End approach and 2 × 250 bp chemistry for the 16S rRNA gene library and 2 × 300 bp for the ciliate protozoa 18S rRNA and fungal ITS1 libraries. The number of samples for each animal group and week that successfully amplified are presented in **Table 1**.

Sequence Data Processing

Sequence data demultiplexing, adaptor removal, and sorting of sequences by barcode were performed by the sequencing provider (Finnish Functional Genomics Centre, Turku). Sequencing data were further processed using Qiime v 1.9.1 (Caporaso et al., 2010). Briefly, the forward and reverse reads were joined using SeqPrep and filtered by quality (>Q20) and length (279–300 bp). Filtered sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using UCLUST (Edgar, 2010). Chimeric reads from bacteria data were removed using ChimeraSlayer (Haas et al., 2011) and from fungal and ciliate protozoa data using usearch61 (Edgar, 2010). The taxonomy of the bacterial OTUs was assigned using Greengenes (gg 13 8 otus) (DeSantis et al., 2006), the archaeal using RIM-DB (Seedorf et al., 2014), ciliate protozoan using ciliate protozoa (Kittelmann et al., 2015), and fungal OTUs using ITS (Koetschan et al., 2014), reference databases. Singleton OTUs were removed. The prokaryote data were evenly subsampled to 17,000, ciliate protozoa to 18,000, and fungal data to 25,000 reads/sample before further analysis to reduce the depth heterogeneity. The sufficient sequencing depth was confirmed by the rarefaction plots (**Supplementary Material 1** and **Supplementary Figure 1**). The sequence reads are available in the Sequence Read Archive (SRA) BioProject PRJNA713003.

Quantification of Microbial Communities

The quantities of bacteria, archaea, ciliate protozoa, and anaerobic fungi were estimated with qPCR by quantifying rRNA gene copy numbers of each taxonomic group in 1 ng of extracted DNA. The bacteria were quantified by amplifying the 16S rRNA gene V4 area (279 bp) using primers 520F and 799r2cor (**Supplementary Table 4**). Amplification reaction (20 μl) contained 0.75 × TB Green Premix Ex Taq II (TaKaRa Bio Inc., China), 0.5 μM of each primer, and 20 ng of DNA. The amplification was done in a StepOnePlus thermocycler (Applied Biosystems, Villebon-sur-Yvette, France) with denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 30 s. For archaea quantification, the 16S rRNA gene (510 bp) was amplified using 896F and 1406R primers (**Supplementary Table 4**). The reaction was carried out at a volume of 10 μl, with a 1 × Power SYBR green PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Life Technologies Ltd., United Kingdom), 0.15 μM of each primer, and 10 ng of template DNA. The quantification of archaea was done in a Viia7 thermocycler (Applied Biosystems) with initial denaturation at 95°C for 10 min, 40 cycles of denaturation

at 95°C for 15 s, and annealing at 60°C for 1 min. Ciliate protozoa were quantified by amplifying the 18S rRNA gene region (223 bp) using 316F and 539R primers (**Supplementary Table 4**). Amplification was carried out at a volume of 10 μl, with a 1 × Power SYBR green PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Life Technologies Ltd., United Kingdom), 0.25 μM of each primer, and 10 ng of template DNA. The quantification was done in a Viia 7 thermocycler (Applied Biosystems) with initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Anaerobic fungi were quantified by amplifying the ITS1 region (120 bp) with FungiF and FungiR primers (**Supplementary Table 4**). The reaction was carried out at a volume of 15 μl, with a 1 × Power SYBR green PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Life Technologies Ltd., United Kingdom), 2 mM of $MgCl_2$, 1 μM of each primer, and 10 ng of template DNA. The quantification of anaerobic fungi was done in a Viia7 thermocycler (Applied Biosystems) with initial denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min. For bacteria and archaea, the melt curve was performed with denaturation at 95°C for 15 s, annealing at 65°C for 15 s, and final denaturation at 95°C for 15 s, with a ramp increment of 0.4°C. For ciliate protozoa and fungi, the melt curve annealing temperature was set to 60°C for 1 min, followed by final denaturation at 95°C for 15 s, with a ramp increment of 0.4°C. All standards and samples were analyzed with three replicates. The absolute quantities of experimental samples were estimated against serial dilutions of DNA standards ranging from 10^2 to 10^9 copies per reaction. A description of the standard preparation is provided in **Supplementary Material 1**. The results were regressed against the logarithmic scale concentration to achieve the standard curve and sample quantities.

Statistical Analyses

The rumen VFA and ammonia-N concentrations, bacterial and archaeal 16S rRNA gene copies, average weekly feed intake, weight gain, and blood IgG levels were analyzed using the mixed procedure, with a repeated approach in SAS version 9.4 (SAS Institute Inc., Cary, NC, United States). Normality was confirmed with the Shapiro–Wilk test. Weight, concentrate, and silage intake data were log-transformed to achieve normal distribution and later reverse transformed for data interpretation. The calf was treated as the experimental unit. Treatment, Week, and Treatment × Week interaction were treated as fixed effects. Pair and Pair × Week interaction were treated as random effects. The fact that samplings were repeated over time and were therefore correlated was taken into account by modeling the correlations between weeks and week variances using appropriate covariance structures selected based on model fit statistics. The effects were estimated using the Residual Maximum Likelihood (REML) method and were declared significant at $p \leq 0.05$. Pairwise comparisons between Week, Treatment, and Treatment × Week interaction were performed using Tukey's test. The copy numbers of ciliate protozoa 18S rRNA gene and fungal ITS1 regions were analyzed with the non-parametric Kruskal–Wallis rank sum test and pairwise comparisons with the Wilcoxon rank sum test.

Microbial community alpha diversity changes in the T- and C-groups over the 8-week period were estimated using the Shannon index, the Simpson index, and the number of observed OTUs, as implemented in Qiime. The statistical analysis was performed using the Kruskal–Wallis rank sum test and pairwise comparisons with the Wilcoxon rank sum test.

To explore the treatment and time effect on the changes in the microbial community structure, between-sample diversity was evaluated as Bray–Curtis dissimilarities following the Hellinger transformation and visualized using principal coordinate analysis (PCoA), as implemented in R packages *Microbiome* (Lahti et al., 2017) and *Phyloseq* (McMurdie and Holmes, 2013). The significance for the Week, Treatment, and Treatment × Week interaction was estimated using distance-based permutational multivariate analysis (Adonis) as implemented in the R package *vegan* (Oksanen et al., 2019) and the pairwise comparisons between the Treatment × Week interaction with permutational multivariate analysis of variance with the false discovery rate adjustment with the R package *RVAideMemoire* (Herve, 2020). Treatment and week effects on individual microbial taxa in the T- and C-groups were evaluated using the ANOVA and mixed procedure as described above. OTUs with relative abundance below 0.1% and detected in less than 6 samples were filtered out from the bacterial and fungal datasets. To achieve normal distribution and deal with 0-values, data were $[\log_2(1 + x)]$ transformed prior to further analysis. Treatment, Week, and the Treatment × Week interaction were treated as fixed effects, and pairwise comparisons were made using Tukey's test.

To explore core microbiome establishment in the T- and C-groups during different stages of development, the core microbiome was defined as a set of OTUs that was present in all calves within a group at a particular week tested. The impact of inoculation on the core microbiome establishment in calves was evaluated by the proportion of OTUs shared between the donor and calves. A more detailed description of statistics is given in **Supplementary Material 1**.

RESULTS

Passive Immunity and General Health

At the beginning of the experiment, blood IgG levels indicated a sufficient passive transfer of IgG after birth, and the concentration was similar in both groups (**Figure 1A**). No significant changes related to the initiation of inoculation were observed in the IgG levels in the T-group at the age of 2 weeks (Treatment: $p > 0.05$, Treatment × Week: $p > 0.05$). The mean IgG levels dropped from a "high" (>24 g/l) in the T-group and "medium" (16–24 g/l) in the C-group to a "low" level (16–8 g/l) in week 3 (Week: $p = 0.0001$) and further declined until week 5, after which they stabilized.

The calves in both groups remained in good general health throughout the experiment. Four calves in the T-group and six calves in the C-group had an incidence of diarrhea that occurred between 2 and 4 weeks of age. One calf from the C-group and two

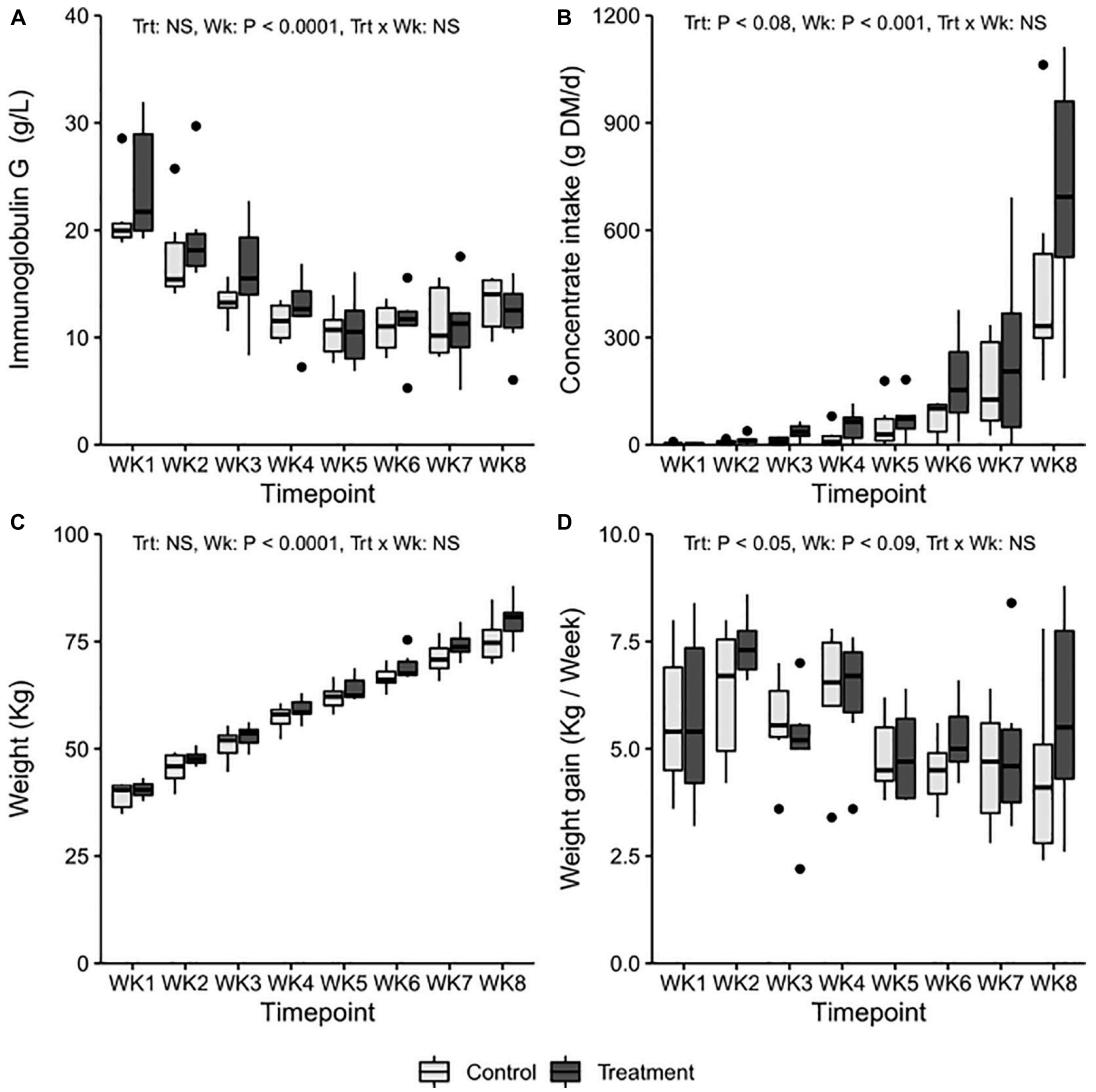


FIGURE 1 | The average weekly **(A)** blood immunoglobulin G levels, **(B)** average daily concentrate DM intake, **(C)** weekly weight (kg), and **(D)** average weight gain in treatment (T-group, dark gray) and control group (C-group, light gray) calves during the 8-week treatment period.

calves from the T-group also had an elevated body temperature associated with diarrhea.

Feed Intake and Growth

The consumption of colostrum, milk, and milk replacer was similar in both groups throughout the experiment (**Supplementary Table 5**). The preferences for different solid feeds varied individually. The T-group tended to eat more concentrates [32.6 ± 1.6, least square means (LSM) ± standard error (SE) g dry matter (DM)/day] than the C-group (17.6 ± 1.6 g DM/day) across the entire treatment period (Treatment: $p = 0.08$) (**Figure 1B**), but the difference was not significant when weekly consumption by group was compared (Treatment × Week: $p > 0.05$).

There were no significant differences in mean weight between the groups at birth (T-group: 34.8 ± 1.5; C-group: 33.4 ± 1.5, LSM ± SE kg). During the following weeks, the mean body weight in the T-group was numerically higher than that in the C-group (Treatment: $p > 0.05$, Week: $p < 0.01$, Treatment × Week: $p > 0.05$) (**Figure 1C**). The T-group gained an average of 350 g more weight per week across the treatment period (Treatment: $p < 0.05$) (**Figure 1D**).

Volatile Fatty Acids

The total ruminal VFA concentration increased with age in both groups but was unaffected by the treatment (**Figure 2**). Week had an effect on most of the individual VFAs. In both groups, the molar proportion of acetate decreased (Week: $p < 0.01$), while propionate, butyrate, and valerate increased (Week: $p < 0.05$) during the 2–8-week period. A significant Treatment × Week interaction indicated a greater decline of acetate molar proportions in the C-group. The molar proportions

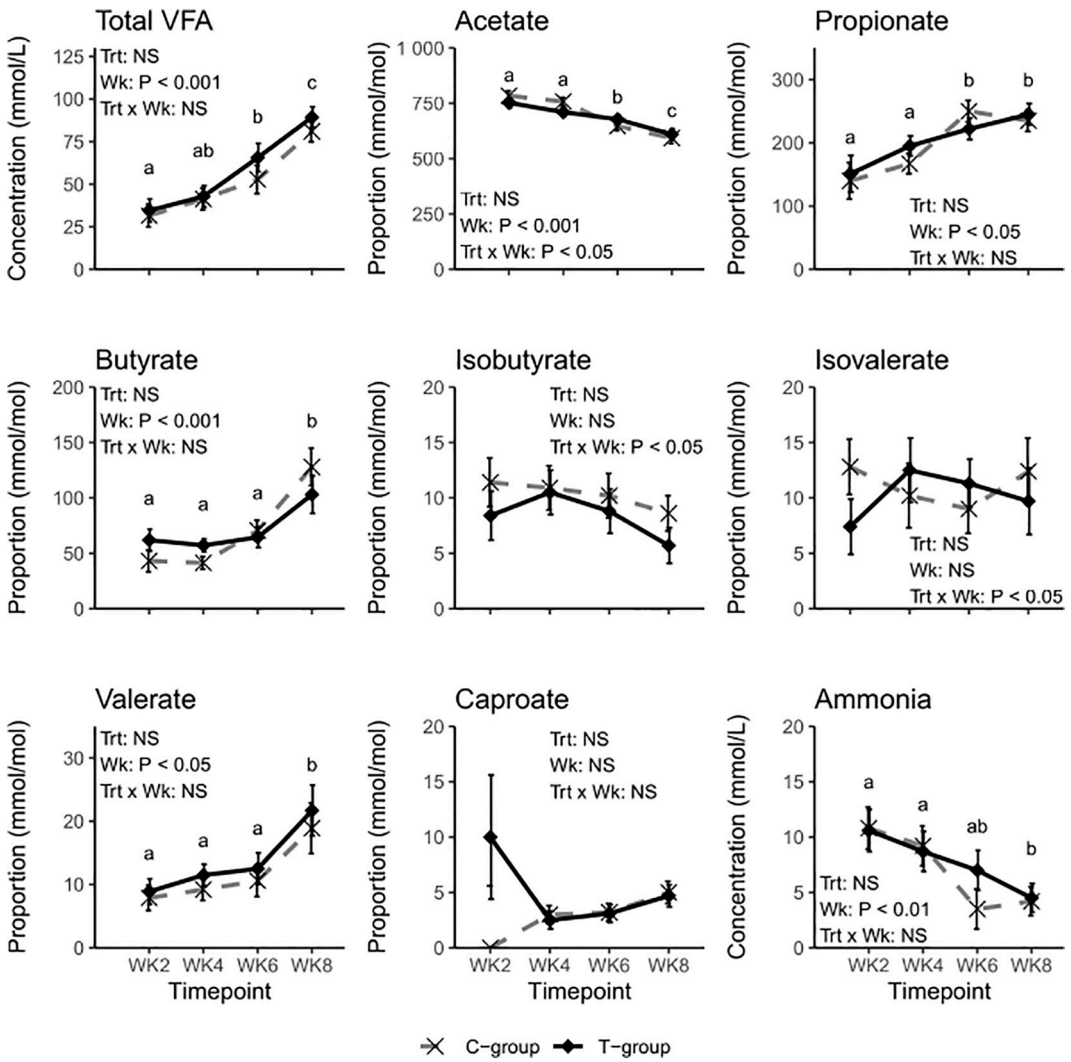


FIGURE 2 | Rumen fermentation characteristics of treatment (T-group, black solid line) and control group (C-group, gray dashed line) calves during the 8-week treatment period. Data for individual volatile fatty acids (VFAs) are presented as molar proportions of total VFA. The significance of Treatment (Trt), Week (Wk), and interaction (Trt × Wk) effects is presented in the figure. The different letters indicate significant differences in the pairwise comparisons of sampling weeks.

of isobutyrate, isovalerate, or caproate were unaffected by the week (Week: $p > 0.05$). Although significant Treatment × Week interactions were observed for isobutyrate and isovalerate, there were no significant differences between the groups. The proportion of ammonia reduced toward the end of the pre-weaning period (Week: $p < 0.01$) but was not significantly affected by the treatment.

Microbial Community Quantities

The treatment had no significant effect on the 16S rRNA gene copy numbers for total bacteria or archaea (Treatment: $p > 0.05$). Nevertheless, the number of bacterial 16S rRNA gene copies was highest in both groups at 2 weeks of age and decreased toward the end of the pre-weaning period, being at its lowest in week 6 (**Figure 3A**). The number of copies in archaea was lowest in week 2 (Week: $p < 0.05$), doubled in concentration in week 4, and remained stable thereafter (**Figure 3B**). The treatment significantly increased the ciliate protozoa 18S rRNA gene copy numbers (Treatment: $p < 0.05$), but due to high variance, no significant differences between the groups were observed (**Figure 3C**). The number of samples no longer observed to have anaerobic fungi increased toward week 8 (**Table 1**), but the copy numbers of the ITS1 region within samples that amplified remained at a similar level throughout the experiment and were unaffected by the treatment (**Figure 3D**).

Sequencing

After quality filtering, the data from 48 calf samples contained 1,329,850 (mean 27,705 ± 5,183) prokaryote reads, and the data from 16 donor pooled inoculum samples contained 807,724 (50,483 ± 9,060) prokaryote reads. From them, 19,891 (414 ± 239) reads in the calves and 42,585 (2,662 ± 631) in

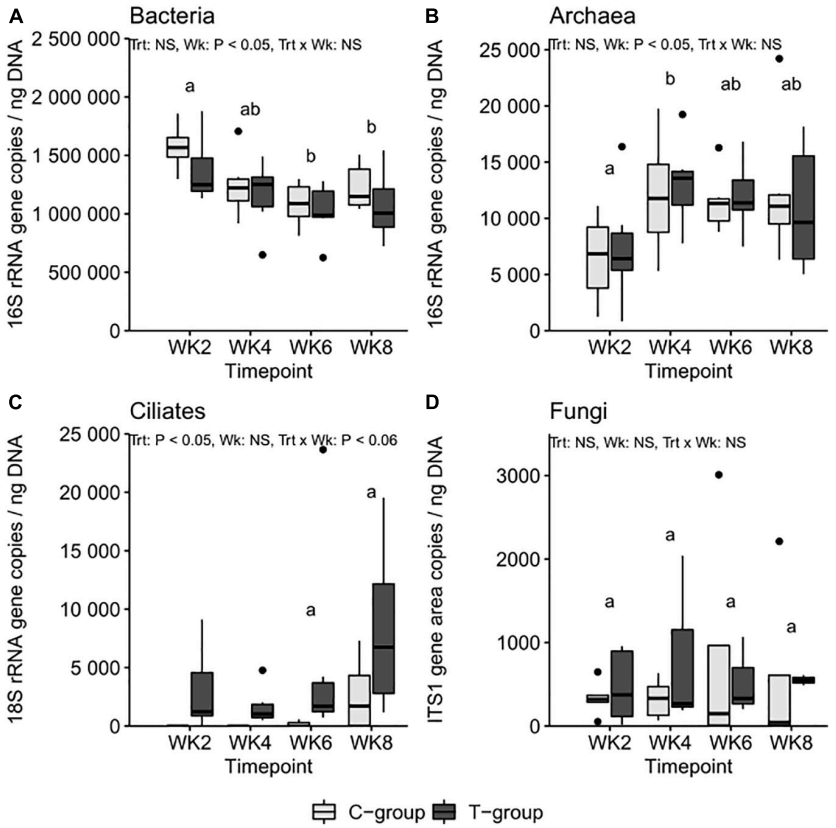


FIGURE 3 | The **(A)** bacteria 16S ribosomal RNA (rRNA), **(B)** archaea 16S rRNA, **(C)** ciliate protozoa 18S, and **(D)** anaerobic fungi internal transcribed spacer 1 (ITS1) region copy numbers per ng of rumen DNA in treatment (T-group) and control (C-group) calves during the 8-week treatment period. The significance of Treatment (Trt), Week (Wk), and interaction (Trt × Wk) effects is presented in the figure. Letters indicate significant differences in the pairwise comparisons of sampling weeks. The C-group remained defaunated until week 6: the statistical tests for rumen ciliate protozoa were therefore performed only for week 6 and week 8 data.

the donor cow were identified as archaeal. The ciliate protozoan data from the calves contained 2,499,249 (83,308 ± 189,856) reads and from the donor contained 1,267,635 (79,227 ± 6,967) reads. The fungal data from the calves contained 2,266,229 (68,674 ± 16,942) reads and the donor contained 1,359,617 (84,976 ± 13,091) reads in total.

Bacteria

The treatment had no significant effect on bacterial alpha diversity. The observed number of OTUs, Shannon diversity, and Simpson's evenness were lowest in both groups in week 2 and increased over the pre-weaning period (in all $p < 0.05$; **Table 1**).

Beta diversity calculated as Bray–Curtis dissimilarities significantly differentiated samples by Week ($p = 0.001$), Treatment ($p = 0.001$), and Treatment × Week interaction ($p = 0.009$) (**Figure 4A** and **Supplementary Table 6**). Week 2 samples in both groups were significantly separated from the later weeks, and the bacterial community structure remained significantly different between the T- and C-groups until week 6. Within the T-group, no significant differences were observed after week 4; in the C-group, after week 6.

The number of bacterial phyla increased from 12 to 17 over the pre-weaning period, but only Firmicutes, Spirochaetes, and Fibrobacteres were significantly affected by the treatment (**Supplementary Table 7**). To better understand bacterial colonization, we grouped bacterial taxa based on their timewise abundance trend in the C-group. We examined the taxa associated with liquid feed digestion, which naturally diminished toward weaning, and taxa associated with solid feed fermentation, which increased in abundance with age and saw differences in colonization dynamics between the groups (**Figure 5** and **Supplementary Table 8**). As a result of inoculation, in week 2, *Fibrobacter*, *Treponema*, CF231, *Selenomonas*, *Sphaerochaeta*, *Pseudobutyrivibrio*, *Anaerovibrio*, *Veillonellaceae* spp., and RFN20 were only observed or were significantly more abundant in the T-group calves. These taxa were also among the most abundant (>0.1%) taxa in the donor samples (**Supplementary Table 8**). At the same time, *Bacteroides*, *Wautersiella*, *Butyricimonas*, genus [*Ruminococcus*] in the family *Lachnospiraceae*, and *Neisseriaceae* spp., which were low abundant in the donor, decreased in abundance earlier in the T-group than the C-group calves (**Figure 5** and **Supplementary Table 8**). Several taxa such as *Campylobacter* and a [*Paraprevotellaceae*] genus in the order Bacteroidales increased in abundance toward the end of the pre-weaning period in the C-group, whereas in the T-group, their abundances

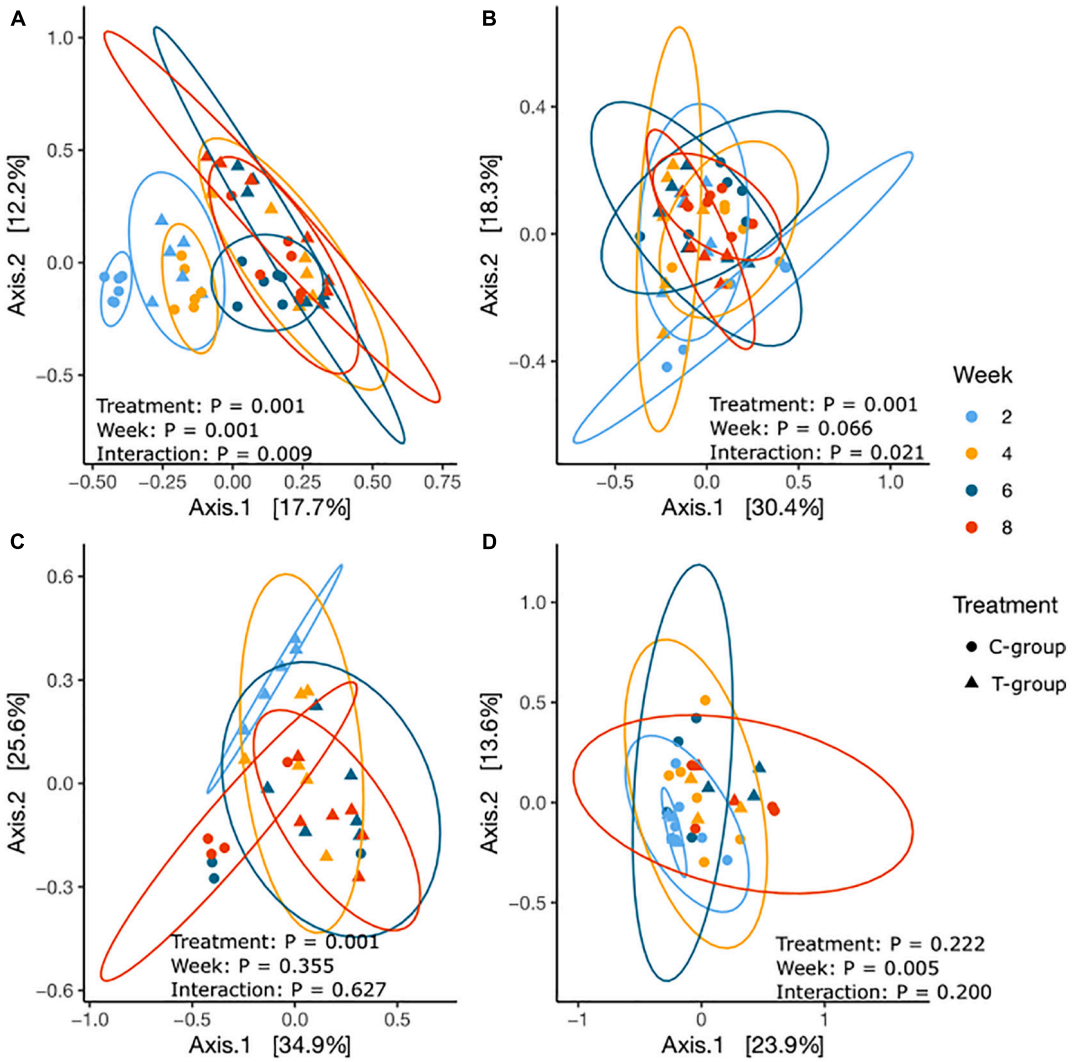


FIGURE 4 | The principal coordinate analysis of Bray–Curtis dissimilarities for **(A)** bacterial, **(B)** archaeal, **(C)** ciliate protozoan, and **(D)** anaerobic fungal communities in treatment (T-group, ▲) and control (C-group, ●) calves at 2 weeks (light blue), 4 weeks (yellow), 6 weeks (dark blue), and 8 weeks (red) of age.

diminished after week 2. A set of bacterial taxa showed no clear age-dependent variation, but its abundance was affected by the treatment. *Bifidobacterium*, *Megasphaera*, and *Mogibacterium* were significantly more abundant in the T-group, whereas the C-group was significantly enriched in *Oscillospira*, *Ruminococcaceae* spp., *Christensenellaceae* spp., and RF39. Where the other aforementioned taxa were also abundant in the donor, *Megasphaera* and *Campylobacter* were low. Nevertheless, in week 8, most of the significant differences between the groups disappeared (**Figure 5** and **Supplementary Table 8**).

Archaea

The number of observed archaeal OTUs was lowest in both groups in week 2 and increased significantly over time ($p < 0.05$), but the T-group had numerically or significantly (week 4; $p < 0.05$) more OTUs. The Shannon diversity was significantly higher in the T-group in week 2 ($p < 0.01$), with no differences observed between the groups in later weeks (**Table 1**).

The Bray–Curtis dissimilarities indicated that the archaeal community structure differed between the T- and C-groups until week 4 ($p < 0.05$), but no longer thereafter. Within the T-group, the archaeal community showed no significant differences between weeks (all $p > 0.05$), whereas in the C-group, the community in week 2 differed significantly from week 8 ($p < 0.05$), suggesting a gradual development (**Figure 4B** and **Supplementary Table 6**).

Twenty archaeal species were identified in the entire dataset (**Supplementary Table 9**). In week 2, calves in the T-group had a taxonomically more diverse archaeal community dominated by *Methanobrevibacter gottschalkii*, *Methanobrevibacter ruminantium*, and *Methanosphaera ISO3-F5*, which were also dominant in the donor samples (**Figure 5** and **Supplementary Table 9**). *Methanomicrobium mobile*, *Methanocorpusculum*

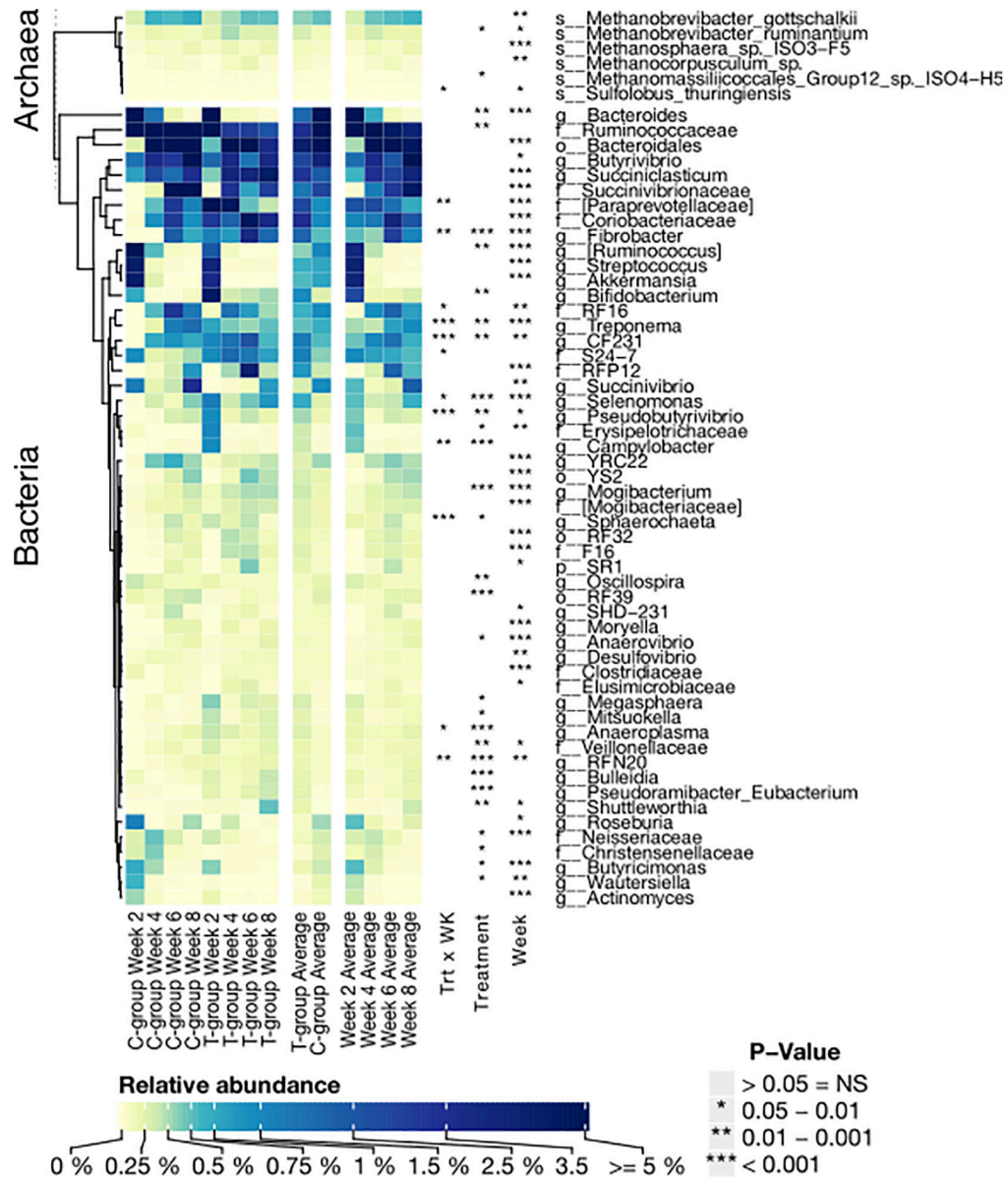


FIGURE 5 | The relative abundance of significantly affected bacterial genera and archaeal species in treatment (T-group) and control group (C-group) calves during the 8-week treatment period. Data are presented as averages of treatment by week, averages of treatment across the dataset, and averages of week across the dataset. The annotation on the right indicates the significance level of Treatment (Trt) and Week (Wk) and their interaction (Trt × Wk). The letter in front of the taxon name indicates the closest identified taxonomic level: species (s); genus (g); family (f); order (o); and phylum (p).

parvum, and several *Methanomassiliicoccales* species were also present in lower abundances in the T-group. In contrast, in the C-group in week 2, not all the calves were colonized with archaea. The dominant *Mbb. gottschalkii* was detected in five calves; two had *Mbb. ruminantium* and *Methanosphaera ISO3-F5*, while *M. mobile* was detected in one animal. *Methanomassiliicoccales* and *Methanocorpusculum* colonized the rumen of the C-group calves after week 4 or later. The abundance of *Mbb. gottschalkii* and *Methanosphaera* sp. *ISO3-F5* increased toward the end of the pre-weaning period (Week: $p < 0.01$), while *Mbb. ruminantium* reached its highest values in week 4 in both groups (Week: $p < 0.05$). Inoculation in the T-group increased the relative abundance of *Mmc.* Group12 sp. ISO4-H5 and *Mbb. ruminantium* (Treatment: $p < 0.05$) (**Figure 5** and **Supplementary Table 9**).

Ciliate Protozoa

The ciliate protozoa alpha diversity, calculated for weeks 6 and 8, was unaffected by the treatment, and the number of observed OTUs, the Shannon index, and Simpson evenness remained at a similar level in both groups (Week: $p > 0.05$, **Table 1**).

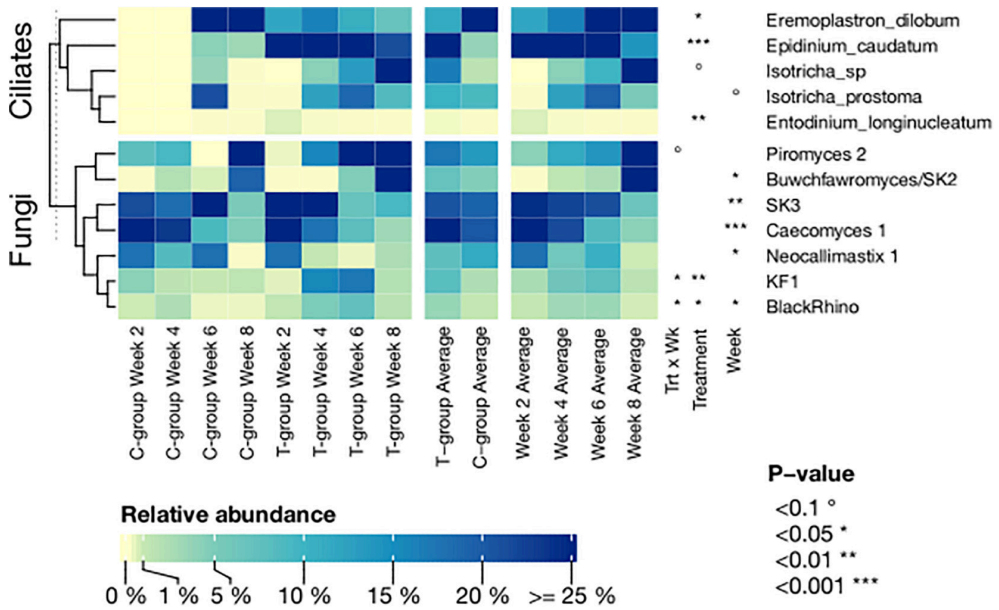


FIGURE 6 | The relative abundance of significantly affected ciliate protozoan and fungal species in treatment (T-group) and control group (C-group) calves during the 8-week treatment period. Data are presented as averages of treatment by week and averages of treatment and week across the dataset. The annotation on the right indicates the significance level of the Treatment (Trt) and Week (Wk) and their interaction (Trt × Wk).

The beta diversity analysis significantly differentiated the T-group ciliate protozoan community in week 2 from the succeeding weeks ($p = 0.001$), but after week 4, the communities no longer differed (**Figure 4C** and **Supplementary Table 6**). In the C-group, ciliate protozoa were detected in three animals in week 6 and in four animals in week 8, and the community compositions did not differ significantly ($p > 0.05$). However, significant differences were observed between the C- and T-groups in week 8 (Treatment: $p = 0.001$) (**Figure 6** and **Supplementary Table 10**).

In total, 25 genera- and 40 species-level ciliate protozoan taxonomic groups were identified in the entire dataset (**Supplementary Table 10**). Donor samples were largely dominated by *Epidinium caudatum*, and apart from few very rare observations, all species observed in both calf groups were also observed in donor samples. In week 2, the T-group was dominated by *E. caudatum*, followed by *Eremoplastron dilobum*, and *Entodinium* sp., which together accounted for 90% of the abundance data (**Figure 6** and **Supplementary Table 10**). In week 4, *Isotricha prostoma* also colonized the rumen and continued to increase in abundance during the following 4 weeks, becoming the dominant species in the T-group in week 8. In contrast, the ciliate protozoan community in the C-group in weeks 6 and 8 was dominated by *E. dilobum*. *I. prostoma* was the second most abundant species in week 6, but in week 8, it was replaced by *Entodinium furca monolobum* (**Figure 6** and **Supplementary Table 10**).

Anaerobic Fungi

The fungal alpha diversity decreased over time, and no significant differences between the groups were observed (Treatment: $p > 0.05$) (**Table 1**). During the later sampling weeks, several samples from both groups failed to produce fungal amplicons.

Beta diversity analysis showed that the fungal communities in week 2 differed from those of later weeks ($p < 0.05$), but there were no significant differences between the T- and C-groups in any sampling week (all: $p > 0.05$) (**Figure 4D** and **Supplementary Table 6**).

In total, 18 species-level fungal groups were observed in the dataset (**Supplementary Table 11**). All taxa observed in both calf groups were also observed in the donor community, which was dominated by *Neocallimastix 1*, SK3, and *Piromyces* 2. In week 2, both the C- and T-groups were dominated by *Caecomyces 1*, SK3, and *Neocallimastix 1*. Over the following weeks, *Caecomyces 1*, *Neocallimastix 1*, and SK3 declined in abundance, while *Piromyces* 2 and *Buwchfawromyces/SK2* became dominant in both groups in week 8. Inoculation significantly increased the abundance of *BlackRhino* (Treatment: $p < 0.05$) in week 4 and KF1 (Treatment: $p < 0.01$) in week 6 in the T-group (**Figure 6** and **Supplementary Table 11**). In week 8, the T- and C-groups differed numerically in the abundance of *Piromyces* 1, which was 16.2% in the C-group and 0.03% in the T-group, but was nearly absent (0.002%) in the donor. However, the fungal abundances were affected by large inter-animal variation (**Supplementary Table 11**).

Core Microbiome

To better understand if there was a limited set of taxa that was important for rumen microbial maturation, and if this set could be influenced by the inoculum, we investigated the core microbiome differences between the groups. Due to continuous changes in rumen microbial colonization over

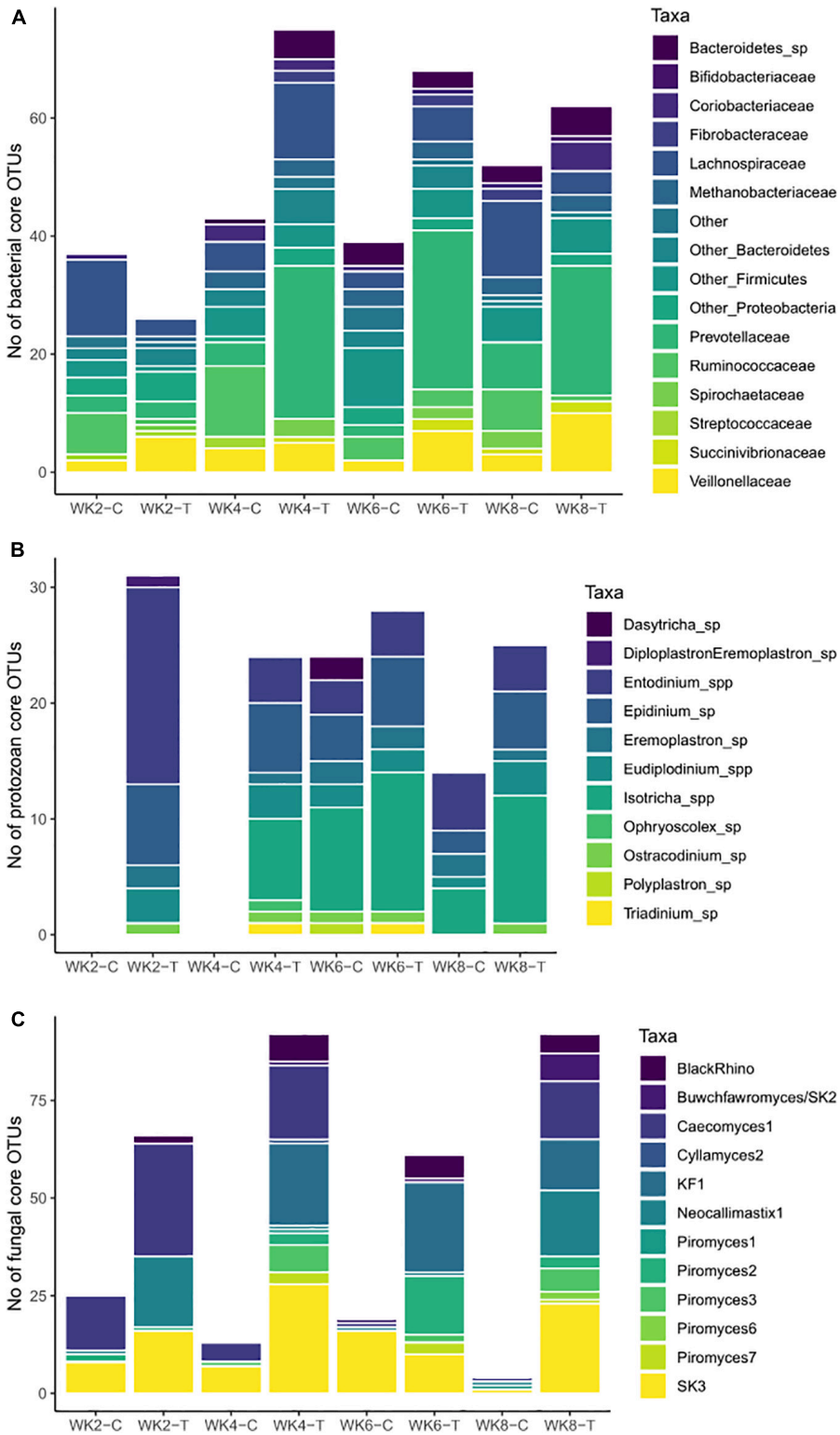


FIGURE 7 | The number of operational taxonomic units (OTUs) and taxonomic composition of **(A)** the core prokaryote community (archaea and bacteria), **(B)** the core ciliate protozoan community, and **(C)** the core anaerobic fungal community in treatment (T) group and control (C) group calves at 2 (WK2), 4 (WK4), 6 (WK6), and 8 (WK8) weeks of age.

the pre-weaning period, a core microbiome was defined for each week and group separately. Only OTUs present in 100% of animals in a particular group in a particular week were included in the core.

Core Bacterial and Archaeal Community Development

Each sampling week had a distinct bacterial core, but we evaluated the speed of maturation by the extent of core OTUs that remained in the core over the subsequent weeks. An earlier establishment of mature microbiome-related taxa was already observed in week 2 in the T-group calves, as 27% of core OTUs in the T-group, compared to 5% in the C-group, were shared with the core in week 8 (**Supplementary Table 12**).

The core bacterial community was composed of 26–78 OTUs, which increased with age and represented an average of 30% of the total bacterial abundance. To a substantial extent, the core OTUs belonged to Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Euryarchaeota phyla, but the number of OTUs per phylum and the OTUs' affiliation at family and genus levels differed between the groups (**Figure 7A** and **Supplementary Table 12**). As a result of inoculation, the number of core OTUs in the T-group tripled between weeks 2 and 4 and remained at an elevated level throughout the rest of the experiment. The increase in core OTUs was caused by the considerable number of *Prevotella* OTUs entering the core, and the dominance of *Prevotella* within Bacteroidetes remained over the following weeks. On the other hand, throughout the whole pre-weaning period, the C-group retained a larger share of Firmicutes core OTUs than the T-group, but the taxonomic composition at family level was age- and group-specific (**Supplementary Table 12**). In the C-group, *Ruminococcaceae* and *Lachnospiraceae* families were dominant in the core in all weeks, while the *Lachnospiraceae* and *Veillonellaceae* families dominated the core in the T-group. The core archaeal OTUs belonged to the *Methanobrevibacter*, *Methanosphaera*, and *Methanomassiliicoccaceae* genera, and their presence in the core was group- and week-specific. The T-group core had *Methanobrevibacter* from week 2, while the C-group had *Methanobrevibacter* only from week 4. The donor cow inoculum had an impact on the establishment of the bacterial core because 16 core OTUs in week 2, 76 OTUs in week 4, and all core OTUs in week 8 were common between the T-group calves and the donor (**Supplementary Table 12**). Proteobacteria-affiliated core OTUs alone were mostly not obtained from the donor, but the genera detected were age-specific. *Succinivibrionaceae* OTUs were observed in the core of the T-group from week 4, while in the C-group, they were observed only in week 8 (**Figure 7A**).

Core Ciliate Protozoan Community Development

The ciliate protozoan core community was more uniform across sampling weeks and represented an average of 94% of the total protozoan abundance. The number of shared OTUs with week 8 in the T-group increased from 42% in week 2 to 75% in week 6. In the C-group, 46% of OTUs were shared between weeks 6 and 8. However, in both groups, the total number of core OTUs decreased with age (**Figure 7B**). Core OTUs affiliated with the *Entodinium*, *Epidinium*, *Diploplastron/Eremoplastron*, *Eudiplodinium/Eremoplastron*, and *Ostracodinium* genera populated the T-group in week 2, while *Isotricha* joined the core community after week 4 (**Supplementary Table 13**). In the C-group, the taxonomic composition of the core in week 6 was similar to the T-group, with the exception of *Polyplastron multivesiculatum* and *Dasytricha ruminantium*. In week 8, the core ciliate protozoa were shared between both calf groups and the donor (**Supplementary Table 13**).

Core Fungal Community Development

There were substantial differences in the anaerobic fungal core community development between the groups (**Figure 7C**). The core in the T-group comprised an average of 83% of the total fungal abundance, while the core in the C-group represented an average of only 38%, suggesting larger inter-animal variation in the C-group calves and a more stable core community composition in the T-group. The effect of inoculation was already observed in week 2, when the T-group calves had 3 times more core OTUs than similar-aged C-group animals, and 59% of these OTUs were shared with the core in week 8 (**Supplementary Table 14**). The effect of inoculation was also observed in the taxonomic diversity. In week 2, both groups' fungal core was dominated by *Caecomyces 1*, *SK3*, and *Neocallimastix 1*. While the fungal taxonomical core in the C-group remained similar over the pre-weaning period, the T-group core in week 4 had OTUs affiliated with 11 genera, with *KF1*, *Buwchfawromyces/SK2*, *Cyllamyces 2*, and *Piromyces 1*, *3*, and *7* joining the core (**Figure 7C** and **Supplementary Table 14**). The taxonomic composition of the T-group core after week 4 remained similar. Nearly all OTUs observed in the T- and C-groups were present in the donor, although some were of low counts.

DISCUSSION

In this study, we used monozygotic twin calves and tested the effect of fresh mature rumen liquid inoculum on rumen microbial community development during the pre-weaning period. Several studies have demonstrated positive effects on phenotypic characteristics and gut development in pre-weaned ruminants (Muscato et al., 2002; Zhong et al., 2014; Cox et al., 2019; Belanche et al., 2020; Palma-Hidalgo et al., 2021) caused by rumen liquid inoculation, but in bovines, the results are inconclusive (Cersosimo et al., 2019; Bu et al., 2020; Yu et al., 2020). The differences in research outcomes may be related to high between-host variation (Mayer et al., 2012). While the host effect can be controlled in twin or triplet experiments in small ruminants (e.g., Belanche et al., 2020; Palma-Hidalgo et al., 2021), twin calving in dairy livestock is rare. In the present study, monozygotic twin calves were therefore created to reduce the genetic host variation. Our results indicate that inoculation enhanced the bacterial and archaeal community establishment and induced differences in ciliate protozoan community composition, but despite continuous inoculation, it

was unable to promote the establishment of a rumen anaerobic fungi community.

Animal Growth and Rumen Fermentation

The inoculum promoted better weight gain in the T-group animals, which was probably associated with an increase in concentrate intake after 6 weeks of age. Our results are in line with those of other studies, which have reported a positive effect of fresh or autoclaved rumen liquid on animals' weight gain (Zhong et al., 2014; Belanche et al., 2020). It is known that butyrate, but also propionate and acetate, even in small doses, stimulates rumen papillae development and rumen epithelia metabolism and absorption (Sander et al., 1959; Sakata and Tamate, 1978; Suárez et al., 2006; Guilloteau et al., 2009; Connor et al., 2014). We therefore hypothesized that the inoculum could stimulate rumen fermentation processes in T-group calves and increase VFA production. However, the better weight gain in the T-group animals in this study could not be explained by the rumen fermentation differences because the VFA profiles between the groups were very similar. The lack of significant differences in VFA proportions was probably a reflection of the lack of a significant difference in the total microbial quantities between the groups. As many microbes can produce the same VFA end products, the VFA proportions were unaffected by the differences between the groups in the rumen microbial community composition.

In contrast with the observations by Rey et al. (2012), in which VFA concentration stabilized around 1 month of age, we observed an increase in VFA concentration in both groups until the end of the experiment. The discrepancies between the studies may be explained by the differences in dietary composition. In the present study, silage was added to the diet in week 7 and may have stimulated fermentation processes in the calves' rumen. The steeper trend in the reduction of acetate in the C-group between weeks 4 and 6 may be a result of increasing concentrate intake, but it also coincides with the first observations of the ciliate protozoa in the C-group calves. The absence of ciliate protozoa has previously been associated with a slight increase in acetate concentration (Newbold et al., 2015). However, due to the small sample size and high interindividual variation, pairwise comparisons failed to show any significant differences between the groups.

Effect of Inoculum on Bacteria Community Development

Immediately after birth, the rumen bacterial community gradually changes from facultative anaerobic bacteria to obligate anaerobes (Jami et al., 2013; Rey et al., 2014). Similarly, in the present study, at 2 weeks of age, the bacterial communities in both calf groups differed from the succeeding weeks. Week 2 bacteria were represented by a broad range of sugar utilizers such as *Bacteroides* (Wexler, 2014), aerobes, or facultative anaerobes, e.g., *Neisseriaceae* spp. (Adeolu and Gupta, 2013) and *Streptococcus* (Stewart et al., 1997), which later reduced in abundance. Moreover, there were also bacteria with special niches among them, such as *Akkermansia* and *Ruminococcus*, capable of utilizing oligosaccharides from milk, mucosa, and saliva (Derrien, 2007; Huang et al., 2011; Bell et al., 2019; Kostopoulos et al., 2020) or being associated with the epimural community (Stewart et al., 1997; Derrien, 2007; Jami et al., 2013; Malmuthuge et al., 2014; Jiao et al., 2015). As the microenvironments during the early life within the rumen change rapidly, many of these taxa reduced in abundance or disappeared earlier from the T-group animals, suggesting that the environment within the T-group rumen was changing more quickly. Our observations are in line with a previous finding in which a faster reduction of several early life-related taxa and the promotion of rumen function and development could be induced with dietary measures (Dias et al., 2017).

In the T-group animals, inoculation stimulated the earlier establishment of taxa related to mature rumen function and fiber degradation, e.g., *Treponema* or the fibrolytic genus *Fibrobacter* (Shinkai et al., 2010; Jewell et al., 2015). The abundance of these taxa increased especially noticeably during the first 4 weeks of life. The positive impact of inoculation was also already visible in the composition of the T-group core community in week 4. The presence of a large number of OTUs in the core, which pertained throughout the rest of the experiment, suggests that the conditions within the T-group calf rumen were sufficient for these mature rumen-related bacterial OTUs to become established and remain functional. Our results support the recent findings of Palma-Hidalgo et al. (2021), who reported that a similar rumen liquid inoculum induced positive effects on rumen bacteria establishment in goats.

We also hypothesized that rumen microbial maturation was related to the reduced Bacteroidetes taxonomic diversity and increased taxonomic diversity of Firmicutes within the rumen. Indeed, in the core communities of both groups, the number of OTUs assigned to the *Prevotella* (Avguštin et al., 1997) genus increased substantially with age, and it became the dominant taxon, replacing nearly all other OTUs assigned to Bacteroidetes. This increase in *Prevotella* occurred earlier (week 4) in the T-group than the C-group (week 8). It has previously been suggested that the relative abundance of *Prevotella* increases with age as a result of increasing fiber content in the diet (Jami et al., 2013; Rey et al., 2014). However, other observations are inconclusive (Li et al., 2012; Dias et al., 2018; Cersosimo et al., 2019). Members of the genus *Prevotella* are broad-range carbohydrate utilizers, and a considerable fluctuation in metabolic capabilities can be expected between species or even between strains within the same species. It is therefore best to evaluate the role of *Prevotella* at the OTU level. It is possible that, as versatile fermenters, *Prevotella* spp. work as hubs in the network of microbial interactions and help maintain the community's interactions. On the other hand, the inoculum treatment resulted in differences in the dominant taxa within the Firmicutes core community. Although the major fiber-degrading taxa were present in the core of both groups in week 8, the T-group had more OTUs assigned to *Veillonellaceae*, whereas the C-group had more OTUs assigned to the *Ruminococcaceae* and *Lachnospiraceae* families. These differences may be related to the competitive advantages provided by the continuous inoculation and availability of feed substrates.

Effect of Inoculum on Archaea Community Development

We hypothesized that by receiving archaea with the inoculum from an adult cow, and with ciliate protozoa and fungi present in the community, hydrogen sources for methanogenesis would be present in T-group animals, creating suitable conditions for hydrogenotrophic archaea growth. However, the treatment did not significantly increase the 16S rRNA gene copy numbers of total archaea. It is possible that factors other than the availability of hydrogen limited the archaea's growth in the developing rumen because the methanogenic community of young calves is known to utilize, e.g., methylamines and methanol for methanogenesis more efficiently than an adult methanogenic community (Friedman et al., 2017). Nevertheless, the inoculum treatment preponed the archaea establishment and increased archaea richness and differences in community composition during the first 4 weeks. Our results are in line with the previous observations by Dias et al. (2017) showing that *Mbb. gottschalkii* is present in milkfed calves, while the abundance of *Methanosphaera* increases with the provision of concentrate. *Methanobrevibacter* is the dominant clade of methanogens in the rumen and uses the hydrogenotrophic pathway to produce methane, whereas methylotrophic *Methanosphaera* can use other substrates such as methanol and methylamines (Morgavi et al., 2010), and may require a more diverse microbial community to produce them. Similarly, in line with the previous results of Friedman et al. (2017), the establishment of *Methanomassiliicoccales* appeared later, suggesting that this archaea might benefit from the more mature microbial community, providing a substrate for energy metabolism. The differences in the archaea richness and community composition between the T- and C-groups during the experiment's first weeks suggest that the archaea from the donor could remain in the rumen possibly due to the more mature microbial community in the T-group, which was able to provide the conditions for a more diverse archaeal community. However, despite the later establishment of archaeal communities in the C-group, by the end of the liquid feeding period, the archaeal composition in both groups was very similar.

Effect of Inoculum on Ciliate Protozoan Community Development

Protozoa are naturally established in the rumen at around 15–30 days of age (Eadie, 1962b; Naga et al., 1969; Cersosimo et al., 2019) and require contact between conspecifics for transmission (Bird et al., 2010). Given that all the calves in the present experiment were kept in individual pens to prevent physical contact, our results suggest that the first adult rumen inoculum was already successful in providing viable ciliate protozoa to the T-group's calves. However, half of the C-group calves also had ciliate protozoa by week 6. Despite no close proximity, the pens were located in a barn where other cows were also housed, so microbial transmission through aerosol droplets in the farm air or manual transmission through unrecorded mistakes in management practices cannot be excluded. Moreover, the calves remained with the dam for a short period after birth, and the dam may have transferred some ciliate protozoa through saliva (Tapio et al., 2016), which was established later, when conditions in the rumen became favorable. With real-time quantitative PCR methods, protozoa have been detected as early as in 1-day-old goats (Abecia et al., 2014), making this hypothesis plausible.

The treatment significantly increased the ciliate protozoan quantity in the T-group animals, estimated with 18S rRNA gene copy numbers. The establishment of a ciliate protozoan community requires a well-functioning bacterial population to be in place (Fonty et al., 1988), and it can further be affected by the inoculum's cell density (Williams and Dinusson, 1972), the type and amount of feed (Kudo et al., 1990; Franzolin and Dehority, 1996; Santra and Pathak, 2001), the pH, and other conditions of the rumen (Eadie, 1962b; Dehority, 2005; Franzolin and Dehority, 2010). The increase in the quantity of rumen ciliates in the T-group may therefore have been affected by several of these factors. Although the ciliate protozoa in the T-group underwent taxonomic changes within the 2–4-week period, these changes did not follow the sequential establishment previously observed in young ruminants, in which *Entodinium* spp. were among the first species to establish, followed by *Diplodinium* and Holotrichs (Bryant et al., 1958; Eadie, 1962b; Naga et al., 1969; Minato et al., 1992; Dias et al., 2017). In the T-group in week 2, *Epidinium* was the dominant protozoan, accounting for 70% of total abundances. Interestingly, it was also the dominant genus in the donor. However, the *Epidinium* decreased in T-group over time due to an increase in the abundance of *Isotricha*, while the *Entodinium* level remained constant. It can be hypothesized that the inoculum provided a significant number of protozoan colonizers, which had well-established community interactions and required less time for reestablishment. The increase in concentrate intake and presence of silage in the diet at the end of the pre-weaning period may also have promoted the rumen microbiome's overall function, thus improving the ciliate community's growth.

Due to different exposures to external microbiome sources, the C-group calves in week 8 were dominated by the *Diplоplastron/Eremoplastron* genus. Previous studies have shown that the A-type and B-type members in a stable ciliate community rarely coexist due to predation and competition for resources (Eadie, 1962a, 1967). While the T-group community included B-type genera like *Eudiplodinium*, *Epidinium*, and *Eremoplastron* obtained from the donor cow, the A-type *P. multivesiculatum* was detected in the C-group core community, suggesting a different seeding source. We hypothesize that the C-group acquired the ciliate protozoan community in a more random manner, requiring several selection steps, which finally led to a community dominated by different taxa than those of the donor cow and the T-group calves.

Effect of Inoculum on Anaerobic Fungi Community Development

The frequent provision of the inoculum did not directly improve the establishment of rumen anaerobic fungi. Although nearly all

the calves had fungi at the beginning of the experiment, only two T-group calves and four C-group calves were observed to have fungi at 8 weeks. The decreasing observations of fungi after the beginning of a solid feed diet have previously been described (Fonty et al., 1987; Dias et al., 2017; Dill-McFarland et al., 2019). The authors speculated that during weaning, as the solid feed intake increased and the competition of carbohydrate sources intensified, the fungal community had to reestablish itself by occupying a different niche in plant structural carbohydrate breakdown. Due to a slower life cycle, the fungi may not have been able to compete for substrate and may have started to diminish. In our study, both groups received the same diet; therefore, other factors may have played a role in the fungal community being more present in the C-group than the T-group. For example, inhibition or predation by other microbial groups may have promoted the vanishment of fungi. Bacteria have been observed to inhibit fungal growth and cellulase activity (Dehority and Tirabasso, 2000), and protozoa are known to be able to ingest and digest fungal zoospores (Morgavi et al., 1994; Miltko et al., 2014). Lower predation pressure would explain why the fungi only started to disappear in the C-group at 6 weeks of age, when the ciliate communities started emerging. Furthermore, fermentation products and changes in pH can influence fungal community development (Joblin and Naylor, 1993; Srinivasan et al., 2001).

Inoculation influenced the taxonomic composition of rumen anaerobic fungi. In line with Dias et al. (2017), *Caecomyces 1* and SK3 dominated the fungal communities of milkfed calves, but fungal taxa underwent changes during the 4–8-week period, when the solid feed intake started to increase. Although the major fungal taxa were similar between the groups, *BlackRhino* and KF1 were more specific to the T-group, while *Piromyces 1* was more abundant in the C-group, possibly indicating different seeding sources, as *BlackRhino* and KF1 were observed in the donor, but *Piromyces 1* only in negligible abundance. Gordon and Phillips (1998) suggested that fungi are probably transferred to the calf through direct contact with the mother or contaminated feeds. This is supported by evidence that fungi have been isolated from the saliva and feces of ruminants (Tapio et al., 2016), and they can be cultivated after prolonged exposure to oxygen, drought, or freezing temperatures (Milne et al., 1989; Davies et al., 1993; McGranaghan et al., 1999) probably due to the production of resistant spores (Brookman et al., 2000).

The positive influence of the inoculum in the T-group animals was observed in the richness of the fungal core community. While the core of the C-group in week 8 was reduced to only 4 OTUs, the core community of the T-group contained 92 OTUs, indicating wide fungal taxonomic diversity in the rumen. It is tempting to speculate that a less diverse fungal community could survive better through the pre-weaning period. As shown previously, a less diverse microbiome may be more efficient because it reduces the various metabolic pathways and interactions between taxa (Kruger Ben Shabat et al., 2016). However, high interindividual variation and the decreasing number of study subjects observed to have fungi may have affected our observations. More research is needed to assess the effects of weaning on the development of the anaerobic fungal community in pre-weaning calves.

CONCLUSION

Our results show that the orally administered microbial inoculum successfully reached the rumen and induced changes in the microbial community structure during the pre-weaning period. However, the inoculum affected the different microbial groups differently: the bacteria and archaea communities matured more quickly than the control calves, and the inoculum improved the establishment of ciliate protozoa, whereas fungal colonization was poor and probably occurred later. Inoculation with adult rumen liquid stimulated weight gain and tended to improve solid feed intake in the T-group during the pre-weaning period. However, these differences between the groups could not be explained by the rumen fermentation parameters.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA713003.

ETHICS STATEMENT

The animal study was reviewed and approved by the National Ethics Committee (ESAVI/5687/04.10.07/2017, Hämeenlinna, Finland).

AUTHOR CONTRIBUTIONS

IT and JV designed the study. IT, PL, and SA conducted the animal experiment and sample collection. HH, MP, and IT performed the laboratory work. HH, SA, and IT analyzed the data. HH and IT wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Natural Resources Institute Finland (Luke) strategic project (41007-00132300) and in part by the Horizon 2020 Research and the Innovation Program MASTER project (grant agreement no. 818368). HH acknowledges the Sustainable Use of Renewable Resources doctoral program of the University of Helsinki for a salary that supported this work.

ACKNOWLEDGMENTS

We thank Tuula-Marjatta Hamama (Luke) for embryo work in creating twin calves, the staff at the research barn in Luke and Tian Nie for the care of experimental animals and assistance in sample collection. We thank Satu Suominen for assistance in data collection, Dominique Graviou (INRAE, France) for

cooperation and advice in qPCR analysis. We thank the Finnish Functional Genomics Centre supported by the University of Turku, Åbo Akademi University, and Biocenter Finland for sequencing. Raisio Agro, Finland, is acknowledged for providing Pikku-Mullin-Herkku calf concentrate for the experiment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.758395/full#supplementary-material

REFERENCES

Abecia, L., Jiménez, E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Pinloche, E., et al. (2017). Natural and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. *PLoS One* 12:e0182235. doi: 10.1371/journal.pone.0182235

Abecia, L., Martín-García, A. I, Martínez, G., Newbold, C. J., and Yáñez-Ruiz, D. R. (2013). Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. *Archaea* 91, 4832–4840. doi: 10.2527/jas2012-6142

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J., et al. (2014). Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Anim. Prod. Sci.* 54:1449. doi: 10.1071/AN14337

Adeolu, M., and Gupta, R. S. (2013). Phylogenomics and molecular signatures for the order *Neisseriales*: proposal for division of the order Neisseriales into the emended family Neisseriaceae and Chromobacteriaceae fam. nov. *Antonie Van Leeuwenhoek* 104, 1–24. doi: 10.1007/s10482-013-9920-6

Ahvenjärvi, S., and Huhtanen, P. (2018). Effects of intraruminal urea-nitrogen infusions on feed intake, nitrogen utilization, and milk yield in dairy cows. *J. Dairy Sci.* 101, 9004–9015. doi: 10.3168/jds.2018-14617

Avguštin, G., Wallace, R. J., and Flint, H. J. (1997). Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. *Int. J. Syst. Bacteriol.* 47, 284–288. doi: 10.1099/00207713-47-2-284

Baldwin, R. L., McLeod, K. R., Klotz, J. L., and Heitmann, R. N. (2004). Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. *J. Dairy Sci.* 87, E55–E65. doi: 10.3168/jds.S0022-0302(04)70061-2

Belanche, A., Palma-Hidalgo, J. M., Nejjam, I., Jiménez, E., Martín-García, A. I., and Yáñ-Ruiz, D. R. (2020). Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. *J. Dairy Sci.* 103, 5047–5060. doi: 10.3168/jds.2019-18002

Belanche, A., Yáñez-Ruiz, D. R., Detheridge, A. P., Griffith, G. W., Kingston-Smith, A. H., and Newbold, C. J. (2019b). Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. *Environ. Microbiol.* 21, 4360–4377. doi: 10.1111/1462-2920.14801

Belanche, A., Cooke, J., Jones, E., Worgan, H. J., and Newbold, C. J. (2019a). Short- and long-term effects of conventional and artificial rearing strategies on the health and performance of growing lambs. *Animal* 13, 740–749. doi: 10.1017/S1751731118002100animal740

Bell, A., Brunt, J., Crost, E., Vaux, L., Nepravishta, R., Owen, C. D., et al. (2019). Elucidation of a sialic acid metabolism pathway in mucus-foraging *Ruminococcus gnavus* unravels mechanisms of bacterial adaptation to the gut. *Nat. Microbiol.* 4, 2393–2404. doi: 10.1038/s41564-019-0590-7

Bird, S. H., Hegarty, R. S., and Woodgate, R. (2010). Modes of transmission of rumen protozoa between mature sheep. *Anim. Prod. Sci.* 50, 414–417. doi: 10.1071/AN09216

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

Bryant, M. P., Small, N., Bouma, C., and Robinson, I. (1958). Studies on the composition of the ruminal flora and fauna of young calves. *J. Dairy Sci.* 41, 1747–1767. doi: 10.3168/jds.S0022-0302(58)91160-3

Bu, D., Zhang, X., Ma, L., Park, T., Wang, L., Wang, M., et al. (2020). Repeated inoculation of young calves with rumen microbiota does not significantly modulate the rumen prokaryotic microbiota consistently but decreases diarrhea. *Front. Microbiol.* 11:1403. doi: 10.3389/fmicb.2020.01403

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *PNAS* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Carballo, O. C., McCoard, S. A., Lowe, K., Ganesh, S. J., Lewis, S. J., and Muetzel, S. (2019). "Rumen microbial composition and fermentation profiles through divergent dietary interventions during early life in calves," in *Proceedings of the 2019 Congress on Gastrointestinal Function, Chicago, 15th-17th of April 2019*, Chicago, IL.

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.* 10:1651. doi: 10.3389/fmicb.2019.01651

Comline, R. S., and Titchen, D. A. (1951). Reflex contraction of the oesophageal groove in young ruminants. *J. Physiol.* 115, 210–226. doi: 10.1113/jphysiol.1951.sp004666

Connor, E. E., Baldwin, R. L., Walker, M. P., Ellis, S. E., Li, C., Kahl, S., et al. (2014). Transcriptional regulators transforming growth factor-β1 and estrogen-related receptor-α identified as putative mediators of calf rumen epithelial tissue development and function during weaning. *J. Dairy Sci.* 97, 4193–4207. doi: 10.3168/jds.2013-7471

Cox, M., Weimer, P., Seinberger, A., Skarlupka, J., and Suen, G. (2019). "Altering the ruminal microbiota in dairy calves using rumen contents dosing," in *Proceedings of the 2019 Congress on Gastrointestinal Function, Chicago, 15th-17th of April 2019*, Chicago, IL, 44–45.

Davies, D. R., Theodorou, M. K., Brooks, A. E., and Trinci, A. P. J. J. (1993). Influence of drying on the survival of anaerobic fungi in rumen digesta and faeces of cattle. *Oxford Academic* 106, 59–63. doi: 10.1111/j.1574-6968.1993.tb05935.x

De Barbieri, I., Hegarty, R. S., Silveira, C., Gulino, L. M., Oddy, V. H., Gilbert, R. A., et al. (2015). Programming rumen bacterial communities in newborn Merino lambs. *Small Rumin. Res.* 129, 48–59. doi: 10.1016/j.smallrumres.2015.05.015

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.

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

Derrien, M. (2007). *Mucin Utilisation and Host Interactions of the Novel Intestinal Microbe Akkermansia Muciniphila*. Dissertation. Netherlands: Wageningen University.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Dias, J., Marcondes, M. I, de Souza, S. M., da Mata e Silva, B. C., Noronha, M. F., Resende, R. T., et al. (2018). Bacterial community dynamics across the gastrointestinal tracts of dairy calves during preweaning development. *Appl. Environ. Microbiol.* 84, 1–12. doi: 10.1128/AEM.02675-17

Dias, J., Marcondes, M. I., Noronha, M. F., Resende, R. T., Machado, F. S., Mantovani, H. C., et al. (2017). Effect of pre-weaning diet on the ruminal archaeal, bacterial, and fungal communities of dairy calves. *Front. Microbiol.* 8:1553. doi: 10.3389/fmicb.2017.01553

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D., and Suen, G. (2019). Diet influences early microbiota development in dairy calves without long-term impacts on milk production. *Appl. Environ. Microbiol.* 85, 1–12.

Eadie, J. M. (1962a). Inter-relationships between certain rumen ciliate protozoa. *J. Gen. Microbiol.* 29, 579–588. doi: 10.1099/00221287-29-4-579

Eadie, J. M. (1962b). The development of rumen microbial populations in lambs and calves under various conditions of management. *J. Gen. Microbiol.* 29, 563–578. doi: 10.1099/00221287-29-4-563

Eadie, J. M. (1967). Studies on the ecology of certain rumen ciliate protozoa. *J. Gen. Microbiol.* 49, 175–194. doi: 10.1099/00221287-49-2-175

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

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

Fonty, G., Gouet, P., Jouany, J. P., and Senaud, J. (1987). Establishment of the microflora and anaerobic fungi in the rumen of lambs. *J. Gen. Microbiol.* 133, 1835–1843. doi: 10.1099/00221287-133-7-1835

Fonty, G., Senaud, J., Jouany, J. P., and Gouet, P. (1988). Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. *Can. J. Microbiol.* 34, 235–241. doi: 10.1139/m88-044

Franzolin, R., and Dehority, B. A. (1996). Effect of prolonged high-concentrate feeding on ruminal protozoa concentrations. *J. Anim. Sci.* 74:2803. doi: 10.2527/1996.74112803x

Franzolin, R., and Dehority, B. A. (2010). Importância do pH na sobrevivência de protozoários no rúmen de bovinos. *Rev. Bras. Zootec.* 39, 2262–2267. doi: 10.1590/S1516-35982010001000023

Friedman, N., Jami, E., and Mizrahi, I. (2017). Compositional and functional dynamics of the bovine rumen methanogenic community across different developmental stages. *Environ. Microbiol.* 19, 3365–3373. doi: 10.1111/1462-2920.13846

Goodrich, J. K., Davenport, E. R., Beaumont, M., Jackson, M. A., Knight, R., Ober, C., et al. (2016). Genetic determinants of the gut microbiome in UK twins. *Cell Host Microbe* 19, 731–743. doi: 10.1016/j.chom.2016.04.017

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., et al. (2014). Human genetics shape the gut microbiome. *Cell* 159, 789–799. doi: 10.1016/j.cell.2014.09.053

Gordon, G. L. R., and Phillips, M. W. (1998). The role of anaerobic gut fungi in ruminants. *Nutr. Res. Rev.* 11, 133–168. doi: 10.1079/NRR19980009

Guilloteau, P., Zabielski, R., David, J. C., Blum, J. W., Morisset, J. A., Biernat, M., et al. (2009). Sodium-butyrate as a growth promoter in milk replacer formula for young calves. *J. Dairy Sci.* 92, 1038–1049. doi: 10.3168/jds.2008-1213

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B., and Franks, A. E. (2015). Presence of selected methanogens, fibrolytic bacteria, and proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS One* 10:e0133048. doi: 10.1371/journal.pone.0133048

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21, 494–504. doi: 10.1101/gr.112730.110

Herr, C. M., and Reed, K. C. (1991). Micromanipulation of bovine embryos for sex determination. *Theriogenology* 35, 45–54. doi: 10.1016/0093-691X(91)90147-6

Herve, M. (2020). *Package "RVAideMemoire". Version 0.9-75*.

Huang, J. Y., Lee, S. M., and Mazmanian, S. K. (2011). The human commensal *Bacteroides fragilis* binds intestinal mucin. *Anaerobe* 17, 137–141. doi: 10.1016/j.anaerobe.2011.05.017

Huhtanen, P. J., Blauwiekel, R., and Saastamoinen, I. (1998). Effects of intraruminal infusions of propionate and butyrate with two different protein supplements on milk production and blood metabolites in dairy cows receiving grass silage-based diet. *J. Sci. Food Agric.* 77, 213–222. doi: 10.1002/(sici)1097-0010(199806)77:2<213::aid-jsfa28>3.0.co;2-6

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

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. Microbiol.* 81, 4697–4710. doi: 10.1128/AEM.00720-15

Jiao, J., Huang, J., Zhou, C., and Tan, Z. (2015). Taxonomic identification of ruminal epithelial bacterial diversity during rumen development in goats. *Appl. Environ. Microbiol.* 81, 3502–3509. doi: 10.1128/AEM.00203-15

Joblin, K. N., and Naylor, G. E. (1993). Inhibition of the rumen anaerobic fungus Neocallimastix frontalis by fermentation products. *Lett. Appl. Microbiol.* 16, 254–256. doi: 10.1111/j.1472-765X.1993.tb01412.x

Khan, M. A., Bach, A., Weary, D. M., and von Keyserlingk, M. A. G. (2016). Invited review: transitioning from milk to solid feed in dairy heifers. *J. Dairy Sci.* 99, 885–902. doi: 10.3168/jds.2015-9975

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

Klein-Jöbstl, D., Quijada, N. M., Dzieciol, M., Feldbacher, B., Wagner, M., Drillich, M., et al. (2019). Microbiota of newborn calves and their mothers reveals possible transfer routes for newborn calves' gastrointestinal microbiota. *PLoS One* 14:e0220554. doi: 10.1371/journal.pone.0220554

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

Koo, H., Hakim, J. A., Crossman, D. K., Lefkowitz, E. J., and Morrow, C. D. (2019). Sharing of gut microbial strains between selected individual sets of twins cohabitating for decades. *PLoS One* 14:e0226111.

Kostopoulos, I., Elzinga, J., Ottman, N., Klievink, J. T., Blijenberg, B., Aalvink, S., et al. (2020). *Akkermansia muciniphila* uses human milk oligosaccharides to thrive in the early life conditions in vitro. *Sci. Rep.* 10:14330. doi: 10.1038/s41598-020-71113-8

Kruger Ben Shabat, S., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., 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

Kudo, H., Cheng, K. J., Imai, S., Han, S. S., and Costerton, J. W. (1990). Effects of feed on the composition of the rumen ciliate protozoal population in cattle and its relationship to cellulolytic ciliate protozoa. *Anim. Feed Sci. Technol.* 29, 159–169. doi: 10.1016/0377-8401(90)90102-E

Lahti, L., Sudarshan, S., and Al, E. (2017). *Tools for Microbiome Analysis in R. Version 1.9.97*.

Li, R. W., Connor, E. E., Li, C., Baldwin Vi, R. L., Sparks, M. E., Baldwin, R. L., et al. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. *Environ. Microbiol.* 14, 129–139. doi: 10.1111/j.1462-2920.2011.02543.x

Malmuthuge, N., Griebel, P. J., and Guan, L. L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves. *Appl. Environ. Microbiol.* 80, 2021–2028. doi: 10.1128/AEM.03864-13

Malmuthuge, N., Li, M., Goonewardene, L. A., Oba, M., and Guan, L. L. (2013). Effect of calf starter feeding on gut microbial diversity and expression of genes involved in host immune responses and tight junctions in dairy calves during weaning transition. *J. Dairy Sci.* 96, 3189–3200. doi: 10.3168/jds.2012-6200

Malmuthuge, N., Liang, G., and Guan, L. L. (2019). Regulation of rumen development in neonatal ruminants through microbial metagenomes and host transcriptomes. *Genome Biol.* 20, 1–16. doi: 10.1186/s13059-019-1786-0

Mäntysaari, P., Liinamo, A. E., and Mäntysaari, E. A. (2012). Energy efficiency and its relationship with milk, body, and intake traits and energy status among primiparous Nordic Red dairy cattle. *J. Dairy Sci.* 95, 3200–3211. doi: 10.3168/jds.2011-4685

Mayer, M., Abenthum, A., Matthes, J. M., Kleeberger, D., Ege, M. J., Hölzel, C., et al. (2012). Development and genetic influence of the rectal bacterial flora of newborn calves. *Vet. Microbiol.* 161, 179–185. doi: 10.1016/j.vetmic.2012.07.023

McCullough, H. (1967). The determination of ammonia in whole blood by a direct colorimetric method. *Clin. Chim. Acta* 17, 297–304. doi: 10.1016/0009-8981(67)90133-7

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

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Meale, S. J., Chaucheyras-Durand, F., Berends, H., Guan, L. L., and Steele, M. A. (2017). From pre- to postweaning: transformation of the young calf's gastrointestinal tract. *J. Dairy Sci.* 100, 5984–5995. doi: 10.3168/jds.2016-12474

Meale, S. J., Popova, M., Saro, C., Martin, C., Bernard, A., Lagree, M., et al. (2021). Early life dietary intervention in dairy calves results in a long-term reduction in methane emissions. *Sci. Rep.* 11:3003. doi: 10.1038/s41598-021-82084-9

Milne, A., Theodorou, M. K., Jordan, M. G. C., King-Spooner, 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

Miltko, R., Bezecki, G., Kowalik, B., and Michaowski, T. (2014). Can fungal zoospores be the source of energy for the rumen protozoa *Eudiplodinium maggii*? *Anaerobe* 29, 68–72. doi: 10.1016/j.anaerobe.2013.08.005

Minato, H., Otsuka, M., Shirasaka, S., Itabashi, H., and Mitsumori, M. (1992). Colonization of microorganisms in the rumen of young calves. *J. Gen. Appl. Microbiol.* 38, 447–456. doi: 10.2323/jgam.38.447

Morgavi, D. P., Forano, E., Martin, C., and Newbold, C. J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal* 4, 1024–1036. doi: 10.1017/S1751731110000546

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* 140, 631–636. doi: 10.1099/00221287-140-3-631

Muscato, T. V., Tedeschi, L. O., and Russell, J. B. (2002). The effect of ruminal fluid preparations on the growth and health of newborn, milk-fed dairy calves. *J. Dairy Sci.* 85, 648–656. doi: 10.3168/jds.S0022-0302(02)74119-2

Naga, M. A., Abou Akkada, A. R., and El-Shazly, K. (1969). Establishment of rumen ciliate protozoa in cow and water buffalo (*Bos bubalus* L.) calves under late and early weaning systems. *J. Dairy Sci.* 52, 110–112. doi: 10.3168/jds.S0022-0302(69)86510-0

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. Micobiol.* 6:1313. doi: 10.3389/fmicb.2015.01313

Oksanen, J., Blanchet, F. G., Friendly, M., Roeland Legendre, P., McGlinn, D., Minchin, P. R., et al. (2019). *vegan: Community Ecology Package. R Package Version 2.5-6*.

Ørskov, E. R., Benzie, D., and Kay, R. N. B. (1970). The effects of feeding procedure on closure of the oesophageal groove in young sheep. *Br. J. Nutr.* 24, 785–795. doi: 10.1079/bjn19700080

Palma-Hidalgo, J. M., Jiménez, E., Popova, M., Morgavi, D. P., Martín-García, A. I., Yáñez-Ruiz, D. R., et al. (2021). Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. *Anim. Microbiome* 3:11. doi: 10.1186/s42523-021-00073-9

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., and Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116, 245–257. doi: 10.1111/jam.12405

Rey, M., Enjalbert, F., and Monteils, V. (2012). Establishment of ruminal enzyme activities and fermentation capacity in dairy calves from birth through weaning. *J. Dairy Sci.* 95, 1500–1512. doi: 10.3168/jds.2011-4902

Rius, A. G., Kittelmann, S., Macdonald, K. A., Waghorn, G. C., Janssen, P. H., and Sikkema, E. (2012). Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture. *J. Dairy Sci.* 95, 5024–5034. doi: 10.3168/jds.2012-5392

Sakata, T., and Tamate, H. (1978). Rumen epithelial cell proliferation accelerated by rapid increase in intraruminal butyrate. *J. Dairy Sci.* 61, 1109–1113. doi: 10.3168/jds.S0022-0302(78)83694-7

Sander, E. G., Warner, R. G., Harrison, H. N., and Loosli, J. K. (1959). The stimulatory effect of sodium butyrate and sodium propionate on the development of rumen mucosa in the young calf. *J. Dairy Sci.* 42, 1600–1605. doi: 10.3168/jds.S0022-0302(59)90772-6

Santra, A., and Pathak, N. N. (2001). The effect of dietary concentrate level on rumen enzyme profile and ciliate protozoa population in cattle fed wheat straw diet. *J. Anim. Feed Sci.* 10, 589–604. doi: 10.22358/jafs/68011/2001

Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P. H. (2014). RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *PeerJ* 2:e494. doi: 10.7717/peerj.494

Shinkai, T., Ueki, T., and Kobayashi, Y. (2010). Detection and identification of rumen bacteria constituting a fibrolytic consortium dominated by *Fibrobacter succinogenes*. *Anim. Sci. J.* 81, 72–79. doi: 10.1111/j.1740-0929.2009.00698.x

Srinivasan, K., Murakami, M., Nakashimada, Y., and Nishio, N. (2001). Efficient production of cellulolytic and xylanolytic enzymes by the rumen anaerobic fungus, Neocallimastix frontalis, in a repeated batch culture. *J. Biosci. Bioeng.* 91, 153–158. doi: 10.1016/S1389-1723(01)80058-X

Stewart, C. S., Flint, H. J., and Bryant, M. P. (1997). "The rumen bacteria," in *The Rumen Microbial Ecosystem*, eds P. N. Hobson and C. S. Stewart (London: Blackie Academic & Professional), 10–72.

Suárez, B. J., Van Reenen, C. G., Gerrits, W. J. J., Stockhofe, N., Van Vuuren, A. M., and Dijkstra, J. (2006). Effects of supplementing concentrates differing in carbohydrate composition in veal calf diets: II. Rumen development. *J. Dairy Sci.* 89, 4376–4386. doi: 10.3168/jds.S0022-0302(06)72484-5

Swanson, E. W., and Harris, J. D. (1958). Development of rumination in the young calf. *J. Dairy Sci.* 41, 1768–1776. doi: 10.3168/jds.S0022-0302(58)91161-5

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. *Nutr. Methodol.* 134, 3378–3384. doi: 10.1093/jn/134.12.3378

Tapio, I., Shingfield, K. J., McKain, N., Bonin, A., Fischer, D., Bayat, A. R., et al. (2016). Oral samples as non-invasive proxies for assessing the composition of the rumen microbial community. *PLoS One* 11:e0151220. doi: 10.1371/journal.pone.0151220

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A core gut microbiome in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540

Wexler, H. M. (2014). "The genus *Bacteroides*," in *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*, eds E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson (Berlin: Springer-Verlag), 459–484. doi: 10.1007/978-3-642-38954-2_129

Williams, P. P., and Dinusson, W. E. (1972). Composition of the ruminal flora and establishment of ruminal ciliated protozoal species in isolated calves. *J. Anim. Sci.* 34, 469–474. doi: 10.2527/jas1972.343469x

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

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J., and Aldridge, B. M. (2018). Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. *Sci. Rep.* 8, 1–14. doi: 10.1038/s41598-018-21440-8

Yu, S., Zhang, G., Liu, Z., Wu, P., Yu, Z., and Wang, J. (2020). Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. *BMC Microbiol.* 20:29. doi: 10.1186/s12866-020-1716-z

Zhong, R. Z., Sun, H. X., Li, G. D., Liu, H. W., and Zhou, D. W. (2014). Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. *Livest. Sci.* 162, 154–158. doi: 10.1016/j.livsci.2013.12.021

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 January 2022
doi: 10.3389/fmicb.2021.821293

Effects of Herbal Tea Residue on Growth Performance, Meat Quality, Muscle Metabolome, and Rumen Microbiota Characteristics in Finishing Steers

Ling Li†, Xiaohong Sun†, Junyi Luo, Ting Chen, Qianyun Xi, Yongliang Zhang and Jiajie Sun**

Guangdong Provincial Key Laboratory of Animal Nutrition Control, Guangdong Laboratory for Lingnan Modern Agriculture, National Engineering Research Center for Breeding Swine Industry, South China Agricultural University, Guangzhou, China

OPEN ACCESS

Edited by:
Robert W. Li, Agricultural Research Service, United States Department of Agriculture (USDA), United States

Reviewed by:
Yury Tatiana Granja-Salcedo, Colombian Corporation for Agricultural Research (AGROSAVIA), Colombia
Carmen Gallo, Austral University of Chile, Chile

***Correspondence:**
Yongliang Zhang
zhangyongliang@scau.edu.cn
Jiajie Sun
jiajiesun@scau.edu.cn

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *24 November 2021*
Accepted: *29 December 2021*
Published: *18 January 2022*

Citation:
Li L, Sun X, Luo J, Chen T, Xi Q, Zhang Y and Sun J (2022) Effects of Herbal Tea Residue on Growth Performance, Meat Quality, Muscle Metabolome, and Rumen Microbiota Characteristics in Finishing Steers. Front. Microbiol. 12:821293. doi: 10.3389/fmicb.2021.821293

Herbal tea residue (HTR) contains various medicinal and nutritional components and is a potential high-quality unconventional source of roughage. In this study, a total of 30 healthy Simmental crossbred finishing steers were equally divided into two groups: CN (fed with a basic diet) and RE (HTR partly replaced *Pennisetum purpureum*). HTR did not alter the growth performance of steers but increased the net meat rate, tenderness, and water-holding capacity and increased the moisture content and oleic acid and linoleic acid concentrations in *longissimus dorsi*. It altered muscle metabolic pathways and improved rumen fermentation by increasing the propionic acid concentration and propionic acid-to-acetic acid ratio. We studied the steers' rumen microbial community composition and determined their correlation with the tested parameters. Certain rumen microorganisms were closely associated with muscle glucolipid metabolites and rumen NH_3-N and volatile fatty acid levels. Our findings suggest that, as a functional roughage source, HTR improved to a certain extent the meat quality of steers by altering the rumen microbial composition and affecting the rumen fatty acid composition and muscle glucolipid metabolism.

Keywords: feed resources, herbal tea residue, muscle metabolism, microbial diversity, beef

INTRODUCTION

Herbal tea, one of the most unique beverages, is a specialty drink developed according to the climatic characteristics of Lingnan in south China (Li et al., 2017). Herbal tea is mainly prepared by decocting Chinese herbal medicines such as herbal jelly, honeysuckle, chrysanthemum, prunella vulgaris, buzha leaf, and licorice (Zhao et al., 2013) and contains various bioactive substances such as flavonoids, organic acids, polysaccharides, alkaloids, and volatile oils (Liu et al., 2011). These substances usually have antibacterial, anti-inflammatory, antioxidant, antiviral, and immune-enhancing medicinal effects (Cao et al., 2014), because of which herbal teas are favored by people in the subtropical region of China.

Herbal tea residue (HTR) is the natural byproduct of preparing herbal tea. With the continuously increasing annual consumption of herbal tea, a large quantity of HTR is produced (Yang and Cui, 2013). To date, the main treatment methods for HTR are landfill, incineration, and stacking (Suthar and Singh, 2011), which not only waste resources but also pollute the environment

(Malkoc and Nuhoglu, 2006). It has been reported that HTR can be used as an adsorbent for heavy metal ions to reduce water pollution (Ahsan et al., 2018). HTR is also a high-quality compost material, which significantly improves the ecological characteristics of soil (Iqbal Khan et al., 2015). Moreover, HTR is essentially a type of Chinese herbal residue (CHR), which still contains a variety of nutrients and functional active substances similar to the raw materials and can be used as animal feed (Abdallah et al., 2019). A previous study suggested that adding 0.5% CHRs to duck diets improved the crude protein content and water-holding capacity of duck meat (Jin-Woo et al., 2017). Supplementation of poultry diets with 3% CHRs improved the nutritional value, sensory quality, and tenderness of meat while reducing the antioxidant status (Kim et al., 2014). Moreover, Chinese herbal feed improves the immune function of dairy cows under heat stress (Shan et al., 2018) and contributes to rumen fermentation and energy metabolism of sheep (Liang et al., 2013).

Many recent reports recommend using a small quantity of CHRs as a functional feed additive. As a type of CHR, HTR is not only rich in protein but also contains crude fiber and trace elements that ruminant require (Xie et al., 2020). Ruminants require approximately 17% dietary crude fiber content (Ding et al., 2020). We therefore hypothesized that HTR could be used as an unconventional feed material to improve growth performance and meat quality in ruminant. In this paper, the HTR was added to the diet of steers to investigate its effects on growth performance, meat quality, muscle metabolome, rumen fermentation and rumen microbial diversity.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

This study was conducted at a large-scale steer farm in Guangxi, China. According to the principle of completely random allocation, 30 healthy Simmental crossbred steers (18 months old and approximately 480 kg per animal) were divided into two groups, namely CN group (fed with basic diet) and RE group (HTR partly replaced *Pennisetum purpureum*). Each experimental group included three replicates, and each replicate contained five animals. All steers were housed individually in an open cowshed at the same time. The basic composition and nutritional level of steer feed are shown in **Table 1**. The chemical composition of HTR was showed in **Supplementary Table 1**. In details, the HTR contained a high water content (75.10%) and the dry matter content was 24.90%. As a proportion of the dry matter content, the crude protein, crude fat, and ash contents were 13.10, 2.60, 6.69%, respectively. The acid detergent fiber and neutral detergent fiber were 39.8 and 54.3% (Zhuang et al., 2021). The experiment lasted for 67 days, including a 7-day pre-feeding period and a 60-day formal study period. During the experiment, the steers were fed regularly at 8 am and 5 pm, and water was available *ad libitum* throughout the experimental period. All animal procedures were approved by the Animal Care Committee at South China Agricultural University.

TABLE 1 | Basic diet composition and nutrient level of finishing steers.

Item	CN	RE
Ingredients		
Corn (%)	23.7	23.7
Bean curd residue (%)	15.0	15.0
Pennisetum purpureum (%)	60.0	10.0
Herbal tea residue (%)	0	50.0
Premix (%)	1.00	1.00
Salt (%)	0.30	0.30
Total (%)	100	100
Nutritional level		
Dry matter (%)	34.6	30.7
Crude protein (%)	9.14	9.70
r Crude fat (%)	2.05	3.00
Neutral detergent fiber (%)	71.2	68.9
Acid detergent fiber (%)	25.2	27.6
Calcium (%)	0.69	0.63
Phosphorus (%)	0.22	0.30
Net energy (MJ/kg)	5.56	5.36

The indicators were calculated on the basis of dry matter. The nutrient contents of the premix were as follows: Zinc, 70–100 mg/kg; Iron, 50–70 mg/kg; Copper, 30–45 mg/kg; Manganese, 6.25–10 mg/kg; Selenium, 0.3–0.5 mg/kg; Iodine, 0.2–1.00 mg/kg; Vitamin A, 7,000–10,000 IU/kg; Vitamin D, 40,000–90,000 IU/kg; Vitamin E, 4,000–5,000 mg/kg. Net energy was a calculated value, and others were measured values. CN, no herbal tea residues; RE, 50% HTRs replaced Pennisetum purpureum.

Measurements and Sampling

The average daily feed intake (ADFI) was recorded once a week, and individual steers were weighed at the beginning and end of the experiment to determine initial weight and average daily gain (ADG). On the last day of the experiment, 200 mL of rumen fluid per individual was obtained from 15 steers in each group before the morning feeding. Whole ruminal samples were collected from steers with a suction strainer (19 mm diameter; 1.5 mm mesh for its filter) and strained through four layers of cheesecloth (Gilbreath et al., 2020). One aliquot (100 mL) was used to determine volatile fatty acids (VFAs) using high-performance liquid chromatography (Actlabs, Ancaster, ON, Canada). Another aliquot (100 mL) was used to extract total genomic DNA for sequencing of rumen microorganisms.

At the end of the experiment, the feed was detained for 12 hours and weighed, and all animals were slaughtered at the same time. Then the carcass weight, eye muscle area (EMA), net meat weight (the weight of muscle and fat in the carcass after bone has been removed), dressing percentage, and net meat percentage were recorded for each animal post slaughter. Dressing percentage is the ratio of carcass weight to live weight at slaughter. Net meat percentage refers to net meat weight as a percentage of carcass weight. After the carcasses were chilled for 45 min at 4°C, the meat color were measured. Following aging for 24 h at 4°C, longissimus dorsi (LD) samples were collected between the 9th and 13th ribs from the right side of the carcasses, of which one was stored at 4°C for subsequent physical analysis, and the other one was frozen for nutrient value analysis.

Meat Quality and Nutritional Composition

The meat color [lightness (L*), redness (a*), and yellowness (b*m)], water-holding capacity (drip loss and cooking loss) and tenderness (shear force) were analyzed. In detail, meat color (average of three randomly selected areas on the sample) was assessed using a Minolta Chroma Meter (CR-300, Dietikon, Switzerland), applying the L*, a*, and b* system (Razminowicz et al., 2006). Drip loss was determined as the weight loss after suspending meat samples (5 × 2 × 3 cm) at 4°C for 24 h. Meat samples in dry polyethylene bags were weighed and heated in a water bath at 85°C for 20 min and then cooled to room temperature (25°C) in running water. The cooked samples were dried and weighed again to calculate cooking loss, expressed as the percentage of uncooked sample weight (%). After measuring the cooking loss, the samples were stored for 24 h at 4°C. Subsequently, shear force was tested with a digital tenderness meter (C-LM3B, Tenovo, Beijing, China), and the average of nine replicates per sample was regarded as the final value (Sales et al., 2020). LD samples were also analyzed for moisture, dry matter, crude protein, crude fat, and ash according to Association of Official Analytical Chemists [AOAC] (2000). The fatty acid composition of frozen samples was measured by fatty acid methyl ester synthesis (O'Fallon et al., 2007). Amino acid levels were determined employing an automatic amino acid analyzer (L-8800; Hitachi, Tokyo, Japan) based on the method described by Yan et al. (2018).

Muscle Metabolome

Non-targeted muscle metabolomics analysis was performed by Novogene Biotechnology (Beijing, China) using LC-MS platform. Specifically, approximately 100 mg of frozen samples were ground and homogenized in 500 μL of 80% methanol containing 0.1% formic acid. The mixtures were kept in an ice bath for 5 min, and then centrifuged at 15,000 × *g* for 10 min at 4°C. After the content of methanol in the supernatant was diluted to 53%, the mixture was centrifuged again (15,000 × *g* for 10 min at 4°C). Then, the supernatant (200 μL) was transferred to an LC-MS sampling vial for LC-MS analysis. Raw data were filtered and aligned by parameter (retention time, mass-to-charge ratio, and peak intensity) selection of Compound Discoverer 3.1 software (Thermo Scientific). The processed data were used to annotate the metabolites using the KEGG, HMDB and LIPID MAPS databases. Moreover, the dataset of two groups was separated with partial least squares discriminant analysis (PLS-DA). The differentially expressed metabolites between two groups are illustrated with a volcano plot.

16S rRNA Gene Sequencing and Annotation Analysis of Rumen Microorganisms

Total genomic DNA was extracted from rumen fluid samples using the SDS method, and the integrity of the extracted DNA was assessed by 1% agarose gel electrophoresis (Black and Foarde, 2007). DNA concentration was determined using Qubit Fluorometer (Invitrogen, Carlsbad, CA). 16S rRNA genes were subsequently amplified using specific primers with barcode (Forward: 5′-GTGCCAGCMGCCGCGG-3′ and Reverse: 5′-GGACTACHVGGGTWTCTAAT-3′) targeting the variable regions V3–V4. The sequencing library was prepared using the gDNA samples using the Illumina TruSeq® DNA PCR-Free Sample Preparation Kit. Qubit and Real-Time PCR System were used to assess the quantity and quality of the sample library. Then, the library constructed was sequenced using NovaSeq6000 platform. Clean reads were obtained from the raw data by strict quality filtering and chimeric sequence removal (Haas et al., 2011). The effective tags of all samples were clustered, and the tags with over 97% similarity were regarded as one operational taxonomic unit (OTU) (Edgar, 2013). According to the Silva 132 database, a representative sequence for each OTU was screened for taxonomic identification based on the Mothur algorithm (Quast et al., 2013). To explore the phylogenetic relationship among different OTUs, multiple sequence alignment was performed using MUSCLE software (Version 3.8.31) (Yuan et al., 2018). All the data were normalized, and the least amount of data were considered as the standard. The subsequent alpha-diversity and beta-diversity analysis were based on the normalized data. Alpha-diversity analysis reflected the complexity and diversity of species for the samples, including the observed species, Simpson, Shannon, Chao1 and ACE indices. For beta-diversity analysis, principal coordinate analysis (PCoA) was performed to obtain the principal coordinates and visualize complex, multidimensional data. Non-metric multi-dimensional scaling (NMDS) was employed to visualize and compare the relationship of the rumen microbial community structure between the two groups. Unweighted pair group method with arithmetic means (UPGMA) clustering was conducted as a type of hierarchical clustering method to interpret the distance matrix using average linkage. Linear discriminant analysis effect size (LEfSe) method was employed to identify statistically significant biomarkers between groups.

Statistical Analysis

Growth performance, carcass characteristics, meat quality, meat nutrition level, and rumen VFA content were analyzed by the independent sample *t*-test using SPSS software 17.0 (IBM Corp., Armonk, NY, United States). The correlation analyses of rumen microbiota with the tested traits were performed using the function cor (x, y, use = "p") and illustrated with function labeledHeatmap (Matrix, xLabels, yLabels) in the R package WGCNA (Langfelder and Horvath, 2008). The data were expressed as mean ± standard error of the mean (SEM), and statistical significance was established at $P < 0.05$.

RESULTS

Growth Performance and Carcass Characteristics

The growth performance and carcass characteristics between the two groups during the finishing phase are listed in **Table 2**. The growth performance parameters initial weight, live weight at slaughter, ADFI, and ADG did not differ between treatments

($P > 0.05$). The carcass weight, dressing percentage, net meat weight, and EMA were not significantly different between groups ($P > 0.05$), but the net meat rate was higher in the RE group than in the CN group (41.72 and 40.28%, respectively; $P < 0.05$).

Meat Quality and Nutritional Composition

The LD quality traits are presented in **Table 3**. Compared with the CN group, the RE group showed a significantly lower drip loss (5.85% vs. 4.45%, $P < 0.01$), cooking loss (29.96% vs. 27.58%, $P < 0.01$) and shear force (65.26 N vs. 48.13 N, $P < 0.01$). For meat color-related parameters (L*, a*, and b*), the values of a* and b* were not significantly different between treatments, but the L* value of the RE group was higher than that of CN group (34.96 and 36.91, respectively). The crude protein and crude fat contents were significantly different between groups. However, the moisture content in the CN and RE groups was 3.30 and 4.69%, respectively, indicating a significant increase ($P < 0.01$) when Simmental steers were fed with diets containing HTR. The content of crude ash was lower in the RE group than in the CN group (0.038% vs. 0.044%, $P < 0.05$; **Table 4**). The amino acid composition in LD was not significantly different between groups (**Supplementary Table 2**). Regarding fatty acid composition (**Table 5**), the content of oleic acid (C18:1n9c) and linoleic acid (C18:2n6t) content accounted for 37.35% and 0.11% of fatty acids in the CN group and 39.74% and 0.18% of fatty acids in the RE group, respectively; their content in the RE group was significantly higher than that in the CN group ($P < 0.05$). Moreover, the ratio of ω-6/ω-3 fatty acids in the RE group was lower than that in the CN group ($P = 0.06$).

Muscle Metabolome

A total of 774 metabolites were detected in the steer muscles, including 519 in the positive ionization mode and 255 in the negative ionization mode (**Supplementary Tables 3A,B**). The annotation results obtained using biological databases (KEGG, HMDB, LIPID MAPS) suggest that these metabolites were mainly involved in the metabolic pathways of lipid metabolism, carbohydrate metabolism, and amino acid metabolism (**Figure 1** and **Supplementary Figure 1**). A total of 21, 6, and 38 metabolites in the positive ion mode were mainly involved in lipid, carbohydrate, and amino acid metabolism pathways, whereas 18, 11, and 19 metabolites in the negative ion mode were associated with lipid, carbohydrate, and amino acid metabolism (**Supplementary Tables 3C,D**). As shown in **Supplementary Figure 2A** (R2Y = 0.84, Q2Y = 0.56) and 2B (R2Y = 0.78, Q2Y = 0.39), the PLS-DA model revealed a clear separation between muscle metabolomes of steers fed with different diets. **Supplementary Tables 3E,F** summarize all the differential metabolites of the two groups in the positive and negative ion mode, as well as their query IDs, *P*-value, and fold change (FC). Based on FC threshold ≥ 2 (or ≤ 0.5) and a *P*-value < 0.05; 90 metabolites, including 30 upregulated and 32 downregulated metabolites in the positive ion mode and 19 upregulated and 9 downregulated metabolites in the negative ion mode, were significantly altered between the two groups (**Figure 2**). Moreover, we found that many differential metabolites were associated with glucose and lipid metabolism pathways. Phosphocholine, linolenic acid, and D-glucose 6-phosphate (G6p) showed significantly lower levels in the muscle in RE animals than in CN animals, whereas adenosine 5′-monophosphate (AMP), androstenedione, arachidonic acid (ARA), caprylic acid, cortisol, cortisone, docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), D-glucarate, histamine,

TABLE 2 | Effects of herbal tea residue feed on the growth performance and carcass characteristics of finishing steers.

Parameter	CN	RE	*P*-value
Initial weight (kg)	479.87 ± 10.99	482.53 ± 6.95	0.24
Live weight at slaughter (kg)	542.91 ± 11.68	547.09 ± 7.56	0.28
Average daily feed intake (kg)	12.64 ± 0.30	12.33 ± 0.28	0.79
Average daily gain (kg)	1.05 ± 0.04	1.08 ± 0.05	0.72
Carcass weight (kg)	307.58 ± 18.02	319.01 ± 16.53	0.65
Dressing percentage (%)	56.54 ± 0.90	57.26 ± 0.46	0.49
Net meat weight (kg)	257.58 ± 18.02	267.58 ± 15.71	0.68
Net meat percentage (%)	40.28 ± 0.38	41.72 ± 0.48	0.04
Eye muscle area (cm^2)	78.82 ± 5.03	81.70 ± 6.09	0.72

The values were calculated as the mean ± standard error of the mean (N = 15). P < 0.05 indicated a significant difference between the two groups; P > 0.05 indicated no significant difference between the two groups. CN, no herbal tea residues; RE, 50% HTR replaced Pennisetum purpureum.

TABLE 3 | Effects of herbal tea residue feed on the meat quality of finishing steers.

Parameter	CN	RE	*P*-value
Drip loss (%)	5.85 ± 0.304	4.45 ± 0.233	0.001
Cooking loss (%)	29.96 ± 0.752	27.58 ± 0.443	0.006
Shear force (N)	65.26 ± 3.404	48.13 ± 2.314	0.001
Meat color			
Lightness (L*)	34.96 ± 0.662	36.91 ± 0.55	0.032
Redness (a*)	18.91 ± 0.723	18.68 ± 0.753	0.823
Yellowness (b*)	9.50 ± 0.534	9.85 ± 0.452	0.624

The values were calculated as the mean ± standard error of the mean (N = 15). The shear force was calculated as the average of nine replicates per sample. P < 0.05 indicated a significant difference between the two groups; P > 0.05 indicated no significant difference between the two groups. CN, no herbal tea residue; RE, 50% HTR replaced Pennisetum purpureum.

TABLE 4 | Effects of herbal tea residue feed on the basic nutritional composition of beef.

Parameter	CN	RE	*P*-value
Moisture (%)	3.30 ± 0.18	4.69 ± 0.16	0.002
Crude protein (%)	86.19 ± 1.38	88.04 ± 1.08	0.33
Crude fat (%)	0.43 ± 0.003	0.46 ± 0.00	0.53
Crude ash (%)	0.044 ± 0.001	0.038 ± 0.002	0.04

The indicators were calculated on the basis of lyophilized samples. The values were calculated as the mean ± standard error of the mean (N = 15). P < 0.05 indicated a significant difference between the two groups; P > 0.05 indicated no significant difference between the two groups. CN, no herbal tea residue; RE, 50% HTR replaced Pennisetum purpureum.

TABLE 5 | Effects of herbal tea residue feed on the fatty acid content of beef (g/100 g).

Fatty acid	CN	RE	*P*-value
Decanoic acid (C10:0)	0.07 ± 0.007	0.06 ± 0.007	0.289
Lauric acid (C12:0)	0.10 ± 0.013	0.08 ± 0.005	0.343
Myristic acid (C14:0)	2.63 ± 0.146	2.41 ± 0.150	0.332
Myristoleic acid (C14:1)	0.44 ± 0.052	0.66 ± 0.108	0.061
Pentadecanoic acid (C15:0)	0.45 ± 0.036	0.46 ± 0.052	0.830
Palmitic acid (C16:0)	26.70 ± 0.453	25.57 ± 0.516	0.123
Palmitoleic acid (C16:1)	3.44 ± 0.141	3.39 ± 0.249	0.851
Heptadecanoic acid (C17:0)	0.97 ± 0.061	1.21 ± 0.130	0.106
10-Heptadecenoic acid (C17:1)	0.65 ± 0.057	0.69 ± 0.034	0.566
Stearic acid (C18:0)	18.18 ± 0.597	17.59 ± 0.651	0.513
Oleic acid (C18:1n9c)	37.345 ± 0.536	39.74 ± 0.855	0.036
Linoleic acid (C18:2n6t)	0.11 ± 0.010	0.18 ± 0.031	0.050
Methyl linoleate (C18:2n6c)	4.27 ± 0.406	4.50 ± 0.618	0.772
α-Linolenic acid (C18:3n3)	0.40 ± 0.050	0.43 ± 0.034	0.577
Arachidic acid (C20:0)	0.15 ± 0.016	0.15 ± 0.008	0.817
11,14,17-Eicosatrienoic acid (C20:3n3)	0.59 ± 0.266	0.53 ± 0.182	0.840
Methyl arachidonic acid (C20:4n6)	1.55 ± 0.329	2.20 ± 0.794	0.454
Eicosapentaenoic acid (C20:5n3)	0.24 ± 0.068	0.25 ± 0.074	0.899
Heneicosanoic acid-methyl ester (C21:0)	0.24 ± 0.062	0.21 ± 0.038	0.696
Docosanoic acid (C22:0)	0.06 ± 0.012	0.05 ± 0.020	0.520
Methyl cis-13,16-docosadienoic acid (C22:2)	0.13 ± 0.032	0.14 ± 0.023	0.896
Nervonic acid (C24:1)	0.21 ± 0.030	0.21 ± 0.055	0.999
Saturated fatty acid (SFA)	49.57 ± 0.439	48.95 ± 0.632	0.437
Unsaturated fatty acid (UFA)	50.24 ± 0.461	51.07 ± 0.738	0.359
Omega-6 (ω-6)	7.07 ± 1.145	6.64 ± 1.373	0.811
Omega-3 (ω-3)	1.18 ± 0.360	1.27 ± 0.302	0.846
ω-6: ω-3	6.98 ± 0.358	5.99 ± 0.334	0.060

The values were calculated as the mean ± standard error of the mean (N = 15). P < 0.05 indicated a significant difference between the two groups; P > 0.05 indicated no significant difference between the two groups. CN, no herbal tea residue; RE, 50% HTR replaced Pennisetum purpureum.

lauric acid, and progesterone showed significantly higher levels in RE animals ($P < 0.05$).

NH_3-N and Volatile Fatty Acid Concentrations in Rumen Fluid

The rumen NH3-N and VFA concentrations are listed in **Table 6**. The concentration of propionic acid in the CN and RE groups was 8.40 and 10.38 mmol/L, respectively, with the RE group showing significantly higher levels than the CN group ($P < 0.05$). The ratio of propionic acid to acetic acid levels in the RE group was also significantly higher than that in the CN group (0.22 and 0.24, respectively; $P < 0.05$). However, there were no significant differences between the CN and RE groups in the concentrations of acetic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid ($P > 0.05$).

16S rRNA Gene Sequencing and Annotation Analysis

The high-throughput sequencing analysis generated a total of 2,442,873 raw reads. On average, each sample yielded approximately 81,429 joined tags, and more than 61.09% of the total joined tags in each sample were processed for subsequent analysis after data filtering, quality control, and chimera removal (**Supplementary Table 4A**). A total of 3,184 OTUs were identified on the basis of 97% nucleotide sequence similarity; of these, 2,611 OTUs were found across all groups and defined as core OTUs. The number of unique OTUs in the CN and RE groups was 251 and 322, respectively (**Figure 3A** and **Supplementary Table 4B**). We annotated all these OTU tags to the Silva132 database and found that 92.09% of the sequences were assigned at the phylum level, whereas 89.42, 81.88, 71.39, 30.59, and 10.27% of the annotated OTUs were assigned at the class, order, family, genus, and species levels, respectively (**Supplementary Table 4C**). Phylogenetic analysis identified the top 10 phyla from the rumen of steers using the QIIME pipeline on the basis of the OTU annotation (**Figures 3B,C**). The dominant phyla in the rumen of steers were Bacteroidetes, Firmicutes, and Euryarchaeota, accounting for 43.95, 31.45, and 7.76%, respectively. And the Firmicutes to Bacteroidetes ratio increased from 0.72 in the CN group to 1.02 in the RE group (**Supplementary Table 5A**). At the genus level, a total of 301 classifiable genera were detected, and nine genera had a relative abundance greater than 1.0%, including *Methanobrevibacter, Anaeroplasma, Bacteroidales, Mycoplasma, Candidatus Saccharimonas, Lachnospiraceae, Ruminococcaceae, Prevotellaceae*, and *Saccharofermentans*. The most abundant genus in the rumen of Simmental steers was *Methanobrevibacter* (4.59%) (**Supplementary Table 5B**).

Changes in Rumen Microorganisms

For the alpha-diversity analysis, we calculated the observed species index, Shannon, Simpson, Chao1, ACE, Good's coverage, PD whole tree indices for each group. Although alpha diversity in RE groups tended to decrease compared with the control, these differences did not significantly affect species level microbial diversity ($P > 0.05$; **Table 7**). The results of the PCoA and NMDS analysis between the groups are shown in **Figure 4A**. The CN and RE samples were separated from each other, which reflects the effect of HTR on the rumen microbial community. The UPGMA clustering tree (**Figure 4B**) measured the similarity in microbial communities between groups according to the degree of their overlap and confirmed the significant structural separation of the rumen microflora between the two groups. The LEfSe analysis was used to identify the biomarkers between the two groups, and 18 differentially abundant taxonomic clades were found, with a Linear Discriminant Analysis (LDA) score higher than 4. The number of biomarkers at the kingdom, phylum, class, order, family, genus and species levels were 2, 4, 5, 3, 2, 1, and 1 respectively (**Figure 5**, left). A total of 11 taxa can be used as biomarkers for CN samples, including Archaea at the kingdom level, Bacteroidetes and Euryarchaeota at the phylum level, Bacteroidia and Methanobacteriales at the class level, Bacteroidales and

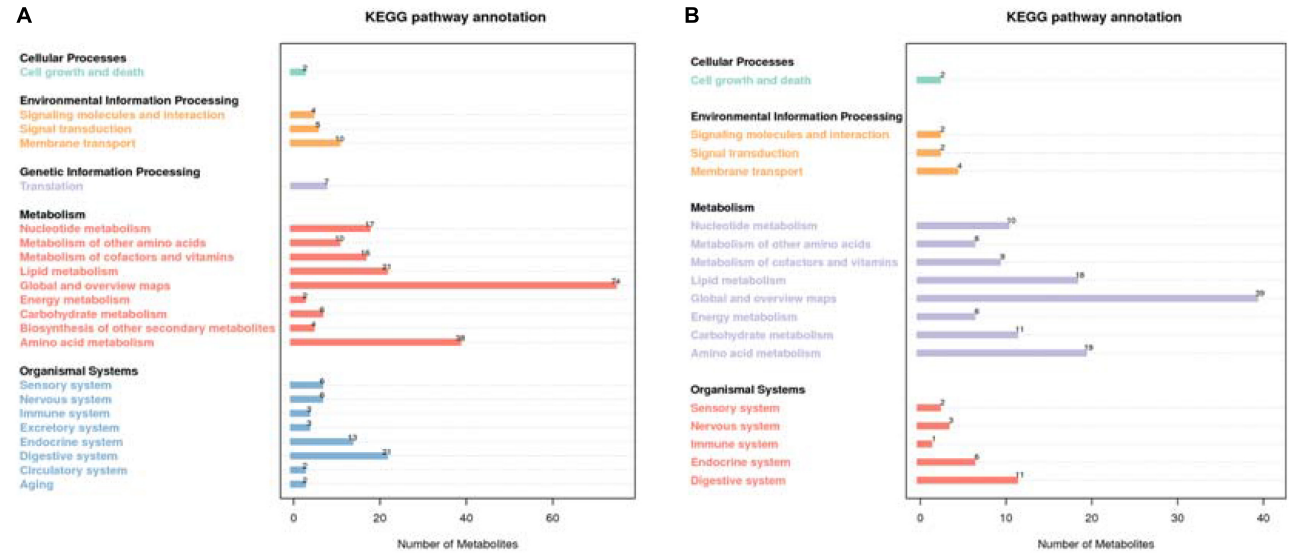


FIGURE 1 | Differential metabolite annotation statistics in the KEGG database in the positive ion mode **(A)** and the negative ion mode **(B)**. The X-axis represents the number of metabolites, and the y-axis represents the KEGG term.

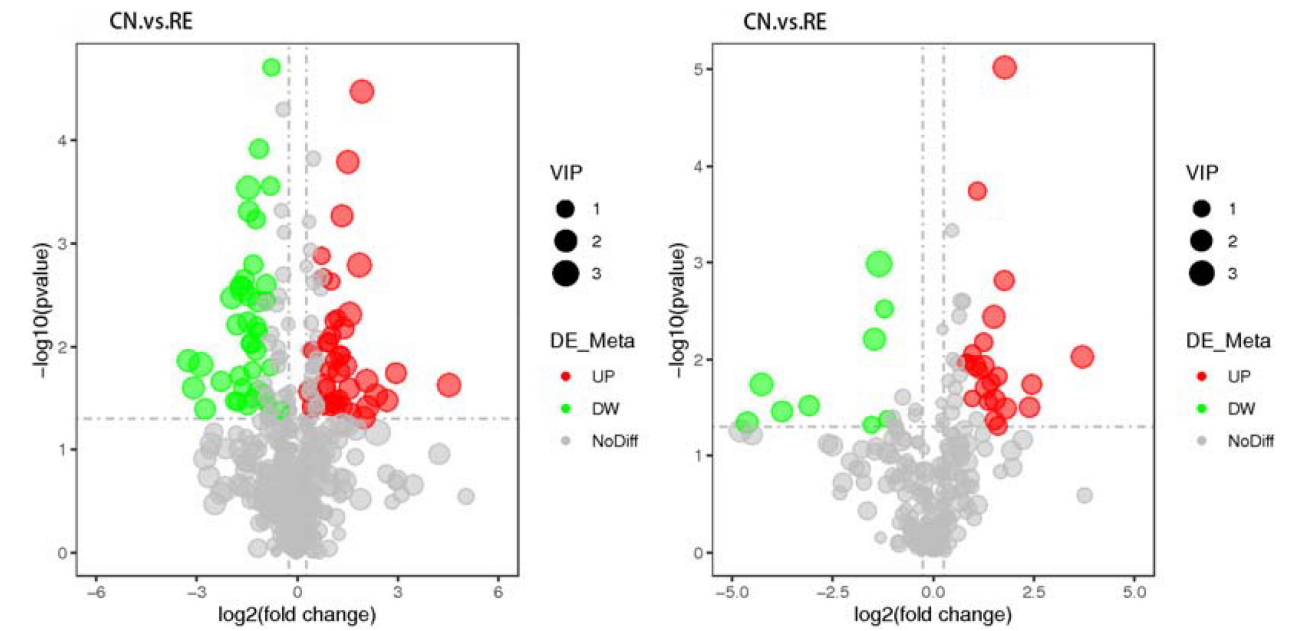


FIGURE 2 | Volcanic map of differential metabolites (positive ion mode on the left and negative ion mode on the right). The X-axis represents the expression multiple change (log2FoldChange) of metabolites in different groups; and the Y-axis represents the significance level of difference (–log10p-value). Each point in the volcanic map represents a metabolite; the significantly upregulated metabolites are represented by red dots, whereas the significantly downregulated metabolites are represented by green dots.

Methanobacteriales at the order level, *Methanobacteriaceae* and *Rikenellaceae* at the family level, *Methanobrevibacter* at the genus level, and *Bacteroidales-bacterium-Bact-22* at the species level. A total of seven taxa can be used as biomarkers for RE samples, including Bacteria at the kingdom level; Proteobacteria and Tenericutes at the phylum level; Mollicutes, Alphaproteobacteria, and Gammaproteobacteria at the class level, and Rickettsiales at the order level (**Figure 5**, right). A total of 23 genera displayed a significant difference in abundance between the CN and RE groups, including 3 upregulated and 20 downregulated genera ($FDR < 0.05$). Specifically, the abundance of *Riemerella* in the CN group and the abundance of *Rikenellaceae*, *Anaerovorax*, *Desulfovibrio*, *Papillibacter*, *Succiniclasticum*, *Veillonellaceae*, *Acetitomaculum*,

TABLE 6 | Effects of herbal tea residue feed on the rumen fermentation parameters of finishing steers.

Metabolite	CN	RE	*P*-value
Ammonia-N (mg/100 mL)	8.80 ± 1.053	11.43 ± 2.435	0.396
Acetic acid (mmol/L)	35.88 ± 2.660	30.58 ± 3.385	0.233
Propionic acid (mmol/L)	8.40 ± 0.343	10.38 ± 0.659	0.019
Isobutyric acid (mmol/L)	0.69 ± 0.109	0.59 ± 0.085	0.457
Butyric acid (mmol/L)	3.89 ± 0.299	3.92 ± 0.436	0.956
Isovaleric acid (mmol/L)	0.74 ± 0.145	0.64 ± 0.075	0.512
Valeric acid (mmol/L)	0.24 ± 0.043	0.19 ± 0.022	0.325
Propionic acid/Acetic acid	0.22 ± 0.007	0.24 ± 0.006	0.023

The values were calculated as the mean ± standard error of the mean (N = 15). $P < 0.05$ indicated a significant difference between the two groups; $P > 0.05$ indicated no significant difference between the two groups. CN, no herbal tea residue; RE, 50% HTR replaced Pennisetum purpureum.

Christensenellaceae, and *Schwartzia* in the RE group was significantly decreased (*FDR* < 0.01; **Supplementary Table 5C**).

Correlation of Rumen Microbiota Abundance With Volatile Fatty Acid and NH3-N Concentration

Pearson correlation analysis was performed to further identify the relationship between the relative abundance of differential bacterial genera identified by 16S rRNA sequencing with rumen VFA and NH3-N concentration (**Supplementary Figure 3**). The concentration of acetic acid, propionic acid, and butyric acid correlated negatively with the relative abundance of *Riemerella* ($P = 0.01$, $P = 0.03$, and $P = 0.03$, respectively) and Moraxella ($P = 0.01$, $P = 0.03$, and $P = 0.04$, respectively). The concentration of propionic acid was negatively correlated with the relative abundance of *Marvinbryantia* ($P = 0.03$). The concentration of isovaleric acid, valeric acid, and NH_3-N showed a significant positive correlation with the relative abundance of *Veillonellaceae* ($P = 0.03$, $P = 0.05$, and $P = 0.02$, respectively), *Olsenella* ($P = 0.008$, $P = 0.01$, and $P = 0.009$, respectively) and *Schwartzia* ($P = 0.01$, $P = 0.002$, and $P = 0.02$, respectively). Additionally, the concentration of isobutyric acid was most highly correlated with the relative abundance of *Olsenella* ($P = 0.002$).

Correlation Between Rumen Microbiota and Muscle Glycolipid Metabolites

We also performed a correlation analysis between differential rumen microorganisms with muscle differential glucolipid metabolites (**Figure 6**). The concentration of caprylic acid, DHA, DPA, glucarate, and lauric acid displayed a strong and positive correlation with relative abundance of *Moraxella* and *Riemerella*, respectively ($P < 0.01$). The concentration of linolenic acid showed a positive correlation with the relative abundance of *Acetitomaculum*, *Anaerovibrio*, *Anaerovorax*, *Blautia*, *Desulfovibrio*, *Howardella*, *Papillibacter*, *Schwartzia*, *Veillonellaceae* ($P < 0.01$) and a negative correlation with the relative abundance of *Riemerella* ($P = 0.01$). The concentration of phosphocholine was positively related to the bacterial abundance of *Anaerovibrio*, *Desulfovibrio*, *Olsenella*, *Papillibacter*, *Rikenellaceae*, *Schwartzia,* and *Veillonellaceae* ($P < 0.01$). In addition, the concentration of G6P correlated strongly and positively with the relative abundance of *Schwartzia* and *Succiniclasticum* ($P < 0.01$).

DISCUSSION

HTR still retain a considerable proportion of the nutrients and bioactive compounds, which has the potential to be used as an unconventional feed resource for ruminant (Xie et al., 2020). The results of the study showed that the HTR as a part of the diet has no adverse effects on the growth performance of finishing steers, and this is in accordance with our early reports of Zhuang et al. (2021). In this study, HTR had no significant effect on carcass weight, dressing percentage and EMA of finishing steers, but significantly improved the net meat percentage. This could be the ample nutrients and active ingredients in HTR to improve the fattening degree of finishing steers (Brscic et al., 2014). Meat quality is an important economic trait of bovine husbandry. Tenderness, water-holding capacity, and color are the vital but highly variable attributes of beef quality (Modzelewska-Kapituła et al., 2018). In the present study, the meat drip loss, cooking loss, and shear force were significantly lower in the RE group than in the CN group. This finding is consistent with the findings reported by Ding et al. (2020), who showed that tea residues increased moisture content and tenderness in pork. The increase in the L * value in RE group was associated with the high moisture content of beef (Barahona et al., 2016). According to our findings, HTR could improve the meat quality of finishing steers to a certain extent.

Regarding the muscle nutrient composition, the content of C18:1n9c and C18:2n6t was higher, and the ω-6/ω-3 ratio was slightly lower when the steers were fed with HTR. C18:1n9c, a monounsaturated fatty acid, can regulate blood lipids and lowers cholesterol (Sales-Campos et al., 2013). C18:2n6t, a type of *trans*-fatty acid produced by ruminants, has a potential protective effect against the development of coronary heart diseases (Salter, 2013). Moreover, a low ω-6/ω-3 ratio in beef is more beneficial for human health as it decreases the risk of heart disease and cancer (Kang, 2004). Ahmed et al. (2016) reported that Chinese herbal medicine feed additive improved the nutritional value of pork by decreasing the ω-6/ω-3 value. Thus, the findings suggest that the beef of the experimental group is more appropriate for human diet.

In this study, HTR did not affect within-sample diversity (species richness and evenness), however, the composition and structure of the rumen microbial community were influenced by HTR. In steers fed with HTR, the abundance of *Bacteroidetes* markedly decreased, whereas the abundance of *Firmicute*s markedly increased, and it became the most abundant phylum. In humans and mice, an increase in the *Firmicutes*-to-*Bacteroidetes* ratio has been correlated with fat deposition in tissues (Ley et al., 2006; Turnbaugh et al., 2006). In our study, the crude fat content of muscle did show an upward trend with an increasing Firmicutes-to-Bacteroidetes ratio. The abundance of the genera *Veillonellaceae*, *Schwartzia,* and *Olsenella* decreased in the rumen

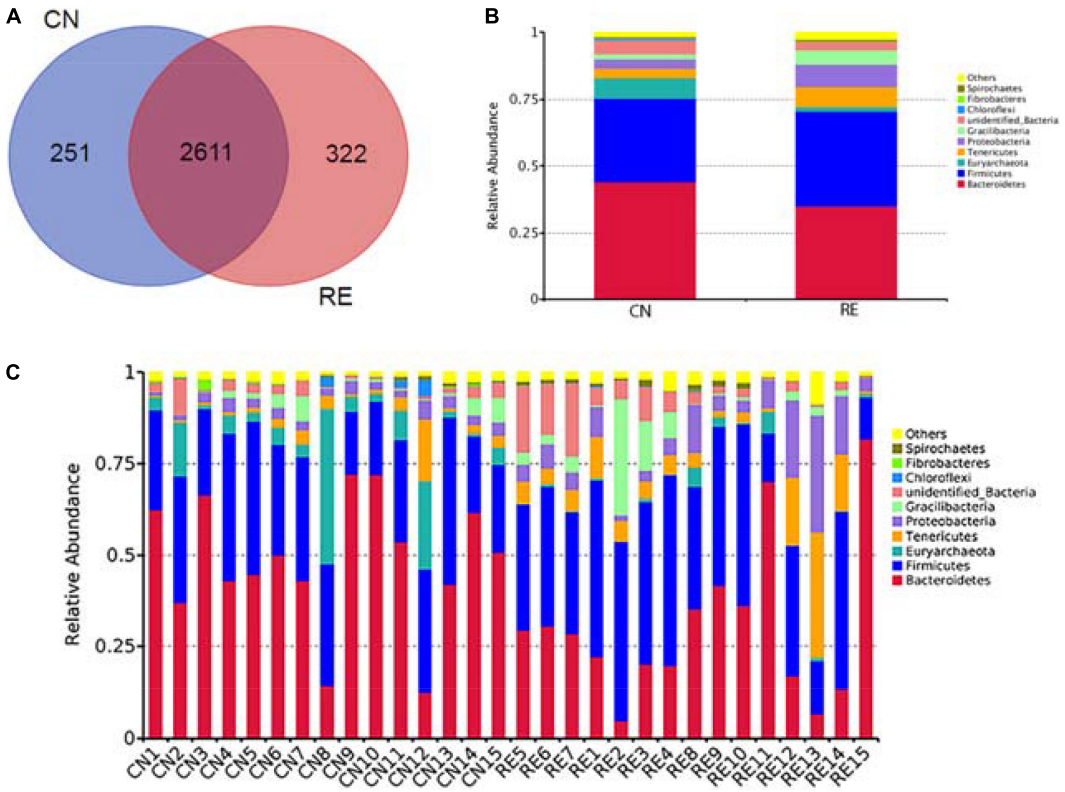


FIGURE 3 | Venn diagram representing the shared and exclusive OTUs at the 97% similarity level between rumen microbiota in the two groups **(A)**. Bar plot shows the relative abundance of rumen microbiota at the phylum level in each group **(B)** and in each sample **(C)**. OTUs, operational taxonomic units; CN, no herbal tea residue (control); RE, 50% HTR replaced *Pennisetum purpureum*.

of Simmental steers fed with HTR. Notably, these genera showed a strong positive correlation with isobutyric acid, valeric acid, and isovaleric acid concentrations. Compared with CN group, isobutyric acid, valeric acid, and isovaleric acid concentrations presented a decreasing trend in the RE group. This finding is consistent with previous findings that these bacteria were positively correlated with rumen VFAs (Kong et al., 2019; Li et al., 2019; Wang et al., 2021). Genus *Marvinbryantia* was also downregulated in the RE group compared with the CN group. The relative abundance of *Marvinbryantia* correlated negatively with the concentrations of propionic acid. Wang et al. (2018) reported that *Marvinbryantia* was an inflammatory bacterium and was negatively correlated with VFA concentration. These findings suggest that HTR reduces the proliferation of inflammatory bacteria. An increase in propionic acid and propionic acid-to-acetic acid ratio was observed in this study, which is often observed as a result of enhanced digestion of fiber and the proliferation of microorganisms in the rumen and may induce changes in metabolic pathways and better rumen fermentation (Christensen et al., 2015). This finding is in agreement with the findings reported by Zhu et al. (2018) and Liang et al. (2019), who suggested that Chinese herbal mixture feed additives improved rumen fermentation, mainly by increasing the concentration of propionic acid and the ratio of propionic acid to acetic acid. Notably, propionate is the main source of glucose for ruminants (den Besten et al., 2013), which may explain the improved carcass characteristics, especially net meat percentage, observed in the RE group in the present study.

Metabolomics analysis showed that the levels of D-glucarate, caprylic acid, lauric acid, DHA, and DPA were higher in the RE group. D-glucarate is oxidized to D-glycerate by glucose oxidase. D-glycerate is a crucial component of pentose phosphate pathway (PPP), which is involved in the first step of glucose

TABLE 7 | Effects of herbal tea residue feed on the alpha diversity indices for bacteria in the ruminal samples of finishing steers.

Items	CN	RE	*P*-value
Observed species	1179.60±50.59	1100.47±56.37	0.305
Shannon	6.81±0.28	6.61±0.28	0.634
Simpson	0.95±0.01	0.95±0.01	0.947
Chao1	1447.77±110.81	1332.69±68.89	0.385
ACE	1426.82±61.86	1353.86±64.65	0.422
Good's coverage	0.99±0.00	0.99±0.00	0.711
PD whole tree	88.93±3.96	87.41±2.90	0.759

The values were calculated as the mean ± standard error of the mean (N = 15). $P < 0.05$ indicated a significant difference between the two groups, and $P > 0.05$ indicated no significant difference between the two groups. CN, no herbal tea residue; RE, 50% HTR replaced Pennisetum purpureum.

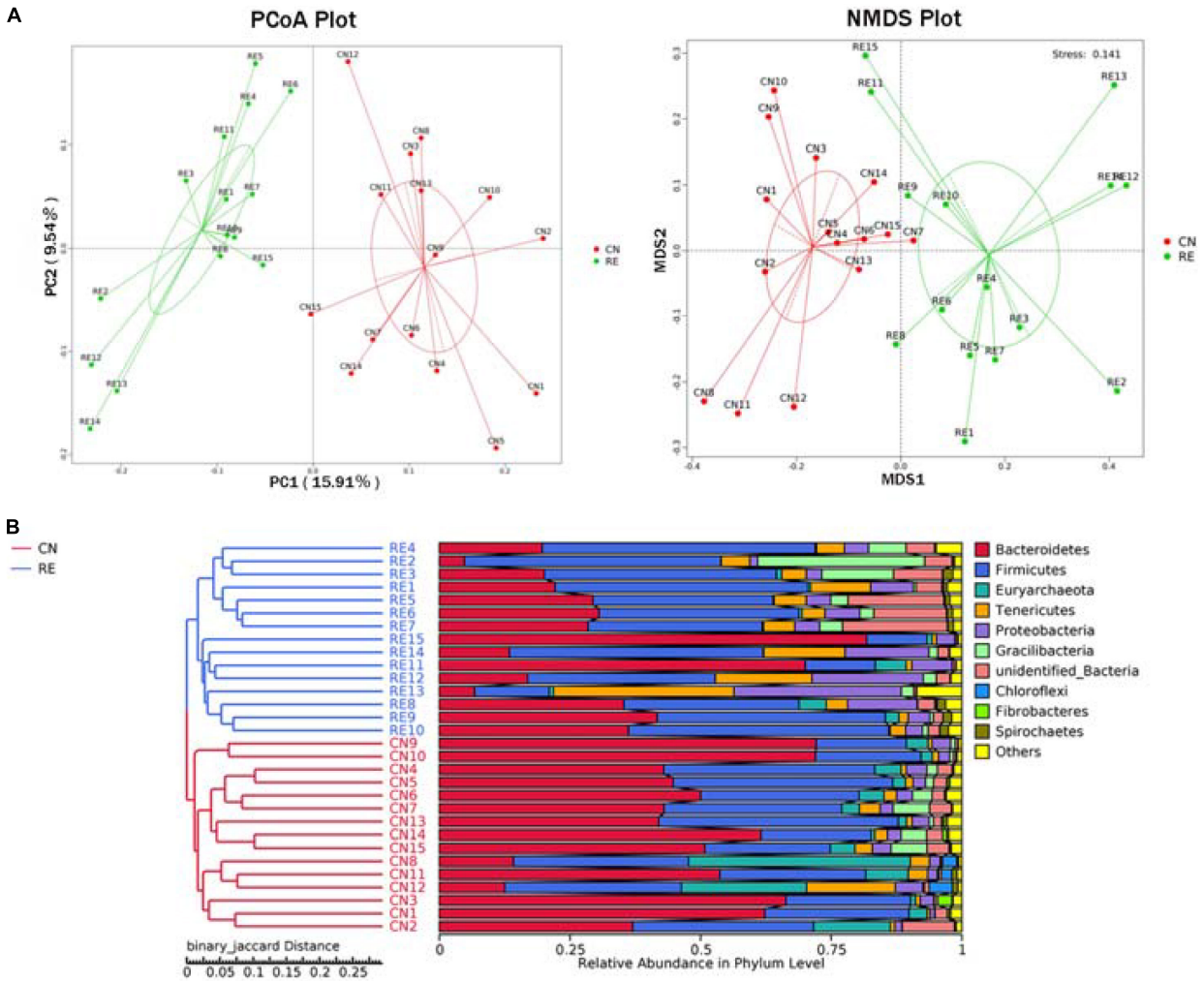


FIGURE 4 | (A) Principal coordinate analysis (PCoA) and non-metric multi-dimensional scaling (NMDS). **(B)** Unweighted pair group method with arithmetic means (UPGMA) cluster tree based on binary Jaccard distance. **(A)** The PCoA was based on the unweighted UniFrac distance, and the NMDS analysis was based on the Bray–Curtis distance. Each point in the figure represents a sample, and the samples in the same group are represented by the same color. **(B)** On the left is the UPGMA cluster tree structure of each sample at the OUT level, and on the right is the relative abundance distribution map of each sample at the phylum level. CN, no herbal tea residues; RE, 50% HTR replaced *Pennisetum purpureum*.

metabolism in the glycolysis branch (Stincone et al., 2015). Medium-chain fatty acids (e.g., caprylic acid and lauric acid) are known to contribute to better beef flavor and odor and improve cholesterol levels (Wilson et al., 2006). DHA and DPA are the most bioactive of ω-3 polyunsaturated fatty acids (PUFAs) and play vital roles in decreasing the hepatic triglyceride content (Pirillo and Catapano, 2013), and PUFAs are known to be beneficial for human health (Russo, 2009). This is consistent with our results of the fatty acid analysis, which showed a reduction in the ω-6: ω-3 ratio. Remarkably, the abundance of *Moraxella* and *Riemerella* increased in the rumen of Simmental steers fed HTR, and the abundance of these bacteria was positively correlated with the levels of D-glucarate, caprylic acid, lauric acid, DHA, and DPA. Currently, there is no information on specific associations between these bacteria and the above muscle metabolites. In addition, the concentration of G6P correlated strongly and positively with the relative abundance of *Succiniclasticum*, which includes starch-degrading bacteria that degrade dietary starch (Fernando et al., 2010; Huws et al., 2016). The abundance of *Anaerovibrio* and *Papillibacter* correlated positively with the concentration of linolenic acid. *Anaerovibrio* participates in the breakdown of fats and sugars (Ouattara et al., 1992; Mannelli et al., 2018). *Papillibacter* belongs to the *Ruminococcaceae* family; members of the *Ruminococcaceae* family are essential for cellulose degradation (Krause et al., 2003). The specific association of *Anaerovibrio* and *Papillibacter* with linolenic acid remains unclear and requires an in-depth investigation in the future. The present findings suggest that HTR improves muscle glucolipid metabolism and rumen fermentation by altering the microbial community composition. However, more systematic studies should be included to reveal the biological associations.

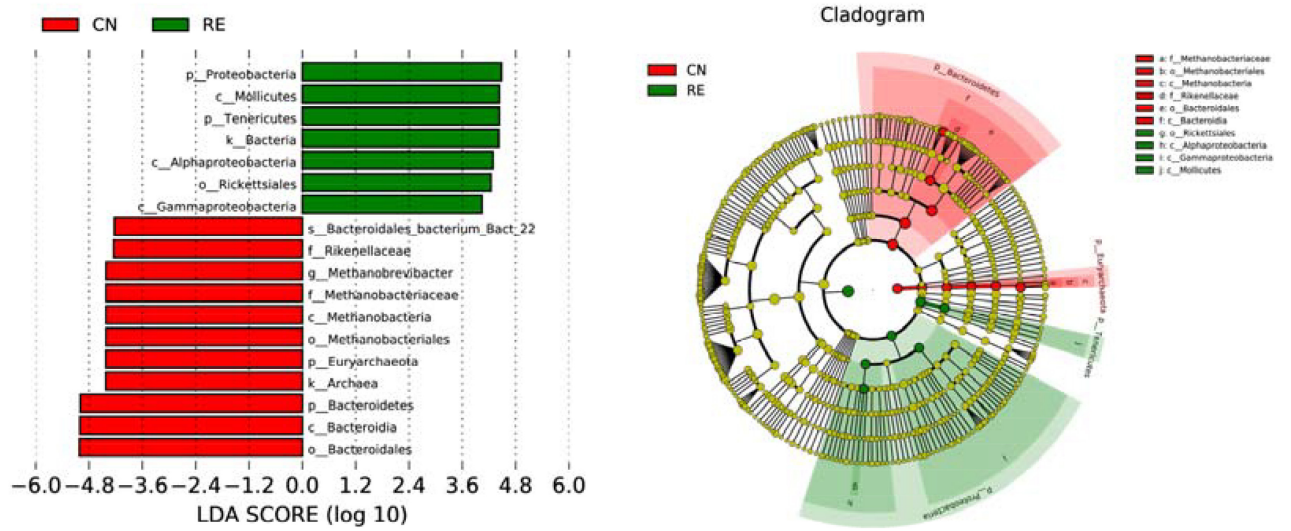


FIGURE 5 | Comparison of the classification of rumen microbiota between two groups by linear discriminant analysis effect size (LefSe) method. The LDA value distribution histogram (left) shows the species with significant differences in abundance in the two groups, and the length of the histogram represents the impact of different species. In the taxonomic cladogram (right), the circles radiating from the inside to the outside represent the classification level from phylum to species. Taxa with enriched levels in CN are shown in red, whereas those with enriched levels in RE are shown in green. The species names indicated by the letters in the picture are shown in the legend on the right.

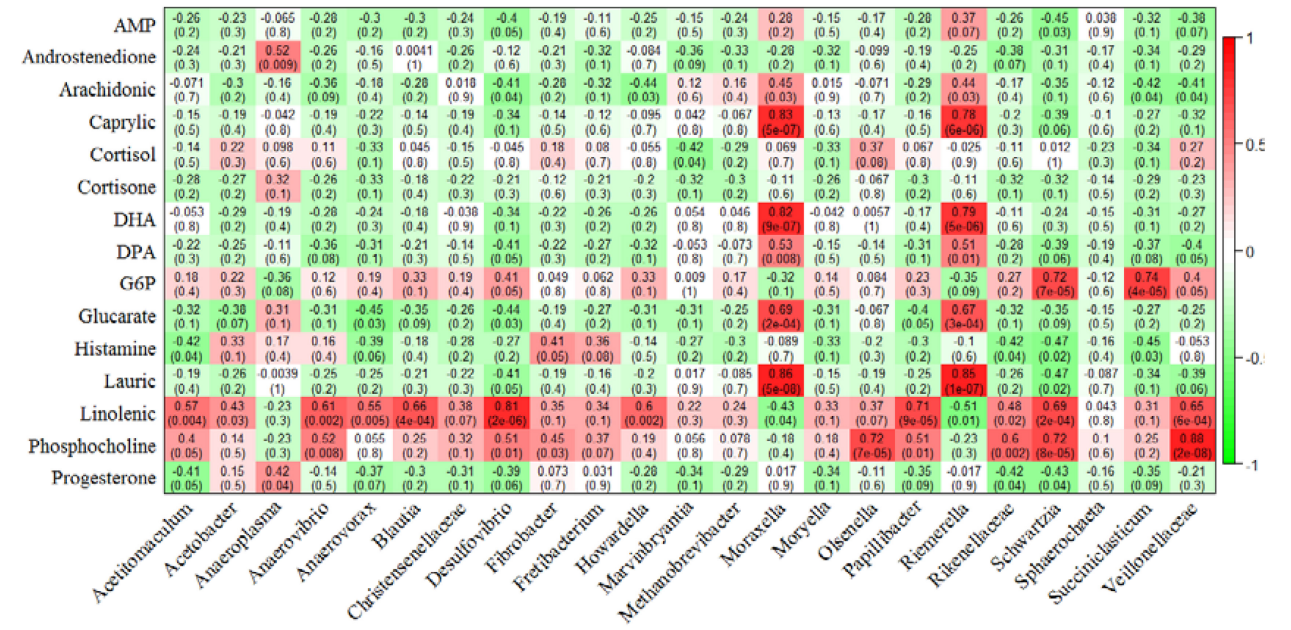


FIGURE 6 | Correlation analysis of rumen microorganisms with beef glycolipid metabolite concentrations. Each cell contains the corresponding correlation and *P*-value. The table is color-coded by correlation according to the color legend. AMP, adenosine monophosphate; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; G6P, glucose-6-phosphate.

CONCLUSION

The present study showed that HTR improved meat quality to a certain extent, influenced the muscle metabolic pathways, and altered the rumen VFA concentration and rumen microbial community composition in Simmental crossbred finishing steers. Moreover, the bacteria were closely associated with muscle glucolipid metabolites and rumen VFA levels of the steers. Our findings suggest that, as a functional roughage, HTR improves the meat quality of steers mainly by altering rumen microbial populations and then affecting rumen fatty acid composition and muscle glucolipid metabolism.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee at South China Agricultural University.

AUTHOR CONTRIBUTIONS

LL: writing-original draft preparation. XS: investigation. JS: data curation and visualization. JL, TC, and QX: supervision. YZ and JS: conceptualization, methodology, writing-reviewing, and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Natural Science Foundation of China Program (Grant Nos. 31802032 and 32072714), the Natural Science Foundation of Guangdong Province (Grant No. 2020A1515010062), the Science and Technology Project of Guangzhou (Grant No. 202002030037), and the Key-Area Research and Development Program of Guangdong Province (2019B110209005).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.821293/full#supplementary-material

Supplementary Figure 1 | Differential metabolites annotation statistics in HMDB **(A)** and LIPID MAPS **(B)** database. The left picture is the positive ion mode, and the right picture is the negative ion mode. The X-axis represents the number of metabolites, and the y-axis represents the term of HMDB or LIPID MAPS.

Supplementary Figure 2 | The PLS-DA scatter plots of each sample in positive (left) and negative (right) modes. The abscissa is the score of the sample on the first principal component; The ordinate is the score of the sample on the second principal component; R2Y represents the explanatory rate of the model, and Q2Y is used to evaluate the predictive ability of the PLS-DA model. When R2Y is greater than Q2Y, the model is well established.

Supplementary Figure 3 | Correlation analysis of rumen microorganisms with rumen volatile fatty acid concentrations. Each cell contains the corresponding correlation and *P*-value. The table is color-coded by correlation according to the color legend.

REFERENCES

Abdallah, A., Zhang, P., Zhong, Q., and Sun, Z. (2019). Application of traditional chinese herbal medicine by-products as dietary feed supplements and antibiotic replacements in animal production. *Curr. Drug Metab.* 20, 54–64. doi: 10.2174/1389200219666180523102920

Ahmed, S. T., Mun, H. S., Islam, M. M., Ko, S. Y., and Yang, C. J. (2016). Effects of dietary natural and fermented herb combination on growth performance, carcass traits and meat quality in grower-finisher pigs. *Meat Sci.* 122, 7–15. doi: 10.1016/j.meatsci.2016.07.016

Ahsan, M. A., Katla, S. K., Islam, M. T., Hernandez-Viezcas, J. A., Martinez, L. M., Díaz-Moreno, C. A., et al. (2018). Adsorptive removal of methylene blue, tetracycline and Cr(VI) from water using sulfonated tea waste. *Environ. Technol. Innov.* 11, 23–40. doi: 10.1016/j.eti.2018.04.003

Association of Official Analytical Chemists [AOAC] (2000). *Association of Official Analytical Chemists*, 17th Edn. Gaithersburg, MD: AOAC.

Barahona, M., Olleta, J., Sañudo, C., Albertí, P., Panea, B., Pérez-Juan, M., et al. (2016). Effects of whole linseed and rumen-protected conjugated linoleic acid enriched diets on beef quality. *Animal* 10, 709–717. doi: 10.1017/s1751731115002591

Black, J., and Foarde, K. (2007). Comparison of four different methods for extraction of *Stachybotrys chartarum* spore DNA and verification by real-time PCR. *J. Microbiol. Methods.* 70, 75–81. doi: 10.1016/j.mimet.2007.03.017

Brscic, M., Prevedello, P., Stefani, A. L., Cozzi, G., and Gottardo, F. (2014). Effects of the provision of solid feeds enriched with protein or nonprotein nitrogen on veal calf growth, welfare, and slaughter performance. *J. Dairy Sci.* 97, 4649–4657. doi: 10.3168/jds.2013-7618

Cao, Y., Ruan, Y., Shen, T., Huang, X., Li, M., Yu, W., et al. (2014). Astragalus polysaccharide suppresses doxorubicin-induced cardiotoxicity by regulating the PI3k/Akt and p38MAPK pathways. *Oxid. Med. Cell. Longev.* 2014:674219. doi: 10.1155/2014/674219

Christensen, R. G., Yang, S. Y., Eun, J. S., Young, A. J., Hall, J. O., and MacAdam, J. W. (2015). Effects of feeding birdsfoot trefoil hay on neutral detergent fiber digestion, nitrogen utilization efficiency, and lactational performance by dairy cows. *J. Dairy Sci.* 98, 7982–7992. doi: 10.3168/jds.2015-9348

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., and Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* 54, 2325–2340. doi: 10.1194/jlr.R036012

Ding, X., Li, H., Wen, Z., Hou, Y., Wang, G., Fan, J., et al. (2020). Effects of fermented tea residue on fattening performance, meat quality, digestive performance, serum antioxidant capacity, and intestinal morphology in fatteners. *Animals* 10:185. doi: 10.3390/ani10020185

Edgar, R. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Fernando, S. C., Purvis, H. T., Najar, F. Z., Sukharnikov, L. O., Krehbiel, C. R., Nagaraja, T. G., et al. (2010). Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl. Environ. Microbiol.* 76, 7482–7490. doi: 10.1128/AEM.00388-10

Gilbreath, K., Nawaratna, G., Wickersham, T., Satterfield, M., Bazer, F., and Wu, G. (2020). Metabolic studies reveal that ruminal microbes of adult steers do not degrade rumen-protected or unprotected L-citrulline. *J. Anim. Sci.* 98, skz370. doi: 10.1093/jas/skz370

Haas, B., Gevers, D., Earl, A., Feldgarden, M., Ward, D., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21, 494–504. doi: 10.1101/gr.112730.110

Huws, S. A., Edwards, J. E., Creevey, C. J., Rees Stevens, P., Lin, W., Girdwood, S. E., et al. (2016). Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS Microbiol. Ecol.* 92:fiv137. doi: 10.1093/femsec/fiv137

Iqbal Khan, M. A., Ueno, K., Horimoto, S., Komai, F., Tanaka, K., and Ono, Y. (2015). Evaluation of the physio-chemical and microbial properties of green tea waste-rice bran compost and the effect of the compost on spinach production. *Plant Prod. Sci.* 10, 391–399. doi: 10.1626/pps.10.391

Jin-Woo, H., Sun, H. C., Yon-Suk, K., Jae-Woong, L., Bo-Im, Y., Sang-Ho, M., et al. (2017). Effects of dietary supplementation of oriental herbal medicine residue and methyl sulfonyl methane on the growth performance and meat quality of ducks. *Anim. Prod. Sci.* 57, 948–957.

Kang, J. X. (2004). Achieving balance in the omega-6/omega-3 ratio through nutrigenomics. *World Rev. Nutr. Diet.* 93, 92–98. doi: 10.1159/000081253

Kim, Y., Lee, G., and Choi, I. (2014). Effects of dietary supplementation of red ginseng marc and α-tocopherol on the growth performance and meat quality of broiler chicken. *J. Sci. Food Agric.* 94, 1816–1821. doi: 10.1002/jsfa.6497

Kong, C., Gao, R., Yan, X., Huang, L., and Qin, H. (2019). Probiotics improve gut microbiota dysbiosis in obese mice fed a high-fat or high-sucrose diet. *Nutrition* 60, 175–184. doi: 10.1016/j.nut.2018.10.002

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

Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 9:559. doi: 10.1186/1471-2105-9-559

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology: human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Li, D., Zheng, X., Duan, L., Deng, S., Ye, W., Wang, A., et al. (2017). Ethnobotanical survey of herbal tea plants from the traditional markets in Chaoshan. *China. J. Ethnopharmacol.* 205, 195–206. doi: 10.1016/j.jep.2017.02.040

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., et al. (2019). 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

Liang, X., Bi, X., Kamruzzaman, M., and Sano, H. (2019). Effect of Chinese herbal medicine on kinetics of plasma phenylalanine, tyrosine and whole body protein synthesis in sheep. *Anim. Sci. J.* 90, 533–538. doi: 10.1111/asj.13180

Liang, X., Yamazaki, K., Kamruzzaman, M., Bi, X., Panthee, A., and Sano, H. (2013). Effects of Chinese herbal medicine on plasma glucose, protein and energy metabolism in sheep. *J. Anim. Sci. Biotechnol.* 4:51. doi: 10.1186/2049-1891-4-51

Liu, H. W., Tong, J. M., and Zhou, D. W. (2011). Utilization of chinese herbal feed additives in animal production. *Agric. Sci. China* 10, 1262–1272. doi: 10.1016/s1671-2927(11)60118-1

Malkoc, E., and Nuhoglu, Y. (2006). Removal of Ni(II) ions from aqueous solutions using waste of tea factory: adsorption on a fixed-bed column. *J. Hazard. Mater.* 135, 328–336. doi: 10.1016/j.jhazmat.2005.11.070

Mannelli, F., Cappucci, A., Pini, F., Pastorelli, R., Decorosi, F., Giovannetti, L., et al. (2018). Effect of different types of olive oil pomace dietary supplementation on the rumen microbial community profile in Comisana ewes. *Sci. Rep.* 8:8455. doi: 10.1038/s41598-018-26713-w

Modzelewska-Kapituła, M., Tkacz, K., Nogalski, Z., Karpińska-Tymoszczyk, M., Draszanowska, A., Pietrzak-Fiećko, R., et al. (2018). Addition of herbal extracts to the Holstein-Friesian bulls' diet changes the quality of beef. *Meat Sci.* 145, 163–170. doi: 10.1016/j.meatsci.2018.06.033

O'Fallon, J., Busboom, J., Nelson, M., and Gaskins, C. (2007). A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. *J. Anim. Sci.* 85, 1511–1521. doi: 10.2527/jas.2006-491

Ouattara, A., Traore, A., and Garcia, J. (1992). Characterization of Anaerovibrio burkinabensis sp. nov a lactate fermenting bacterium isolated from rice field soils. *Int. J. Syst. Bacteriol.* 42, 390–397.

Pirillo, A., and Catapano, A. L. (2013). Omega-3 polyunsaturated fatty acids in the treatment of hypertriglyceridaemia. *Int. J. Cardiol.* 170, S16–S20. doi: 10.1016/j.ijcard.2013.06.040

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Razminowicz, R., Kreuzer, M., and Scheeder, M. (2006). Quality of retail beef from two grass-based production systems in comparison with conventional beef. *Meat Sci.* 73, 351–361. doi: 10.1016/j.meatsci

Russo, G. L. (2009). Dietary n-6 and n-3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention. *Biochem. Pharmacol.* 77, 937–946. doi: 10.1016/j.bcp.2008.10.020

Sales, F., Bravo-Lamas, L., Realini, C., Lira, R., Aldai, N., and Morales, R. (2020). Grain supplementation of calves as an alternative beef production system to pasture-finished steers in Chilean Patagonia: meat quality and fatty acid composition. *Transl. Anim. Sci.* 4, 352–362. doi: 10.1093/tas/txz188

Sales-Campos, H., Souza, P. R. D., Peghini, B. C., da Silva, J. S., and Cardoso, C. R. (2013). An overview of the modulatory effects of oleic acid in health and disease. *Mini Rev. Med. Chem.* 13, 201–210. doi: 10.2174/1389557511313020003

Salter, A. M. (2013). Dietary fatty acids and cardiovascular disease. *Animal* 7, 163–171. doi: 10.1017/S1751731111002023

Shan, C., Guo, J., Sun, X., Li, N., Yang, X., Gao, Y., et al. (2018). Effects of fermented Chinese herbal medicines on milk performance and immune function in late-lactation cows under heat stress conditions. *J. Anim. Sci.* 96, 4444–4457. doi: 10.1093/jas/sky270

Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M. C., Campbell, K., Cheung, E., et al. (2015). The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biol. Rev. Camb. Philos. Soc.* 90, 927–963. doi: 10.1111/brv.12140

Suthar, S., and Singh, D. (2011). Phytotoxicity of composted herbal pharmaceutical industry wastes. *Environ. Sci. Pollut. Res. Int.* 19, 3054–3059. doi: 10.1007/s11356-012-0985-yv

Turnbaugh, P., Ley, R., Mahowald, M., Magrini, V., Mardis, E., and Gordon, J. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Wang, B., Wang, Y., Zuo, S., Peng, S., Wang, Z., Zhang, Y., et al. (2021). Untargeted and Targeted Metabolomics Profiling of Muscle Reveals Enhanced Meat Quality in Artificial Pasture Grazing Tan Lambs via Rescheduling the Rumen Bacterial Community. *J. Agric. Food Chem.* 69, 846–858. doi: 10.1021/acs.jafc.0c06427

Wang, H., Ji, Y., Yin, C., Deng, M., Tang, T., Deng, B., et al. (2018). Differential analysis of gut microbiota correlated with oxidative stress in sows with high or low litter performance during lactation. *Front. Microbiol.* 9:1665. doi: 10.3389/fmicb.2018.01665

Wilson, T. A., Kritchevsky, D., Kotyla, T., and Nicolosi, R. J. (2006). Structured triglycerides containing caprylic (8:0) and oleic (18:1) fatty acids reduce blood cholesterol concentrations and aortic cholesterol accumulation in hamsters. *Biochim. Biophys. Acta* 1761, 345–349. doi: 10.1016/j.bbalip

Xie, Y., Chen, Z., Wang, D., Chen, G., Sun, X., He, Q., et al. (2020). Effects of Fermented Herbal Tea Residues on the Intestinal Microbiota Characteristics of Holstein Heifers Under Heat Stress. *Front. Microbiol.* 11:1014. doi: 10.3389/fmicb.2020.01014

Yan, J., Liu, P., Xu, L., Huan, H., Zhou, W., Xu, X., et al. (2018). Effects of exogenous inosine monophosphate on growth performance, flavor compounds, enzyme activity, and gene expression of muscle tissues in chicken. *Poult. Sci.* 97, 1229–1237. doi: 10.3382/ps/pex415

Yang, X., and Cui, X. (2013). Adsorption characteristics of Pb (II) on alkali treated tea residue. *Water Resour. Ind.* 3, 1–10. doi: 10.1016/j.wri.2013.05.003

Yuan, M., Li, D., Zhang, Z., Sun, H., An, M., and Wang, G. (2018). Endometriosis induces gut microbiota alterations in mice. *Hum. Reprod.* 33, 607–616. doi: 10.1093/humrep/dex372

Zhao, J., Deng, J., Chen, Y., and Li, S. (2013). Advanced phytochemical analysis of herbal tea in China. *J. Chromatogr.* 1313, 2–23. doi: 10.1016/j.chroma.2013.07.039

Zhu, Z., Song, Z. H., Cao, L. T., Wang, Y., Zhou, W. Z., Zhou, P., et al. (2018). Effects of traditional Chinese medicine formula on ruminal fermentation, enzyme activities and nutrient digestibility of beef cattle. *Anim. Sci. J.* 89, 661–671. doi: 10.1111/asj.12978

Zhuang, X., Chen, Z., Sun, X., Li, F., Luo, J., Chen, T., et al. (2021). Fermentation quality of herbal tea residue and its application in fattening cattle under heat stress. *BMC Vet. Res.* 17:348. doi: 10.1186/s12917-021-03061-y

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 February 2022
doi: 10.3389/fmicb.2021.769438

Performance, Rumen Microbial Community and Immune Status of Goat Kids Fed *Leucaena leucocephala* Post-weaning as Affected by Prenatal and Early Life Nutritional Interventions

*Einar Artiles-Ortega[1,2], Orelvis Portal[3,4], Jeyamalar Jeyanathan[1], Beydis Reguera-Barreto[2], Pedro Yoelvys de la Fé-Rodríguez[2], Raciel Lima-Orozco[2,4] and Veerle Fievez[1]**

[1] *Laboratory for Animal Nutrition and Animal Product Quality, Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium,* [2] *Departamento de Medicina Veterinaria y Zootecnia, Facultad de Ciencias Agropecuarias, Universidad Central "Marta Abreu" de Las Villas, Santa Clara, Cuba,* [3] *Departamento de Biología, Facultad de Ciencias Agropecuarias, Universidad Central "Marta Abreu" de Las Villas, Santa Clara, Cuba,* [4] *Centro de Investigaciones Agropecuarias, Facultad de Ciencias Agropecuarias, Universidad Central "Marta Abreu" de Las Villas, Santa Clara, Cuba*

OPEN ACCESS

Edited by:
Robert W. Li, Agricultural Research Service, United States Department of Agriculture (USDA), United States

Reviewed by:
Juan J. Loor, University of Illinois Urbana-Champaign, United States
Neeta Agarwal, Indian Veterinary Research Institute (IVRI), India

***Correspondence:**
Veerle Fievez
Veerle.Fievez@UGent.be

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *02 September 2021*
Accepted: *28 December 2021*
Published: *16 February 2022*

Citation:
Artiles-Ortega E, Portal O, Jeyanathan J, Reguera-Barreto B, de la Fé-Rodríguez PY, Lima-Orozco R and Fievez V (2022) Performance, Rumen Microbial Community and Immune Status of Goat Kids Fed Leucaena leucocephala Post-weaning as Affected by Prenatal and Early Life Nutritional Interventions. Front. Microbiol. 12:769438. doi: 10.3389/fmicb.2021.769438

Leucaena leucocephala represents a local protein source in tropical ruminant diets. However, its full exploitation is impaired by mimosine, unless it is degraded by the rumen microbial community. Recently, the ruminal bacterial communities of newborns were persistently modified through prenatal or postnatal dietary interventions. Such early-life interventions might enhance adaptation of ruminants to *Leucaena leucocephala*, which was investigated using a 2 × 2 factorial design trial that tested both supplementation of *L. leucocephala* in the late pregnancy diet of goat does, and supplementation of live yeast to their newborns. The composition of ruminal bacteria, immune status, as well as organic matter digestibility (OMD) and performance of kids were studied during and after the intervention. Ten pregnant goats were divided into two groups: the D+ and D– groups, which either received or did not receive 30 g of *L. leucocephala* forage meal during the last 7 ± 0.5 weeks of gestation. Twins from each goat were divided into the K+ and K– group (supplemented with or without 0.2 g/d of live yeast from day 3 until weaning at 8 weeks). Rumen samples were collected from 4-, 8-, 14-, and 20-weeks old kids to assess the bacterial community, while immune parameters (white blood cells, immunoglobulin M and G, and chitotriosidase activity) were measured in blood and saliva sampled at 4-, 8-, and 20-weeks. We found a stimulatory effect of the prenatal exposure on the post-weaning dry matter intake of the *L. leucocephala* supplemented diet, resulting in a higher daily gain and final body weight at 20 weeks in the D+ *vs*. D– group (406 *vs*. 370 g DM/d, 85.4 vs. 78.6 g/d, and 15.2 vs. 13.8 kg, respectively). Moreover, *Ruminococcus* represented

a greater proportion of the rumen bacterial community of the D+ vs. D– kids (5.1 vs. 1.6%). Differences in the immune status were relatively small and not thought to be a driving factor of differences in animal performance. Furthermore, postnatal supplementation of live yeast favored maturation of the rumen bacterial community (i.e., greater abundance of Bacteroidetes, in particular *Prevotella*, and reduced abundance of Firmicutes) and protozoa colonization. Concomitantly, OMD was enhanced post-weaning, suggesting effects of the early-life intervention persisted and could have affected animal performance.

Keywords: goat kids, early life intervention, bacterial community, immune status, performance, digestibility

INTRODUCTION

Due to the increasing demand for animal-derived food and the restricted availability of good quality forage and concentrates, there is a need to find cheap and readily available alternative feed sources to support livestock production in tropical countries (Kim et al., 2019). Protein-rich leaves from legume trees are such feed resources (Aye and Adegun, 2013) with *Leucaena leucocephala* as one of the highly productive, palatable, and most widely used legumes in tropical agropastoral systems (Vega et al., 2016; Ahmed et al., 2018). Nevertheless, *L. leucocephala* contains toxic secondary metabolites, i.e., L-mimosine [(*S*)-α-Amino-β-[1-(3-hydroxy-4-oxopyridine)] propionic acid] and its digestive intermediates (isomers of hydroxypyridone; 2,3 and 3,4-DHP). The toxicity of these plant secondary metabolites could be alleviated through degradation by rumen microbes (Akingbade et al., 2001; Angarita et al., 2015). Indeed, in several studies, performed in tropical countries, the rumen microbial community of ruminants fed *L. leucocephala* contained some DHP degrading bacteria such as *Synergistes jonesii* (Allison et al., 1990), *Streptococcus lutetiensis*, *Clostridium butyricum,* and *Lactobacillus vitulinus* (Dominguez-Bello and Stewart, 1991; Derakhshani et al., 2016). Recently, own research (unpublished data) also showed that *in vitro* degradation of *L. leucocephala* forage meal as well as L-mimosine itself was highly influenced by the origin of the inoculum and was more extensive with inoculum from Cuban goats compared to Belgian sheep.

The transinoculation of a DHP-degrading rumen inoculum from *Leucaena*-adapted animals to non-adapted animals contributed to the detoxification of L-mimosine and its intermediates in non-adapted animals (Jones and Megarrity, 1986; Akingbade et al., 2001). However, in other research transinoculation did not show any effect on the detoxification of L-mimosine (Vaithiyanathan et al., 2005) or the detoxification effect was lost after a relatively short period with *L. leucocephala*-free diets (Graham et al., 2013). This may be due to the fact that the rumen microbiome of adult ruminants is difficult to manipulate due to the resistance of the indigenous microflora against the colonization of foreign bacterial strains (Weimer et al., 2010). In contrast, the proliferating microbial community in the rumen of young ruminants seems more moldable and several studies showed short and medium-term persistency of early life microbial manipulation (Yanez-Ruiz et al., 2010; Belanche et al., 2020). In this respect, active yeasts, used as feed additives in young ruminants, improved rumen microbial activity and particularly stimulated the growth and activity of fiber-degrading bacteria (Chaucheyras-Durand et al., 2008). Indeed, yeast supplementation as probiotic was shown to create favorable conditions allowing the earlier establishment of cellulolytic bacteria and ciliate protozoa in the rumen of newborn lambs (Chaucheyras-Durand and Fonty, 2002).

Moreover, the early-life interest has been extended to prenatal modulation of the microbial community through maternal feeding during the gestation period (Faubladier et al., 2013; Codagnone et al., 2019; Xiong et al., 2019). In ruminants, only a few studies investigated the effect of a prenatal treatment on the developing microbiome of the offspring (De Barbieri et al., 2015a,b). Nevertheless in these studies the offspring was raised with their mother during the lactation period, which compromised the separation of the prenatal effects from the mother's influence during lactation. In addition, enhanced post-weaning intake of *Chromolaena odorata* by goat kids has been related to an *in utero* exposure period with maternal ingestion of *C. odorata* (Hai et al., 2013).

In the current study, we hypothesized that the use of *L. leucocephala*, fed after weaning, would be optimized by either or both a prenatal and postnatal treatment. The prenatal treatment included the dietary supply of *L. leucocephala* to mother goats during late pregnancy whereas live yeast supplementation until weaning was tested as postnatal treatment. The objective of this study was to assess their effects on feed intake and growth of the kids as well as on the composition of the ruminal bacterial community and their immune status during their development from 4 to 20 weeks. We also determined apparent digestibility at the age of 20 weeks.

MATERIALS AND METHODS

The experiment was in accordance with the recommendations of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (approval number EC2015/12) for a similar experiment conducted at the Laboratory of Animal Nutrition and Animal Product Quality of Ghent University. The current experiment was conducted in the Laboratory of Animal Nutrition and the Veterinary Clinic, Facultad de Ciencias

Agropecuarias at Universidad Central "Marta Abreu" de Las Villas (UCLV), Cuba.

Animals, Treatments and Experimental Design

Prenatal Treatment

Ten pregnant goats (35 ± 5 kg BW) with twin pregnancies were selected from a commercial farm (total of 60 goats), where does conceived by natural mating. Twin pregnancies were identified ultrasonically 2 months after mating. The ten goats were randomly allocated to two experimental groups (D+: *L. leucocephala* [LL] supplemented group, and D–: Control group) during the last 7 ± 0.5 weeks of gestation (**Figure 1**). Each group was housed in separate stables with individual pens (1.0 × 1.5 m; width × length with *ad libitum* access to water). Despite their individual housing, goats had visual contact. Feed was offered twice a day, with diets consisting of 2.0 kg DM/head/day of star grass (*Cynodon nlemfuensis*) hay, and 0.369 ± 0.03 kg DM/head/day of concentrate (**Supplementary Table S1**). The forage meal of *L. leucocephala* was offered only to the D+ group.

The *L. leucocephala* cv. Cunningham used to prepare the forage meal was grown on the dairy farm "Tres Caminos" (22°25′22.9″ N, 80°03′13″ W) at the "Desembarco del Granma" cooperative, located in the Santa Clara municipality, central region of Cuba. The average precipitation, temperature and relative humidity during the cropping period were 47.5 ± 42.13 mm, 26.3 ± 2.02°C and 82.5 ± 4.19%, respectively. The legume forage was harvested freshly from young branches around noon, at the end of the rainy season (between the 3rd and 27th October 2018). The material selected for this research (a pool of leaves without branches) was dried in a furnace at 65°C for three days, then chopped to a particle size of 0.1 cm and stored in a dry area until use.

Postnatal Treatment

After birth, kids (twins) were separated from their mother immediately after the first intake of colostrum (first day), and were divided into two groups which were provided with milk replacer (125 g/L, Kalvowin, Polmass S.A., Poland [**Supplementary Table S2**]) twice a day until weaning (8 weeks of age). All kids had *ad libitum* access to water. One group of kids received 0.2 g of yeast (Yea Sac®I-1026 [2 × 10^9 CFU/g live *Saccharomyces cerevisiae*], Alltech, Deinze, Belgium) per head from day 3 until weaning [K+] and the other group did not receive yeast [K–]. This resulted in four experimental groups: D+K+, D+K–, D–K+ and D–K– (n = 5) as illustrated in **Figure 1**. The determination of the number of goat kids required for this experiment was based on former experiments studying persistency of early life treatments and a sample size per treatment of 4 (e.g., Wang et al., 2017), 5 (e.g., Hai et al., 2013; Wang X. et al., 2019), 8 (e.g., Abecia et al., 2018; Zhang et al., 2019), up to 10 animals (e.g., Debruyne et al., 2018). To determine the minimum sample size, a power analysis was performed using GPower version 3.1.9.7 (**Supplementary Table S3**). Finally, a sample size of 4–7 per treatment group was calculated to be required for a power of 80% and a type I error α of 0.05. The yeast product (small dry pellets exclusively containing yeast cells) was suspended in 10 mL of saline solution (LABIOFAM SA, Cuba) and was introduced to the rumen of the K+ kids daily before

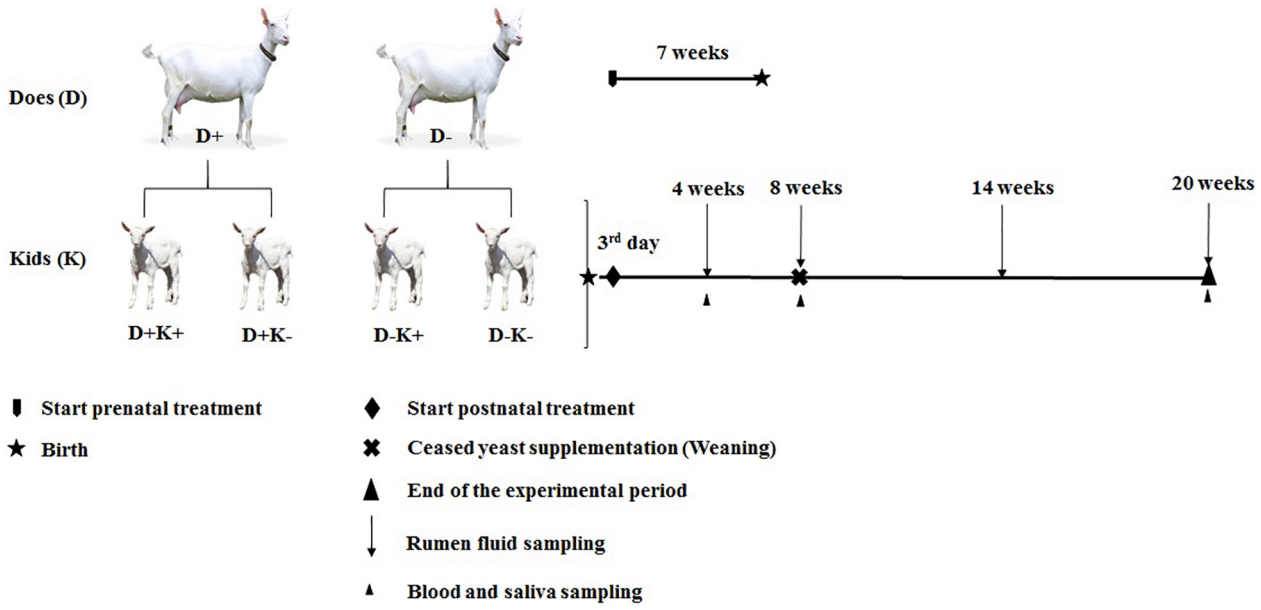


FIGURE 1 | Experimental design and sampling and data collection schedule of goat kids. D+, *L. leucocephala* [LL] supplemented group of does; D–, control group of does; K+, kids treated with yeast; K–, untreated kids. Four experimental groups; D+K+, yeast supplemented kids from LL supplemented group of does; D–K+, yeast supplemented kids from Control group of does; D+K–, non-supplemented kids from LL supplemented group of does; and D–K–, non supplemented kids from Control group of does.

milk feeding using an esophageal tube connected to a syringe (Chaucheyras-Durand and Fonty, 2002). One kid from each of the D–K+ and D–K– groups died during the first 4 weeks of life (esophageal groove dysfunction and ruminal bloat). These deaths were not related to the experimental conditions or treatments. Furthermore, data from one kid of the D–K– group at 4-weeks of age were removed due to clinical signs of digestive disorders around the time of sampling. These problems lasted shortly and the kid recovered well afterward. Accordingly, data of the later time points were maintained in the dataset.

From day 22 until weaning (8 weeks), kids were offered star grass hay *ad libitum* and a Lacto pre-starter (50–200 g/kid; RALTEC TC-01, Serveram S.L., Barcelona, Spain [**Supplementary Table S2**]). After weaning, all kids received star grass hay [2/3 of the offered dietary DM] and forage meal of *L. leucocephala* [supplying 30% of the CP requirements] mixed with commercial concentrate. Feed was provided twice a day (at 8:00 and 17:00) according to the kids' nutritive requirements (Kearl, 1982). Adjustments were made biweekly, after determining body weight before the morning feeding. Feed refusals was collected just prior to the distribution of the next feeding. The refused feed were collected and dried in an oven at 65°C for 72 h, pooled by animal/day, ground and sieved through a 1 mm screen, and stored until analysis. Average daily gain was calculated by the difference between the final and initial body weight divided by the number of days in the corresponding period. Dry matter intake (DMI) was measured daily by weighing the feed offered and refused, and the feed conversion efficiency (FCE) was calculated by the feed intake divided by the body weight gain in the same period.

Sampling of Ruminal Content, Saliva, Blood, and Colostrum

Ruminal contents were sampled at 4, 8, 14, and 20 weeks of age before the morning feeding, using an esophageal tube. The collected rumen fluid (2 mL) was immediately snap frozen in liquid nitrogen and stored at −80°C for microbial analysis. Saliva and blood were sampled before the morning feeding at 4, 8, and 20 weeks of age (**Figure 1**). Saliva was collected immediately before blood sampling by using a forcep and a small piece of absorbing sponge. The sponge was held into the kids' mouth for 1 min to stimulate saliva production. Saliva was removed from the sponge by squeezing into a 10 mL pipet tip placed into the collection tube, and aliquots of approximately 100 μL were stored at −20°C. Blood samples of does (at kidding) and their kids (at 4, 8, and 20 weeks of age) were taken from jugular vein puncture in 20 mL assay tubes with and without anticoagulant (10% EDTA; 100 μL/mL of blood; Sigma-Aldrich, Diegem, Belgium). White blood cells (WBC) were counted using the Neubauer chamber under a binocular microscope (NOVEL, NOV-XSZ-107T, Beijing, China). Afterward, the leftover blood was centrifuged at 1500 × *g* for 5 min at room temperature to obtain plasma. Blood samples without anticoagulant (10 mL) were stored overnight at 4°C and centrifuged at 1500 × *g* for 10 min at room temperature to obtain serum. Serum aliquots of 2 mL were stored at −20°C. Colostrum (20 mL) was collected during the first hour after delivery and stored at −20°C. Due to the high viscosity of colostrum, removal of casein was required prior to the immunoglobulin determination by ELISA (Alves et al., 2015). For this, frozen samples of colostrum were thawed slowly in an ice bath and centrifuged at 490 × *g* for 1 h at 4°C to precipitate casein. The supernatant was diluted in a 0.15 M NaCl solution (pH 4.6) up to its original volume and kept overnight at 4°C. Then, the samples were centrifuged at 11,000 × *g* for 15 min at 4°C, and the supernatant (containing antibodies) was aliquoted and stored at −20°C in 2 mL polyethylene tubes.

Bacterial Community Analyses

DNA Extraction

Total genomic DNA was extracted by repeated bead beating (Mini-Bead-beater 8, BioSpec Inc., Bartlesville, United Kingdom) plus column purification method (Yu and Morrison, 2004). The yield and quality of extracted DNA was determined using a NanoDrop spectrophotometer (VWR International BVBA, Leuven, Belgium). Extracted DNA was used for bacterial 16S rRNA gene amplicon sequencing and quantitative real time PCR (qPCR).

For amplicon sequencing, extracted gDNA was submitted to Macrogen Sequencing Service (Macrogen, Seoul, South Korea) for library preparation and bacterial 16S rRNA gene amplicon sequencing (V3–V4 region, primers: 344F and 806R; Klindworth et al., 2013). Preparation of the amplicons barcoded library was based on the Illumina 16S metagenomic sequencing library preparation protocol[1] and the sequencing was performed using Illumina MiSeq V3-technology (2 × 300 bp).

Bacterial 16S rRNA Gene Amplicon Sequencing: Pipeline and Data Treatment

The amplicon sequencing dataset was demultiplexed and barcodes were clipped off by the sequence provider. Reads were processed and analyzed using the Quantitative Insights into Microbial Ecology (QIIME1) bioinformatics pipeline version 1.9.1 (Caporaso et al., 2010b). Forward and reverse reads were merged using the fastq-join method (Aronesty, 2011), after which primer removal and quality filtering was performed using QIIME1. The subsequent analysis, picking Operational Taxonomic Units (OTU), assigning taxonomy, inferring phylogeny and creating OTU tables, were also performed by QIIME1. The sequences were clustered into OTU using the open-reference OTU picking workflow with a 97% similarity threshold using UCLUST, and chimeras were removed using UCHIME (Edgar, 2010). Representative OTU sequences were aligned to the Greengenes 97% core OTU set (v13_8; DeSantis et al., 2006), with a minimum percent identity of 97% using the PyNast algorithm (Caporaso et al., 2010a) with QIIME1 default parameters. Quality filtering resulted in an average 66 699 ± 11 067 reads per sample. Rarefaction analyses were performed using QIIME1, which indicated that the sequencing depth is enough to analyze the bacterial communities in all samples (data not shown). Both alpha diversity (Chao 1, PD whole tree, observed OTU, Shannon index, and dominance) and beta diversity (based on Bray–Curtis dissimilarity; Bray

[1] https://support.illumina.com

and Curtis, 1957) indices were determined using QIIME1. The principal coordinate (PCoA) plots were generated from Bray Curtis dissimilarity matrices, and the non-parametric permutational MANOVA-based statistical test ANOSIM and ADONIS were used to determine differences in overall microbial community between treatments. To analyze the differences in taxa abundance between treatments, a first screening was performed using Kruskal–Wallis test in QIIME1 (Caporaso et al., 2010b). Taxa that showed differences between treatment were further analyzed using a non-parametric factorial ANOVA approach using art function in ARTool R package version 0.10.7 (Wobbrock et al., 2011). Core successional microbes were generated using the QIIME software package with a script core_diversity_analyses.py. Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) database under accession number PRJNA757729.

Quantification of Major Microbial Groups (Quantitative Real Time PCR)

The abundance of the 16S rRNA gene of total bacteria, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, and *Synergistes jonesii*, 5.8S rRNA gene of anaerobic fungi (*Neocallimastigales*), and 18S rRNA gene of protozoa were quantified by qPCR. The primers used for the qPCR are given in **Supplementary Table S4**.

The qPCR reactions were assayed in a 12.5 μL reaction mixture containing 6.25 μL of Maxima®SYBR Green/ROX qPCR Master Mix (2X) (ThermoFischer Scientific, Waltham, MA, United States), 1 μL of primer mixture containing 0.5 μM of each primer, DNA (20 ng) and molecular water. Amplification of each target group was carried out in a two-step cycling protocol (StepOne^{TM} Real Time PCR System, Applied Biosystems, CA, United States) with the following program: initial denaturation at 95°C for 10 min, 35 cycles at 95°C for 15 s (denaturation), 60°C for 1 min (annealing/extension). The melting curve was built by measuring the fluorescence emissions with increased temperature from 60 to 95°C with ramps of 0.5°C every 15 s. Duplicate qPCR quantification was performed on 20 ng of extracted DNA. A plasmid containing a single copy of the targeted gene of each microorganism was used as qPCR standards for each target. The copy numbers in the standards were calculated based on the DNA concentrations determined by the NanoDrop. External standards were prepared and used in every qPCR run to enumerate the gene copies in the samples. The absolute quantity of each group of microorganisms was calculated using the respective standards and expressed as corresponding gene copies/mL of sample (Staroscik, 2004).

Colostrum, and Plasma Chitotriosidase Activity

Colostrum and plasma chitotriosidase (ChT) activity was measured as described by Arguello et al. (2008). Briefly, 1 μL of sample (either colostrum or plasma) was incubated with 100 μL of 22 mM artificial ChT substrate (4-methylumbelliferyl-D-*N*, *N*′, *N*″ triacetylchitotriose, Sigma-Aldrich) in 0.5 M citrate phosphate buffer (pH 5.2) for 15 min at 37°C. The reaction was stopped by 5 mL of 0.5 M Na_2CO_3-$NaHCO_3$ buffer (pH 10.7). Fluorescence was measured at 365 nm excitation and 450 nm emission (Fluorimeter Infinite 200, Tecan, Switzerland). The ChT activity was quantified as nanomoles of substrate hydrolyzed per milliliter (U/mL) (Moreno-Indias et al., 2012). Each sample was tested in triplicate and a valid result was considered when the standard deviation was less than 10% of the average.

Immunoglobulin M and Immunoglobulin G Concentrations in Saliva and Serum of Kids, and in Colostrum

Immunoglobulin M (IgM) and immunoglobulin G (IgG) were quantified as mg/mL of sample using commercial ELISA kits (Life Diagnostic Inc., West Chester, PA, United States). Pilot tests were performed with samples of each experimental condition to determine a suitable sample dilution fitting within the kit's quantitation interval. For IgM quantitation, serum, saliva and colostrum were diluted at 1:25000, 1:500, and 1:25000, respectively. For IgG quantitation the dilutions were 1:150000, 1:2000, and 1:450000 for serum, saliva, and colostrum, respectively. Samples were individually tested in triplicate by measuring the optical density at 450 nm in microplate reader Infinite 200 (Tecan, Switzerland). A valid result was considered when the standard deviation was less than 10% of the average.

In vivo Assays to Assess the Feed Digestibility

The apparent dry matter (DM) and organic matter (OM) digestibility was assessed *in vivo* according to the recommendations of Azevedo et al. (2014). Briefly, three kids from each group were allocated in metabolic cages for 8 days at 20 weeks of age (3 days of adaptation). Animals were fed as described in the post-natal treatment section. All animals had free access to water. Feed refusals were collected just prior to the distribution of the next feeding. Feces were collected daily at 08:00 h from the fecal bags attached to the cage and weighed. Prior to the feces collection, kids were removed from the metabolic cages, which were cleaned completely. Urine and feces were separated and contact was avoided through a urine device collector (Lima et al., 2011). The refused feed and feces were collected and dried in an oven at 65°C for 72 h, pooled by animal/day, ground and sieved through a 1 mm screen, and stored until analysis.

Intake was calculated by difference between feed offered and feed refusal. Dry Matter Digestibility (DMD) and Digestible Organic Matter in DM (DOMD) were calculated based on Briceño-Poot et al. (2012).

Statistical Analysis

A non-parametric factorial ANOVA approach using the art function in the ARTool R package version 0.10.7 (Wobbrock et al., 2011) in the R statistical computing environment (version 3.6.1) was used for the analysis of the data obtained. Prenatal treatment, postnatal treatment and their interaction were used as fixed effects, and mother's identity (kids delivered by the same doe) was used as random factor. The BH procedure (Benjamini and Hochberg, 1995) was used for multiple comparisons and treatment effects were declared significant at $P < 0.05$ and a trend toward significance at $0.05 \leq P < 0.10$. In addition, Spearman Rank non-parametric correlation was performed to check the

correlation between different taxa (at genus level) and the average daily gain [ADG], and FCE using SPSS 21.0 (SPSS, 2012).

RESULTS

Age Is the Major Determinant of Variation in Rumen Bacterial Community Composition, Animal Performance and Immune Status

Diversity in the ruminal bacterial community structure of goat kids generally increased until 14 weeks of age, while dominance decreased (**Supplementary Table S5**). When all samples were visualized using a PCoA plot at bacterial OTU level, samples clustered based on the age of the kids (**Supplementary Figure S1**). Samples of 14-week and 20-week-old goat kids clustered together, and this cluster was away from the samples of the younger kids along PC1, which explained 35% of the variation. Bacterial OTU of 4- and 8-weeks-old kids clustered separately from each other along the PC2 axis, which only explained 8% of the variation. Particularly, samples from 8-weeks-old kids were very diverse, with some clustering separately from samples of 4-weeks-old kids while others were not separated.

In total, 12 bacterial phyla were identified across the four age groups, with Bacteroidetes and Firmicutes being the dominant phyla, irrespective of age (**Figure 2**). The relative abundance of the phylum Firmicutes was higher ($P < 0.05$) at the age of 4 and 8 weeks compared with 14 and 20 weeks, mainly due to the increased relative abundance of the genera *Solibacillus*, *Bacillus*, Uncul_Bacillaceae and Uncul_Ruminococcaceae at younger age, while Uncul_Lachnospiraceae were relatively more abundant in older kids. The relative abundance of the phylum Bacteroidetes remained similar at 4 and 8 weeks of age, but became more important ($P < 0.05$) at 14 and 20 weeks of age, mainly due to the increased relative abundance of the genus *Prevotella* and uncultured Bacteroidales.

The gene copy numbers of total bacteria, *Neocallimastigales* (fungi), protozoa, and specific bacteria were significantly influenced by age (**Supplementary Table S6**). Virtually, no protozoa were present in the rumen fluid of kids at 4 and 8 weeks of age, irrespective of the experimental treatment. The 16S rRNA gene copy number of total bacteria increased ($P < 0.05$) after weaning (14 and 20 weeks) compared with 4 and 8 weeks. The copy numbers of *Neocallimastigales* remained similar at 4 and 8 weeks, decreased ($P < 0.05$) at 14 weeks, but increased to the level of 4 weeks at the age of 20 weeks. Additionally, *R. flavefaciens* and *S. ruminantium* were higher ($P < 0.05$) after weaning compared with preweaning. The 16S rRNA gene copy numbers of *S. ruminantium* and *F. succinogenes* were highest ($P < 0.05$) at 14 weeks. The abundance of 16S rRNA genes of *R. albus* was higher ($P < 0.05$) after weaning compared with 4 weeks. The copy numbers of *S. jonesii* increased ($P < 0.05$) till 14 weeks, but decreased to the level of 8 weeks at the age of 20 weeks.

Additionally, some of the studied animal performance parameters (BW, DMI, and FCE) increased with age as expected ($P < 0.05$, **Supplementary Table S7**). The immune status of kids (Total WBC, eosinophils, monocytes, ChT activity, and the concentration of IgG and IgM in serum and saliva) was also influenced by age ($P < 0.05$), except for the lymphocyte and neutrophil proportions.

Effects of Early Life Nutritional Intervention on Bacterial Community Composition Were More Evident After Weaning

The postnatal treatment continued until weaning. After weaning, all kids received *L. leucocephala* forage meal (30% of the requirements of CP) mixed with a commercial concentrate twice a day. The effects of prenatal and postnatal treatments on bacterial community composition were more evident after weaning (14 and 20 weeks), once the postnatal treatment was ceased.

Generally, prenatal treatment did not influence any of the alpha diversity indices measured (**Table 1**). However, at the age of 8 weeks, dominance tended to be lower in D+ kids as compared with D– kids ($P = 0.08$). A postnatal effect was observed in alpha diversity indices only at 20 weeks, 12 weeks after the treatment ceased: the Shannon index was higher and the dominance was lower in K+ kids as compared with K– kids ($P < 0.05$). Furthermore, interaction effects were observed on alpha diversity indices at the age of 4 weeks: diversity (PD_Whole tree) and richness (Chao1 index) were ($P < 0.05$) or tended to be ($P = 0.09$) higher in D+K+ and D–K– kids compared with D+K– kids, while dominance was higher in D+K+ kids compared with D–K+ kids.

There were no prenatal, postnatal nor interaction effects on the bacterial community structure at 4 and 8 weeks (**Figures 3A–F**), which was confirmed by the ANOSIM and ADONIS analysis ($P > 0.05$). However, at 4 weeks of age, the K+ kids showed lower PC2 values (close to 0 or negative), except for one observation. In contrast, K– kids showed positive PC2 values, except for 2 observations. Inter-animal variation within each treatment seemed to be higher in younger animals as compared with animals at post-weaning ages (**Figures 3G–L**). Indeed, treatment effects were more evident at 14 weeks (**Figures 3G–I**) and 20 weeks (**Figures 3J–L**), which was confirmed by ANOSIM and ADONIS analysis ($P < 0.05$), except for the prenatal effect at 14 weeks ($P = 0.116$). At 14 weeks of age, K+ kids clustered away from K– kids along the PC1 axis, which explained 16% of the variation (**Figure 3I**). Furthermore, K+ kids showed lower individual variability as compared with the K– group at this age (**Figure 3I**). A clear clustering was also observed between D+K– and D–K– kids along the PC2 axis (13.9% of variation explained) in which D–K– kids were more diverse compared with D+K– kids (**Figure 3G**). At 20 weeks, clustering according to the prenatal treatment (D+ *vs.* D–) was obvious along the PC1 axis (**Figure 3K**), in which the majority of the D+ kids showed a positive PC1 value and high individual variation, whereas all D– kids showed lower PC1 values (negative or closer to 0) and less individual variation. Notably, at the age of 20 weeks, D+K+ kids clustered away from the other treatment groups along the PC1

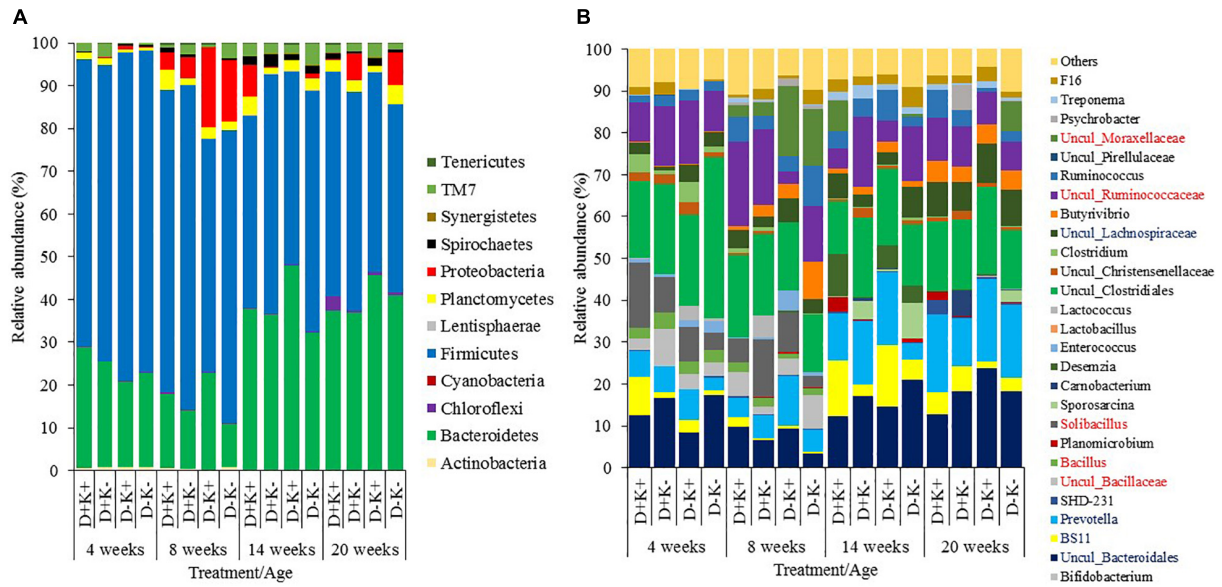


FIGURE 2 | Relative abundance of rumen bacteria (%) in goat kids at phyla **(A)** and genus **(B)** level (relative abundance >1%) of goat kids at 4, 8, 14, and 20 weeks of age. Genera presented in the legend in blue or red font, indicate higher abundance at older or younger ages, respectively. Although the genera Solibacillus, Bacillus, Uncul_Bacillaceae were significantly higher in weeks 4 and 8 compared to weeks 14 and 20, this could not be visualized in weeks 14 and 20 due to too limited abundance.

axis, which explained 18% of the variation (**Figure 3J**). The other three groups clustered separately from each other along the PC2 axis, which explained 12% of the variation.

Differences in the relative abundance of bacteria were analyzed at phylum and genus levels (**Figure 2**). Statistics are presented separately by age group in **Supplementary Table S8**, where only taxa representing more than 1% of the total bacterial community in at least one treatment group are shown. No effects of treatments were observed at phylum level and only few genera showed differences at 4 and 8 weeks of age. At 4 weeks, the relative abundance of Uncul_Lachnospiraceae was higher ($P < 0.05$), and the relative abundance of Uncul_Bacteroidales tended to be lower ($P = 0.07$) in K+ kids compared with K– kids. Additionally, the relative abundance of Uncul_Lachnospiraceae tended to be higher ($P = 0.07$) in D– kids compared with the D+ kids. At 8 weeks, the relative abundance of the genus *Clostridium* was higher in D+ kids ($P < 0.05$) compared with D– kids. The relative abundance of *Butyrivibrio* was lower ($P = 0.05$), while BS11 tended to be higher ($P = 0.07$) in K+ kids compared with K– kids.

After weaning, treatment differences were observed at phylum and genus levels, although all animals were given the same diet (**Figure 2** and **Supplementary Table S8**). No prenatal effects were observed at 14 weeks. Firmicutes were affected by the postnatal treatment and tended to be lower in K+ kids compared with K– kids ($P = 0.07$) at 14 weeks of age. At genus level, K+ kids showed higher ($P < 0.05$) relative abundance of BS11 and lower abundance of Uncul_Ruminococcaceae as compared with K– kids. Additionally, the abundance of the genus *Desemzia* tended to be higher ($P = 0.07$) and of Uncul_Christensenellaceae tended to be lower ($P = 0.10$) in K+ kids compared with K- kids. Moreover, some interactions were observed: *Prevotella* and Uncul_Lachnospiraceae showed the highest and lowest abundance, respectively, in untreated kids (D–K–), while single treated kids (D+K– and D–K+) showed the lowest and highest abundances. Inversely, D+K+ kids showed the lowest abundance of Uncul_Clostridiales, while single treated kids showed the highest abundances.

At 20 weeks, the relative abundance of the phylum Chloroflexi was higher ($P > 0.05$) in D+ kids and tended to be higher ($P = 0.06$) in K+ kids compared with D– and K– kids, respectively, particularly due to the higher ($P_{interaction} < 0.05$) relative abundance of this phylum in D+K+ kids compared with D+K– kids. The relative abundance of the phylum TM7 was higher in K+ kids ($P < 0.05$) compared with K– kids, particularly in prenatally untreated kids ($P_{interaction} = 0.06$). In the current microbial dataset, only one genus has been identified within the phylum TM7 and Chloroflexi (i.e., F16 and SHD-231, respectively). As such these genera determined the effect of their respective phyla. The relative abundance of the phylum Proteobacteria tended to be higher ($P = 0.08$) in K– kids compared to K+ kids. At the age of 20 weeks, the genus *Ruminococcus* showed a higher ($P < 0.05$) relative abundance in D+ kids compared with D– kids and also tended to be higher ($P = 0.05$) in D+K+ kids compared with D–K+ and D–K– kids. The genera *Prevotella* tended to be higher ($P = 0.07$) and Uncul_Christensenellaceae tended to be lower ($P = 0.05$) in K+ compared with K– kids at the age of 20 weeks. The latter was in line with the observation at 14 weeks, while other postnatal differences at 14 and 20 weeks varied.

The qPCR data suggested neither the bacterial nor fungal (*Neocallimastigales*) numbers were affected by the treatments at younger ages (**Table 2**). Protozoa were observed only after

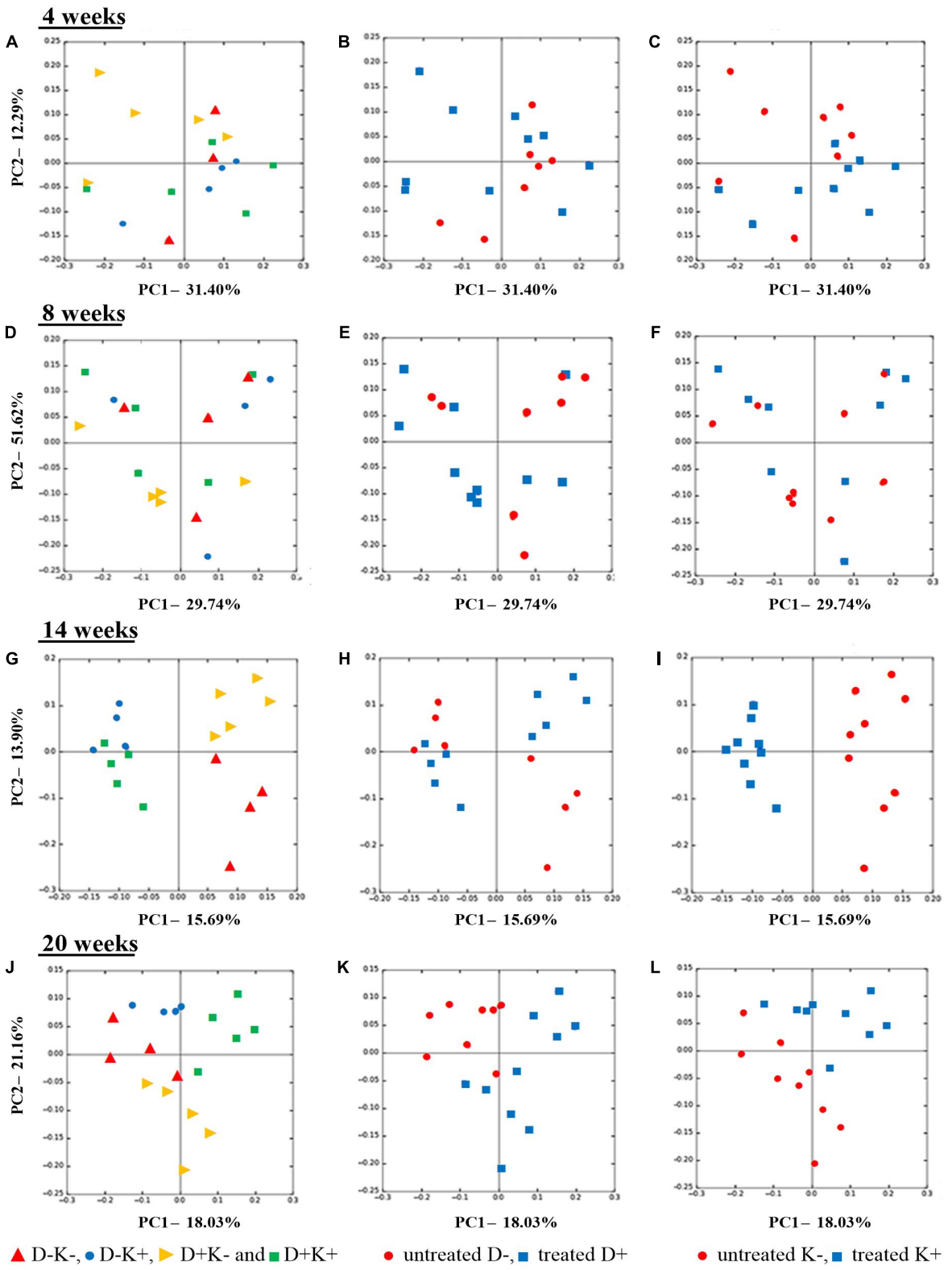


FIGURE 3 | Principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity indices at OTU level, of the rumen bacterial community structure of goat kids pre weaning (4 weeks), at weaning (8 weeks) and post weaning (14 and 20 weeks). Kids were treated prenatally through supplementation of *L. leucocephala* forage meal in their mothers' diet during the last 7 weeks of pregnancy (D+), postnatally until weaning with yeast (K+), or not (D– and K–). Left Figures **(A,D,G,J)** present all four treatments separately while middle **(B,E,H,K)** and right **(C,F,I,L)** figures visualize prenatal and postnatal treatments, respectively.

TABLE 1 | Mean (standard deviation) of α-diversity indices (Chao1, PD_Whole_tree, Observed OTU, Shannon index and dominance) characterizing the rumen bacterial community structure of goat kids at 4, 8, 14, and 20 weeks of age.

Alpha diversity indices	Treatments				*P*-value		
	D+K+	D+K–	D–K+	D–K–	Prenatal	Postnatal	Interaction
4 weeks							
Chao1	716 (156.2)	629 (228.6)	671 (80.2)	738 (49.6)	0.555	0.501	0.094
PD_Whole_tree	67.5^{a} (14.22)	58.1^{b} (18.86)	63.6^{ab} (7.80)	68.0^{a} (3.25)	0.455	0.466	0.042
Observed OTU	631 (158.3)	566 (208.1)	613 (78.9)	662 (27.2)	0.364	0.671	0.149
Shannon index	5.59 (0.628)	5.89 (0.917)	6.30 (0.308)	5.80 (0.261)	0.356	0.830	0.290
Dominance	0.10^{a} (0.049)	0.06^{ab} (0.032)	0.04^{b} (0.011)	0.08^{ab} (0.014)	0.507	0.918	0.046
8 weeks							
Chao1	820 (151.9)	829 (161.7)	782 (82.3)	786 (33.3)	0.409	0.853	0.248
PD_Whole_tree	76.2 (11.86)	75.1 (13.31)	70.1 (7.12)	70.8 (1.67)	0.278	0.178	0.801
Observed OTU	746 (130.2)	734 (170.7)	673 (93.3)	701 (27.6)	0.372	0.430	0.747
Shannon index	6.29 (0.385)	6.01 (0.882)	5.32 (0.452)	5.81 (0.654)	0.148	0.851	0.109
Dominance	0.04 (0.012)	0.06 (0.057)	0.09 (0.024)	0.08 (0.059)	0.084	0.361	0.114
14 weeks							
Chao1	1016 (69.7)	967 (68.2)	999 (56.8)	984 (79.2)	0.990	0.378	0.631
PD_Whole_tree	90.7 (6.48)	88.6 (5.13)	91.1 (5.15)	89.6 (6.54)	0.699	0.645	0.942
Observed OTU	908 (95.3)	876 (62.3)	908 (72.0)	890 (66.3)	0.751	0.521	0.782
Shannon index	6.17 (0.839)	6.63 (0.155)	6.47 (0.398)	6.43 (0.817)	0.798	0.407	0.619
Dominance	0.07 (0.045)	0.04 (0.003)	0.05 (0.009)	0.06 (0.051)	0.772	0.414	0.888
20 weeks							
Chao1	991 (39.0)	970 (37.4)	1035 (124.0)	969 (46.0)	0.669	0.318	0.745
PD_Whole_tree	89.7 (2.68)	89.9 (3.42)	94.3 (8.28)	89.5 (3.62)	0.446	0.440	0.475
Observed OTU	895 (35.6)	883 (42.5)	942 (101.0)	889 (41.5)	0.497	0.398	0.635
Shannon index	6.83 (0.161)	6.17 (0.553)	6.92 (0.189)	6.58 (0.437)	0.209	0.001	0.179
Dominance	0.03 (0.006)	0.07 (0.035)	0.03 (0.005)	0.04 (0.023)	0.200	0.009	0.186

Different letters in the same row (^{a,b}) indicate significant differences among treatments ($P < 0.05$) according to the BH procedure (Benjamini and Hochberg, 1995).

weaning and were higher in K+ kids compared with K– kids at 14 and 20 weeks. Protozoa numbers were also higher in D+K+ kids compared with K– kids (D+K– and D–K–) at 14 weeks. 16S rRNA gene copies of *F. succinogenes* and *R. albus* tended to be higher ($P = 0.10$ and $P = 0.09$, respectively) in K+ kids compared with K– kids at 4 weeks of age. At the age of 8 weeks, 16S rRNA gene copies of *R. albus* tended to be higher in D+ kids compared with D– kids ($P = 0.10$). From 14 weeks of age onward, some differences were observed in the quantitative abundance of *S. jonesii*, but the prenatal *L. leucocephala* supplementation to mother goats did not enhance the abundance of this genus. Also at 20 weeks of age, the abundance of *S. ruminantium* was higher ($P < 0.05$) in K+ kids compared with K– kids and tended to be higher in D+ kids ($P = 0.09$) compared with D– kids. Additionally, the quantity of *S. ruminantium* was highest in D+K+ kids.

Core Successional Microbiome

The core microbiome (present in 80% of all animals) was characterized at the age of 4, 8, 14, and 20 weeks irrespective of the treatment (**Supplementary Figure S2**). In total, eight core phyla were identified across the four age groups. Similar to the overall microbiome, core Firmicutes and Bacteroidetes were the dominant phyla irrespective of age. Other predominant core bacteria (relative abundance > 1% in at least one of the age groups) belonged to the phyla Planctomycetes and TM7. Contrary to the overall observations, the relative abundance of core Bacteroidetes decreased with age ($P < 0.01$) mainly as a result of the decreasing relative abundance of core OTU within the genera Uncul_Bacteroidales and *Prevotella*, except at 14 weeks of age where an increase was observed of the relative abundance of the core BS11 genus (phylum Bacteroidetes). In contrast, the relative abundance of core Firmicutes increased with age ($P < 0.01$), except at 14 weeks of age mainly due to the decreased relative abundance of the core OTU within the genus Uncul_Clostridiales.

Only 49 OTU, observed at the age of 4 weeks, persisted until 20 weeks. These core OTU belonged to three phyla: Actinobacteria, Bacteroidetes, and Firmicutes (**Table 3**). The relative distribution of the core-persistent microbiome mainly depended on the age (**Supplementary Table S9**), but some bacterial phyla and genera showed prenatal and/or postnatal

TABLE 2 | Mean (standard deviation) of absolute abundance of target rumen microbes determined by real-time PCR (log_{10}/mL rumen fluid) of goat kids at 4, 8, 14, and 20 weeks of age.

Index	Treatments				*P*-value		
	D+K+	D+K–	D–K+	D–K–	Prenatal	Postnatal	Interaction
4 weeks							
Total Bacteria	12.6 (0.17)	12.5 (0.18)	12.4 (0.31)	12.3 (0.15)	0.247	0.583	0.798
Fungi[1]	9.8 (0.19)	9.8 (0.26)	9.7 (0.39)	9.4 (0.21)	0.128	0.408	0.380
Protozoa	Nd	nd	nd	nd	–	–	–
F. succinogenes	10.2 (0.81)	9.22 (0.84)	10.3 (0.43)	10.3 (0.41)	0.250	0.096	0.218
R. flavefasciens	8.7 (0.50)	8.1 (1.77)	9.1 (0.79)	9.2 (0.64)	0.306	0.732	0.701
R. albus	9.7 (1.87)	7.9 (1.52)	9.1 (1.73)	7.9 (0.75)	0.872	0.088	0.769
S. ruminantium	11.7 (0.45)	11.5 (0.32)	11.6 (0.33)	11.5 (0.42)	0.785	0.355	0.788
S. jonesii	8.9 (0.53)	8.0 (1.05)	8.7 (0.38)	8.5 (0.63)	0.806	0.274	0.400
8 weeks							
Total Bacteria	12.6 (0.19)	12.6 (0.20)	12.6 (0.19)	12.5 (0.28)	0.671	0.659	0.283
Fungi	9.8 (0.19)	10.3 (1.07)	10.2 (1.15)	9.7 (0.14)	0.727	0.939	0.427
Protozoa	nd	nd	nd	nd	–	–	–
F. succinogenes	10.5 (0.59)	10.9 (1.03)	10.9 (0.80)	10.5 (0.54)	0.671	0.659	0.283
R. flavefasciens	8.5 (0.60)	8.2 (0.71)	9.2 (0.73)	9.0 (0.77)	0.727	0.939	0.427
R. albus	9.8 (0.55)	10.3 (1.06)	9.0 (1.30)	8.5 (1.29)	0.105	0.835	0.190
S. ruminantium	11.4 (0.23)	11.5 (0.39)	11.4 (0.23)	11.5 (0.44)	0.969	0.652	0.912
S. jonesii	8.7 (0.82)	8.6 (0.93)	9.1 (0.39)	8.9 (0.50)	0.450	0.862	0.972
14 weeks							
Total Bacteria	12.9 (0.25)	13.1 (0.37)	13.2 (0.22)	12.9 (0.33)	0.700	0.785	0.205
Fungi	8.0 (0.51)	8.1 (0.50)	8.0 (0.52)	7.6 (0.34)	0.510	0.217	0.177
Protozoa	11.1^{a} (0.42)	9.74^{c} (0.31)	10.8^{ab} (0.07)	10.5^{b} (0.19)	0.159	<0.001	0.001
F. succinogenes	11.0 (0.43)	11.2 (0.51)	11.4 (0.77)	10.9 (0.58)	0.701	0.817	0.351
R. flavefasciens	9.0 (0.46)	10.1 (0.73)	9.8 (0.91)	9.3 (1.12)	0.818	0.310	0.083
R. albus	10.1 (0.30)	10.8 (0.69)	10.5 (0.63)	10.0 (0.71)	0.535	0.536	0.149
S. ruminantium	12.0 (0.29)	12.4 (0.36)	12.4 (0.26)	12.1 (0.40)	0.979	0.505	0.080
S. jonesii	9.4^{b} (0.26)	9.7^{a} (0.15)	9.7^{a} (0.15)	9.5^{ab} (0.29)	0.911	0.404	0.033
20 weeks							
Total Bacteria	13.0 (0.22)	12.9 (0.11)	12.9 (0.13)	12.9 (0.12)	0.412	0.812	0.401
Fungi	9.8 (0.38)	9.3 (0.35)	9.5 (0.28)	9.4 (0.18)	0.537	0.117	0.327
Protozoa	11.3 (0.38)	10.5 (0.51)	11.1 (0.50)	10.7 (0.26)	0.803	0.008	0.510
F. succinogenes	10.6 (0.34)	10.2 (0.20)	10.7 (0.35)	10.6 (0.33)	0.121	0.248	0.296
R. flavefasciens	9.7 (0.68)	9.5 (0.60)	9.6 (1.15)	9.2 (0.49)	0.757	0.265	0.657
R. albus	10.1 (0.54)	9.8 (0.13)	9.9 (0.22)	9.9 (0.73)	0.881	0.455	0.505
S. ruminantium	12.2^{a} (0.18)	11.7^{b} (0.16)	11.7^{b} (0.21)	11.8^{b} (0.17)	0.089	0.016	0.005
S. jonesii	8.6^{bc} (0.07)	8.5^{c} (0.10)	8.7^{a} (0.09)	8.6^{b} (0.19)	<0.001	<0.001	<0.001

Values with different superscripts in same row (^{a,b,c}) differ significantly ($P < 0.05$) among treatments according to the BH procedure (Benjamini and Hochberg, 1995).
[1]Fungi was determined based on the order Neocallimastigales.

treatment effects. In contrast to the overall bacteriome, at phylum level, there was a lower relative abundance ($P < 0.05$) of core Bacteroidetes (solely represented by OTU of the genus Uncul_Bacteroidales) in K+ kids as compared with K– kids at 4 weeks of age. Core Firmicutes showed an interaction effect at the age of 4 weeks and were relatively least abundant in D+K+ kids ($P < 0.05$). This was mainly due to a tendency for lower relative abundance of the core OTU in the family Uncul_Clostridiales ($P = 0.08$). Additionally, the relative abundance of the core Uncul_Ruminococcaceae at 4 weeks of age was lowest in D–K– ($P < 0.05$). The relative abundance of core-bacteria belonging to the genera *Ruminococcus, Oscillospira*, and Uncul_Lachnospiraceae, Ruminococcaceae, Mogibacteriaceae, Christenellaceae, Clostridiaceae, showed some treatment differences at 8, 14, or 20 weeks of age (**Table 3**). The relative abundance of the core OTU belonging to the genera Uncul_Lachnospiraceae and Uncul_Ruminococcaceae was increased by the postnatal treatment at week 20,

TABLE 3 | Mean (standard deviation) of the relative abundance (%) of the core-persistent microbiome (phyla [in **bold**] and genus level) which were observed in 80% of the goat kids irrespective of treatment at 4 weeks of age and that persisted through 8 and 14 weeks of age till 20 weeks of age.

Taxa	Treatments				*P*-value		
	D+K+	D+K–	D–K+	D–K–	Prenatal	Postnatal	Interaction
4 weeks							
Actinobacteria (Uncul_Coriobacteriaceae*)	0.1 (0.11)	0.1 (0.09)	0.2 (0.18)	0.5 (0.56)	0.267	0.394	0.216
Bacteroidetes (Uncul_Bacteroidales*)	1.7 (1.34)	11.9 (8.18)	4.1 (4.40)	7.8 (13.73)	0.614	0.030	0.204
Firmicutes	9.5^{b} (2.80)	14.7^{a} (2.93)	18.2^{a} (6.06)	11.6^{ab} (7.64)	0.564	0.589	0.032
Uncul_Clostridiales	4.9 (2.12)	9.9 (3.47)	8.1 (3.69)	6.4 (4.69)	0.938	0.265	0.083
Uncul_Mogibacteriaceae	0.1 (0.04)	0.4 (0.03)	0.1 (0.04)	0.0 (0.01)	0.900	0.089	0.078
Uncul_Christensenellaceae	0.8 (0.70)	1.5 (1.15)	1.3 (1.86)	0.7 (0.91)	0.433	0.605	0.299
Uncul_Clostridiaceae	0.1 (0.09)	0.1 (0.14)	0.1 (0.06)	0.0 (0.03)	0.412	0.167	0.624
Uncul_Lachnospiraceae	1.4 (1.16)	0.2 (0.40)	1.9 (1.59)	1.7 (1.68)	0.218	0.168	0.137
Uncul_Ruminococcaceae	2.1^{b} (1.56)	2.5^{b} (0.90)	6.2^{a} (5.09)	1.5^{b} (1.84)	0.084	0.054	0.043
Oscillospira	0.0 (0.03)	0.1 (0.09)	0.0 (0.01)	0.0 (0.04)	0.688	0.553	0.724
Ruminococcus	0.0 (0.05)	0.4 (0.80)	0.4 (0.39)	1.2 (0.73)	0.060	0.018	0.307
Other_Ruminococcaceae	0.0 (0.03)	0.0 (0.01)	0.0 (0.01)	0.0 (0.00)	0.718	0.241	0.786
8 weeks							
Actinobacteria (Uncul_Coriobacteriaceae*)	0.1 (0.13)	0.0 (0.01)	0.1 (0.10)	0.1 (0.12)	0.578	0.508	0.445
Bacteroidetes (Uncul_Bacteroidales*)	1.4 (1.58)	1.0 (1.52)	1.5 (1.84)	0.1 (0.06)	0.841	0.274	0.644
Firmicutes	11.7 (3.47)	16.8 (16.69)	11.9 (8.73)	10.7 (3.30)	0.510	0.418	0.801
Uncul_Clostridiales	5.3 (1.73)	6.5 (5.22)	7.4 (9.70)	4.6 (1.70)	0.628	0.859	0.567
Uncul_Mogibacteriaceae	0.1 (0.07)	0.1 (0.07)	0.1 (0.05)	0.2 (0.24)	0.577	0.403	0.192
Uncul_Christensenellaceae	0.2 (0.11)	0.3 (0.28)	0.1 (0.05)	0.1 (0.03)	0.017	0.477	0.561
Uncul_Clostridiaceae	0.3^{a} (0.20)	0.1^{b} (0.11)	0.2^{ab} (0.31)	0.2^{ab} (0.17)	0.849	0.440	0.012
Uncul_Lachnospiraceae	1.1 (0.96)	0.6 (0.35)	2.4 (1.99)	1.3 (0.56)	0.064	0.213	0.447
Uncul_Ruminococcaceae	1.3 (1.19)	6.7 (12.21)	0.6 (0.37)	0.8 (0.82)	0.402	0.502	0.473
Oscillospira	0.0 (0.02)	0.0 (0.03)	0.0 (0.01)	0.0 (0.02)	0.199	0.967	0.543
Ruminococcus	3.2 (5.03)	2.1 (2.54)	1.2 (2.01)	3.5 (4.80)	0.872	0.936	0.838
Other_Ruminococcaceae	0.0 (0.01)	0.3 (0.61)	0.0 (0.01)	0.0 (0.04)	0.424	0.410	0.407
14 weeks							
Actinobacteria (Uncul_Coriobacteriaceae*)	0.0 (0.01)	0.0 (0.01)	0.0 (0.01)	0.1 (0.12)	0.400	0.093	0.150
Bacteroidetes (Uncul_Bacteroidales*)	0.6 (0.55)	0.4 (0.27)	0.3 (0.24)	0.2 (0.13)	0.261	0.861	0.845
Firmicutes	10.8 (4.86)	10.7 (4.72)	9.24 (1.475)	11.2 (4.01)	0.855	0.864	0.787
Uncul_Clostridiales	6.4 (1.88)	7.1 (3.90)	7.3 (1.51)	5.7 (1.49)	0.827	0.224	0.223
Uncul_Mogibacteriaceae	0.1 (0.05)	0.1 (0.04)	0.0 (0.02)	0.0 (0.01)	0.986	0.938	0.819
Uncul_Christensenellaceae	0.2 (0.13)	0.2 (0.13)	0.2 (0.15)	0.2 (0.11)	0.707	0.402	0.642
Uncul_Clostridiaceae	0.1 (0.10)	0.2 (0.16)	0.0 (0.02)	0.2 (0.18)	0.371	0.045	0.609
Uncul_Lachnospiraceae	2.8^{a} (2.73)	0.6^{b} (0.191)	0.6^{ab} (0.30)	3.0^{a} (3.05)	0.727	0.536	0.006
Uncul_Ruminococcaceae	0.9 (0.29)	2.7 (1.50)	0.9 (0.17)	2.0 (1.59)	0.210	0.001	0.195
Oscillospira	0.0 (0.02)	0.1 (0.02)	0.0 (0.01)	0.0 (0.02)	0.099	0.010	0.726
Ruminococcus	0.4 (0.36)	0.8 (1.09)	0.2 (0.19)	0.8 (0.47)	0.632	0.228	0.669
Other_Ruminococcaceae	0.0 (0.03)	0.1 (0.03)	0.0 (0.06)	0.1 (0.06)	0.584	0.086	>0.999
20 weeks							
Actinobacteria (Uncul_Coriobacteriaceae*)	0.0 (0.01)	0.0 (0.01)	0.0 (0.01)	0.0 (0.03)	0.936	0.643	0.544
Bacteroidetes (Uncul_Bacteroidales*)	0.6^{a} (0.19)	0.3^{b} (0.18)	0.4^{b} (0.19)	0.5^{ab} (0.25)	0.878	0.161	0.045
Firmicutes	14.0 (4.93)	12.9 (4.38)	17.2 (7.44)	10.7 (2.97)	>0.999	0.279	0.471
Uncul_Clostridiales	7.9 (4.99)	8.7 (3.79)	10.2 (7.82)	7.1 (2.75)	0.877	0.871	0.434
Uncul_Mogibacteriaceae	0.0 (0.03)	0.1 (0.02)	0.1 (0.02)	0.1 (0.09)	0.082	0.008	0.377
Uncul_Christensenellaceae	0.5 (0.41)	0.6 (0.32)	0.6 (0.28)	0.4 (0.12)	0.914	0.956	0.213

(Continued)

TABLE 3 | Continued

Taxa	Treatments				*P*-value		
	D+K+	D+K–	D–K+	D–K–	Prenatal	Postnatal	Interaction
Uncul_Clostridiaceae	0.1 (0.05)	0.1 (0.05)	0.1 (0.06)	0.1 (0.03)	0.728	0.578	0.927
Uncul_Lachnospiraceae	2.1 (1.46)	1.4 (0.92)	3.5 (0.96)	1.7 (0.52)	0.138	0.030	0.462
Uncul_Ruminococcaceae	2.8 (0.70)	1.7 (0.75)	2.6 (1.12)	1.1 (0.49)	0.533	0.015	0.342
Oscillospira	0.0 (0.03)	0.0 (0.02)	0.0 (0.02)	0.0 (0.04)	0.878	0.521	0.426
Ruminococcus	0.16 (0.176)	0.17 (0.168)	0.03 (0.027)	0.02 (0.035)	0.020	0.895	0.772
Other_Ruminococcaceae	0.17 (0.109)	0.07 (0.037)	0.17 (0.063)	0.12 (0.106)	0.588	0.106	0.858

Values with different superscripts in same row (^{a,b}) differ significantly ($P < 0.05$) among treatments according to the BH procedure (Benjamini and Hochberg, 1995).
**The phyla of Actinobacteria and Bacteroidetes, only include one core genus, i.e., Uncul_Coriobacteriaceae and Uncul_Bacteroidales, respectively.*

12 weeks after the treatment was ceased. Core OTU of the genus Uncul_Coriobacteriaceae (phylum Actinobacteria) colonized the rumen at 4 weeks but their abundance decreased with age (**Supplementary Table S9**) while no treatment effect was observed.

Animal Performance and Immune Status

Animal performance data over the complete trial are presented in **Table 4**, while detailed data at intermediate ages are shown in **Supplementary Table S10**. Birth weight of kids was not affected by feeding *L. leucocephala* to mother goats in the last seven weeks of gestation. Both prenatal and postnatal treatments influenced the body weight in this trial. Throughout the whole experiment, D+ kids were heavier ($P < 0.05$) or tended to be heavier (at 4 weeks) compared with D– kids. Body weight was higher in K+ kids at 20 weeks ($P < 0.05$) and tended to be higher at 4 ($P = 0.06$) and 14 weeks ($P = 0.07$) compared with K– kids. Growth was higher in D+K+ kids ($P = 0.05$) compared with the other treatment groups in the first 4 weeks of life. Moreover, ADG was higher in K+ as compared with K– kids ($P < 0.05$) in the first 4 weeks and over the 20 weeks of life, and tended to be higher over the 14 weeks of life ($P = 0.10$) in K+ kids compared with K– kids. The DMI tended to be lower in D–K+ group of kids as compared with the other groups at this age. After weaning, the DMI were higher ($P < 0.05$) and tended to be higher ($P = 0.07$) in D+ compared with D– kids at 20 and 14 weeks, respectively. The FCE was higher ($P < 0.05$) in K– kids compared with K+ kids throughout the experimental period, except at the age of 8 weeks.

The total WBC and their components, mainly monocytes and eosinophils, were also influenced by prenatal and postnatal treatments. Notably, the total WBC count was higher in 8- and 20-weeks old K+ kids, as well as in D+ kids at 20 weeks as compared with K– kids and D– kids, respectively ($P < 0.05$). The concentration of serum IgG (sIgG) and IgM [in serum (sIgM) and saliva (mIgM)] and chitotriosidase activity were influenced by prenatal and postnatal treatments (**Supplementary Table S10**). In particular, the concentration of sIgG was higher in D– and K+ kids at 8 and 20 weeks compared with D+ and K– kids. At 4 weeks, D+K+ and D–K– kids had higher concentrations of sIgG compared with the other two groups (**Supplementary Table S10**). In general, an opposite effect was observed in the concentration of mIgG (salivary IgG). The K+ kids showed higher concentrations of sIgM at 4 and 8 weeks and lower concentrations of sIgM at 20 weeks compared with K– kids. The concentrations of sIgM were higher in D+ kids at 4 weeks mainly due to the higher concentration of sIgM in D+K+ kids compared with the other groups. The concentrations of mIgM were higher in K+ and D+ kids at 8 weeks compared with K– and D– kids. An opposite effect was observed at 20 weeks: the concentration of mIgM was higher in K– and D– kids compared with K+ and D+ kids. There were also some interaction effects in the concentrations of sIgM and mIgM. The ChT activity was higher in D+K+ and D–K– kids compared with the other two groups at 4 weeks. At 8 weeks of age, the ChT activity was lower in D–K+ kids compared with D+K+ and D–K– kids ($P < 0.05$), while the ChT activity was higher in D+K+ compared with D+K– kids ($P < 0.05$) at 20 weeks. The ChT activity was higher in D– kids at 4 weeks and K+ kids at 20 weeks compared with D+ and K– kids, respectively.

Correlation analysis between the relative abundances of core bacterial genera (present in 80% of the animals) with ADG and FCE are given in **Supplementary Table S11**. At the age of 4 and 8 weeks, there were some positive and negative correlations between the relative abundance of some of the minor core genera with ADG and FCE. Among the major genera, some correlations were observed at 14 and 20 weeks of age, but none of the correlations were consistent at different time points: BS11 negatively correlated with FCE and Uncul_Ruminococcaceae correlated positively with FCE at 14 weeks of age. At 20 weeks, Uncul_Clostridiales and Uncul_Ruminococcaceae correlated positively with ADG, while Uncul_Bacteroidales, *Coprococcus* and *Pseudobutyrivibrio* correlated negatively. Additionally, Uncul_Clostridiales correlated negatively while Uncul_Bacteroidales correlated positively with FCE.

In vivo Digestibility Trial

The *in vivo* digestibility assay was performed at the age of 20 weeks to assess the effects of the early-life intervention on the digestibility of diets with 30% of the dietary protein provided by *L. leucocephala* forage meal (**Table 4**). The DM intake and the digestible OM intake were higher in D+ kids compared with D– kids ($P < 0.01$), while the apparent digestibility of DM and the apparent digestibility of OM tended to be higher in D+

TABLE 4 | Mean (standard deviation) of body weight (BW) of goat kids at 20 weeks of age and average daily gain (ADG), dry matter intake (DMI) and feed conversion efficiency (FCE) over the first 20 weeks of life (all kids) as well as mean (standard deviation) of DM, OM and digestible OM intake and apparent digestibility of DM and OM obtained during the digestibility trial of 5 days at 20 weeks of age (n = 3 per treatment).

Items	Treatments				*P*-value		
	D+K+	D+K–	D–K+	D–K–	Prenatal	Postnatal	Interaction
Performance parameters (all animals)							
BW (kg) (20 weeks)	16.2 (1.00)	14.1 (1.16)	14.5 (1.00)	13.2 (0.48)	0.024	0.006	0.456
ADG (g/d) (birth-20 weeks)	92.4 (5.89)	78.5 (6.95)	81.6 (6.99)	75.6 (4.31)	0.046	0.004	0.445
DMI (g/d) (birth-20 weeks)	418 (25.8)	393 (36.7)	371 (26.4)	368 (22.3)	0.050	0.135	0.761
FCE (kg/kg) (birth-20 weeks)	4.8 (0.10)	5.3 (0.13)	4.8 (0.12)	5.2 (0.14)	0.914	<0.001	0.945
Digestibility trial (n = 3 per treatment)							
Intake (g/d)							
DM	1036 (2.7)	964 (96.6)	886 (70.2)	863 (8.9)	0.003	0.195	0.500
OM	951 (72.4)	885 (102.2)	814 (81.6)	792 (39.5)	0.112	0.591	>0.999
Digestible OM	655 (19.6)	533 (88.1)	515 (45.0)	460 (21.5)	0.009	0.007	0.409
Apparent digestibility (g/kg)							
DM	709 (17.9)	629 (38.4)	657 (4.8)	608 (19.7)	0.077	0.001	0.262
OM	633 (17.3)	554 (37.7)	581 (4.7)	533 (19.4)	0.077	0.001	0.262

DM, dry matter; OM, organic matter.

kids (P = 0.08). Postnatally treated kids (K+) showed higher digestible OM intake, and higher apparent digestibility of DM and OM compared with K– kids (P < 0.01). Furthermore, no interaction effects on the intake nor digestibility of DM or OM were observed.

DISCUSSION

Because of the resistance of the indigenous rumen microbiome of adult ruminants against the colonization by foreign bacterial strains (Weimer et al., 2010), prenatal or early in life modification of microbial communities of the gastrointestinal tract through nutritional interventions has been proposed to improve animal production (Abecia et al., 2013; Jami et al., 2014). Furthermore, early life microbial modulation may persist over a longer period and/or increase the resilience of the microbial community against similar perturbations later in life (Abecia et al., 2014, 2018; Debruyne et al., 2018). In this trial, we assessed whether the animal performance, immune status and microbial community of goat kids were influenced by a prenatal treatment (does fed with *L. leucocephala* forage meal) and/or postnatal intervention (supplementation of active yeast to kids). Additionally, we checked whether these effects would extend beyond the duration of the treatments, when the kids were fed *L. leucocephala* forage meal post-weaning.

At the end of the experimental period (20 weeks), both the pre- as well as the postnatal treatments resulted in an increased body weight and average daily gain. For the prenatal treatment, this could be linked to an enhanced feed intake post-weaning, presumably related to an *in utero* exposure through the maternal ingestion of the same feedstuff. This is in line with previous observations in goats where stimulatory effects of prenatal exposure were demonstrated on the feed intake of diets containing *C. odorata*. This plant has nutritionally valuable leaves (e.g., CP exceeding 200 g/kg DM) but a strong and repellent smell (Hai et al., 2012, 2013). Later work of the same group (Hai et al., 2016) confirmed the concept of *in utero* learning as feeding behavior of the offspring did not change when *C. odorata* was supplied to the mother goats during the lactation period only. In contrast to *C. odorata*, *L. leucocephala* supplemented in the current study does not contain odorous compounds. Nevertheless, the presence in *L. leucocephala* of the toxic compound mimosine, could impair digestibility, DMI and animal performance.

As mimosine is a plant secondary metabolite, we hypothesized that systemic responses (e.g., immune response) could occur in non-adapted animals, which could impact DMI and growth. As such, prenatal treatment was expected to alleviate this systemic response. However, at week 20, white blood cell concentrations tended to be lowest in non-treated kids (D–K–) and from weaning onward, serum IgG-levels were lower when kids had been treated prenatally. Nevertheless, differences were relatively small and were not thought to be a driving factor of differences in body weight, ADG and DMI. Additionally, the colostrum of *L. leucocephala*-treated mother goats showed a higher IgM concentration than untreated goats and had a considerably higher amount of IgG (**Supplementary Table S12**). Ruminant newborns receive immunoglobulin through passive transfer from colostrum (Tizard, 2013) and may show retarded growth when colostrum quantity or quality (e.g., immunoglobulin concentration) is too low (Robison et al., 1988; Elsohaby et al., 2019). In the current study, kids did not differ in BW at birth and 4 weeks of age, but D+ kids were heavier throughout the rest of the experimental period, although at 20 weeks of age an additive effect of yeast supplementation was observed.

Furthermore, the enhanced DMI and/or ADG observed in the D+ animals after weaning, could have been related to the trend of enhanced digestibility of the feed, which was assessed at the end of the experiment. As mimosine impairs rumen degradability

(Artiles-Ortega et al., 2021), inclusion of *L. leucocephala* in the does' diet was hypothesized to enhance the inoculation of mimosine-degrading bacteria in goat kids. *S. jonesii*, the first species that had been identified to possess mimosine-degrading properties, belonging to the phylum Synergistetes, was present in all goat kids in low quantities (less than 1% of relative abundance), which is in line with previous reports (Jami et al., 2013; Wang et al., 2016; Wang L. et al., 2019). Strikingly, at 20 weeks of age, the highest absolute abundance of *S. jonesii* (based on qPCR) was obtained in D– kids, which is hard to explain biologically. However, this treatment effect has not been consistently observed throughout the former samplings, while also the biological relevance of the difference (0.1 log units) seems minor. Meanwhile, other mimosine-degrading species have been identified [*Streptococcus lutetiensis*, *Clostridium butyricum,* and *Lactobacillus vitulinus* (Dominguez-Bello and Stewart, 1991; Derakhshani et al., 2016)]. No qPCR data have been generated for these species. The metataxonomic analysis indicated the genera *Streptococcus* and *Lactobacillus* to be present in low quantities in all goat kids (less than 1% of relative abundance) without differences between treatment groups. In addition, the genus *Clostridium* decreased with age irrespective of the prenatal treatment. Nevertheless, during the second month, the highest relative abundance was observed in D+K+ kids. Hence, it seems unlikely that feeding of *L. leucocephala* during pregnancy has enhanced the inoculation and proliferation of mimosine-degrading bacteria in the offspring. As such, changes in these bacteria could not be linked to variation in DMI and ADG, or digestibility of the diets. Other authors have suggested dietary supplementation of *L. leucocephala* to increase cellulolytic and proteolytic bacteria while reducing the protozoa population (Galindo et al., 2005, 2007, 2009). qPCR data of the current study did not suggest consistent changes in protozoal numbers in D+ kids, nor in representative key cellulolytic bacteria (*Fibrobacter succinogenes*, *R. albus,* and *R. flavefaciens*), while, at the age of 20 weeks the relative abundance of *Ruminococcus* increased in treated kids. The genus *Ruminococcus* includes important fiber degrading species, which are one of the early colonizers in the rumen (Koike and Kobayashi, 2001; Denman and McSweeney, 2006). As such, it is not surprising to observe a prenatal effect in this bacterial genus.

The rumen bacterial richness and diversity increased with age irrespective of pre- or postnatal treatments. Furthermore, there were changes in bacterial composition which linked to the animal's age. This is in line with Furman et al. (2020), who demonstrated that age globally affects bacterial composition independent of diet. As age is the major determinant of the microbial composition particularly during the pre-weaning period, early life treatment effects can be masked by age-related changes. In our study, the postnatal effects on bacterial composition were more obvious after weaning (14 and 20 weeks), once the rumen fully developed and the bacterial composition was more stable. At phylum level, the importance of Bacteroidetes is known to increase with age, while Firmicutes are decreasing (Jami et al., 2013; Abecia et al., 2017). This development could be accelerated by yeast supplementation immediately after birth. Recent work showed that yeast fed to cattle early in life enhanced bacterial diversity in the rumen, which persisted throughout the trial (Newbold and Ramos-Morales, 2020). In our study, Firmicutes (14 weeks) and Proteobacteria (20 weeks) tended to be less abundant while the relative abundance of the genus *Prevotella* (phylum Bacteroidetes) tended to be higher at 20 weeks in K+ kids, which is in line with the observations reported by Newbold and Ramos-Morales (2020) and Peng et al. (2020). According to AlZahal et al. (2017), yeast supplementation did not affect the family Ruminococcaceae. However, in the current study, the relative abundance of Uncul_Ruminococcaceae (the most abundant genus in the Ruminococcaceae family) decreased in K+ kids at 14 weeks, with a concomitant increase of the genus BS11. A similar observation was reported by Welty et al. (2019), who suggested that both families possibly occupy the same niche. Additionally, the core Uncul_Lachnospiraceae and Uncul_Ruminococcaceae (the most abundant genus in the Lachnospiraceae and Ruminococcaceae families, respectively) were higher in K+ kids 12 weeks after the yeast supplementation ceased, while there were no differences in these genera in the overall microbiome at this age.

Furthermore, the qPCR analysis showed protozoa remained absent in the rumen at 4 and 8 weeks of age in this trial, which may be linked to the removal of the kids from their mother after colostrum ingestion. Similar observations were reported previously in goats raised with milk replacers (Abecia et al., 2017; Debruyne et al., 2018; Belanche et al., 2019). However, *S. cerevisiae* supplementation to K+ kids enhanced the protozoal abundance, similar to the observations by Miranda et al. (1996), while earlier colonization of protozoa took place in the rumen of yeast supplemented lambs (Chaucheyras-Durand and Fonty, 2002). This suggests that yeast supplementation favored the maturation of the rumen microbial ecosystem, which is in line with Chaucheyras-Durand et al. (2019). Furthermore, yeast supplementation favors fibrolytic bacteria (Pinloche et al., 2013; Chaucheyras-Durand et al., 2019; Peng et al., 2020). In this sense, it was observed that *F. succinogenes* and *R. albus* tended to be higher in K+ kids compared to K– kids at 4 weeks, which suggests that colonization of these species was favored by yeast supplementation. We also observed an increased abundance of *S. ruminantium* in K+ kids at 20 weeks, in line with earlier reports in adult ruminants (Pinloche et al., 2013). *Fibrobacter succinogenes*, *R. albus,* and *R. flavefaciens* were quantified to assess the effect on cellulolytic bacteria, since these species are presently recognized as the major cellulolytic bacterial species found in the rumen (Koike and Kobayashi, 2001), whereas *S. ruminantium* was quantified as a representative rumen amylolytic bacterial species (Mackie et al., 1978) and *S. jonesii* was quantified as a major L-mimosine and DHP degrading species found in the rumen (McSweeney et al., 2019).

Yeast supplementation concomitantly enhanced animal performance: ADG and FCE were improved in K+ kids at 4 weeks of age, which persisted (or tended to persist) after weaning. As a result, the BW was higher in these kids. Such persistent effects of yeast supplementation had been observed

before by Chaucheyras-Durand and Fonty (2001). Chaucheyras-Durand et al. (2019) suggested a link between the enhanced performance and the improvement of the microbial colonization in the maturing rumen through the use of yeast as a feed additive. They suggested the development of a microbial ecosystem toward a more efficient fiber degradation. In line with these suggestions, enhanced dry matter and organic matter digestibility by K+ kids compared to K– kids has been observed in our study.

CONCLUSION

Overall, our findings show predominant age-related changes during the bacterial colonization, which could have masked prenatal and early life treatment effects on the ruminal bacterial composition. Nevertheless, such effects became more evident after weaning. The current study supports the stimulatory effects of prenatal exposure and post-natal supplementation of yeast on the intake of a *L. leucocephala* supplemented diet, reflected in an improved ADG and BW. Postnatal supplementation of yeast favored maturation of the rumen bacterial ecosystem (i.e., greater importance of Bacteroidetes, in particular *Prevotella*, and reduced abundance of Firmicutes) and protozoa colonization. Concomitantly, animal performance parameters and DM and OM digestibility were enhanced even post-weaning, when the supplementation was ceased, suggesting effects of the early-life intervention persisted later in life.

DATA AVAILABILITY STATEMENT

The data is publicly available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA757729.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical commission of the Faculty of Veterinary Medicine, Ghent University, Belgium (approval number EC2015/12), respectively, following the European Directive (EU) No 241/2014. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

VF, RL-O, and EA-O conceived and designed the experiments. EA-O and BR-B conducted the *in vivo* experiment. EA-O, OP, and JJ performed the bacterial analysis. PF-R and EA-O performed the immunological analysis. EA-O performed the statistical analysis, interpreted the data, and wrote the manuscript. JJ, RL-O, and VF corrected the manuscript and jointly supervised all this work. All authors have read and agreed to the published version of the manuscript and to be accountable for all aspects of the work.

FUNDING

This research was supported by the special research fund for developing countries (BOF scholarship: BOF.DCV.2014.0009.01) of Ghent University, Belgium, Universidad Central "Marta Abreu" de Las Villas (UCLV), Cuba (Project No. 10042) and National Program of Basic Sciences (Project No: P223LH001-025), and the VLIR-UOS partner program (ZEIN2015RIP29) for Institutional University Cooperation between UCLV, Cuba, and Flemish universities.

ACKNOWLEDGMENTS

Special acknowledgments to the staff of the Laboratory for Animal Nutrition and Animal Product Quality of Ghent University, and the Laboratory for Animal Nutrition and the Clinical Laboratory at Facultad de Ciencias Agropecuarias (UCLV), Cuba, for the technical assistance during this research, and to Alltech Belgium® for providing us the live yeast supplement.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.769438/full#supplementary-material

REFERENCES

Abecia, L., Jiménez, E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Pinloche, E., et al. (2017). Natural and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. *PLoS One* 12:e0182235. doi: 10.1371/journal.pone.0182235

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, I., Pinloche, E., Creevey, C., et al. (2018). Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Front. Microbiol.* 9:2227. doi: 10.3389/fmicb.2018.02227

Abecia, L., Martin-Garcia, A. I., Martinez, G., Newbold, C. J., and Yanez-Ruiz, D. R. (2013). Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. *J. Anim. Sci.* 91, 4832–4840. doi: 10.2527/jas.2012-6142

Abecia, L., Waddams, K. E., Martinez-Fernandez, G., Martin-Garcia, A. I., Ramos-Morales, E., Newbold, C. J., et al. (2014). An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by Archaea. *Archaea* 2014:841463. doi: 10.1155/2014/841463

Ahmed, M., Jusoh, S., Alimon, A., Ebrahimi, M., and Samsudin, A. (2018). Nutritive and anti-nutritive evaluation of Kleinhovia hospita, *Leucaena leucocephala* and *Gliricidia sepium* with respect to their effects on in vitro rumen fermentation and gas production. *Trop. Anim. Sci. J.* 41, 128–136. doi: 10.5398/tasj.2018.41.2.128

Akingbade, A. A., Nsahlai, I. V., Bonsi, M. L. K., Morris, C. D., and Du Toit, L. P. (2001). Reproductive performance of South African indigenous goats inoculated with DHP-degrading rumen bacteria and maintained on *Leucaena*

leucocephala/grass mixture and natural pasture. *Small Rumin. Res.* 39, 73–85. doi: 10.1016/S0921-4488(00)00174-7

Allison, M. J., Hammond, A. C., and Jones, R. J. (1990). Detection of ruminal bacteria that degrade toxic dihydroxypyridine compounds produced from mimosine. *Appl. Environ. Microbiol.* 56, 590–594. doi: 10.1128/AEM.56.3.590-594.1990

Alves, A. C., Alves, N. G., Ascari, I. J., Junqueira, F. B., Coutinho, A. S., Lima, R. R., et al. (2015). Colostrum composition of santa ines sheep and passive transfer of immunity to lambs. *J. Dairy Sci.* 98, 3706–3716. doi: 10.3168/jds.2014-7992

AlZahal, O., Li, F., Guan, L. L., Walker, N. D., and Mcbride, B. W. (2017). Factors influencing ruminal bacterial community diversity and composition and microbial fibrolytic enzyme abundance in lactating dairy cows with a focus on the role of active dry yeast. *J. Dairy Sci.* 100, 4377–4393. doi: 10.3168/jds.2016-11473

Angarita, E., Molina, I., Villegas, G., Mayorga, O., Chará, J., and Barahona, R. (2015). Quantitative analysis of rumen microbial populations by qPCR in heifers fed on *Leucaena leucocephala* in the Colombian tropical dry forest. *Acta Sci. Anim. Sci.* 37, 135–142. doi: 10.4025/actascianimsci.v37i2.24836

Arguello, A., Castro, N., Batista, M., Moreno-Indias, I., Morales-Delanuez, A., Sanchez-Macias, D., et al. (2008). Chitotriosidase activity in goat blood and colostrum. *J. Dairy Sci.* 91, 2067–2070. doi: 10.3168/jds.2007-0826

Artiles-Ortega, E., de la Fé-Rodríguez, P. Y., Reguera-Barreto, B., Lima-Orozco, R., and Fievez, V. (2021). In vitro rumen degradability of tropical legumes and their secondary metabolites depends on inoculum source. *Res. Square* [Preprint]. doi: 10.21203/rs.3.rs-1090076/v1

Aronesty, E. (2011). *ea-utils: Command-Line Tools for Processing Biological Sequencing Data. Expression Analysis*. Durham: University of Minnesota.

Aye, P., and Adegun, M. (2013). Chemical composition and some functional properties of Moringa, Leucaena and Gliricidia leaf meals. *Agric. Biol. J. North Am.* 4, 71–77. doi: 10.5251/abjna.2013.4.1.71.77

Azevedo, E. B., Poli, C. H. E. C., David, D. B., Amaral, G. A., Fonseca, L., Carvalho, P. C. F., et al. (2014). Use of faecal components as markers to estimate intake and digestibility of grazing sheep. *Livest. Sci.* 165, 42–50. doi: 10.1016/j.livsci.2014.04.018

Belanche, A., Palma-Hidalgo, J. M., Nejjam, I., Jiménez, E., Martín-García, A. I., and Yáñez-Ruiz, D. R. (2020). Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. *J. Dairy Sci.* 103, 5047–5060. doi: 10.3168/jds.2019-18002

Belanche, A., Yáñez-Ruiz, D. R., Detheridge, A. P., Griffith, G. W., Kingston-Smith, A. H., and Newbold, C. J. (2019). Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. *Environ. Microbiol.* 21, 4360–4377. doi: 10.1111/1462-2920.14801

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of southern wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Briceño-Poot, E. G., Ruiz-González, A., Chay-Canul, A. J., Ayala-Burgos, A. J., Aguilar-Pérez, C. F., Solorio-Sánchez, F. J., et al. (2012). Voluntary intake, apparent digestibility and prediction of methane production by rumen stoichiometry in sheep fed pods of tropical legumes. *Anim. Feed Sci. Technol.* 176, 117–122. doi: 10.1016/j.anifeedsci.2012.07.014

Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chaucheyras-Durand, F., Ameilbonne, A., Auffret, P., Bernard, M., Mialon, M.-M., Dunière, L., et al. (2019). Supplementation of live yeast based feed additive in early life promotes rumen microbial colonization and fibrolytic potential in lambs. *Sci. Rep.* 9, 19216–19216. doi: 10.1038/s41598-019-55825-0

Chaucheyras-Durand, F., and Fonty, G. (2001). Establishment of cellulolytic bacteria and development of fermentative activities in the rumen of gnotobiotically-reared lambs receiving the microbial additive Saccharomyces cerevisiae CNCM I-1077. *Reprod. Nutr. Dev.* 41, 57–68. doi: 10.1051/rnd:2001112

Chaucheyras-Durand, F., and Fonty, G. (2002). Influence of a probiotic yeast (*Saccharomyces cerevisiae* CNCM I-1077) on microbial colonization and fermentations in the rumen of newborn lambs. *Microb. Ecol. Health Dis.* 14, 30–36. doi: 10.1080/089106002760002739

Chaucheyras-Durand, F., Walker, N. D., and Bach, A. (2008). Effects of active dry yeasts on the rumen microbial ecosystem: past, present and future. *Anim. Feed Sci. Technol.* 145, 5–26. doi: 10.1016/j.anifeedsci.2007.04.019

Codagnone, M. G., Stanton, C., O'mahony, S. M., Dinan, T. G., and Cryan, J. F. (2019). Microbiota and neurodevelopmental trajectories: role of maternal and early-life nutrition. *Ann. Nutr. Metab.* 74(Suppl. 2), 16–27. doi: 10.1159/000499144

De Barbieri, I., Hegarty, R. S., Silveira, C., Gulino, L. M., Oddy, V. H., Gilbert, R. A., et al. (2015a). Programming rumen bacterial communities in newborn Merino lambs. *Small Rumin. Res.* 129, 48–59. doi: 10.1016/j.smallrumres.2015.05.015

De Barbieri, I., Hegarty, R. S., Silveira, C., and Oddy, V. H. (2015b). Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of Merino lambs. *Small Rumin. Res.* 129, 37–47. doi: 10.1016/j.smallrumres.2015.05.017

Debruyne, S., Ruiz-González, A., Artiles-Ortega, E., Ampe, B., Van Den Broeck, W., De Keyser, E., et al. (2018). Supplementing goat kids with coconut medium chain fatty acids in early life influences growth and rumen papillae development until 4 months after supplementation but effects on in vitro methane emissions and the rumen microbiota are transient. *J. Anim. Sci.* 96, 1978–1995. doi: 10.1093/jas/sky070

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

Derakhshani, H., Corley, S. W., and Al Jassim, R. (2016). Isolation and characterization of mimosine, 3, 4 DHP and 2, 3 DHP degrading bacteria from a commercial rumen inoculum. *J. Basic Microbiol.* 56, 580–585. doi: 10.1002/jobm.201500590

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Dominguez-Bello, M. G., and Stewart, C. S. (1991). Characteristics of a rumen Clostridium capable of degrading mimosine, 3(oh)-4-(1h)-pyridone and 2,3 dihydroxypyridine. *Syst. Appl. Microbiol.* 14, 67–71. doi: 10.1016/S0723-2020(11)80363-2

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Elsohaby, I., Cameron, M., Elmoslemany, A., Mcclure, J. T., and Keefe, G. (2019). Effect of passive transfer of immunity on growth performance of preweaned dairy calves. *Can. J. Vet. Res.* 83, 90–96.

Faubladier, C., Julliand, V., Danel, J., and Philippeau, C. (2013). Bacterial carbohydrate-degrading capacity in foal faeces: changes from birth to pre-weaning and the impact of maternal supplementation with fermented feed products. *Br. J. Nutr.* 110, 1040–1052. doi: 10.1017/S0007114512006162

Furman, O., Shenhav, L., Sasson, G., Kokou, F., Honig, H., Jacoby, S., et al. (2020). Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. *Nat. Commun.* 11:1904. doi: 10.1038/s41467-020-15652-8

Galindo, J., Delgado, D., Pedraza, R., and García, D. E. (2005). Impacto de los árboles, los arbustos y otras leguminosas en la ecología ruminal de animales que consumen dietas fibrosas. *Past. Forraj.* 28, 59–68.

Galindo, J., García, C., Marrero, Y., Castillo, E., Aldana, A. I., Torres, V., et al. (2007). Efecto de la composición del pastizal de *Leucaena leucocephala* con

gramíneas en la población microbiana ruminal de toros. *Rev. Cubana Cien. Agríc.* 41, 145–148.

Galindo, J., Marrero, Y., Ruiz, T., González, N., Díaz, A., Aldama, A. I., et al. (2009). Efecto de una mezcla múltiple de leguminosas herbáceas y *Leucaena leucocephala* en la población microbiana y productos fermentativos del rumen de añojos mestizos de Cebú. *Rev. Cub. Cien. Agríc.* 43, 260–264.

Graham, S. R., Dalzell, S. A., Ngu, N. T., Davis, C. K., Greenway, D., Mcsweeney, C. S., et al. (2013). Efficacy, persistence and presence of *Synergistes jonesii* in cattle grazing leucaena in Queensland: on-farm observations pre- and post-inoculation. *Anim. Prod. Sci.* 53, 1065–1074. doi: 10.1071/AN12301

Hai, P. V., Everts, H., Tien, D. V., Schonewille, J. T., and Hendriks, W. H. (2012). Feeding Chromonaela odorata during pregnancy to goat dams affects acceptance of this feedstuff by their offspring. *Appl. Anim. Behav. Sci.* 137, 30–35. doi: 10.1016/j.applanim.2012.01.010

Hai, P. V., Schonewille, J. T., Dam Van, T., Everts, H., and Hendriks, W. H. (2016). Exposure to a novel feedstuff by goat dams during pregnancy and lactation versus pregnancy alone does not further improve post-weaning acceptance of this feedstuff by their kids. *J. Sci. Food Agric.* 96, 2215–2219. doi: 10.1002/jsfa.7338

Hai, P. V., Schonewille, J. T., Tien, D. V., Everts, H., and Hendriks, W. H. (2013). Improved acceptance of Chromonaela odorata by goat kids after weaning is triggered by in utero exposure but not consumption of milk. *Appl. Anim. Behav. Sci.* 146, 66–71. doi: 10.1016/j.applanim.2013.03.011

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Jami, E., White, B. A., and Mizrahi, I. (2014). Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS One* 9:e85423. doi: 10.1371/journal.pone.0085423

Jones, R. J., and Megarrity, R. G. (1986). Successful transfer of DHP-degrading bacteria from Hawaiian goats to Australian ruminants to overcome the toxicity of Leucaena. *Austr. Vet. J.* 63, 259–262. doi: 10.1111/j.1751-0813.1986.tb02990.x

Kearl, L. C. (1982). *Nutrient Requirements of Ruminants in Developing Countries*. Logan: International Feedstuffs Institute.

Kim, S. W., Less, J. F., Wang, L., Yan, T., Kiron, V., Kaushik, S. J., et al. (2019). Meeting global feed protein demand: challenge, opportunity, and strategy. *Annu. Rev. Anim. Biosci.* 7, 221–243. doi: 10.1146/annurev-animal-030117-014838

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

Koike, S., and Kobayashi, Y. (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.* 204, 361–366. doi: 10.1111/j.1574-6968.2001.tb10911.x

Lima, R., Díaz, R. F., Castro, A., and Fievez, V. (2011). Digestibility, methane production and nitrogen balance in sheep fed ensiled or fresh mixtures of sorghum–soybean forage. *Livest. Sci.* 141, 36–46. doi: 10.1016/j.livsci.2011.04.014

Mackie, R. I., Gilchrist, F. M. C., Robberts, A. M., Hannah, P. E., and Schwartz, H. M. (1978). Microbiological and chemical changes in the rumen during the stepwise adaptation of sheep to high concentrate diets. *J. Agric. Sci.* 90, 241–254. doi: 10.1017/S0021859600055313

McSweeney, C. S., Padmanabha, J., Halliday, M. J., Hubbard, B., Dierens, L., Denman, S. E., et al. (2019). Detection of Synergistes jonesii and genetic variants in ruminants from different geographical locations. *Trop. Grassl. Forr. Trop.* 7, 154–163. doi: 10.17138/tgft(7)154-163

Miranda, R. L. A., Mendoza, M. G. D., Bárcena-Gama, J. R., González, M. S. S., Ferrara, R., Ortega, C. M. E., et al. (1996). Effect of Saccharomyces cerevisiae or *Aspergillus oryzae* cultures and NDF level on parameters of ruminal fermentation. *Anim. Feed Sci. Technol.* 63, 289–296. doi: 10.1016/S0377-8401(96)01008-5

Moreno-Indias, I., Morales-Delanuez, A., Hernández-Castellano, L. E., Sánchez-Macías, D., Capote, J., Castro, N., et al. (2012). Docosahexaenoic acid in the goat kid diet: effects on immune system and meat quality1. *J. Anim. Sci.* 90, 3729–3738. doi: 10.2527/jas.2011-4351

Newbold, C. J., and Ramos-Morales, E. (2020). Review: Ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal* 14, s78–s86. doi: 10.1017/S1751731119003252

Peng, Q.-H., Cheng, L., Kang, K., Tian, G., Al-Mamun, M., Xue, B., et al. (2020). Effects of yeast and yeast cell wall polysaccharides supplementation on beef cattle growth performance, rumen microbial populations and lipopolysaccharides production. *J. Integr. Agric.* 19, 810–819. doi: 10.1016/S2095-3119(19)62708-5

Pinloche, E., Mcewan, N., Marden, J. P., Bayourthe, C., Auclair, E., and Newbold, C. J. (2013). The effects of a probiotic yeast on the bacterial diversity and population structure in the rumen of cattle. *PLoS One* 8:e67824. doi: 10.1371/journal.pone.0067824

Robison, J. D., Stott, G., and Denise, S. (1988). Effects of passive immunity on growth and survival in the dairy heifer. *J. Dairy Sci.* 71, 1283–1287. doi: 10.3168/jds.S0022-0302(88)79684-8

SPSS (2012). *Software for Windows, Release 21.0, Inc*. Chicago, IL: Property of IBM Corporation.

Staroscik, A. (2004). Calculator for determining the number of copies of a template. *URI Genom. Sequen. Center* 19:2012.

Tizard, I. (2013). *Veterinary Immunology*, 9th Edn. St. Louis, MO: Elsevier Inc.

Vaithiyanathan, S., Sheikh, Q., and Kumar, R. (2005). Effect of transinoculation of goat rumen liquor on degradation and metabolism of mimosine in sheep fed with *Leucaena leucocephala* leaves. *Asian-Australas. J. Anim. Sci.* 18, 332–339.

Vega, A. M., Herrera, R. S., Torres, V., Lamela, L., Montejo, I., Santana, A., et al. (2016). Performance of replacement cuban charolais females in a silvopastoral system compared to a monoculture. *Cuban J. Agric. Sci.* 50, 51–59.

Wang, L., Xu, Q., Kong, F., Yang, Y., Wu, D., Mishra, S., et al. (2016). Exploring the goat rumen microbiome from seven days to two years. *PLoS One* 11:e0154354. doi: 10.1371/journal.pone.0154354

Wang, L., Zhang, K., Zhang, C., Feng, Y., Zhang, X., Wang, X., et al. (2019). Dynamics and stabilization of the rumen microbiome in yearling Tibetan sheep. *Sci. Rep.* 9:19620. doi: 10.1038/s41598-019-56206-3

Wang, X., Martin, G. B., Wen, Q., Liu, S., Zhang, J., Yu, Y., et al. (2019). Linseed oil and heated linseed grain supplements have different effects on rumen bacterial community structures and fatty acid profiles in cashmere kids1. *J. Anim. Sci.* 97, 2099–2113. doi: 10.1093/jas/skz079

Wang, Y., Cao, P., Wang, L., Zhao, Z., Chen, Y., and Yang, Y. (2017). Bacterial community diversity associated with different levels of dietary nutrition in the rumen of sheep. *Appl. Microbiol. Biotechnol.* 101, 3717–3728. doi: 10.1007/s00253-017-8144-5

Weimer, P. J., Stevenson, D. M., Mantovani, H. C., and Man, S. L. C. (2010). Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. *J. Dairy Sci.* 93, 5902–5912. doi: 10.3168/jds.2010-3500

Welty, C. M., Wenner, B. A., Wagner, B. K., Roman-Garcia, Y., Plank, J. E., Meller, R. A., et al. (2019). Rumen microbial responses to supplemental nitrate. II. Potential interactions with live yeast culture on the prokaryotic community and methanogenesis in continuous culture. *J. Dairy Sci.* 102, 2217–2231. doi: 10.3168/jds.2018-15826

Wobbrock, J. O., Findlater, L., Gergle, D., and Higgins, J. J. (2011). "The aligned rank transform for nonparametric factorial analyses using only anova procedures," in *Proceedings of the SIGCHI Conference on Human Factors in Computing Systems*, Vancouver, BC, 143–146.

Xiong, Y., Pang, J., Lv, L., Wu, Y., Li, N., Huang, S., et al. (2019). Effects of maternal supplementation with rare earth elements during late gestation and lactation on performances, health, and fecal microbiota of the sows and their offspring. *Animals* 9:738. doi: 10.3390/ani9100738

Yanez-Ruiz, D. R., Macias, B., Pinloche, E., and Newbold, C. J. (2010). The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. *FEMS Microbiol. Ecol.* 72, 272–278. doi: 10.1111/j.1574-6941.2010.00852.x

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 36, 808–812. doi: 10.2144/04365st04

Zhang, Q., Li, C., Niu, X., Zhang, Z., Li, F., and Li, F. (2019). The effects of milk replacer allowance and weaning age on the performance, nutrients digestibility, and ruminal microbiota communities of lambs. *Anim. Feed Sci. Technol.* 257:114263. doi: 10.1016/j.anifeedsci.2019.114263

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 March 2022
doi: 10.3389/fmicb.2022.780073

Effect of Long-Term Supplementation With Silkworm Pupae Oil on the Methane Yield, Ruminal Protozoa, and Archaea Community in Sheep

Govindasamy Thirumalaisamy[1,2], Pradeep Kumar Malik[1], Shraddha Trivedi[1], Atul Purushottam Kolte[1] and Raghavendra Bhatta[1]*

[1] ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India, [2] ICAR-National Dairy Research Institute, Karnal, India

OPEN ACCESS

Edited by:
Robert W. Li,
Agricultural Research Service (USDA), United States

Reviewed by:
Takehiro Nishida,
Obihiro University of Agriculture and Veterinary Medicine, Japan
Paul E. Smith,
Teagasc Grange Animal and Bioscience Research Department (ABRD), Ireland
Yutaka Uyeno,
Shinshu University, Japan

***Correspondence:**
Pradeep Kumar Malik
malikndri@gmail.com
orcid.org/0000-0002-6581-4772

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: 20 September 2021
Accepted: 27 January 2022
Published: 08 March 2022

Citation:
Thirumalaisamy G, Malik PK, Trivedi S, Kolte AP and Bhatta R (2022) Effect of Long-Term Supplementation With Silkworm Pupae Oil on the Methane Yield, Ruminal Protozoa, and Archaea Community in Sheep. Front. Microbiol. 13:780073. doi: 10.3389/fmicb.2022.780073

Supplementation with lipids and oils is one of the most efficient strategies for reducing enteric methane emission. However, high costs and adverse impacts on fiber degradation restrict the use of conventional oils. Silkworm pupae, a non-conventional oil source rarely used for human consumption in India, could be one of the cheaper alternatives for methane mitigation. The objective of this study was to investigate the effect on sheep of long-term supplementation (180 days) of silkworm pupae oil (SWPO) with two distinct supplementation regimes (daily and biweekly) on daily enteric methane emission, methane yield, nutrient digestibility, rumen fermentation, ruminal archaea community composition, and protozoal population. The effect of the discontinuation of oil supplementation on enteric methane emission was also investigated. Eighteen adult male sheep, randomly divided into three groups (n = 6), were provisioned with a mixed diet consisting of 10.1% crude protein (CP) and 11.7 MJ/kg metabolizable energy formulated using finger millet straw and concentrate in a 55:45 ratio. SWPO was supplemented at 2% of dry matter intake (DMI) in test groups either daily (CON) or biweekly (INT), while no oil was supplemented in the control group (CTR). DMI (p = 0.15) and CP (p = 0.16) in the CON and INT groups were similar to that of the CTR group; however, the energy intake (MJ/kg) in the supplemented groups (CON and INT) was higher (p < 0.001) than in CTR. In the CON group, body weight gain (kg, p = 0.02) and average daily gain (g, p = 0.02) were both higher than in the CTR. The daily methane emission in the CON (17.5 g/day) and INT (18.0 g/day) groups was lower (p = 0.01) than the CTR group (23.6 g/day), indicating a reduction of 23–25% due to SWPO supplementation. Similarly, compared with the CTR group, methane yields (g/kg DMI) in test groups were also significantly lower (p < 0.01). The transient nature of the anti-methanogenic effect of SWPO was demonstrated in the oil discontinuation study, where daily methane emission reverted to pre-supplementation levels after a short period. The recorded methanogens were affiliated to the families *Methanobacteriaceae*, *Methanomassilliicoccaceae*, and *Methanosarcinaceae*. The long-term supplementation

of oil did not induce any significant change in the rumen archaeal community, whereas minor species such as *Group3b* exhibited differing abundance among the groups. *Methanobrevibacter*, irrespective of treatment, was the largest genus, while *Methanobrevibacter gottschalkii* was the dominant species. Oil supplementation in CON and INT compared with CTR decreased ($p < 0.01$) the numbers of total protozoa ($\times 10^7$ cells/ml), *Entodiniomorphs* ($\times 10^7$ cells/ml), and *Holotrichs* ($\times 10^6$ cells/ml). SWPO continuous supplementation (CON group) resulted in the largest reduction in enteric methane emission and relatively higher body weight gain ($p = 0.02$) in sheep.

Keywords: archaea, long-term feeding, methane yield, sheep, silkworm pupae oil

INTRODUCTION

The global livestock sector accounts for 14.5% of anthropogenic greenhouse gas emissions (Gerber et al., 2013). Enteric fermentation alone is accountable for 87–97 Tg of methane produced on an annual basis (Chang et al., 2019). Apart from contributing to global warming, the enteric methane emissions from livestock are also responsible for 2–12% of dietary energy loss (Johnson and Johnson, 1995), where every liter of methane carries 39.5 kJ of energy from the host animal (Guan et al., 2006).

The rumen is an ideal habitat for protozoa, where they live in close association with prokaryotic microbes (Newbold et al., 2015). After bacteria, protozoa are the second most abundant microbes, constituting up to 50% of the rumen biomass (Newbold et al., 2015) with the overall dominance of ciliated protozoa (Morgavi et al., 2010). Rumen protozoa are not essential for animal survival, yet they perform important functions such as protein breakdown, bacterial predation (Williams and Coleman, 1992), reduction of the shedding of potential pathogens (Newbold et al., 2015), lipid metabolism, and shifts in volatile fatty acids production (Eugène et al., 2004). However, their presence in the rumen negatively impacts the energy efficiency of the rumen ecosystem (Newbold et al., 2015). One of the major functions of rumen protozoa is to transfer hydrogen to other microbes, particularly to the methanogens (Li et al., 2018). Methanogens belong to the phylum *Euryarchaeota* (Balch et al., 1979), constituting 3–4% of the rumen microbiota (Yanagita et al., 2000). Rumen protozoa associated methanogens are responsible for 37% of enteric methane emissions (Machmüller et al., 2003). It has been reported that the counts of rumen protozoa are linearly related to methane emissions; however, methanogenesis is also regulated by other mechanisms independent of protozoa (Guyader et al., 2014).

Oil supplementation is a promising method for increasing diet density. In addition, supplementation with lipids effectively reduces enteric methane production (Morgavi et al., 2010). Oil supplementation is considered a promising approach for enteric methane mitigation in ruminants through a shift in rumen fermentation or microbiota composition (Vargas et al., 2020). Commonly used edible oils such as linseed, rapeseed, palm, and canola are expensive (Government of India, 2021). Silkworm pupae oil (SWPO) is non-conventional, inexpensive, and adequately available in India (Srinivas et al., 2019). The oil contains reasonably good combinations of both unsaturated and saturated fatty acids (Thirumalaisamy et al., 2020). Polyunsaturated fatty acids reduce the abundance of protozoa and, therefore, can be one of the potential mitigation options for reducing enteric methane emissions (Guyader et al., 2017).

Recently, the supplementation of SWPO in a short-term study decreased the enteric methane emission of sheep (Thirumalaisamy et al., 2020). Recently, Jayanegara et al. (2020) evaluated oils from five different insect sources for their impact on *in vitro* methane and concluded that mealworm and cricket oil supplementation at 5% effectively decreased methane production by 26–33%.

Due to adaptability of the rumen ecosystem, the methane inhibition is usually short-lived (Mathison et al., 1998), and animals return to normal methane emissions after withdrawal of ameliorating supplement from the diet (Hristov et al., 2013). Although this is a known issue, nevertheless, very few studies were undertaken to evaluate the impact of long-term supplementation on methane emission. Therefore, studies are needed to establish the persistency of methane mitigation in the long term (Beauchemin et al., 2020). Previous research (Thirumalaisamy et al., 2020) has shown that the short-term supplementation of SWPO significantly reduces enteric methane emission, which encouraged us to evaluate the effect over the long term. We hypothesized that long-term supplementation of SWPO may decrease methane emissions and methane yield by altering the archaeal community composition and reducing ruminal protozoa. Therefore, the current study was designed to investigate (1) the effects of long-term supplementation of SWPO in two distinct supplementation regimes (daily and biweekly) on daily enteric methane emission, methane yield (g/kg dry matter intake), nutrient digestibility, rumen fermentation, ruminal archaea diversity, and protozoal numbers in sheep, and (2) whether discontinuation of oil supplementation affects methane emission.

MATERIALS AND METHODS

Animals and Feeding

The animal study was approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry, and Dairying, Government of India (approval no. 25/14/2017-CPCSEA). All procedures

were carried out as per the guidelines of the Institute Animal Ethics Committee.

A 180-day-long animal study was carried out in (n = 18) 18-month-old male *Mandya* sheep (BW 24.1 ± 0.27 kg), randomly divided into three groups of six. The animals were housed in a well-ventilated shed constructed with half cemented walls and half iron mesh. The iron mesh was fixed into the cemented walls at 1.8 m above the ground on the east and west sides. The sheep were housed in individual pens having free access to clean drinking water and feed throughout the day. Initially, all the animals were dewormed with anthelminthic fenbendazole at 5 mg/kg BW. The animals were offered a total mixed ration (TMR) formulated with 55% finger millet straw (*Eleusine coracana*) and 45% concentrate mixture. The TMR was formulated to meet nutrient requirement as per ICAR (2013) feeding standards . The concentrate mixture was prepared using maize grain (320 g/kg), wheat bran (400 g/kg), soybean meal (130 g/kg), groundnut cake (120 g/kg), mineral mixture (20 g/kg), and salt (10 g/kg). The TMR offered to the animals in different groups was similar in all nutritional aspects and contained 10.1% crude protein (CP) and 11.7 MJ/kg metabolizable energy (ME). The TMR was offered *ad libitum* twice a day at 09:00 and 14:00, and clean drinking water was accessible throughout the day.

The oil extracted from dried silkworm pupae using the solvent extraction method (*n*-hexane) was purchased from a local vendor in the suburb of Bangalore and stored in an airtight container. The SWPO was filled in a plastic container (2 L; NJ Phillips, Australia) connected to an automatic oral drenching gun (NJ Phillips, part no SH46) for administering the required volume. The oil was supplemented at 2% of dry matter intake either daily (CON) or biweekly (INT), whereas animals in the control group (CTR) were offered TMR without oil. Thus, the animals in the INT group received oil daily for 1 week and then this was discontinued for the subsequent week.

Dry matter intake (DMI, g/day) was recorded at monthly interval for three consecutive days throughout the experiment to adjust SWPO volume (w/v) in the CON and INT groups.

Chemical Analysis

The chemical composition of the feed ingredients, TMR, refusals, and feces were analyzed in triplicate. The dry matter content in the samples was determined as per AOAC (2012) in a hot air oven at 100°C for 12 h, and dried samples were ground using a Cyclotec mill. For determining total ash, the samples were initially burnt in crucibles on a hot plate and later transferred to a muffle furnace at 550°C for 4 h (AOAC, 2012). Organic matter (OM) was determined by subtracting the total ash from the initial dry weight of the sample and expressed as a percentage. Crude protein (CP, N × 6.25) was determined as per AOAC (2005) using an automatic nitrogen analyzer (Gerhardt, Germany). The crude fiber (CF) and fiber fractions such as neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using an automatic fiber analyzer (Fibretherm FT12, Gerhardt, Germany) in accordance with AOAC (2005) and Van Soest et al. (1991). The ether extract (EE) was estimated using Soxtherm (Gerhardt, Germany) as per the standard method (AOAC, 2005). The gross energy content (kcal/100 g DM) of TMR and feed ingredients was calculated following the equation of Crisan and Sands (1978), and expressed as metabolizable energy (MJ/kg DM) after considering digestible and urinary losses. The chemical composition (g/kg DM) is presented in **Table 1**.

Ammonia-N concentration in the ruminal fluid samples was determined as per Conway (1957) following the microdiffusion technique. The microdiffusion cell has two chambers—1 ml of the mixed boric acid (2%) indicator (methyl red 66 mg, methylene blue 33 mg in 100 ml alcohol) was pipetted into the inner chamber while an equal volume of saturated sodium carbonate was placed in the outer chamber of the disc. A measure of 1 ml of strained ruminal fluid was pipetted into the outer chamber directly opposite to the sodium carbonate, and then the microdiffusion cell was immediately covered with the lid. To allow the mixing of the contents of the outer chamber, the disc was gently rotated and incubated for 2 h at room temperature. After the incubation, the contents of the inner chamber were titrated against 0.01 N sulfuric acid until the color turned pink. Ammonia-N was determined using the following formula:

$$\text{Ammonia} - \text{N}\ \left(\text{mg/dl}\right)\ =\ \text{ml of}\ 0.001\ \text{N}H_2SO_4\ \times\ 1$$

Volatile fatty acids (VFA) concentration in the ruminal fluid samples was determined according to Filípek and Dvoøák (2009) using a gas chromatograph (Agilent 7890B, Santa Clara, United States). In brief, preserved metaphosphoric acid mixed supernatant samples were thawed at room temperature, and after a short spinning at 13,000 rpm for 5 min, about 1.5 ml of the sample was transferred to GC screw vials (2 ml; Agilent Technologies). The screw vials containing fluid samples and the VFA standard and washing solvent were placed in the automatic injector port (G4513A). The injector dispensed a fixed volume (1 μl) of the sample while maintaining the 20:1 splitting ratio. The injector was equipped with a glass liner containing glass wool to separate dirt particles from the sample. Nitrogen gas (N_2) at a flow rate of 2 ml per min was used as a carrier gas. The gas chromatograph was equipped with the FFAP column (CP7485, 25 m × 0.32 mm × 0.30 μm; Agilent Technologies) and flame ionization detector (FID). The following conditions were maintained during the analysis: temperature program, 59–250°C (20°C/min, 10 min); injector temperature, 230°C; detector temperature, 280°C. The analysis time was approximately 16.7 min. The concentration of individual volatile fatty acids was determined using the following formula:

$$\text{VFA con.(mmol)}\ =\ \frac{\text{Peak area of sample}\ \times\ \text{Conc. of standard}\ \times \text{dilution}}{\text{Peak area of standard}}$$

Total metabolic hydrogen produced was calculated using the concentration of volatile fatty acids as per the equation of Marty and Demeyer (1973) and hydrogen utilized (%) in methane was calculated based on daily enteric methane emission.

$$\text{Total 2H produced}\ =\ 2\text{A} + \text{P} + 4\text{B} + 3\text{V}$$

TABLE 1 | Chemical composition (g/kg DM) of feed ingredients and TMR.

Attributes	Ingredients/diet						
	Finger millet straw	Concentrate	Maize	Soybean meal	Groundnut cake	Wheat bran	TMR
OM	910	942	982	914	942	982	928
CP	36.5	193	91.5	447	424	141	101
EE	10.3	32.2	47.4	101	57.9	47.4	20.1
NDF	670	460	514	498	354	514	618
ADF	508	131	93.1	262	224	93.1	357
CF	330	43.9	26.8	94.1	106	268	255
TA	89.7	58.0	18.3	85.8	57.7	18.3	71.7
NFE	429	579	711	266	262	711	452
ME* (MJ/kg)	11.6	11.6	12.8	9.50	10.7	12.8	11.7

*OM, organic matter; CP, crude protein (N_2 × 6.25); EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; CF, crude fiber; TA, total ash; TMR, total mixed ration. Nitrogen-free extract (NFE) was calculated by subtracting the %CP + %EE + %CF + %TA + %moisture from 100. ME is the metabolizable energy, calculated from the gross energy of feed by multiplying by 0.82. *The energy value of TMR plus oil was 12.24 MJ.*

Body Weight, Nutrient Digestibility, and Methane Measurement

During the entire experimental period (180 days), before morning feeding the animals were weighed at monthly intervals on a digital balance (Blue Bird, 300 kg). Their total weight gain (kg) was calculated as the difference between final and initial live-weight, and average daily gain (ADG, g/day) was calculated by dividing total weight gain by the number of experimental days.

A digestibility trial was conducted for 10 days between days 171 and 180, and apparent nutrient digestibility was determined. In brief, the quantity of daily TMR offered, feed refusals, and feces were recorded and the representative samples were collected from the individual animal. The DMI (g/day) of the individual animal was calculated by subtracting the quantity of dry refusals from the feed offered. The energy intake was calculated by considering daily DMI (g/day) and energy content of TMR (ME, MJ/kg DM), as described under chemical analysis. The digestibility coefficient of various nutrients was determined by using the following equation:

$$\text{Digestibility coefficient} = \frac{\text{Nutrient intake} - \text{Excretion of nutrient}}{\text{Intake of nutrient}}$$

The sulfur hexafluoride (SF_6) tracer technique (Berndt et al., 2014) was employed for the quantification of daily enteric methane emission for 10 consecutive days (days 171–180). During methane measurements, animals in the INT group received SWPO from days 171 to 175, whereas the remaining 5 days (176–180) were under the supplementation break. The brass permeation tubes (8.5 mm diameter, 34 mm long, 4.8 mm wide bore, and 30 mm deep with blind hole) fitted with a Swagelok nut (7 mm diameter) were used as a source of SF_6. A Teflon septum (0.24 mm PTFE) was placed below the SS frit (3/8″ OD, 2 μm pore size), and the tubes were closed. The permeation tubes were charged with 750 ± 48.18 mg SF_6 (99.9%) in liquid nitrogen and retained in an incubator at 39°C for 70 days. The tubes were monitored and weighed weekly on a balance (Denver, Germany; 210 g accuracy ± 0.1 mg) and calibrated for release over 8 weeks. The release rate was calculated by linear regression considering the difference in tube weights during the calibration period. The calibrated tubes were placed in the rumen 10 days before the commencement of daily methane measurements. The mean SF_6 release rate from the permeation tubes in this study was 3.47 ± 0.46 mg/day. The PVC canister for the background sample was connected to the nylon and capillary tubes (Supelco, Cat#56712-U, ID 1/16) and Quick connectors (Swagelok, Cat#B-QC4-D-200) assembled by following the standard guidelines (Williams et al., 2014). The canister for the daily background sample was hung on the iron wire mesh fixed in the cemented wall on the east side. The vacuumized PVC canisters (> 95 kPa) were tied individually to collect breath samples for 24 h. Similar timings were maintained for tying and removal of the canisters throughout the measurement period. The post-collection canister pressure was measured by a digital pressure meter (Leo 2, Keller, Switzerland). Thereafter, the breath and background samples in the canister were diluted (2.15–3.0-fold) with high-purity N_2 gas, and pressure was measured to calculate the dilution factor.

The successive subsamples were collected in a gas airtight glass syringe (Hamilton, 1 ml) for analyzing the methane and SF_6 concentrations using a gas chromatograph (GC 2010 plus; Shimadzu, Japan). The GC was fitted with a FID and electron capture detector for the analysis of methane and SF_6 concentrations, respectively. In brief, the following chromatographic conditions were maintained for the SF_6 analysis: inlet temperature of 100°C, column temperature of 40°C, detector temperature of 250°C, airflow rate of 400 ml/min, hydrogen flow rate 40 ml/min, and nitrogen flow rate of 30 ml/min. In contrast, the following conditions were applied for the methane analysis: inlet temperature of 100°C, column temperature of 60°C, detector temperature of 150°C, airflow rate of 400 ml/min, hydrogen flow rate of 40 ml/min, and nitrogen flow rate of 30 ml/min. Methane (ppm) and SF_6 (ppt) concentrations were calculated using the canister pressures at different time points (Lassey et al., 2014) with a slight modification for local elevation and atmospheric pressure:

$$[G_S] = \frac{90 - \tau_f}{\tau_e - \tau_s} \times [G_A]$$

G_S represented the concentration of methane (ppm) or SF_6 (ppt) at an atmospheric pressure of 90 kPa and elevation of 920 m. τ_f (kPa) was the final vacuum in the canister after N_2 dilution, τ_s (kPa) is the post-sampling vacuum in the canister, τ_e is the vacuum in the evacuated canister, and G_A is the concentration of methane (ppm) or SF_6 (ppt) in the samples presented to the GC.

Daily enteric methane emissions were calculated using the equation of Moate et al. (2014).

$$R_{CH4} = R_{SF6}\frac{[CH_4]_M - [CH_4]_{BG}}{[SF_6]_M - [SF_6]_{BG}} \times \frac{MW_{CH_4}}{MW_{SF_6}} \times 1,000$$

R_{CH4} is the CH_4 emission (g/day), R_{SF6} is the SF_6 release rate from the tubes (mg/day), $[CH_4]_M$–$[CH_4]_{BG}$ is the methane concentration (ppm) in the sample and background, $[SF_6]_M$–$[SF_6]_{BG}$ is the methane concentration (ppm) in the sample and background, and MW_{CH4} and MW_{SF6} represent the molecular mass of CH_4 and SF_6, respectively.

The DMI (g/day) along with daily methane emission (g/day) recorded over 10 days during methane measurement trials were used for the calculation of methane yield (MY, g/kg DMI). Daily methane emission (g/day) was divided by the mean DMI (g/day) over the measurement period. After 180 days, oil supplementation for the CON and INT groups was discontinued, and all the animals received a similar basal diet without SWPO. After 30 days of oil withdrawal, daily enteric methane emission was again quantified for 10 days along with the recording of DMI (g/day). The enteric methane emission was measured following the method described previously in this section.

Ruminal Fluid Collection

The day that the methane measurements ended (day 180), rumen fluid samples were collected 3 h post-feeding from each sheep using a nylon stomach tube (length 1 m). The stomach tube and handheld vacuum pump (Mityvac 8,000; Lincoln Industrial, St. Louis, United States) were connected to a sample collection vessel. The first 30 ml of rumen fluid was discarded to avoid saliva contamination and then 45 ml of rumen fluid was collected and strained through a muslin cloth. The tubes containing strained ruminal fluid samples for DNA isolation and fermentation parameters (two sets of 15 ml each) were placed on ice for immediate transportation to the laboratory. Another set of tubes containing 15 ml of strained fluid were transported to the laboratory without placement in an icebox for protozoal enumeration. The first subset was centrifuged at 13,600 × *g* for 15 min, and after adding 2–3 drops of saturated $HgCl_2$ and metaphosphoric acid (25%) in 1:4 (v/v), the supernatant was preserved for ammonia-N and VFA estimation, respectively. The second subset of strained fluid was used for protozoal enumeration on the same day, whereas the third subset of ruminal fluid was centrifuged at low speed (1,000 rpm, 5 min) and the supernatant was preserved at −80°C until used in the process of DNA isolation.

Protozoal Enumeration

The protozoa in the rumen fluid were enumerated as per the method of Kamra and Agarwal (2003) using a Neubauer counting chamber. In brief, a 5-ml sample was pipetted into a screw cap tube containing an equal volume of 37% formaldehyde. Two drops of methyl green (70 μl) and glacial acetic acid (2 ml) diluted to 100 ml with distilled water were added to it and stored overnight at room temperature. The ruminal protozoa were enumerated in 30 microscopic fields. Ruminal protozoa enumeration and morphological identification were carried out under a phase-contrast microscope (Nikon Eclipse, Japan) at ×10 objective. The ruminal protozoa based on the morphology and cilia distribution were categorized as *Entodiniomorphs* and *Holotrichs* (Hungate, 1966). The protozoal numbers in the rumen fluid were calculated using the following formula:

$$N = \frac{n \times A \times D}{a \times v}$$

where N is the number of protozoa (cells) in 1 ml of rumen fluid, n is the average cell count per microscopic field, A is the area of the slide on which the diluted rumen fluid sample spread, D is the dilution of rumen fluid, a is the area of the microscopic field, and v is the volume of rumen fluid in the cavity. The protozoal numbers were expressed as $\times 10^7$ or $\times 10^6$ cells/ml.

Following the methane measurements in the oil discontinuation study, the rumen protozoa in each group were enumerated as per the method described previously.

DNA Isolation

The samples were initially centrifuged at 13,400 × *g* and 4°C for 10 min, and the pellet was retained by discarding the supernatant. Thereafter, 1 ml of lysis buffer as described by Yu and Morrison (2004) was added to dissolve the pellet by gentle pipetting. The contents were transferred to a 2-ml sterile screw cap tube with an O-ring (BioSpec, United States) and contained 0.5 g (0.1 mm diameter) pre-sterilized zirconia beads (BioSpec, United States). The RBB+C method of Yu and Morrison (2004) was employed for genomic DNA isolation. Genomic DNA quality was checked using 0.8% agarose gel electrophoresis, while the DNA concentration was ascertained with Qubit 4.0 (Thermo Fisher Scientific, Waltham, United States).

Library Preparation and Sequencing

Amplicon libraries were prepared using a Nextra XT kit (Illumina Inc., San Diego, United States). The archaea-specific primer sequences *Arch-344F* (Wemheuer et al., 2012) and *Arch-806R* (Takai and Horikoshi, 2000) were synthesized along with Illumina recommended adapters and error-correcting barcodes unique to each sample. The PCR amplification was performed as follows: initial denaturation at 95°C for 3 min followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. A reaction without template was used as a negative control, whereas the *Methanobrevibacter smithii* DNA template was used as a positive control during the PCR amplification. All 18 amplicon libraries were purified with AMPureXP beads (Beckman Coulter Life Sciences, United States) and analyzed individually on a 4,200 Tape Station (Agilent Technologies, United States). The libraries were multiplexed (10–20 pM of

each) and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) using MiSeq reagent kit v3, and 2 × 300 bp paired-end reads were generated to obtain approximately 0.1 million sequences per library.

Statistical Analysis

All the data were checked for a normal (Gaussian) distribution in GraphPad Prism version 9 (GraphPad Software, San Diego, United States) using the D'Agostino–Pearson normality test at the 0.05 alpha level. The data pertaining to daily methane emissions, nutrient digestibility, fermentation, and rumen protozoa were analyzed in SPSS version 21.0 (IBM SPSS, United States) using ANOVA with the following model.

$$Y_{ij} = \mu + A_i + \varepsilon_{ij}$$

Y_{ij} represents individual observation, μ represents the population mean, A_i represents the treatment effect, and ε_{ij} represents experimental error. The difference among means was compared using Tukey's *post hoc* method and considered significant at $p \leq 0.05$. The impact of SWPO withdrawal and previous methane emissions were compared with paired *t*-tests in GraphPad Prism version 9. The correlation matrix between multiple variables was performed in GraphPad Prism version 9, and Pearson correlation coefficients (r) considering Gaussian distributions were calculated at the 95% CI.

Bioinformatics Analysis

The amplicon sequences were processed using DADA2 v1.16 (Callahan et al., 2016) in R v4.0.2. The sequencing quality was assessed using the functions plotQualityProfile, dereplication, denoising, and merging. Chimeras were removed from the filtered reads using the function removeBimeraDenovo. Fasta files compatible with DADA2 for taxonomy assignments were generated using Rumen and Intestinal Methanogen-DB (Seedorf et al., 2014). Furthermore, the archaea-specific database RIM-DB was used for taxonomy classification, and the annotated taxonomy table from DADA2 was imported into phyloseq (McMurdie and Holmes, 2013) in R. The ASVs (amplicon sequence variants) with low abundance were pruned and rarefied to the lowest numbers (31,044 sequences) to examine the archaeal diversity measures. The rarefaction curve was plotted using the vegan package V2.0-7 (Oksanen et al., 2013), and the Shannon index and *post hoc* comparisons were performed using Wilcoxon rank sum tests. The multivariate homogeneity of group dispersion was tested using the betadisper function of the vegan package. The ASV count data were scaled to per 10,000 sequences, taxonomically annotated at various ranks, and then the relative abundance plots were generated using ggplot2 (Wickham, 2011). Differential abundance was calculated using DESeq2 and significance analysis was performed using the Wald parametric test with Benjamini–Hochberg correction (Love et al., 2014). The diet effect on the diversity of methanogens at different taxonomic ranks was studied across groups and tested for significance using PERMANOVA with 999 permutations. The core microbiome analysis was performed in microbiome V1.4.1 (Lahti and Shetty, 2012) in R by keeping a minimum prevalence and detection threshold of 50% and 0.01, respectively.

RESULTS

Intake, Digestibility, and Average Daily Gain

The mean DMI in the CTR (884 g/day), CON (900 g/day), and INT (893 g/day) groups was similar ($p = 0.15$), and there was no adverse impact of the long-term (180 days) SWPO supplementation on the DMI in the sheep (**Table 2**). The mean DMI during withdrawal period in CTR (967 g/day), CON (1,014 g/day), and INT (988 g/day) groups was also similar ($p = 0.176$). The intake (g/day) of OM, CP, NDF, and ADF in the CON and INT groups did not differ from the CTR group. However, the intake of EE in the CON (36.2 g/day) and INT (36.0 g/day) groups was significantly higher ($p < 0.01$) than in the CTR group (18.0 g/day). Similarly, the ME intake in CON (11.0 MJ/day) and INT (10.7 MJ/day) groups was also significantly higher ($p < 0.01$) than in the CTR group (10.3 MJ/day). The apparent digestibility (%) of all the nutrients except EE in the CON and INT groups was comparable with the CTR group.

TABLE 2 | Effect of silkworm pupae oil supplementation on intake and nutrient digestibility.

Attributes	CTR	CON	INT	SEM	*P*-value
Initial BW (kg)	24.1	24.1	24.1	0.273	0.996
Final BW (kg)	29.9^{a}	33.0^{b}	30.8^{ab}	0.492	0.022
BW gain (kg)	5.87	8.88	6.63	0.552	0.058
ADG (g/day)	32.7^{a}	49.3^{b}	36.9^{ab}	3.07	0.024
Intake (g/day)					
DM	884	900	893	3.42	0.155
OM	818	832	826	3.11	0.151
CP	96.1	96.7	96.5	0.13	0.157
NDF	507	518	513	2.29	0.152
ADF	295	304	300	1.74	0.153
EE	18.0^{a}	36.2^{b}	36.0^{b}	0.83	< 0.0001
Energy* (MJ/day)	10.3^{a}	11.0^{c}	10.7^{b}	0.05	< 0.0001
Apparent digestibility (%)					
DM	68.8	67.2	67.9	0.29	0.093
OM	69.7	68.4	68.7	0.28	0.127
CP	67.6	65.9	65.6	0.42	0.109
NDF	63.1	61.7	62.8	0.34	0.210
ADF	48.3	48.0	48.7	0.48	0.842
EE	78.6^{a}	87.6^{b}	88.0^{b}	0.46	< 0.0001
Methane emission					
Daily methane (g/day)	23.6^{c}	17.5^{a}	18.0^{ab}	0.918	0.008
Methane yield (g/kg DMI)	26.7^{c}	19.3^{a}	20.3^{ab}	0.101	0.004
Methane (g/day) during withdrawal period#	23.3	22.8	24.4	2.06	0.208

*CTR, control; CON, daily oil supplementation; INT, biweekly oil supplementation; DM, dry matter; OM, organic matter; CP, crude protein ($N_2 \times 6.25$); NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract. Mean values bearing different superscripts in a row differ significantly ($p < 0.05$). *Estimated using dry matter intake (g/day) and feed energy (ME MJ/kg). #Measurement after 30 days of the withdrawal of oil supplementation.*

Long-term feeding of SWPO led to a higher (p = 0.02) body weight in the CON group (33.0 kg) compared with the CTR group (29.9 kg). However, the difference in body weight between CTR and INT as well as CON and INT after 180 days of SWPO feeding was similar. A significant difference (p = 0.02) in the ADG between CON (49.3 g/day) and CTR (32.7 g/day) was recorded in this study. On the other hand, the difference in ADG (g/day) in sheep between the CTR-INT and CON-INT groups was non-significant. In the present study, a positive correlation (r = 0.53) between the ME intake (MJ/day) and ADG (g/day) was recorded. During the entire experimental period of 180 days, the animals consumed 3.24 and 1.61 L of SWPO in the CON and INT groups, respectively. The corresponding cost of the daily SWPO in the CON and INT groups was INR 0.72 and 0.36, respectively (1 US$ = 75 INR).

Methane Emissions

Daily enteric methane emissions in the CON (17.5 g/day) and INT (18.0 g/day) groups were significantly lower (p < 0.01) than in the CTR group (23.6 g/day). Data on daily methane emissions (g/day) revealed a reduction of about 23–25% in the CON and INT groups due to the daily or biweekly supplementation of SWPO (**Table 2**). However, the daily enteric methane emission (g/day) between the CON and INT groups did not differ significantly (p = 0.008). Similarly, the difference in daily enteric methane emission (g/day) between the supplementation and non-supplementation days in the INT group was not significant (p = 0.77). A uniform comparison of methane emission (MY) among the groups also revealed a significant reduction (p < 0.01) in the MY in CON (19.3 g/kg DMI) and INT (20.3 g/kg DMI) groups compared with that of the CTR group (26.7 g/kg DMI); however, the MY (g/kg DMI) between the CON and INT groups was non-significant. There was a weak negative correlation (r = -0.05; p = 0.80) between the MY and DMI (**Figure 1**). MY was positively correlated with protozoa numbers (r = 0.36) and acetate (r = 0.25). The enteric methane emissions (g/day) among the sheep of different groups were similar (p = 0.208) during the withdrawal period (**Table 2**).

Ammonia, Volatile Fatty Acids, and Metabolic Hydrogen

The ruminal ammonia concentration in the CON and INT groups compared with the CTR group (19.7 mg/100 ml) was significantly lower (p < 0.01). Similarly, the ammonia concentration was also different (p < 0.01) between the CON and INT groups. On the other hand, total volatile fatty acid (TVFA) production was similar (p = 0.99). Despite the similar TVFA concentration, the propionate production with reference to CTR (11.3 mmol) was higher (p < 0.01) in the SWPO supplemented CON (13.0 mmol) and INT (12.3 mmol) groups (**Table 3**). However, variation in the concentration of other VFA between the groups was non-significant. The A/P ratio in the CON and INT groups was significantly lower (p < 0.01) than that in the CTR group.

In the CTR, CON, and INT groups of the present study, the production of metabolic hydrogen was 130, 128.5, and 128.6 mmol; however, the utilization of produced metabolic hydrogen in methanogenesis was 73, 54, and 56% in the respective groups. Results from the study indicated that 16–18% less metabolic hydrogen was utilized in CON and INT groups on account of reduced methanogenesis.

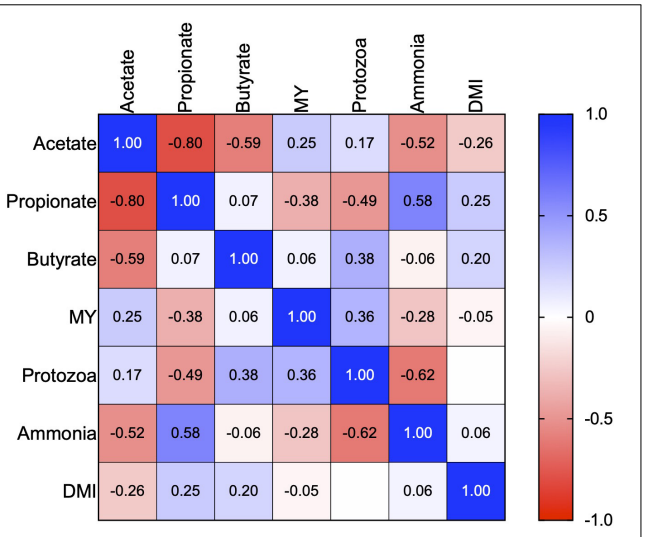


FIGURE 1 | Correlation matrix (Pearson) between MY, ruminal protozoa, DMI, and individual volatile fatty acids. The scalar colors symbolize the positivity (closer to 1, blue squares) or negativity (closer to -1, red squares) of the correlation. MY is the methane yield, and DMI is dry matter intake in the correlation matrix.

TABLE 3 | Effect of oil supplementation on the rumen fermentation and protozoan numbers.

Attributes	CTR	CON	INT	SEM	*P*-value
NH_3-N (mg/100 ml)	19.7^c	18.8^b	17.9^a	0.20	< 0.001
Total VFA (mM)	68.5	68.7	68.4	1.29	0.997
VFA (mmol)					
Acetate	51.9	50.5	50.7	1.03	0.850
Propionate	11.3^a	13.0^b	12.3^b	0.25	0.006
Iso-butyrate	0.75	0.63	0.68	0.04	0.516
Butyrate	3.49	3.51	3.58	0.17	0.977
Iso-valerate	0.82	0.77	0.80	0.04	0.900
Valerate	0.24	0.23	0.27	0.01	0.077
A/P ratio	4.59^c	3.87^a	4.12^b	0.08	< 0.001
Ruminal protozoa					
Total ($\times 10^7$ cells/ml)	9.26^c	6.51^b	5.30^a	0.282	< 0.001
Entodiniomorphs ($\times 10^7$ cells/ml)	8.87^c	6.25^b	5.08^a	0.275	< 0.001
Holotrichs ($\times 10^6$ cells/ml)	3.87^b	2.54^a	2.19^a	0.118	< 0.001
Withdrawal					
Total ($\times 10^7$ cells/ml)	9.21	8.62	8.69	0.130	0.113
Entodiniomorphs ($\times 10^7$ cells/ml)	9.01	8.40	8.48	0.123	0.092
Holotrichs ($\times 10^6$ cells/ml)	0.73	0.78	0.75	0.031	0.743

CTR, control (no oil supplementation); CON, daily oil feeding; INT, biweekly oil feeding; NH_3-N, ammonia nitrogen; VFA, volatile fatty acid. Mean values bearing different superscripts in the same row differ significantly (p < 0.05).

Rumen Protozoa

Supplementation of SWPO lowered ($p < 0.01$) the numbers of total protozoa ($\times 10^7$ cells/ml) in the CON (6.51) and INT (5.30) groups compared with the CTR group (9.21). Similarly, the numbers of *Entodiniomorphs* ($\times 10^7$ cells/ml) and *Holotrichs* ($\times 10^6$ cells/ml) in the CON and INT groups relative to the CTR group were low ($p < 0.01$) (**Table 3**). Moreover, the difference in the total protozoa, *Entodiniomorphs*, and *Holotrichs* between the CON and INT groups was also significant ($p < 0.01$). The ammonia and ruminal protozoa ($r = -0.62$), and propionate and protozoa ($r = -0.49$) negatively correlated (**Figure 1**). Data from the withdrawal study indicated similar numbers of total protozoa ($p = 0.11$), *Entodiniomorphs* ($p = 0.09$), and *Holotrichs* ($p = 0.74$) among the groups.

Archaeal Diversity

Overall, 7,406,185 paired-end raw reads (mean 411,454) per sample were generated in this study. After quality filtering and chimeric removal, a total of 4,735,849 paired-end reads (263,103 ± 38,445) were retained for further analysis comparing ruminal archaea diversity. The filtered reads were categorized into 69 archaeal ASVs (**Supplementary Figure 1**). At the order level, the *Methanobacteriales* represented the most significant fraction of the ruminal archaea; however, no significant differences in abundance of archaea at the order level between the CON, CTR, and INT groups was observed (**Figure 2** and **Supplementary File 1**). Similarly, methanogens in this study were affiliated to three families, namely *Methanobacteriaceae*, *Methanomassilliicoccaceae*, and *Methanosarcinaceae*. *Methanobacteriaceae* constituted 89.8% (range 88.6–91.0%) of the total rumen archaea and their distribution remains unaffected with the oil supplementation.

Similarly, at the genus level, *Methanobrevibacter* was the most prominent genera in the sheep rumen; however, their proportion was not significantly different ($p = 0.98$) between the three groups. Despite their limited proportions, the abundance of *Group9* ($p = 0.04$) and *Methanobacterium* ($p = 0.04$) was significantly different among the groups. The *group9* archaea were less abundant in the CTR group (0.12%) compared with the CON (0.26%) and INT (0.71%) groups, whereas the *Methanobacterium* proportion in the CON group (0.91%) was significantly lower ($p = 0.04$) than in the CTR (2.10%) and INT (3.24%) groups. At the species level, the proportions of *Group3b* ($p = 0.06$), *Group*9 ($p = 0.03$), and *Methanobacterium* ($p = 0.04$) were different between the groups (**Figure 2**). The results for the archaeal proportion in the present study indicated that ∼98% of the ruminal archaea remain unaffected by the oil supplementation, and only a minor fraction of the archaea (< 2%) showed a proportional increase or decrease (**Figure 2**). Moreover, the Shannon index, which estimates alpha diversity, did not reveal any significant differences in the archaeal diversity between the groups (**Figure 3**).

Core Archaeome

At a minimum prevalence of 50% and a minimum detection threshold of 0.01, each group's core archaeome was computed at the genus (**Figure 4A**) and species levels (**Figure 4B**). *Methanobrevibacter*, *Group12*, *Methanobacterium*, and *Methanosphaera* constituted the core archaeome across the groups. However, *Group9* was exclusively present in the CTR and INT groups, whereas *Group10* was present only in the CTR group. Comparison of archaeome at the species level revealed the presence of the *Methanobrevibacter gottschalkii clade*, *Group12* sp. *ISO4-H5*, *Methanobrevibacter millerae*, the *Methanobrevibacter ruminantium clade*, *Methanobrevibacter wolinii*, *Methanosphaera IS03-F5*, and *Methanobacterium* sp. across the groups. The differences were observed in the proportion of *Group10* sp. and *Group9* sp. These two species were exclusively absent in the CON group. This analysis revealed that the core archaeome in the CON group was distinct from the CTR and INT groups.

DISCUSSION

SWPO supplementation resulted in additional body weight gains of 3.1 and 0.9 kg in the CON and INT groups, respectively, as compared with the CTR group. Supplementation of SWPO in CON group led to an additional ADG (16.6 g/day). The lower body weight (kg) and ADG (g/day) in our study were mainly due to the maturity of the experimental animals (18 months). Body weight gain and ADG would have been higher if the animals were growing (i.e., at the age of 3–6 months).

Lipid supplementation is one of the established methods for reducing enteric methane emission (Pinares-Patiño et al., 2016). The study also established that the SWPO supplementation in daily (CON) or biweekly (INT) regimes was equally effective in reducing methane emissions. Methane yields (g/kg DMI) reported here were consistent with previous reports (Pinares-Patino et al., 2014; Charmley et al., 2016; Williams et al., 2019). The reduction in daily methane emissions (g/day) due to long-term supplementation of SWPO was 5–8% higher than a previous short-term study (Thirumalaisamy et al., 2020). The supplementation of lipids mitigate methane emission via inhibition of hydrogen-producing microbes (Tapio et al., 2017), impeding microbial colonization and increasing hydrogen sequestration (van Nevel and Demeyer, 1992) and a shift in microbiota (Vargas et al., 2020). However, the extent of methane reduction depends on the source of the lipids and the fatty acid composition. A significant reduction (23–26%) in daily enteric methane emissions due to SWPO supplementation demonstrated its anti-methanogenic effectiveness. A recent study (Jayanegara et al., 2020) reported that supplementation with 5% insect oils effectively reduced *in vitro* methane production. Like cricket and mealworm oil (Jayanegara et al., 2020), the SWPO also possesses a relatively higher proportion of unsaturated fatty acids (65.5%) than saturated. Oleic (C18:1) and linolenic (C18:3) are two primary unsaturated fatty acids in SWPO that aggregately represent 58.4% of the total fatty acids (Thirumalaisamy et al., 2020). One plausible explanation for the reduction in methane emission can be the high degree of fatty acid unsaturation.

The negative correlation between DMI (g/day) and MY (g/kg DMI) is consistent with previous studies (Muetzel, 2011;

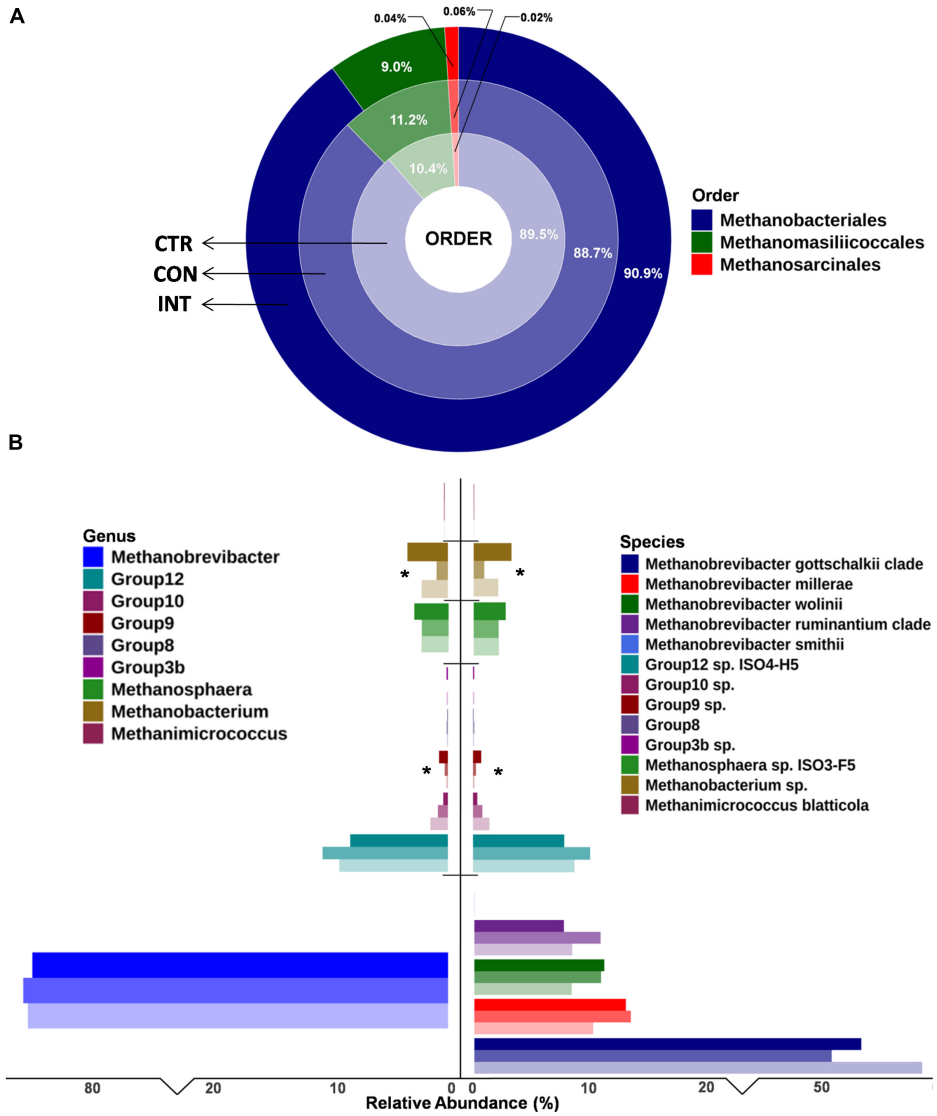


FIGURE 2 | Ruminal archaea diversity in sheep from three different groups at **(A)** order level and **(B)** genus and species level. The increasing intensity of the same color shows the abundance in the CTR, CON, and INT groups. For instance, light blue indicates CTR, medium blue indicates CON, and dark blue indicates the INT group. *Denotes the significance in abundance among groups. CTR, control group (no oil); CON, daily oil supplementation; INT, biweekly oil supplementation.

Hristov and Melgar, 2020). In addition, the higher energy intake (ME MJ/day) in test groups due to oil supplementation could be one of the reasons for the reduction in methane emissions. However, Bayat et al. (2018) did not observe any difference in MY due to the variable energy density.

Total VFA and acetate production were unaffected by the SWPO supplementation. However, a shift in the fermentation pattern with more propionate and a decreased acetate to propionate ratio was apparent in this study. The higher propionate production and lower acetate to propionate ratio without any apparent change in total VFA and acetate concurs with the findings of previous studies (Jalè et al., 2006; Vargas et al., 2020). The reduction in rumen methanogenesis in the CON and INT groups could be due to the additional propionate production, which utilized spared H_2. Fat supplementation is believed to be toxic to the rumen protozoa (Machmüller et al., 1998); however, the negative impact is not uniform across fat/oil sources and varies with the degree of unsaturation (Jenkins, 1993). The negative impact of SWPO supplementation on protozoa was in this study can be attributed to the high degree of unsaturation in the oil (Thirumalaisamy et al., 2020). It is well established that defaunation leads to the proliferation of succinate-producing bacteria (Kurihara et al., 1978; Ungerfeld et al., 2020), resulting in more propionate production (Eugène et al., 2004; Li et al., 2018). This was also substantiated by our results demonstrating a negative correlation between rumen protozoa and propionate production.

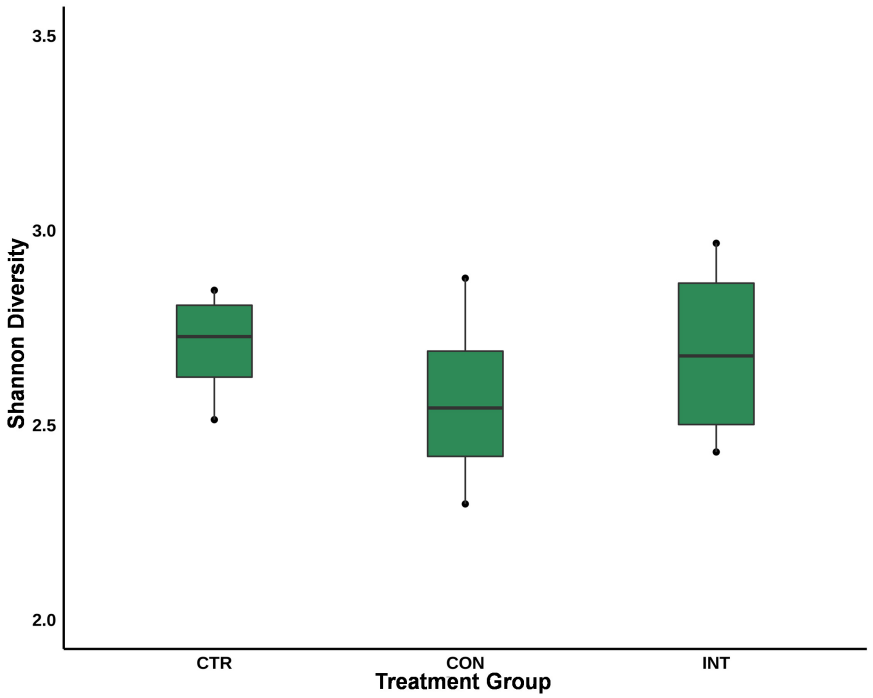


FIGURE 3 | Alpha diversity of the ruminal archaea in sheep supplemented with silkworm pupae oil. CTR, control group (no oil); CON, daily oil supplementation; INT, biweekly oil supplementation.

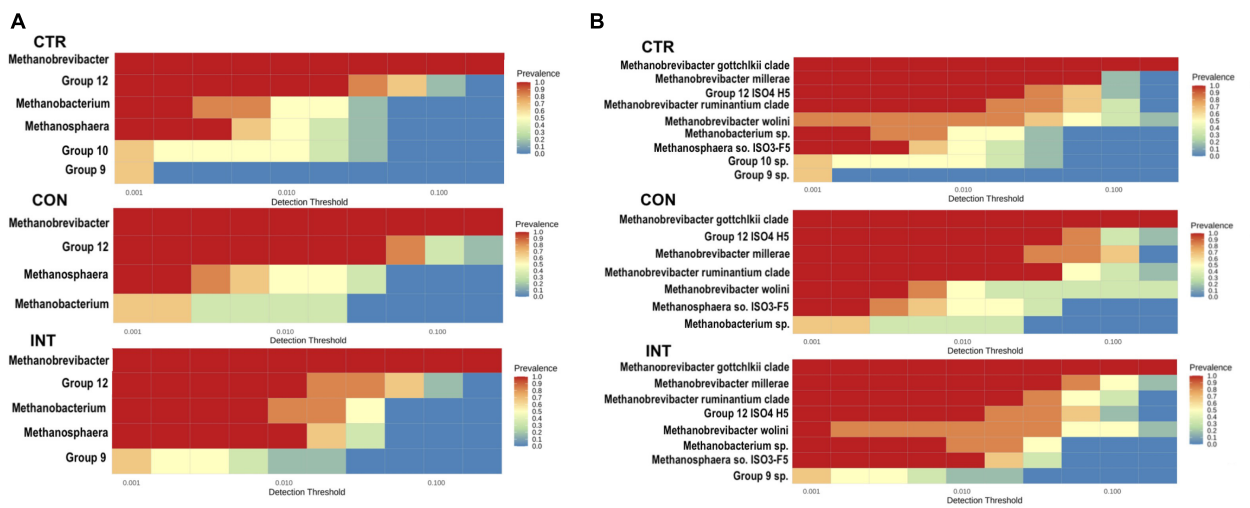


FIGURE 4 | Ruminal archaea representing the core microbiome at a minimum prevalence of 50%. **(A)** Represents the core microbiome at the genus level in the CTR, CON, and INT groups, while **(B)** represents the core microbiome at the species level in the respective groups. The color gradients indicate the variability in prevalence. CTR, control; CON, daily oil supplementation; INT, biweekly oil supplementation.

After methanogenesis, propionate is considered another major sink for H_2 removal (Janssen, 2010). The H_2 spared from reduced methanogenesis was, however, not completely redirected to propionate production, indicating that a major portion of H_2 remains unaccounted for. These findings are consistent with previous reports (Anderson et al., 2010; Ungerfeld, 2015), implying that caproate and formate may be two other major sinks for H_2 utilization.

Rumen protozoa are not essential for animal survival, but due to their functional association with methanogens (Newbold et al., 2015), they held accountable for nearly 37% of methane emissions (Machmüller et al., 2003). Results indicated that the SWPO supplementation effectively reduced the protozoal

numbers with a concurrent decrease in enteric methane emission. After the withdrawal of the oil supplementation, an increase in both the protozoal numbers and methane emission confirmed their involvement in methane emission. This is consistent with a previous report (Sutton et al., 1983) confirming that the discontinuation feeding with oils increased protozoa numbers to the pre-supplementation level. The role of rumen protozoa in bacterial predation is well known (Williams and Coleman, 1992) and *Entodiniomorphs* are more efficient predators than are the *Holotrichs* (Belanche et al., 2012). Although we have not explored the bacterial community in the present study, a shift in the bacterial community due to the reduction in predation by protozoa is expected. In a meta-analysis, Newbold et al. (2015) reported a 9% increase in the bacterial populations due to defaunation. One of the most consistent consequences of defaunation is the reduction in ammonia concentration (Newbold et al., 2015), which is also evident in the current study. This could be explained by the reduced bacterial breakdown due to the lower protozoal numbers (Williams and Coleman, 1992); however, it needs further confirmation.

There was little impact of protozoal reduction on the methanogens community. This contradicts a previous study (Tan et al., 2020) that reported a significant effect of protozoa reduction on the methanogen community. In the current study, *Methanobrevibacter*, despite being the prominent archaeal genus, was similarly distributed between test (CON and INT) and CTR groups. This is consistent with an earlier short-term study (Thirumalaisamy et al., 2020) that evaluated SWPO at a similar level. Similarly, the distribution of the prominent species *Methanobrevibacter gottschalkii* was also uniform among the groups. These findings indicate that the core methanogens in sheep remain unaffected by the daily or biweekly supplementation of SWPO. A previous study (St-Pierre et al., 2015) also confirmed the dominance of *Methanobrevibacter* in the sheep rumen. Only minor methanogens such as *Group3b*, *Group9*, and *Methanobacterium* having a distribution frequency of $< 2\%$ in the archaeal community were affected with the SWPO supplementation. *Group3b* methanogen was recently identified in the rumen (Jin et al., 2017) but with a limited distribution of $\sim$0.2%. The contribution of *Group3b* to rumen methanogenesis is yet to be investigated.

SWPO is readily accessible in India (Srinivas et al., 2019) and cheaper than conventional edible oil sources (Government of India, 2021). Animals in the CON and INT groups consumed 3.24 and 1.61 L of oil during the experimental period of 180 days. The daily financial cost (INR) for feeding SWPO in the CON and INT groups was only 0.72 and 0.36, respectively (1 US$ = 75 INR). Based on the prevailing price of mutton (INR 600/kg), sheep in the CON group, due to the SWPO supplementation, had an additional ADG equivalent of INR 9.96 per day. However, the supplementation in the INT group led to an additional ADG of 4.2 g—only INR 2.52 per day. Thus, it would be far more economical for the farmers to feed SWPO daily (CON) at the recommended level of 2%. Although we attempted the SWPO supplementation through automatic oral drenching in the present study, it may not be practicable in a farm setting. Adding SWPO to concentrate mixture would be the most practical option on a farm, which requires further investigation.

CONCLUSION

From the present study, it can be concluded that the supplementation of SWPO as 2% of DMI over the long term, with daily or biweekly supplementing, decreased daily methane emissions of sheep by 23–26%. The supplementation of SWPO, either through daily or biweekly regimes, was equally effective in reducing methane emissions; however, the daily supplementation of SWPO at the recommended level led to a significant increase in body weight gain and average daily gain compared with animals in the control and biweekly groups. It can be inferred from the discontinuation study that the daily supplementation of SWPO is required to achieve a persistent reduction in enteric methane emission, and withdrawal of the oil supplementation led to an increase in enteric methane emission to the pre-supplementation level. The oil did not significantly change the archaeal community composition but persistently decreased the ruminal protozoa numbers. The feeding of SWPO may lead to a relatively higher average daily gain in growing sheep and, therefore, could result in a net profit. Drenching of oil may not be practically feasible in the farm settings; therefore, further studies will be required to investigate the methane mitigation potential of including SWPO into formulated concentrates.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institute Animal Ethics Committee, National Institute of Animal Nutrition and Physiology.

AUTHOR CONTRIBUTIONS

PM, AK, and RB conceived and designed the study. GT performed the methane measurement, digestibility studies, executed the chemical composition, and ruminal fluid analysis. ST and AK performed the molecular bioinformatics analysis, and visualization of the data. All authors participated in the data analysis and article writing.

FUNDING

The authors were thankful to the Indian Council of Agricultural Research, New Delhi, for providing the financial support to carry out this research under the outreach methane project entitled "Estimation of Methane Emission under Different Feeding Systems and Development of Mitigation Strategies."

ACKNOWLEDGMENTS

We would like to thank the Director of the Institute for providing all the logistical support for the study. We are also thankful to Dr. K. P. Suresh, ICAR-NIVEDI, Bengaluru, for helping with the data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.780073/full#supplementary-material

Supplementary Figure 1 | Rarefaction plot (**A**, before; **B**, after) showing the archaeal richness in different groups of sheep receiving feed with or without silkworm pupae oil.

Supplementary File 1 | Read stats, OTUs taxonomy and percent abundance.

REFERENCES

Anderson, R. C., Huwe, J. K., Smith, D. J., Stanton, T. B., Krueger, N. A., Callaway, T. R., et al. (2010). Effect of nitroethane, dimethyl-2-nitroglutarate and 2-nitromethyl-propionate on ruminal methane production and hydrogen balance in vitro. *Bioresour. Technol.* 101, 5345–5349. doi: 10.1016/j.biortech.2009.11.108

AOAC (2005). *Official method of Analysis. Association of Officiating Analytical Chemists*. Washington DC: AOAC.

AOAC (2012). *Official method of Analysis. Association of Officiating Analytical Chemists*. Washington DC: AOAC.

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., and Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* 43, 260–296. doi: 10.1128/mmbr.43.2.260-296.1979

Bayat, A. R., Tapio, I., Vilkki, J., Shingfield, K. J., and Leskinen, H. (2018). Plant oil supplements reduce methane emissions and improve milk fatty acid composition in dairy cows fed grass silage-based diets without affecting milk yield. *J. Dairy Sci.* 101, 1136–1151. doi: 10.3168/jds.2017-13545

Beauchemin, K. A., Ungerfeld, E. M., Eckard, R. J., and Wang, M. (2020). Fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal* 14, s2–s16.

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

Berndt, A., Boland, T. M., Deighton, M. H., Gere, J. I., Grainger, C., Hegarty, R. S., et al. (2014). "Guidelines for use of sulphur hexafluoride (SF6) tracer technique to measure enteric methane emissions from ruminants," in *New Zealand Agricultural Greenhouse Gas Research Centre*, ed. M. Lambert (New Zealand: Ministry for Primary Industries), doi: 10.13140/2.1.2271.8241

Callahan, B. J., Sankaran, K., Fukuyama, J. A., McMurdie, P. J., and Holmes, S. P. (2016). Bioconductor workflow for microbiome data analysis: from raw reads to community analyses. *F1000Research* 5:492. doi: 10.12688/F1000RESEARCH.8986.1

Chang, J., Peng, S., Ciais, P., Saunois, M., Dangal, S. R. S., Herrero, M., et al. (2019). Revisiting enteric methane emissions from domestic ruminants and their δ13CCH4 source signature. *Nat. Commun.* 10, 1–14. doi: 10.1038/s41467-019-11066-3

Charmley, E., Williams, S. R. O., Moate, P. J., Hegarty, R. S., Herd, R. M., Oddy, V. H., et al. (2016). A universal equation to predict methane production of forage-fed cattle in Australia. *Anim. Prod. Sci.* 56, 169–180. doi: 10.1071/AN15365

Conway, E. J. (1957). *Micro-diffusion Analysis And Volumetric Error*, 4th Edn. London Crossby: Lockwood and Sons Ltd.

Crisan, E. V., and Sands, A. (1978). "Nutritional value of edible mushroom," in *The Biology and Cultivation of Edible Mushrooms*, eds S. Chang and W. Hayer (New York, NY: Academic Press), 137–168.

Eugène, M., Archimède, H., and Sauvant, D. (2004). Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. *Livest. Prod. Sci.* 85, 81–97. doi: 10.1016/S0301-6226(03)00117-9

Filípek, J., and Dvoøák, R. (2009). Determination of the volatile fatty acid content in the rumen liquid: comparison of gas chromatography and capillary isotachophoresis. *Acta Vet. Brno* 78, 627–633. doi: 10.2754/avb200978040627

Gerber, P. J., Hristov, A. N., Henderson, B., Makkar, H., Oh, J., Lee, C., et al. (2013). Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. *Animal* 7(Suppl. 2), 220–234. doi: 10.1017/S1751731113000876

Government of India (2021). *Ministry of Consumer Affairs, Food and Public Distribution*. Available online at: https://consumeraffairs.nic.in/comparativetestbyconsumersa/edible-vegetable-oil. (acessed December 8, 2021).

Guan, H., Wittenberg, K. M., Ominski, K. H., and Krause, D. O. (2006). Efficacy of ionophores in cattle diets for mitigation of enteric methane. *J. Anim. Sci.* 84, 1896–1906. doi: 10.2527/jas.2005-652

Guyader, J., Eugène, M., Nozière, 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

Guyader, J., Ungerfeld, E. M., and Beauchemin, K. A. (2017). Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). *Front. Microbiol.* 8:393. doi: 10.3389/fmicb.2017.00393

Hristov, A. N., and Melgar, A. (2020). Short communication: relationship of dry matter intake with enteric methane emission measured with the GreenFeed system in dairy cows receiving a diet without or with 3-nitrooxypropanol. *Animal* 14, s484–s490. doi: 10.1017/S1751731120001731

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.

Hungate, R. E. (1966). *The Rumen And Its Microbes*. New York, NY: Academic Press Inc,.

ICAR (2013). *Nutrient Requirements of Cattle and Buffalo. Indian Council of Agricultural Research*. New Delhi: Indian Council of Agricultural Research.

Jalè, D., Potkański, A., Szumacher-Strabel, M., Kowalczyk, J., and Cieślak, A. (2006). The effect of a high forage diet and different oil blends on rumen fermentation *in vitro*. *J. Anim. Feed Sci.* 15, 141–144. doi: 10.22358/jafs/70429/2006

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

Jayanegara, A., Gustanti, R., Ridwan, R., and Widyastuti, Y. (2020). Fatty acid profiles of some insect oils and their effects on in vitro bovine rumen fermentation and methanogenesis. *Ital. J. Anim. Sci.* 19, 1311–1318. doi: 10.1080/1828051X.2020.1841571

Jenkins, T. C. (1993). Lipid Metabolism in the Rumen. *J. Dairy Sci.* 76, 3851–3863. doi: 10.3168/jds.S0022-0302(93)77727-9

Jin, W., Cheng, Y., and Zhu, W. (2017). The community structure of *Methanomassiliicoccales* in the rumen of Chinese goats and its response to a high-grain diet. *J. Anim. Sci. Biotechnol.* 8, 1–10. doi: 10.1186/s40104-017-0178-0

Johnson, K. A., and Johnson, D. E. (1995). Methane emissions from cattle. *J. Anim. Sci.* 73, 2483–2492. doi: 10.2527/1995.7382483x

Kamra, D. N., and Agarwal, N. (2003). "Rumen protozoa," in *Techniques in Rumen Microbiology*, eds D. N. Kamra and N. Agarwal (Bareilly: Centre of Advance Studies in Animal Nutrition, Indian Veterinary Research Institute, Izatnagar), 35–45.

Kurihara, Y., Takechi, T., and Shibata, F. (1978). Relationship between bacteria and ciliate protozoa in the rumen of sheep fed on a purified diet. *J. Agric. Sci.* 90, 373–381. doi: 10.1017/S0021859600055489

Lahti, L., and Shetty, S. (2012). *Microbiome@GitHub*. Available online at: url: https://github.com/microbiome/microbiome (accessed July 31, 2020).

Lassey, K. R., Martin, R. J., Williams, S. R. O., Berndt, A., Iwaasa, A. D., Hegarty, R. S., et al. (2014). "Analysis of breath samples," in *Guidelines For Use Of Sulphur Hexafluoride (Sf6) Tracer Technique To Measure Enteric Methane Emissions From Ruminants*, ed. M. G. Lambert (New Zealand: New Zealand Agricultural Greenhouse Gas Research Centre), 89–112.

Li, Z., Deng, Q., Liu, Y., Yan, T., Li, F., Cao, Y., et al. (2018). Dynamics of methanogenesis, ruminal fermentation and fiber digestibility in ruminants following elimination of protozoa: a meta-analysis. *J. Anim. Sci. Biotechnol.* 9, 1–9. doi: 10.1186/s40104-018-0305-6

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. doi: 10.1186/s13059-014-0550-8

Machmüller, A., Ossowski, D. A., Wanner, M., and Kreuzer, M. (1998). Potential of various fatty feeds to reduce methane release from rumen fermentation in vitro (Rusitec). *Anim. Feed Sci. Technol.* 71, 117–130. doi: 10.1016/S0377-8401(97)00126-0

Machmüller, A., Soliva, C. R., and Kreuzer, M. (2003). Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion. *Br. J. Nutr.* 90, 529–540. doi: 10.1079/bjn2003932

Marty, R. J., and Demeyer, D. I. (1973). The effect of inhibitors of methane production of fermentation pattern and stoichiometry in vitro using rumen contents from sheep given molasses. *Br. J. Nutr.* 30, 369–376. doi: 10.1079/bjn19730041

Mathison, G. W., Okine, E. K., McAllister, T. A., Dong, Y., Galbraith, J., and Dmytruk, O. I. N. (1998). Reducing methane emissions from ruminant animals. *J. Appl. Anim. Res.* 14, 1–28. doi: 10.1080/09712119.1998.9706212

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Moate, P. J., Richard, S., Williams, O., Deighton, M. H., Pryce, J. E., Hayes, B. J., et al. (2014). "Mitigation of enteric methane emissions from the Australian dairy industry," in *Proceedings of the 5th Australasian Dairy Science Symposium*, Hamilton New Zealand, 121–140. doi: 10.13140/2.1.2172.4489

Morgavi, D. P., Forano, E., Martin, C., and Newbold, C. J. (2010). Erratum: microbial ecosystem and methanogenesis in ruminants. *Animal* 4, 1024–1036. doi: 10.1017/S1751731112000407

Muetzel, S. (2011). *Effect Of Level Of Intake On Methane Production Per Kg Of Dry Matter Intake.* MAF Technical Paper No: 2011/95. New Zealand: Ministry of Agriculture and Forestry, AgResearch.

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

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., et al. (2013). *Package 'Vegan'."* Community Ecology Package, Version 2, no. 9, 1–295.

Pinares-Patiño, C. S., Franco, F. E., Molano, G., Kjestrup, H., Sandoval, E., MacLean, S., et al. (2016). Feed intake and methane emissions from cattle grazing pasture sprayed with canola oil. *Livest. Sci.* 184, 7–12. doi: 10.1016/j.livsci.2015.11.020

Pinares-Patino, C., Williams, S. R. O., Martin, C., Swainson, N. M., Berndt, A., Molano, G., et al. (2014). "Data quality assurance and quality control," in *Guidelines For Use Of Sulphur Hexaflouride Tracer Technique To Measure Enteric Methane Emission From Ruminants*, ed. M. G. Lambert (New Zealand: New Zealand Agricultural Greenhouse Gas Research Centre), 166.

Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P. H. (2014). RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea fromthe rumen and other intestinal environments. *PeerJ* 2:e494. doi: 10.7717/peerj.494

Srinivas, G., Nidoni, U., Ct, R., and Kt, R. (2019). Supercritical fluid extraction of pupae oil from mulberrysilkworm (Bombyx Supercritical fluid extraction of pupae oilfrom mulberry silkworm (*Bombyx mori L.*). *J. Pharmacogn. Phytochem.* 8, 4507–4513.

St-Pierre, B., Cersosimo, L. M., Ishaq, S. L., and Wright, A. D. G. (2015). Toward the identification of methanogenic archaeal groups as targets of methane mitigation in livestock animals. *Front. Microbiol.* 6:776. doi: 10.3389/fmicb.2015.00776

Sutton, J. D., Knight, R., McAllan, A. B., and Smith, R. H. (1983). Digestion and synthesis in the rumen of sheep given diets supplemented with free and protected oils. *Br. J. Nutr.* 49, 419–432. doi: 10.1079/bjn19830051

Takai, K., and Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl. Environ. Microbiol.* 66, 5066–5072. doi: 10.1128/AEM.66.11.5066-5072.2000

Tan, C., Ramírez-Restrepo, C. A., Shah, A. M., Hu, R., Bell, M., Wang, Z., et al. (2020). The community structure and microbial linkage of rumen protozoa and methanogens in response to the addition of tea seed saponins in the diet of beef cattle. *J. Anim. Sci. Biotechnol.* 11:80. doi: 10.1186/s40104-020-00491-w

Tapio, I., Snelling, T. J., Strozzi, F., and Wallace, R. J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8:7. doi: 10.1186/s40104-017-0141-0

Thirumalaisamy, G., Malik, P. K., Kolte, A. P., Trivedi, S., Dhali, A., and Bhatta, R. (2020). Effect of silkworm (Bombyx mori) pupae oil supplementation on enteric methane emission and methanogens diversity in sheep. *Anim. Biotechnol.* 33, 128–140. doi: 10.1080/10495398.2020.1781147

Ungerfeld, E. M. (2015). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Front. Microbiol.* 6:37. doi: 10.3389/fmicb.2015.00037

Ungerfeld, E. M., Evans, P., Hoedt, E. C., Li, Z., Zhang, Z., Guan, L., et al. (2020). Comparative microbiome analysis reveals the ecological relationships between rumen methanogens, acetogens, and their hosts ecology of methanogens and acetogens in the rumen. *Front. Microbiol.* 1:1311. doi: 10.3389/fmicb.2020.01311

van Nevel, C. J., and Demeyer, D. I. (1992). Influence of antibiotics and a deaminase inhibitor on volatile fatty acids and methane production from detergent washed hay and soluble starch by rumen microbes in vitro. *Anim. Feed Sci. Technol.* 37, 21–31. doi: 10.1016/0377-8401(92)90117-O

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597.

Vargas, J. E., Andrés, S., López-Ferreras, L., Snelling, T. J., Yáñez-Ruíz, D. R., García-Estrada, C., et al. (2020). Dietary supplemental plant oils reduce methanogenesis from anaerobic microbial fermentation in the rumen. *Sci. Rep.* 10:1613. doi: 10.1038/s41598-020-58401-z

Wemheuer, B., Wemheuer, F., and Daniel, R. (2012). RNA-based assessment of diversity and composition of active archaeal communities in the German Bight. *Archaea* 2012:695826. doi: 10.1155/2012/695826

Wickham, H. (2011). ggplot2. *Wiley Interdiscip. Rev. Comput. Stat.* 3, 180–185. doi: 10.1002/wics.147

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

Williams, S. R. O., Hannah, M. C., Jacobs, J. L., Wales, W. J., and Moate, P. J. (2019). Volatile fatty acids in ruminal fluid can be used to predict methane yield of dairy cows. *Animals* 9:1006. doi: 10.3390/ani9121006

Williams, S. R. O., Moate, P. J., and Deighton, M. H. (2014). "Sampling background air," in *Guidelines For Use Of Sulphur Hexaflouride (Sf6) Tracer Technique To Measure Enteric Methane Emissions From Ruminants*, ed. M. G. Lambert (New Zealand: New Zealand Agricultural Greenhouse Gas Research Centre), 81–88.

Yanagita, K., Kamagata, Y., Kawaharasaki, M., Suzuki, T., Nakamura, Y., and Minato, H. (2000). Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of Methanomicrobium mobile by fluorescence in situ hybridization. *Biosci. Biotechnol. Biochem.* 64, 1737–1742. doi: 10.1271/bbb.64.1737

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 36, 808–812. doi: 10.2144/04365st04

Conflict of Interest: The 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.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 April 2022
doi: 10.3389/fmicb.2022.855565

Differences in the Composition of the Rumen Microbiota of Finishing Beef Cattle Divergently Ranked for Residual Methane Emissions

*Paul E. Smith[1,2], Alan K. Kelly[2], David A. Kenny[1] and Sinéad M. Waters[1]**

[1] Teagasc, Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Meath, Ireland, [2] UCD School of Agricultural and Food Science, University College Dublin, Dublin, Ireland

With the advent of high throughput technology, it is now feasible to study the complex relationship of the rumen microbiota with methanogenesis in large populations of ruminant livestock divergently ranked for enteric emissions. Recently, the residual methane emissions (RME) concept has been identified as the optimal phenotype for assessing the methanogenic potential of ruminant livestock due to the trait's independence from animal productivity but strong correlation with daily methane emissions. However, there is currently a dearth of data available on the bacterial and archaeal microbial communities residing in the rumens of animals divergently ranked for RME. Therefore, the objective of this study was to investigate the relationship between the rumen microbiota and RME in a population of finishing beef cattle. Methane emissions were estimated from individual animals using the GreenFeed Emissions Monitoring system for 21 days over a mean feed intake measurement period of 91 days. Residual methane emissions were calculated for 282 crossbred finishing beef cattle, following which a ∼30% difference in all expressions of methane emissions was observed between high and low RME ranked animals. Rumen fluid samples were successfully obtained from 268 animals during the final week of the methane measurement period using a trans-oesophageal sampling device. Rumen microbial DNA was extracted and subjected to 16S rRNA amplicon sequencing. Animals ranked as low RME had the highest relative abundances ($P < 0.05$) of lactic-acid-producing bacteria (*Intestinibaculum*, *Sharpea*, and *Olsenella*) and *Selenomonas*, and the lowest ($P < 0.05$) proportions of *Pseudobutyrivibrio*, *Butyrivibrio,* and *Mogibacterium*. Within the rumen methanogen community, an increased abundance ($P < 0.05$) of the genus *Methanosphaera* and *Methanobrevibacter* RO clade was observed in low RME animals. The relative abundances of both *Intestinibaculum* and *Olsenella* were negatively correlated ($P < 0.05$) with RME and positively correlated with ruminal propionate. A similar relationship was observed for the abundance of *Methanosphaera* and the *Methanobrevibacter* RO clade. Findings from this study highlight the ruminal abundance of bacterial genera associated with the synthesis of propionate *via* the acrylate pathway, as well as the methanogens *Methanosphaera* and members of the *Methanobrevibacter* RO clade as potential microbial biomarkers of the methanogenic potential of beef cattle.

Keywords: rumen, microbiota, 16S rRNA, cattle, methane

OPEN ACCESS

Edited by:
Christina D. Moon, AgResearch Ltd., New Zealand

Reviewed by:
Marc D. Auffret, Royal Agrifirm Group, Netherlands
Timothy John Snelling, Harper Adams University, United Kingdom

***Correspondence:**
Sinéad M. Waters
Sinead.Waters@teagasc.ie

Specialty section:
This article was submitted to Microorganisms in Vertebrate Digestive Systems, a section of the journal Frontiers in Microbiology

Received: *15 January 2022*
Accepted: *10 March 2022*
Published: *29 April 2022*

Citation:
Smith PE, Kelly AK, Kenny DA and Waters SM (2022) Differences in the Composition of the Rumen Microbiota of Finishing Beef Cattle Divergently Ranked for Residual Methane Emissions. Front. Microbiol. 13:855565. doi: 10.3389/fmicb.2022.855565

INTRODUCTION

Recently, the Intergovernmental Panel on Climate Change (IPCC) has identified a rapid and sustained reduction in global methane production as a necessity to mitigate against the current increase in global temperatures (IPCC, 2021). At present, a third of the global anthropogenic methane emissions originate from ruminant livestock (Saunois et al., 2020). Indeed, enteric methane is accountable for ∼6% of global anthropogenic greenhouse gas (GHG) emissions (Gerber et al., 2013; Beauchemin et al., 2020) and ∼19% of Ireland's national GHG emissions profile (Duffy et al., 2021). As a result, there has been an increased interest in the development of methane mitigation strategies for ruminant livestock.

To reduce the GHG emissions profile of the livestock industry, numerous authors have advocated the potential of genetic selection to achieve permanent and accumulative reductions to the methane output of future livestock generations (Wall et al., 2010; Pickering et al., 2015; de Haas et al., 2017; Beauchemin et al., 2020). To date, most investigations examining the relationship between the rumen microbiota and methane output have been conducted on animals ranked for daily methane emissions (DME; g/day; Danielsson et al., 2017) or methane yield (MY; g/kg of DMI; Kittelmann et al., 2014; Shi et al., 2014; Roehe et al., 2016). However, the direct genetic selection of animals for reduced DME or MY is unlikely to be implemented as part of a breeding strategy due to the antagonistic relationship of both traits with animal productivity (Bird-Gardiner et al., 2017; Renand et al., 2019; Smith et al., 2021).

Residual methane emissions (RME), defined as the difference between an animal's actual and expected methane output based on its level of feed intake and body weight (Bird-Gardiner et al., 2017), has recently been advocated as the optimal trait for identifying low methane emitting cattle due to the trait's phenotypic and genetic independence of animal productivity but strong correlation with DME (Herd et al., 2014; Manzanilla-Pech et al., 2016; Bird-Gardiner et al., 2017; Donoghue et al., 2020; Smith et al., 2021). Indeed, supported by moderate heritability estimates for the trait (Manzanilla-Pech et al., 2016; Donoghue et al., 2020), selecting animals for a low RME phenotype has the potential to reduce the methanogenic output of individual animals, without compromising the productivity of future generations of livestock.

Recently, our group observed a 30% difference in methane output along with shifts in theoretical hydrogen (H) production and a varied expression of microbial fermentation pathways associated with propionate production, yet a similar level of animal productivity, in cattle ranked low for RME (Smith et al., 2021). However, to date, there has been no investigation on the effect of ranking animals for RME on the composition of the rumen microbiota.

The abundance and fermentative activity of individual members of the rumen microbiota are influenced by fluctuating H dynamics in the rumen (Janssen, 2010), with methanogenesis recognised as one of the primary metabolic processes regulating dissolved ruminal dihydrogen (H_2) concentrations (McAllister and Newbold, 2008; Morgavi et al., 2010). While the genetic factors controlling methanogenesis are yet to be determined, the composition of the rumen microbiota has explained 15–40% of the variation in methane output in some studies (Difford et al., 2018; Wallace et al., 2019). This suggests it may be possible to discover potential rumen microbial biomarkers that are reflective of the methanogenic potential of an animal. Indeed, the discovery of rumen microbial signatures associated with methanogenesis may benefit the identification of low-methane emitting animals by reducing the number of animals required to undergo long and expensive methane measurement estimation periods, as part of the development of an environmentally focused breeding programme.

Therefore, the objective of this study was to investigate the composition of the rumen microbiota in animals, phenotypically divergent in RME, in an effort to identify rumen microbial biomarkers indicative of the methanogenic potential of an animal.

MATERIALS AND METHODS

All animal procedures used in this study were approved by the Teagasc Animal Ethics Committee and conducted using procedures consistent with the experimental licence (AE19132/P078) issued by the Irish Health Products Regulatory Authority in accordance with European Union legislation (Directive 2010/63/EU) for the protection of animals used for scientific purposes.

Animal Model

This experiment was conducted as part of a larger study designed to investigate the effects of ranking finishing beef cattle, in terms of RME, on enteric emissions and animal productivity. A detailed description of the animal model, measurements recorded, and derivation of traits has been presented in Smith et al. (2021).

Briefly, over a period of 18 months, data were obtained from 282 commercial beef cattle (steers = 128 and heifers = 154) enrolled in a feed efficiency performance test. Cattle were the progeny of AI bulls under evaluation as part of the Gene Ireland Breeding Program[1], and were recruited from commercial breeding herds based on factors including sire, breed, genetic merit, pedigree, age, and performance tested under standardised conditions at the Irish Cattle Breeding Federation (ICBF) national beef bull progeny test station (Tully, Co., Kildare, Ireland). Cattle included in this study originated from continental late-maturing beef dams (Charolais, Limousin, or Simmental), sired by early maturing (EM) or late maturing (LM) sire breeds. The proportion of EM and LM sired animals was 25 and 75%, respectively.

Eligible cattle entered the test centre in groups of 40–75 animals, hereby referred to as "batches," and underwent a minimum 100-day feed efficiency performance test. Starting in January 2019 and finishing in July 2020, animals from seven consecutive batches were included in this study. Upon arrival at the facility, cattle were allocated to indoor pens (6.1 m × 4.6 m) bedded with peat. Cattle were separated based on gender and initially penned in groups of five to six depending on their initial

[1]https://www.icbf.com/?page_id=12900

weight and age. Cattle were offered a 30-day adjustment period to allow dietary acclimatisation and adaption to the facilities. During the adjustment phase, animals were fitted with a RFID tag (HDX EID Tag, Allflex Livestock Intelligence, Dallas, TX, United States). Once tagged, the pen size was increased by opening the gates between adjacent pens to accommodate 11–30 animals per pen. The mean age and body weight of animals at the beginning of the measurement period were 441 days (SD = 49 days) and 476 kg (SD = 67 kg), respectively. Steers and heifers averaged 476 (SD = 46 days) and 410 (SD = 27 days) days of age while LM and EM averaged 442 (SD = 51 days) and 435 (SD = 43 days) days of age at the commencement of the measurement period, respectively.

Cattle were offered the same total mixed ration (TMR) *ad libitum*, which consisted of 77% concentrates and 23% hay (see **Supplementary Table 1**), and underwent a mean feed intake measurement period of 91 days (71–128 days). Enteric emissions (methane and carbon dioxide) were estimated over a 21-day period during a mean feed efficiency test period of 91 days using the GreenFeed Emissions Monitoring system (GEM; C-Lock Inc., Rapid City, SD, United States). Following the completion of the measurement period, animals were slaughtered in a commercial abattoir.

Rumen Fluid Collection

During the last week of the enteric emissions measurement period, samples of rumen fluid (25–50 ml) were collected from 268 animals before feeding using the transoesophageal rumen sampling device (FLORA rumen scoop; Guelph, ON, Canada). Feed was restricted from animals for a minimum of 2 h before sampling. Samples were divided across two 25-ml tubes with ruminal fluid pH measured immediately using a digital pH meter (Orion SA 720; Thermo Fisher Scientific, Waltham, MA, United States). Following this, 500 μl of rumen fluid was pipetted into 2-ml cyrotubes (Sarstedt, Co., Wexford, Ireland) containing autoclaved zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm) and immediately preserved *via* snap-freezing in liquid nitrogen along with the remaining rumen fluid contained in 25 ml tubes. On the same day of sampling, samples were transported 61 km to the Teagasc research facility (Teagasc Grange, Dunsany, Co., Meath, Ireland) on dry ice and stored at −80°C until further molecular analysis was conducted.

DNA Extraction and Library Preparation

Eight samples were misplaced resulting in microbial DNA being extracted from 260 samples of 500 μl of frozen rumen fluid sample using a modified version of repeated bead beating and column purification method (Yu and Morrison, 2004), as previously described (McGovern et al., 2018; Smith et al., 2020a,b). A blank extraction control was subjected to the same procedure as rumen fluid samples for each extraction kit. DNA quality was assessed using agarose gels (0.8%) and a 1-kb DNA ladder (Bioline GmbH, Luckenwalde, Germany). The concentration of extracted DNA was quantified on the Nanodrop 1000 spectrophotometer and diluted to 100 and 5 ng/μl before running agarose gels and PCR amplification.

Using 12.5 ng of extracted rumen microbial DNA, amplicon libraries (n = 260) were generated by performing two rounds of PCR amplification as outlined in the Illumina Miseq *16S Sample Preparation Guide* with minor modifications to cycle length, as outlined in McGovern et al. (2018) and Smith et al. (2020c). In addition, six amplicon libraries were generated to assess sequencing run performance and library preparation. Three amplicon libraries were generated using the ZymoBIOMICS^{TM} Microbial Community DS (Zymo Research Corp., Irvine, CA, United States). An additional three libraries were synthesised using the synthetic rumen amplicon sequencing standard, as described by Smith et al. (2020c).

The first round of PCR amplification, targeting the V4 hypervariable region of the 16S rRNA gene, was performed using the 515F/806R primers (Caporaso et al., 2011) designed with Nextera overhang adapters and 2x KAPA Hifi HotStart ReadyMix DNA polymerase (Roche Diagnostics, West Sussex, United Kingdom). Cycle conditions were as follows: 95°C for 3 min, 20 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and then 72°C for 5 min.

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, United Kingdom). A negative control subjected to the same procedures as rumen amplicon samples was performed for each purification kit. Following purification, amplicons were subject to a second round of PCR to permit the attachment of dual indices and Illumina sequencing adapters using the Nextera XT indexing kit (Illumina, San Diego, CA, United States). Cycle conditions for the second round of PCR were 95°C for 3 min, 8 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and then 72°C for 5 min followed by an additional PCR purification with the QIAquick PCR Purification Kit (Qiagen, Manchester, United Kingdom). Confirmation of amplicon generation was conducted visually on a 2% agarose gel. Amplicons were split across three separate runs, pooled together in equal concentration, and subject to gel purification using the Qiagen Gel Extraction Kit (Qiagen, Manchester, United Kingdom) to remove adapter primers followed by further purification to remove any residues of agarose using the QIAquick PCR purification kit (Qiagen, Manchester, United Kingdom). In total, 276 samples were sequenced (n = 260 rumen samples; n = 6 positive controls; n = 11 negative controls).

Pooled sample purity and quantity were analysed on the Nanodrop 1000 with further validation on the Qubit fluorometer and using the KAPA SYBR FAST universal kit with Illumina Primer Premix (Roche Diagnostics, West Sussex, United Kingdom). Following this, the library pool was diluted and denatured as per the Illumina Miseq *16S Sample Preparation Guide* with sequencing conducted on the Illumina MiSeq using the 500 cycle version 2 MiSeq reagent kit (Illumina, San Diego, CA, United States) over three separate runs.

Rumen Metabolite Analysis

Short-chain fatty acid concentrations in rumen fluid samples were measured using a Varian Saturn 2000 GC 450 (Varian, Middelburg, The Netherlands). A detailed description of sample

preparation, the extraction of VFA, and the cycle conditions utilized were previously described (Smith et al., 2021).

Sequencing Analysis

Amplicon sequence data were processed in R (version 4.0.2) using DADA2 (version 1.18.0) and submitted to the pipeline as described by Callahan et al. (2016) with minor modifications. Quality checks of both forward and reverse reads were initiated followed by the filtering and trimming of poor quality reads and removal of primer sequences using the trimLeft function. Identical sequences were combined using the dereplication function followed by the merging of forward and reverse reads. An ASV table was then constructed following which chimeric sequences were removed and taxonomy assigned to sequences variants using the RefSeq + RDP (NCBI RefSeq 16S rRNA database supplemented by RDP; release date 06/11/2020) downloaded from the DADA2 website. A bootstrapping threshold of 80 was applied for taxonomic classification by incorporating minBoot = 80 as part of the assignTaxonomy function. Sample metadata, sequence taxonomy, and ASVs were combined into a phyloseq object using phyloseq (version 1.34.0; McMurdie and Holmes, 2013) for further analysis. Ten rumen amplicon samples were removed because they had a significantly low sequencing depth. A rarefication curve was plotted for the remaining rumen samples (n = 250). Based on a plateauing of the generated rarefication curve (see **Supplementary Figure 1**), samples were rarefied to the lowest sequencing depth of all samples (26,366 reads per sample). Following this, alpha (Shannon and Simpson) diversity was calculated for each sample. For comparisons of beta diversity, as well as differential abundance analysis, ASVs which were not present in >5% of the samples were removed before calculating the relative abundance, based on rarefied reads.

To determine the proportion of rumen methanogens belonging to the SGMT or RO clade, ASVs assigned to the *Methanobrevibacter* genus were further classified by conducting an online NCBI BLAST search against the RefSeq database[2].

Data and Statistical Analysis

Before assessing differences in the bacterial and archaeal structure amongst RME groups, the homogeneity of group dispersions was assessed between low and high-ranked animals. Following this, PERMANOVA tests based on Bray-Curtis dissimilarities, 9,999 permutations, and a significance level of ($P < 0.05$) were implemented to determine if the bacterial and archaeal structure differed amongst high and low RME animals. Both the assessment of the homogeneity of group dispersions and PERMANOVA analysis were carried using the R package vegan (Oksanen et al., 2019) (version 2.5.7) implemented through microbiome (Lahti and Shetty, 2017) (version 1.12.0). The R package plotly (version 4.9.3) was used to generate 3D NMDS plots based on Bray-Curtis dissimilarities.

Statistical comparisons of the relative abundance of the rumen bacteria and archaea between the RME groups was conducted on ASVs with a mean relative abundance greater than 0.5% in at least one RME group using the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC; version 9.4). The statistical model used included the fixed effect of RME group (high, medium, and low), breed maturity/genotype (LM and EM), gender (steer and heifer), and their interactions. Non-statistically significant ($P > 0.10$) interactions were subsequently excluded from the final model. Age and initial body weight at the start of the performance test were included as covariates, and a contemporary group was incorporated as a random effect in the statistical model. The residuals of each model were normally distributed. Differences among means were determined by F-tests using Type III sums of squares. The PDIFF option and the Tukey test were applied to evaluate pairwise comparisons between means. Mean values were considered to be different when $P < 0.05$ and a tendency when $P \geq 0.05$ and <0.10. The associations among the microbial abundances with performance and fermentation parameters were determined through partial correlations, adjusted for gender, breed maturity, and contemporary group using the MANOVA/PRINTE statement within the GLM procedure of SAS. Correlation coefficients were classified as strong ($r > 0.6$), moderate (r between 0.4 and 0.6), or weak ($r < 0.4$), respectively.

RESULTS

Animal Performance

Statistical comparisons of feed intake and emissions traits amongst the RME groups are presented in **Supplementary Table 2**. Low RME animals produced 17.69 and 30.42% less ($P < 0.05$) DME in comparison to animals ranked as medium and high for RME, respectively. Similarly, the low RME animals had lower ($P < 0.05$) daily carbon dioxide emissions (DCE; kg/day) than animals ranked as medium and high. Low RME animals had the lowest ($P < 0.05$) MY and methane intensity (MI; g/kg of carcass weight) of the RME groups. A difference of 29.73 and 29.63% for MY and MI was detected amongst the low and high RME groups, respectively.

DNA Extraction and Sequencing Performance

After quality filtering, merging, and removal of chimeric sequences, an average of 67,970 ± 27,857 reads per rumen sample were generated across the three runs. Correlations between the composition of libraries generated from the DS standards and the theoretical composition of the ZymoBIOMICS^{TM} ranged from r_s 0.95 to 0.97. In addition, the correlation of the synthetic sequencing standard with the composition reported in Smith et al. (2020c) ranged from r_s 0.89 to 0.90. Negative extraction controls generated on average 50 reads per sample (range of 13–93) after quality filtering, merging, and removal of chimeric sequences. DNA extraction and sequencing performance were deemed satisfactory based on the strong concordance of the microbial compositions in both standards with that of their theoretical composition and the low number of reads obtained for negative controls.

[2]https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch

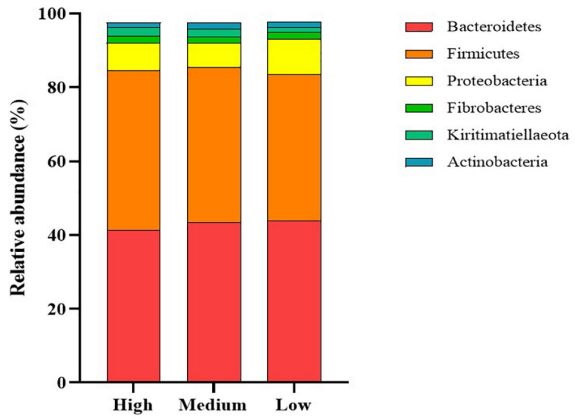


FIGURE 1 | Stack plot comparing differences, in the relative abundances of the six most abundant rumen bacterial phyla, between cattle ranked as high, medium, and low for residual methane emissions.

Rumen Microbial Composition

The dominant members of the bacterial community, within each group at the phylum and family levels, are displayed in **Figures 1**, **2**. At the phylum level, the rumen bacterial community was dominated by *Firmicutes* and *Bacteroidetes*, which had a mean combined relative abundance of 84.58%. *Proteobacteria* was the next most abundant bacterial phylum (7.70%) followed by *Fibrobacteres* (1.89%), *Kiritimatiellaeota* (1.86%), and *Actinobacteria* (1.53%). *Prevotellaceae* was the most abundant bacterial family observed, with a mean relative abundance of 45.73% across all samples. The families *Lachnospiraceae, Ruminococcaceae,* and *Acidaminococcaceae* contributed to 35.72% of the bacterial community composition. *Prevotella* was the primary genus of bacteria observed with a mean relative abundance of 55.05% across all samples, followed by *Succiniclasticum* (11.33%), *Ruminococcus* (9.28%), *Fibrobacter* (4.15%), and *Succinivibrio* (2.27%).

The genera *Methanobrevibacter* and *Methanosphaera* accounted for 93.87 and 5.09%, respectively, of the archaeal community across all samples. Within the *Methanobrevibacter* genus, the relative abundance of members of SGMT and RO clade was 51.31 and 46.58%, respectively, with the remaining 2.11% of species identified as *M. boviskoreani.*

Effect of Residual Methane Emissions Ranking on Bacterial Community Composition

Comparisons in the bacterial community, at the genus level, between RME groups, are presented in **Table 1**. The overall bacterial community structure did not differ between high and low-ranked RME animals (PERMANOVA; $P = 0.87$) (**Figure 3**). In addition, no difference in *alpha* diversity was detected at the species level between the RME groups ($P > 0.05$). At the phylum level, an increased ($P < 0.05$) relative abundance of *Proteobacteria* was observed in the low compared to high RME animals. The opposite was observed for the abundance of *Kiritimatiellaeota,* with an increased ($P < 0.05$) abundance observed in the high RME group. No other bacterial phyla were impacted by RME ranking.

At the family level, the proportions of *Ruminococcaceae*, *Succinivibrionaceae,* and *Clostridiales Family XIII Incertae Sedis* were increased ($P < 0.05$) in the high compared to low RME ranked animals. The proportions of the bacterial families, *Lactobacillaceae*, *Erysipelotrichaceae,* and *Selenomonadaceae* were greater ($P < 0.05$) in low RME groups in comparison to their high counterparts.

Within the bacterial family *Lachnospiraceae*, greater ($P < 0.05$) proportions of both *Pseudobutyrivibrio* and *Butyrivibrio* were

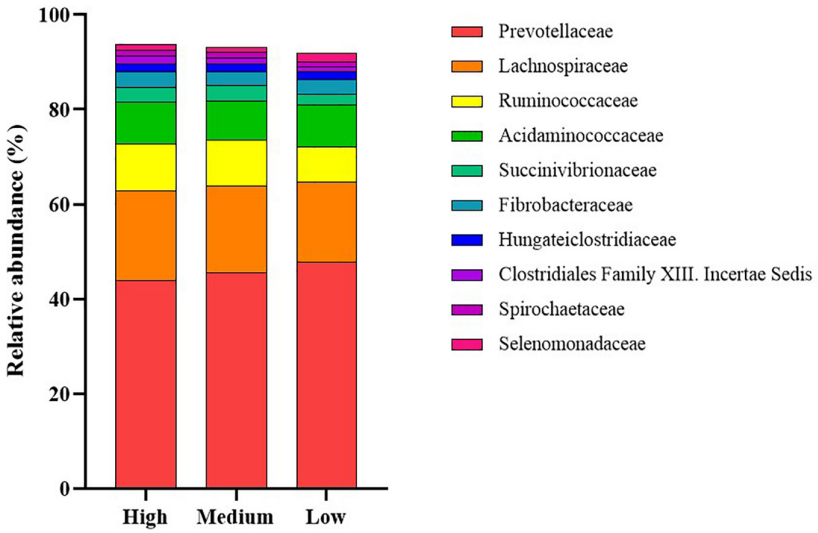


FIGURE 2 | Stack plot comparing differences, in the relative abundances of the ten most abundant rumen bacterial families, between cattle ranked as high, medium, and low for residual methane emissions.

TABLE 1 | Characterization of the rumen bacterial genera in finishing beef cattle ranked for residual methane.

Bacteria genus	RME Ranking[1]			SEM[2]	*P*-value
	High *n* = 80	Medium *n* = 96	Low *n* = 74		
Anaeroplasma	0.71	0.77	0.76	0.11	0.59
Bifidobacterium	1.63	1.81	1.72	0.3	0.77
Butyrivibrio	1.35[a]	1.12[a]	0.80[b]	0.19	<0.001
Eubacterium	0.55	0.56	0.68	0.07	0.06
Fibrobacter	4.35	4.21	4.08	0.5	0.77
Intestinibaculum	0.37[a]	0.50[a]	0.86[b]	0.1	<0.0001
Mogibacterium	1.63[a]	1.16[b]	0.97[b]	0.16	<0.0001
Olsenella	0.53[a]	0.74[a]	1.02[b]	0.13	<0.0001
Prevotella	53.15	54.55	55.71	1.74	0.23
Pseudobutyrivibrio	0.69[a]	0.52[b]	0.43[b]	0.09	<0.001
Ruminobacter	1.77	1.61	1.08	0.43	0.08
Ruminococcus	9.92	9.3	8.65	0.67	0.16
Selenomonas	0.74[a]	0.85[a]	1.21[b]	0.15	<0.001
Sharpea	1.27[a]	1.75[ab]	1.86[b]	0.26	0.03
Succiniclasticum	11.48	11.26	10.51	0.69	0.23
Succinivibrio	2.78	2.41	1.84	0.46	0.41
Treponema	1.63	1.61	1.56	0.17	0.82

[1] *High = RME was > 0.5 SD above the mean; Medium = RME was ± 0.5 SD above and below the mean; Low = RME was > -0.5 SD below the mean.*
[2] *SEM = pooled standard error.*
[a,b] *Least squares means within main effect and a row with different superscripts differ.*

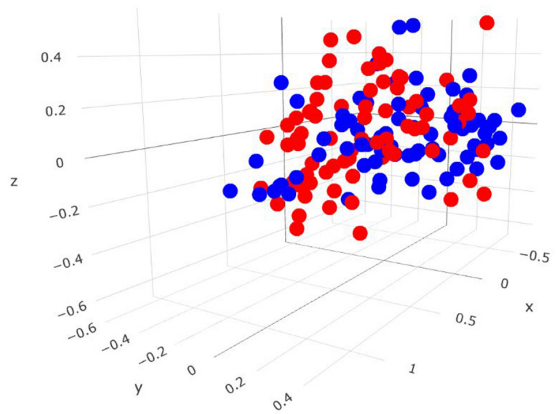


FIGURE 3 | Three-dimensional Bray-Curtis NMDS plot highlighting differences in the bacterial community composition between animals ranked as high and low for residual methane emissions. Red = low RME; blue = high RME.

present in high RME ranked animals. Animal ranking for RME had a significant influence on the genus *Mogibacterium* ($P < 0.05$), which was significantly increased in high RME ranked animals. The proportion of the genus *Ruminobacter* tended ($P = 0.08$) to be higher in high RME animals. The bacterial genera *Intestinibaculum*, *Olsenella*, *Selenomonas,* and *Sharpea* had increased ($P < 0.05$) relative abundances in low RME ranked animals. In addition, the abundance of *Eubacterium* tended ($P = 0.06$) to be affected by RME ranking, with a decreased proportion of the genus observed in the high and medium ranked animals relative to low RME counterparts.

Relationship of Rumen Bacteria With Enteric Emissions and Rumen Fermentation

Partial correlation analysis amongst the relative abundance of rumen bacterial genera with both enteric emissions traits and VFAs are presented in **Tables 2**, **3**. The relative abundances of *Intestinibaculum*, *Olsenella,* and *Selenomonas* were negatively correlated ($P < 0.05$) with all methane phenotypes and positively correlated with propionate percentage. In addition, both *Intestinibaculum* and *Olsenella* were negatively associated ($P < 0.05$) with A:P ratio, along with *Olsenella* sharing a negative relationship ($P < 0.05$) with butyrate percentage. *Prevotella* abundance was negatively correlated ($P < 0.05$) with DME, RME, and MI. The abundances of *Butyrivibrio*, *Pseudobutyrivibrio*, *Mogibacterium,* and *Succiniclasticum* were positively correlated ($P < 0.05$) with all methane emission traits. Furthermore, the relative abundances of both *Butyrivibrio* and *Mogibacterium* were negatively associated ($P < 0.05$) with propionate percentage. The abundances of both *Ruminobacter* and *Ruminococcus* were positively correlated ($P < 0.05$) with both DME and RME. Both the proportions of *Fibrobacter* and *Treponema* were negatively associated with total SCFA production.

Effect of Residual Methane Emissions Ranking on Archaeal Community Composition

Comparisons in the archaeal community at the genus level and within the *Methanobrevibacter* clades between RME groups are presented in **Table 4**. Based on PERMANOVA analysis, a

TABLE 2 | Correlation coefficients amongst rumen bacterial genera and traits associated with enteric emissions.

	DME	DCE	RME	MY	MI
Anaeroplasma	− 0.02	− 0.04	− 0.04	− 0.07	0.01
Bifidobacterium	0.05	− 0.01	0.03	0.00	0.10
Butyrivibrio	0.35***	0.10	0.30***	0.24**	0.28***
Eubacterium	− 0.08	0.03	− 0.13*	− 0.10	− 0.01
Fibrobacter	0.06	0.02	0.07	0.03	0.01
Intestinibaculum	− 0.30***	0.01	− 0.36***	− 0.38***	− 0.27***
Mogibacterium	0.38***	0.07	0.38***	0.38***	0.35***
Olsenella	− 0.30***	0.00	− 0.32***	− 0.29***	− 0.31***
Prevotella	−0.19**	−0.09	− 0.13*	− 0.09	− 0.15*
Pseudobutyrivibrio	0.32***	0.12†	0.27***	0.22**	0.28***
Ruminobacter	0.13*	0.01	0.16*	0.15*	0.12†
Ruminococcus	0.23**	0.12†	0.14*	0.09	0.23**
Selenomonas	− 0.25***	− 0.02	− 0.23**	− 0.18**	− 0.24**
Sharpea	− 0.05	− 0.01	− 0.09	− 0.10	− 0.06
Succiniclasticum	0.13*	0.03	0.14*	0.15*	0.13*
Succinivibrio	0.12†	0.01	0.13*	0.09	0.11†
Treponema	0.11†	0.10	0.04	− 0.01	0.03

DME, daily methane production; DCE, daily carbon dioxide production; RME, residual methane emissions; MY, methane yield; MI, methane intensity.
†$P < 0.10$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

TABLE 3 | Correlation coefficients amongst rumen bacterial genera with rumen fermentation characteristics.

	Total SCFA (mM)	Acetate (%)	Butyrate (%)	Propionate (%)	A:P
Bifidobacterium	0.21**	−0.14*	0.18**	−0.03	−0.02
Butyrivibrio	−0.08	0.05	0.01	−0.16*	0.11
Eubacterium	0.06	−0.05	−0.04	0.04	−0.09
Fibrobacter	−0.24**	0.05	0.00	−0.03	0.02
Intestinibaculum	−0.08	−0.15*	−0.11	0.21**	−0.25**
Mogibacterium	−0.07	0.00	0.13†	−0.15*	0.11
Olsenella	−0.08	−0.04	−0.22**	0.16*	−0.16*
Prevotella	0.04	0.02	−0.04	0.10	−0.01
Pseudobutyrivibrio	−0.12†	0.04	0.02	−0.14†	0.09
Ruminobacter	−0.04	0.02	−0.05	0.02	−0.01
Ruminococcus	0.10	−0.09	0.05	−0.04	−0.02
Selenomonas	0.04	−0.06	0.03	0.14*	−0.09
Sharpea	0.03	−0.10	−0.04	0.03	−0.13
Succiniclasticum	0.03	0.09	0.09	−0.15	0.10
Succinivibrio	0.04	0.05	0.02	−0.08	0.05
Treponema	−0.18**	0.06	0.00	−0.09	0.06

A:P = acetate to propionate ratio.
†P < 0.10.
** P < 0.05.*
*** P < 0.01.*

TABLE 4 | Characterization of the rumen methanogens in finishing beef cattle ranked for residual methane.

	RME ranking[1]				
Rumen methanogens	**High** *n = 80*	**Medium** *n = 96*	**Low** *n = 74*	**SEM[2]**	***P*-value**
Genus					
Methanobrevibacter	94.01	93.64	93.6	0.32	0.27
Methanosphaera	4.93[a]	5.22[a]	5.79[b]	0.39	<0.01
Methanobrevibacter clades					
RO	45.25[a]	48.07[ab]	53.92[b]	4.57	<0.01
SGMT	52.11	49.6	45.59	4.45	0.08

[1] High = RME was > 0.5 SD above the mean; Medium = RME was ± 0.5 SD above and below the mean; Low = RME was > -0.5 SD below the mean.
[2] SEM = pooled standard error.
[a,b] Least squares means within main effect and a row with different superscripts differ.

tentative difference in the structure of the archaeal community was detected amongst the high and low RME groups ($P = 0.07$), although no clear separation was observed (**Figure 4**). The relative abundance of *Methanobrevibacter* did not differ amongst the RME groups ($P > 0.05$). Within *Methanobrevibacter*, the abundance of the SGMT clade was not affected by RME ranking. However, an increased ($P < 0.05$) relative abundance of the RO clade was observed in the low compared to high RME animals. The relative abundance of *Methanosphaera* was increased ($P < 0.05$) in the low relative to high RME animals.

Relationship of Rumen Methanogens With Enteric Emissions and Rumen Fermentation

Partial correlation analysis amongst the relative abundance of rumen methanogens and with both enteric emissions and VFAs are presented in **Tables 5**, **6**. The RO clade was negatively correlated ($P < 0.05$) with all methane traits, butyrate percentage, and A:P ratio, but was positively associated with propionate percentage. The opposite relationships were observed for the relative abundance of the SGMT clade, which was positively correlated ($P < 0.05$) with RME, MY, butyrate percentage, and A:P ratio, but negatively correlated with propionate percentage. *Methanosphaera* was negatively correlated ($P < 0.05$) with all methane phenotypes, acetate percentage, and the A:P ratio but positively correlated with propionate percentage.

A strong negative correlation ($r = -0.80$; $P < 0.0001$) between the relative abundance of *Methanobrevibacter* and *Methanosphaera* was observed. A similar relationship was detected between the relative abundance of the SGMT and RO clade ($r = -0.93$; $P < 0.0001$).

DISCUSSION

Recently, RME has been identified as the optimal phenotype for identifying low methane emitting ruminants, with our group having recently reported an ∼30% difference in methane output, but comparable levels of animal productivity between high and low RME ranked animals (Smith et al., 2021). Indeed, the independence of RME from animal productivity makes it the ideal phenotype for examining the inherent biological mechanisms influencing methanogenesis. However, before this study, there had been no attempts to investigate the key rumen microbes associated with inter animal divergence in RME.

Animals ranked as low for RME had increased abundances of the lactic-acid-producing bacteria *Intestinibaculum*, *Olsenella,* and *Sharpea.* In addition, the abundances of *Intestinibaculum* and *Olsenella* were negatively associated with all methane phenotypes, but correlated positively with ruminal propionate percentage. This suggests that an increased abundance of both *Intestinibaculum* and *Olsenella* may play a role in reducing the dissolved H_2 concentrations in the rumen *via* the redirection of metabolic H toward the acrylate pathway. Although *Intestinibaculum* and *Olsenella* are predominantly lactic-acid-producing bacteria (Kim et al., 2019) and not deemed to be among the potent producers of propionate in the rumen, members of *Selenomonas* are capable of fermenting lactate to

TABLE 5 | Correlation coefficients amongst rumen methanogens and traits associated with enteric emissions.

	DME	DCE	RME	MY	MI
Genus					
Methanobrevibacter	0.06	0.00	0.10	0.12†	0.10
Methanosphaera	−0.20**	−0.03	−0.23**	−0.22**	−0.19**
Methanobrevibacter clades					
RO	−0.14*	0.12†	−0.26***	−0.28***	−0.13*
SGMT	0.08	−0.15*	0.20**	0.23**	0.08

DME, daily methane production; DCE, daily carbon dioxide production; RME, residual methane emissions; MY, methane yield; MI, methane intensity.
†P < 0.10.
** P < 0.05.*
*** P < 0.01.*
**** P < 0.001.*

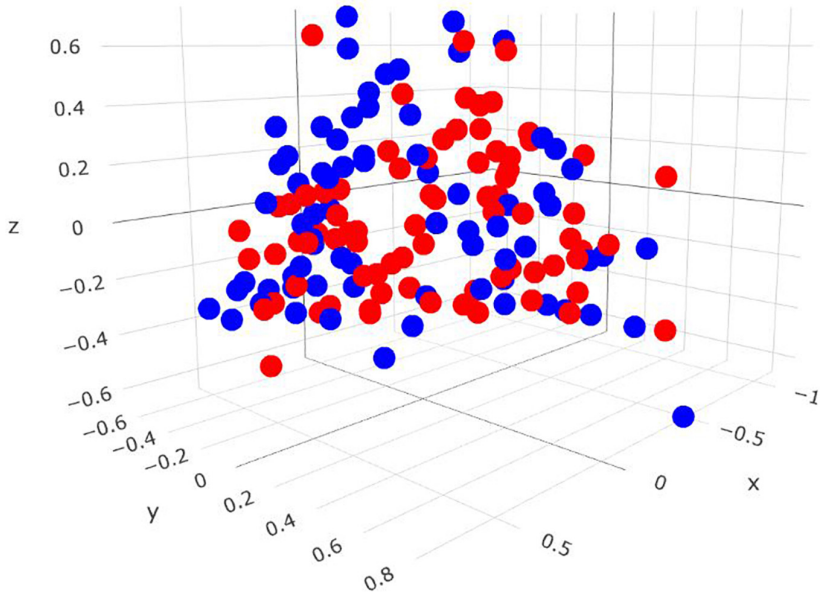


FIGURE 4 | Three-dimensional Bray-Curtis NMDS plot highlighting differences in the archaeal community composition between animals ranked as high and low for residual methane emissions. Red = low RME; blue = high RME.

propionate (Evans and Martin, 1997; Denman et al., 2015). This possibly explains the observed relationship of *Selenomonas* with propionate synthesis and methane output. Although this symbiotic relationship between lactic-acid-producing bacteria and *Selenomonas* will need further interpretation, it gives credence to the concept of "microbial teams" having a role in redirecting metabolic H to lactate and propionate, as proposed by Ungerfeld (2020).

Metabolic H can also be redirected toward butyrate production when H_2 concentrations are high in the rumen; however, this will still result in a net production of H (Janssen, 2010). Elevated relative abundances of the genera *Pseudobutyrivibrio* and *Butyrivibrio*, known producers of formate, butyrate, acetate, and H_2 (Van Gylswyk et al., 1996, Kelly et al., 2010; Emerson and Weimer, 2017; Palevich et al., 2017) in the rumen, were observed in high RME animals. Data generated *in vitro* suggests the existence of a symbiotic relationship between the species of *Butyrivibrio* and *Methanobrevibacter* associated with the utilisation of formate during hydrogenotrophic methanogenesis (Leahy et al., 2010). *Methanobrevibacter* species are also postulated to be capable of adhering to the surface of some *Butyrivibrio* species, which may benefit H_2 transfer from bacteria to archaea, akin to the symbiotic relationship observed between ruminal protozoa and methanogens (Ng et al., 2016). As such, the catabolic activities of some members of the *Butyrivibrio* genus may indirectly support methanogenesis through the supply of formate to ruminal methanogens.

The positive relationship of *Mogibacterium* with RME follows a similar relationship to that of previous findings, showing an increased abundance of *Mogibacterium* to be associated with high MY cattle (Wallace et al., 2015). As this genus has been identified as being asaccharolytic (Nakazawa et al., 2000), its contribution to ruminal methanogenesis will require further investigation.

Traditionally, the expression of the methyl coenzyme M reductase (*mcr*) gene in the rumen has been advocated as a more credible biomarker of ruminal methanogenesis in comparison to metataxonomic-based applications (Wilkins et al., 2015). Moreover, recent evidence is supportive of the abundance of individual members of the rumen methanogen community as indicators for the methanogenic potential of an animal (Tapio et al., 2017; Ramayo-Caldas et al., 2020). Similar to the findings in this study, an elevated proportion of the *Methanobrevibacter* RO clade and *Methanosphaera* has been associated with a reduced methane output in sheep (Shi et al., 2014; Kittelmann et al., 2014) and dairy cows (Danielsson et al., 2017). Martínez-Álvaro et al. (2020) suggested an increased diversity of the methanogen community and methanogenesis pathway expression to contribute to a reduced MY in cattle. In this study, the structure of the methanogen community tended to differ amongst RME groups, with the abundance of *Methanosphaera* and the *Methanobrevibacter* RO clade increased

TABLE 6 | Correlation coefficients amongst rumen methanogens with rumen fermentation characteristics.

	Total SCFA (mM)	Acetate (%)	Butyrate (%)	Propionate (%)	A:P
Genus					
Methanobrevibacter	0.12†	0.07	0	− 0.06	0.12†
Methanosphaera	− 0.05	− 0.14*	0.01	0.14*	− 0.22**
Methanobrevibacter clades					
RO	0.03	− 0.1	− 0.29***	0.18*	− 0.21**
SGMT	0.01	0.08	0.26**	− 0.15*	0.17*

A:P = acetate to propionate ratio.
†P < 0.10.
** P < 0.05.*
*** P < 0.01.*
**** P < 0.001.*

in low RME animals. Members of the *Methanosphaera* genus predominately produce methane *via* the reduction of methanol (Tapio et al., 2017). In addition, the division of the two clades of *Methanobrevibacter* was proposed based on the ability of the SGMT clade to synthesise two isomers of the *mcr* gene, *mcrI* and *mcrII*, with only *mcrI* expressed by the RO clade (Tapio et al., 2017). A fluctuation in the abundance of methanogens, with differing methanogenesis pathways, between high and low methane emitting animals is often perceived as competition for methanogenesis substrates amongst ruminal methanogens for H_2 (Morgavi et al., 2010). Consistent with this, a negative correlation between the relative abundances of SGMT and RO, as well as opposing correlations with RME was observed in this study, which may suggest competition amongst these members of the *Methanobrevibacter* genus for H_2.

Furthermore, the reduction of methanol *via* methylotrophic methanogenesis has a lower H_2 requirement in comparison to the hydrogenotrophic synthesis of methane, with the energetic advantage methylotrophic methanogens possess at low H_2 pressure detailed by Feldewert et al. (2020). The availability of dissolved H_2 in the rumen is also postulated to regulate the expression of the *mcr*, with *mcrII* only expressed when the quantity of H_2 is high (Reeve et al., 1997) which, as depicted by Danielsson et al. (2017), gives the SGMT clade a competitive advantage in the presence of a greater availability of H_2. Therefore, as more H_2 is diverted to additional H sinks under high H_2 concentrations, there will be a reduced production of H_2 from fermentation, which, in the current experiment, may have increased the competitiveness of *Methanosphaera* in the rumen of low RME animals. Similarly, when dissolved H_2 concentrations are kept low by an increased rate of methanogenesis and H_2 producing fermentation pathways are favoured, the SGMT clade may have a competitive advantage over members of the RO clade as more H_2 is produced during fermentation. Subsequently, the relationship between *Methanosphaera* and the abundances of the SGMT and RO clade with DME and RME observed in this study would suggest that the methanogenic output of an animal, at least in part, influences the composition of the rumen methanogen community, likely as a result of H dynamics in the rumen.

Recently, the abundances of some members of the rumen methanogen community associated with RME in this study have been proposed to be influenced by the genetics of the host. For example, the ratio of *Mbb. gottschalkii* to *Mbb. ruminantium* in the rumen has an estimated heritability of 0.17 (Li et al., 2019), while the abundance of *Methanosphaera* may also be regulated by host genetics (Difford et al., 2018). Based on the prospects of host genetics regulating the abundance of the methanogen community, it is likely the host may elicit some control over the rate of ruminal methanogenesis and/or concentration of dissolved H_2. However, further investigation will be required to uncover the means by which the host regulates control over the prevailing conditions within the rumen.

Under the intensive finishing conditions deployed in this experiment, a baseline assessment of the relationship of the rumen microbiota with RME is presented. Furthermore, this study has identified some of the key microbial genera to target as part of the development of environmentally focused breeding programmes and future antimethanogenic dietary supplements. However, further metagenomic and metatranscriptomic analysis will be required to uncover both the rumen microbial genes and metabolic pathways associated with a low RME phenotype. Nonetheless, the abundance of a small cohort of rumen microbial genera has been identified as potential microbial biomarkers for methanogenesis, albeit the consistency of their relationship with RME will require further assessment across different diet types.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA797238.

ETHICS STATEMENT

The animal study was reviewed and approved by Teagasc Animal Ethics Committee and licenced by Irish Health Products Regulatory Authority in accordance with European Union legislation (Directive 2010/63/EU), for the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

SW, AK, DK, and PS conceived and designed the experiments and interpreted the results and wrote the manuscript. PS performed the experiments. PS and AK analysed the data. SW contributed reagents, materials, and analysis tools. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the FACCE ERA-GAS "RumenPredict" grant (16/RD/ERAGAS/1RUMENPREDICT-ROI2017) and Horizon 2020 "MASTER" grant (contract no: 818368). The use of equipment purchased through the Department of Agriculture, Food and the Marine STILMULUS fund project "GreenBreed" (17/S/235) is acknowledged. PS was funded by a Teagasc Walsh Scholarship (RMIS 0364).

ACKNOWLEDGMENTS

We would like to acknowledge and thank Stephen Conroy for the use of the facilities at the Irish Cattle Breeding Federation (ICBF) Progeny Performance Test Centre.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.855565/full#supplementary-material

Supplementary File 1 | Amplicon sequence variants (ASV) table of rumen bacteria and archaea genera identified in this study.

REFERENCES

Beauchemin, K. A., Ungerfeld, E. M., Eckard, R. J., and Wang, M. (2020). Fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal* 14, 2–16. doi: 10.1017/S1751731119003100

Bird-Gardiner, T., Arthur, P. F., Barchia, I. M., Donoghue, K. A., and Herd, R. M. (2017). Phenotypic relationships among methane production traits assessed under ad libitum feeding of beef cattle. *J. Anim. Sci.* 95, 4391–4398. doi: 10.2527/jas2017.1477

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

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Müller, B., Schnürer, A., et al. (2017). Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Front. Microbial.* 8:226. doi: 10.3389/fmicb.2017.00226

de Haas, Y., Pszczola, M., Soyeurt, H., Wall, E., and Lassen, J. (2017). Invited review: phenotypes to genetically reduce greenhouse gas emissions in dairying. *J. Dairy Sci.* 100, 855–870. doi: 10.3168/jds.2016-11246

Denman, S. E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., and McSweeney, C. S. (2015). Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. *Front. Microbiol.* 6:1087. doi: 10.3389/fmicb.2015.01087

Difford, G. F., Plichta, D. R., Løvendahl, P., Lassen, J., Noel, S. J., Højberg, O., et al. (2018). Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genet.* 14:e1007580. doi: 10.1371/journal.pgen.1007580

Donoghue, K. A., Bird-Gardiner, T., Herd, R. M., Hegarty, R. S., and Arthur, P. F. (2020). Genetic variance and covariance components for carbon dioxide production and postweaning traits in Angus cattle. *J. Anim. Sci.* 98:skaa253. doi: 10.1093/jas/skaa253

Duffy, P., Black, K., Fahey, D., Hyde, B., Kehoe, A., Murphy, J., et al. (2021). *National Inventory Report 2019, Greenhouse Gas Emissions 1990 - 2019 Reported To The United Nations Framework Convention On Climate Change*. Ireland: Environmental Protection Agency (Ireland).

Emerson, E. L., and Weimer, P. J. (2017). Fermentation of model hemicelluloses by *Prevotella* strains and *Butyrivibrio fibrisolvens* in pure culture and in ruminal enrichment cultures. *Appl. Microbiol. Biotechnol.* 101, 4269–4278. doi: 10.1007/s00253-017-8150-7

Evans, J. D., and Martin, S. A. (1997). Factors affecting lactate and malate utilization by *Selenomonas ruminantium*. *Appl. Environ. Microbiol.* 63, 4853–4858. doi: 10.1128/aem.63.12.4853-4858.1997

Feldewert, C., Lang, K., and Brune, A. (2020). The hydrogen threshold of obligately methyl-reducing methanogens. *FEMS Microbiol. Lett.* 367:fnaa137. doi: 10.1093/femsle/fnaa137

Gerber, P. J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., et al. (2013). *Tackling Climate Change Through Livestock: A Global Assessment Of Emissions And Mitigation Opportunities*. Rome: Food and Agriculture Organization of the United Nations (FAO),.

Herd, R. M., Arthur, P. F., Donoghue, K. A., Bird, S. H., Bird-Gardiner, T., and Hegarty, R. S. (2014). Measures of methane production and their phenotypic relationships with dry matter intake, growth, and body composition traits in beef cattle. *J. Anim. Sci.* 92, 5267–5274. doi: 10.2527/jas.2014-8273

IPCC (2021). *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press. In Press.

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

Kelly, W. J., Leahy, S. C., Altermann, E., Yeoman, C. J., Dunne, J. C., Kong, Z., et al. (2010). The glycobiome of the rumen bacterium *Butyrivibrio proteoclasticus* B316T highlights adaptation to a polysaccharide-rich environment. *PLoS One* 5:11942. doi: 10.1371/journal.pone.0011942

Kim, J. S., Choe, H., Lee, Y. R., Kim, K. M., and Park, D. S. (2019). *Intestinibaculum porci gen. nov.*, sp. nov., a new member of the family *Erysipelotrichaceae* isolated from the small intestine of a swine. *J. Microbiol.* 57, 381–387. doi: 10.1007/s12275-019-8631-8

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

Lahti, L., and Shetty, S. (2017). *Tools for Microbiome Analysis in R. Version 1.9.19*. Available online at: https://microbiome.github.io/tutorials/ (accessed September 13, 2021).

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., 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

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., et al. (2019). 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

Manzanilla-Pech, C. I., De Haas, Y., Hayes, B. J., Veerkamp, R. F., Khansefid, M., Donoghue, K. A., et al. (2016). Genomewide association study of methane emissions in Angus beef cattle with validation in dairy cattle. *J. Anim. Sci.* 94, 4151–4166. doi: 10.2527/jas.2016-0431

Martínez-Álvaro, M., Auffret, M. D., Stewart, R. D., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., et al. (2020). Identification of complex rumen microbiome interaction within diverse functional niches as mechanisms affecting the variation of methane emissions in bovine. *Front. Microbiol.* 11:659. doi: 10.3389/fmicb.2020.00659

McAllister, T. A., and Newbold, C. J. (2008). Redirecting rumen fermentation to reduce methanogenesis. *Aust. J. Exp. Agric.* 48, 7–13. doi: 10.1071/ea07218

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/full

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Morgavi, D. P., Forano, E., Martin, C., and Newbold, C. J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal* 4, 1024–1036. doi: 10.1017/S1751731110000546

Nakazawa, F., Sato, M., Poco, S. E., Hashimura, T., Ikeda, T., Kalfas, S., et al. (2000). Description of *Mogibacterium pumilum* gen. nov., sp. nov. and *Mogibacterium vescum* gen. nov., sp. nov., and reclassification of *Eubacterium timidum* (Holdeman et al. 1980) as *Mogibacterium timidum* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* 50, 679–688. doi: 10.1099/00207713-50-2-679

Ng, F., Kittelmann, S., Patchett, M. L., Attwood, G. T., Janssen, P. H., Rakonjac, J., et al. (2016). An adhesin from hydrogen-utilizing rumen methanogen *Methanobrevibacter ruminantium* M1 binds a broad range of hydrogen-producing microorganisms. *Environ. Microbiol.* 18, 3010–3021. doi: 10.1111/1462-2920.13155

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2019). *Vegan: Community Ecology Package. R Package*.

Palevich, N., Kelly, W. J., Leahy, S. C., Altermann, E., Rakonjac, J., and Attwood, G. T. (2017). The complete genome sequence of the rumen bacterium *Butyrivibrio hungatei* MB2003. *Stand. Geno. Sci.* 12, 1–14. doi: 10.1186/s40793-017-0285-8

Pickering, N. K., Oddy, V. H., Basarab, J., Cammack, K., Hayes, B., Hegarty, R. S., et al. (2015). Animal board invited review: genetic possibilities to reduce enteric methane emissions from ruminants. *Animal* 9, 1431–1440. doi: 10.1017/S1751731115000968

Ramayo-Caldas, Y., Zingaretti, L., Popova, M., Estellé, J., Bernard, A., Pons, N., et al. (2020). Identification of rumen microbial biomarkers linked to methane emission in Holstein dairy cows. *J. Anim. Breed. Genet* 137, 49–59. doi: 10.1111/jbg.12427

Reeve, J. N., Nölling, J., Morgan, R. M., and Smith, D. R. (1997). Methanogenesis: genes, genomes, and who's on first? *J. Bacterial.* 179:5975. doi: 10.1128/jb.179.19.5975-5986.1997

Renand, G., Vinet, A., Decruyenaere, V., Maupetit, D., and Dozias, D. (2019). Methane and carbon dioxide emission of beef heifers in relation with growth and feed efficiency. *Animals* 9:1136. doi: 10.3390/ani9121136

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

Saunois, M., Stavert, A. R., Poulter, B., Bousquet, P., Canadell, J. G., Jackson, R. B., et al. (2020). The global methane budget 2000–2017. *Earth Syst. Sci. Data.* 12, 561–623. doi: 10.1111/gcb.15901

Shi, W. B., Moon, C. D., Leahy, S. C., Kang, D. W., 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

Smith, P. E., Enriquez-Hidalgo, D., Hennessy, D., McCabe, M. S., Kenny, D. A., Kelly, A. K., et al. (2020a). Sward type alters the relative abundance of members of the rumen microbial ecosystem in dairy cows. *Sci. Rep.* 10, 1–10. doi: 10.1038/s41598-020-66028-3

Smith, P. E., Waters, S. M., Kenny, D. A., Boland, T. M., Heffernan, J., and Kelly, A. K. (2020b). Replacing barley and soybean meal with by-products, in a pasture based diet, alters daily methane output and the rumen microbial community in vitro using the rumen simulation technique (RUSITEC). *Front. Microbiol.* 11:1614. doi: 10.3389/fmicb.2020.01614/full

Smith, P. E., Waters, S. M., Expósito, R. G., Smidt, H., Carberry, C. A., and McCabe, M. S. (2020c). Synthetic sequencing standards: a guide to database choice for rumen microbiota amplicon sequencing analysis. *Front. Microbiol.* 11:606825. doi: 10.3389/fmicb.2020.606825

Smith, P. E., Waters, S. M., Kenny, D. A., Kirwan, S. F., Conroy, S., and Kelly, A. K. (2021). Effect of divergence in residual methane emissions on feed intake and efficiency, growth and carcass performance, and indices of rumen fermentation and methane emissions in finishing beef cattle. *J. Anim. Sci.* 99:skab275. doi: 10.1093/jas/skab275

Tapio, I., Snelling, T. J., Strozzi, F., and Wallace, R. J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8:7. doi: 10.1186/s40104-017-0141-0

Ungerfeld, E. M. (2020). Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Front. Microbiol.* 11:589. doi: 10.3389/fmicb.2020.00589

Van Gylswyk, N. O., Hippe, H., and Rainey, F. A. (1996). *Pseudobutyrivibrio ruminis* gen. nov., sp. nov., a butyrate-producing bacterium from the rumen that closely resembles *Butyrivibrio fibrisolvens* in phenotype. *Int. J. Syst. Evol.* 46, 559–563. doi: 10.1099/00207713-46-2-559

Wall, E., Simm, G., and Moran, D. (2010). Developing breeding schemes to assist mitigation of greenhouse gas emissions. *Animal* 4, 366–376. doi: 10.1017/S175173110999070X

Wallace, R. J., Rooke, J. A., McKain, N., Duthie, C. A., Hyslop, J. J., Ross, D. W., et al. (2015). The rumen microbial metagenome associated with high methane production in cattle. *BMC Genom.* 16:839. doi: 10.1186/s12864-015-2032-0

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

Wilkins, D., Lu, X. Y., Shen, Z., Chen, J., and Lee, P. K. (2015). Pyrosequencing of mcrA and archaeal 16S rRNA genes reveals diversity and substrate preferences of methanogen communities in anaerobic digesters. *Appl. Environ. Microbiol.* 8, 604–613. doi: 10.1128/AEM.02566-14

Yu, Z., and Morrison, M. (2004). Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 70, 4800–4806. doi: 10.1128/aem.70.8.4800-4806.2004

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 May 2022
doi: 10.3389/fmicb.2022.892605

Metabolite Profile, Ruminal Methane Reduction, and Microbiome Modulating Potential of Seeds of *Pharbitis nil*

*Rajaraman Bharanidharan[1], Krishnaraj Thirugnanasambantham[2,3,4], Ridha Ibidhi[2], Myunggi Baik[1], Tae Hoon Kim[5], Yookyung Lee[6] and Kyoung Hoon Kim[2,5]**

[1] Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul, South Korea, [2] Department of Ecofriendly Livestock Science, Institutes of Green-Bio Science and Technology, Seoul National University, Pyeongchang, South Korea, [3] Pondicherry Centre for Biological Science and Educational Trust, Villupuram, India, [4] Department of Biotechnology, Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences, Chennai, India, [5] Department of International Agricultural Technology, Graduate School of International Agricultural Technology, Seoul National University, Pyeongchang, South Korea, [6] National Institute of Animal Sciences, Rural Development Administration, Jeonju, South Korea

We identified metabolites in the seeds of *Pharbitis nil* (PA) and evaluated their effects on rumen methanogenesis, fiber digestibility, and the rumen microbiome *in vitro* and *in sacco*. Four rumen-cannulated Holstein steers (mean body weight 507 ± 32 kg) were used as inoculum donor for *in vitro* trial and live continuous culture system for *in sacco* trial. PA was tested *in vitro* at doses ranging from 4.5 to 45.2% dry matter (DM) substrate. The *in sacco* trial was divided into three phases: a control phase of 10 days without nylon bags containing PA in the rumen, a treatment phase of 11 days in which nylon bags containing PA (180 g) were placed in the rumen, and a recovery phase of 10 days after removing the PA-containing bags from the rumen. Rumen headspace gas and rumen fluid samples were collected directly from the rumen. PA is enriched in polyunsaturated fatty acids dominated by linoleic acid (C18:2) and flavonoids such as chlorogenate, quercetin, quercetin-3-*O*-glucoside, and quinic acid derivatives. PA decreased ($p < 0.001$) methane (CH_4) production linearly *in vitro* with a reduction of 24% at doses as low as 4.5% DM substrate. A quadratic increase ($p = 0.078$) in neutral detergent fiber digestibility was also noted, demonstrating that doses < 9% DM were optimal for simultaneously enhancing digestibility and CH_4 reduction. *In sacco*, a 50% decrease ($p = 0.087$) in CH_4 coupled with an increase in propionate suggested increased biohydrogenation in the treatment phase. A decrease ($p < 0.005$) in ruminal ammonia nitrogen (NH_3-N) was also noted with PA in the rumen. Analysis of the rumen microbiome revealed a decrease ($p < 0.001$) in the Bacteroidetes-to-Firmicutes ratio, suggesting PA to have antiprotozoal potential. At the genus level, a 78% decrease in *Prevotella* spp. and a moderate increase in fibrolytic *Ruminococcus* spp. were noted in the treatment phase. *In silico* binding of PA metabolites to *cyclic GMP-dependent protein kinase* of *Entodinium caudatum* supported the antiprotozoal effect of PA. Overall, based on its high nutrient value and antiprotozoal activity, PA could probably replace the ionophores used for CH_4 abatement in the livestock industry.

Keywords: methane, rumen, *in silico*, culture systems, PUFA, quercetin, *Entodinium caudatum*

OPEN ACCESS

Edited by:
George Tsiamis, University of Patras, Greece

Reviewed by:
Gabriel De La Fuente Oliver, Universitat de Lleida, Spain
Ana Margarida Pereira, University of the Azores, Portugal

***Correspondence:**
Kyoung Hoon Kim
khhkim@snu.ac.kr

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *09 March 2022*
Accepted: *04 April 2022*
Published: *09 May 2022*

Citation:
Bharanidharan R, Thirugnanasambantham K, Ibidhi R, Baik M, Kim TH, Lee Y and Kim KH (2022) Metabolite Profile, Ruminal Methane Reduction, and Microbiome Modulating Potential of Seeds of Pharbitis nil. Front. Microbiol. 13:892605. doi: 10.3389/fmicb.2022.892605

INTRODUCTION

Ruminal CH_4 production, in addition to its adverse effect on the environment, represents the loss of 3–10% of the gross energy intake of the animal (Appuhamy et al., 2016). Over the past 50 years, ruminant nutritionists have developed numerous strategies for enteric CH_4 abatement (Beauchemin et al., 2020). A meta-analysis showed that dietary supplementation of ionophore antibiotics (e.g., monensin) decreases CH_4 production by up to 15% and increases feed efficiency in beef cattle (Appuhamy et al., 2013). However, the use of ionophores has been banned in many countries, including South Korea, due to concern over their residues in livestock products (Maron et al., 2013; Lee et al., 2018). Similarly, consumer acceptance of 3-nitrooxypropanol, although a promising CH_4 inhibitor, will influence the acceptance of inorganic synthetic compounds (Beauchemin et al., 2020). Therefore, studies have focused on plant-based organic strategies such as supplementing lipids and oil seeds rich in polyunsaturated fatty acids (PUFAs) or additives rich in flavonoids, tannins, and saponins, which reportedly reduce CH_4 production (Hassan et al., 2020; Ku-Vera et al., 2020). Nevertheless, the adverse effects of PUFAs and other phyto-additives on nutrient digestibility, rumen fermentation, and animal performance have raised concerns over the feasibility of such strategies (Beauchemin et al., 2020). Natural seaweeds and seaweed bioactives can reduce CH_4 production with fewer adverse effects on animal productivity (Abbott et al., 2020). However, concern over the safety of feeding bromoform-containing seaweeds to livestock, their associated toxicity to the environment (i.e., ozone depletion), and their net carbon footprint necessitates the discovery of new potential natural feed additives for CH_4 abatement (Glasson et al., 2022).

Our previous *in vitro* screening study involving 152 plant extracts reported that ethanolic extracts from the seeds of *Pharbitis nil* (Pharbitis semen; PA) can decrease CH_4 by up to 37% (Bharanidharan et al., 2021a). *Pharbitis nil* (Convolvulaceae) is an annual climbing herb widely distributed throughout Korea, Japan, and China, and PA is used as a purgative agent and in treating digestive disorders (Kim et al., 2020). Previous phytochemical investigations have reported the isolation of bioactive chemical constituents such as resin glycosides (Ono, 2017), monoterpenoids (Lee et al., 2017), diterpenoids (Ki et al., 2009; Woo et al., 2017), triterpenoid saponins (Jung et al., 2008), flavonoids, chlorogenic acid derivatives (Saito et al., 1994), and phenolic compounds (Kim et al., 2011) from different parts of *P. nil*. Similarly, we reported the enrichment of PUFAs in PA using gas chromatography-mass spectroscopy (GC-MS) without derivatization (Bharanidharan et al., 2021a). However, a complete secondary metabolite and fatty acid (FA) profile of PA is needed to understand their effects on methanogenesis and fermentation. Similarly, studies on the dose response effect of PA on CH_4 production and fermentation characteristics are needed to optimize the additive dosage such that minimal adverse effects on rumen fermentation and nutrient digestibility are achieved. Furthermore, nutritional interventions with high dietary PUFAs should be evaluated, including their *in vivo* effect on the rumen microbiota based on their toxicity toward major cellulolytic bacteria (Beauchemin et al., 2020).

Artificial rumen replacements such as *in vitro* continuous or batch culture systems do not adequately simulate *in vivo* rumen gas and volatile fatty acid (VFA) production due to various factors such as lack of VFA absorption and feed passage *in vitro* (Krishnamoorthy et al., 2005; Amanzougarene and Fondevila, 2020). *In sacco* experimental approaches involving live continuous culture systems (LCCSs) have been used to assess the digestibility of feedstuffs (Krizsan et al., 2013). However, to the best our knowledge, only our previous work (Kim et al., 2016) has used this approach for the initial assessment of feed additives in terms of their effects on CH_4 production and microbial population. Moreover, LCSS-based approaches have yet to be validated.

Here, we profiled PA metabolites using ultra-performance liquid chromatography high-resolution mass spectroscopy (UPLC-HRMS/MS) and GC-MS; evaluated the *in vitro* effect of ground PA on CH_4 production, fermentation, and digestibility; and evaluated *in sacco* the effect of PA on CH_4 production, fermentation, and rumen microbial dynamics using an LCCS.

MATERIALS AND METHODS

Animals, Basal Diet, and Plant Material

Four cannulated Holstein steers (mean body weight 507 ± 32 kg), cared for in accordance with guidelines of the Animal Ethical Committee, Seoul National University, Republic of Korea (approval number SNU-210615-1), were used for the *in vitro* and *in sacco* trials. A diet comprising tall fescue hay and commercial concentrate pellets (33:67, w/w) was used as the basal diet of the donor steers and as substrate for *in vitro* incubations. The animals were fed 5 kg dry matter (DM) of the basal diet twice daily at 09:00 and 17:00 and had *ad libitum* access to fresh water. PA was purchased from a local market in Dongdaemun-gu, Seoul, Republic of Korea. The diet material and PA were dried in a forced-air oven at 65°C for 72 h to estimate the DM content, ground to pass through a 1-mm screen (Thomas Scientific Model 4, Swedesboro, NJ, United States), and stored at −20°C until *in vitro* incubations and chemical analyses. The ingredient and nutrient compositions of the basal diet/substrate are presented in **Table 1**, and its FA composition can be found in **Supplementary Table 1**.

Chemical Analyses

The feed and PA samples were dried in a forced-air oven at 65°C for 72 h to estimate DM content and then ground to pass through a 1-mm screen (Thomas Scientific Model 4). The organic matter content was determined after ashing at 600°C for 3 h using a Nabertherm LE 14/11/R7 Compact Muffle Furnace (Lilienthal, Germany) (Undersander et al., 1993). The ether extract (EE) content was determined using an ANKOM^{XT15} Extractor (Ankom Technology Corp., Fairport, NY, United States) following a filter bag procedure (AM-5-04; 2001) with petroleum ether as the solvent. Neutral detergent fiber (NDF) and acid detergent fiber contents were measured

TABLE 1 | Ingredients and chemical composition of the basal diet.

Ingredient composition, g/kg DM	
Concentrate	
Broken corn	8.4
Wheat	112.2
Sodium bicarbonate	5.5
Rice bran	44.3
Salt	2.0
Molasses	17.9
Ammonium chloride	1.0
CMS	9.9
Corn flake	132.0
DDGS	70.0
Soybean Hull	11.9
Amaferm[1]	0.4
Corn Gluten Feed	132.0
Limestone	21.6
Palm kernel meal	96.8
Mineral-vitamin mixture[2]	1.3
Roughage	
Tall fescue hay	333.0
Chemical Composition, g/kg DM	
Diet/Substrate	
Organic matter (OM)	942.5
Crude protein (CP)	112.5
Ether extract (EE)	43.5
Neutral detergent fiber (aNDFom)[3]	400.5
Acid detergent fiber (ADFom)[4]	202.0
Gross energy (GE, MJ/kg)	18.5

CMS, Condensed Molasses Soluble; DDGS, Dried Distiller's Grains with Solubles.
[1]Amaferm: A fermentation extract of Aspergillus oryzae (Biozyme Enterprises Inc., MO, United States).
[2]Nutrients per kg of additive (Grobic-DC; Bayer HealthCare, Leverkusen, Germany): Vit. A, 2,650,000 IU; Vit. D3, 530,000 IU; Vit. E, 1,050 IU; Niacin, 10,000 mg; Mn, 4,400 mg; Zn, 4,400 mg; Fe, 13,200 mg; Cu, 2,200 mg; I, 440 mg; Co, 440 mg.
[3]Neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash.
[4]Acid detergent fiber excluding residual ash.

using a filter bag technique with an Ankom A2000 Fiber Analyzer (ANKOM Technology Corp.). The neutral detergent fiber content was analyzed using heat-stable amylase and expressed exclusive of residual ash (Van Soest et al., 1991). The analytical method for acid detergent fiber was based on Van Soest (1973), and the results are presented exclusive of residual ash. The nitrogen (N) content was determined using the Kjeldahl method (Kjeltec Auto Sampler System, 8400 Analyzer; Foss, Sweden) as described by (AOAC International, 2016). Crude protein (CP) was calculated as 6.25 × N. The gross energy content was determined using an automatic isoperibol calorimeter (6400EF, Parr Instrument Company, Moline, IL, United States). Concentrations of FAs in the feed and PA were analyzed using the direct methylation method of O'Fallon et al. (2007) and an Agilent 7890B GC system (Agilent Technologies, Santa Clara, CA, United States) with a flame ionization detector as described previously (Bharanidharan et al., 2021b). The FA content was expressed as mg/100 g DM.

Metabolite Extraction and Sample Preparation for Ultra-Performance Liquid Chromatography High-Resolution Mass Spectroscopy

Pharbitis nil samples (10 g) were ground into a homogenous fine powder using a mortar and pestle in liquid nitrogen, followed by extraction with 2 L of methanol-water (80:20, v/v) with continuous stirring for 24 h at room temperature. The resultant extract was filtered using Whatman No. 2 filter paper, and the residue was re-extracted using 1 L of methanol-water (80:20, v/v) with continuous stirring for 12 h at room temperature. Both extracts were pooled and concentrated using a rotary vacuum evaporator (Heidolph Instruments, Schawabatch, Germany) followed by freeze drying (FDFC-12012, Operon, Seoul, South Korea) for 48 h. The dry extract (yield 3.2% w/w) was stored at −80°C until analysis. For UPLC-HRMS analysis, 20 mg of dry extract was dissolved in 2 mL of methanol-water (80:20, v/v) via sonication for 10 min (VCX130, SONICS Vibra-Cell™, Newtown, CT, United States), and the solution was passed through a 0.22-μm polyvinylidene fluoride syringe filter (Millex-GV, Millipore® , Darmstadt, Germany). The filtered samples were dried completely using a nitrogen evaporator (HyperVap HV-300, Labogene, Seoul, South Korea), and the dried extracts were reconstituted (5 mg/mL) in 80% HPLC-grade methanol (Sigma-Aldrich, St. Louis, MO, United States) in an autosample vial prior to analysis.

Ultra-Performance Liquid Chromatography High-Resolution Mass Spectroscopy Analysis for Untargeted Metabolomics

UPLC separation was performed using an UltiMate™ 3000 (Thermo Scientific, Waltham, MA, United States) UPLC system with a CORTECS C18 (2.1 mm × 150 mm, 1.6 μm; Waters, Milford, MA, United States) UPLC column. The flow rate was 0.3 mL/min, and the solvent system consisted of 0.1% aqueous formic acid (A) and acetonitrile with 0.1% formic acid (B). Linear gradient elution was applied as follows: 1% B at 0–1.0 min, 30% B at 1.0–25.0 min, 30-100% B at 25–50 min, and 100-1% B at 50–60 min. The column temperature was held at 45°C, and the injection volume was 5 μL.

Electrospray ionization (ESI)-MS was carried out using a hybrid triple quadrupole time-of-flight (TripleTOF® 5600 + ; AB Sciex, Framingham MA, United States) mass spectrometer with MS1 and MS2 data recorded in both positive and negative ionization modes. The ion spray voltage in positive and negative mode was 5.5 and −4.5 kV, respectively. The desolvation gas (N_2) temperature was set to 500°C. For MS acquisition, the nebulizer gas (N_2), heating gas (N_2), and curtain gas (N_2) flow rates were 50, 50, and 25 psi, respectively. A declustering potential of ±60 V, collision energy of ±35 V, and collision energy spread of ±10 V were applied, in both positive and negative ionization modes. The MS analysis alternated between MS full scanning and information-dependent acquisition scanning.

Elements version 2.1.1 software (Proteome Software Inc., Portland, OR, United States) was used to process raw ion chromatograms. Raw data files were converted to mz5 format using ProteoWizard version pwiz_Reader_ABI: 3.0.9987. Raw profile data and converted data were imported into Elements software for peak identification, alignment, feature extraction, and area normalization, with separate analyses used for positive and negative ionization mode. Feature finding was conducted over a mass range of 30–1500 m/z as described previously (Lim, 2020).

In vitro Effects of *Pharbitis nil* on CH_4 Production and Digestibility

Approximately 300 mL of ruminal fluid was collected from each donor steer before the morning feeding and strained through four layers of muslin before getting pooled into a prewarmed flask flushed with O_2-free CO_2. The fluid was then diluted with O_2-free buffer (McDougall, 1948; adjusted to pH 7.0) at a ratio of 1:2 (v/v) and placed in a water bath pre-heated to 39°C with continuous CO_2 flushing. The *in vitro* trial was performed as described in Bharanidharan et al. (2021a) to test the effect of ground PA. Briefly, incubation was carried out with three replicates with each comprising 30 mL of rumen fluid mixture in 60-mL serum bottles containing 210 mg DM of substrate. The experimental setup comprised a blank (i.e., only rumen fluid mixture without substrate and ground PA), a control (i.e., rumen fluid mixture with substrate but without ground PA), a positive control (i.e., rumen fluid mixture with substrate and 30 ppm monensin; CAS No. 22373-78-0, Sigma-Aldrich), and dietary treatments (i.e., rumen fluid mixture with substrate and 4.5, 9.0, 13.6, 18.1, 22.6, or 45.2% DM ground PA). After 24 h of incubation, the total gas production was measured using water displacement apparatus (Fedorah and Hrudey, 1983). CH_4 concentration in the headspace gas and VFAs in the medium were determined using the Agilent 7890B GC system (Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector using methods described by Bharanidharan et al. (2021a). The pH was measured using a pH meter (model AG 8603; Seven Easy pH, Mettler-Toledo, Schwerzenbach, Switzerland) and the NH_3-N concentration was determined using a modified colorimetric method (Chaney and Marbach, 1962).

In parallel procedures, another fermentation run was conducted to determine the effects of PA on *in vitro* DM and NDF digestibility. Incubation was carried out with three replicates with each comprising 60 mL of rumen fluid mixture in 120-mL serum bottles containing 420 mg DM of substrate. The same experimental setup with the same dietary treatments as above was used for the trial. After 24 h of incubation, the incubation medium was transferred to 50-mL centrifuge tubes. Any particles attached to the walls of the serum bottles were washed off with distilled H_2O and transferred to centrifuge tubes. The tubes were centrifuged at 3000 × *g* (ScanSpeed 1580R, Labogene, Seoul, South Korea) for 20 min, and the supernatants were discarded. The tubes containing the pellets were dried in a forced-air oven at 65°C for 48 h to determine the residual DM weights and NDF digestibility. The digestibility of DM and NDF was calculated based on the proportion of the initial weight incubated lost. The DM content of the blank was used for normalization. The incubation procedure was repeated three times in separate weeks to assess the repeatability.

In sacco Effects of *Pharbitis nil* on CH_4 Production and Rumen Microbial Dynamics

The same four cannulated Holstein steers used as rumen fluid donors were allocated to individual feeders equipped with steel stanchions, and the experiment was carried out as described previously (Kim et al., 2016). A total of twelve polyethylene/nylon bags (10 × 20 cm, pore size 300 ± 20 μm, FILTRA-BAG®, Mfr. No: EFT-1250A, Thomas Scientific) were filled with 60 g of unground PA each (three bags per steer). Each bag was sealed using a heat sealer and placed in a large retaining sac (20 × 30 cm, pore size 3 × 5 mm). A 3-m-long nylon cable was attached to one end of the retaining sac, and a cannula stopper was attached to the other end. The steers were fed the basal diet during a 14-day adaptation period before beginning the trial. The experiment was divided into three phases: a control phase of 10 days without sacs containing PA in the rumen, a treatment phase of 11 days in which three sacs containing PA were placed in three positions in the rumen (total 180 g per steer), and a recovery phase of 10 days after the sacs containing PA were removed from the rumen. The steers were locked in position by metal stanchions and allowed to stand until sampling was complete to minimize changes in ruminal volume and shape. Sampling for ruminal headspace gas was conducted on 11 occasions beginning 3 days before the initiation of the treatment phase (day 0). Gas samples from four steers were collected on days −3, −2, −1, 3, 5, 7, 9, 11, 14, 17, and 21 of the feeding period. On day 12, the steers began the recovery phase. Samples of rumen head space gas were taken at 1, 2, and 3 h after morning feeding by passing a 20-mL graduated syringe connected to a two-way stopcock (KOVAX, Seoul, South Korea) into the rumen through the cannula stopper. Six samples (three each during rumen contraction and relaxation) per sampling period were collected and immediately transferred to a vacuum tube (ref 364979, BD Vacutainer, Becton Dickinson, NJ, United States) for CH_4 analysis as described previously (Bharanidharan et al., 2021a). Ruminal fluid samples were collected on days −1, 3, 11, and 21 at 3.5 h after morning feeding. After immediately measuring the pH using a pH meter (model AG 8603; Seven Easy pH, Mettler-Toledo, Schwerzenbach, Switzerland), ruminal fluid was transferred to a 50-mL centrifuge tube and centrifuged at 12,000 × *g* for 10 min (Supra-22K, Hanil Science Industrial, Gimpo, South Korea). The supernatant was transferred to a 15-mL centrifuge tube and stored at −20°C until analysis of NH_3-N and VFA concentrations as described previously (Bharanidharan et al., 2018).

For microbial analysis, ruminal fluid samples collected on days −1, 11, and 21 were snap-frozen in liquid nitrogen and stored at −80°C until DNA extraction and next-generation sequencing using previously described methods and primers (Bharanidharan et al., 2018). Primers targeting the variable

region V4 (515F/806R) were used because they cover both bacterial and archaeal populations, producing an amplicon size amenable for Illumina MiSeq sequencing (Caporaso et al., 2011; Kozich et al., 2013). The raw Illumina MiSeq reads generated were demultiplexed according to their barcodes, and the sequences were quality-filtered (≥Q20) based on the quality control process in Quantitative Insights into Microbial Ecology (QIIME) version 1.9.0 software with a filtered length of ≥ 200 bp as described previously (Bharanidharan et al., 2021c). The processed paired reads were concatenated into a single read, and each single read was screened for operational taxonomic unit (OTU) picking using the *de novo* clustering method CD-HIT-OTU embedded in QIIME 1.9.0 with reference to the National Center for Biotechnology Information (NCBI) database (NCBI_16S_20190602, 97% nucleotide identity).

In silico Effects of *Pharbitis nil* Metabolites Against *Entodinium caudatum*

Cyclic GMP (cGMP)-dependent protein kinase (cGK), the central regulator of cGMP signaling in malaria parasites and a potential target for new antimalarial drugs (Baker et al., 2020), was used to evaluate the potential antiprotozoal effects of major PA metabolites. Because the rumen protozoa-specific protein sequence of cGK was not available in non-redundant databases, we used a manual method to determine the protein sequence. The partial mRNA sequence of cGK 9-1(XM_001017123) from *Tetrahymena thermophila* SB210, a widely used protozoal model, was retrieved from the NCBI database (accessed on 02 September 2021). The non-annotated genome assembly of *E. caudatum* MZG-1 (NBJL00000000.3) was downloaded from the NCBI database (accessed on 02 September 2021), and a local nucleotide database was created in BioEdit version 7.2.5 (Scripps Research, La Jolla, CA, United States) (Hall, 1999). A local nucleotide BLAST (BLASTn) search of the *T. thermophila* SB210 cGK nucleotide sequence was performed against the *E. caudatum* strain MZG-1 genome database. The result revealed nucleotide homology with *E. caudatum* strain MZG-1 (NBJL03004678.1). Furthermore, a protein homolog of the *E. caudatum* strain MZG-1 protein was searched for using the BLASTx algorithm[1]. The results revealed that the sequence of *E. caudatum* strain MZG-1 was 67% homologous (*e*-value = 0) to *Bacillus* cyclic nucleotide-binding domain-containing protein (MBP3841481) and its corresponding nucleotide (JAGAWU010000032) sequences. The corresponding aligned nucleotide sequence of *E. caudatum* strain MZG-1 was extracted and translated to an amino-acid sequence using the BLASTx algorithm. The amino-acid sequence was confirmed to encode cGK and was later used as the query for structure prediction.

The primary structural parameters of the *E. caudatum* strain MZG-1 specific cGK (query) protein were determined using ProtParam[2]. The physicochemical characters such as molecular weight, theoretical isoelectric point, amino-acid composition, extinct coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity were computed. Secondary structure conformational parameters related to the presence of α-helices, β-turns, extended strands, and random coils were computed using the self-optimized prediction method with alignment (SOPMA) tool[3]. The similarity of the query protein with publicly available homologs was assessed by searching against non-redundant databases, including NCBI and Protein Data Bank (PDB) databases. The online protein structure prediction tool PS[2] [4] was used to predict the three-dimensional structure of the protein. Superimposition of predicted models of the query onto the template was performed using SALIGN[5] and visualized using the UCSF Chimera package (Pettersen et al., 2004). The models were analyzed based on the DOPE score, and their stereochemical quality was validated by generating a Ramachandran plot using PROCHECK[6] on the SAVES server[7].

For molecular docking studies, the chemical structures of the major metabolites (ligands) of PA (dicaffeoyl quinic acid, quercetin-3-*O*-glucoside, chlorogenate, palmitic acid, linoleic acid, and oleic acid) were retrieved from the PubChem compound database[8]. The retrieved ligand structures in .sdf format were converted to .pdb format using PyMOL version 1.7.4.5 software (DeLano Scientific LLC, San Carlos, CA, United States). Docking analysis was carried out using AutoDockTools version 1.5.4 and AutoDock version 4.2 software (Scripps Research Institute Molecular Graphics Laboratory, La Jolla, CA, United States) as described previously (Arokiyaraj et al., 2015). The docked complexes were visualized using Discovery Studio Visualizer version 4.5 (BIOVIA, San Diego, CA, United States).

Statistical Analyses

In vitro data were analyzed using the PROC MIXED procedure of SAS version 9.4 (SAS Institute, Cary, NC, United States) with a Tukey-Kramer adjustment. The dose was considered a fixed effect and each incubation run a random effect. Linear, quadratic, and cubic components of the response to increasing doses of PA were evaluated using orthogonal polynomial contrasts. The CONTRAST option of the MIXED procedure uses the coefficient matrix generated in PROC IML for unequally spaced treatments. Pearson's correlation coefficients were calculated to identify correlations between substrate FA concentration, CH_4 production, fermentation characteristics, and digestibility using the PROC CORR function in SAS.

In sacco data were also analyzed using the PROC MIXED procedure of SAS. The statistical model for rumen microbial composition data included treatment type and period as fixed effects and each animal as a random effect. The headspace CH_4 concentration, ruminal fermentation characteristics (pH,

[1]http://blast.ncbi.nlm.nih.gov/Blast.cgi

[2]https://web.expasy.org/protparam/

[3]http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html

[4]http://ps2.life.nctu.edu.tw/

[5]http://salilab.org/salign

[6]http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/

[7]https://saves.mbi.ucla.edu/

[8]https://pubchem.ncbi.nlm.nih.gov/

VFAs, and NH_3), and microbial abundance were subjected to repeated-measures analysis of variance. The statistical model for fermentation parameters used the same model with the inclusion of day as a fixed effect. Within the period, each day was taken as a repeated measurement, and the treatment × day interaction was not evaluated. For the CH_4 data, both day and time were included as fixed effects in the model and were considered repeated measurements. The treatment, day, and time effects as well as treatment × day and treatment × time interactions were evaluated. The Akaike information criterion was used to identify the covariance structure with the best fit, and the autoregressive and compound symmetry covariance structures were used to analyze the fermentation characteristics and CH_4 data, respectively. Means were calculated using the LSMEANS function and were compared using the PDIFF option in SAS. Significant treatment effects were detected by pairwise comparisons employing Tukey's test. Regarding differences according to treatment, $p < 0.05$ was taken to indicate significance and $0.05 < p < 0.1$ was considered to indicate a trend toward significance. To identify bacterial lineages that drive the clustering of microbial communities in different phases, we performed principal component analysis (PCA) using the fviz_pca_biplot function in the FactoMineR package (Husson et al., 2020) in R version 4.0.3 software (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Pharbitis nil Chemical Composition

The proximate and FA composition of PA are shown in **Table 2**. The CP, NDF, and EE contents of PA were 235.0, 421.0, and 129.4 g/kg DM, respectively. The analysis of the FA composition of PA revealed enrichment of saturated FAs (SFAs; 3.5 g/kg DM), monounsaturated fatty acids (MUFAs; 2.3 g/kg DM), and PUFAs (5.1 g/kg DM) dominated by linoleic acid (C18:2; 4.5 g/kg DM). The omega-6:omega-3 FA ratio was 10.2 with enrichment of omega-6 FAs (4.6 g/kg DM).

TABLE 2 | Proximate and fatty acid composition of seeds of *Pharbitis nil*.

Proximate composition (g/kg DM)	
OM	948.0
CP	235.0
EE	129.4
aNDFom[1]	421.0
ADFom[2]	160.0
GE, MJ/kg	20.5
Fatty acid composition (mg/100 g DM)	
Caprylic acid (C8:0)	4.69
Capric acid (C10:0)	12.50
Myristic acid (C14:0)	23.81
Pentadecylic acid (C15:0)	4.03
Palmitic acid (C16:0)	2308.26
Palmitoleic acid (C16:1)	44.70
Margaric acid (C17:0)	10.44
Ginkgolic acid (C17:1)	0.61
Stearic acid (C18:0)	795.82
Elaidic acid (*trans* 9 C18:1)	30.79
Oleic acid (*cis* 9 C18:1)	2253.04
Trans-linoleic acid (C18:2n6t)	28.42
Linoleic acid (C18:2n6c)	4513.31
Arachidic acid (C20:0)	147.67
Gamma-Linolenic acid (C18:3n6)	60.80
Gondoic acid (C20:1n9)	7.07
Alpha linolenic acid (C18:3n3)	453.34
Heneicosylic acid (C21:0)	6.11
Eicosadienoic acid (C20:2n6)	8.29
Behenic acid (C22:0)	86.17
Tricosylic acid (C23:0)	2.33
Arachidonic acid (C20:4n6)	10.18
Lignoceric acid (C24:0)	51.39
SFA	3448.53
MUFA	2336.21
Omega-6	4620.99
Omega-3	453.34
Omega6:3	10.19
PUFA	5074.33
Trans fat	1534.24
Total fatty acids (mg/100 g DM)	10863.76

[1]*Acid detergent fiber expressed excluding residual ash.*
[2]*Neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash.*
OM, organic matter; CP, crude protein; EE, ether extract; GE, gross energy; SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids.
SFA = C10:0 + C11:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C24:0.
MUFA = C14:1n5 + C16:1n7 + C17:1n7 + C18:1n7 + C18:1n9 + C20:1n9 + C22:1n9 + C24:1n9.
Omega-6 = C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:2n6 + C22:4n6.
Omega-3 = C18:3n3 + C22:6n3.
PUFA = C18:2n6 + C18:2c9,t11 + C18:3n3 + C18:3n6 + C20:2n6 + C20:3n3 + C20:3n6 + C20:4n6 + C20:5n3 + C22:2n6 + C22:4n6 + C22:5n3 + C22:6n3.

Pharbitis nil Metabolite Profile

Altogether, 147 significant analytes were identified in the PA methanolic extract, 59 of which were identified in ESI positive ion mode (**Figure 1A** and **Table 3**), 54 in ESI negative ion mode (**Figure 1B** and **Table 4**), and 34 in both ESI positive and negative ion mode (**Table 5**). The identified analytes can be classified into saccharides and their derivatives, phenolic compounds and their derivatives, alkaloids, flavonoids, triterpenoids, coumarins, quinic acids, FAs, organosulfonic acids, and others. FAs and their derivatives, especially linoleic acid derivatives, were the main constituents in PA. Analytes such as caffeic acid, chlorogenate, quercetin, quercetin-3-*O*-glucoside, betaine, wilforlide A, 1,28-dicaffeoyloctacosanediol, hederagenin base + O-dHex-Hex-Hex, soyasaponin *V*, osmanthuside *H*, ginsenoside Ro, cinncassiol D2 glucoside, sarasinoside A1, and dioctyl sulfosuccinate were present in high proportions. Precursor organic acids including nicotinic acid, coumaric acids, dicaffeoylquinic acid, echinocystic acid, koetjapic acid, linoelaidic acid, and bovinic acid were identified. Finally, the phytosterols fucosterol, campesterol, and sitosterol were detected.

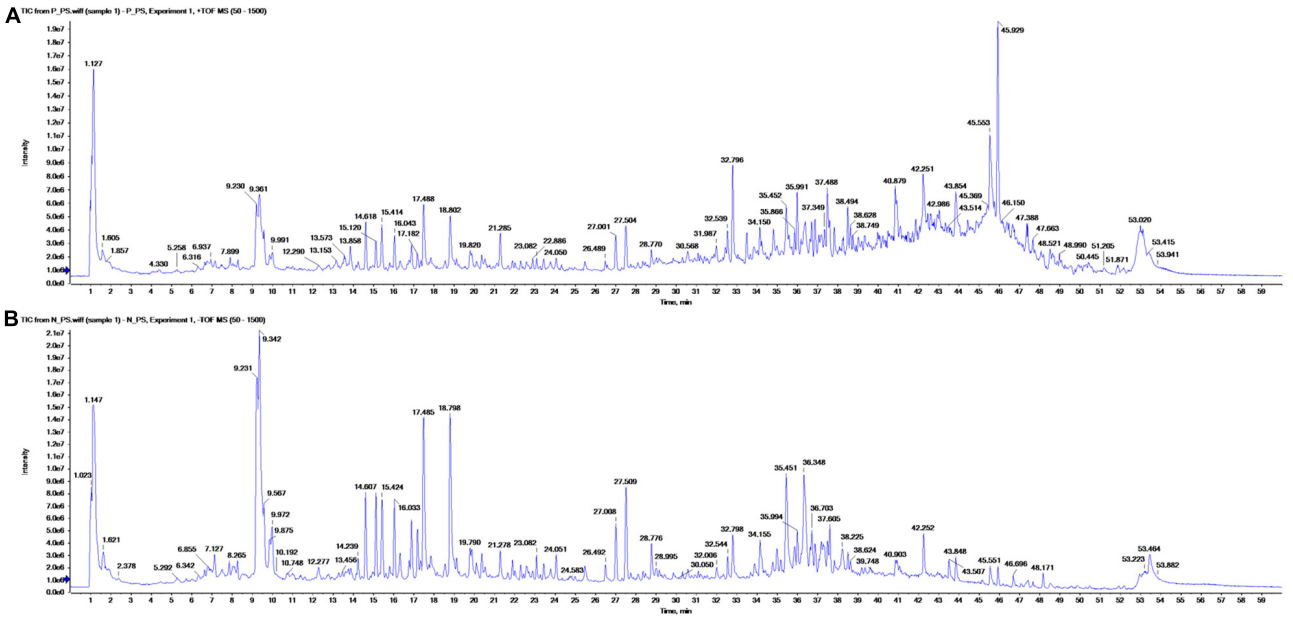


FIGURE 1 | Total ion chromatograms of metabolites in the methanol extract of *Pharbitis nil* seeds in positive **(A)** and negative ionization **(B)** mode from ultra-performance liquid chromatography high-resolution mass spectroscopy analysis.

In vitro Effects of *Pharbitis nil* on CH_4 Production, Fermentation, and Digestibility

Our results negate a dose–response relationship between PA and *in vitro* CH_4 production, fermentation parameters, and digestibility (**Table 6**). Dietary SFA, MUFA, and PUFA concentrations increased with an increasing PA dose, whereas total gas production decreased linearly ($p < 0.001$). The production of CH_4 (mmol/g DM) decreased by 23.7–69.1% compared with the control at doses of 4.5–45.2% DM. At doses $\leq$ 9% DM, enhancement of DM digestibility (DMD; $p = 0.052$) and NDF digestibility (NDFD; $p = 0.073$) by 9.6–15.2% was noted compared to the control, in which 31.1–50.5% decreases ($p < 0.001$) in CH_4 production expressed as mmol/g digestible DM (CH_4/dDM) or NDF (CH_4/dNDF) were observed. At doses $\geq$ 13.6% DM, DMD and NDFD decreased but remained higher than in the control. Although DM and NDF degradation was unaffected at low doses of PA, the production of total VFAs and NH_3-N decreased ($p < 0.001$). Conversely, the molar proportions of the VFAs propionate and butyrate increased linearly ($p < 0.001$) with an increasing dose. By contrast, the proportions of acetate, iso-butyrate, valerate, and iso-valerate decreased linearly ($p < 0.001$).

Pearson's correlation coefficients for the tested parameters are listed in **Table 7**. Strong negative associations of dietary FA concentrations with total gas production ($r = -0.85$; $p < 0.005$), total CH_4 production ($r = -0.85$; $p < 0.005$), and CH_4 production per g dNDF ($r = -0.76$; $p < 0.005$) were noted *in vitro*. The dietary FA concentration was strongly associated with propionate production ($r = 0.89$; $p < 0.005$) and the NH_3-N concentration ($r = -0.71$; $p < 0.005$). However, no association ($p > 0.05$) was noted between the dietary FA concentration and DMD or NDFD. The proportion of propionate was strongly negatively associated with total CH_4 production ($r = -0.95$; $p < 0.005$) and CH_4 production per g dNDF ($r = -0.89$; $p < 0.005$).

In sacco Effects of *Pharbitis nil* on the Rumen Headspace CH_4 Concentration, Fermentation, and the Rumen Microbiome

In sacco, the average CH_4 concentration in the rumen headspace gas sample decreased ($p = 0.087$) from 11.3 to 5.5% in the treatment phase and then steadily increased to 8.9% in the recovery phase (**Figure 2**). An interaction between treatment and sampling day ($p < 0.05$) was noted for the CH_4 concentration. No interaction ($p > 0.05$) between treatment and sampling time was noted. Ruminal fluid pH and NH_3-N concentration decreased ($p < 0.005$) and remained relatively low at the end of the treatment phase, then increased in the recovery phase (**Table 8**). The total VFA concentration ($p < 0.05$) and the proportions of propionate ($p = 0.089$) and butyrate ($p = 0.078$) increased during the treatment phase and recovered toward the control level during the recovery phase. The acetate proportion exhibited the opposite pattern ($p < 0.05$).

Analysis of the rumen microbial reads at a depth of 46,860 quality reads yielded an average of 886 OTUs (range 711–985 OTUs; **Supplementary Figure 1**). Diversity analysis of the rumen microbiota revealed that the total microbial species richness (alpha diversity) was unaffected by PA (**Supplementary Figure 1**). Analysis of the rumen microbial composition revealed marked shifts in relative abundance at the phylum and species levels in the post-treatment period (**Figures 2**, **3**

TABLE 3 | Metabolites in the total methanolic extract of *P. nil* seeds identified using ultra-performance liquid chromatography high-resolution mass spectroscopy (UPLC-HRMS/MS) in positive ion mode.

Retention time (min)	Class	Analyte name	Formula	MW (g/mol)	log_{10} Ion Intensity
1.09	Antioxidant	Betaine	$C_5H_{11}NO_2$	117.10	6.05
1.13	Saccharide	Sucrose	$C_{12}H_{22}O_{11}$	342.10	5.84
1.55	Pyridine carboxylic acids	Nicotinic acid	$C_6H_5NO_2$	123.00	5.75
2.00	Aromatic amino acid	Tyrosine	$C_9H_{11}NO_3$	181.10	5.23
2.71	Ribonucleoside	Adenosine	$C_{10}H_{13}N_5O_4$	267.10	5.64
4.38	Aromatic amino acid	Phenylalanine	$C_9H_{11}NO_2$	165.10	5.92
4.40	Aralkylamines	Phenylethanolamine	$C_8H_{11}NO$	137.10	5.66
9.22	Cinnamate ester and tannin	Chlorogenate	$C_{16}H_{18}O_9$	354.10	5.84
9.57	Phenethyl alcohol glycosides	Darendoside A	$C_{19}H_{28}O_{11}$	432.20	6.79
13.86	Hydroxycinnamic acids	*p*-coumaric acid	$C_9H_8O_3$	164.00	5.44
15.13	Unknown	NCGC00380707-01_C26H42O11_(5xi,6alpha,7alpha,9xi,16xi)-16-(beta-D-Glucopyranosyloxy)-6,7,17-trihydroxykauran-19-oic acid	$C_{26}H_{42}O_{11}$	530.30	6.09
15.41	Flavonoid	Quercetin	$C_{15}H_{10}O_7$	302.00	5.47
16.89	Gamma-lactone	Marrubiin	$C_{20}H_{28}O_4$	332.20	5.61
17.93	Hydroxycinnamic acids	*Cis*-caffeic acid	$C_9H_8O_4$	180.00	5.30
21.29	Hydroxycinnamic acids	*p*-Coumaric acid	$C_9H_8O_3$	164.00	5.42
22.90	Alkaloid	Ergocristine	$C_{35}H_{39}N_5O_5$	609.30	4.86
24.11	Unknown	Gabapentin related compound D	$C_{18}H_{29}NO_3$	307.20	5.34
26.98	Triterpenoids	Jujubasaponin IV	$C_{48}H_{78}O_{18}$	942.50	4.83
27.01	Triterpenoids	Echinocystic acid	$C_{30}H_{48}O_4$	472.40	5.87
27.50	Phenolic glycosides	Wilforlide A	$C_{30}H_{46}O_3$	454.30	5.63
28.76	Steroid acids	Koetjapic acid	$C_{30}H_{46}O_4$	470.30	5.31
29.02	Fatty acyls	9-KODE	$C_{18}H_{30}O_3$	294.20	5.17
29.02	LCFA	(Z)-5,8,11-trihydroxyoctadec-9-enoic acid	$C_{18}H_{34}O_5$	330.20	5.68
32.52	Unknown	NCGC00385219-01_C17H20O4_3,6,9-Tris(methylene)-2-oxododecahydroazuleno[4,5-b]furan-8-yl acetate	$C_{17}H_{20}O_4$	288.10	4.80
32.80	Coumaric acids and derivatives	1,28-Dicaffeoyloctacosanediol	$C_{46}H_{70}O_8$	750.50	5.01
32.85	Fatty acyls	15-Ketoprostaglandin E1	$C_{20}H_{32}O_5$	352.20	5.23
33.85	Amines	Phytosphingosine	$C_{18}H_{39}NO_3$	317.30	5.39
33.87	MCFA	9(10)-Epoxy-12Z-octadecenoic acid	$C_{18}H_{32}O_3$	296.20	5.62
34.32	LCFA	Vernolic acid	$C_{18}H_{32}O_3$	296.20	6.35
34.54	LCFA	Linolenic acid	$C_{18}H_{30}O_2$	278.20	5.99
34.38	Glycerophosphocholines	Lysophosphatidylcholine(18:3)	$C_{26}H_{48}NO_7P$	517.30	4.66
35.42	Oxo fatty acid	12(13)Ep-9-KODE	$C_{18}H_{30}O_4$	310.20	4.95
35.45	LCFA	Linoelaidic acid	$C_{18}H_{32}O_2$	280.20	5.98
35.81	Monoglyceride	2-monopalmitin	$C_{19}H_{38}O_4$	330.30	4.68
35.86	Amines	Desferrioxamine H	$C_{20}H_{36}N_4O_8$	460.30	6.44
35.98	Unknown	Asperhenamate_120258	$C_{32}H_{30}N_2O_4$	506.20	5.75
36.00	Glycerophosphocholines	1-Linoleoyl-lysophosphatidylcholine	$C_{26}H_{50}NO_7P$	519.30	6.96
36.35	Triterpenoids	Ganoderic acid eta	$C_{30}H_{44}O_8$	532.30	6.70
36.39	Glycerophosphocholines	1-palmitoyl-lysophosphatidylcholine	$C_{24}H_{50}NO_7P$	495.30	5.77
36.85	Monoglyceride	Glyceryl linolenate	$C_{21}H_{36}O_4$	352.30	5.83
37.48	Glycerophosphocholines	Oleoyl-lysophosphatidylcholine	$C_{26}H_{52}NO_7P$	521.30	7.05
39.16	Fatty amide	Linoleoyl ethanolamide	$C_{20}H_{37}NO_2$	323.30	5.68
39.33	Glycerophosphocholines	Stearoyl lysophosphatidylcholine	$C_{26}H_{54}NO_7P$	523.40	6.35
40.86	Monoglyceride	1-Linoleoylglycerol	$C_{21}H_{38}O_4$	354.30	6.44
40.93	Fatty amide	Oleoyl Ethanolamide	$C_{20}H_{39}NO_2$	325.30	5.19
41.89	Glycerophosphocholines	1-eicosanoyl-sn-glycero-3-phosphocholine	$C_{28}H_{58}NO_7P$	551.40	5.15
42.18	Unknown	Monoacyl glycerol	$C_{21}H_{40}O_4$	356.30	5.47
42.23	Fatty acyls	Bovinic acid	$C_{18}H_{32}O_2$	280.20	6.74
42.74	Chlorins	Pheophorbide A	$C_{35}H_{36}N_4O_5$	592.30	5.52

(Continued)

TABLE 3 | (Continued)

Retention time (min)	Class	Analyte name	Formula	MW (g/mol)	log_{10} Ion Intensity
43.14	Unknown	NCGC00380823-01!2-(14-methylpentadecanoylamino)-3-phenylpropanoic acid	$C_{25}H_{41}NO_3$	403.30	4.80
43.62	Fatty amide	*N*-Oleyl-Leucine	$C_{24}H_{45}NO_3$	395.30	4.62
44.28	Ester	13S-Hydroxy-9Z,11E-octadecadienoic acid, methyl ester	$C_{19}H_{34}O_3$	310.30	4.89
45.15	Stigmastanes	Fucosterol	$C_{29}H_{48}O$	412.40	5.70
45.77	Unknown	Digalactosyldiacylglycerols-36:6	$C_{51}H_{84}O_{15}$	936.60	5.73
45.89	Ergostane steroids	Campesterol	$C_{28}H_{48}O$	400.40	5.37
45.94	Fatty amide	Erucamide	$C_{22}H_{43}NO$	337.30	7.64
46.73	Stigmastanes	Sitosterol	$C_{29}H_{50}O$	414.40	5.92
47.36	Triterpenoids	Friedelin	$C_{30}H_{50}O$	426.40	4.67
48.54	Diglycerides	Diacylglycerol(18:3n6/18:1n9)	$C_{39}H_{68}O_5$	616.50	5.53

MCFA, medium chain fatty acids; LCFA, long chain fatty acids.

and **Supplementary Table 2**). Decreased abundances of sequences assigned to the phyla Bacteroidetes ($p < 0.05$), Spirochaetes ($p < 0.05$), and Proteobacteria ($p = 0.062$) and an increase ($p < 0.05$) in the phylum Firmicutes were observed post-treatment (**Figures 3**, **4** and **Supplementary Table 2**). Consequently, a decrease ($p < 0.005$) in the Bacteroidetes:Firmicutes ratio was noted (**Figure 5**). Regarding beta diversity, PCA revealed that all rumen samples from steers at different phases clustered separately, with 43.9% of the variation explained (**Figure 6**). PA led to a 78% decrease ($p < 0.05$) in the abundance of OTUs assigned to the most abundant bacterial species, *Prevotella ruminicola* (18.1%), compared to the control phase (**Supplementary Table 2**). Other species in the families *Prevotellaceae* and *Paraprevotellaceae* exhibited decreases of similar magnitude. In terms of fibrolytic microorganisms, OTUs assigned to *Butyrivibrio fibrisolvens*, *Ruminococcus albus, Ruminococcus bromii,* and *Ruminococcus lactaris* increased ($p < 0.05$) several fold post-treatment (**Supplementary Table 2**). The abundance of *Streptococcus* sp. increased several-fold. Although the abundance of Phylum Euryarchaeota was unchanged, OTUs assigned to the genus *Methanobrevibacter* and *Methanosphaera* increased ($p < 0.05$) post-treatment (**Supplementary Table 2**).

In silico Analysis of the Effects of Active Metabolites From *Pharbitis nil* on cGK

The results of a primary structural evaluation of the deduced cGK protein using ProtParam are shown in **Supplementary Tables 3, 4**. Evaluation of secondary structure conformational parameters using the SOPMA tool indicated that cGK is composed of 29.06% loops, 46.39% helices, 16.83% extended strands, and 7.72% β-turns (**Supplementary Figure 2**). Homology modeling of cGK performed based on the crystal structure of the A chain of *Plasmodium vivax* Sal-1 cGK (PDB ID: 4RZ7) as the template yielded sequence identity and *e*-values of 41.8 and 2e-94, respectively (**Supplementary Figure 3A**). The predicted model of cGK was superimposed on the cGK template (PDB ID: 4RZ7; **Supplementary Figure 3B**). PROCHECK analysis to assess the quality of the predicted model using a Psi/Phi Ramachandran plot indicated that 85.2% of the residues were in the most-favored regions, 10.1% were within additionally allowed regions, and 2.3% were in disallowed regions (**Supplementary Table 5** and **Supplementary Figure 3C**). The G-factor for the modeled cGK was −0.27, revealing the predicted model to be of high quality (**Supplementary Table 6**). Therefore, model validation suggested that the model adequately represented the native protein.

Analysis of docked protein-ligand complexes based on binding affinity revealed that the predicted protein model of *E. caudatum* cGK has the highest binding energies of −6.83, −6.75, and −5.92 kcal/mol with dicaffeoyl quinic acid, quercetin-3-*O*-glucoside, and chlorogenate, respectively (**Supplementary Table 7**). Long-chain FAs such as linoleic acid, oleic acid, and palmitic acid also exhibited fair binding to cGK with moderate energy values of −4.75, −4.52, and −4.14 kcal/mol, respectively (**Supplementary Table 7**). Hydrogen bond interactions between the ligands and the active residues of cGK were noted (**Figures 7A–F** and **Supplementary Table 7**).

DISCUSSION

Research on plant-based natural feed additives has been promoted due to restrictions on the non-therapeutic use of antibiotics in livestock production. Research into natural resources with high nutritional value and bioactive compounds can enhance productivity and mitigate the environmental footprint of livestock systems. Following our previous work (Bharanidharan et al., 2021a), we investigated for the first time the nutritive value and secondary metabolites of PA. We found high contents of CP, NDF, fat, and FAs in PA, suggesting that it has high nutritive value. The greater contents of FAs identified using GC-MS and FA derivatives identified using UPLC-HRMS/MS corroborate our prior finding (Bharanidharan et al., 2021a) that 60% of the compounds identified using GC-MS were derivatives of long-chain FAs. The fat content (129 g/kg DM) of PA is similar to that of grapeseed (Kapcsándi et al., 2021), whereas the FA profile (C16:0 [21%], C18:0 [21%], C18:2 [41%]) is comparable with that of sunflower seeds (Akkaya, 2018).

TABLE 4 | Metabolites in the total methanolic extract of *P. nil* seeds identified using UPLC-HRMS/MS in negative ion mode.

Retention time (min)	Class	Analyte name	Formula	MW (g/mol)	log_{10} Ion Intensity
1.07	Saccharide	Sorbitol	$C_6H_{14}O_6$	182.10	5.54
1.09	Saccharide	Gluconate	$C_6H_{12}O_7$	196.10	6.58
1.12	Saccharide	Raffinose	$C_{18}H_{32}O_{16}$	504.20	6.28
1.14	Saccharide	Sucrose	$C_{12}H_{22}O_{11}$	342.10	6.24
2.16	Peptides	Glutathione disulfide	$C_{20}H_{32}N_6O_{12}S_2$	612.20	4.61
6.96	Amino acids	Tryptophan	$C_{11}H_{12}N_2O_2$	204.10	5.39
9.09	Alkyl-phenylketones	3,4-Dihydroxyacetophenone	$C_8H_8O_3$	152.00	4.78
9.12	Phenolic glycoside	Feruloyl Hexoside (isomer of 847)	$C_{16}H_{20}O_9$	401.11	5.15
9.58	Coumaric acid esters	Osmanthuside H	$C_{19}H_{28}O_{11}$	432.20	6.54
9.93	Cinnamate ester and tannin	Chlorogenate	$C_{16}H_{18}O_9$	354.10	6.84
12.11	Aromatic amino acid tyrosine	Tyrosine	$C_9H_{11}NO_3$	181.10	5.18
12.94	Phenyl propanoid	Coumarin + 1O	$C_9H_6O_3$	162.00	4.67
15.12	Terpene glycosides	Cinncassiol D2 glucoside	$C_{26}H_{42}O_{11}$	530.30	6.52
15.38	Lignan glycosides	Liriodendrin	$C_{34}H_{46}O_{18}$	742.30	5.09
16.30	Flavanones	Silydianin	$C_{25}H_{22}O_{10}$	482.10	6.12
17.19	Flavonoid glycosides	5,7-dihydroxy-6-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-8-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-2-one	$C_{21}H_{26}O_{15}$	518.10	5.22
18.16	Flavonoid glycosides	Isorhamnetin 3-galactoside	$C_{22}H_{22}O_{12}$	478.10	5.38
18.79	Quinic acids	4,5-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.10	6.01
19.78	Triterpene saponins	Soyasaponin V	$C_{48}H_{78}O_{19}$	958.50	5.89
19.87	Phenolic glycoside	Lipedoside A	$C_{29}H_{36}O_{14}$	608.20	6.04
20.92	Triterpene sapogenins	Soyasaponin Ba	$C_{48}H_{78}O_{19}$	958.50	4.67
21.21	Quinic acids	4,5-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.10	5.21
22.01	Triterpene saponins	Ginsenoside Ro	$C_{48}H_{76}O_{19}$	956.50	5.12
23.43	Triterpene saponins	3-Glc-Gal-GlcUA-Soyasapogenol B	$C_{48}H_{78}O_{19}$	958.50	5.78
23.77	Unknown	[(4R,5S,6R,6aS,7R,10aR,11bR)-5-acetyloxy-6-hydroxy-10a-methoxy-4,7,11b-trimethyl-9-oxo-1,2,3,4a,5,6,6a,7,11,11a-decahydronaphtho[2,1-f][1]benzofuran-4-yl]methyl acetate	$C_{25}H_{36}O_8$	464.20	5.33
24.05	Triterpene glycosides	3-(Rha(1-2)Glu(1-2)Glu-28-Glu Hederagenin	$C_{54}H_{88}O_{23}$	1104.60	5.78
25.47	Triterpene glycosides	3-Rha(1-2)Gal(1-2)GluA-Soyasaponenol B	$C_{48}H_{78}O_{18}$	942.50	5.57
26.49	Fatty acid esters	26-(2-Glucosyl-6-acetylglucosyl]-1,3,11,22-tetrahydroxyergosta-5,24-dien-26-oate	$C_{42}H_{66}O_{17}$	842.40	5.88
27.01	Triterpene saponins	Hederagenin base + O-dHex-Hex-Hex	$C_{48}H_{78}O_{18}$	942.50	6.37
27.51	Unknown	NCGC00381017-01_C48H78O18_beta-D-Glucopyranose, 1-*O*-[(3beta,5xi,9xi)-3-[(6-deoxy-3-*O*-beta-D-glucopyranosyl-alpha-L-mannopyranosyl)oxy]-23-hydroxy-28-oxoolean-12-en-28-yl]	$C_{48}H_{78}O_{18}$	942.50	6.49
28.34	Monoterpenoids	Soyasapogenol B base + O-HexA-HexA-Hex + Me + Acetyl	$C_{51}H_{80}O_{21}$	1028.50	5.39
28.48	Monoterpenoids	Soyasapogenol B base + O-HexA-Hex	$C_{42}H_{68}O_{14}$	796.50	4.89
28.83	Unknown	NCGC00169139-03_C42H66O15_1-*O*-[(3beta,5xi,9xi,18xi)-3-(beta-D-Glucopyranuronosyloxy)-23-hydroxy-28-oxoolean-12-en-28-yl]-beta-D-glucopyranose	$C_{42}H_{66}O_{15}$	810.40	4.92
28.88	Triterpene saponins	Bayogenin base + O-Hex	$C_{36}H_{58}O_{10}$	650.40	4.55
29.00	Fatty acyls	5-(oleoyloxy)octadecanoicacid	$C_{18}H_{32}O_4$	312.20	6.15
30.31	Fatty acyls	9-HPODE_RT1	$C_{18}H_{32}O_4$	312.20	5.96
30.58	Triterpene saponins	Elatoside K	$C_{53}H_{84}O_{23}$	1088.50	5.03
32.01	Triterpene saponins	Camelliasaponin A1	$C_{58}H_{92}O_{25}$	1188.60	5.40
32.24	Macrolide	Zearalenone	$C_{18}H_{22}O_5$	318.10	5.09
32.81	Fatty acyls	13-HPODE	$C_{18}H_{32}O_4$	312.20	6.12
34.21	Glycerophospholipids	LPI 18:3	$C_{27}H_{47}O_{12}P$	594.30	5.75
35.19	Unknown	(E,2S,3R,4R,5S)-4-acetyloxy-2-amino-3,5,14-trihydroxyicos-6-enoic acid	$C_{22}H_{41}NO_7$	431.30	6.03
35.48	Glycerophospholipids	LPI 18:2	$C_{27}H_{49}O_{12}P$	596.30	6.89
35.99	Glycerophospholipids	LPS 21:1	$C_{27}H_{52}NO_9P$	565.30	6.27
36.41	Glycerophospholipids	Lysophosphatidylcholine(16:0)	$C_{24}H_{50}NO_7P$	495.30	5.01

(Continued)

TABLE 4 | (Continued)

Retention time (min)	Class	Analyte name	Formula	MW (g/mol)	log_{10} Ion Intensity
36.70	LCFA	Linolenic acid	$C_{18}H_{30}O_2$	278.20	6.31
36.64	Glycerophospholipids	LPI 18:1	$C_{27}H_{51}O_{12}P$	598.30	5.64
36.80	Glycerophospholipids	LPG 18:2	$C_{24}H_{45}O_9P$	508.30	5.19
37.05	Glycerophospholipids	LPC 18:1	$C_{26}H_{52}NO_7P$	521.30	5.69
39.33	Glycerophospholipids	LPC 18:0	$C_{26}H_{54}NO_7P$	523.40	5.62
39.64	Glycerophospholipids	LPI 18:0	$C_{27}H_{53}O_{12}P$	600.30	5.86
42.25	Organosulfonic acid	Dioctyl sulfosuccinate	$C_{20}H_{38}O_7S$	422.20	5.31
43.84	Organosulfonic acid	Dioctyl sulfosuccinate	$C_{20}H_{38}O_7S$	422.20	4.77
47.66	LCFA	FAHFA 36:4	$C_{36}H_{62}O_4$	558.50	5.21

LCFA, long chain fatty acids.

Moreover, PA, as a source of omega-3 and -6 FAs (predominantly linoleic acid), can promote animal and human health (Haug et al., 2007). Plant secondary metabolites (PSMs) such as polyphenols reportedly benefit the health and productivity of ruminants (Olagaray and Bradford, 2019). The identification of flavonoids such as osmanthuside H, *p*-coumaric acid, quercetin, quercetin-3-*O*-glucoside, quercetin-4-*O*-glucoside, caffeic acid, ferulic acid, chlorogenic acid, and their derivatives in PA is consistent with previous studies (Saito et al., 1994; Kim et al., 2011). However, several metabolites (including betaine, wilforlide A, 1,28-dicaffeoyloctacosanediol, ginsenoside Ro, and other saturated and unsaturated FAs) are reported for the first time in this study. Notably, dioctyl sulfosuccinate, an anionic surfactant that is used as a laxative and stool softener, was identified in PA for the first time. PA is used in traditional medicine for its gastroprokinetic effect (Kim et al., 2020). In addition, the identified metabolites, including the FAs, have been shown to exhibit antimicrobial activity against bacteria (McGaw et al., 2002; Vasta et al., 2019) and protozoa (Matsumoto et al., 1991; Ayemele et al., 2020). Therefore, these compounds could be used to manipulate the rumen microbiome to mitigate CH_4 emissions and optimize feed efficiency.

It is valuable to identify and assess the effects of metabolites present in plant materials on rumen fermentation characteristics and microbes than testing the raw plant material directly.

It is because the environmental factors such as soil conditions could greatly affect the concentration of the active metabolite in that plant. This in turn would influence the optimal dose determination and practical application of the plant material. However, it is also important to consider the synergic effects from other components which could be achieved when plant raw materials are used directly. Because the effects of crude PA extract was investigated at one dose in our previous study (Bharanidharan et al., 2021a), we used raw plant material at range of doses here. The decrease in CH_4 production in response to PA *in vitro* confirms our previous observation (Bharanidharan et al., 2021a). At doses as low as 4.5% DM, PA decreased the production of CH_4 (mmol/g DM incubated) by 24% *in vitro*, similar to 30 ppm monensin. The same effect was evident *in sacco*; the CH_4 concentration in the rumen headspace gas decreased by 50% after 10 days. However, this could not be considered as a decrease in absolute CH_4 production, since the total gas production was not quantified. This observed effect of PA on methanogenesis might have been related to the higher concentrations of FAs (3.8–5.8% DM) *in vitro*. The maximum decrease in CH_4 production was 69% *in vitro*, and there were strong negative associations between the FA concentration and gas parameters, as reported previously (Patra, 2013). Patra (2013) suggested that for each percentage point increase in fat in the diet, CH_4 emission decreased by 3.77% *in vivo* with a maximum reduction of 15.1% at 6% dietary DM, which is lower than our finding. It is possible that other PSMs in PA might have contributed to the effect (Ku-Vera et al., 2020). In addition, *in vitro* culture systems can overestimate such effects compared to *in vivo* systems (Machmüller, 2006).

The inhibitory effects of fats, FAs, and other PSMs on rumen methanogenesis are related to altered H_2 thermodynamics in the rumen caused by a decrease in the populations of ciliate protozoa, their associated methanogens and other H_2 producing bacteria (Newbold et al., 2015; Toprak, 2015). Although the protozoal population was not quantified in the current study, the decrease in methanogenesis in the current study could be attributed to the 90% decrease in total ciliate protozoan population as observed upon addition of ethanol extract of PA in our previous *in vitro* study (Bharanidharan et al., 2021a). Generally, rumen protozoa are associated with and engulf rumen bacteria and archaea (Park and Yu, 2018). Rumen protozoa degrade the proteins of engulfed microbial cells into oligopeptides and free amino acids, which are deaminated to produce free NH_3-N, leading to inefficient dietary N utilization (Newbold et al., 2015). A recent study also reported that the presence or absence of different protozoal genera differentially modulate rumen prokaryotic community ecological structure (Solomon et al., 2021). Therefore, in this study, the shift in Bacteroidetes to Firmicutes ratio in the treatment phase could also be partially related to the defaunation effect of PA. However, other reasons including the specific binding of FAs in PA to the lipid bilayer membrane of Gram-negative bacteria that leads to cell death (McGaw et al., 2002) could also be attributed to the microbial shift. Huws et al. (2015) reported a 65% decrease in the *Prevotella* population upon adding C18:3-rich flax seed oil to the diet, supporting our hypothesis. Consistent with our results, Park et al. (2019) indicated a decrease in the Bacteroidetes:Firmicutes ratio as well as decreased *Prevotella* and increased *Streptococcus*

TABLE 5 | Metabolites in the total methanolic extract of *P. nil* seeds identified using UPLC-HRMS/MS in both positive and negative ion modes.

Retention time (min)	Class	Analyte name	Formula	MW (g/mol)	log_{10} Ion Intensity	
					Positive	Negative
1.11	Aminoacids	Arginine	$C_6H_{14}N_4O_2$	174.10	6.64	4.88
1.62	Antioxidant	Citrate	$C_6H_8O_7$	192.00	5.74	4.80
1.80	Alpha-amino acid	D-Glutamine	$C_5H_{10}N_2O_3$	146.10	5.99	5.12
1.93	Aminoacids	*N*-acetylglutamate	$C_7H_{11}NO_5$	189.10	5.98	5.98
6.06	Vitamin	Vitamin B5	$C_9H_{17}NO_5$	219.10	5.50	4.89
6.63	Purine nucleosides	*N6*-Succinyladenosine	$C_{14}H_{17}N_5O_8$	383.10	6.55	6.11
7.13	Cinnamate ester and a tannin	Chlorogenate	$C_{16}H_{18}O_9$	354.10	6.26	6.52
9.37	Hydroxycinnamic acids	*Cis*-caffeic acid	$C_9H_8O_4$	180.00	7.17	5.86
14.13	Antioxidant	Ferulate	$C_{10}H_{10}O_4$	194.10	5.07	5.02
14.43	Unknown	NCGC00380707-01_C26H42O11_(5xi,6alpha,7alpha,9xi,16xi)-16-(beta-D-Glucopyranosyloxy)-6,7,17-trihydroxykauran-19-oic acid	$C_{26}H_{42}O11$	530.30	5.52	5.88
15.42	Antioxidant	Quercetin-3-*O*-glucoside	$C_{21}H_{20}O_{12}$	464.10	6.18	6.46
16.03	Unknown	NCGC00380707-01_C26H42O11_(5xi,6alpha,7alpha,9xi,16xi)-16-(beta-D-Glucopyranosyloxy)-6,7,17-trihydroxykauran-19-oic acid	$C_{26}H_{42}O_{11}$	530.30	6.03	6.50
16.05	Antioxidant	Quercetin 3-galactoside	$C_{21}H_{20}O_{12}$	464.10	5.42	6.12
16.14	Antioxidant	Quercetin-4-*O*-glucoside	$C_{21}H_{20}O_{12}$	464.10	5.42	6.15
17.49	Polyphenols	1,3-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.10	7.14	7.17
17.50	Polyphenols	Dicaffeoyl quinolactone	$C_{25}H_{22}O_{11}$	498.10	7.02	7.13
17.77	Flavonoid glycosides	Nepitrin	$C_{22}H_{22}O_{12}$	478.10	5.08	5.40
18.82	Polyphenols	3,5-Dicaffeoylquinic acids	$C_{25}H_{24}O_{12}$	516.10	6.93	7.11
18.99	Glycosyloxyisoflavone	Tectoridin	$C_{22}H_{22}O_{11}$	462.10	5.73	5.59
32.78	Resinoside	Sarasinoside A1	$C_{62}H_{100}N_2O_{26}$	1288.70	5.74	5.95
33.89	MCFA	9(10)-Epoxy-12Z-octadecenoic acid	$C_{18}H_{32}O_3$	296.20	5.35	6.02
34.15	LCFA	9,10-DiHOME	$C_{18}H_{34}O_4$	314.20	6.10	6.44
35.67	Glycerophospholipids	1-Linoleoyl-lysophosphatidylserine	$C_{24}H_{44}NO_9P$	521.30	5.12	5.22
35.73	Glycerophospholipids	1-Palmitoylglycerophosphoinositol	$C_{25}H_{49}O_{12}P$	572.30	4.90	6.00
36.22	MCFA	9(10)-Epoxy-12Z-octadecenoic acid	$C_{18}H_{32}O_3$	296.20	4.93	6.31
36.25	LCFA	Linolenic acid	$C_{18}H_{30}O_2$	278.20	6.63	3.00
36.74	Glycerophoethanolamines	LysoPE(16:0/0:0)	$C_{21}H_{44}NO_7P$	453.30	6.13	5.92
37.35	Glycerophoethanolamines	LysoPE(18:1(9Z)/0:0)	$C_{23}H_{46}NO_7P$	479.30	6.26	5.96
37.38	Fatty acyls	9-KODE	$C_{18}H_{30}O_3$	294.20	6.65	5.97
37.39	Fatty acyls	13-KODE	$C_{18}H_{30}O_3$	294.20	6.65	5.97
37.60	Fatty acyls	Linoelaidic acid	$C_{18}H_{32}O_2$	280.20	5.75	6.41
37.62	Fatty acyls	Bovinic acid	$C_{18}H_{32}O_2$	280.20	6.14	6.41
38.23	Unknown	Dihydrocelastryl Diacetate	$C_{33}H_{44}O_6$	536.30	3.08	5.50
39.15	Glycerophoethanolamines	LysoPE(18:0/0:0)	$C_{23}H_{48}NO_7P$	481.30	5.30	5.25

MCFA, medium chain fatty acids; LCFA, long chain fatty acids.

abundances upon inhibition of *E. caudatum*. However, it could not be completely related to the results observed in the current study as different *Entodinium* spp. that exhibit different predatory behavior are represented in different proportions in the rumen (Kišidayová et al., 2021). Furthermore, the decrease in ruminal NH_3-N in this study indicates reduced microbial protein turnover due to the inhibition of protozoa (Dai and Faciola, 2019). However, *Prevotella* species reportedly degrade dietary protein to produce NH_3-N (Griswold et al., 1999); thus, the decrease in their relative abundance might have contributed to the reduced NH_3-N. Although rumen protozoa population is associated with butyrate production, the increase in butyrate proportion upon adding PA *in vitro* may be partially related with the increase in relative abundance of butyrate producers such as *Butyrivibrio fibrisolvens* and *Pseudobutyrivibrio ruminis* in treatment phase of *in sacco* trial. The strong binding of PUFAs such as oleic acid, palmitic acid, and linoleic acid to cGK of *E. caudatum in silico* further provided insight into the antiprotozoal effect of PA, as these FAs are reportedly toxic to rumen protozoa (Dohme et al., 2008). This is the first study to predict the structure of *E. caudatum* cGK, which is involved in cell replication and other cell-cycle processes. Furthermore, in an evaluation of *Calotropis gigantea* extract rich in quercetin, quercetin-3-*O*-glucoside, and other hydrocinnamic acid derivatives for its effect on protozoa, Ayemele et al. (2021) reported a 50% decrease in the abundance of *E. caudatum*

TABLE 6 | Dose–response effect of *P. nil* seeds on *in vitro* methane (CH_4) production, fermentation parameters, and digestibility ($n_{replicate}$ = 4).

Item	Control	Monensin	*P. nil* seeds treatment (% substrate DM)						SEM	*p*-value	
			4.5	9.0	13.6	18.1	22.6	45.2		Linear	Quadratic
Total fatty acids, mg/g DM incubated	34.9	34.9	38.1	41.0	43.7	46.2	48.5	57.9			
PUFA	13.3	13.3	14.9	16.4	17.7	19.0	20.2	24.9			
MUFA	10.2	10.2	10.8	11.3	11.8	12.2	12.6	14.3			
SFA	11.4	11.4	12.4	13.3	14.2	15.0	15.7	18.6			
pH	6.3	6.4	6.4	6.4	6.4	6.4	6.4	6.4	0.01	0.030	0.175
Gas, mmol/g DM incubated	6.9	5.9	5.9	5.0	5.5	4.5	4.7	3.8	0.17	<0.0001	<0.0001
CH_4, mmol/mol gas	162.9	142.9	145.8	127.6	121.0	105.2	101.1	88.3	2.57	<0.0001	<0.0001
CH_4, mmol/g DM incubated	1.1	0.8	0.9	0.7	0.7	0.5	0.5	0.4	0.03	<0.0001	<0.0001
CH_4, mmol/g NDF incubated	2.8	2.1	2.1	1.6	1.6	1.2	1.2	0.9	0.08	<0.0001	<0.0001
CH_4, mmol/g DDM incubated	2.1	1.5	1.4	1.0	1.1	0.8	0.8	0.7	0.10	<0.0001	<0.0001
CH_4, mmol/g DNDF incubated	6.4	4.7	4.5	3.2	3.3	2.5	2.6	2.0	0.34	<0.0001	<0.0001
Total VFA, mmol/g DM incubated	9.6	10.5	9.4	8.7	9.2	9.3	10.1	9.2	0.21	0.531	<0.0001
Acetate, %	47.8	46.0	47.9	46.1	44.5	39.6	34.6	33.2	1.05	<0.0001	0.338
Propionate, %	26.8	30.0	29.1	34.0	35.9	38.2	38.4	41.9	0.78	<0.0001	<0.0001
Isobutyrate, %	1.8	1.5	1.7	1.5	1.6	1.2	1.1	1.1	0.07	<0.0001	0.600
Butyrate, %	17.8	16.0	16.1	13.9	13.3	18.4	24.4	22.3	1.24	<0.0001	<0.0001
Isovalerate, %	4.7	5.6	4.1	3.6	3.9	2.2	1.2	1.2	0.32	<0.0001	0.304
Valerate, %	1.1	1.0	1.0	0.9	0.9	0.4	0.3	0.3	0.06	<0.0001	0.371
Acetate: propionate	1.8	1.6	1.7	1.4	1.2	1.1	0.9	0.8	0.05	<0.0001	<0.0001
NH_3-N, mg/g DM incubated	41.9	38.6	37.4	27.7	28.7	29.2	34.3	27.1	0.64	<0.0001	<0.0001
DMD, mg/g DM incubated	550.7	572.0	603.8	634.6	610.7	603.4	587.8	536.2	25.65	0.472	0.052
NDFD, mg/g NDF incubated	437.0	457.4	480.1	504.0	485.4	479.2	466.8	426.4	28.41	0.627	0.073

SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; NDF, neutral detergent fiber; DDM, digestible dry matter; DNDF, digestible NDF; DMD, dry matter digestibility; NDFD, NDF digestibility.

TABLE 7 | Pearson correlation coefficients for associations between dietary fatty acid content, CH_4 production, fermentation parameters, and digestibility.

	Gas, mmol/g DM	CH_4, mmol/g DM	CH_4, mmol/g DDM	CH_4, mmol/g DNDF	Total VFA	Acetate	Propionate	DMD	NDFD	pH	NH_3-N
Total fatty acids	−0.85**	−0.85**	−0.76**	−0.76**	−0.26	−0.88**	0.89**	−0.23	−0.19	0.13	−0.71**
SFA	−0.85**	−0.85**	−0.76**	−0.76**	−0.26	−0.88**	0.89**	−0.23	−0.19	0.13	−0.71**
MUFA	−0.85**	−0.85**	−0.76**	−0.76**	−0.26	−0.88**	0.89**	−0.23	−0.19	0.13	−0.71**
PUFA	−0.85**	−0.85**	−0.76**	−0.76**	−0.26	−0.88**	0.89**	−0.23	−0.19	0.13	−0.71**
NH_3-N	0.81**	0.82**	0.82**	0.81**	0.57**	0.49*	−0.81**	−0.19	−0.14	−0.30	
pH	−0.49*	−0.46*	−0.56*	−0.52*	−0.10	−0.19	0.30	0.37†	0.26		
NDFD	−0.12	−0.09	−0.28	−0.34	−0.18	0.16	−0.04	0.86**			
DMD	−0.05	−0.08	−0.31	−0.30	−0.25	0.24	−0.04				
Propionate	−0.92**	−0.95**	−0.90**	−0.89**	−0.23	−0.87**					
Acetate	0.80**	0.83**	0.73**	0.73**	−0.10						
Total VFA	0.28	0.21	0.26	0.25							
CH_4, mmol/g DNDF	0.95**	0.96**	0.99**								
CH_4, mmol/g DDM	0.94**	0.97**									
CH_4, mmol/g DM	0.97**										

SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; DDM, digestible dry matter; DNDF, digestible NDF; DMD, dry matter digestibility; NDFD, NDF digestibility.
†*P < 0.1; *P < 0.05; **P < 0.005; ***P < 0.001.*

coupled with a decrease in NH_3-N. This is consistent with the strong binding of quercetin-3-*O*-glucoside to *E. caudatum* cGK. Jin et al. (2021) reported the antimethanogenic potential of caffeic acid, in agreement with the large proportion of that flavonoid in PA in this study. Therefore, the major metabolites of PA may be responsible, at least in part, for inhibiting *E. caudatum*, thereby decreasing CH_4 and NH_3-N production.

Despite the symbiotic relationship between protozoa and methanogens (Levy and Jami, 2018), protozoal inhibition increased methanogen abundance, consistent with our previous

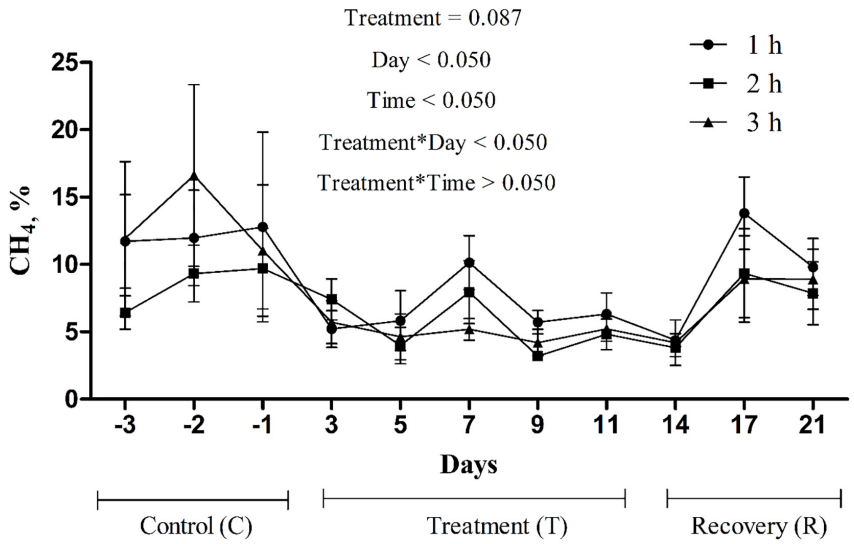


FIGURE 2 | Changes in methane (CH_4) concentration in rumen headspace gas due to the addition of *P. nil* seeds (n = 4 heads).

study (Bharanidharan et al., 2021a). Similar responses to defaunation (Mosoni et al., 2011) and the addition of oils (Nur Atikah et al., 2018) have been reported. The proportion of methanogens that associates with protozoa is not large (Belanche et al., 2014), and other planktonic methanogens in the rumen might not be affected by PA. The decrease in CH_4 caused by feed with increased lipid content is related to the increased production of propionic acid via biohydrogenation of unsaturated FAs (Hook et al., 2010). This is consistent with the strong associations between dietary PUFA content, propionate proportion, and CH_4 production. This suggests that propionate synthesis was the major hydrogen sink despite the greater abundance of methanogens, which use H_2 for CH_4 production. Similarly, a monensin-mediated decrease in CH_4 via increased biohydrogenation was caused by the inhibition of protozoa, whereas the effect on methanogens was minimal (Hook et al., 2010). In addition, the abundance of *B. fibrisolvens*, which is involved in the initial step of rumen biohydrogenation of PUFA to vaccenic acid (McKain et al., 2010), increased in the treatment phase. Moreover, decreased CH_4 production due to linseed and coconut oil rich in PUFAs is reportedly not clearly linked to methanogenic gene abundance or changes in the archaeal community (Patra and Yu, 2013; Martin et al., 2016). It is also possible that the PA metabolites hindered the gene activity of methanogens for reducing H_2 to CH_4 in the rumen. However, after withdrawing PA (recovery phase) *in sacco*, the CH_4 concentration in the headspace gas and other fermentation parameters started reverting to their initial values. This might have been due to the recovery of rumen microbes to their initial composition as a result of probable increase in rumen protozoal population in absence of PA. *In vitro* batch or continuous culture systems cannot be used to evaluate short- and long-term responses together with re-adaptation of microbes, and typically do not yield consistent results, unlike *in sacco* methods involving LCCSs. However, the outward and inward flow of gases through the cannula needs to be considered if this method is to be used to evaluate the effects of plant materials on methanogenesis and other fermentation parameters (Wang et al., 2019). In addition, lack of models for quantifying the total gas production has limited the LCCS approach to determine only the rumen headspace CH_4 concentration rather than the absolute CH_4 production.

TABLE 8 | Effects of *P. nil* seeds on fermentation parameters *in sacco* in a live continuous culture system (n = 4 heads).

Item/Days	Control	Treatment		Recovery	SEM	*p*-value	
	Day -1	Day 3	Day 11	Day 21		Treatment	Day
Ruminal pH	6.8	6.6	6.1	6.6	0.06	0.051	0.001
Total VFA (mM)	94.0	105.5	126.1	106.3	2.50	0.013	0.003
Acetate, %	45.9	44.9	41.6	44.9	0.65	0.509	0.027
Propionate, %	27.9	29.6	30.3	28.9	0.96	0.180	0.400
Butyrate, %	17.2	17.2	20.8	17.5	0.73	0.913	0.012
Isobutyrate, %	1.7	1.5	1.0	1.6	0.09	0.435	0.003
Valerate, %	1.9	1.7	1.7	1.7	0.09	0.482	0.946
Isovalerate, %	5.5	5.2	4.6	5.3	0.39	0.658	0.232
Acetate: propionate	1.7	1.5	1.4	1.6	0.06	0.254	0.006
NH_3-N, mg/dL	18.8	14.0	12.3	18.3	0.95	0.002	0.048

Methane mitigation strategies involving plant additives are useful only if nutrient digestibility and other fermentation parameters are not concomitantly compromised. Dietary supplementation of lipids and the elimination of rumen protozoa are associated with decreased nutrient digestibility (Patra, 2013; Dai and Faciola, 2019). However, DMD and NDFD were increased by PA, likely because of the increased populations of fibrolytic bacteria such as *B. fibrisolvens*, *R. albus, R. bromii,* and *R. lactaris*. This is in agreement with the increase in *R. albus* and *R. flavefaciens* observed after defaunation

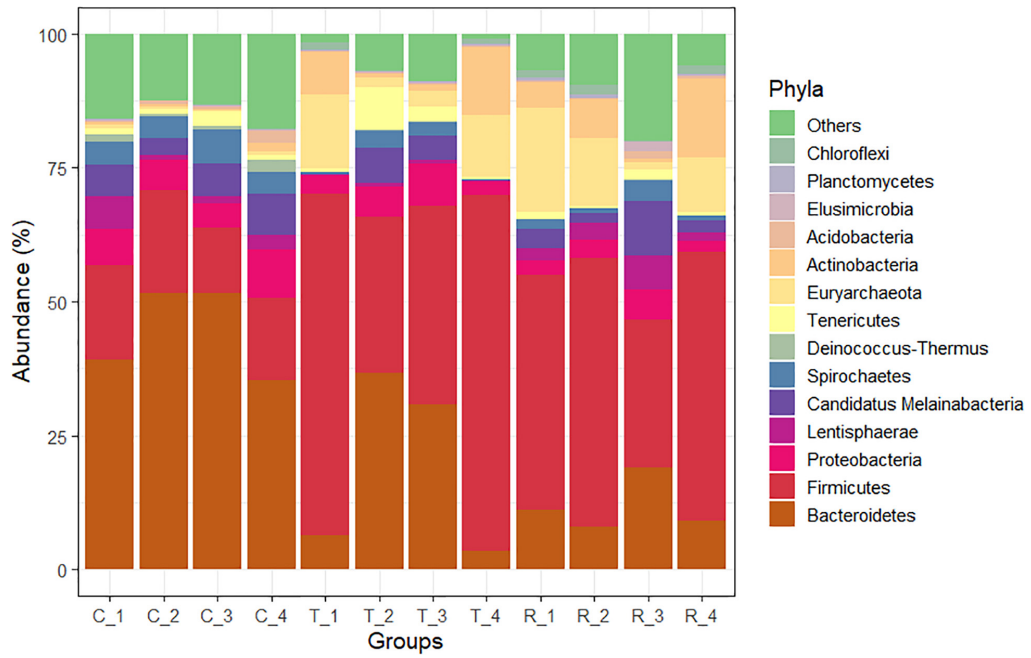


FIGURE 3 | Phylum-level changes in the rumen microbiome of cannulated Holstein steers due to *P. nil* seeds. C, control; T, treatment; R, recovery.

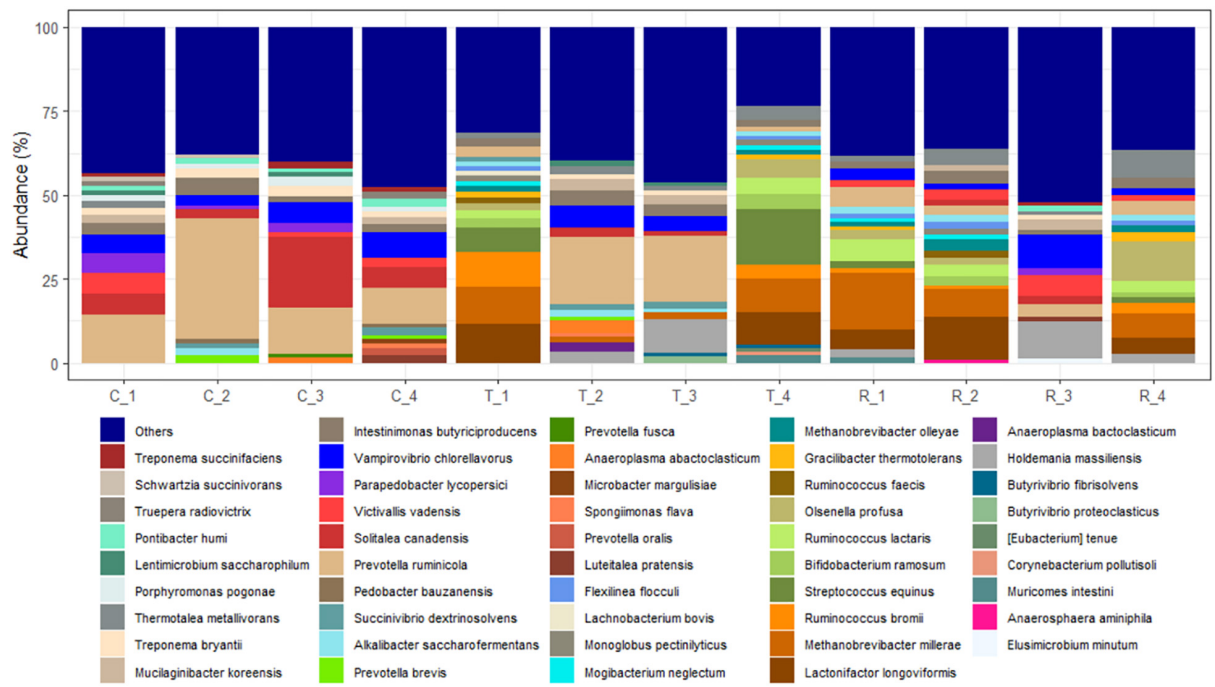


FIGURE 4 | Species-level changes in the rumen microbiome of cannulated Holstein steers due to *P. nil* seeds. C, control; T, treatment; R, recovery.

(Mosoni et al., 2011; Park et al., 2019) and the addition of oil to a sheep diet (Adeyemi et al., 2016). Doreau et al. (2009) noted that dietary addition of oils did not negatively affect organic matter fermentation in the rumen, consistent with this work and a prior defaunation study (Park et al., 2019). It is also possibly a result of the protozoal groups inhibited; *Epidinium*, *Polyplastron*, and *Eudiplodinium* are cellulolytic, whereas *Entodinium* is weakly hemicellulolytic and so its contribution to fiber digestion is minimal (Takenaka et al., 2004). However, in this study, the magnitude of increase in digestibility declined at

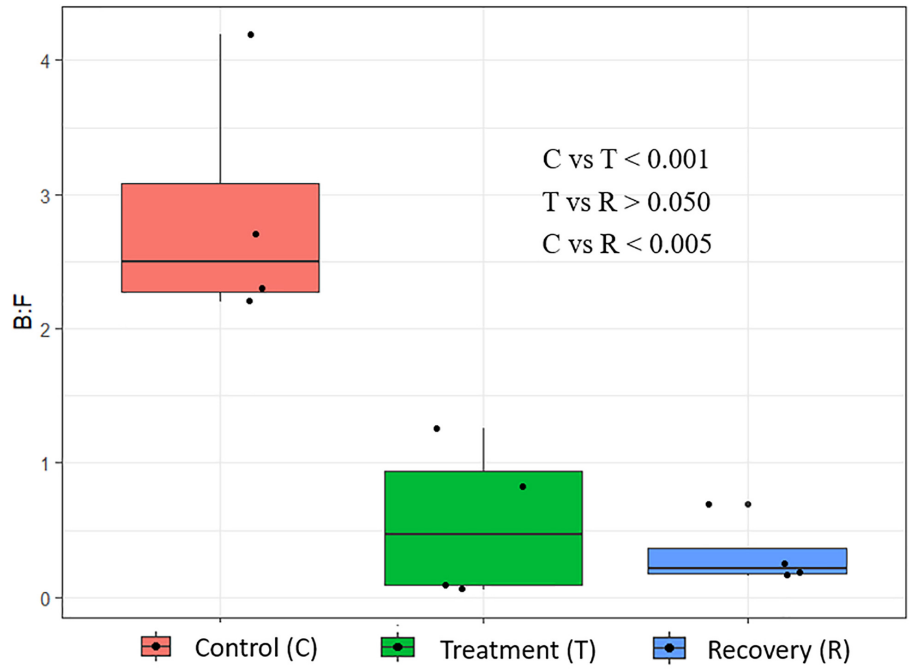


FIGURE 5 | Changes in the Bacteroidetes:Firmicutes ratio in the rumen of cannulated Holstein steers due to *P. nil* seeds.

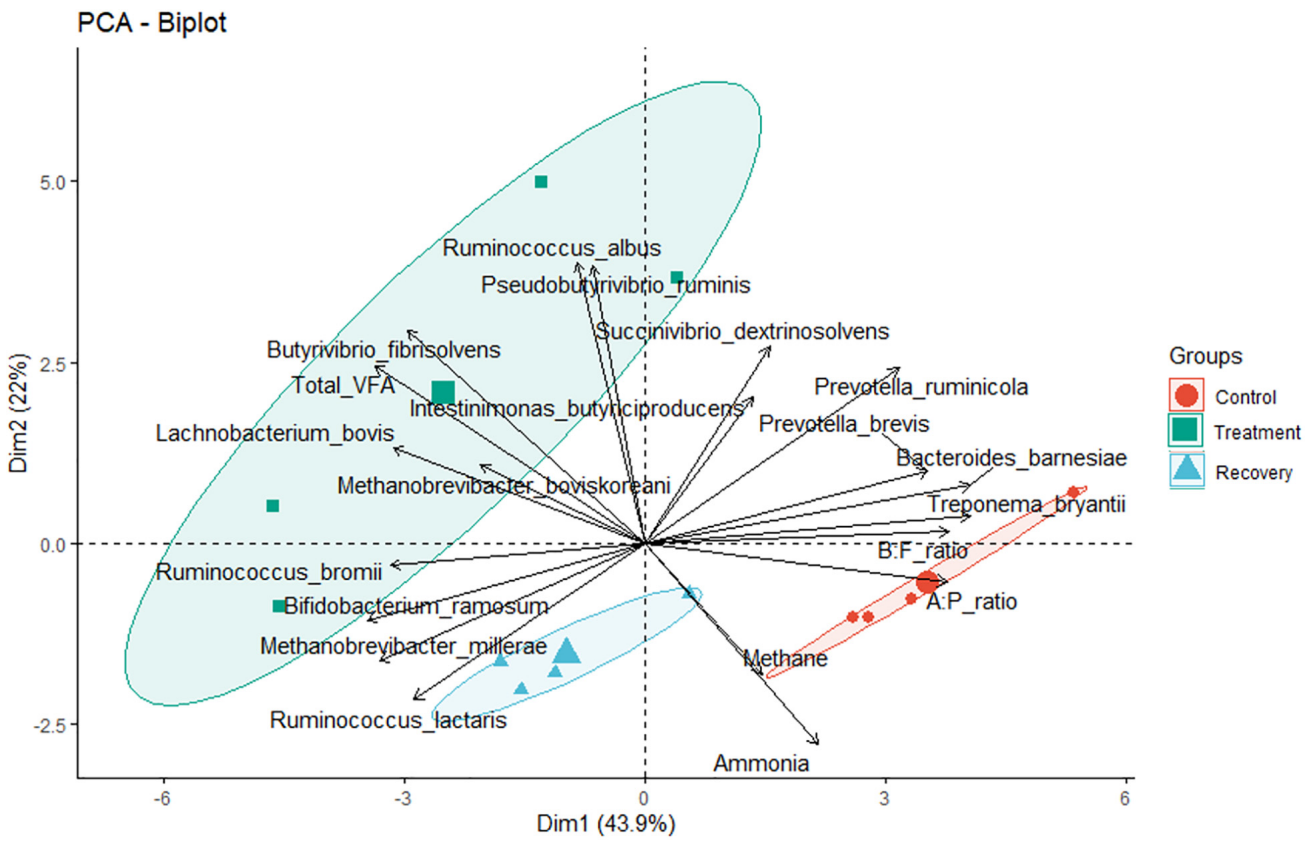


FIGURE 6 | Principal component analysis results reflecting correlations between rumen bacterial/archaeal communities, CH_4 yield, and fermentation parameters *in sacco*.

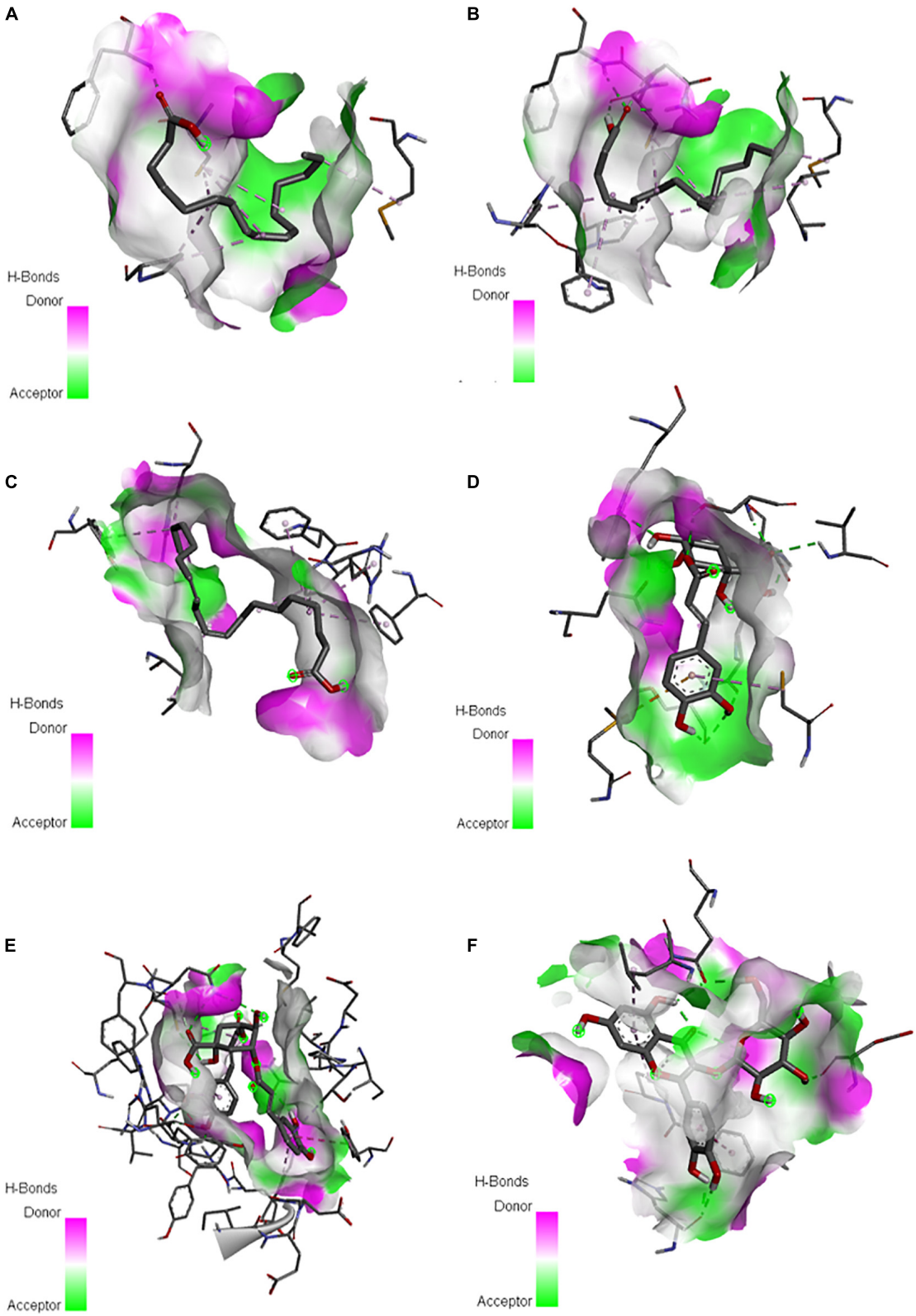


FIGURE 7 | Putative binding sites of palmitic acid **(A)**, oleic acid **(B)**, linoleic acid **(C)**, chlorogenate **(D)**, dicaffeoyl quinic acid **(E)**, and quercetin-3-*O*-glucoside **(F)** from *P. nil* seeds with cyclic GMP-dependent protein kinase of the rumen protozoan *Entodinium caudatum*.

doses > 9% DM (>4.1% FAs), thereby suggesting that a dose of < 9% DM is optimal for decreasing CH_4 production *in vitro* while enhancing digestibility. Moreover, meta-analyses (Hess et al., 2008; Knapp et al., 2014) have shown that dietary addition of up to 6% fats does not affect feed digestibility. Although an increase in digestibility was noted, the decrease in total VFA concentration and increase in pH suggest inhibition of rumen microbial fermentation *in vitro*, consistent with our previous study (Bharanidharan et al., 2021a). However, an inverse effect was observed *in sacco*—a decrease in ruminal pH and an increase in total VFAs. This variation in fermentation characteristics between culture systems further suggests the importance of using LCCSs, as an increase in the partial pressure of headspace gas inhibits fermentation in *in vitro* batch culture systems (Yang, 2017).

CONCLUSION

Our results highlight the advantages of LCCSs for *in sacco* investigations over *in vitro* batch culture systems for studying fermentation characteristics. We determined the optimal dose of PA for decreasing CH_4 production *in vitro* and the doses that modulate fermentation characteristics and digestibility. At doses < 9% DM, PA may be a potential additive for mitigating livestock CH_4 emissions as it also increased the DMD and NDFD *in vitro*. PA contains bioactive compounds such as PUFAs and flavonoids that may reduce the rumen ciliate protozoan population, potentially decreasing intra-ruminal protein recycling and increasing biohydrogenation. However, re-adaptation of rumen microbes after the withdrawal of supplementation hampers commercial application of this strategy. Thus, the treatment duration and effects on individual protozoan genera should be addressed in future studies. Considering its high nutritive value, PA can be used in total mixed rations or as a substitute for grains in concentrate feed, making it a new source of functional feed for ruminants. However, *in vivo* studies are needed to evaluate other important nutritional traits, such as palatability, growth performance, nutrient digestibility, energy partitioning, and N utilization.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI, PRJNA789417 (https://www.ncbi.nlm.nih.gov/sra/PRJNA789417).

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use of Seoul National University (SNU-210615-1).

AUTHOR CONTRIBUTIONS

RB and KK designed and conceptualized the experiment. RB performed the management of steers, *in vitro* and *in sacco* trial, and sample collection. RB, KT, RI, and TK performed laboratory analyses. KT performed the *in silico* docking analysis. MB and YL supervised the experiment. RB organized the data, performed the microbial data processing, bioinformatics, statistical analyses and visualization. RB wrote the first draft of the manuscript including tables and figures, which was revised and edited by RB and KK. All authors read and approved the final manuscript.

FUNDING

The present study was supported by the National Institute of Animal Science, Ministry of Rural Development Administration, South Korea (research project no. PJ0149402022).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.892605/full#supplementary-material

REFERENCES

Abbott, D. W., Aasen, I. M., Beauchemin, K. A., Grondahl, F., Gruninger, R., Hayes, M., et al. (2020). Seaweed and seaweed bioactives for mitigation of enteric methane: challenges and opportunities. *Animals* 10, 1–28. doi: 10.3390/ani10122432

Adeyemi, K. D., Ahmed, M. A., Jotham, S., Roslan, N. A., Jahromi, M. F., Samsudin, A. A., et al. (2016). Rumen microbial community and nitrogen metabolism in goats fed blend of palm oil and canola oil. *Ital. J. Anim. Sci.* 15, 666–672. doi: 10.1080/1828051X.2016.1222245

Akkaya, M. R. (2018). Prediction of fatty acid composition of sunflower seeds by near-infrared reflectance spectroscopy. *J. Food Sci. Technol.* 55, 2318–2325. doi: 10.1007/s13197-018-3150-x

Amanzougarene, Z., and Fondevila, M. (2020). Fitting of the in vitro gas production technique to the study of high concentrate diets. *Animals* 10, 1–13. doi: 10.3390/ani10101935

AOAC International (2016). *Official Methods of Analysis. 20th ed.* Arlington, VA: AOAC Int.

Appuhamy, J. A. D. R. N., France, J., and Kebreab, E. (2016). Models for predicting enteric methane emissions from dairy cows in North America, Europe, and Australia and New Zealand. *Glob. Chang. Biol.* 22, 3039–3056. doi: 10.1111/gcb.13339

Appuhamy, J. A. D. R. N., Strathe, A. B., Jayasundara, S., Wagner-Riddle, C., Dijkstra, J., and France, J. (2013). Anti-methanogenic effects of monensin in dairy and beef cattle: a meta-analysis. *J. Dairy Sci.* 96, 5161–5173. doi: 10.3168/jds.2012-5923

Arokiyaraj, S., Choi, S. H., Lee, Y., Bharanidharan, R., Hairul-Islam, V. I., Vijayakumar, B., et al. (2015). Characterization of ambrette seed oil and its mode of action in bacteria. *Molecules* 20, 384–395. doi: 10.3390/molecules20010384

Ayemele, A. G., Ma, L., Li, X., Yang, P., Xu, J., Yu, Z., et al. (2021). Identification of bioactive phytochemicals from six plants: mechanistic insights into the inhibition of rumen protozoa, ammoniagenesis, and α-glucosidase. *Biology* 10:1055. doi: 10.3390/biology10101055

Ayemele, A. G., Ma, L., Park, T., Xu, J., Yu, Z., and Bu, D. (2020). Giant milkweed (Calotropis gigantea): a new plant resource to inhibit protozoa and decrease

ammoniagenesis of rumen microbiota in vitro without impairing fermentation. *Sci. Total Environ.* 743:140665. doi: 10.1016/j.scitotenv.2020.140665

Baker, D. A., Matralis, A. N., Osborne, S. A., Large, J. M., and Penzo, M. (2020). Targeting the Malaria Parasite cGMP-Dependent Protein Kinase to Develop New Drugs. *Front. Microbiol.* 11:602803. doi: 10.3389/fmicb.2020.602803

Beauchemin, K. A., Ungerfeld, E. M., Eckard, R. J., and Wang, M. (2020). Review: fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal* 14, S2–S16. doi: 10.1017/S1751731119003100

Belanche, A., de la Fuente, G., and Newbold, C. J. (2014). Study of methanogen communities associated with different rumen protozoal populations. *FEMS Microbiol. Ecol.* 90, 663–677. doi: 10.1111/1574-6941.12423

Bharanidharan, R., Arokiyaraj, S., Baik, M., Ibidhi, R., Lee, S. J., Lee, Y., et al. (2021a). In vitro screening of east asian plant extracts for potential use in reducing ruminal methane production. *Animals* 11:1020. doi: 10.3390/ani11041020

Bharanidharan, R., Thirugnanasambantham, K., Ibidhi, R., Bang, G., Jang, S. S., Baek, Y. C., et al. (2021b). Effects of Dietary Protein Concentration on Lipid Metabolism Gene Expression and Fatty Acid Composition in 18-23-Month-Old Hanwoo Steers. *Animals* 11:3378. doi: 10.3390/ANI11123378

Bharanidharan, R., Lee, C. H., Thirugnanasambantham, K., Ibidhi, R., Woo, Y. W., Lee, H. G., et al. (2021c). Feeding Systems and Host Breeds Influence Ruminal Fermentation. *Front. Microbiol.* 12:701081. doi: 10.3389/fmicb.2021.701081

Bharanidharan, R., Arokiyaraj, S., Kim, E. B., Lee, C. H., Woo, Y. W., Na, Y., et al. (2018). Ruminal methane emissions, metabolic, and microbial profile of Holstein steers fed forage and concentrate, separately or as a total mixed ration. *PLoS One* 13:e0202446. doi: 10.1371/journal.pone.0202446

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U. S. A.* 108, 4516–4522. doi: 10.1073/PNAS.1000080107

Chaney, A. L., and Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clin. Chem.* 8, 130–132. doi: 10.1093/clinchem/8.2.130

Dai, X., and Faciola, A. P. (2019). Evaluating Strategies to Reduce Ruminal Protozoa and Their Impacts on Nutrient Utilization and Animal Performance in Ruminants – A Meta-Analysis. *Front. Microbiol.* 10:2648. doi: 10.3389/fmicb.2019.02648

Dohme, F., Machmüller, A., Wasserfallen, A., and Kreuzer, M. (2008). Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. *Lett. Appl. Microbiol.* 32, 47–51. doi: 10.1111/j.1472-765x.2001.00863.x

Doreau, M., Aurousseau, E., and Martin, C. (2009). Effects of linseed lipids fed as rolled seeds, extruded seeds or oil on organic matter and crude protein digestion in cows. *Anim. Feed Sci. Technol.* 150, 187–196. doi: 10.1016/j.anifeedsci.2008.09.004

Fedorah, P. M., and Hrudey, S. E. (1983). A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. *Environ. Technol. Lett.* 4, 425–432. doi: 10.1080/09593338309384228

Glasson, C. R. K., Kinley, R. D., de Nys, R., King, N., Adams, S. L., Packer, M. A., et al. (2022). Benefits and risks of including the bromoform containing seaweed Asparagopsis in feed for the reduction of methane production from ruminants. *Algal Res.* 64:102673. doi: 10.1016/j.algal.2022.102673

Griswold, K. E., White, B. A., and Mackie, R. I. (1999). Diversity of extracellular proteolytic activities among Prevotella species from the rumen. *Curr. Microbiol.* 39, 187–194. doi: 10.1007/s002849900443

Hall, T. A. (1999). BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucleic Acids Symp. Ser.* 41, 95–98.

Hassan, F. U., Arshad, M. A., Ebeid, H. M., ur Rehman, M. S., Khan, M. S., Shahid, S., et al. (2020). Phytogenic Additives Can Modulate Rumen Microbiome to Mediate Fermentation Kinetics and Methanogenesis Through Exploiting Diet–Microbe Interaction. *Front. Vet. Sci.* 7:575801. doi: 10.3389/fvets.2020.575801

Haug, A., Høstmark, A. T., and Harstad, O. M. (2007). Bovine milk in human nutrition - A review. *Lipids Health Dis.* 6, 1–16. doi: 10.1186/1476-511X-6-25

Hess, B. W., Moss, G. E., and Rule, D. C. (2008). A decade of developments in the area of fat supplementation research with beef cattle and sheep. *J. Anim. Sci.* 86, E188–E204. doi: 10.2527/jas.2007-0546

Hook, S. E., Wright, A. D. G., and McBride, B. W. (2010). Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* 2010:945785. doi: 10.1155/2010/945785

Husson, F., Josse, J., Le, S., and Maintainer, J. M. (2020). Multivariate exploratory data analysis and data mining. *Cran* 1, 1–130.

Huws, S. A., Kim, E. J., Cameron, S. J. S., Girdwood, S. E., Davies, L., Tweed, J., et al. (2015). Characterization of the rumen lipidome and microbiome of steers fed a diet supplemented with flax and echium oil. *Microb. Biotechnol.* 8, 331–341. doi: 10.1111/1751-7915.12164

Jin, Q., You, W., Tan, X., Liu, G., Zhang, X., Liu, X., et al. (2021). Caffeic acid modulates methane production and rumen fermentation in an opposite way with high-forage or high-concentrate substrate in vitro. *J. Sci. Food Agric.* 101, 3013–3020. doi: 10.1002/jsfa.10935

Jung, D. Y., Ha, H., Lee, H. Y., Kim, C., Lee, J. H., Bae, K. H., et al. (2008). Triterpenoid saponins from the seeds of Pharbitis nil. *Chem. Pharm. Bull.* 56, 203–206. doi: 10.1248/cpb.56.203

Kapcsándi, V., Hanczné Lakatos, E., Sik, B., Linka, L. Á, and Székelyhidi, R. (2021). Characterization of fatty acid, antioxidant, and polyphenol content of grape seed oil from different Vitis vinifera L. varieties. *OCL Oilseeds Fats Crop Lipids* 28:30. doi: 10.1051/ocl/2021017

Ki, H. K., Sang, U. C., and Kang, R. L. (2009). Diterpene glycosides from the seeds of Pharbitis nil. *J. Nat. Prod.* 72, 1121–1127. doi: 10.1021/np900101t

Kim, K. H., Arokiyaraj, S., Lee, J., Oh, Y. K., Chung, H. Y., Jin, G. D., et al. (2016). Effect of rhubarb (Rheum spp.) root on in vitro and in vivo ruminal methane production and a bacterial community analysis based on 16S rRNA sequence. *Anim. Prod. Sci.* 56, 402–408. doi: 10.1071/AN15585

Kim, K. H., Ha, S. K., Choi, S. U., Kim, S. Y., and Lee, K. R. (2011). Bioactive phenolic constituents from the seeds of Pharbitis nil. *Chem. Pharm. Bull.* 59, 1425–1429. doi: 10.1248/cpb.59.1425

Kim, Y. S., Kim, J. W., Ha, N. Y., Kim, J., and Ryu, H. S. (2020). Herbal Therapies in Functional Gastrointestinal Disorders: a Narrative Review and Clinical Implication. *Front. Psychiatry* 11:601. doi: 10.3389/fpsyt.2020.00601

Kišidayová, S., Durkaj, D., Mihaliková, K., Váradyová, Z., Puchalska, J., Szumacher-Strabel, M., et al. (2021). Rumen Ciliated Protozoa of the Free-Living European Bison (Bison bonasus, Linnaeus). *Front. Microbiol.* 12:658448. doi: 10.3389/fmicb.2021.658448

Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P., and Tricarico, J. M. (2014). Invited review: enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. *J. Dairy Sci.* 97, 3231–3261. doi: 10.3168/JDS.2013-7234

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-13

Krishnamoorthy, U., Rymer, C., and Robinson, P. H. (2005). The in vitro gas production technique: limitations and opportunities. *Anim. Feed Sci. Technol.* 123, 1–7. doi: 10.1016/j.anifeedsci.2005.04.015

Krizsan, S. J., Janèík, F., Ramin, M., and Huhtanen, P. (2013). Comparison of some aspects of the in situ and in vitro methods in evaluation of neutral detergent fiber digestion. *J. Anim. Sci.* 91, 838–847. doi: 10.2527/jas.2012-5343

Ku-Vera, J. C., Jiménez-Ocampo, R., Valencia-Salazar, S. S., Montoya-Flores, M. D., Molina-Botero, I. C., Arango, J., et al. (2020). Role of Secondary Plant Metabolites on Enteric Methane Mitigation in Ruminants. *Front. Vet. Sci.* 7:584. doi: 10.3389/fvets.2020.00584

Lee, H. J., Cho, S. H., Shin, D., and Kang, H. S. (2018). Prevalence of antibiotic residues and antibiotic resistance in isolates of chicken meat in Korea. *Korean J. Food Sci. Anim. Resour.* 38, 1055–1063. doi: 10.5851/kosfa.2018.e39

Lee, S. R., Moon, E., and Kim, K. H. (2017). Neolignan and monoterpene glycoside from the seeds of Pharbitis nil. *Phytochem. Lett.* 20, 98–101. doi: 10.1016/j.phytol.2017.04.019

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

Lim, J. H. (2020). *Relationship between Glossiness and Cooking Liquid Components of Temperate japonica Rice*. Available Online at: https://s-space.snu.ac.kr/handle/10371/166743 (accessed on 25 Feb, 2022).

Machmüller, A. (2006). Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. *Agric. Ecosyst. Environ.* 112, 107–114. doi: 10.1016/j.agee.2005.08.010

Maron, D. F., Smith, T. J. S., and Nachman, K. E. (2013). Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Glob. Health* 9:48. doi: 10.1186/1744-8603-9-48

Martin, C., Ferlay, A., Mosoni, P., Rochette, Y., Chilliard, Y., and Doreau, M. (2016). Increasing linseed supply in dairy cow diets based on hay or corn silage: effect on enteric methane emission, rumen microbial fermentation, and digestion. *J. Dairy Sci.* 99, 3445–3456. doi: 10.3168/jds.2015-10110

Matsumoto, M., Kobayashi, T., Takenaka, A., and Itabashi, H. (1991). Defaunation effects of medium-chain fatty acids and their derivatives on goat rumen protozoa. *J. Gen. Appl. Microbiol.* 37, 439–445. doi: 10.2323/jgam.37.439

McDougall, E. I. (1948). The composition and output of sheep's saliva. *Biochem. J.* 43, 99–109. doi: 10.1042/bj0430099

McGaw, L. J., Jäger, A. K., and Van Staden, J. (2002). Antibacterial effects of fatty acids and related compounds from plants. *S. Afr. J. Bot.* 68, 417–423. doi: 10.1016/S0254-6299(15)30367-7

McKain, N., Shingfield, K. J., and Wallace, R. J. (2010). Metabolism of conjugated linoleic acids and 18: 1 fatty acids by ruminal bacteria: products and mechanisms. *Microbiology* 156, 579–588. doi: 10.1099/mic.0.036442-0

Mosoni, P., Martin, C., Forano, E., and Morgavi, D. P. (2011). Long-term defaunation increases the abundance of cellulolytic ruminococci and methanogens but does not affect the bacterial and methanogen diversity in the rumen of sheep. *J. Anim. Sci.* 89, 783–791. doi: 10.2527/jas.2010-2947

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

Nur Atikah, I., Alimon, A. R., Yaakub, H., Abdullah, N., Jahromi, M. F., Ivan, M., et al. (2018). Profiling of rumen fermentation, microbial population and digestibility in goats fed with dietary oils containing different fatty acids. *BMC Vet. Res.* 14:334. doi: 10.1186/S12917-018-1672-0/TABLES/5

O'Fallon, J. V., Busboom, J. R., Nelson, M. L., and Gaskins, C. T. (2007). A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. *J. Anim. Sci.* 85, 1511–1521. doi: 10.2527/jas.2006-491

Olagaray, K. E., and Bradford, B. J. (2019). Plant flavonoids to improve productivity of ruminants – A review. *Anim. Feed Sci. Technol.* 251, 21–36. doi: 10.1016/j.anifeedsci.2019.02.004

Ono, M. (2017). Resin glycosides from Convolvulaceae plants. *J. Nat. Med.* 71, 591–604. doi: 10.1007/s11418-017-1114-5

Park, T., and Yu, Z. (2018). Do Ruminal Ciliates Select Their Preys and Prokaryotic Symbionts? *Front. Microbiol.* 9:1710. doi: 10.3389/fmicb.2018.01710

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

Patra, A. K. (2013). The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: a meta-analysis. *Livest. Sci.* 155, 244–254. doi: 10.1016/j.livsci.2013.05.023

Patra, A. K., and Yu, Z. (2013). Effects of coconut and fish oils on ruminal methanogenesis, Fermentation, And abundance and diversity of microbial populations in vitro. *J. Dairy Sci.* 96, 1782–1792. doi: 10.3168/jds.2012-6159

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., et al. (2004). UCSF Chimera - A visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612. doi: 10.1002/jcc.20084

Saito, N., Cheng, J., Ichimura, M., Yokoi, M., Abe, Y., and Honda, T. (1994). Flavonoids in the acyanic flowers of Pharbitis nil. *Phytochemistry* 35, 687–691. doi: 10.1016/S0031-9422(00)90588-0

Solomon, R., Wein, T., Levy, B., Eshed, S., Dror, R., Reiss, V., et al. (2021). Protozoa populations are ecosystem engineers that shape prokaryotic community structure and function of the rumen microbial ecosystem. *ISME J.* 16, 1187–1197. doi: 10.1038/s41396-021-01170-y

Takenaka, A., Tajima, K., Mitsumori, M., and Kajikawa, H. (2004). Fiber Digestion by Rumen Ciliate Protozoa. *Microbes Environ.* 19, 203–210. doi: 10.1264/jsme2.19.203

Toprak, N. N. (2015). Do fats reduce methane emission by ruminants? - A review. *Anim. Sci. Pap. Rep.* 33, 305–321.

Undersander, D., Mertens, D. R., and Thiex, N. (1993). *Forage analysis procedures*. Omaha, NE: National Forage Testing Association.

Van Soest, P. J. (1973). Collaborative Study of Acid-Detergent Fiber and Lignin. *J. AOAC Int.* 56, 781–784. doi: 10.1093/jaoac/56.4.781

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Vasta, V., Daghio, M., Cappucci, A., Buccioni, A., Serra, A., Viti, C., et al. (2019). Invited review: plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: experimental evidence and methodological approaches. *J. Dairy Sci.* 102, 3781–3804. doi: 10.3168/jds.2018-14985

Wang, R., Wang, M., Zhang, X. M., Wen, J. N., Ma, Z. Y., Long, D. L., et al. (2019). Effects of rumen cannulation on dissolved gases and methanogen community in dairy cows. *J. Dairy Sci.* 102, 2275–2282. doi: 10.3168/jds.2018-15187

Woo, K. W., Park, K. J., Choi, S. Z., Son, M. W., Kim, K. H., and Lee, K. R. (2017). A New ent-kaurane Diterpene Glycoside from Seeds of Pharbitis nil. *Chem. Nat. Compd.* 53, 468–471. doi: 10.1007/s10600-017-2024-1

Yang, W. (2017). "Factors Affecting Rumen Fermentation Using Batch Culture Technique," in *Fermentation Processes*, ed. A. F. Jozala (London: IntechOpen), doi: 10.5772/64207

Conflict of Interest: The 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 June 2022
doi: 10.3389/fmicb.2022.889618

Methane Reduction Potential of Brown Seaweeds and Their Influence on Nutrient Degradation and Microbiota Composition in a Rumen Simulation Technique

*Susanne Künzel[1,2†], Timur Yergaliyev[1,2†], Katharina J. Wild[1,2], Hanna Philippi[1,2], Asta H. Petursdottir[3], Helga Gunnlaugsdottir[3,4], Chris K. Reynolds[5], David J. Humphries[5], Amélia Camarinha-Silva[1,2] and Markus Rodehutscord[1,2]**

[1] Institute of Animal Science, University of Hohenheim, Stuttgart, Germany, [2] Hohenheim Center for Livestock Microbiome Research, University of Hohenheim, Stuttgart, Germany, [3] Matís, Reykjavík, Iceland, [4] Faculty of Food Science and Nutrition, School of Health Sciences, University of Iceland, Reykjavík, Iceland, [5] School of Agriculture, Policy and Development, University of Reading, Reading, United Kingdom

OPEN ACCESS

Edited by:
Diego P. Morgavi,
INRAE
Clermont-Auvergne-Rhône-Alpes,
France

Reviewed by:
Gilbert Kamgan Nkuekam,
University of Pretoria, South Africa
Cátia Pacífico,
Biome Diagnostics GmbH, Austria
Stephanie A. Terry,
Lethbridge Research Center,
Agriculture and Agri-Food Canada,
Canada

***Correspondence:**
Markus Rodehutscord
inst450@uni-hohenheim.de

† These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: 04 March 2022
Accepted: 17 May 2022
Published: 28 June 2022

Citation:
Künzel S, Yergaliyev T, Wild KJ, Philippi H, Petursdottir AH, Gunnlaugsdottir H, Reynolds CK, Humphries DJ, Camarinha-Silva A and Rodehutscord M (2022) Methane Reduction Potential of Brown Seaweeds and Their Influence on Nutrient Degradation and Microbiota Composition in a Rumen Simulation Technique. Front. Microbiol. 13:889618. doi: 10.3389/fmicb.2022.889618

This study aimed to investigate the effects of two brown Icelandic seaweed samples (*Ascophyllum nodosum* and *Fucus vesiculosus*) on *in vitro* methane production, nutrient degradation, and microbiota composition. A total mixed ration (TMR) was incubated alone as control or together with each seaweed at two inclusion levels (2.5 and 5.0% on a dry matter basis) in a long-term rumen simulation technique (Rusitec) experiment. The incubation period lasted 14 days, with 7 days of adaptation and sampling. The methane concentration of total gas produced was decreased at the 5% inclusion level of *A. nodosum* and *F. vesiculosus* by 8.9 and 3.6%, respectively ($P < 0.001$). The total gas production was reduced by all seaweeds, with a greater reduction for the 5% seaweed inclusion level ($P < 0.001$). Feed nutrient degradation and the production of volatile fatty acids and ammonia in the effluent were also reduced, mostly with a bigger effect for the 5% inclusion level of both seaweeds, indicating a reduced overall fermentation (all $P \leq 0.001$). Microbiota composition was analyzed by sequencing 16S rRNA amplicons from the rumen content of the donor cows, fermenter liquid and effluent at days 7 and 13, and feed residues at day 13. Relative abundances of the most abundant methanogens varied between the rumen fluid used for the start of incubation and the samples taken at day 7, as well as between days 7 and 13 in both fermenter liquid and effluent ($P < 0.05$). According to the differential abundance analysis with q2-ALDEx2, in effluent and fermenter liquid samples, archaeal and bacterial amplicon sequence variants were separated into two groups ($P < 0.05$). One was more abundant in samples taken from the treatment without seaweed supplementation, while the other one prevailed in seaweed supplemented treatments. This group also showed a dose-dependent response to seaweed inclusion, with a greater number of differentially abundant members between a 5% inclusion level and unsupplemented samples than

between a 2.5% inclusion level and TMR. Although supplementation of both seaweeds at a 5% inclusion level decreased methane concentration in the total gas due to the high iodine content in the seaweeds tested, the application of practical feeding should be done with caution.

Keywords: seaweed, macro algae, rumen, methane, Rusitec, microbiota, 16S rRNA gene

INTRODUCTION

The rapidly growing global population brings serious challenges to the food industry. While ruminant livestock are vital in sustaining food security by converting inedible plant matter into meat and dairy products, they significantly contribute to global methane (CH_4) emissions, a potent greenhouse gas (Lassey, 2007). These CH_4 emissions are mainly related to fermentation processes orchestrated by the rumen microbiome. Diet composition is one of the major factors influencing rumen microbial communities (de Menezes et al., 2011; Henderson et al., 2015) and is therefore linked to CH_4 production by ruminants.

Feed production does not need arable land, which may help us cope with the increasing demand for animal products. Seaweed could be an alternative animal feed material that has already been used for thousands of years in coastal areas (Evans and Critchley, 2014). Some seaweed species can also affect ruminal CH_4 production even at a low inclusion level in the feed. Pronounced effects were reported for *Asparagopsis taxiformis* grown in the Pacific Ocean, with CH_4 formation reduced by up to 99% with seaweed inclusion $\leq$5% *in vitro* (Kinley et al., 2016; Machado et al., 2018; Roque et al., 2019). Numerous studies have demonstrated that the effect of seaweed supplementation on methanogenesis was associated with a modified rumen microbiome (Molina-Alcaide et al., 2017; Roque et al., 2019; Abbott et al., 2020). In the rumen, hydrogenotrophic methanogens produce CH_4 by using hydrogen (H_2) for carbon dioxide (CO_2) reduction through the Wolfe cycle (Leahy et al., 2010; Thauer, 2012). Accordingly, the composition of archaeal methanogens and abundances of hydrogen-producing bacteria were identified as key factors associated with levels of CH_4 emissions in ruminants (Tapio et al., 2017). Considering the relevance of H_2 for CH_4 production, manipulating H_2 production or its utilization pathways through diet and microbiome composition is an approach that may provide new insights into the development of CH_4 mitigation strategies.

Seaweeds are a very heterogeneous group of feeding substances, and their application in animal feeding has been restricted due to a lack of information about species-specific nutritive value. Several factors influence nutrient and bioactive compounds in seaweed, such as species, season, and site of harvesting (Paiva et al., 2018; Britton et al., 2021). To the best of our knowledge, most studies concerning seaweed in ruminant nutrition were conducted with species harvested in the Pacific Ocean. Since there is also a high variation of Atlantic seaweeds, there is a need for information about these seaweeds. The current study aimed to investigate the effect of two Icelandic seaweed samples on *in vitro* gas and methane production, nutrient degradation, and microbiota composition using a continuous long-term rumen simulation technique (Rusitec). Both seaweeds are endemic to Iceland and are also available in large quantities in other European countries. Our hypothesis was that two common and abundant North Atlantic seaweed species could reduce CH_4 formation to the point where their abundance and ease of access would make them a viable option as a ruminant feed substance. A secondary objective was to investigate changes in microbiota composition over time in the *in vitro* system fed with or without seaweed.

MATERIALS AND METHODS

Treatments

Two samples of seaweed naturally occurring in Iceland were studied for their effect on TMR formulated for cattle in a Rusitec system (Czerkawski and Breckenridge, 1977). The TMR was composed of 20% corn grain, 20% soybean meal, 40% corn silage, and 20% grass silage and served as the control treatment. One seaweed was *Ascophyllum nodosum* (AN), harvested in August 2018, and the other was *Fucus vesiculosus* (FV), harvested in June 2019. Both seaweeds were used at 2.5% (AN2.5 and FV2.5) and 5% (AN5 and FV5) inclusion in the TMR on a dry matter (DM) basis in exchange for TMR. The seaweeds and all ingredients of the TMR were dried and ground to pass a 1 mm screen. The analyzed nutrient composition, bromoform, and total phenolic content (TPC) of the seaweeds and TMR and the calculated nutrient composition of the experimental treatments are shown in **Table 1**.

Rumen Content and Synthetic Saliva

Rumen content was collected from three rumen-fistulated non-lactating Jersey cows before the morning feeding. Animals had free access to water and a diet composed of 33% corn silage, 33% grass silage, 23% grass hay, 10% barley straw, and 1% mineral mixture (on a DM basis). During the daytime, the cows were kept on pasture. Two liters of rumen fluid were taken from each cow into prewarmed thermos flasks, comprised of 1 L pumped from the liquid phase and 1 L squeezed out from the solid phase. Additionally, 200 g of squeezed solid phase from each cow was

Abbreviations: ADFom, acid detergent fiber on ash-free basis; AN, *Ascophyllum nodosum*; aNDFom, Neutral detergent fiber on ash free-basis; A:P, acetate to propionate ratio; ASV, amplicon sequence variant; CH_4, methane; CO_2, carbon dioxide; CP, crude protein, DM, dry matter; E, effluent; EMPS, estimated microbial protein synthesis; FL, fermenter liquid; FR, feed residues; FV, *Fucus vesiculosus*; H_2, hydrogen; LAM, liquid-associated microbes; SAM, solid-associated microbes; OTU, operational taxonomic units; PCoA, principle-coordinate analysis; PCR, polymerase chain reaction; r, correlation coefficient; RF, rumen fluid; RSP, rumen solid phase; TMR, total mixed ration; TPC, total phenolic content; VFA, volatile fatty acids.

TABLE 1 | Nutrient composition, bromoform, total phenolic content of the used seaweeds, and five experimental diets.

	DM %	OM %	CA %	CP %	ADFom %	aNDFom %	EE %	$CHBr_3$ μg/kg	TPC
									Phloroglucinol equivalent g/100 g sample
A. nodosum	93.0	70.0	30.0	10.7	18.6	24.1	2.0	8.0	7.9
F. vesiculosus	89.5	75.0	25.0	9.3	25.0	19.8	1.8	<0.8	7.4
Treatments									
TMR	91.8	94.0	6.0	17.8	14.8	29.6	2.9	n.a.	n.a.
AN2.5[1]	91.8	93.4	6.6	17.6	14.9	29.5	2.8	–	–
AN5[1]	91.8	92.8	7.2	17.4	15.0	29.3	2.8	–	–
FV2.5[1]	91.7	93.6	6.4	17.6	15.0	29.4	2.8	–	–
FV5[1]	91.7	93.1	6.9	17.4	15.3	29.1	2.8	–	–

AN, Ascophyllum nodosum; FV, Fucus vesiculosus (both with 2.5 or 5% inclusion level); TMR, total mixed ration; DM, dry matter; OM, organic matter; CA, crude ash; CP, crude protein; ADFom, acid detergent fiber on ash free basis; aNDFom, neutral detergent fiber on ash free basis; EE, ether extract; $CHBr_3$, bromoform; TPC, total phenolic content; n.a., not analyzed.

[1] *Values are calculated with respective proportions of TMR and seaweed based on the analyzed values of the ingredients.*

transferred into prewarmed, isolated containers. Rumen fluid was strained through two layers of cheesecloth and mixed with equal parts from the donor animals. Subsequently, rumen fluid was mixed with a buffer solution (1:1), flushed with CO_2, and stirred at 39°C until fermenters of the Rusitec were inoculated. The buffer solution was prepared according to the suggested composition of artificial saliva by McDougall (1948) with the addition of ^{15}N enriched NH_4Cl (0.0378 g/L; 104 mg ^{15}N/g N) used for the calculation of microbial protein synthesis.

Rusitec System

Each run lasted 14 days (days 0–13), with 7 days of adaptation, followed by 7 days of sampling. Ten fermenters were arranged side by side, with five fermenters sharing one circulation thermostat (Lauda ECO E 4 S, Lauda-Königshofen, Germany) and one buffer pump (Ismatec IPC ISM 931, Wertheim, Germany) (**Figure 1**). Each of the five treatments was replicated in two fermenters for two runs each, resulting in four replications per treatment. Fermenters were randomized in each run. The circulation thermostat was used as a block design, and each treatment was used once in each block. Due to technical arrangements, an additional empty fermenter was connected to each circulation thermostat. The glass fermenters were kept at a constant temperature of 39°C with a heating jacket. The buffer pump continuously infused buffer solution into each fermenter at a daily rate of 75% of the fermenter's capacity (950 ml) to stimulate salivation. Each fermenter contained a feed container with continuous vertical movement ensured by a lift motor to simulate rumen motility (10–12 strokes/min; **Figure 2**). The fluid effluent was separated from the fermentation gas in a glass cylinder and then collected in 1 L glass bottles. The glass cylinder and effluent bottles were placed in a 4°C tempered water bath. Gaseous effluent was passed through gas counters (BlueVCount, BlueSens gas sensor GmbH, Herten, Germany) to measure gas production via gas-tight tubes and was subsequently collected quantitatively in gas-tight five-layered plastic-aluminum bags (Dr.-Ing. Ritter Apparatebau GmbH & Co., KG, Bochum, Germany). The methane concentration of total gas production was measured using an infrared CH_4 analyzer from the gas collected in the plastic-aluminum bags (PRONOVA Analysentechnik GmbH & Co., KG, Berlin, Germany).

Feed and solid rumen content were weighed into nylon bags (120 mm × 70 mm) with a 100 μm pore size and closed with cable ties. To start the system, one feed bag containing 15 g of the respective treatment and one feed bag containing 60 g of solid rumen content were put into the container of each fermenter. After 24 h, the bag with rumen content was replaced by another feed bag. Subsequently, each feed bag was removed after 48 h, rinsed with 50 (adaptation period) or 100 ml (sampling period) of buffer solution, squeezed moderately, and replaced by a new bag containing the specific treatment. The resulting liquid was returned to the respective fermenter.

Sampling and Chemical Analyses

The total gas production and CH_4 concentration of total gas were measured in 24 h intervals during the sampling period. The temperature, pH, and redox potential of the fermenter fluid were measured daily in the fermenter before the feed bags were changed (SenTix ORP, WTW, Weilheim in Oberbayern, Germany; BlueLine 14 pH IDS, SI Analytics, Mainz, Germany). From days 7 to 13, a sample from the fermenter liquid phase (FL; 30 ml/d) was taken daily and pooled by the fermenter to obtain the fraction of liquid-associated microbes (LAM) by centrifugation according to Boguhn et al. (2006), with minor modifications. In brief, the suspension was centrifuged twice at 2,000 × *g* for 5 min at 4°C. Then, the supernatant was centrifuged three times at 15,000 × *g* for 15 min at 4°C. Afterward, samples were freeze-dried and pulverized with a ball mill (MM 400; Retsch GmbH, Haan, Germany). On day 13, solid-associated microbes (SAM) were separated from the feed residues (FR), as described by Ranilla and Carro (2003). Ammonia nitrogen (NH_3-N), ^{15}N abundance, and volatile fatty acids (VFA) were analyzed in the effluent (E), which was weighed and sampled daily (70 ml/d), pooled by the fermenter, and stored at −20°C until it was centrifuged for 15 min at 24,000 × *g*. The analysis of VFA was performed by vacuum

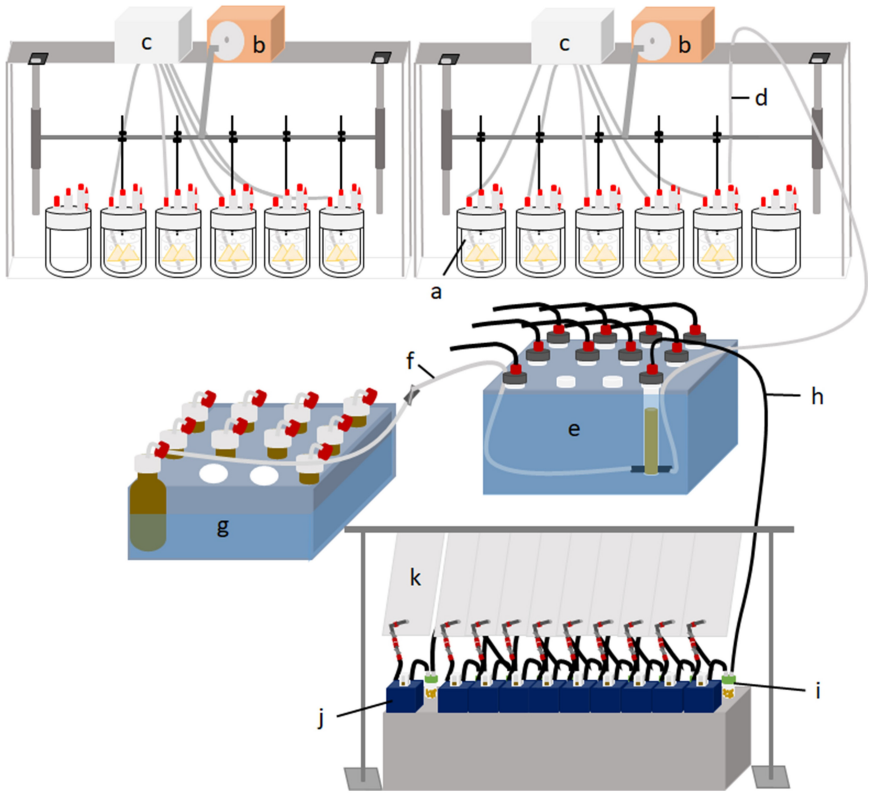


FIGURE 1 | Schematic diagram of the Rusitec set up with the 12 fermenters (a) connected to a lift motor (b), a buffer pump (c), and a gas-tight tube for the effluent (d). The effluent is separated in glass cylinders in a cooling water bath (e) into fluid effluent (f), which is collected in glass bottles in a cooling water bath (g), and gaseous effluent (h). The gaseous effluent is passed through a cold trap with an H_2S absorber granulate (i), measured with gas counters (j), and sampled for methane analysis in plastic bags (k).

distillation and gas chromatography (Hewlett-Packard 6890; Agilent, Waldbronn, Germany) measurement, as described by Wischer et al. (2013). NH_3-N was analyzed using Kjeldahl steam distillation with phosphate buffer (Vapodest 50, Gerhardt, Königswinter, Germany). The daily production of VFA and NH_3-N was calculated by relating the analyzed concentrations to the daily measured amount of E. The removed feed bags were dried for 24 h at 65°C, weighed, and pooled by the fermenter to determine nutrient degradation from days 7 to 12. Feed and FR were analyzed according to official methods in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 1976) for DM (method 3.1), crude protein (CP; method 4.1.1), neutral detergent fiber on an ash-free basis (aNDFom; method 6.5.1), and acid detergent fiber on an ash-free basis (ADFom; method 6.5.2). Seaweeds and TMR were additionally analyzed for crude ash (method 8.1) and ether extract (method 5.1.1). Degradation of nutrients was calculated as the difference between the input and output of each fermenter in relation to input and expressed as a percentage.

Bromoform was analyzed in seaweeds according to method 8260B (EPA, 1996) by ALS Global (Miami, FL, United States). For the analysis of TPC, dried seaweeds were extracted in Milli-Q water (1:7 sample:water) for 1 h at room temperature (400 rpm). The TPC was determined on the sample supernatant according to the method by Singleton and Rossi (1965) adapted to the microplate format. For the analysis of heavy metals and minerals in the seaweeds, an ultraWAVE acid digestion system (Milestone Inc., Italy) and an Agilent 7900 quadrupole inductively coupled plasma mass spectrometer (Agilent Technologies, Singapore) were used according to NMKL (2007). Analyzed heavy metals and minerals are shown in **Supplementary Table 1**. Feed, FR, freeze-dried particle-free E, LAM, SAM, and NH_4Cl were analyzed for ^{15}N abundance using an elemental analyzer (EA, 1108; Carlo Erba Instruments, Biberach, Germany) combined with an isotope mass spectrometer (MS Finnigan MAT; ThermoQuest Italia S.p.A., Milan, Italy). SAM was additionally analyzed for total N.

Microbiota composition was analyzed in rumen solid phase (RSP) and rumen fluid (RF) from the liquid phase of each cow in both experimental runs ($n = 6$), and the mixture of rumen fluid from all cows and buffer solution, treated as RF replicates ($n = 2$) on day 0. On days 7 and 13, 1 ml of FL and E were taken from each fermenter for the analysis of microbiota composition ($n = 40$). Feed residues from both feed bags remaining for 24 and 48 h in the fermenter were sampled on day 13 ($n = 40$). Additionally, the liquid after SAM treatment of both bags was sampled on day 13 ($n = 20$). Samples were stored at −20°C immediately after collection.

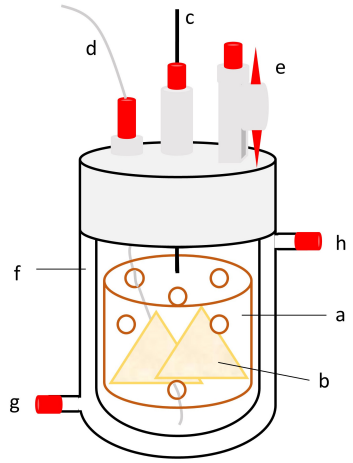


FIGURE 2 | Schematic diagram of a Rusitec fermenter with a feed container (a) containing feed bags (b). The feed container is connected via a bar (c) to a lift motor. Buffer is consistently infused through a tube (d) connected to the buffer pump. The connection to the effluent (e) is closable to make it possible to remove the fermenter. The heating jacket (f) is connected via an inflow (g) and outflow (h) to a circulation thermostat.

Target Amplicon Sequencing

DNA was extracted with the commercial DNA extraction kit FastDNA^{TM} Spin Kit for soil (MP Biomedicals, LLC, Solon, OH, United States), following the manufacturer's instructions. DNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and stored at −20°C. Two sequencing libraries were prepared as previously described to assess bacterial (V1–V2 region; Kaewtapee et al., 2017) and archaeal communities (Arch349 and Arch806 primers; Lee et al., 2012; Deusch et al., 2017). In brief, targeted regions of the 16S rRNA gene were amplified by a first polymerase chain reaction (PCR) using forward primers with 6-nt barcodes and a 2-nt linker attached and both primers were complementary to the Illumina adapters. Then, 1 µl of the resulting product was used as a template for a second PCR, this time using a reverse primer containing a specific sequence to multiplex and index primers. Obtained amplicons were checked by agarose gel electrophoresis and normalized using the SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, United States). Sequencing was performed on the 250 bp paired-end Illumina NovaSeq 6000 platform.

Calculations

The estimated microbial protein synthesis (EMPS) was calculated in accordance with Boguhn et al. (2006) and Hildebrand et al. (2011). In brief, the daily outflow of microbial N from a fermenter (N_m; mg/d) was calculated as the sum of N originating from the LAM in the effluent (N_{LAM-E}; mg/d) and SAM (N_{SAM}; mg/d) fractions:

$$N_m = N_{LAM-E} + N_{SAM} \quad (1)$$

Therefore, N_{LAM-E} was calculated with the sum of the daily input of ^{15}N via buffer solution and feed ($^{15}N_{in}$; µg/d), the sum of the daily output of ^{15}N via FR and NH_3-N ($^{15}N_{out}$; µg/d), and the analyzed abundance of ^{15}N in N_{LAM-E} ($^{15}N_{LAM-E}$; µg/mg N):

$$N_{LAM-E} = \frac{^{15}N_{in} - {}^{15}N_{out}}{^{15}N_{LAM-E}} \quad (2)$$

The N_{SAM} was calculated using the amount of N in FR (N_{FR}; mg/day), the analyzed abundances of ^{15}N in the FR N ($^{15}N_{FR}$; µg/mg N), the feed N ($^{15}N_{feed}$; µg/mg N), SAM N ($^{15}N_{SAM}$, µg/mg N), and the assumed natural abundance of ^{15}N in unlabeled N_{SAM} (3.66303 µg/mg):

$$N_{SAM} = N_{FR} \times \frac{^{15}N_{FR} - {}^{15}N_{Feed}}{^{15}N_{SAM} - 3.66303} \quad (3)$$

Microbial CP (CP_m; mg/d) was calculated as N_m (Eq. 1) multiplied by 6.25:

$$CP_m = N_m \times 6.25 \quad (4)$$

In the next step, EMPS [g/kg degraded organic matter (OM)] was determined using the CP_m (mg/d), the degraded organic matter (OM_{deg}; mg/d), and the amount of OM originating from SAM in the FR (OM_{SAM}; mg/d):

$$EMPS = \frac{CP_m}{OM_{deg} + OM_{SAM}} \quad (5)$$

Where OM_{SAM} was calculated as described by Boguhn et al. (2006) with the amount of N_{SAM} (Eq. 3), the analyzed N concentration in SAM ($N_{\%SAM}$; %), the concentration of ash in SAM [12%, Boguhn et al. (2006)], and the proportion of DM in the isolated SAM fraction [0.93, Boguhn et al. (2006)]:

$$OM_{SAM} = \frac{N_{SAM}}{N_{\%SAM}} \times (100 - 12) - 0.93 \quad (6)$$

Statistical Analyses and 16S Bioinformatics

Statistical analysis of gas data, nutrient degradation, NH_3-N, VFA, and EMPS was done with a one-way ANOVA in SAS 9.4 using the mixed procedure. The treatment was the fixed effect, and the run, circulation thermostat, fermenter, and day were used as random effects. When treatment differences were identified, multiple *t*-tests (Fisher's LSD test) were used to distinguish between treatments. The residuals were checked graphically for the normal distribution and homogeneity of variance.

Fastq files were demultiplexed with Sabre[1] and processed by Qiime2 (v.2021.2/8; Bolyen et al., 2019). Primers were removed by the q2-cutadapt plugin (Martin, 2011). Reads were quality filtered, error corrected, dereplicated, and merged by the q2-dada2 plugin (Callahan et al., 2016). Taxonomy assignment of produced amplicon sequence variants (ASVs) was performed using VSEARCH-based consensus (Rognes et al., 2016) and pre-fitted sklearn-based classifiers (Pedregosa et al., 2011) against the Silva SSU-rRNA database (v.138.1, 16S 99%; Quast et al., 2013), formatted by RESCRIPt (Robeson et al., 2021). Reads

[1]https://github.com/najoshi/sabre

from organelles and unassigned sequences were removed from the analysis. A phylogenetic tree was constructed with the q2-phylogeny plugin, implementing MAFFT 7.3 (Katoh and Standley, 2013) and FastTree 2.1 (Price et al., 2010). For diversity assessment, ASV tables were rarefied to 3580 (archaea) and 5856 (bacteria) sampling depths. Alpha diversity was estimated by Faith's phylogenetic diversity (Faith, 1992) and Shannon's entropy (Shannon, 1948) indices. For beta diversity, Bray–Curtis (Bray and Curtis, 1957) and Jaccard (Jaccard, 1912) distances were employed. Ordination of the beta-diversity distances was implemented with a principal-coordinate analysis (PCoA; Halko et al., 2011). Differences in diversity metrics were tested with the Kruskal–Wallis *H*-test (Kruskal and Wallis, 1952; alpha diversity and relative abundances), followed by Dunn's pairwise test (Dunn, 1964) and a PERMANOVA test (Anderson, 2001; beta diversity) with 999 permutations. The obtained *P*-values were corrected for multiple comparisons using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995).

For correlation analysis, ASV tables were filtered to remove genera with less than 1% relative abundances. Absolute abundances of the remaining genera were correlated using "Sparse Cooccurrence Network Investigation for Compositional Data" or the SCNIC tool (Shaffer et al., 2020). Genera were correlated using the SparCC method (Friedman and Alm, 2012) and with numerical metadata using the Spearman correlation (Dodge, 2010). All *P*-values were considered to be significant if $\leq$0.05 (except for q2-ALDEx2 output) and correlation coefficients (r) if their absolute values were $\geq$0.3.

The q2-ALDEx2 differential abundance plugin was used to test the effect of seaweed supplementation on ASVs raw counts (relative abundance $\geq$1%) with a significance threshold of Wilcoxon test 0.1 (Fernandes et al., 2013, 2014). Sequences are available at the European Nucleotide Archive (ENA) under accession number PRJEB50942 and bioinformatics[2].

RESULTS

Gas Production, Nutrient Degradation, and Microbial Protein Synthesis

The total gas production was decreased by both seaweeds compared to TMR alone, with a greater effect for the 5% seaweed inclusion level ($P < 0.001$, **Table 2**). No significant difference between AN and FV was detected for total gas production. For the CH_4 concentration of total gas and the methane production per g of degraded OM, only the 5% inclusion level of the two seaweeds showed a difference compared to TMR alone ($P < 0.001$ and 0.017, respectively). The reduction was bigger for AN5 (8.9 and 16.9% reduction for CH_4 concentration and CH_4/g degraded OM) compared to FV5 (3.6 and 11.2% reduction for CH_4 concentration and CH_4/g degraded OM).

Seaweed supplementation also decreased the degradation of all analyzed nutrients in the feed bags ($P < 0.001$, **Table 3**). Only for ADFom, AN2.5 showed no significant difference to TMR alone. In all other cases, nutrient degradation was significantly lower with seaweed supplementation. The 5% inclusion level caused at least a numerically greater reduction than the 2.5% inclusion. The highest decrease compared to TMR alone was observed for CP, with a minimum reduction of 8.9% percentage points. The two seaweed products only differed at the 5% inclusion level for DM and OM degradation, where FV showed a greater decrease than AN. The EMPS was decreased by both seaweeds, with a greater effect of the 5% inclusion level ($P < 0.001$, **Table 3**). No significant difference was detected between the inclusion of AN and FV.

TABLE 2 | Total gas production and methane concentration of the produced gas (days 7–13) and methane production per g of degraded organic matter (OM; days 7–12) of the five treatments.

	Total gas (mL/d)	CH_4 (% of total gas)	CH_4/degraded OM (mL/g)
TMR	1386^{a}	16.9^{a}	36.7^{a}
AN2.5	1238^{b}	16.6^{ab}	35.1^{ab}
AN5	1114^{c}	15.4^{c}	30.5^{c}
FV2.5	1177^{bc}	16.6^{ab}	35.9^{ab}
FV5	1114^{c}	16.3^{b}	32.6^{bc}
Pooled SEM	*35.9*	*2.01*	*4.66*
P	<0.001	<0.001	0.017

AN, Ascophyllum nodosum; FV, Fucus vesiculosus (both with 2.5 or 5% inclusion level); TMR, total mixed ration.
$^{a-c}$Means within a column not showing a common superscript differ ($P < 0.05$).

TABLE 3 | Nutrient degradation in the feed bags of the five treatments (days 7–12) and estimated microbial protein synthesis (days 7–13).

	Degraded					EMPS
	DM	OM	CP	ADFom	aNDFom	
	%					mg/g degraded OM
TMR	44.8^{a}	44.3^{a}	39.5^{a}	14.0^{a}	24.7^{a}	120^{a}
AN2.5	41.2^{b}	40.7^{b}	30.6^{b}	11.5^{ab}	21.8^{b}	109^{b}
AN5	39.5^{c}	39.2^{b}	25.7^{c}	8.5^{c}	18.7^{c}	100^{c}
FV2.5	40.4^{bc}	40.0^{b}	30.4^{b}	11.1^{b}	20.5^{bc}	110^{b}
FV5	37.7^{d}	37.3^{c}	25.1^{c}	9.7^{bc}	18.9^{c}	100^{c}
Pooled SEM	*0.66*	*0.73*	*0.82*	*0.99*	*0.89*	*10.8*
P	<0.001	<0.001	<0.001	0.001	<0.001	<0.001

AN, Ascophyllum nodosum; FV, Fucus vesiculosus (both with 2.5 or 5% inclusion level); TMR, total mixed ration; DM, dry matter; OM, organic matter; CP, crude protein; ADFom, acid detergent fiber on ash free basis; aNDFom, neutral detergent fiber on ash free basis; EMPS, estimated microbial protein synthesis.
$^{a-d}$Means within a column not showing a common superscript differ ($P < 0.05$).

Fermentation Characteristics

The temperature ($P = 0.576$; 38.6°C) and redox potential measured in FL (−241 mV; $P = 0.062$) did not differ among the treatments. The pH in the fermenter was different between the TMR alone (pH = 6.82) and the seaweed supplemented treatments (all pH = 6.85; $P = 0.004$). At both supplementation levels, seaweed decreased analyzed NH_3-N and VFA production compared to TMR alone, except for propionate production in FV2.5 (**Table 4**; $P \leq 0.001$). For isovalerate and valerate, the 5% inclusion level of both seaweeds showed a less pronounced reduction than the 2.5% inclusion level. For butyrate, this was

[2]https://github.com/timyerg/Kunzel_rstc_2019

TABLE 4 | NH_3-N and VFA production analyzed in the effluent of the five treatments (days 7–13).

	NH_3-N	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	VFA_{total}	C2:C3
	mmol/d	mmol/d	mmol/d	mmol/d	mmol/d	mmol/d	mmol/d	mmol/d	
TMR	5.40^{a}	19.7^{a}	7.00^{a}	0.40^{a}	7.85^{a}	1.40^{a}	3.08^{a}	39.3^{a}	2.86^{a}
AN2.5	3.39^{b}	17.3^{b}	6.56^{cd}	0.28^{b}	5.27^{c}	0.82^{c}	2.29^{d}	32.5^{bc}	2.63^{b}
AN5	2.73^{d}	16.4^{c}	6.57^{d}	0.26^{c}	5.86^{b}	0.89^{b}	2.97^{b}	32.9^{b}	2.47^{bc}
FV2.5	3.42^{b}	16.7^{c}	6.83^{ab}	0.27^{c}	5.35^{c}	0.77^{d}	2.45^{c}	32.4^{bc}	2.42^{cd}
FV5	2.84^{c}	15.4^{d}	6.78^{bc}	0.26^{c}	5.39^{c}	0.89^{b}	2.98^{b}	31.7^{c}	2.25^{d}
Pooled SEM	*0.140*	*0.71*	*0.335*	*0.008*	*0.469*	*0.069*	*0.177*	*0.83*	*0.062*
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

AN, Ascophyllum nodosum; FV, Fucus vesiculosus (both with 2.5 or 5% inclusion level); TMR, total mixed ration.
$^{a-d}$Means within a column not showing a common superscript differ ($P < 0.05$).

only the case for seaweed AN and acetate only for seaweed FV. For NH_3-N and the other VFA, the decrease was equal to or greater for the 5% inclusion level than the 2.5%.

Alpha and Beta Diversity

After sequencing and demultiplexing, a total of 4,843,225 and 5,851,973 read pairs were obtained for archaeal and bacterial datasets, respectively. Quality filtering, denoising, merging of paired reads, and chimera removal resulted in 2,648,201 archaeal and 4,237,733 bacterial ASVs. Archaeal Faith's PD and Shannon indices were approximately equal in RF and RSP at the start of the experiment (**Figures 3A,C** and **Supplementary Table 2**) and failed to reject the null hypothesis when tested against each other (Faith's PD $P = 0.881$, Shannon $P = 0.531$). Archaeal phylogenetic diversity was greater in the Rusitec samples compared to the inoculum; meanwhile, Shannon entropy fluctuated in the system among sampling days. Compared to RF, Faith's PD was greater in E and FL at day 7 ($P = 0.001$ and 0.044) and in E at day 13 ($P = 0.026$). E phylogenetic diversity at day 13 decreased compared to day 7 ($P = 0.048$). Shannon entropy at day 7 was greater in E than RF ($P = 0.006$). The entropy of E and FL sample types was reduced from days 7 to 13 ($P = 0.001$). At day 13, both metrics demonstrated no differences between FR and SAM samples (all $P > 0.05$) but were greater in FR samples from bags with a 48-h incubation period compared to 24 h (Faith's PD $P = 0.028$, Shannon $P = 0.001$). In the bacterial dataset, the alpha diversity of samples at day 0 (RF and RSP) was also at the same level (all $P > 0.05$) (**Figures 3B,D** and **Supplementary Table 2**). Unlike archaea, bacterial communities demonstrated no changes in E and FL samples compared by sampling days and the initial RF at day 0 (all $P > 0.05$). Meanwhile, the Shannon index decreased in E and FL at day 7 ($P = 0.029$ and 0.017) and in E at day 13 ($P = 0.019$). Both tested alpha diversity metrics of FR and SAM revealed no differences between these sample types (all $P > 0.05$) but were lower for both of them than in all samples from days 0 to 7 and E and FL at day 13 (all $P < 0.05$). When testing FR samples based on incubation time, no differences were observed for phylogenetic diversity, but Shannon entropy was lower in 48-h samples ($P = 0.026$).

In archaea, seaweed additives affected the Shannon entropy of E at days 7 and 13 and FL at day 13 (**Supplementary Figure 1**). At both days 7 and 13, diets AN5, FV2.5, and FV5 resulted in lower entropy than the TMR alone (all $P < 0.05$). In FL, the Shannon index of FV2.5 and FV5 samples was also lower than that of TMR (all $P < 0.05$). Among bacteria, no significant

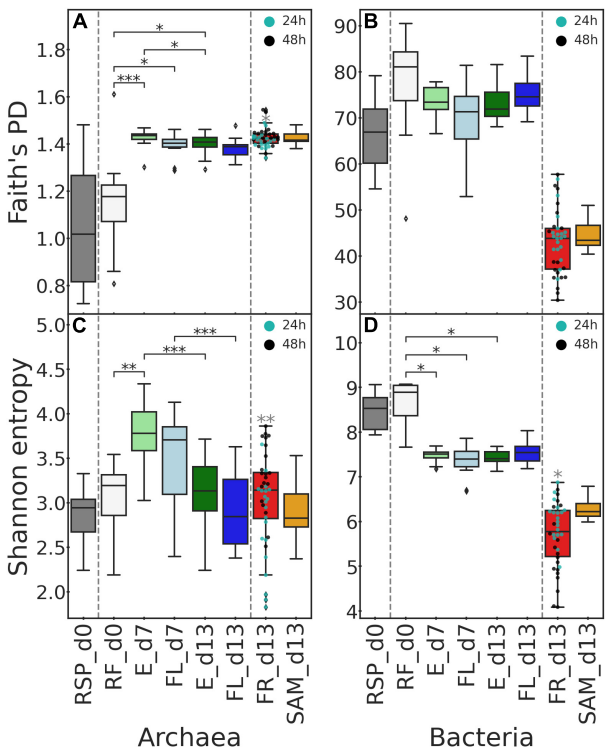


FIGURE 3 | Alpha diversity indices by sample type and sampling day. Faith's phylogenetic diversity **(A,B)** and Shannon entropy index **(C,D)** are plotted for archaea **(A,C)** and bacteria **(B,D)**. Boxplots visualize the alpha diversity metric distribution across all samples inside a given group. Gray dashed lines subdivide boxes into groups, where pairwise comparisons were performed. Significant after Benjamini–Hochberg correction, *P*-values of Dunn's pairwise test are denoted by black asterisks. For FR samples, additional (dots) were plotted, indicating samples taken from bags with 24 and 48 h incubation time. Gray asterisks are plotted above FR samples if the Kruskal–Wallis test indicated a significant difference between samples with different incubation times. Significant *P*-values denoted as: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

changes between treatments were observed according to both alpha diversity metrics (all $P > 0.05$).

Concerning beta diversity, PCoA analysis (**Figure 4**) was performed on the ASVs, using Jaccard and Bray–Curtis distances. Archaeal and bacterial samples clustered into three big groups (RF and RSP from day 0 as one group, E and FL samples from days 7 to 13 as another one, and FR and SAM from day 13 as the third), with clearer clusters in the archaeal dataset. PERMANOVA analysis revealed that microbial composition was affected by the sample type (all $P < 0.05$), sampling day (all $P < 0.05$), and between FR samples incubated for 24 and 48 h (all $P < 0.01$), except for archaeal Jaccard distances.

Seaweed inclusion affected archaeal beta diversity according to both Jaccard and Bray–Curtis distances of E samples at days 7 and 13 and FL at day 13 (all $P < 0.05$). Additionally, Bray–Curtis distances were also affected in FR and SAM samples (all $P < 0.05$). Pairwise tests with correction for multiple comparisons found differences in Bray–Curtis distances of FR samples between the FV5 diet and TMR alone and AN2.5 (all $P = 0.025$). No effect of seaweed on bacterial beta diversity was detected (all $P > 0.05$).

Archaeal and Bacterial Taxonomy Composition

Most of the archaeal sequences (≈ 95% of the reads) were assigned to *Methanomicrobium, Methanobrevibacter*, unclassified to genus level members of the Methanomethylophilaceae family, *Candidatus Methanomethylophilus,* and *Methanimicrococcus* (**Figure 5A**). *Methanobrevibacter* was the most abundant genus in RSP and RF samples, with a gradual shift toward *Methanomicrobium* dominance in E and FL. Considering all samples, regardless of the treatment by sample type and day (**Supplementary Table 3**), relative abundances of *Methanomicrobium* were barely detectable in the samples that served as inoculum RF and RSP but increased up to the dominance in samples from the Rusitec (all $P < 0.05$). On the contrary, *Methanobrevibacter* ratios mostly decreased in the Rusitec samples (all $P < 0.05$, except for E at day 7 compared to RF: $P = 0.065$). Moreover, *Methanomicrobium* relative abundances in E and FL were also greater at day 13 than at day 7 ($P = 0.01$ and 0.001); meanwhile, by the same comparison, the presence of *Methanobrevibacter* at day 13 was lower for E ($P = 0.03$). Except for SAM, the proportions of unclassified *Methanomethylophilaceae* in RF were lower than in Rusitec sample types (all $P < 0.05$). Methanomicrobium in FL at day 13 ($P = 0.023$), unidentified Methanomethylophilaceae in E at day 7 ($P = 0.029$), Methanimicrococcus in E at day 7 ($P = 0.023$), and FL at day 13 ($P = 0.008$) had higher relative abundances in FV5 samples than TMR alone (**Supplementary Table 4**). At day 13, *Methanimicrococcus* was also more abundant in FV2.5 samples from SAM ($P = 0.043$).

On average, among bacteria, *Lactobacillus* showed the highest abundance across all samples, followed by *Pseudoscardovia*, unclassified Bacteroidales, marked by Silva database as F082 (further F082), *Limosilactobacillus*, and *Prevotella* (**Figure 5B**). The high abundance of *Lactobacillus* was mostly observed in FR and SAM samples. In E and FL samples, F082 dominated the community. *Prevotella* accounted for the highest abundance in the inoculum (day 0) but was less abundant in E, FL, FR, and SAM (all $P < 0.05$) compared to RF, except for E at day 7. Compared with sample types and sampling days (**Supplementary Table 3**), relative abundances of *Lactobacillus, Pseudoscardovia,* and F082 were about the same in E and FL at both days 7 and 13 and increased their proportions compared to cow samples (all $P < 0.05$, except for *Pseudoscardovia* at day 13) after incubation in the Rusitec. Notably, *Lactobacillus* and *Pseudoscardovia* were poorly represented in RF and RSP. Treatment AN5 decreased relative abundances of F082 in E samples on day 7 ($P = 0.025$) and FR on day 13 ($P = 0.005$) compared to TMR alone. Meanwhile, *Ruminobacter* proportions in E ($P = 0.044$) and SAM samples ($P = 0.05$) increased at day 13. FV5 also increased relative abundances of *Ruminobacter* in FL samples at day 13 ($P = 0.022$) (**Supplementary Table 4**).

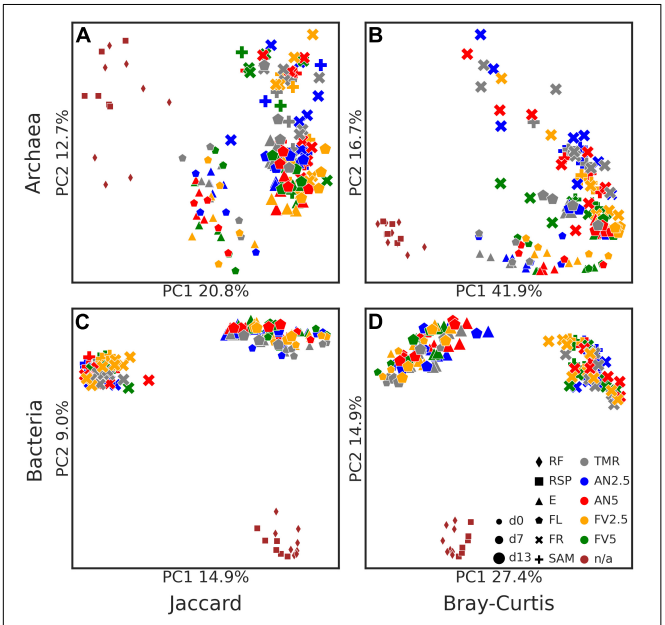


FIGURE 4 | Principal coordinates analysis (PCoA) plots of beta diversity metrics. Jaccard **(A,C)** and Bray–Curtis **(B,D)** distances are plotted for archaea **(A,B)** and bacteria **(C,D)**. Points are sized by the sampling day (days 0–13), shaped by the sample type, and colored by treatments [as stated in the legend on subplot **(D)**]. Label "n/a" in the legend means no treatment was applied.

Effect of Seaweed Additives on the Microbial Amplicon Sequence Variant Abundances

In E and FL sample types, 10 archaeal and 10 bacterial ASVs were differentially abundant in seaweed-supplemented diets compared to TMR alone (**Figure 6**). In E, archaeal ASV with the first four characters in corresponding id digest 7f9f (further indicated in round brackets), assigned to *Candidatus Methanomethylophilus*, was more abundant in treatments AN5 ($P = 0.054$) and FV5 ($P = 0.049$). *Methanobrevibacter wolinii* ASVs (80e7, b43f) were less abundant in FV5-supplemented treatments ($P = 0.013$, 0.01);

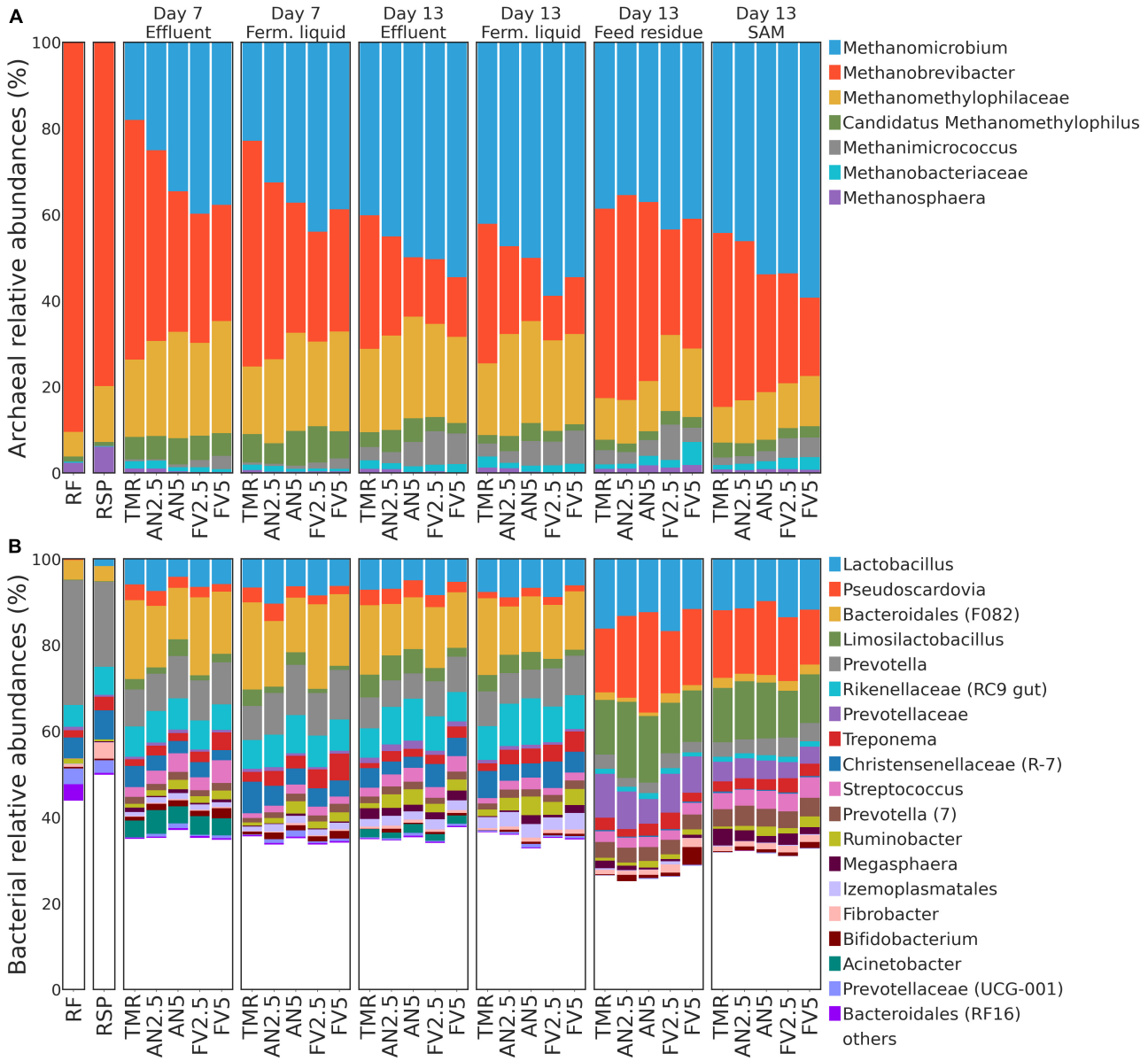


FIGURE 5 | Stacked taxonomy bar plots. Relative abundances of **(A)** archaeal and **(B)** bacterial genera are plotted for each sample type and sampling day by treatments (only genera with relative abundance ≥3.5% among at least one column were included). If certain features were not annotated to genus level, the last available taxonomy unit was shown. Minor Silva annotation identifiers, such as clades, taxonomic groups, clones, and others not included in commonly used nomenclature, are indicated in the round brackets.

meanwhile, *Methanomicrococcus* (48e8) abundances increased (P = 0.002). Unclassified *Methanomethylophilaceae* ASVs (d2f4, 8b4b, 9af5, 898c, and 37c0) were mostly associated with seaweed-supplemented samples, with one ASV (c795) more abundant in TMR alone and decreased when the FV2.5 (Wilcoxon P = 0.008) was added. In FL, *Methanomicrococcus* (48e8) (P = 0.005) and *Methanomethylophilaceae* (9af5, d2f4) (P = 0.067, 0.069) were more abundant in FV5 diet. *Methanomethylophilaceae* (d2f4) also increased when FV2.5 seaweed was added (P = 0.052).

Among bacteria of E and FL sample types, ASVs (7718, ea75), assigned to the Bacteroidales F082 group, *Pseudoscardovia* (9a4e), *Atopobium* (e1d3), *Prevotellaceae* (8969), and *Succiniclasticum* (1814) were mostly associated with TMR alone. However, *Prevotellaceae* (8969) (P = 0.083) and *Pseudoscardovia* (9a4e) (P = 0.085) were more abundant in FL samples of AN5. *Prevotella* (30d0) was more abundant in AN2.5, AN5, and FV5 samples (P = 0.002, 0.001, and 0.011 in FL and 0.002, 0.003, and 0.005 in E). *Rikenellaceae* RC9 gut group (81ff, 01fe) also increased in E and FL sample types when AN seaweed was added (P in the range of 0.002–0.003). Finally, in E unclassified *Spirochaetaceae* (8023) was more abundant in AN5 (P = 0.004) and FV5 (P = 0.04) supplemented diets.

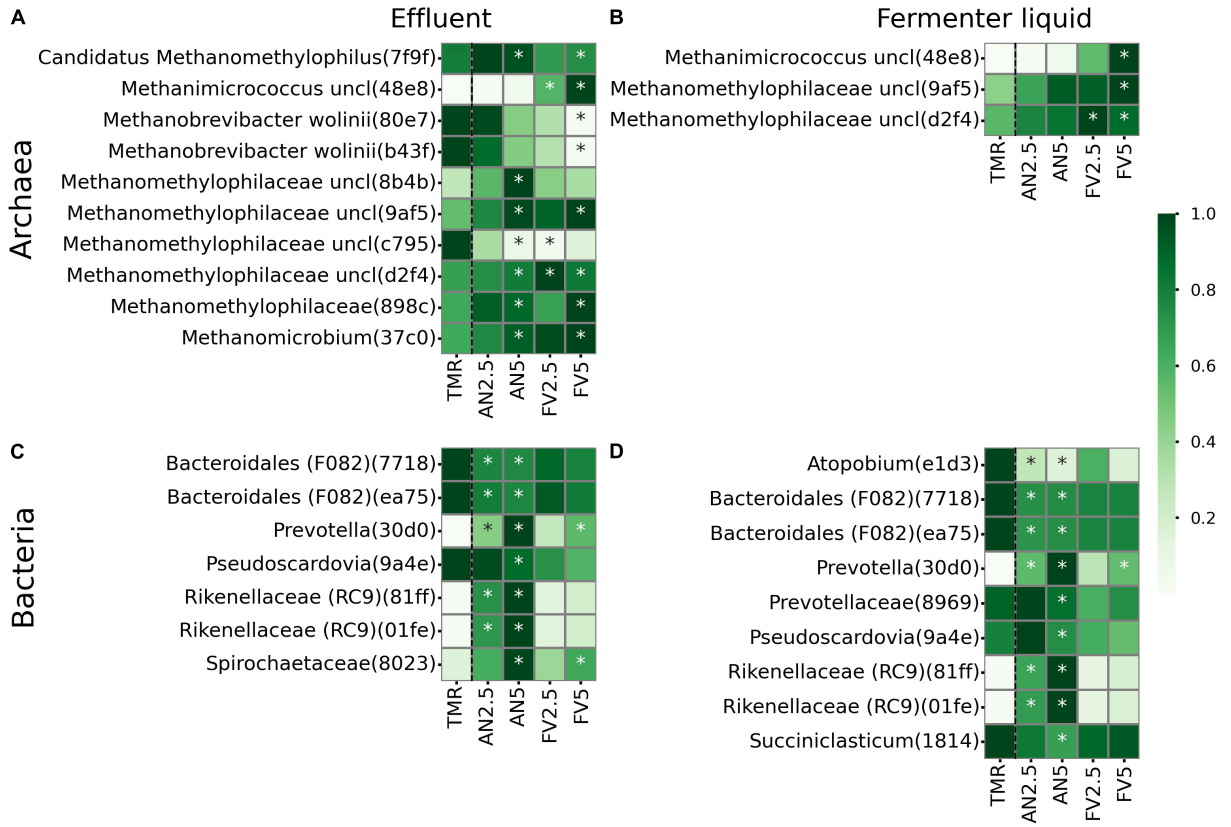


FIGURE 6 | Relative abundances of differentially abundant features according to ALDEx2. Relative abundances are normalized by rows. Archaeal **(A,B)** and bacterial **(C,D)** features from effluent **(A,C)** and fermenter liquid **(B,D)** sample types. All seaweed additives were compared with TMR. ALDEx2 significant abundances are marked by asterisks. All supplements were compared to TMR alone and separated by black dashed lines.

Correlations

A correlation analysis was performed to investigate potential relationships between microbiota genera, total gas, CH_4 concentration, and VFA production (**Supplementary Tables 5–7**). At the genus level, correlations of microbial abundances with total gas production, CH_4 concentration, and VFA were performed for E, FL, and FR sample types (**Supplementary Figure 2**), with only significant correlations plotted (absolute value of Spearman $r \geq 0.3$ and $P \leq 0.05$). The largest number of genera that negatively correlated with the above-mentioned traits was detected in E samples; meanwhile, in FL and FR, positive correlations prevailed.

DISCUSSION

Seaweed Effects

A significant reduction of CH_4 concentration in the total gas was found to be caused by the supplementation of both seaweed species at the 5% inclusion level. However, the effect was not as high as previously reported for *A. taxiformis* (Kinley et al., 2016; Machado et al., 2018; Roque et al., 2019). Additionally, the lower CH_4 concentration in total gas was accompanied by a reduced rate of overall fermentation. The microbiota produced not only less methane but also fewer metabolites relevant to animals. This was indicated by the decreased total gas production, VFA, NH_3-N, and nutrient degradation. Reduced fermentation and nutrient degradation would generally mean a diminished amount of nutrients and energy available for the host animal. However, in the case of CP, a reduced degradation could benefit the animal because the ruminal undegradable protein could be used as a bypass protein like it was already shown for other seaweed species (Tayyab et al., 2016). The reduced CP degradation may have resulted from the formation of complexes between the phlorotannins contained in both seaweeds and other proteins (Belanche et al., 2016) and resulted in a lower EMPS, but more research is needed to confirm this.

The CH_4 reduction effect of *A. taxiformis* is attributed to the presence of bromoform (Machado et al., 2016), which is known to be a carcinogen (National Toxicology Program, 1989). The suggested modes of action in the brown seaweeds AN and FV used in the present study were likely related to phlorotannins as the bromoform content [8.0 (AN) and <0.8 μg/kg DM (FV)] was negligibly lower compared to the one analyzed by Machado et al. (2016) for *A. taxiformis* (1,723,000 μg/kg DM). The TPC, an indicator of phlorotannin content, was very similar in both seaweeds (7.9 vs. 7.4 phloroglucinol equivalent g/100 g sample). The greater CH_4 reduction with the supplementation of AN

compared to FV could be explained by either the slightly greater TPC or the greater bromoform content in this sample, even if it is still very low. However, both species showed the same CH_4 concentration of total gas at the low seaweed inclusion level. Both bromoform and terrestrial tannins influence methanogens (Cieslak et al., 2014; Machado et al., 2018). Additionally, it has been shown that the composition of archaeal methanogens is associated with CH_4 production (Tapio et al., 2017). Consistent with this finding, our study showed that the decline in methane concentration of total gas in seaweed supplemented samples was accompanied by the separation of archaeal and bacterial ASVs in a dose-dependent manner, indirectly supporting the importance of methanogen composition. Interestingly, AN5 seaweed supplementation resulted in more differentiation of microbial ASVs abundances and a stronger reduction of CH_4 concentration when compared to TMR than FV5.

The treatment showing the highest CH_4 reduction in this study, AN5, also contained the highest iodine concentration (**Supplementary Table 1**). The iodine content of AN was more than 10 times greater than that of FV. Therefore, the application of practical feeding should be done with caution. Iodine toxicity in dairy cows is reported by the National Research Council (U.S.) (2001) for a concentration of 5 mg iodine/kg dietary DM. The European Food Safety Authority recommends a maximum iodine content of 2 mg/kg of complete feed (EFSA, 2013). In the current experiment, the iodine concentration was 35 mg/kg DM for treatment AN2.5 and 2.75 mg/kg DM for treatment FV2.5. Additionally, it was shown that the iodine concentration in milk follows a dose-response relationship with iodine intake (Antaya et al., 2015; Newton et al., 2021). Humans, especially children, are even more sensitive to iodine poisoning than ruminants (Zimmermann et al., 2005). Therefore, the seaweed inclusion levels used in the present study do not apply to practical ruminant feeding, except for FV2.5. Seaweed supplementation at an exceptional iodine inclusion level would therefore not significantly reduce methane compared to TMR.

Methanogenesis

In our study, *Prevotella*, unclassified members of *Prevotellaceae* and *Clostridia* negatively correlated with CH_4 concentration (**Supplementary Table 6**) and archaeal methanogens (**Supplementary Table 7**). *Prevotella* spp. and other members of the *Prevotellaceae* family are known for their ability to consume H_2 and produce propionate (Strobel, 1992; Mitsumori et al., 2012; Denman et al., 2015), which in turn is negatively associated with CH_4 formation and able to act as an alternative hydrogen sink (Ungerfeld, 2015). Inverse associations of *Prevotella* spp. with CH_4 concentration were previously reported (Mitsumori et al., 2012; Aguilar-Marin et al., 2020). Positive correlations with CH_4 concentration were observed for *Fibrobacter, Lactobacillus, Pseudoramibacter, Olsenella, Shuttleworthia,* and unclassified *Oscillospiraceae* (NK4A214). *Fibrobacter,* a *fibrolytic* bacteria that produce formate, can also be utilized by methanogens as a substrate for CH_4 production (Rychlik and May, 2000). Positive correlations of the *Fibrobacter* genus with CH_4 formation were previously reported (Wang et al., 2016). The addition of some *Lactobacillus* and *Limosilactobacillus* spp. (*L. mucosae*) in an *in vitro* rumen fermentation technique led to the rise of CH_4 production (Soriano et al., 2014). *Pseudoramibacter* can utilize carbohydrates as an energy source (Deusch et al., 2017), producing VFA during fermentation, including butyrate, acetate, formate, and hydrogen (Palakawong Na Ayudthaya et al., 2018), and increasing methane production by methanogens. *Olsenella* contains genes encoding choline trimethylamine lyase (Kelly et al., 2019) and is involved in CH_4 production (Broad and Dawson, 1976; Neill et al., 1978). *Shuttleworthia* is positively correlated with CH_4 formation in dairy heifers (Cunha et al., 2019) and produces acetate and butyrate through glucose fermentation (Downes et al., 2002). *Oscillospiraceae* members were linked with acetate production (Tanca et al., 2017) and, in our study, correlated with acetate, the A:P ratio, and CH_4 concentration. In general, those correlations are consistent with previous studies. It was shown that the addition of AN to an *in vitro* fermentation system led to a decrease in the growth of *Fibrobacter succinogenes* (positively correlated with CH_4 concentration) and an increase in *Prevotella bryantii* (negatively correlated with CH_4 concentration) (Wang et al., 2009). As it was reviewed by Abbott et al. (2020), it is possible that seaweeds modulate the growth of cellulolytic rumen bacteria by altering polysaccharide availability (Lee et al., 2019).

Adaptation of Microbiota Composition

Although we allowed rumen microbiota to adapt for 7 days in the Rusitec prior to sampling, archaeal community distribution demonstrated a shift in the dominant genera at the end of the adaptation period and revealed a variation between the samplings at days 7 and 13. Partially, it can be explained by lower metabolite digestibility in the Rusitec than *in vivo* conditions and lack of or low protozoa counts (Martínez et al., 2010; Hristov et al., 2012). Relative abundances of *Methanobrevibacter*, which is the most abundant methanogen genus in cow samples, continuously decreased in the Rusitec. This tendency was somehow more profound in seaweed-treated samples, although no statistically significant differences with TMR were detected. In the Rusitec samples, dominance gradually shifted from *Methanobrevibacter* to *Methanomicrobium* by day 13. Although it has been shown that the Rusitec provides a stable condition for the methanogens (Lengowski et al., 2016), additional studies with longer *in vitro* incubation periods may be performed to investigate *Methanobrevibacter/Methanomicrobium* ratio dynamics in such systems since abundances of *Methanobrevibacter* relative to other archaea may affect CH_4 production (Cunha et al., 2019). When the total gas production and methane concentration are additionally statistically analyzed not only by treatment but also by sampling day and the interaction between treatment and day, it becomes evident that both traits increased during the experimental period ($P = 0.005$ and <0.001, respectively; **Supplementary Table 8**). This coincides with changes in the proportion of the most abundant methanogens in the system (*Methanobrevibacter* abundances decreased, while *Methanomicrobium* increased) between days 7 and 13 and indicates that adaptation of the microbes to the system was not finalized after day 7. Previous studies have shown a decrease in the protozoa population, also known as a methane producer, in

Rusitec fermenters over time (Ziemer et al., 2000; Martínez et al., 2010; Morgavi et al., 2012). Protozoa were not analyzed in the current study, but this potential shift could also have led to an altered archaeal community distribution.

Conclusion

The North Atlantic seaweed species used herein can modify the microbiota in the rumen toward reduced methane production. However, methane reduction was not great, and the seaweed inclusion in the ration caused reduced *in vitro* fermentation overall. Therefore, the tested seaweed species are not a viable option to be used as feed for ruminants to mitigate methane production.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the European Nucleotide Archive (ENA) repository, accession number PRJEB50942 (https://www.ebi.ac.uk/ena/browser/view/PRJEB50942).

ETHICS STATEMENT

The animal study was reviewed and approved by the Regierungspräsidium Stuttgart, Germany.

AUTHOR CONTRIBUTIONS

SK, KW, MR, AC-S, HG, and AP conceived and designed the experiment. SK, KW, and HP performed the experiments. SK, KW, HP, and MR performed the *in vitro* data analysis. TY and AC-S performed the microbial data analysis. AP and HG carried out chemical data analysis. SK and TY drafted the manuscript. All authors revised and approved the manuscript revisions.

FUNDING

This study was funded by the EIT Food, the innovation community on the food of the European Institute of Innovation and Technology (EIT), a body of the EU, under Horizon 2020, the EU Framework Programme for Research and Innovation via the activity SeaCH4NGE [seaweed supplementation to mitigate methane (CH4) emissions by cattle; No. 19084].

ACKNOWLEDGMENTS

We gratefully acknowledge the support of Christiane Horn, Helga Ott, Sibylle Rupp, Helga Terry, Brynja Einarsdóttir, Branka Borojevic, and Natasa Desnica in conducting the chemical analyses. We also acknowledge support by the High Performance and Cloud Computing Group at the Zentrum für Datenverarbeitung of the University of Tübingen, the state of Baden-Württemberg through bwHPC, and the German Research Foundation (DFG) through grant no. INST 37/935-1 FUGG.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.889618/full#supplementary-material

Supplementary Figure 1 | Alpha diversity indices by treatment, sample-type and day of sampling. Faith's phylogenetic diversity and Shannon entropy index are plotted for archaea and bacteria. Each subplots represents one of alpha diversity metrics at a certain day of sampling. Boxplots visualize alpha diversity metrics distribution across all treatments inside of a certain group. Kruskal–Wallis *H*-test was performed to test the differences between treatments alpha diversity and corresponding *P*-values are plotted on the top of subplots. Significant *P*-values denoted as '*' if $P \leq 0.05$.

Supplementary Figure 2 | SCNIC based correlations of microbial relative abundances among effluent, fermenter liquid and feed residue sample types with total gas production, methane as a percentage of total gas [CH4(%)], ammonium nitrogen in effluent (NH_3-N), VFA production and acetate to propionate ratio (A:P ratio). Only correlation coeffitients (*r*) with absolute values $\geq$0.3 and adjusted *P*-values $\leq$ 0.05 are plotted. Positive correlations are colored in read and negative in blue.

REFERENCES

Abbott, D. W., Aasen, I. M., Beauchemin, K. A., Grondahl, F., Gruninger, R., Hayes, M., et al. (2020). Seaweed and seaweed bioactives for mitigation of enteric methane: challenges and opportunities. *Animals* 10:2432. doi: 10.3390/ani10122432

Aguilar-Marin, S. B., Betancur-Murillo, C. L., Isaza, G. A., Mesa, H., and Jovel, J. (2020). Lower methane emissions were associated with higher abundance of ruminal Prevotella in a cohort of colombian buffalos. *BMC Microbiol.* 20:364. doi: 10.1186/s12866-020-02037-6

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

Antaya, N. T., Soder, K. J., Kraft, J., Whitehouse, N. L., Guindon, N. E., Erickson, P. S., et al. (2015). Incremental amounts of Ascophyllum nodosum meal do not improve animal performance but do increase milk iodine output in early lactation dairy cows fed high-forage diets. *J. Dairy. Sci.* 98, 1991–2004. doi: 10.3168/jds.2014-8851

Belanche, A., Jones, E., Parveen, I., and Newbold, C. J. (2016). A metagenomics approach to evaluate the impact of dietary supplementation with Ascophyllum nodosum or Laminaria digitata on rumen function in rusitec fermenters. *Front. Microbiol.* 7:299. doi: 10.3389/fmicb.2016.00299

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Boguhn, J., Kluth, H., and Rodehutscord, M. (2006). Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. *J. Dairy Sci.* 89, 1580–1591. doi: 10.3168/jds.S0022-0302(06)72225-1

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

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of southern wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Britton, D., Schmid, M., Revill, A. T., Virtue, P., Nichols, P. D., Hurd, C. L., et al. (2021). Seasonal and site-specific variation in the nutritional quality of temperate seaweed assemblages: implications for grazing invertebrates and the commercial exploitation of seaweeds. *J. Appl. Phycol.* 33, 603–616. doi: 10.1007/s10811-020-02302-1

Broad, T. E., and Dawson, R. M. (1976). Role of choline in the nutrition of the rumen protozoon entodinium caudatum. *J. Gen. Microbiol.* 92, 391–397. doi: 10.1099/00221287-92-2-391

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

Cieslak, A., Zmora, P., Pers-Kamczyc, E., Stochmal, A., Sadowinska, A., Salem, A. Z., et al. (2014). Effects of two sources of tannins (Quercus L. and Vaccinium vitis idaea L.) on rumen microbial fermentation: an in vitro study. *Ital. J. Anim. Sci.* 13:3133. doi: 10.4081/ijas.2014.3133

Cunha, C. S., Marcondes, M. I., Veloso, C. M., Mantovani, H. C., Pereira, L. G. R., Tomich, T. R., et al. (2019). Compositional and structural dynamics of the ruminal microbiota in dairy heifers and its relationship to methane production. *J. Sci. Food Agric.* 99, 210–218. doi: 10.1002/jsfa.9162

Czerkawski, J. W., and Breckenridge, G. (1977). Design and development of a long-term rumen simulation technique (rusitec). *Br. J. Nutr.* 38, 371–384. doi: 10.1079/bjn19770102

de Menezes, A. B., Lewis, E., O'Donovan, M., O'Neill, B. F., Clipson, N., and Doyle, E. M. (2011). Microbiome analysis of dairy cows fed pasture or total mixed ration diets. *FEMS Microbiol. Ecol.* 78, 256–265. doi: 10.1111/j.1574-6941.2011.01151.x

Denman, S. E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., and McSweeney, C. S. (2015). Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. *Front. Microbiol.* 6:1087. doi: 10.3389/fmicb.2015.01087

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

Dodge, Y. (2010). *The Concise Encyclopedia of Statistics*. New York, NY: Springer.

Downes, J., Munson, M. A., Radford, D. R., Spratt, D. A., and Wade, W. G. (2002). Shuttleworthia satelles gen. nov., sp. nov., isolated from the human oral cavity. *Int. J. Syst. Evol.* 52, 1469–1475. doi: 10.1099/00207713-52-5-1469

Dunn, O. J. (1964). Multiple comparisons using rank sums. *Technometrics* 6, 241–252. doi: 10.2307/1266041

EFSA (2013). Scientific Opinion on the safety and efficacy of iodine compounds (E2) as feed additives for all species: calcium iodate anhydrous and potassium iodide, based on a dossier submitted by HELM AG. *EFSA J.* 11:3101. doi: 10.2903/j.efsa.2013.3101

EPA (1996). Method 8260B: Volatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS) (Washington, DC: United States Environmental Protection Agency).

Evans, F. D., and Critchley, A. T. (2014). Seaweeds for animal production use. *J. Appl. Phycol.* 26, 891–899. doi: 10.1007/s10811-013-0162-9

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* 61, 1–10. doi: 10.1016/0006-3207(92)91201-3

Fernandes, A. D., Macklaim, J. M., Linn, T. G., Reid, G., and Gloor, G. B. (2013). ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-Seq. *PLoS One* 8:e67019. doi: 10.1371/journal.pone.0067019

Fernandes, A. D., Reid, J. N., Macklaim, J. M., McMurrough, T. A., Edgell, D. R., and Gloor, G. B. (2014). Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* 2:15. doi: 10.1186/2049-2618-2-15

Friedman, J., and Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLoS Comput. Biol.* 8:e1002687. doi: 10.1371/journal.pcbi.1002687

Halko, N., Martinsson, P.-G., Shkolnisky, Y., and Tygert, M. (2011). An algorithm for the principal component analysis of large data sets. *SIAM J. Sci. Comput.* 33, 2580–2594. doi: 10.1137/100804139

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

Hildebrand, B., Boguhn, J., and Rodehutscord, M. (2011). Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation. *Animal* 5, 537–546. doi: 10.1017/S1751731110002156

Hristov, A. N., Lee, C., Hristova, R., Huhtanen, P., and Firkins, J. L. (2012). A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. *J. Dairy Sci.* 95, 5299–5307. doi: 10.3168/jds.2012-5533

Jaccard, P. (1912). The distribution of the flora in the alpine zone. *New Phytol.* 11, 37–50. doi: 10.1111/j.1469-8137.1912.tb05611.x

Kaewtapee, C., Burbach, K., Tomforde, G., Hartinger, T., Camarinha-Silva, A., Heinritz, S., et al. (2017). Effect of *Bacillus* subtilis and *Bacillus* licheniformis supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs. *J. Anim. Sci. Biotechnol.* 8:37. doi: 10.1186/s40104-017-0168-2

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kelly, W. J., Leahy, S. C., Kamke, J., Soni, P., Koike, S., Mackie, R., et al. (2019). Occurrence and expression of genes encoding methyl-compound production in rumen bacteria. *Anim. Microbiome* 1:15. doi: 10.1186/s42523-019-0016-0

Kinley, R. D., de Nys, R., Vucko, M. J., Machado, L., and Tomkins, N. W. (2016). The red macroalgae Asparagopsis taxiformis is a potent natural antimethanogenic that reduces methane production during in vitro fermentation with rumen fluid. *Anim. Prod. Sci.* 56, 282–289. doi: 10.1071/AN15576

Kruskal, W. H., and Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. *J. Am. Stat. Assoc.* 47, 583–621. doi: 10.2307/2280779

Lassey, K. R. (2007). Livestock methane emission: from the individual grazing animal through national inventories to the global methane cycle. *Agric. For. Meteorol.* 142, 120–132. doi: 10.1016/j.agrformet.2006.03.028

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., 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

Lee, H. J., Jung, J. Y., Oh, Y. K., Lee, S.-S., Madsen, E. L., and Jeon, C. O. (2012). Comparative survey of rumen microbial communities and metabolites across one caprine and three bovine groups, using bar-coded pyrosequencing and ^{1}H nuclear magnetic resonance spectroscopy. *Appl. Environ. Microbiol.* 78, 5983–5993. doi: 10.1128/AEM.00104-12

Lee, S. J., Jeong, J. S., Shin, N. H., Lee, S. K., Kim, H. S., Eom, J. S., et al. (2019). Impact of Ecklonia stolonifera extract on in vitro ruminal fermentation characteristics, methanogenesis, and microbial populations. Asian Australas. *J. Anim. Sci.* 92, 1864–1872. doi: 10.5713/ajas.19.0092

Lengowski, M. B., Zuber, K. H. R., Witzig, M., Möhring, J., Boguhn, J., and Rodehutscord, M. (2016). Changes in rumen microbial community composition during adaption to an in vitro system and the impact of different forages. *PLoS One* 11:e0150115. doi: 10.1371/journal.pone.0150115

Machado, L., Magnusson, M., Paul, N. A., Kinley, R., de Nys, R., and Tomkins, N. (2016). Identification of bioactives from the red seaweed Asparagopsis taxiformis that promote antimethanogenic activity in vitro. *J. Appl. Phycol.* 28, 3117–3126. doi: 10.1007/s10811-016-0830-7

Machado, L., Tomkins, N., Magnusson, M., Midgley, D. J., de Nys, R., and Rosewarne, C. P. (2018). In vitro response of rumen microbiota to the antimethanogenic red macroalga Asparagopsis taxiformis. *Microb. Ecol.* 75, 811–818. doi: 10.1007/s00248-017-1086-8

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200

Martínez, M. E., Ranilla, M. J., Tejido, M. L., Saro, C., and Carro, M. D. (2010). Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and rusitec fermenters. II. protozoa population and diversity of bacterial communities. *J. Dairy Sci.* 93, 3699–3712. doi: 10.3168/jds.2009-2934

McDougall, E. I. (1948). Studies on ruminant saliva. 1. the composition and output of sheep's saliva. *Biochem. J.* 43, 99–109. doi: 10.1042/bj0430099

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., et al. (2012). Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated

methane analogue. *Br. J. Nutr.* 108, 482–491. doi: 10.1017/S0007114511005794

Molina-Alcaide, E., Carro, M. D., Roleda, M. Y., Weisbjerg, M. R., Lind, V., and Novoa-Garrido, M. (2017). In vitro ruminal fermentation and methane production of different seaweed species. *Anim. Feed Sci. Technol.* 228, 1–12. doi: 10.1016/j.anifeedsci.2017.03.012

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

National Research Council (U.S.) (2001). *Nutrient Requirements of Dairy Cattle*. Washington D.C: National Academy Press.

National Toxicology Program (1989). Toxicology and carcinogenesis studies of tribromomethane (bromoform) (CAS No. 75-25-2) in F344/N rats and B6C3F1 mice (gavage studies). *Natl. Toxicol. Program. Tech. Rep. Ser.* 350, 1–198.

Neill, A. R., Grime, D. W., and Dawson, R. M. (1978). Conversion of choline methyl groups through trimethylamine into methane in the rumen. *Biochem. J.* 170, 529–535. doi: 10.1042/bj1700529

Newton, E. E., Pétursdóttir, Á. H., Ríkharðsson, G., Beaumal, C., Desnica, N., Giannakopoulou, K., et al. (2021). Effect of dietary seaweed supplementation in cows on milk macrominerals, trace elements and heavy metal concentrations. *Foods* 10:1526. doi: 10.3390/foods10071526

NMKL (2007). *Trace Elements - As, Cd, Hg, Pb and other Elements. Determination by ICP-MS After Pressure Digestion (NMKL 186)* (Bergen: Nordic Committee on Food Analysis).

Paiva, L., Lima, E., Neto, A. I., and Baptista, J. (2018). Seasonal variability of the biochemical composition and antioxidant properties of Fucus spiralis at two Azorean islands. *Mar. Drugs* 16:248. doi: 10.3390/md16080248

Palakawong Na Ayudthaya, S., van de Weijer, A. H. P., van Gelder, A. H., Stams, A. J. M., Vos, W. M., de, et al. (2018). Organic acid production from potato starch waste fermentation by rumen microbial communities from dutch and thai dairy cows. *Biotechnol. Biofuels* 11: 13. doi: 10.1186/s13068-018-1012-4

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.

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ranilla, M. J., and Carro, M. D. (2003). Diet and procedures used to detach particle-associated microbes from ruminal digesta influence chemical composition of microbes and estimation of microbial growth in rusitec fermenters. *J. Anim. Sci.* 81, 537–544. doi: 10.2527/2003.812537x

Robeson, M. S., O'Rourke, D. R., Kaehler, B. D., Dillon, M. R., and Bokulich, N. A. (2021). RESCRIPt: reproducible sequence taxonomy reference database management. *PLoS Comput. Biol.* 17:e1009581. doi: 10.1371/journal.pcbi.1009581

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584

Roque, B. M., Brooke, C. G., Ladau, J., Polley, T., Marsh, L. J., Najafi, N., et al. (2019). Effect of the macroalgae Asparagopsis taxiformis on methane production and rumen microbiome assemblage. *Anim. Microbiome* 1:3. doi: 10.1186/s42523-019-0004-4

Rychlik, J. L., and May, T. (2000). The effect of a methanogen, methanobrevibacter smithii, on the growth rate, organic acid production, and specific ATP activity of three predominant ruminal cellulolytic bacteria. *Curr. Microbiol.* 40, 176–180. doi: 10.1007/s002849910035

Shaffer, M., Thurimella, K., and Lozupone, C. A. (2020). SCNIC: sparse correlation network investigation for compositional data. *BioRxiv* [Preprint]. doi: 10.1101/2020.11.13.380733

Shannon, C. E. (1948). A mathematical theory of communication. *Bell Labs Tech. J.* 27, 379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x

Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144–158.

Soriano, A. P., Mamuad, L. L., Kim, S.-H., Choi, Y. J., Jeong, C. D., Bae, G. S., et al. (2014). Effect of Lactobacillus mucosae on in vitro rumen fermentation characteristics of dried brewers grain, methane production and bacterial diversity. Asian Australas. *J. Anim. Sci.* 27, 1562–1570. doi: 10.5713/ajas.2014.14517

Strobel, H. J. (1992). Vitamin B12-dependent propionate production by the ruminal bacterium Prevotella ruminicola 23. *Appl. Environ. Microbiol.* 58, 2331–2333. doi: 10.1128/aem.58.7.2331-2333.1992

Tanca, A., Fraumene, C., Manghina, V., Palomba, A., Abbondio, M., Deligios, M., et al. (2017). Diversity and functions of the sheep faecal microbiota: a multi-omic characterization. *Microb. Biotechnol.* 10, 541–554. doi: 10.1111/1751-7915.12462

Tapio, I., Snelling, T. J., Strozzi, F., and Wallace, R. J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8:7. doi: 10.1186/s40104-017-0141-0

Tayyab, U., Novoa-Garrido, M., Roleda, M. Y., Lind, V., and Weisbjerg, M. R. (2016). Ruminal and intestinal protein degradability of various seaweed species measured in situ in dairy cows. *Anim. Feed Sci. Technol.* 213, 44–54. doi: 10.1016/j.anifeedsci.2016.01.003

Thauer, R. K. (2012). The wolfe cycle comes full circle. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15084–15085. doi: 10.1073/pnas.1213193109

Ungerfeld, E. M. (2015). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Front. Microbiol.* 6:37. doi: 10.3389/fmicb.2015.00037

Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (1976). *Handbuch Der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch), BD. III Die chemische Untersuchungs von Futtermitteln mit 1.-8. Ergänzuungslieferung (1983-2012)*. Darmstadt: VDLUFA-Verlag.

Wang, J., Liu, M., Wu, Y., Wang, L., Liu, J., Jiang, L., et al. (2016). Medicinal herbs as a potential strategy to decrease methane production by rumen microbiota: a systematic evaluation with a focus on perilla frutescens seed extract. *Appl. Microbiol. Biotechnol.* 100, 9757–9771. doi: 10.1007/s00253-016-7830-z

Wang, Y., Alexander, T. W., and McAllister, T. A. (2009). In vitro effects of phlorotannins from Ascophyllum nodosum (brown seaweed) on rumen bacterial populations and fermentation. *J. Sci. Food Agric.* 89, 2252–2260. doi: 10.1002/jsfa.3717

Wischer, G., Boguhn, J., Steingaß, H., Schollenberger, M., Hartung, K., and Rodehutscord, M. (2013). Effect of monensin on *in vitro* fermentation of silages and microbial protein synthesis. *Arch. Anim. Nutr.* 67, 219–234. doi: 10.1080/1745039X.2013.793050

Ziemer, C. J., Sharp, R., Stern, M. D., Cotta, M. A., Whitehead, T. R., and Stahl, D. A. (2000). Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. *Environ. Microbiol.* 2, 632–643. doi: 10.1046/j.1462-2920.2000.00146.x

Zimmermann, M. B., Ito, Y., Hess, S. Y., Fujieda, K., and Molinari, L. (2005). High thyroid volume in children with excess dietary iodine intakes. *Am. J. Clin. Nutr.* 81, 840–844. doi: 10.1093/ajcn/81.4.840

Conflict of Interest: The 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.

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 22 July 2022
DOI 10.3389/fmicb.2022.920427

OPEN ACCESS

EDITED BY
Christina D. Moon,
AgResearch Ltd, New Zealand

REVIEWED BY
Maggie Weinroth,
US National Poultry Research Centre (USDA-ARS), United States
Peng Guo,
Hubei Academy of Agricultural Sciences, China

*CORRESPONDENCE
Sara Ricci
nutrition@vetmeduni.ac.at

†PRESENT ADDRESS
Cátia Pacífico,
Biome Diagnostics GmbH,
Vienna, Austria

SPECIALTY SECTION
This article was submitted to Microorganisms in Vertebrate Digestive Systems, a section of the journal Frontiers in Microbiology

RECEIVED 14 April 2022
ACCEPTED 27 June 2022
PUBLISHED 22 July 2022

CITATION
Ricci S, Pacífico C, Castillo-Lopez E, Rivera-Chacon R, Schwartz-Zimmermann HE, Reisinger N, Berthiller F, Zebeli Q and Petri RM (2022) Progressive microbial adaptation of the bovine rumen and hindgut in response to a step-wise increase in dietary starch and the influence of phytogenic supplementation. *Front. Microbiol.* 13:920427. doi: 10.3389/fmicb.2022.920427

Progressive microbial adaptation of the bovine rumen and hindgut in response to a step-wise increase in dietary starch and the influence of phytogenic supplementation

Sara Ricci[1]*, Cátia Pacífico[1†], Ezequias Castillo-Lopez[1], Raul Rivera-Chacon[1], Heidi E. Schwartz-Zimmermann[2], Nicole Reisinger[3], Franz Berthiller[2], Qendrim Zebeli[1] and Renee M. Petri[4]

[1]Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock, Department for Farm Animals and Veterinary Public Health, Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Vienna, Austria, [2]Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock, Department of Agrobiotechnology (IFA-Tulln), Institute of Bioanalytics and Agro-Metabolomics, University of Natural Resources and Life Sciences, Vienna, Austria, [3]DSM, BIOMIN Research Center, Tulln, Austria, [4]Agriculture and Agri-Food Canada, Sherbrooke Research and Development Centre, Sherbrooke, QC, Canada

Microbial composition and activity in the gastrointestinal tract (GIT) of cattle has important implications for animal health and welfare, driving the focus of research toward ways to modify their function and abundance. However, our understanding of microbial adaption to nutritional changes remains limited. The aim of this study was to examine the progressive mechanisms of adaptation in the rumen and hindgut of cattle receiving increasing amounts of starch with or without dietary supplementation of a blended phytogenic feed additive (PFA; containing menthol, thymol and eugenol). We used 16S rRNA gene amplicon sequencing to assess the microbial composition and predicted metabolic pathways in ruminal solid and liquid digesta, and feces. Furthermore, we employed targeted liquid chromatography-mass spectrometry methods to evaluate rumen fluid metabolites. Results indicated a rapid microbial adaptation to diet change, starting on the second day of starch feeding for the particle associated rumen liquid (PARL) microbes. Solid rumen digesta- and feces-associated microbes started changing from the following day. The PARL niche was the most responsive to dietary changes, with the highest number of taxa and predicted pathways affected by the increase in starch intake, as well as by the phytogenic supplementation. Despite the differences in the microbial composition and metabolic potential of the different GIT niches, all showed similar changes toward carbohydrate metabolism. Metabolite measurement confirmed the high prevalence of glucose and volatile fatty acids (VFAs) in the rumen due to the increased substrate availability and metabolic activity of the microbiota. Families *Prevotellaceae*, *Ruminococcaceae* and *Lachnospiraceae* were found to be positively correlated with carbohydrate metabolism, with the

latter two showing wide-ranging predicted metabolic capabilities. Phytogenic supplementation affected low abundant taxa and demonstrated the potential to prevent unwanted implications of feeding high-concentrate diet, such as reduction of microbial diversity. The inclusion of 50% concentrate in the diet caused a major shift in microbial composition and activity in the GIT of cattle. This study demonstrated the ability of microorganisms in various GIT niches to adjust differentially, yet rapidly, to changing dietary conditions, and revealed the potential beneficial effects of supplementation with a PFA during dietary adaptation.

KEYWORDS

cattle, feces, microbial activity, concentrate diet, grain, phytogenic additive, metabolomics, microbiota

Introduction

The importance of microbiota composition and activity, in terms of the complex mechanisms regulating animal health, metabolic function and production, has come into focus in recent years (O'Hara et al., 2020). Among the many different factors that can affect microbiota structure and function, including genetics, environment, and metabolic state, diet remains the key driver of change (Deusch et al., 2017; De Angelis et al., 2020). In dairy cattle, diet composition is critical for ensuring that high energy requirements for milk production are met (Zebeli et al., 2008). Research has focused on increasing the efficiency of feed utilization, avoiding severe negative energy balance, improving animal health and welfare, and reducing methane emissions, all through modulation of the rumen and hindgut microbiota (Humer et al., 2018b; Petri et al., 2018; Matthews et al., 2019). Changes in dietary composition, especially during the period of adaptation, pose an increased risk for dysbiosis of the gut microbiota (David et al., 2014). When a microbial niche is subject to an external perturbation, such as an oversupply of substrates, there is a system of adaptations in metabolic activity driving changes in ecological composition until a new level of homeostasis is reached. In gastrointestinal microbial ecosystems, this new steady state can be detrimental for the host resulting in inflammation and illness (Sommer et al., 2017; Lachnit et al., 2019).

Compared with other carbohydrate sources in dairy diets, starch is rapidly degraded by ruminal bacteria into glucose, which is transformed into pyruvate and then into end-products essential for the animals, such as acetate, propionate, and butyrate (Hoover and Miller, 1991; Mills et al., 1999; Tester et al., 2004). Supplying increased amounts of starch provides the cow with the additional energy required for milk production. However, an excessive amount of starch causes an accumulation of organic acids in the rumen due to a lack of buffering mechanisms, which can affect both the stability of the gut microbiota and animal health (Aschenbach et al., 2011). In fact, under lower pH values, some bacterial species thrive to the detriment of others, and the metabolic profile changes as a result (Russell and Rychlik, 2001; Ametaj et al., 2010). Such conditions can cause the proliferation of pathogens, as well as the production of harmful compounds (Kleen et al., 2003; Plaizier et al., 2012). The resulting dysbiotic status, if prolonged, can cause subsequent health issues, such as subacute ruminal acidosis (SARA) and inflammation in cattle (Plaizier et al., 2008; Khafipour et al., 2016). Previous research has demonstrated the capacity of microbiota to adapt and change in response to dietary variations (Petri et al., 2013; Wetzels et al., 2016), as well as their resilience potential (Weimer, 2015). Nevertheless, there is still a lack of understanding with regard to the progressive adaptation mechanisms of the GIT microbiota to increases in dietary starch. Furthermore, studies employing phytogenic feed additives (PFA) as supplement to high concentrate feeding have successfully prevented significant shifts in microbiota composition and reduced the production of harmful metabolites (Cardozo et al., 2006; Neubauer et al., 2018). While these phytogenic compounds can result in immediate changes to rumen fermentation, with the potential to alter the hindgut as well, and to help in the recovery of a perturbed ecosystem, the mechanisms of action remain largely unknown (Neubauer et al., 2018; Zhou et al., 2019).

The aim of our study was to increase the understanding of how a rapid shift in substrates affects the microbiota inhabiting different GIT niches in dairy cattle, by investigating the daily adaptation of the microorganisms, as well as their predicted metabolism and metabolite production, to increase amounts of readily fermentable carbohydrates introduced with the diet. Furthermore, we aimed to examine if supplementation with a blended PFA would mitigate and prevent a possible dysbiosis, by modulating the proliferation and activity of the ruminal and fecal microbiota.

Materials and methods

Experiment design and animal housing

The trial was conducted as part of a larger experiment at the research farm of the University of Veterinary Medicine, Vienna. The main experimental design and diets have been described in detail by Rivera-Chacon et al. (2022). In brief, the trial was a crossover design with two runs, separated by a 4-week washout period. Nine Holstein non-lactating rumen-cannulated cows (mean body weight: 992 ± 73 kg, mean age: 10.0 ± 0.8 years) were divided into two groups (control—CON and treatment—PHY). The groups were balanced for body weight, with five cows assigned to the PHY and four to the CON group in the first run. The treatments were inverted in the second run (four cows PHY and five cows CON). Animals were group-housed, and each cow had access to an individual feed bunk (through computer-regulated access gates). A period of adaptation to the grouping, feeders and basal diet occurred for 1 week before the trial started. The feeding protocol of the adaptation consisted in a step-wise replacement of the forage proportion (75% grass silage, 15% corn silage, and 10% grass hay in dry matter basis) with concentrate, starting with 10% concentrate mixture on day 1 and reaching 60% on day 6 (dry matter basis). The concentrate mixture (30.22% barley, 18.1% triticale, 23.08% bakery by-products, 24.0% rapeseed meal, 3.0% molasses, 0.8% mineral-vitamin premix for dairy cattle, 0.5% limestone, and 0.3% salt) was the same for the two groups, with the treatment group receiving a blended phytogenic feed additive at 400 mg/kg (dry matter basis) (Digestarom®, a mixture of herbs and spices that contains menthol, thymol and eugenol, DSM Austria GmbH). To maintain a stable intake of PHY additive throughout the trial, from day 1 to day 6, the corresponding quantities of PFA were supplemented directly into the rumen through the cannula. Diet was offered as a total mixed ration and provided once daily by an automatic feeding system (Trioliet Triomatic T15, Oldenzaal, The Netherlands), and was available *ad libitum*, along with water and mineral blocks. Orts were removed and fresh feed was delivered every morning. Daily feed intake was recorded automatically (Insentec B.V., Marknesse, The Netherlands). Starch intake was calculated based on the dry matter intake (DMI), and the chemical composition of feed ingredients was analyzed at the start and end of dietary adaptation.

Ruminal pH measurements and conventional microbial fermentation products

Ruminal pH was measured every 15 mins with the Lethbridge Research Centre Ruminal pH Measurement System (LRCpH; Dascor Inc., CA, USA) (Penner et al., 2006). Specific calculations, as well as conversion of measured millivolts to pH, were similar to Castillo-Lopez et al. (2014b), but a pH threshold of 5.8 was used to assess ruminal acidification. All samples were collected daily, 4 h after morning feeding. Samples of ruminal fluid for VFA, ammonia and lactate analyses were collected from the ventral sac of the rumen using a 20-ml sterile syringe and stored at −20°C. Composition of VFAs was measured using gas chromatography (GC-2010 PLUS, Shimadzu) applying the protocol described by Castillo-Lopez et al. (2021). The concentration of ammonia was measured using an indophenol colorimetric method on a U3000 spectrophotometer (INULA GmbH, Vienna, Austria) (Weatherburn, 1967). Thawed samples were centrifuged at 15,115 × *g* for 10 min. Ammonia and phenol were oxidized by sodium hydroxide in the presence of sodium nitroprusside and dichloroisocyanuric acid. Absorbance was measured at 655 nm after 90 min of reaction. D-Lactate was measured with the Megazyme K-DATE assay (Megazyme Int., Ireland), according to the manufacturer's instructions.

Ruminal metabolomics

Samples of rumen fluid for metabolomics analyses were collected 4 h after the morning feeding from the ventral sac of the rumen, snap frozen in liquid nitrogen and stored at −80°C. Metabolites were determined with anion-exchange chromatography-high resolution mass spectrometry (IC-HR-MS) and high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Carboxylic acids, sugar phosphates, and sugars were analyzed by anion exchange chromatography on a Dionex Integrion HPIC system (Thermo Scientific) coupled to a Thermo Scientific Q Exactive Orbitrap mass spectrometer. Sample preparation consisted of shaking 20 µl of rumen fluid with 980 µl of acetonitrile/water (80:20, v/v) at 4°C for 10 min, centrifugation at 14,350 × *g* for 10 min and tenfold dilution of the supernatants with acetonitrile/water (20:80, v/v).

Biogenic amines were determined by LC-MS/MS after derivatization with phenyl isothiocyanate (PITC). Sample preparation was performed in 96-well plates using a modified protocol based on Biocrates' MxP® Quant 500 kit (Innsbruck, Austria). Briefly, a 10 µl aliquot of rumen fluid sample or different volumes of calibration stock solutions containing between 0.009 and 9 mg/L of all analytes and 30 µl of internal standard solution containing 10 mg/L ^{13}C-putrescine in acetonitrile:water (50:50, v/v) were pipetted into a 96-well plate and evaporated to dryness under a stream of nitrogen. Subsequently, 50 µl of derivatization reagent (ethanol:water:pyridine:PITC 31.7:31.7:31.7:5.0, v/v/v/v) was added and the plate was covered, shaken for 20 s, and placed in the dark at room temperature for derivatization of amines. After 1 h of derivatization, the derivatization reagent was evaporated under nitrogen. Finally, analytes were extracted by shaking in 300 µl of methanol containing 4.9 mM ammonium acetate for

30 min and the extracts were centrifuged. One aliquot of the extract was used directly for LC-MS/MS measurement, while another aliquot was diluted at 1:25 with methanol prior to measurement because of substantial concentration differences of the analytes in the rumen fluid.

The chromatographic and mass spectrometric conditions as well as the quantification approaches and the used solvents and reagents are described in detail in the supplementary data in Supplementary Material. Selected reaction monitoring (SRM) for LC-MS/MS analysis is given in Supplementary Table S1.

Microbiota analyses

Sample collection

Samples for microbiota analyses were collected aseptically 4 h after morning feeding, following an approach similar to Castillo-Lopez et al. (2014a). To collect samples of solid digesta and particle associated rumen liquid (PARL) (Tafaj et al., 2004), a handful of digesta was sampled from four different locations (dorsal, cranial and caudal mat and ventral sac). PARL samples were collected in a beaker by squeezing the digesta sample through four layers of sterile gauze, while solid digesta were sampled with tweezers. Fecal samples were collected aseptically from the rectum using disposable rectal exploration gloves. All samples were collected in duplicate, immediately snap-frozen in liquid nitrogen, and subsequently stored at −80°C. Samples of rumen content were collected for 6 days, while feces were sampled for an additional seventh day, to account for digestive passage rate.

DNA extraction, sequencing, and sequences analysis

A total of 108 samples were collected for both the PARL and digesta niches, and 126 samples were collected from feces. DNA was extracted using DNeasy PowerSoil Kit (Qiagen, Germany) with additional pre-processing steps for mechanical and enzymatic lysis (Neubauer et al., 2018). Details about the protocol are given in supplementary data in the Supplementary Material. The samples were sent to an external laboratory (Microsynth, Balgach, Switzerland) for targeted 16S rRNA gene sequencing. The primers 341F-ill (5′-CCTACGGGNGGCWGCAG-3′) and 802R-ill (5′-GACTACHVGGGTATCTAATCC-3′) were used to target the V3-V4 hypervariable regions of the bacterial 16S rRNA gene, with an expected product of approximately 460 bp (Klindworth et al., 2013). Libraries were prepared adding barcodes and Illumina adaptors through 16S Nextera two-step PCR. Equimolar pools of samples were sequenced using a 250 bp paired-end reads protocol for Illumina MiSeq sequencing platform. Demultiplexing, trimming of adaptors and reads merging was performed by Microsynth. Quality of the merged reads was inspected using FASTQC (Andrews and Babraham Bioinformatics, 2010), and the merged sequences were analyzed with software QIIME 2 (v. 2020.2) (Bolyen et al., 2019). Sequences were filtered for quality (PHRED score 20) before denoising with Deblur (Amir et al., 2017). Reads were trimmed at 385 nucleotides for rumen samples, which resulted in the loss of one sample from PARL ($n = 107$). Reads from fecal samples were trimmed at 400 nucleotides. All digesta and fecal samples passed the quality filtering and denoising ($n = 108$ and $n = 126$, respectively). The resulting tables were further filtered to exclude mitochondrial contamination. Taxonomy was assigned with a Naive Bayes classifier trained for the specific 16S rRNA gene target regions against the SILVA 132 99% OTU reference database (Quast et al., 2012). Chloroplasts were found with very low relative frequencies (<0.01%) in all three matrices analyzed. Alpha and beta diversity were calculated after samples with <1,000 reads were discarded ($n = 105$ for PARL, $n = 107$ for digesta and $n = 121$ for feces after filtering). The filtered amplicon sequence variants (ASVs) tables were used to calculate abundance-based coverage estimator (ACE) (Chao and Yang, 1993), Chao1 (Chao, 1984), Faith's phylogenetic diversity (Faith and Baker, 2006) and Shannon index (Shannon, 1948), as well as weighted and unweighted UniFrac distances (Lozupone et al., 2007) per each matrix. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) was run using the QIIME2 plugin (v. 2019.10), with the default options (average NSTI was 0.18 for rumen samples and 0.36 for fecal samples) (Bolyen et al., 2019; Caicedo et al., 2020). Pathways names are reported according to MetaCyc Metabolic Pathway Database (Caspi et al., 2020).

Statistical analyses

Datasets for ruminal pH, intake, microbial fermentation products and alpha diversity were checked for normal distribution with PROC UNIVARIATE procedure in SAS. When a variable was not normally distributed, PROC TRANSREG was run with Box-Cox model, to evaluate the best transformation to be applied. The presence of outliers was assessed calculating a simple linear regression with the PROC REG procedure, as well as Cook's distance (Cook's D) on the regression model and diagnostics on residuals. Values with a Cook's D above 0.8 were removed from the dataset for downstream analyses. Data were further analyzed using the PROC MIXED procedure of SAS with cow, experimental run, day, treatment and the interaction between day and treatment as fixed effects and cow within run as random effect. Cow within run was also considered as repeated measure, and *post-hoc* Tukey correction for *P*-values was applied. The animal as fixed effect was excluded from the model for the metabolomics data. Differences in beta diversity matrices (weighted and unweighted UniFrac distance) were calculated in QIIME2 using ADONIS

(tested for adaptation day, treatment and their interaction). Analysis of the composition of microbiota was evaluated using Microbiome Multivariable Associations with Linear Models (MaAsLin2) package in R (Mallick et al., 2021). Differential abundance was calculated using Centered Log-Ratio (CLR) normalization and LM method, with adaptation day and treatment as fixed effects and individual animal and run as random effects. False Discovery Rate (FDR) was calculated with default parameters (Benjamini–Hochberg method) (Benjamini and Hochberg, 1995). Sequences were further analyzed applying the QIIME 2 plugin q2-longitudinal, performing a random forest regression aiming to predict the adaptation day on the basis of the microbiota composition ("longitudinal maturity-index") (Bokulich et al., 2018). The group that did not receive the phytogenic feed additive was used as control, using 0.4 as fraction of samples to be excluded from the training set. PICRUSt2 results were analyzed using MaAsLin2, applying the same model used for the amplicon sequences. Metabolome data were analyzed using MetaboAnalyst (v. 5.0) to evaluate the effect of the progressive days and of the phytogenic feed additive on the rumen metabolite profile (Chong et al., 2019). Quantitative Enrichment Analysis was performed to identify enriched pathways during the days and due to the treatment. Spearman correlations were calculated between significantly affected taxa and metabolites as well as the 50 most abundant pathways detected *via* PICRUSt, using rcorr function of R package Hmisc (Harrell and Dupont, 2020). Further correlations were run between significantly affected genera and a subset of pathways selected within the dataset for their role in the metabolism of acetate, propionate, butyrate, ammonia, and lactate. Network analyses were performed through Model-based Integration of Metabolite Observations and Species Abundances 2 (MIMOSA2) web application (Noecker et al., 2016). Representative sequences for each ASV and absolute abundances of metabolites were used with PICRUSt KO genomes and KEGG metabolic model, with a similarity threshold of 0.99 and rank-based estimation. MIMOSA2 web application with such settings maps sequences to Greengenes database, which has not been updated since 2013. Previous research has demonstrated overall accordance between databases up to the family level (Sierra et al., 2020). Thus, results for network prediction are presented and discussed at the family level. Briefly, the MIMOSA2 framework estimates metabolic potential (CMP) scores based on the community composition, which variation is then compared to variation in measured metabolite abundances per each sample. Predictions of metabolite levels are validated through a regression model, and contributions are calculated as variation in metabolite abundance explained by a specific taxon. Contributions were considered significant with $P \leq 0.05$ and FDR < 0.1. For all other statistical analyses, significance was considered at $P \leq 0.05$ and tendencies were considered for $0.05 < P \leq 0.10$.

Results

Ruminal pH and feed intake

Results for feed intake and ruminal pH are given in detail in Table 1. Feed intake increased ($P < 0.01$) on the last days of experiment. Starch inclusion in the diet was steadily incremented, with starch intake gradually increasing from day 1 at 0.98 ± 0.12 kg to reach 3.55 ± 0.12 kg on day 6 ($P < 0.01$). Ruminal pH decreased with the progressive inclusion of concentrate in the diet ($P < 0.01$), with a minimum value below 5.8 on average on day 6 of experiment. Time with pH below 5.8 and acidosis index (determined by calculating the time that ruminal pH was below 5.8 per kg DMI) both increased along with the adaptation day ($P = 0.01$) (Khiaosa-ard et al., 2018).

Microbiota alpha and beta diversity

Solid digesta

ACE and Shannon index decreased to reach the minimum values on day 6 for both PHY and control group (Figure 1). All other alpha diversity parameters evaluated were affected by the adaptation day ($P < 0.01$) (Supplementary Table S2). The PHY showed an effect for ACE, Chao1, and Faith's phylogenetic diversity ($P = 0.09$, $P = 0.09$ and $P = 0.03$, respectively). ADONIS revealed a significant impact of the adaptation day on both weighted and unweighted UniFrac analyses, while the PHY treatment showed an effect only for the unweighted UniFrac ($P < 0.01$) (Figure 2A). There was no significant effect for the interaction between the PHY and the adaptation days.

PARL

Alpha diversity indexes in PARL decreased over the adaptation days ($P < 0.01$), with lower values reached on day 5 (Figure 1; Supplementary Table S2). The PHY treatment did not affect the alpha diversity. Beta diversity in PARL was affected by the adaptation day ($P < 0.01$), while Unweighted UniFrac was affected by the PHY treatment ($P = 0.03$) (Figure 2B). The interaction between PHY and adaptation day was not significant.

Feces

Alpha diversity was affected by the adaptation day (Figure 1), with values decreasing over the 7 days for both groups (Supplementary Table S3). The PHY affected both ACE and Chao1 indexes ($P = 0.08$). ADONIS for beta diversity in feces revealed an effect of the adaptation day for both distance matrices ($P < 0.01$), but no effect of the interaction between PHY and day. The PHY treatment affected only unweighted UniFrac, showing a tendency ($P = 0.08$) (Figure 2C).

10.3389/fmicb.2022.920427

TABLE 1 Nutrient intake and ruminal pH parameters during a 6-day adaptation to a high concentrate diet.

Intake, kg[1]	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	SEM[2]	*P*-values[3]		
								Day	PHY	Day*PHY
Dry matter	12.4	11.3	11.9	11.7^{x}	13.3^{y}	13.8	0.65	<0.01	0.97	0.73
Starch	0.98^{a}	1.33^{b}	1.85^{a}	2.24^{b}	3.05^{a}	3.55^{b}	0.12	<0.01	0.53	0.76
Non fiber carbohydrates	2.50	2.68^{a}	3.24^{b}	3.57^{b}	4.53^{a,x}	5.00^{y}	0.20	<0.01	0.88	0.81
Neutral detergent fiber	6.16^{a}	5.24^{b}	5.11	4.60	4.79	4.50	0.28	<0.01	0.81	0.70
Ruminal pH[1]										
Minimum pH	6.32^{a}	6.10^{b}	6.10^{b}	5.98^{a}	5.89^{a}	5.76^{b}	0.06	<0.01	0.71	0.95
Maximum pH	6.76	6.74^{a}	6.58^{b}	6.61^{b}	6.48^{a}	6.39	0.05	<0.01	0.56	0.71
Time below pH 5.8 (min/d)	33.3	2.50	18.3	128	161^{a}	320^{b}	68.9	0.01	0.23	0.49
Acidosis index[4]	2.35	0.21	1.14	2.07	3.39^{a}	16.4^{b}	5.41	0.01	0.61	0.82

Cows were divided in two groups supplemented or not with a phytogenic feed additive (PHY). The percentage of concentrate was incremented daily, starting from 10% on day 1 to reach 60% on day 6.

[1] Data shown as least square means.

[2] The largest standard error of the mean.

[3] *P*-values for the effect of day, phytogenic treatment (PHY) and the day × treatment interaction (Day*PHY).

[4] Acidosis index was determined by calculating the time that ruminal pH was below 5.8 per kg DMI.

[a,b] Values with different superscripts indicate a significant difference ($P \leq 0.05$) between consecutive days.

[x,y] Values with different superscripts indicate a tendency for difference ($0.05 < P \leq 0.10$) between consecutive days.

Microbiota composition and differential abundance

Solid digesta

A total of 4,148,399 reads were grouped into 19,324 features and assigned to 21 phyla, of which the two most abundant were *Firmicutes* and *Bacteroidetes*, which accounted together for more than 80% of all the reads (Supplementary Figure S1). The most represented families are presented in Figure 3A. *Prevotella* 1 (12.7%) was the most abundant genus on average across all samples, followed by *Rikenellaceae* RC9 gut group (6.1%) and *Lachnospiraceae* NK3A20 group (5.4%) (Figure 3C). Most phyla were significantly affected by the increasing amount of concentrate in the diet starting from day 5, with the highest frequency of *Firmicutes* (59.9%) on day 6 ($P < 0.01$) and of *Bacteroidetes* on day 5 (30.5%) ($P < 0.01$). Effects at the family level were appreciable mainly from day 4. Family *Lactobacillaceae* decreased over the 6 days ($P = 0.03$), while *Erysipelotrichaceae* tended to increase from day 4 ($P < 0.01$). *Lachnospiraceae*, *Prevotellaceae* and *Ruminococcaceae* were also affected by the concentrate included in the diet, with the first two families increasing by day 6 ($P < 0.01$) and the latter decreasing ($P < 0.01$). Genera *Prevotella* 1 ($P = 0.08$), *Lachnospiraceae* NK3A20 group ($P < 0.01$), *Ruminococcus* 2 ($P = 0.02$), and *Selenomonas* 1 ($P < 0.01$) increased over the experimental days. *Ruminococcaceae* NK4A214 group increased over the first 4 days (4.1%; $P < 0.01$) and decreased again on day 6 (3.6%; $P < 0.01$). Similarly, *Lachnospiraceae* XPB1014 group, ND3007 group, and NK4A136 group decreased over the experimental days, from day 5 ($P < 0.01$, $P < 0.01$, and $P = 0.01$, respectively). The PHY treatment increased the relative frequency of *Ruminococcaceae* UCG-005 (0.28 and 0.20% in PHY and control group, respectively; $P = 0.01$) and *Ruminococcaceae* V9D2013 group (0.07 and 0.05% in PHY and control group, respectively; $P = 0.04$). *Ruminiclostridium* 9 (0.12 and 0.21% in PHY and control group, respectively; $P = 0.10$) and *Alloprevotella* (0.02% and 0.03% in PHY and control group, respectively; $P = 0.08$) tended to be more abundant in the control group. The 50 most important features detected with the maturity index prediction were used to build a heatmap showing the trend for their frequency over the 6 days of sampling (Supplementary Figure S3A). Among these, more than half were assigned to families *Lachnospiraceae* and *Ruminococcaceae*, and in particular to genera *Lachnospiraceae* NK3A20 group and *Ruminococcaceae* NK4A214 group (Supplementary Table S7). Both genera were also identified as differentially abundant, together with *Prevotella* 1 and other *Lachnospiraceae* genera (XPB1014 group, ND3007 group, NK4A136 group).

PARL

For PARL, 4,493,095 reads were grouped into 18,557 features. A total of 24 phyla were detected, and as for digesta,

the two most abundant were *Firmicutes* and *Bacteroidetes* (Supplementary Figure S1). Figure 3B shows the relative abundance of the most abundant families. *Prevotella* 1 (16.1%) and *Lachnospiraceae* NK3A20 group (4.2%) were among the most abundant genera on average across all samples, together with *Ruminococcaceae* NK4A214 group (4.8%) (Figure 3C). The progressive inclusion of concentrates in the diet significantly affected the relative abundance of phyla and of families, mainly from day 3. Family *Lactobacillaceae* and genus *Lactobacillus* decreased over the days ($P < 0.01$), while *Streptococcaceae* increased only on day 6 (0.11%, $P = 0.05$). Similarly, *Succinivibrionaceae* tended to increase only on day 5 ($P = 0.06$). Families *Lachnospiraceae*, *Ruminococcaceae,* and *Prevotellaceae* showed fluctuations over the experimental days. Genus *Prevotella* 1 started to decline on day 4 (16.4%) to day 5 (15.9%) ($P = 0.03$), while *Prevotellaceae* UCG-001 and *Prevotellaceae* NK3B31 group increased over the experimental days ($P < 0.01$). *Lachnospiraceae* NK3A20 group and *Ruminococcaceae* NK4A214 group's relative frequency increased from day 2 ($P = 0.01$ and $P < 0.01$, respectively). *Burytivibrio* 2 and *Pseudobutyrivibrio* increased from day 5 ($P = 0.03$ and $P < 0.01$, respectively). The PHY supplementation had more impact on PARL than digesta microbiota composition: 14 families and 36 genera were significantly affected by the treatment. Families *Veillonellaceae* ($P < 0.01$) and *Clostridiales* Family XI ($P < 0.01$) had higher relative frequency in the control group. The PHY treatment increased the frequency of genera *Ruminococcaceae* UCG-011 (1.1 and 0.8% in PHY and control group, respectively; $P < 0.01$), *Ruminococcaceae* UCG-005 (0.3% and 0.2% in PHY and control group, respectively; $P = 0.02$), and *Lachnospiraceae* UCG-006 (0.13% and 0.11% in PHY and control group, respectively; $P = 0.04$). Supplementary Figure S3B shows the 50 most important taxa, identified with the random forest regression and their variation over the sampling days per each group, control or treatment. As for digesta, most of the features were classified as belonging to families *Lachnospiraceae* and *Ruminococcaceae* (Supplementary Table S7). Features classified as *Prevotella* 1, *Butyrivibrio* 2, and *Lactobacillus* were identified as part of the key taxa in the adaptation to the new diet both by the random forest and the differential expression analysis.

Feces

Reads for fecal samples (1,426,602) were assigned to 10,211 features. Features were grouped in 15 phyla, of which only three had a relative abundance above 1% (*Firmicutes*, *Bacteroidetes* and *Spirochaetes*, 76.9, 17.7, and 1.3%, respectively) (Supplementary Figure S1). Most represented families are shown in Figure 4A. *Ruminococcaceae* UCG-005, *Romboutsia* and *Christensenellaceae* R-7 group were the most abundant genera on average across all samples (8.4, 7.2, and 6.7%, respectively) (Supplementary Figure S2). Effects on phyla were mostly visible from day 3 or 4, while *Tenericutes* showed an increase only on day 7 ($P < 0.01$). Phylum *Epsilonbacteraeota,* to which family *Campylobacteraceae* belongs, tended to decrease on day 5 ($P = 0.06$), disappearing in the last 2 days. Most of the families were significantly affected by the diet changes from day 3. Family *Ruminococcaceae* tended to decrease over the 7 days, significantly from day 5 ($P < 0.01$). In parallel, *Lachnospiraceae* increased from 14.3% on day 1 to 21% on day 7 (significant increment from day 3, $P = 0.05$). The progressive inclusion of concentrate in the diet affected 109 genera. Genera *Lachnospiraceae* AC2044 group ($P = 0.02$), *Ruminobacter* ($P = 0.04$), *Blautia* ($P = 0.03$), *Butyrivibrio* ($P = 0.01$), *Treponema* 2 ($P < 0.01$), and *Ruminococcus* 2 ($P = 0.03$) increased already from day 2. *Ruminococcus* 1 decreased on day 2 (0.4%; $P = 0.02$) and increased again on day 6 (1.4%; $P = 0.04$). The PHY supplementation tended to increase the abundance of families *Paludibacteriaceae*, *Campylobacteraceae,* and *Clostridiales* Family XIII in the fecal samples (Figure 4B). At the genus level, the PHY treatment increased the abundance of low abundant taxa, such as *[Eubacterium] ventriosum* group (0.04% and 0.02% in PHY and control group, respectively; $P < 0.01$), *Prevotellaceae* UCG-003 (0.6% and 0.3% in PHY and control group, respectively; $P < 0.01$), *Lachnospiraceae* UCG-007 (0.08% and 0.02% in PHY and control group, respectively; $P = 0.02$), and *Ruminococcaceae* UCG-011 (0.08% and 0.04% in PHY and control group, respectively; $P = 0.06$). Random forest regression (Supplementary Figure S3C) identified the key component of the fecal microbiota as mainly belonging to families *Lachnospiraceae* and *Ruminoccaceae* (Supplementary Table S7). Family *Clostridiales* Family XIII and genus *Prevotellaceae* UCG-003 were confirmed to play an important role in the adaptation to a new diet by both the random forest and the differential abundance analysis.

Microbial activity parameters

The concentration of total VFAs was impacted by dietary adaptation day (Table 2, $P < 0.01$). Acetate, propionate, and butyrate concentration increased over the 6 days of experiment ($P < 0.01$), reaching the highest values on day 5. Isobutyrate and isovalerate concentrations decreased from day 1 to day 6 ($P < 0.01$). There was an interaction between PHY treatment and experimental day for propionate concentration ($P = 0.04$), and a tendency toward interaction for total VFA concentration ($P = 0.08$). Ammonia concentration in the rumen fluctuated over the days ($P = 0.05$). Values tended to increase on day 2, to decrease again on day 3, reaching the lowest in the PHY group (19.22 ± 3.12 mg/dl). Ammonia concentration tended to be higher on day 6 for the PHY group, with a tendency for an interaction between days and treatment ($P = 0.06$). D-Lactate concentration showed high numerical concentrations on days 4 and 5, but no significant effects of diet or treatment.

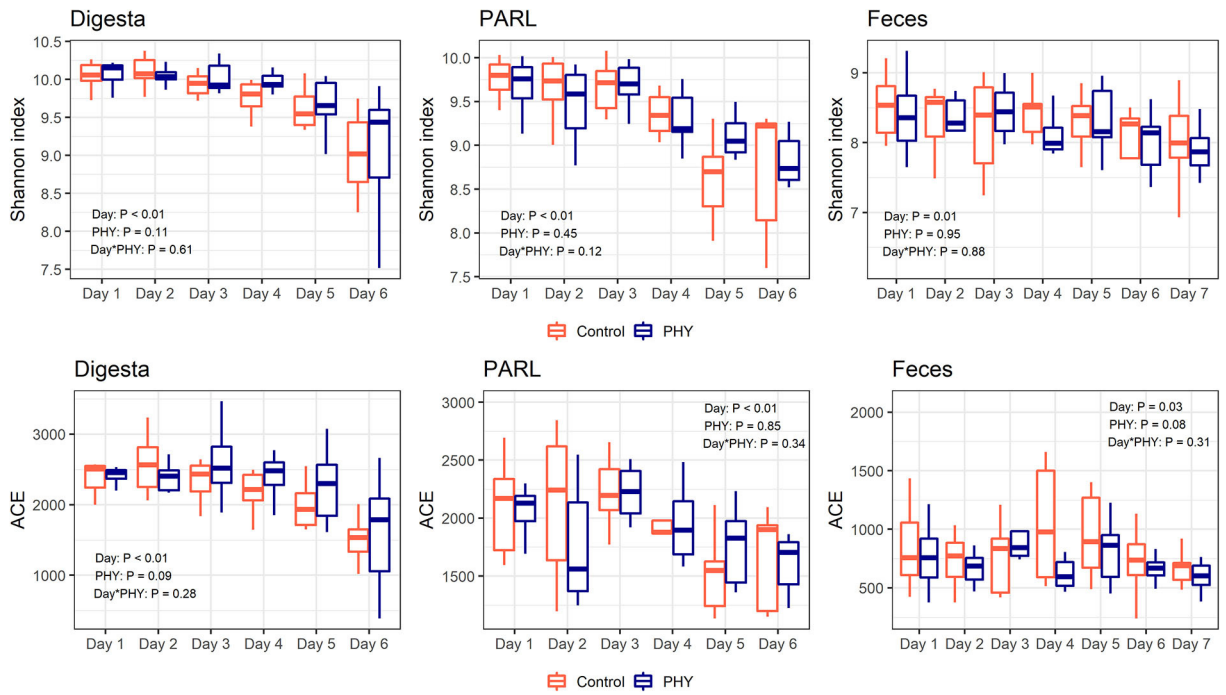


FIGURE 1
Alpha-diversity indices in the three analyzed matrices, for control and treatment (PHY) groups. Digesta, solid digesta; PARL, particle associated rumen liquid; ACE, Abundance-based Coverage Estimator. Samples were collected for six consecutive days for rumen content and for an additional seventh day for feces. *P*-values are presented for the effect of day, phytogenic treatment (PHY) and the day × treatment interaction (Day*PHY).

All the metabolites measured were affected by the dietary adaptation day (Figure 5A). There was an interaction between PHY treatment and adaptation day for 3-hydroxybutyric acid ($P = 0.01$) and disaccharides ($P = 0.01$). Furthermore, there was a trend for interaction for ribose and galactose-1-phosphate ($P = 0.08$). 3-(3-hydroxyphenyl) propionic acid tended to be affected by the treatment ($P = 0.09$) (Supplementary Table S4). Principal component analysis (PCA) of the metabolites showed a clear separation of the last 2 days of adaptation from the previous four, as shown in Figure 5B. This variance was explained mainly by three compounds: acetic acid, butyric acid, and glucose. The same compounds, with the addition of propionic acid, were found to be important features for the discrimination between PHY and control group, although the groups did not cluster separately. A total of 13 biogenic amines were identified and analyzed in the rumen fluid. The majority were affected by the adaptation day, with the exception of histamine, sarcosine, and gamma-aminobutyric acid (Figure 6). The latter showed considerably lower concentrations in the PHY group compared to the control ($P = 0.05$). There was an interaction between adaptation day and PHY treatment for spermidine ($P = 0.03$).

Pathways prediction analysis

Quantitative enrichment analysis showed effects of the day on several pathways, notably phenylalanine metabolism, pentose phosphate pathway, glycolysis/gluconeogenesis, starch and sucrose metabolism, and galactose, fructose and mannose metabolism ($P < 0.01$). The PHY treatment showed some tendencies, specifically for propanoate metabolism, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism and pyruvate metabolism ($P = 0.10$).

The relative abundances of the most abundant pathways predicted by PICRUSt which were shared by all three matrices for the 6 days of experiment are shown in Figure 7. All most abundant pathways were affected by the concentrate inclusion in the diet, starting from day 2 in PARL and from day 4 or 5 in digesta. Similarly, in feces, changes in the 50 most abundant pathways were evident from day 3 or 4. The PHY treatment affected 13, 46, and 12 pathways in digesta, PARL, and feces, respectively. Pathways PWY-7013 [(S)-propane-1,2-diol degradation], PWY-5676 (acetyl-CoA fermentation to butanoate II), and PWY-6588 (pyruvate

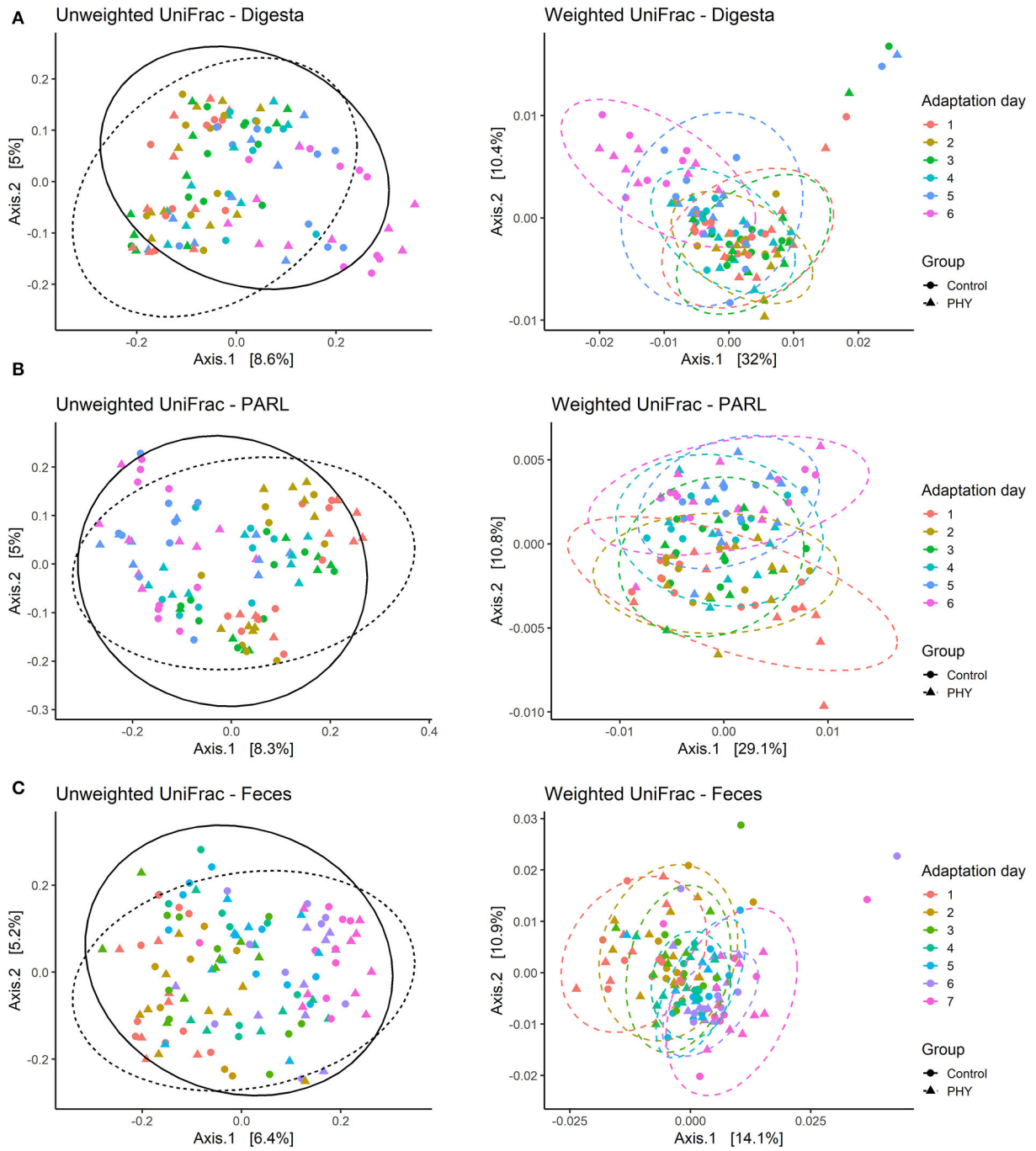


FIGURE 2
Beta-diversity for the three matrices analyzed. Graphs show weighted and unweighted UniFrac analysis over the experimental days for control and treatment (PHY) groups. **(A)** In solid digesta samples (Digesta), adaptation day impacted both weighted and unweighted UniFrac ($P < 0.01$), while the PHY treatment showed an effect only for the unweighted UniFrac ($P < 0.01$). **(B)** Beta-diversity in PARL was affected by the adaptation day ($P < 0.01$), while only unweighted UniFrac was affected by the PHY treatment ($P = 0.03$). **(C)** In feces, adaptation day had an effect on both beta diversity matrices ($P < 0.01$), while the treatment only showed a trend for unweighted UniFrac ($P = 0.08$).

fermentation to acetone) were enhanced by the treatment both in digesta and PARL samples. Pathway P163-PWY (L-lysine fermentation to acetate and butanoate) was affected by the PHY additive in all three matrices investigated, being more expressed in the PHY group in digesta and PARL, but less expressed in feces.

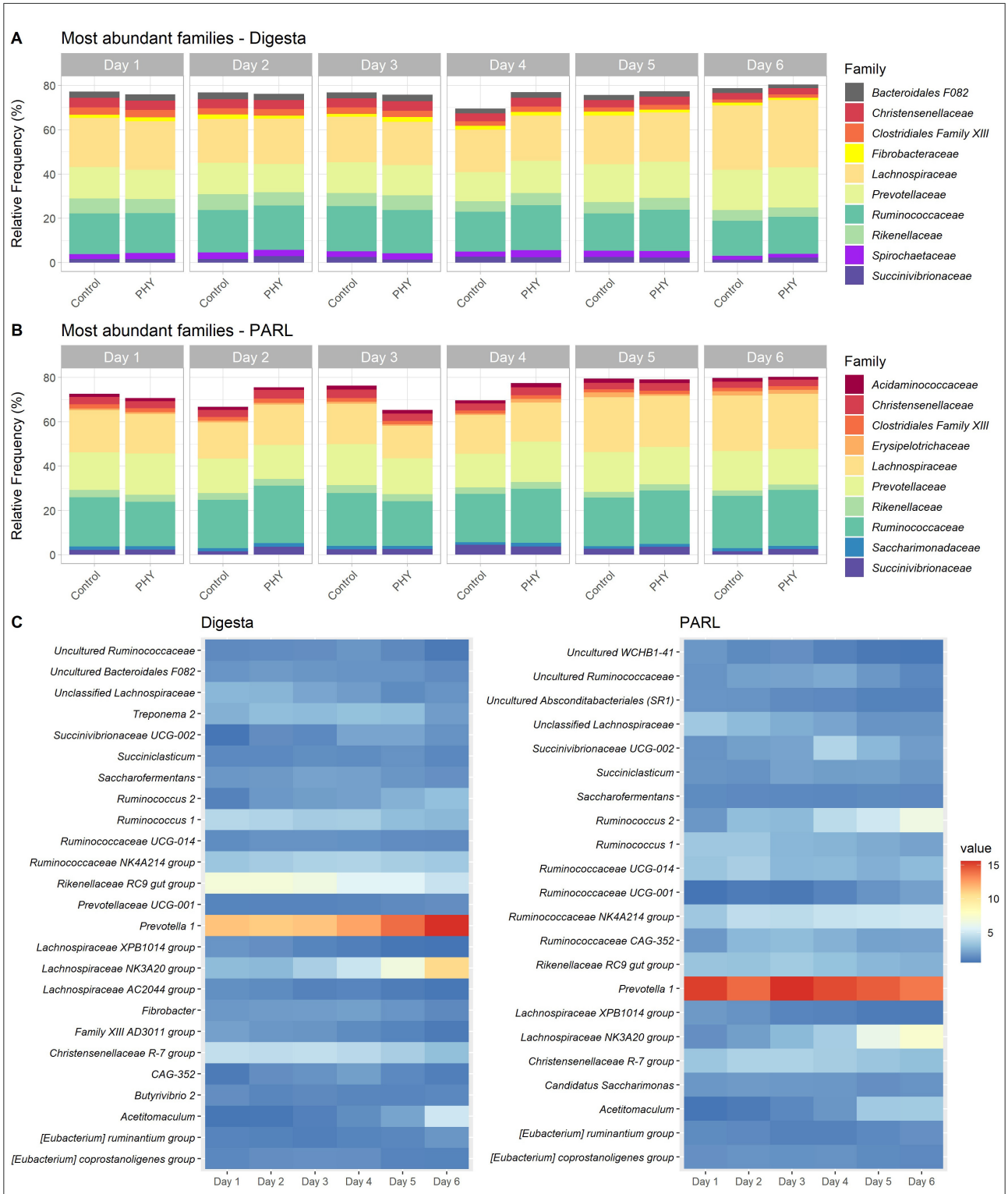


FIGURE 3
Microbiota composition of the rumen samples. Bar plots showing the mean relative frequency of the 10 most abundant families (across all samples) in **(A)** solid digesta and **(B)** particle associated rumen liquid (PARL) over the experimental days and between the two experimental groups (PHY and control). The heatmaps **(C)** show the mean relative frequency of the 25 most abundant genera across all samples for solid digesta (Digesta) and particle associated rumen liquid (PARL) over the experimental days.

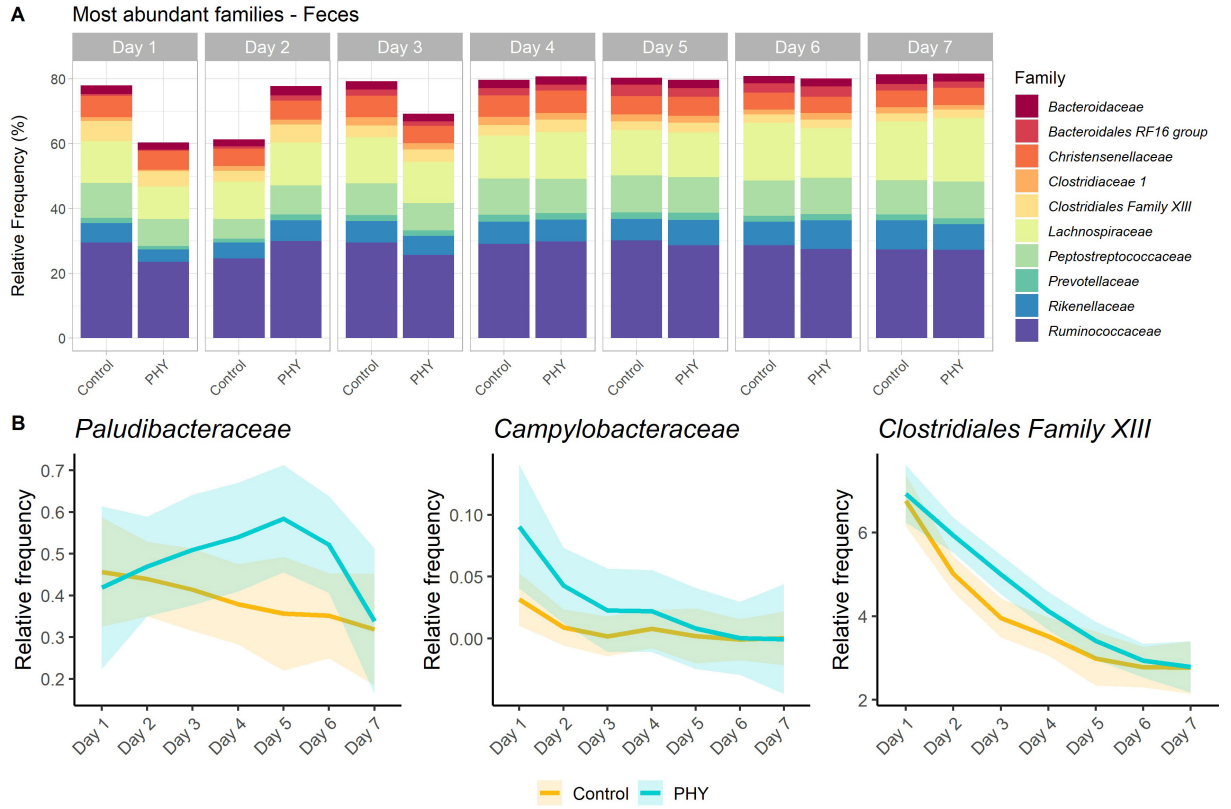


FIGURE 4
Microbiota composition of the fecal samples. **(A)** Barplot showing the mean relative frequency of the 10 most abundant families (across all samples) over the experimental days and between the two experimental groups (PHY and control). **(B)** Plots showing the different relative frequency trends over the experimental days between the treatment (PHY) and control group. The PHY increased the relative abundance of *Paludibacteriaceae* ($P = 0.06$), *Campylobacteraceae* ($P = 0.09$) and *Clostridiales Family XIII* ($P = 0.09$).

Network analysis

Spearman correlation between differentially abundant taxa and metabolites revealed positive correlations between members of the family *Lachnospiraceae* and *Ruminococcaceae* and carbohydrates, both in digesta and in PARL samples, although with relatively low R scores (Figure 8). Three genera identified in digesta samples (*Streptococcus*, *FD2005* and *Lachnobacterium*) were positively correlated with D-lactate concentration measured in the rumen fluid, although with low R scores ($P < 0.01$; R scores = 0.55, 0.30, and 0.37, respectively). The same genera were also correlated with the pathways associated with lactate, such as ANAEROFRUCAT-PWY [homolactic fermentation (fructose fermentation to lactate)], P122-PWY [heterolactic fermentation (lactate heterofermentation)], P124-PWY [*Bifidobacterium* shunt (glucose fermentation to lactate (*Bifidobacteria*)], P461-PWY (hexitol fermentation to lactate, formate, ethanol and acetate), PWY-5100 (pyruvate fermentation to acetate and lactate II), and PWY-6641 (superpathway of sulfolactate degradation) (Supplementary Figure S4).

For digesta, MIMOSA2 calculated the CMP scores for five metabolites, including acetate, D-Mannose 6-phosphate, and phenylacetic acid, for a total of 1243 taxa involved (Supplementary Table S5). In PARL samples, a total of 1061 taxa were found to be contributing to the variation of four metabolites, including succinate and sedoheptulose 7-phosphate (Supplementary Table S6). CMP scores were calculated also for D-glucose 6-phosphate and lactate in both PARL and digesta. Both ruminal niches hosted *Ruminococcaceae* and *Succinivibrionaceae* as major taxa driving differences in lactate concentration across samples, both encoding for K01069 (hydroxyacylglutathione hydrolase), which contributes to form D-lactate from carbohydrate metabolism. However, no taxon could explain more than 1% of the variance for lactate. The variation in D-glucose 6-phosphate concentration appeared to be driven mostly by *Erysipelotrichaceae* and *Ruminococcaceae* in PARL samples. In digesta samples, most of the variance for this metabolite were explained by *Lachnospiraceae*, followed by *Erysipelotrichaceae* and *Ruminococcaceae*. The variation in D-mannose-6-phosphate concentration was mainly contributed by *Erysipelotrichaceae*, *Ruminococcaceae*, *Lachnospiraceae,* and

TABLE 2 Concentration of volatile fatty acids (VFA), ammonia, and D-lactate measured in the rumen fluid.

	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6			*P*-values[3]		
LSM[1]	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM[2]	Day	PHY	Day*PHY
Total VFA, mM	105^{a}	84.2^{a}	121^{b}	127^{b}	143	124	147^{x}	131^{x}	$163^{y,a}$	$153^{y,a}$	128^{b}	142^{b}	7.26	<0.01	0.11	0.08
Acetate, mM	67.9^{a}	59.1^{a}	77.9^{b}	81.8^{b}	92.2	81.6	94.7	86.5	96.0^{a}	96.0^{a}	75.0^{b}	84.5^{b}	5.19	<0.01	0.42	0.17
Propionate, mM	18.2^{a}	14.3^{a}	20.8^{b}	22.4^{b}	27.0	22.6	27.3^{x}	22.8^{x}	30.7^{y}	27.1^{y}	25.2	27.4	1.56	<0.01	0.12	0.04
Butyrate, mM	11.8^{a}	9.21^{a}	13.9^{b}	14.7^{b}	15.5	13.0	17.3^{a}	14.9^{a}	24.0^{b}	21.9^{b}	20.4	23.6	1.09	<0.01	0.17	0.11
Valerate, mM	2.14^{a}	1.89^{a}	2.34^{b}	2.48^{b}	2.77	2.31	2.56	2.44	2.55	2.73	2.39	2.57	0.18	0.01	0.71	0.12
Isovalerate, mM	2.58^{x}	2.46^{x}	2.84^{y}	2.93^{y}	2.69	2.40	2.40	2.35	2.52^{a}	2.62^{a}	1.90^{b}	2.04^{b}	0.21	<0.01	0.90	0.68
Isobutyrate, mM	1.89^{x}	1.72^{x}	1.98^{y}	2.12^{y}	1.88	1.77	1.64	1.71	1.75^{a}	1.77^{a}	1.37^{b}	1.33^{b}	0.14	<0.01	0.92	0.40
Ratio acetate:propionate	3.71	3.86	3.74	3.70	3.43	3.69	3.45^{a}	3.84^{a}	3.02^{b}	3.55^{b}	3.01	3.18	0.16	<0.01	0.10	0.40
Ammonia, mg/dL	26.5	25.8	28.6	32.7	27.6	19.2	29.7	24.1	26.8	27.2	16.5	26.5	3.12	0.05	0.99	0.06
D-Lactate, mM	0.11	0.12	0.07	0.07	0.08	0.11	0.11	0.24	0.20	0.09	0.03	0.15	0.16	0.27	0.20	0.14

Values were measured 4 h after the morning feeding, during adaptation to a high concentrate diet. The animals were divided in two groups, of which one received a phytogenic feed additive (PHY), while the other served as control (CON).

[1] Least square means.

[2] The largest standard error of the mean.

[3] *P*-values for the effect of day, phytogenic treatment (PHY) and the day × treatment interaction (Day*PHY).

^{a,b} Values with different superscripts indicate a significant difference ($P \leq 0.05$) between consecutive days.

^{x,y} Values with different superscripts indicate a tendency for difference ($0.05 < P \leq 0.10$) between consecutive days.

Coriobacteriaceae. The taxa most associated with variation in glucose-1-phosphate concentration were *Ruminococcaceae* and *Lachnospiraceae*, despite explaining <1% of the variation. In PARL, families *Prevotellaceae* and *Succinivibrionaceae* were identified as the main taxa putatively responsible for the variation of sedoheptulose-7-phosphate, through K03271 (D-sedoheptulose-7-phosphate isomerase). Three biogenic amines (spermidine, 5-aminovaleric acid, and spermine) were associated with 1,226 taxa in digesta samples, and with 1,155 taxa in PARL. In addition, significant associations were found in PARL samples also for beta-alanine, putrescine, cadaverine, and phenethylamine (Supplementary Table S6). In both niches, *Ruminococcaceae* and *Lachnospiraceae* were putatively responsible for the main variation for all the biogenic amines, except for putrescine and phenethylamine, for which the main producers were identified as *Methanobacteriaceae* and *Anaerolinaceae*, respectively.

Discussion

Rapid adaptive response of the microbiota

The aim of this study was to evaluate the day-by-day adaptation of the gastrointestinal microbiota in cattle fed increasing amounts of readily fermentable carbohydrates, and the effects of diet supplementation with a blended PFA, through the analysis of composition, metabolism, and predicted pathways. These results will help to elucidate the mechanisms of adaptation of microbiota undertaken when challenged with a dietary change, particularly if there is a critical point in the adaptive response, over where the system becomes unbalanced and changes toward dysbiosis (Sommer et al., 2017).

The gradual inclusion of starch in the diet and the phytogenic additive altered microbiota composition and metabolic activity in all GIT niches evaluated in our experiment. Previous studies have not shown day-to-day alterations of the microbial composition in rumen content nor in feces, whereas we demonstrated a rapid adaptation of the microbiota to changing feeding conditions (Plaizier et al., 2017; Huang et al., 2020). This could be due to the different feeding programs of the animals, or the sampling schedule employed in the other experiments. While other studies looking at the difference between days have allowed between 7 and 40 days of adaptation prior to sampling, in our experiment the goal was to assess the impact of dietary change and therefore, diet did not remain constant throughout the experiment. This unique look at the process of microbial adaptation allow for new insights into the evolution of SARA and potential points for modulating ecological shifts to improve animal health. However, we monitored the microbiota over the first 6 days of adaptation to a new diet, and it is recognized that further adaptations of

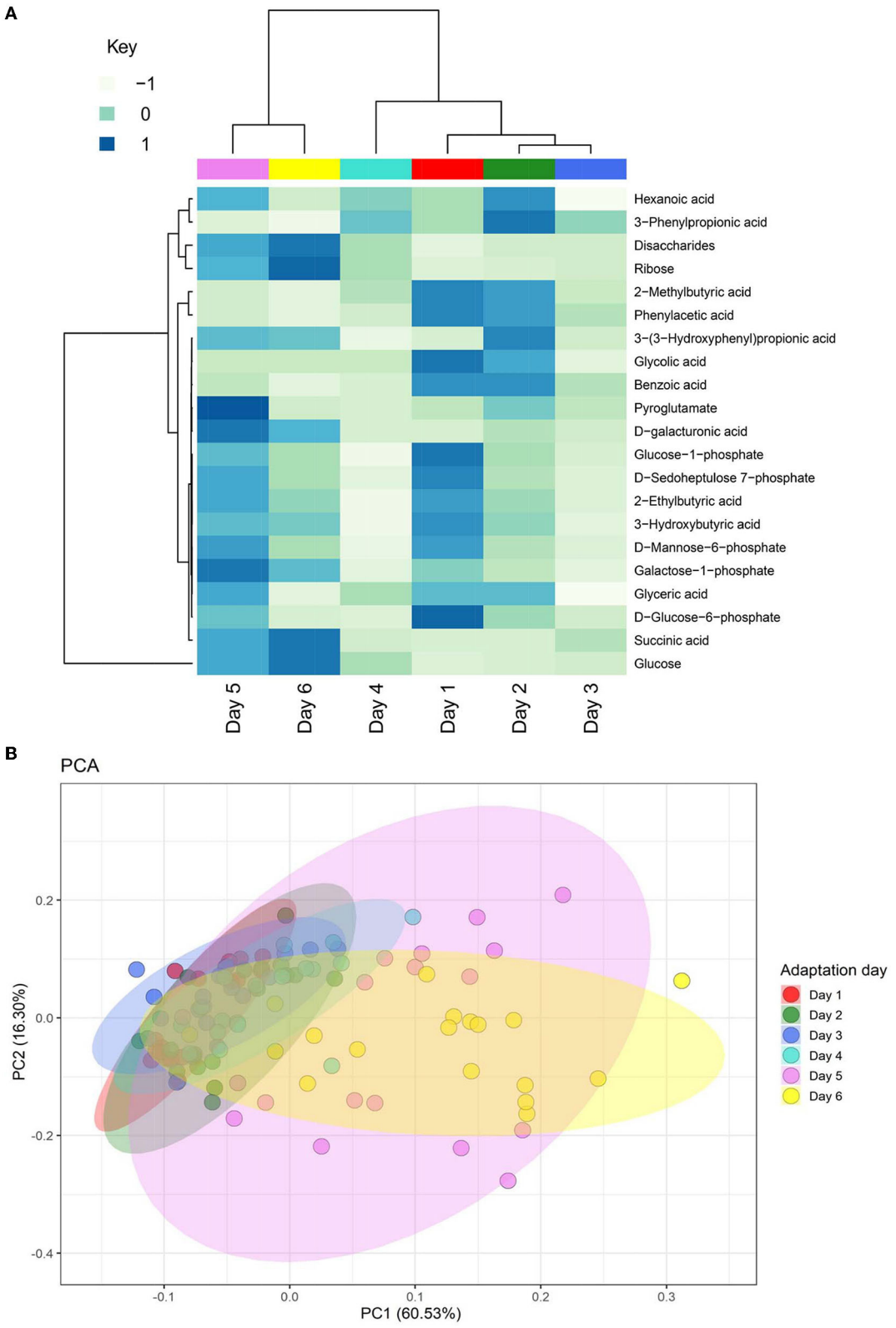
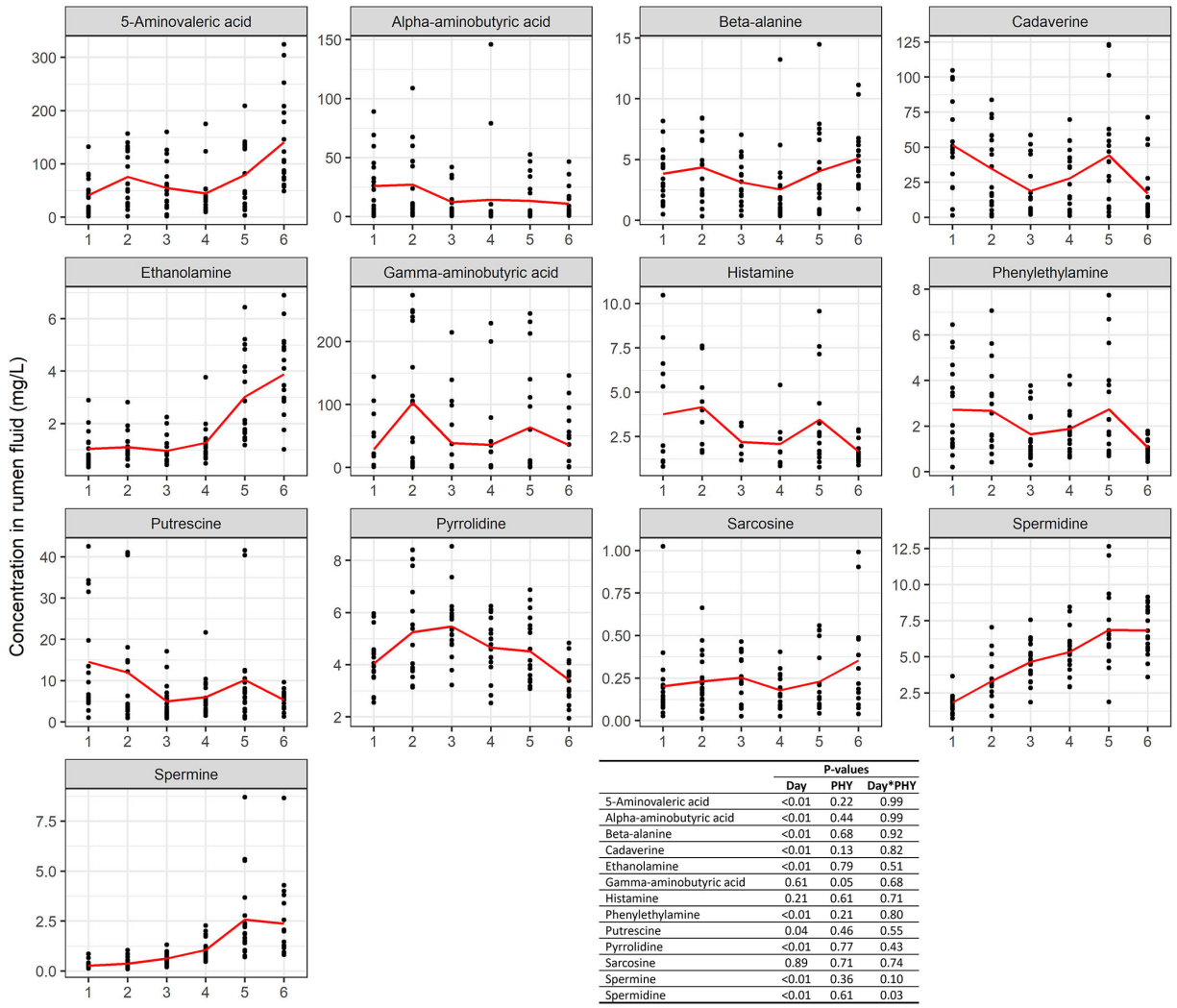


FIGURE 5
Heatmap **(A)** showing the change in concentration of metabolites measured in the rumen fluid across the six experimental days. Rows were scaled to have mean zero and standard deviation one, to normalize the different concentrations of each metabolite. Values range from −1

(Continued)

FIGURE 5
(lowest concentration measured) to 1 (highest concentration measured). The colors below the dendrogram correspond to the experimental days. The dendrogram shows a separate cluster for the last two days of experiment (days 5 and 6). Principal Component Analysis (PCA) **(B)** showing the variance of the metabolite composition in the rumen fluid. The graph is based on the normalized values calculated in MetaboAnalyst over the six experimental days.



	P-values		
	Day	PHY	Day*PHY
5-Aminovaleric acid	<0.01	0.22	0.99
Alpha-aminobutyric acid	<0.01	0.44	0.99
Beta-alanine	<0.01	0.68	0.92
Cadaverine	<0.01	0.13	0.82
Ethanolamine	<0.01	0.79	0.51
Gamma-aminobutyric acid	0.61	0.05	0.68
Histamine	0.21	0.61	0.71
Phenylethylamine	<0.01	0.21	0.80
Putrescine	0.04	0.46	0.55
Pyrrolidine	<0.01	0.77	0.43
Sarcosine	0.89	0.71	0.74
Spermine	<0.01	0.36	0.10
Spermidine	<0.01	0.61	0.03

FIGURE 6
Concentration of biogenic amines measured in the rumen fluid 4 h after the morning feeding. The red line represents the trend of the mean values over the 6 days of experiment. *P*-values are presented for Day, treatment (PHY) and interaction between Day and treatment (Day*PHY).

the microbiota are likely after this time, as the community fully adapts to the diet over the following days.

Niche-specific responses

Interestingly, PARL was the quickest and most responsive to dietary changes, with the highest number of taxa and predicted pathways affected by the increasing concentrate intake and the phytogenic additive. Despite the overall similarities in microbial composition, our results suggest a different metabolic activity occurring in PARL and in solid digesta. Variation in reaction to dietary challenges in the different ruminal niches is a well-known phenomenon (Metzler-Zebeli et al., 2015; Schären et al., 2017). It is suggested that the differences in bacterial activity in the rumen, within the same phylum and family, could be due to the accessibility of the substrates, depending on the solubility of the ingested material (Rubino et al., 2017; Hart et al.,

10.3389/fmicb.2022.920427

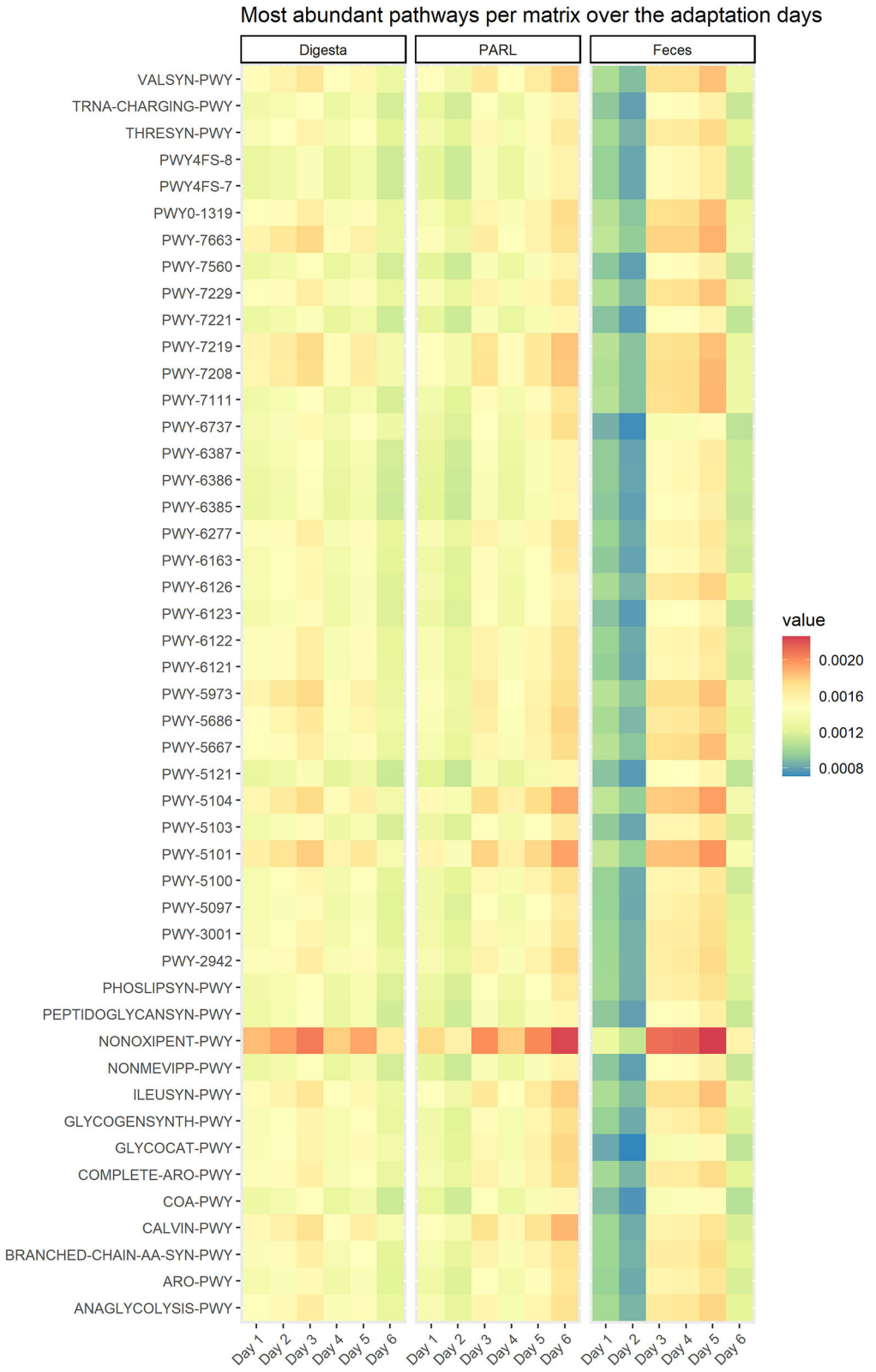


FIGURE 7
Relative abundance of the most abundant pathways for the three analyzed matrices. Pathways were predicted *via* PICRUSt2 based on the 16S rRNA gene amplicon sequencing of samples of solid digesta (Digesta), particle associated rumen liquid (PARL) and feces.

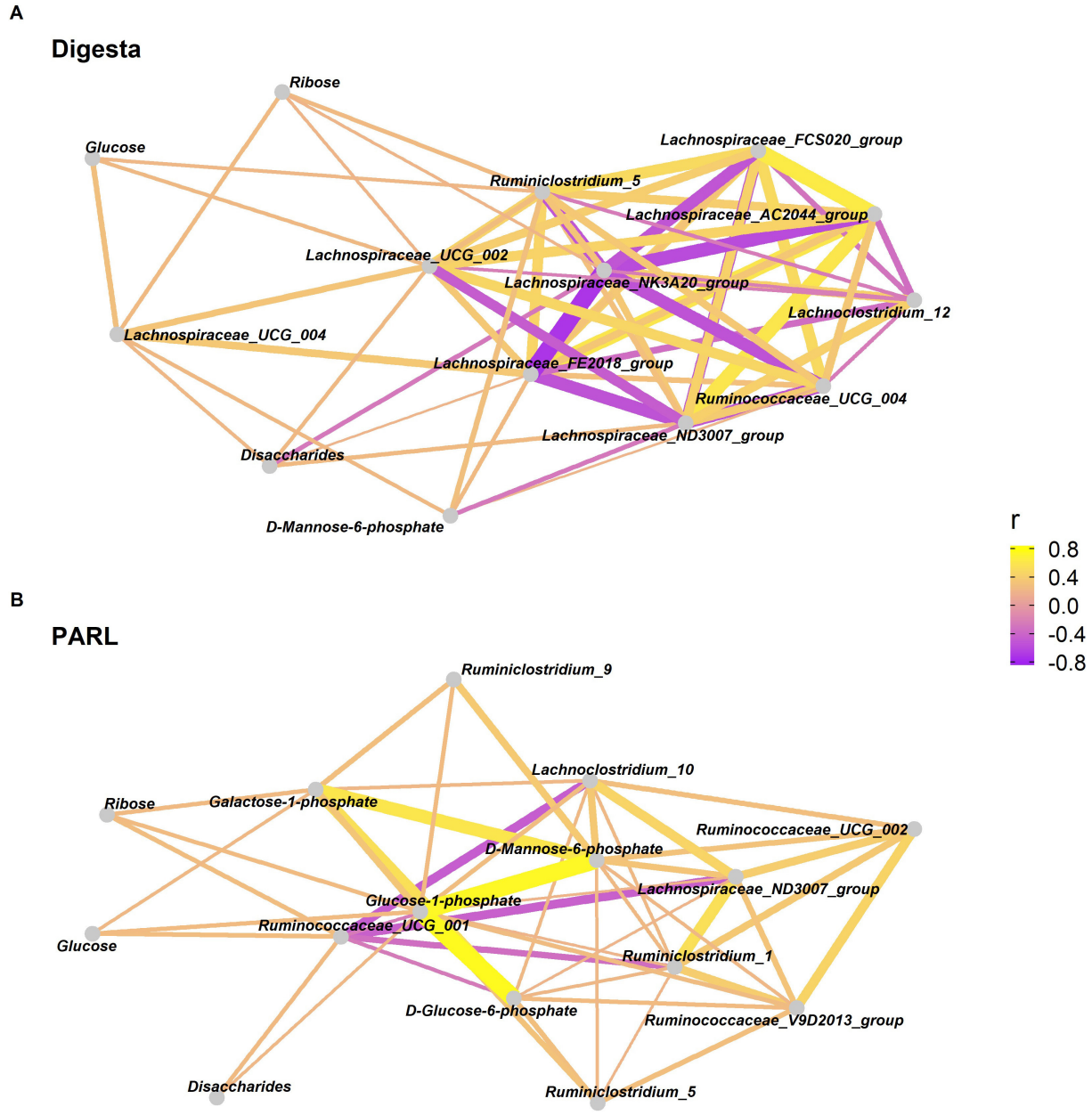


FIGURE 8
Network analysis based on Spearman correlations between carbohydrates and microbes in the rumen. Correlations were calculated between genera belonging to families *Lachnospiraceae* and *Ruminococcaceae* and carbohydrates in **(A)** solid digesta (Digesta) and **(B)** particle associated rumen liquid (PARL). Correlation coefficients are indicated by color (purple = negative, yellow = positive) and line thickness.

2018). Thus, the macroscopic differences between solid and liquid digesta could explain the different responses to the high concentrate feeding found in our study. Differences detected in the time of reaction to the newly introduced substrates between the two niches suggest a slower adaptation of solid digesta to dietary changes, probably due to the microcolonies formed by the bacteria on the fiber particles in the ruminal mat, causing them to be more refractory to rapid adjustments (Cheng et al., 1981).

It is recognized that the microorganisms that colonize the distinct sections of the GIT are substantially different, but we found some similarities in the adaptation to the dietary challenge between the ruminal and the fecal microbiota (Ozbayram et al., 2018; Holman and Gzyl, 2019), with a delay in the

changes for the hindgut. As expected, the increase in concentrate intake reduced the alpha diversity indices for all three matrices analyzed. Reduced richness and diversity are a common finding in cattle-fed high-concentrate diet and it is believed that such a reduction could limit the microorganisms' capacity of resources utilization (Plaizier et al., 2017). However, it has previously been suggested that a reduction in diversity can actually increase the efficiency in utilization of substrates in ruminants (Shabat et al., 2016; Belanche et al., 2019), and our findings confirm that the gastrointestinal microbiota can rapidly adapt its metabolic capability to face newly available substrate, despite the species diversity reduction.

Since the total mean retention time of ingested feed in cattle is at least 24 h, it was expected that changes in microbial activity and composition in feces would be evident with ∼1 day of delay in respect to the ruminal environment (Hartnell and Satter, 1979; Mambrini and Peyraud, 1997). This parallel shaping of the microbial population in the rumen and in feces is well shown by the beta diversity graphs, in which separated clusters are noticeable for the last 2 days of experiment, being days 5 and 6 for the ruminal niches and days 6 and 7 for the fecal samples.

While Plaizier et al. (2017) found the composition of the fecal microbiota to be relatively stable over time during SARA, we demonstrated a daily adaptation of fecal microorganisms to the increment in readily digestible concentrate in the diet, with altered composition and activity. Nevertheless, some researchers suggest that the response of the large intestine to high-concentrate feeding might be not consistent among experiments (Kotz et al., 2020).

Predominant role of *Ruminococcaceae* and *Lachnospiraceae* in ruminal adaptation to a highly fermentable diet

Starch, the main non-structural carbohydrate included in our experimental diet, is degraded into glucose or glucose-1-phosphate in the rumen (Hoover and Miller, 1991; Mills et al., 1999). While the overall concentration of glucose tended to increase over the 6 days in our experiment, glucose-1-phosphate decreased. Previous studies have confirmed that increased concentrate intake results in a higher concentration of glucose and VFAs in the rumen, due to the augmented level of readily fermentable carbohydrates introduced with the diet (Ametaj et al., 2010; Saleem, 2012). The decreased concentration of glucose-1-phosphate found in our study could suggest a high metabolization rate through glycolysis, or possible accumulation in the form of glycogen (Lou et al., 1997; Mills et al., 1999; Hackmann, 2015). The main putative producers of glucose-1-phosphate in our study were identified as belonging to families *Ruminococcaceae* and *Lachnospiraceae* in digesta samples, through cellobiose phosphorylase (K00702), which catalyzes the reaction that transforms plant-derived cellobiose into D-glucose (Hamura et al., 2012). In fact, members of these two taxa used to be considered mainly fibrolytic bacteria (Bickhart and Weimer, 2018; Holman and Gzyl, 2019), but certainly their enzymatic apparatus allows them to metabolize more readily available sugars as well. A previous metagenomic study found several putative carbohydrate-active enzymes to be associated with both families in solid digesta (Wang et al., 2013), and in our experiment both families were confirmed as connected with the metabolism of carbohydrates by the network analysis, albeit with moderate correlations. In addition, random forest regression analysis identified several ASVs classified as belonging to families *Ruminococcaceae* and *Lachnospiraceae* to be highly descriptive of the adaptation of the microbiota composition in the two ruminal niches over the experimental days. Therefore, despite the limits and the predictive nature of our analyses, all approaches used strongly supported the major role of these two taxa, suggesting a highly adaptive metabolic potential and strong plasticity in response to progressive increases in dietary starch. Our predictive analysis also associated *Ruminococcaceae* and *Lachnospiraceae* with the variance of acetate, succinate, and phenylacetic acid, highlighting the need to better investigate the metabolic potential of these families and their adaptive roles in the ruminal environment.

Diet shapes ruminal microbial metabolism and composition

Our analyses revealed that the main drivers of the shift in metabolites composition, noticeable over the last 2 days of experiment, were glucose, acetic acid, and butyric acid. Both PICRUSt and quantitative enrichment analysis appeared to confirm the importance of these metabolites, with the most abundant pathways being related with starch metabolism, for instance NONOXIPENT-PWY (pentose phosphate pathway (non-oxidative branch) I), PWY-5100 (pyruvate fermentation to acetate and lactate II), GLYCOGENSYNTH-PWY (glycogen biosynthesis I, from ADP-D-Glucose), and PWY-6737 (starch degradation V). According to the function prediction, families *Erysipelotrichaceae* and *Lachnospiraceae* synthesized most of the acetate in digesta samples. Deusch et al. (2017) suggested a role of *Erysipelotrichaceae* in the production of lactate in the rumen, while Chen et al. (2021) found a negative correlation between members of this family and acetate production. This is in contrast with our results, demonstrating the need to further investigate the metabolic capabilities of the *Erysipelotrichaceae* family.

Genera *Bacillus*, *Prevotella*, *Ruminobacter*, and *Selenomonas* were identified in literature as main microorganisms producing propionate in the rumen, expressing high levels of succinate-CoA synthetase (Wang et al., 2020). Although the same pathway

was not identified among the most abundant groups in our experiment, succinic acid concentration tended to increase in parallel with propionate concentration. Furthermore, bacteria belonging to family *Prevotellaceae* (in particular *Prevotellaceae* NK3B31 group and *Prevotellaceae* UCG-001) and *Selenomonas* 1 were found to increase over the 6 days in PARL and digesta, confirming their importance in high-concentrate diet digestion in the rumen. While *Prevotella* was not associated with succinic acid production in PARL samples in our study, family *Veillonellaceae*, to which genus *Selenomonas* belongs, was indicated as one of the major putative synthesizers. However, *Prevotella* plays a main role in carbohydrate metabolism and their role as main functional group in planktonic microbiota in the production of butyrate during a high-concentrate feeding regime was also confirmed (Wirth et al., 2018; Wang et al., 2020).

Microbial metabolism produces potentially harmful metabolites in response to the dietary change

Some microbial by-products could pose a risk for animal health when their concentration is increased and remains at high levels. The reshaping of ruminal microflora in high-concentrate feeding is often accompanied by increased production of potentially harmful compounds, such as lipopolysaccharide (LPS), lactic acid, and biogenic amines (Khafipour et al., 2009; Saleem, 2012). Increased concentrations of ethanolamine are a common finding when cows are fed high energy diets, and augmented epithelial turnover and bacterial cell lysis were indicated as possible causes (Saleem, 2012; Zhang et al., 2017).

Sedoheptulose 7-phosphate is part of the pentose phosphate pathways associated with carbohydrate metabolism, as well as a fundamental element for LPS synthesis in Gram-negative bacteria (Taylor et al., 2008). In PARL samples, *Prevotellaceae* and *Succinivibrionaceae*, both Gram-negative and having increased frequency in the first 4 days of experiment, were the two families more strongly associated with the variation of sedoheptulose 7-phosphate. It is possible that the decreased concentration of the metabolite was due to its utilization, through D-sedoheptulose-7-phosphate isomerase (K03271), for the formation of LPS for the outer membrane of the bacteria.

The abundance of known lactic acid producing groups, such as *Streptococcaceae*, *Pseudobutyrivibrio* and *Butyrivibrio* 2, significantly increased in the rumen only on day 6 (Mackie and Gilchrist, 1979; Hernandez et al., 2008). We found a positive correlation between lactic acid and *Streptococcus* in digesta samples, as well as with genera *Lachnobacterium* and *FD2005*, belonging to family *Lachnospiraceae*. Both have already been associated with SARA in goats (Chen et al., 2021), but the production of this potentially harmful metabolite is reported only for members of the genus *Lachnobacterium* (Whitford et al., 2001). Interestingly, our results indicated that families *Ruminococcaceae* and *Succinivibrionaceae*, while not being generally associated with lactate production, have the potential to produce lactate, and could play a role along with other microbes in the susceptibility of an animal to SARA.

Effects of the PFA on microbiota of the GIT

In our study, the phytogenic addition resulted in a higher abundance of *Paludibacteriaceae*, *Campylobacteraceae,* and *Clostridiales* Family XIII in the fecal samples. These families are typically described as members of the core microbiota in a healthy GIT (Dong et al., 2016; Plaizier et al., 2017; Holman and Gzyl, 2019). Although some strains belonging to the latter two taxa are considered as pathogens, their shedding seems to be more associated with seasonality, husbandry, or individuality, rather than with families relative abundance in the feces (Sproston et al., 2011; Dong et al., 2016). Genus *Paludibacter*, on the other hand, has been previously described as negatively affected by the high amount of carbohydrates in the diet, suggesting a positive role played by the phytogenic additive in preserving a healthy microbiota.

The phytogenic addition resulted in higher alpha diversity indices, especially toward the end of the experiment. This is an interesting result, as it seems that high diversity is a key factor in microbiota resilience (Sommer et al., 2017). Albeit affecting only relatively small abundant taxa, as confirmed by the significant difference found only for unweighted UniFrac, the phytogenic additive demonstrated to have the potential to alter the microbial activity in the rumen and to preserve a diverse microbiota across the GIT. Spermine and spermidine were previously reported as increased due to high concentrate feeding, but the level of both amines in the rumen was successfully reduced with supplementation with a phytogenic additive in a previous study (Humer et al., 2018a). In our experiment, only spermidine concentration was reduced by the phytogenic additive. Although biogenic amines receive a lot of attention especially in food contamination, research has not yet completely elucidated how substances, such as thymol, menthol, or eugenol, impact this aspect of microbial metabolism (Naila et al., 2010; Özogul et al., 2015).

However, the modifications in bacterial composition and activity were not enough to cause major fluctuations in the production of the other metabolites over the experimental period. It is possible that these changes could be more pronounced in animals undergoing a more severe dietary disruption. Further research is necessary to investigate the mechanism of action of the phytogenic additive under conditions of increasing severity, in order to understand

its potential toward mitigation of dysbiosis in animals fed concentrate-rich diets.

Conclusion

Rapid diet changes and increasing amounts of starch are responsible for rapid shifts in microbiota composition and activity in cattle GIT. Microorganisms inhabiting the rumen and hindgut of dairy cattle demonstrated a high adaptation capacity, with the effects of perturbation of the ecosystems starting to be visible with inclusion of 50% concentrate in the diet. As expected, readily fermentable carbohydrates increased glucose, VFAs, and related metabolites and pathways both in the rumen and in feces, with bacteria belonging to families *Ruminococcaceae* and *Lachnospiraceae* showing a great plasticity and capacity to adapt rapidly to sudden dietary shifts. Supplementation with a PFA that includes menthol, thymol, and eugenol showed potential beneficial effects by increasing the diversity of the microbiota, despite affecting only relatively small abundant taxa. Lastly, our work contributes to better understanding of PARL: this lesser studied digesta fraction, whose microbiota is at the interface between solid and liquid rumen content, is highly important in the utilization of nutrients. Therefore, modifying its composition through the use of feed additives could be a target for the development of nutritional interventions to improve digestion and rumen health in cattle.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, BioProject PRJNA769001.

Ethics statement

The animal study was reviewed and approved by Institutional Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and the Austrian national authority according to the law for animal experiments (Protocol Number: BMBWF-68.205/0003-V/3b/2019).

Author contributions

QZ leads the CD laboratory and acquired funding with RP, NR, HS-Z, and FB. QZ, RP, and NR designed the experiment. SR, RR-C, RP, and EC-L performed the experimental trial. SR and CP performed the DNA extractions. HS-Z carried out the metabolomics analyses. SR performed bioinformatics and statistical analyses, with the help of QZ, RP, and CP. SR wrote the manuscript, that was revised and approved by all authors. All authors contributed to the article and approved the submitted version.

Funding

This work has been funded by the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock, the Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation for Research, Technology and Development.

Acknowledgments

The authors would like to thank the staff at the Vetmeduni VetFarm, as well as T. Enzinger, E. Draxler, P. Kheirandish, M. Reiter, M. Aigensberger, P. Rennhofer, and A. Ferretti for their very appreciated help and assistance during the experiment. We also want to greatly acknowledge the laboratory staff at the Institute of Animal Nutrition, A. Dockner, S. Leiner, M. Hollmann, S. Sharma, and A. Sener-Aydemir (Vetmeduni, Vienna) for their help with lab analyses.

Conflict of interest

NR is employed at DSM, BIOMIN Research Center, a company that manufactures feed additives and financially supports the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock. However, the authors declare that this had no impact on the analysis or interpretation of results.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.920427/full#supplementary-material

References

Ametaj, B. N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M. J., Dunn, S. M., et al. (2010). Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics* 13, 583–594. doi: 10.1007/s11306-010-0227-6

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., et al. (2017). Deblur rapidly resolves single-nucleotide community sequence patterns. *mSystems* 2, e00191–e00116. doi: 10.1128/mSystems.00191-16

Andrews, S., and Babraham Bioinformatics (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available online at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed February 12, 2020).

Aschenbach, J. R., Penner, G. B., Stumpff, F., and Gäbel, G. (2011). Ruminant nutrition symposium: role of fermentation acid absorption in the regulation of ruminal pH. *J. Anim. Sci.* 89, 1092–1107. doi: 10.2527/jas.2010-3301

Belanche, A., Kingston-Smith, A. H., Griffith, G. W., and Newbold, C. J. (2019). A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. *Front. Microbiol.* 10, 122. doi: 10.3389/fmicb.2019.00122

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Bickhart, D. M., and Weimer, P. J. (2018). Symposium review: host–rumen microbe interactions may be leveraged to improve the productivity of dairy. *Cows* 101, 10. doi: 10.3168/jds.2017-13328

Bokulich, N. A., Dillon, M. R., Zhang, Y., Rideout, J. R., Bolyen, E., Li, H., et al. (2018). q2-longitudinal: longitudinal and paired-sample analyses of microbiome data. *mSystems* 3, e00219–e00218. doi: 10.1128/mSystems.00219-18

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

Caicedo, H. H., Hashimoto, D. A., Caicedo, J. C., Pentland, A., and Pisano, G. P. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 669–673. doi: 10.1038/s41587-020-0550-z

Cardozo, P. W., Calsamiglia, S., Ferret, A., and Kamel, C. (2006). Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet. *J. Anim. Sci.* 84, 2801–2808. doi: 10.2527/jas.2005-593

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., et al. (2020). The MetaCyc database of metabolic pathways and enzymes: a 2019 update. *Nucl. Acids Res.* 48, D445–D453. doi: 10.1093/nar/gkz862

Castillo-Lopez, E., Ramirez Ramirez, H. A., Klopfenstein, T. J., Hostetler, D., Karges, K., Fernando, S. C., et al. (2014a). Ration formulations containing reduced-fat dried distillers grains with solubles and their effect on lactation performance, rumen fermentation, and intestinal flow of microbial nitrogen in Holstein cows. *J. Dairy Sci.* 97, 1578–1593. doi: 10.3168/jds.2013-6865

Castillo-Lopez, E., Rivera-Chacon, R., Ricci, S., Petri, R. M., Reisinger, N., and Zebeli, Q. (2021). Short-term screening of multiple phytogenic compounds for their potential to modulate chewing behavior, ruminal fermentation profile, and pH in cattle fed grain-rich diets. *J. Dairy Sci.* 104, 4271–4289. doi: 10.3168/jds.2020-19521

Castillo-Lopez, E., Wiese, B. I., Hendrick, S., McKinnon, J. J., McAllister, T. A., Beauchemin, K. A., et al. (2014b). Incidence, prevalence, severity, and risk factors for ruminal acidosis in feedlot steers during backgrounding, diet transition, and finishing1. *J. Anim. Sci.* 92, 3053–3063. doi: 10.2527/jas.2014-7599

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* 11, 265–270.

Chao, A., and Yang, M. C. K. (1993). Stopping rules and estimation for recapture debugging with unequal failure rates. *Biometrika* 80, 193–201. doi: 10.1093/biomet/80.1.193

Chen, X., Su, X., Li, J., Yang, Y., Wang, P., Yan, F., et al. (2021). Real-time monitoring of ruminal microbiota reveals their roles in dairy goats during subacute ruminal acidosis. *NPJ Biofilms Microb.* 7, 45. doi: 10.1038/s41522-021-00215-6

Cheng, K.-J., Fay, J. P., Coleman, R. N., Milligan, L. P., and Costerton, J. W. (1981). Formation of bacterial microcolonies on feed particles in the Rumen. *Appl. Environ. Microbiol.* 41, 298–305. doi: 10.1128/aem.41.1.298-305.1981

Chong, J., Wishart, D. S., and Xia, J. (2019). Using metaboanalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr. Protoc. Bioinforma.* 68, e86. doi: 10.1002/cpbi.86

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563. doi: 10.1038/nature12820

De Angelis, M., Ferrocino, I., Calabrese, F. M., De Filippis, F., Cavallo, N., Siragusa, S., et al. (2020). Diet influences the functions of the human intestinal microbiome. *Sci. Rep.* 10, 4247. doi: 10.1038/s41598-020-61192-y

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

Dong, H.-J., Kim, W., An, J.-U., Kim, J., and Cho, S. (2016). The fecal microbial communities of dairy cattle shedding shiga toxin–producing *Escherichia coli* or *Campylobacter jejuni*. *Foodborne Pathog. Dis.* 13, 7. doi: 10.1089/fpd.2016.2121

Faith, D. P., and Baker, A. M. (2006). Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evol. Bioinforma.* 2, 117693430600200. doi: 10.1177/117693430600200007

Hackmann, T. J. (2015). Maximizing efficiency of rumen microbial protein production. *Front. Microbiol.* 6, 16. doi: 10.3389/fmicb.2015.00465

Hamura, K., Saburi, W., Abe, S., Morimoto, N., Taguchi, H., Mori, H., et al. (2012). Enzymatic characteristics of cellobiose phosphorylase from *Ruminococcus albus* NE1 and kinetic mechanism of unusual substrate inhibition in reverse phosphorolysis. *Biosci. Biotechnol. Biochem.* 76, 812–818. doi: 10.1271/bbb.110954

Harrell, F. E. J., and Dupont, C. (2020). *Hmisc: Harrell Miscellaneous*. Available online at: https://CRAN.R-project.org/package=Hmisc (accessed November 29, 2020).

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

Hartnell, G. F., and Satter, L. D. (1979). Determination of rumen fill, retention time and ruminal turnover rates of ingesta at different stages of lactation in dairy cows. *J. Anim. Sci.* 48, 381–392. doi: 10.2527/jas1979.482381x

Hernandez, J. D., Scott, P. T., and Shephard, R. W. (2008). The characterization of lactic acid producing bacteria from the rumen of dairy cattle grazing on improved pasture supplemented with wheat and barley grain. *J. Appl. Microbiol.* 10, 1754–1763. doi: 10.1111/j.1365-2672.2007.03696.x

Holman, D. B., and Gzyl, K. E. (2019). A meta-analysis of the bovine gastrointestinal tract microbiota. *FEMS Microbiol. Ecol.* 95, fiz072. doi: 10.1093/femsec/fiz072

Hoover, W. H., and Miller, T. K. (1991). Rumen digestive physiology and microbial ecology. *Vet. Clin. N. Am. Food Anim. Pract.* 7, 311–325. doi: 10.1016/S0749-0720(15)30801-X

Huang, S., Ji, S., Yan, H., Hao, Y., Zhang, J., Wang, Y., et al. (2020). The day-to-day stability of the ruminal and fecal microbiota in lactating dairy cows. *MicrobiologyOpen* 9, e990. doi: 10.1002/mbo3.990

Humer, E., Kröger, I., Neubauer, V., Schedle, K., Reisinger, N., and Zebeli, Q. (2018a). Supplementing phytogenic compounds or autolyzed yeast modulates ruminal biogenic amines and plasma metabolome in dry cows experiencing subacute ruminal acidosis. *J. Dairy Sci.* 101, 9559–9574. doi: 10.3168/jds.2018-14744

Humer, E., Petri, R. M., Aschenbach, J. R., Bradford, B. J., Penner, G. B., Tafaj, M., et al. (2018b). Invited review: practical feeding management recommendations to mitigate the risk of subacute ruminal acidosis in dairy cattle. *J. Dairy Sci.* 101, 872–888. doi: 10.3168/jds.2017-13191

Khafipour, E., Krause, D. O., and Plaizier, J. C. (2009). A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci.* 92, 1060–1070. doi: 10.3168/jds.2008-1389

Khafipour, E., Li, S., Tun, H. M., Derakhshani, H., Moossavi, S., and Plaizier, J. C. (2016). Effects of grain feeding on microbiota in the digestive tract of cattle. *Anim. Front.* 6, 13–19. doi: 10.2527/af.2016-0018

Khiaosa-ard, R., Pourazad, P., Aditya, S., Humer, E., and Zebeli, Q. (2018). Factors related to variation in the susceptibility to subacute ruminal acidosis in early lactating Simmental cows fed the same grain-rich diet. *Anim. Feed Sci. Technol.* 238, 111–122. doi: 10.1016/j.anifeedsci.2018.02.005

Kleen, J. L., Hooijer, G. A., Rehage, J., and Noordhuizen, J. P. T. M. (2003). Subacute ruminal acidosis (SARA): a review. *J. Vet. Med. Ser. A* 50, 406–414. doi: 10.1046/j.1439-0442.2003.00569.x

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. *Nucl. Acids Res.* 41, e1–e1. doi: 10.1093/nar/gks808

Kotz, A., Azevedo, P. A., Khafipour, E., and Plaizier, J. C. (2020). Effects of the dietary grain content on rumen and fecal microbiota of dairy cows. *Can. J. Anim. Sci.* 101, 1–13. doi: 10.1139/cjas-2020-0122

Lachnit, T., Bosch, T. C. G., and Deines, P. (2019). Exposure of the host-associated microbiome to nutrient-rich conditions may lead to dysbiosis and disease development—an evolutionary perspective. *mBio* 10, e00355-19. doi: 10.1128/mBio.00355-19

Lou, J., Dawson, K. A., and Strobel, H. J. (1997). Glycogen biosynthesis via UDP-glucose in the ruminal bacterium prevotella bryantii B14. *Appl. Environ. Microbiol.* 63, 5. doi: 10.1128/aem.63.11.4355-4359.1997

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi: 10.1128/AEM.01996-06

Mackie, R. I., and Gilchrist, F. M. C. (1979). Changes in lactate-producing and lactate-utilizing bacteria in relation to pH in the rumen of sheep during stepwise adaptation to a high-concentrate diet. *Appl. Environ. Microbiol.* 38, 9. doi: 10.1128/aem.38.3.422-430.1979

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., et al. (2021). Multivariable association discovery in population-scale meta-omics studies. *Microbiology* 17, e1009442. doi: 10.1101/2021.01.20.427420

Mambrini, M., and Peyraud, J. (1997). Retention time of feed particles and liquids in the stomachs and intestines of dairy cows. Direct measurement and calculations based on faecal collection. *Reprod. Nutr. Dev.* 37, 427–442. doi: 10.1051/rnd:19970404

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P. W., and Cotter, P. D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* 10, 115–132. doi: 10.1080/19490976.2018.1505176

Metzler-Zebeli, B. U., Khol-Parisini, A., Gruber, L., and Zebeli, Q. (2015). Microbial populations and fermentation profiles in rumen liquid and solids of Holstein cows respond differently to dietary barley processing. *J. Appl. Microbiol.* 13, 1502–1514. doi: 10.1111/jam.12958

Mills, J., France, J., and Dijkstra, J. (1999). A review of starch digestion in the lactating dairy cow and proposals for a mechanistic model: 1. Dietary starch characterisation and ruminal starch digestion. *J. Anim. Feed Sci.* 8, 291–340. doi: 10.22358/jafs/68938/1999

Naila, A., Flint, S., Fletcher, G., Bremer, P., and Meerdink, G. (2010). Control of biogenic amines in food-existing and emerging approaches. *J. Food Sci.* 75, R139–R150. doi: 10.1111/j.1750-3841.2010.01774.x

Neubauer, V., Petri, R., Humer, E., Kröger, I., Mann, E., Reisinger, N., et al. (2018). High-grain diets supplemented with phytogenic compounds or autolyzed yeast modulate ruminal bacterial community and fermentation in dry cows. *J. Dairy Sci.* 101, 2335–2349. doi: 10.3168/jds.2017-13565

Noecker, C., Eng, A., Srinivasan, S., Theriot, C. M., Young, V. B., Jansson, J. K., et al. (2016). Metabolic model-based integration of microbiome taxonomic and metabolomic profiles elucidates mechanistic links between ecological and metabolic variation. *mSystems* 1, e00013–15. doi: 10.1128/mSystems.00013-15

O'Hara, E., Neves, A. L. A., Song, Y., and Guan, L. L. (2020). The role of the gut microbiome in cattle production and health: driver or passenger? *Annu. Rev. Anim. Biosci.* 8, 199–220. doi: 10.1146/annurev-animal-021419-083952

Ozbayram, E., Ince, O., Ince, B., Harms, H., and Kleinsteuber, S. (2018). Comparison of rumen and manure microbiomes and implications for the inoculation of anaerobic digesters. *Microorganisms* 6, 15. doi: 10.3390/microorganisms6010015

Özogul, F., Kacar, Ç., and Hamed, I. (2015). Inhibition effects of carvacrol on biogenic amines formation by common food-borne pathogens in histidine decarboxylase broth. *LWT Food Sci. Technol.* 64, 50–55. doi: 10.1016/j.lwt.2015.05.027

Penner, G. B., Beauchemin, K. A., and Mutsvangwa, T. (2006). An evaluation of the accuracy and precision of a stand-alone submersible continuous ruminal pH measurement system. *J. Dairy Sci.* 89, 2132–2140. doi: 10.3168/jds.S0022-0302(06)72284-6

Petri, R. M., Kleefisch, M. T., Metzler-Zebeli, B. U., Zebeli, Q., and Klevenhusen, F. (2018). Changes in the rumen epithelial microbiota of cattle and host gene expression in response to alterations in dietary carbohydrate composition. *Appl. Environ. Microbiol.* 84, e00384–e00318. doi: 10.1128/AEM.00384-18

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J., et al. (2013). Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis. *Appl. Environ. Microbiol.* 79, 3744–3755. doi: 10.1128/AEM.03983-12

Plaizier, J. C., Khafipour, E., Li, S., Gozho, G. N., and Krause, D. O. (2012). Subacute ruminal acidosis (SARA), endotoxins and health consequences. *Anim. Feed Sci. Technol.* 172, 9–21. doi: 10.1016/j.anifeedsci.2011.12.004

Plaizier, J. C., Krause, D. O., Gozho, G. N., and McBride, B. W. (2008). Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. *Vet. J.* 176, 21–31. doi: 10.1016/j.tvjl.2007.12.016

Plaizier, J. C., Li, S., Danscher, A. M., Derakshani, H., Andersen, P. H., and Khafipour, E. (2017). Changes in microbiota in rumen digesta and feces due to a grain-based subacute ruminal acidosis (SARA) challenge. *Microb. Ecol.* 74, 485–495. doi: 10.1007/s00248-017-0940-z

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucl. Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Rivera-Chacon, R., Castillo-Lopez, E., Ricci, S., Petri, R. M., Reisinger, N., and Zebeli, Q. (2022). Supplementing a phytogenic feed additive modulates the risk of subacute rumen acidosis, rumen fermentation and systemic inflammation in cattle fed acidogenic diets. *Animals* 12, 1201. doi: 10.3390/ani12091201

Rubino, F., Carberry, C. M., Waters, S., Kenny, D., McCabe, M. S., et al. (2017). Divergent functional isoforms drive niche specialisation for nutrient acquisition and use in rumen microbiome. *ISME J.* 11, 932–944. doi: 10.1038/ismej.2016.172

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

Saleem, F. (2012). A metabolomics approach to uncover the effects of grain diets on rumen health in dairy. *Cows* 95, 18. doi: 10.3168/jds.2012-5403

Schären, M., Kiri, K., Riede, S., Gardener, M., Meyer, U., Hummel, J., et al. (2017). Alterations in the rumen liquid-, particle- and epithelium-associated microbiota of dairy cows during the transition from a silage- and concentrate-based ration to pasture in spring. *Front. Microbiol.* 8, 744. doi: 10.3389/fmicb.2017.00744

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., 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

Shannon, C. E. (1948). A mathematical theory of communication. *Bell Syst. Tech. J.* 27, 379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x

Sierra, M. A., Li, Q., Pushalkar, S., Paul, B., Sandoval, T. A., Kamer, A. R., et al. (2020). The influences of bioinformatics tools and reference databases in analyzing the human oral microbial community. *Genes* 11, 878. doi: 10.3390/genes11080878

Sommer, F., Anderson, J. M., Bharti, R., Raes, J., and Rosenstiel, P. (2017). The resilience of the intestinal microbiota influences health and disease. *Nat. Rev. Microbiol.* 15, 630–638. doi: 10.1038/nrmicro.2017.58

Sproston, E. L., Ogden, I. D., MacRae, M., Dallas, J. F., Sheppard, S. K., Cody, A. J., et al. (2011). Temporal variation and host association in the campylobacter population in a longitudinal ruminant farm study. *Appl. Environ. Microbiol.* 77, 6579–6586. doi: 10.1128/AEM.00428-11

Tafaj, M., Junck, B., Maulbetsch, A., Steingass, H., Piepho, H.-P., and Drochner, W. (2004). Digesta characteristics of dorsal, middle and ventral rumen of cows fed with different hay qualities and concentrate levels. *Arch. Anim. Nutr.* 58, 325–342. doi: 10.1080/0003942041233 1273259

Taylor, P. L., Blakely, K. M., de Leon, G. P., Walker, J. R., McArthur, F., Evdokimova, E., et al. (2008). Structure and function of sedoheptulose-7-phosphate isomerase, a critical enzyme for lipopolysaccharide biosynthesis and a target for antibiotic adjuvants. *J. Biol. Chem.* 283, 2835–2845. doi: 10.1074/jbc.M70616 3200

Tester, R. F., Karkalas, J., and Qi, X. (2004). Starch—composition, fine structure and architecture. *J. Cereal Sci.* 39, 151–165. doi: 10.1016/j.jcs.2003.12.001

Wang, L., Hatem, A., Catalyurek, U. V., Morrison, M., and Yu, Z. (2013). Metagenomic insights into the carbohydrate-active enzymes carried by the microorganisms adhering to solid digesta in the rumen of cows. *PLOS ONE* 8, 11. doi: 10.1371/journal.pone.0078507

Wang, L., Zhang, G., Li, Y., and Zhang, Y. (2020). Effects of high forage/concentrate diet on volatile fatty acid production and the microorganisms involved in VFA production in cow rumen. *Animals* 10, 223. doi: 10.3390/ani10020223

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* 39, 971–974. doi: 10.1021/ac60252a045

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

Wetzels, S. U., Mann, E., Metzler-Zebeli, B. U., Pourazad, P., Qumar, M., Klevenhusen, F., et al. (2016). Epimural indicator phylotypes of transiently-induced subacute ruminal acidosis in dairy cattle. *Front. Microbiol.* 7, 274. doi: 10.3389/fmicb.2016.00274

Whitford, M. F., Yanke, L. J., Forster, R. J., and Teather, R. M. (2001). Lachnobacterium bovis gen. nov., sp. nov., a novel bacterium isolated from the rumen and faeces of cattle. *Int. J. Syst. Evol. Microbiol.* 51, 1977–1981. doi: 10.1099/00207713-51-6-1977

Wirth, R., Kádár, G., Kakuk, B., Maróti, G., Bagi, Z., Szilágyi, Á., et al. (2018). The planktonic core microbiome and core functions in the cattle rumen by next generation sequencing. *Front. Microbiol.* 9, 2285. doi: 10.3389/fmicb.2018.02285

Zebeli, Q., Dijkstra, J., Tafaj, M., Steingass, H., Ametaj, B. N., and Drochner, W. (2008). Modeling the adequacy of dietary fiber in dairy cows based on the responses of ruminal pH and milk fat production to composition of the diet. *J. Dairy Sci.* 91, 2046–2066. doi: 10.3168/jds.2007-0572

Zhang, R., Zhu, W., Jiang, L., and Mao, S. (2017). Comparative metabolome analysis of ruminal changes in Holstein dairy cows fed low- or high-concentrate diets. *Metabolomics* 13, 74. doi: 10.1007/s11306-017-1204-0

Zhou, R., Wu, J., Zhang, L., Liu, L., Casper, D. P., Jiao, T., et al. (2019). Effects of oregano essential oil on the ruminal pH and microbial population of sheep. *PLOS ONE* 14, e0217054. doi: 10.1371/journal.pone.0217054

Frontiers in Microbiology

Explores the habitable world and the potential of microbial life

The largest and most cited microbiology journal which advances our understanding of the role microbes play in addressing global challenges such as healthcare, food security, and climate change.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact