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A Comparison of Three Artificial Rumen Systems for Rumen Microbiome Modeling

Claire A. Shaw [1,†], Yuna Park [1,†], Maria Gonzalez [1], Rich A. Duong [1], Pramod K. Pandey [2], Charles G. Brooke [1,†] and Matthias Hess [1,*]

[1] Systems Microbiology & Natural Products Laboratory, University of California, Davis, CA 95616, USA; cgbrooke@ucdavis.edu (C.G.B.)
[2] School of Veterinary Medicine, University of California, Davis, CA 95616, USA; pkpandey@ucdavis.edu
* Correspondence: mhess@ucdavis.edu; Tel.: +1-(530)-752-8809
† These authors contributed equally to this work.

Abstract: The rumen contains a complex mixture of microbes, crucial for the animal's ability to degrade feed. Some of the feed-derived carbon is released as methane, a potent greenhouse gas, into the atmosphere. There is growing interest in reducing the loss of feed-derived carbon, making it available to the animal and improving animal productivity. Artificial rumen systems (ARSs) have been widely used to evaluate novel feed additives in terms of their ability to reduce methane production in the rumen and their effect on the rumen microbiome function prior to conducting resource-intensive animal trials. While the value of ARSs is widely acknowledged, it remains unclear which of these in vitro systems simulate the natural system most accurately. Here, we evaluated three different ARSs and compared them to in vivo rumen metrics. The results showed that all systems were capable of maintaining stable pH, redox potential, and temperature over time. The batch-style ARS simulated the rumen over 48 h. The semi-continuous ARS mimicked the volatile fatty acid profile and microbiota of the in vivo rumen for up to 120 h. Similarly, all ARSs maintained the prokaryotic and eukaryotic rumen populations over the duration of the study, with the semi-continuous ARS maintaining the natural rumen microbiome more accurately and for up to 120 h. In sum, our results suggest that three of the widely used ARSs simulate the rumen ecosystem adequately for many short-term rumen microbiome studies, with the more advanced semi-continuous ARS being more accurate when rumen simulation is extended to over 48 h.

Keywords: artificial rumen systems; greenhouse gases; methane; microbial ecology; rumen microbiome; rumen modeling

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1. Introduction

Ruminants, such as cattle, sheep, and goats, are an important source of high-quality protein, and they will be crucial for providing food safety to the growing global population. When managed correctly, ruminants can utilize land that is unsuitable for row crop agriculture [1], decrease the risk of wildfire [2], and facilitate the conservation of native birds and plants [3]. Ruminants can also utilize byproducts from plant-based agriculture, such as almond hulls and citrus pulp [4], which would otherwise contribute to the growing waste stream from food production [5]. These agricultural byproducts possess the potential to address one of the major environmental challenges associated with ruminant livestock, namely, the microbially mediated production of enteric methane (CH_4). The complex microbial community in the rumen facilitates the conversion of complex plant carbohydrates, otherwise inaccessible to the ruminant animal, into metabolic intermediates, such as volatile fatty acids (VFAs) and hydrogen [6,7].

Whereas VFAs and H_2 can be utilized further by some rumen microbes or the host animal, rumen methanogens reduce carbon dioxide (CO_2), with the help of hydrogen (H_2),

under anaerobic rumen conditions into CH_4, which is then emitted into the atmosphere, primarily through the esophagus [8]. In the atmosphere, CH_4 acts as a greenhouse gas with a global warming potential that is 28 times more potent than that of CO_2 on a 100-year time scale [9], and the production of feed-derived CH_4 has been estimated to account for a loss of up to 12% of the total feed energy consumed by the ruminant animal [10]. Therefore, it is understandable that there has been a continuous interest in identifying improved feed formulations and novel additives that redirect feed-derived carbon away from CH_4 towards animal protein. Artificial (in vitro) rumen systems have been used for many years to make the ruminant industry more sustainable and to screen feed formulations and potential feed additives, such as ionophores, biologically active compounds sourced from plants, dietary lipids, and exogenous enzymes, for their impact on the rumen ecosystem and the host animal [11–15]. In vitro rumen models have been used extensively over several decades in the area of rumen microbiology and rumen nutrition, and whereas in early years, in vitro rumen models were used to only test changes in simple feed parameters, in recent years, the combination of more advanced in vitro systems with advanced omics and analytical approaches have made in vitro rumen systems very useful tools to enhance our understanding of rumen function and changes in rumen function in response to dietary changes at the molecular and the microbial level [16,17].

The central piece of artificial rumen systems (ARSs) is a vessel that is inoculated with rumen fluid and usually also some rumen solids that are collected from one or more donor animals. The inoculum is then usually complemented with a basal feed mixture, artificial saliva buffer, and the substrate (e.g., feed additive) under investigation. Regardless of whether the ARS is being run as a batch or semi-continuous system, they all aim to maintain an environment comparable to the rumen, and capable of sustaining the microbial populations typically found within it, by maintaining anaerobic conditions throughout the incubation period. Simplistic batch systems are usually composed of vessels filled with rumen-derived content, which are either placed in a water bath or surrounded with a heating jacket to maintain an operating temperature of 37 °C, representative of the rumen temperature. Advanced batch systems are fitted with a pressure sensor and valve allowing for a real-time measurement of gas production [18]. Batch systems are usually run for a relatively short incubation period, typically between 2 and 48 h, whereas more complex semi-continuous ARSs, such as the rumen simulation technique (RUSITEC), have a continuous influx of saliva buffer and effluent tubing, which enables the maintaining of experimental conditions for up to multiple weeks [19]. Semi-continuous systems are also engineered to allow for repeated feeding throughout the incubation period, and they simulate the addition of saliva in the animal, which makes these systems useful platforms for gaining insights into the long-term effects of diets and feed additives that have the potential to alter the rumen function towards a decreased CH_4 production.

Despite the value of these in vitro rumen systems, there are significant challenges associated with the results from these ARSs. The extent to which in vitro-obtained results can be compared and applied to predict an in vivo response varies by study and often depends on the type of ARS (e.g., continuous, semi-continuous, batch fermentation systems) that is employed for these initial scoping assays but also on other parameters such as operational practices (and differences in these practices) and the diet of the animal from which the inoculum for the ARS was obtained. Even less obvious differences (e.g., the time of sampling, sampling location within the rumen, handling of inoculum) can have profound implications on the observed microbial response, and subsequently on how well the in vitro response will mimic the in vivo response [20,21]. The batch systems used in this experiment and the batch systems that have been discussed above are only able to replicate the rumen environment for a relatively limited period, mostly due to the lack of buffering and the subsequent buildup of waste metabolites [22], but they remain valuable for economical screening efforts. The semi-continuous RUSITEC platforms, though advantageous when performing extended rumen fermentation experiments, are by no means without shortcomings. More specifically, semi-continuous culture platforms can

be expensive and time-consuming to set up and run, and similar to batch systems, they have been shown to be unsuitable for maintaining the protozoan rumen population [22]. A recent review by Vinyard and Faciola [22] covers the advantages and pitfalls of some of the most common in vitro rumen platforms more thoroughly.

Although all in vitro rumen systems are unable to perfectly mimic the rumen environment, they remain an integral part of reducing overall costs and providing a higher throughput screening method for studies that center on rumen function. To provide guidance on which ARSs might be the most suitable for addressing specific objectives, we compared three in vitro rumen models, one batch system and two semi-continuous systems, to each other and to the donor animal from which the rumen fluid was obtained to determine the ability of each system to maintain an environment capable of supporting natural rumen microbiota and function. The three engineered systems were run in parallel for five days using an initial inoculum, sourced from a single donor animal, and parameters including temperature, pH, gas production, VFA production, and microbiome assemblage were evaluated. Understanding both the limitations and the advantages of each system is necessary to improve such artificial models, increase the reliability of each, and optimize their usage for testing the effects of potential methane-mitigating substrates for use in ruminant agriculture.

2. Materials and Methods

2.1. Rumen Fluid Collection

To inoculate the engineered rumen systems and collect daily samples for comparison to the artificial models, rumen content was collected from a rumen-fistulated Holstein cow housed at the UC Davis Dairy Teaching and Research Facility. The donor animal was fed a dry cow total mixed ration (TMR) composed of 46.7% wheat hay, 30% alfalfa hay, 20% almond hulls, and 3.3% mineral pellet (Supplemental Table S1). Rumen content collection was performed in accordance with the Institution of Animal Care and Use Committee (IACUC) at the University of California, Davis, under protocol number 21,117. Four liters of rumen fluid and 90 g of rumen solids were collected prior to morning feeding on the first day of the experiment. Solids were collected by hand from the rumen and placed in a separate sterile container from the fluid for transport to the lab. Approximately 100 mL of rumen fluid was collected every morning in 24 h increments for five days from the same animal for comparative VFA and microbiome analysis with the in vitro and in vivo system. Rumen content was collected via a fistula and using a perforated PVC pipe, 500 mL syringe, and Tygon tubing (Saint-Gobain North America, Malvern, PA, USA). The collected rumen samples were immediately strained through cheesecloth into two 4 L pre-warmed vacuum-insulated containers. Redox potential and pH of the collected rumen content were recorded at the dairy, and then containers were transported to the laboratory.

2.2. Feed Collection and Preparation

The same dry cow total mixed ration (TMR) fed to the donor Holstein cow was used as substrate in the in vitro rumen systems to control for diet differences. The feed was provided by and collected from the UC Davis Dairy Teaching and Research Facility, with all components mixed as they are normally presented to the pen (Supplemental Table S1). Feed was ground in the laboratory using an Oster 14-Speed blender (SunBeam, Boca Raton, FL, USA), mixed, and subsequently dried at 55 °C for 72 h using a Model 10 Quincy Lab Oven (Quincy Lab Inc, Chicago, IL, USA). Feed was stored in airtight containers at 4 °C until used over the course of this experiment.

2.3. In Vitro Rumen Systems

Three in vitro models (Figure 1) simulating the rumen were set up and run in parallel for 120 h in the laboratory. The first model was the Ankom RF Gas Production System (Ankom Technology RF Gas Production System, Macedon, NY, USA) in a 300 mL vessel (Figure 1A). The second was a semi-continuous fermentation system, using 1 L Polypropy-

lene (PP) vessels (Figure 1B), based on the rumen simulation technique (RUSITEC) developed by Czerkawski and Breckenridge [19]. The third was a semi-continuous fermentation system, also based on the RUSITEC, in which the 1 L PP vessels were replaced with vessels manufactured from tri-clamp food-grade stainless steel (Figure 1C).

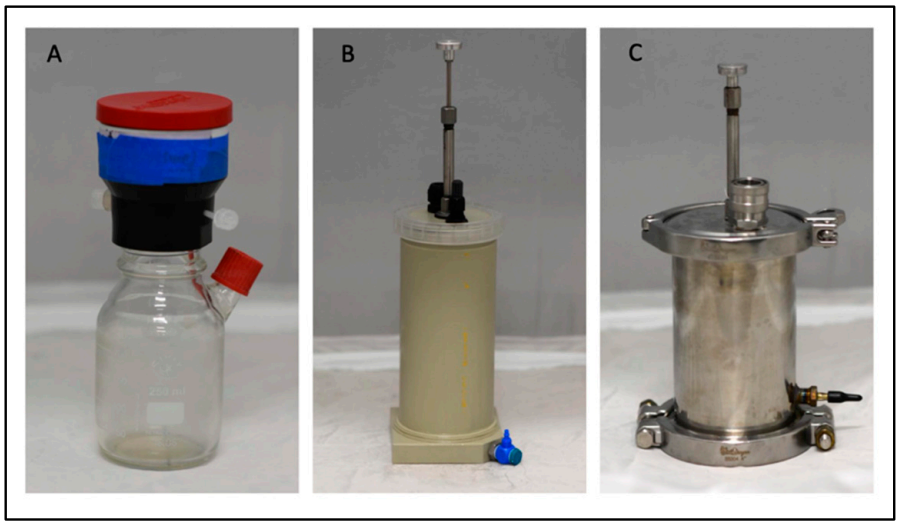

Figure 1. Vessels for in vitro rumen fermentation. Ankom batch fermentation system (**A**). Polypropylene fermentation vessel of the RUSITEC PP system (**B**) and stainless steel fermentation system for the RUSITEC Prime platform (**C**).

2.4. Experimental Design

Rumen fluid was collected from the animal at 8 a.m. after feeding and was used to inoculate all vessels of each model within 1 h after the rumen fluid was collected. Experiments in the Ankom-based systems and both RUSITEC-based systems were run in triplicate (Figure 2).

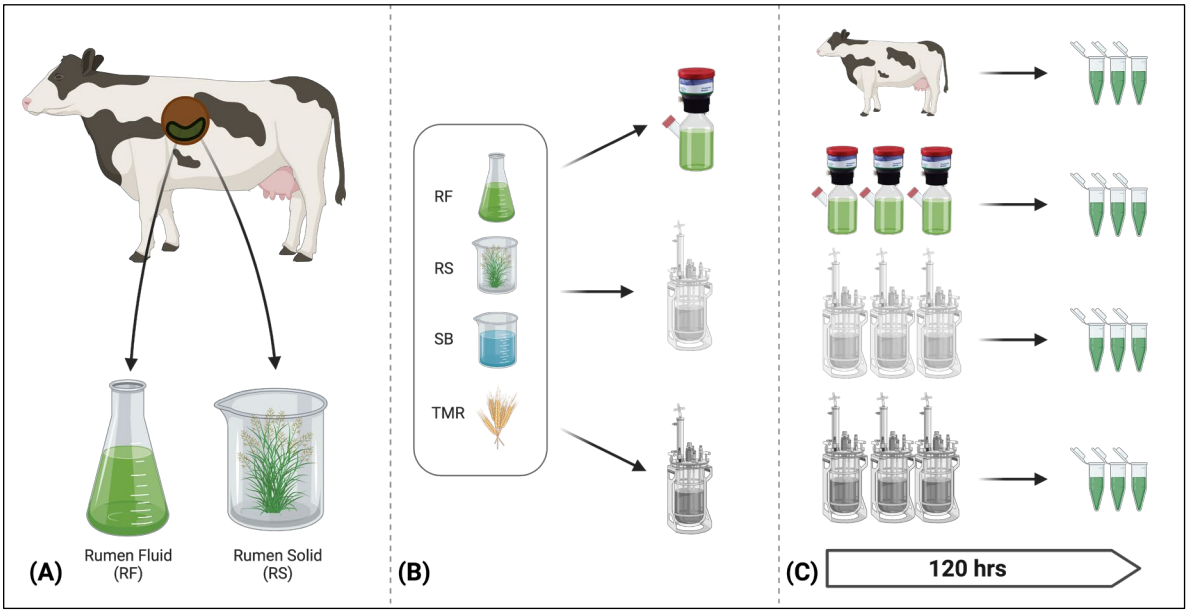

Figure 2. Experimental Outline. (**A**) Rumen fluid (RF) and solid (RS) were collected from a fistulated Holstein cow and (**B**) mixed with saliva buffer (SB) and total mixed ration (TMR). Aliquots of the rumen content/buffer/feed mix were transferred to the three different artificial rumen systems under evaluation. (**C**) Rumen content/buffer/feed mix was performed in triplicates at 37 °C for a total of 120 h. Aliquots for subsequent data collection and analyses were collected from the individual vessels and the donor animal every 24 h.

2.5. Ankom Gas Production System

ANKOM systems were run according to protocols previously established by the Hess lab [11]. Each 300 mL vessel contained 150 mL of rumen fluid and 50 mL of an artificial saliva buffer [23] and received feed and solids at a ratio of 1 g feed or solid per 75 mL of fluids, resulting in 2.67 g of rumen solids and 2.67 g of the ground dry cow TMR in individual concentrate bags. Each vessel was fitted with an Ankom head unit, with a foil gas bag (Restek, Bellefonte, PA, USA) fitted to the pressure release valve to collect produced gasses. Vessel content was kept at average rumen temperature by placing the vessels in a circulating water bath at 37 °C.

2.6. RUSITEC, Polypropylene (PP) Vessels

RUSITEC methods were adapted from Czerkawski and Breckenridge [19]. Three PP RUSITEC vessels were each filled with 562.5 mL of rumen fluid and 187.5 mL of the artificial saliva buffer, totaling 750 mL. These vessels received 10 g of rumen solids and 10 g of the same dry cow TMR. Individual vessels received 0.39 mL/min of sterile artificial saliva buffer via individual tubing and a peristaltic pump throughout the experiment. Gas produced was captured in foil gas bags attached to each individual reactor. Effluent fluid was collected into individual overflow vessels, and these overflow vessels were chilled in ice to prevent the production of additional fermentation products. RUSITEC vessels were incubated in a 37 °C water bath, and contents were mixed continuously via piston agitation to simulate rumen conditions.

2.7. RUSITEC Prime, Stainless Steel Vessels

All three stainless steel RUSITEC vessels were incubated under the same conditions and received uniform amounts of rumen fluid, rumen solids, saliva buffer, and feed equal to the PP vessels mentioned above.

2.8. Sample Collection

Conductivity, pH, and temperature measurements were taken every 24 h with an OHAUS ST300 portable pH meter that was calibrated on a three-point curve using OHAUS standards (OHAUS, Parsippany, NJ, USA). Vessels were fed every 24 h, and each feed bag remained in the designated vessel for a total of 48 h to simulate rumen retention time and ensure the fiber-adherent fraction of the microbiome had sufficient time to transfer to the new feed. Liquid and gas samples were collected daily in 24 h increments post feeding, with samples from the animal collected at 8 a.m. post feeding. Sample collection from vessels was performed at 24 h increments at 9:30 a.m. Fluid samples from each vessel were collected in triplicate in 1.5 mL tubes, flash frozen in liquid nitrogen, and stored at −80 °C until further processed. After each feeding and fluid collection, vessels were individually purged with N_2 gas to maintain anaerobic conditions. Gas bags were also collected every 24 h for total gas production analysis and CH_4 and CO_2 concentrations. Gas volumes were measured by manual expulsion of each bag through a flow meter (MGC-1 V3.3, Ritter, Bochum, Germany).

2.9. Volatile Fatty Acid and Greenhouse Gas Analysis

To analyze volatile fatty acid concentrations, rumen fluid samples were prepared by using 1/5th volume of 25% metaphosphoric acid and centrifuging. After centrifugation at 2000 rpm for 10 min (Eppendorf Centrifuge 5417C, Eppendorf, Hamburg, Germany), the supernatant was filtered through a 0.22 µm filter and stored in autosampler vials at 4 °C until analysis. The GC conditions were as follows: analytical column RESTEK Rxi®, 5 ms (30 m × 0.25 mm I.D. × 0.25 µm) film thickness; the oven temperature was set to 80 °C for 0.50 min and followed by a 20 °C/min ramp rate until 200 °C, holding the final temperature for 2 min; carrier gas was high-purity helium at a flow rate of 2.0 mL/min; and the FID was held at 250 °C. A 1 µL sample was injected through Split/Splitless Injectors (SSL), with the injector base temperature set at 250 °C. Split flow and split ratio were programmed at 200

and 100 mL/min, respectively. To develop calibration curves, certified reference standards (RESTEK, Bellefonte, PA, USA) were used. All analyses were performed using a Thermo TriPlus Autosampler and Thermo Trace GC Ultra (Thermo Electron Corporation, Rodano Milan, Italy).

Methane and CO_2 were measured using an SRI Gas Chromatograph (8610C, SRI, Torrance, CA, USA) fitted with a $3' \times 1/8''$ stainless steel Haysep D column and a flame ionization detector with methanizer (FID-met). The oven temperature was held at 90 °C for 5 min. Carrier gas was high-purity hydrogen at a flow rate of 30 mL/min. The FID was held at 300 °C. A 1 mL sample was injected directly onto the column. Calibration curves were developed with an Airgas certified CH_4 and CO_2 standard (Airgas USA, Sacramento, CA, USA).

2.10. Statistics

Prism 9 Version 9.4.1 (GraphPad, Boston, MA, USA) was used to analyze and graph the data. More specifically, ROUT was used to test for the presence of outliers, including multiple outliers, that were present in the datasets [24]. Due to the single data point for the in vivo sample, in vivo data were excluded from outlier testing. Two-way ANOVA was used to evaluate the differences in the basic parameters (pH and redox), the VFA profiles, and the gas production data.

2.11. DNA Extractions

DNA extractions were performed with 300 uL of each fluid sample using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following manufacturer's directions. Extracted DNA quantity and quality were evaluated using a nanodrop (Thermo Scientific Nanodrop 2000, ThermoFisher Scientific, Pleasanton, CA, USA), and then, DNA was stored at −20 °C until PCR amplification and sequencing.

2.12. PCR Amplification, Library Preparation, and Sequencing

PCR amplification, library preparation, and sequencing were performed by the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory (Lemont, IL, USA) according to the following protocol. Briefly, PCR amplicon libraries targeting the 16S rRNA encoding gene present in metagenomic DNA were produced using a barcoded primer set adapted for the Illumina HiSeq2000 and MiSeq [25]. DNA sequence data were generated using Illumina paired-end sequencing at the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory. Specifically, the V4 region of the 16S rRNA gene (515F-806R) was PCR-amplified with region-specific primers that include sequencer adapter sequences used in the Illumina flow cell [25,26]. 515F: AATGATAC-GGCGACCACCGAGATCTACACGCTXXXXXXXXXXXXTATGGTAATTGTG TGYCAGCMGCCGCGTAA; 806R: CAAGCAGAAGACGGCATACGAGATAGTCAGCCAG CCGG-ACTACNVGGGTWTCTAAT. The forward amplification primer contained a twelve-base barcode sequence to support pooling of up to 2167 different samples in each lane [25,26]. Each 25 µL PCR reaction contained 9.5 µL of MO BIO PCR Water (Certified DNA-Free, Mo bio, Carlsbad, CA, USA), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2× concentration, 1× final, (Quanta Bio, Beverly, MA, USA)), 1 µL Golay barcode-tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. The conditions for PCR were as follows: 94 °C for 3 min to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were then quantified using PicoGreen (Invitrogen, Waltham, MA, USA) and a plate reader (InfiniteÒ 200 PRO, Tecan, Männedorf, Switzerland). Once quantified, volumes of each of the products were pooled into a single tube so that each amplicon was represented in equimolar amounts. This pool was then cleaned up using AMPure XP Beads (Beckman Coulter, Brea, CA, USA) and then quantified using a fluorometer (Qubit, Invitrogen, Waltham, MA, USA). After quantification, the molarity of the pool was deter-

mined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike for sequencing on the Illumina MiSeq (Illumina, San Diego, CA, USA). Amplicons were sequenced on a 251bp × 12bp × 251bp MiSeq run using customized sequencing primers and procedures [25]. Genomic DNA was amplified using an ITS barcoded primer set, adapted for the Illumina HiSeq2000 and MiSeq [27]. ITS1f: AATGATACGGCGACCACCGAGATCTACACGGCTTGGTCATTTAGAGGAA-GTAA; ITS2: CAAGCAGAAGACGGCATACGAGATNNNNNNNNNNCGGCTGCGTT-CTTCATCGATGC. Each 25 µL PCR reaction contained 9.5 µL of MO BIO PCR Water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2× concentration, 1× final), 1 µL Golay barcode-tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. The conditions for PCR were also as follows: 94 °C for 3 min to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were quantified using PicoGreen (Invitrogen) and a plate reader. Once quantified, different volumes of each of the products were pooled into a single tube so that each amplicon was represented equally. This pool was then cleaned up using AMPure XP Beads (Beckman Coulter, Indianapolis, ID, USA) and then quantified using a fluorometer (Qubit, Invitrogen, Waltham, MA, USA). After quantification, the molarity of the pool was determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike for 2 × 251 bp sequencing on the Illumina MiSeq.

2.13. Microbiome Analysis

Qiime2 2022.2 [28] was used to process the raw reads, assign taxonomy, and perform diversity analyses. Reads were demultiplexed and quality-filtered using the q2-demux plugin [25]. DADA2 [29], along with R 4.1.3 [30], was used to denoise the quality-filtered sequences and pair the 16S reads. Internal transcribed spacer (ITS) sequences were run unpaired with the forward reads only, as the reverse-read quality of ITS reads was too low for pairing. Denoised sequences were aligned and sorted into amplicon sequence variants (ASVs) using MAFFT Fast Tree [31] and assigned taxonomy using SciKit-learn [32]. Bacterial 16S sequences were classified using the SILVA 138.1 database at 99% sequence identity [33], and fungal ITS sequences were classified using the UNITE V8.3 database at 99% sequence identity [34]. Bray–Curtis and Weighted Unifrac plots (Supplemental Figures S1–S4) were generated using the q2-diversity plugin and visualized using QIIME2view. Faith's PD and Pielou's evenness diversity analyses were calculated using the q2-diversity plugin and the MAFFT Fast Tree output [35,36].

3. Results

The pH and redox potential of the fluid samples from the in vivo sample and the in vitro vessels were measured every 24 h for a total incubation time of 120 h (Figure 3). Whereas the in vivo pH remained steady at 7.08 (SD ± 0.11) throughout the experiment, the pH value within the Ankom systems declined from 6.3 to 5.4 (average SD ± 0.05) over the incubation period. Both the semi-continuous RUSITEC systems maintained a consistent pH (Figure 3A). The redox potential of the rumen fluid in vivo remained negative, ranging between −13 mV and −1 mV, and the redox potential for all in vitro was in the positive range ($p < 0.0001$) (Figure 3B). The batch (Ankom) system displayed the most divergence from in vivo measurements (−9.4 mV; SD ± 6.84), with a vessel average of 74.6 mV (SD ± 3.10) over the total incubation period. Both the semi-continuous RUSITEC systems maintained lower redox potentials than the Ankom system but still diverged from the in vivo measurements, with an average of 24.86 mV (SD ± 2.68) for the RUSITEC PP vessels and 24.66 mV (SD ± 1.48) for RUSITEC prime reactors. A detailed breakdown of the pH and redox potential measurements is provided in Supplemental Table S2.

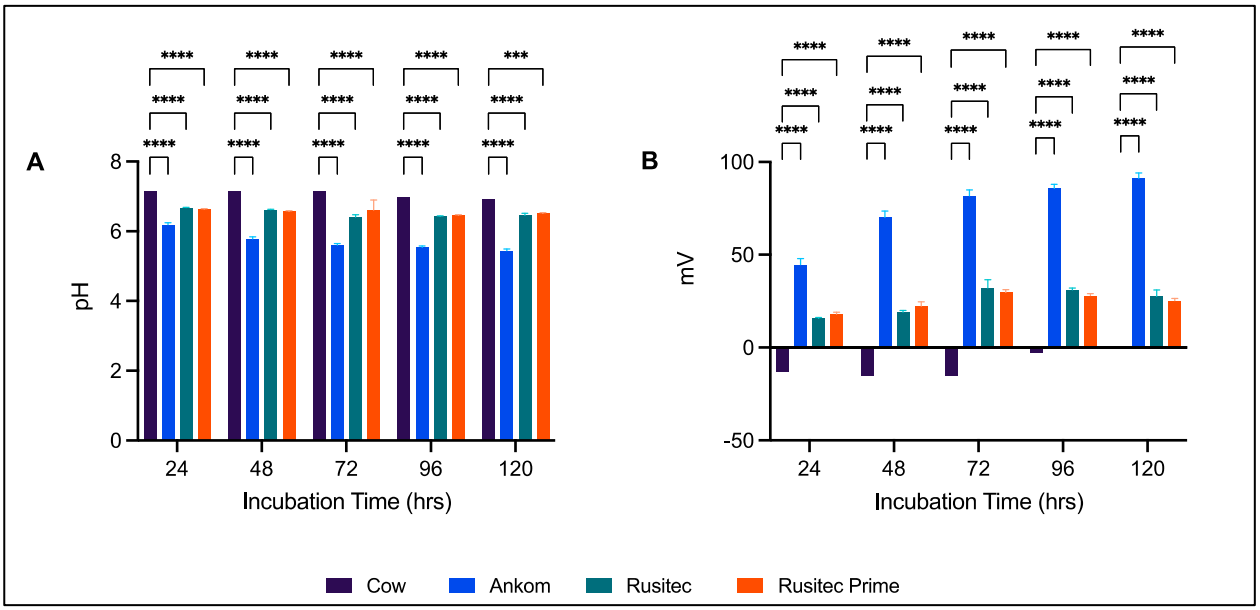

Figure 3. Physical fermentation parameters. Content of the in vitro rumen systems and the cow's rumen were collected in 24 h intervals, and pH (**A**) and conductivity (**B**) of the contents were determined. Statistical significance: *** = $p < 0.0005$; **** = $p < 0.00005$.

The temperature of the vessel fluid and of the rumen fluid was recorded every 24 h (Supplemental Table S3). The rumen fluid temperature of the donor animal maintained a consistent temperature of ~39 °C, and the temperatures of the rumen fluid in all in vitro systems was lower. The average temperatures for the Ankom vessels were between 32.6 °C and 35.5 °C, between 34.3 °C and 35.8 °C, and between 33.4 °C and 36.1 °C for the RUSITEC PP and RUSITEC Prime vessels, respectively.

3.1. Gas Production

Carbon dioxide (CO_2) and methane (CH_4) were measured for each in vitro vessel at 24 h intervals over a total of 120 h (Figure 4). The Ankom systems displayed a notable level of variation for both CO_2 and CH_4 production throughout the entire incubation period (Figure 4A,B). The gas production within the Ankom vessels differed significantly from the gas production in the RUSITEC vessels, with significant differences after 24 h and 72 h for CO_2 and after 24 h for CH_4 ($p < 0.05$). The variation in CO_2 and CH_4 between the Ankom vessels was higher than the variation within the other platforms across all time points. At 24 h, the standard deviation for the Ankom vessel's CO_2 production was 11.892 mL/g*dry matter, compared with 2.048 mL/g*dry matter for the RUSITEC PP and 1.047 mL/g*dry matter for the RUSITEC prime. On average, the deviation in the gas concentrations of both CO_2 and CH_4 within each platforms' replicates was lowest amongst the RUSITEC prime vessels. Both the RUSITEC systems displayed an increase in CO_2 output between 48 h and 120 h, following a consistent production between the initiation of the experiment (0 h) and 48 h. A drop in the CH_4 production was observed for both RUSITEC systems after 48 h with consistent, but low, levels of CH_4 after 72, 96, and 120 h. A detailed breakdown of the CO_2 and CH_4 measurements are provided in Supplemental Table S4.

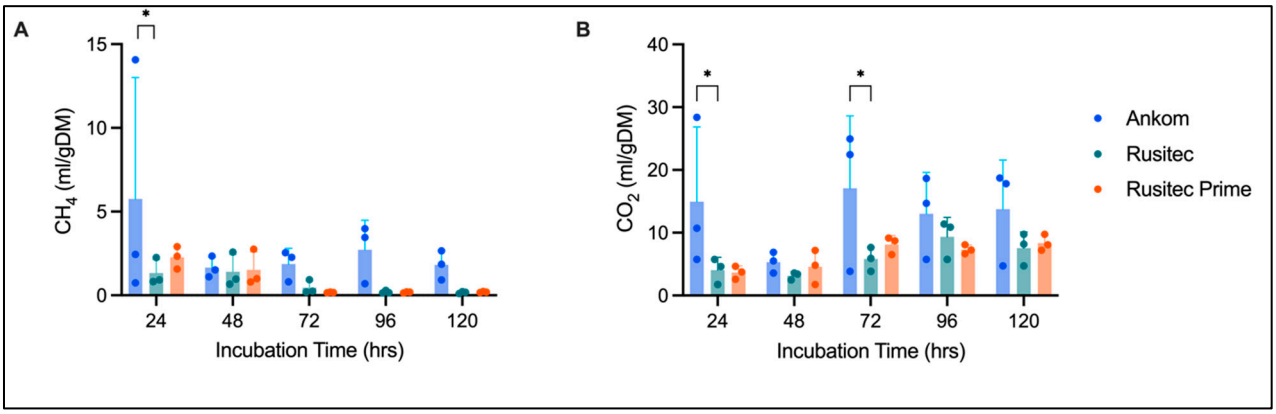

Figure 4. Gas production profiles. Methane (CH_4) (**A**) and carbon dioxide (CO_2) (**B**) production during in vitro fermentation was determined in 24 h intervals via gas chromatography. Statistical significance: * = $p < 0.05$.

3.2. VFA Comparison

Volatile fatty acid (VFA) profiles were analyzed for each in vitro vessel and the in vivo sample at 24 h intervals over 120 h (Figure 5). The total VFA concentration was transiently significant ($p < 0.05$) between the rumen and the Ankom system at 24, 48, and 120 h (Figure 5A). The VFA concentrations within the RUSITEC PP and RUSITEC prime reactors also differed significantly ($p < 0.0005$ and $p < 0.05$, respectively) from the VFA concentrations that were observed in vivo at 24 h. The total VFA concentration in the Ankom units increased over the incubation period and fluctuated slightly at the beginning of the experiment in the RUSITEC systems, but total VFAs reached equilibrium after 72 h in the RUSITEC systems (Figure 5A). Isobutryic acid was generally the most abundant VFA across the cow and all vessels, and propionic acid was the second most abundant. Acetic acid concentrations followed a similar trend to the total VFA concentrations over the entire experiment, with a steady increase in the Ankom units and stabilization after 72 h in both RUSITEC systems. Significant differences between in vivo samples and all three in vitro platforms were detected 24 h after the initiation of rumen fermentation, with $p < 0.05$, $p < 0.005$, $p < 0.05$ for Ankom, RUSITEC PP, RUSITEC prime, respectively. Importantly, the differences were no longer significant after 48 h (Figure 5B). Isovaleric acid and propionic acid production within the Ankom reactors differed significantly from the in vivo production after 72, 96, and 120 h (Figure 5E,F). The isobutyric and valeric acid profiles differed only at 24 and 48 h (Figure 5D,G). Valeric acid displayed great variation at 48 h across the different vessels, including between individual vessels within the same system, but appeared to reach more of an equilibrium at 72 h and beyond. There was a high single-vessel variation at the 48 h timepoint. In summary, both of the RUSITEC platforms maintained VFA profiles over the 120 h incubation period, with the in vitro VFA profiles relatively consistent with the in vivo VFA profiles. A detailed breakdown of the VFA measurements is provided in Supplemental Table S5.

3.3. 16S rRNA Gene Sequencing Results

A total of 6,565,549 reads were generated from a total of 49 samples, with a mean of 133,991 (SD ± 41,962) reads and a minimum sample read count of 25,840. The DNA from one RUSITEC PP vessel at 120 h consistently failed to pass quality control and therefore had to be excluded from further analysis. After quality filtering, pairing, and removal of chimeric sequences, an average of 78.48% (SD ± 3.78) of reads remained per sample (Supplemental Table S6). The in vivo rumen bacterial community was stable across the 120 h study period and was primarily composed of Firmicutes (33.59% SD ± 3.79) and Bacteroidota (52.98% SD ± 3.50) (Table 1, Figure 6A). The Ankom vessels maintained similar bacterial profiles at 24 and 48 h, but members belonging to Firmicutes dominated the community at 72 h and beyond, contributing >90% of the prokaryotic reads at 120 h

(Table 1, Figure 6A). A detailed breakdown of the prokaryotic population during in vivo and in vitro fermentation and its temporal succession is summarized in Table 1.

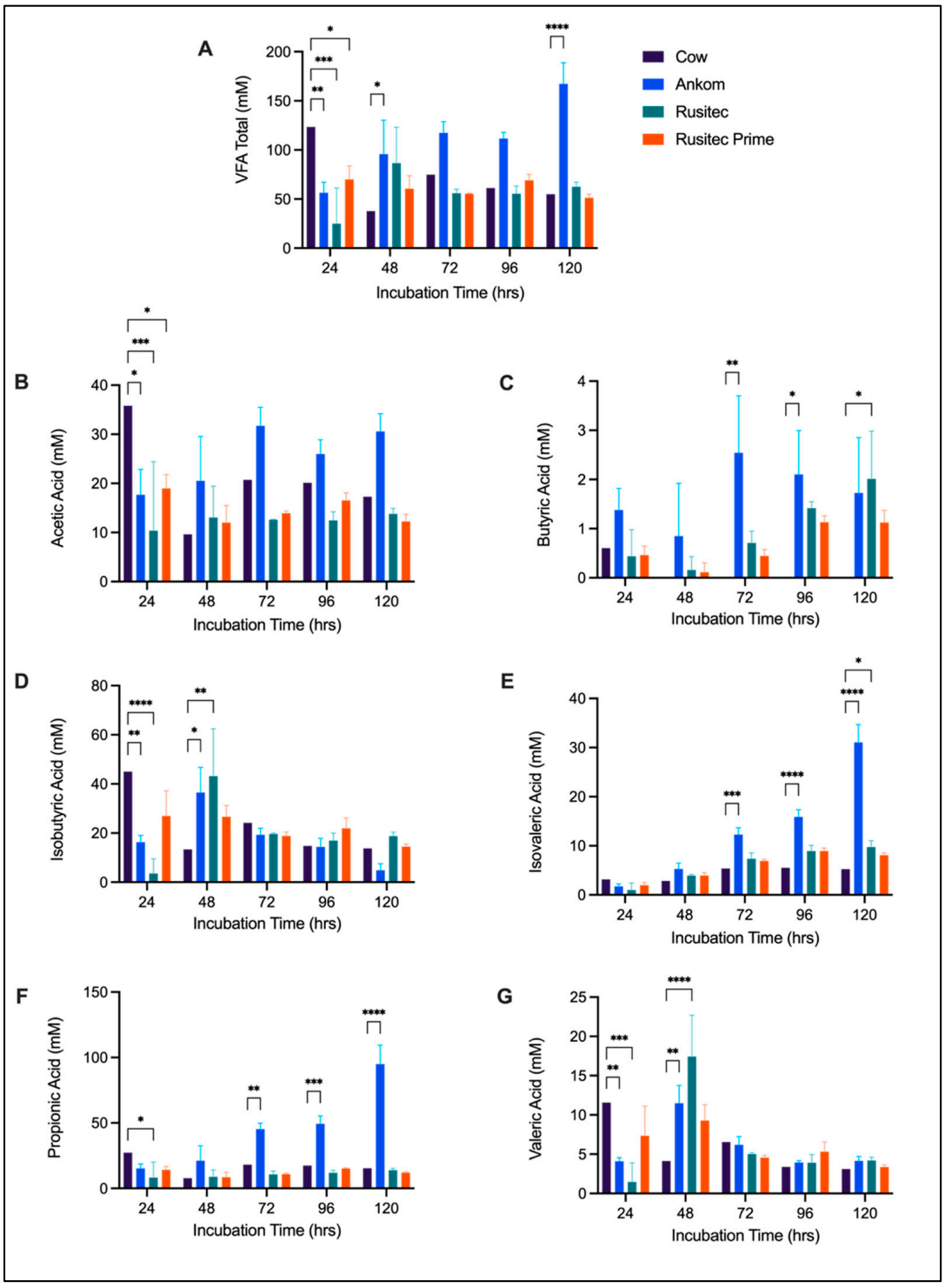

Figure 5. Volatile fatty acid profiles. Total volatile fatty acid production (**A**), as well as production of acetic acid (**B**), butyric acid (**C**), isobutyric acid (**D**), isovaleric acid (**E**), propionic acid (**F**), and valeric acid (**G**) were determined every 24 h over a total period of 120 h. Statistical significance: * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$, **** = $p < 0.00005$.

Table 1. Mean and standard deviation of relative abundances of prokaryotic phyla.

	Firmicutes	*Bacteroidota*	*Proteobacteria*	*Verrucomicrobiota*	*Euryarchaeota*	*Thermoplasmatota*	*Spirochaetota*	*Fibrobacterota*	*Patescibacteria*	*Cyanobacteria*	*Actinobacteriota*	*Chloroflexi*
Cow-24 h [1]	34.34238	52.09	0.81	4.18	1.95	0.27	1.34	1.32	0.76	1.45	0.27	0.53
Cow-48 h [1]	30.95768	58.68	0.52	2.33	1.01	0.32	2.02	0.89	0.90	1.28	0.23	0.28
Cow-72 h [1]	37.34452	50.87	0.70	2.27	1.34	0.41	2.68	1.20	0.88	0.84	0.24	0.41
Cow-96 h [1]	36.784	49.67	0.76	2.99	1.71	0.36	2.27	1.58	0.97	1.34	0.23	0.38
Cow-120 h [1]	28.51376	53.59	1.37	4.42	1.63	0.55	3.08	1.98	0.86	2.69	0.13	0.35
Cow-Total [2]	33.59 ± 3.79	52.98 ± 3.5	0.83 ± 0.32	3.24 ± 1.01	1.53 ± 0.36	0.38 ± 0.11	2.28 ± 0.66	1.39 ± 0.41	0.87 ± 0.08	1.52 ± 0.7	0.22 ± 0.05	0.39 ± 0.09
Ankom-24 h	18.39 ± 1.68	62.39 ± 2.14	2.78 ± 0.79	9.96 ± 0.94	1.09 ± 0.33	0.54 ± 0.11	0.67 ± 0.11	0.07 ± 0.04	0.59 ± 0.09	2.44 ± 0.19	0.15 ± 0.06	0.28 ± 0.05
Ankom-48 h	41.87 ± 3.64	37.1 ± 2.45	1.71 ± 0.22	10.69 ± 1.02	2.61 ± 0.27	0.24 ± 0.09	0.95 ± 0.27	0.04 ± 0.01	0.28 ± 0.04	1.81 ± 0.48	0.71 ± 0.03	1.16 ± 0.05
Ankom-72 h	70.25 ± 6.23	13.67 ± 5.14	1.13 ± 0.41	7.18 ± 0.97	2.47 ± 0.38	0.12 ± 0.04	0.66 ± 0.29	0.02 ± 0	0.23 ± 0.06	1.08 ± 0.49	1.2 ± 0.2	1.26 ± 0.1
Ankom-96 h	82.51 ± 0.77	6.47 ± 1.11	0.68 ± 0.08	3.86 ± 0.43	2.2 ± 0.23	0.11 ± 0.07	0.47 ± 0.14	0.01 ± 0.01	0.17 ± 0.06	0.9 ± 0.43	1.12 ± 0.22	0.96 ± 0.2
Ankom-120 h	91.82 ± 2.01	2.71 ± 0.78	0.17 ± 0.03	1.61 ± 0.42	1.47 ± 0.54	0.05 ± 0.04	0.18 ± 0.11	0 ± 0	0.07 ± 0.04	0.44 ± 0.12	0.85 ± 0.11	0.44 ± 0.07
Ankom-Total [2]	81.52 ± 9.93	7.62 ± 5.51	0.66 ± 0.47	4.22 ± 2.49	2.05 ± 0.57	0.09 ± 0.06	0.44 ± 0.27	0.01 ± 0.01	0.16 ± 0.08	0.8 ± 0.44	1.05 ± 0.23	0.88 ± 0.38
RUSITEC PP-24 h	30.26 ± 4.35	54.37 ± 2.54	2.23 ± 0.78	5.53 ± 0.85	1.46 ± 0.68	1.37 ± 0.39	0.61 ± 0.13	0.04 ± 0.03	0.84 ± 0.08	1.27 ± 0.73	0.11 ± 0.05	0.56 ± 0.22
RUSITEC PP-48 h	35.55 ± 1.12	41.99 ± 0.68	3.64 ± 0.28	5.16 ± 0.51	1.26 ± 0.01	2.43 ± 0.39	3.27 ± 0.68	0.82 ± 0.46	0.7 ± 0.05	1.84 ± 0.38	0.18 ± 0.07	0.98 ± 0.12
RUSITEC PP-72 h	45.06 ± 8.38	35.72 ± 3.82	5.23 ± 1.86	3.47 ± 2.25	0.97 ± 0.4	1.9 ± 0.9	2.47 ± 0.96	0.78 ± 0.33	0.23 ± 0.12	1.65 ± 1.2	0.21 ± 0.13	0.88 ± 0.36
RUSITEC PP-96 h	48.26 ± 9.7	34.75 ± 6.67	5.29 ± 1.39	2.01 ± 0.14	0.53 ± 0.15	1.56 ± 0.61	2.5 ± 1.07	1.14 ± 0.66	0.06 ± 0.03	2.16 ± 1.73	0.32 ± 0.08	0.56 ± 0.12
RUSITEC PP-120 h	42.13 ± 1.28	40.43 ± 1.29	3.64 ± 0.49	1.5 ± 0.05	0.56 ± 0.08	1.48 ± 0.08	2.7 ± 0.23	1.1 ± 0.01	0.03 ± 0.01	4.07 ± 0.51	0.58 ± 0.07	0.68 ± 0.03
RUSITEC PP-Total [2]	40.12 ± 8.79	41.53 ± 8.18	4.03 ± 1.57	3.68 ± 1.9	0.98 ± 0.5	1.77 ± 0.63	2.28 ± 1.15	0.75 ± 0.54	0.4 ± 0.35	2.06 ± 1.27	0.26 ± 0.17	0.74 ± 0.25
RUSITEC Prime-24 h	23.89 ± 3.29	57.27 ± 2.73	3.47 ± 0.44	6.62 ± 0.29	1.41 ± 0.74	1.59 ± 0.56	0.69 ± 0.16	0.19 ± 0.05	1 ± 0.1	2.12 ± 0.2	0.06 ± 0.04	0.42 ± 0.1
RUSITEC Prime-48 h	32.5 ± 2.3	42.9 ± 2	4.74 ± 0.23	8.32 ± 0.65	1.55 ± 0.4	1.83 ± 0.18	2.09 ± 0.19	0.5 ± 0.22	0.91 ± 0.04	1.95 ± 0.08	0.19 ± 0.06	0.68 ± 0.1
RUSITEC Prime-72 h	39.5 ± 3.89	36.33 ± 1.2	4.76 ± 1.18	7.4 ± 2.32	1.34 ± 0.3	1.93 ± 0.54	2.6 ± 0.61	1.32 ± 1.5	0.43 ± 0.14	1.65 ± 0.45	0.22 ± 0.07	1.14 ± 0.22
RUSITEC Prime-96 h	44.16 ± 1.49	33.73 ± 2.43	4.2 ± 0.11	6.2 ± 1.2	1.61 ± 0.51	2.22 ± 0.73	2.46 ± 0.13	0.66 ± 0.24	0.25 ± 0.06	1.12 ± 0.25	0.21 ± 0.08	1.5 ± 0.46
RUSITEC Prime-120 h	47.1 ± 2.37	32.75 ± 1.08	4.97 ± 0.82	4.48 ± 0.25	1.14 ± 0.27	2.38 ± 0.45	2.86 ± 0.19	0.66 ± 0.09	0.09 ± 0.02	0.85 ± 0.57	0.27 ± 0.01	0.97 ± 0.34
RUSITEC Prime-Total [2]	37.43 ± 8.99	40.6 ± 9.53	4.43 ± 0.81	6.6 ± 1.68	1.41 ± 0.44	1.99 ± 0.53	2.14 ± 0.84	0.67 ± 0.7	0.54 ± 0.38	1.54 ± 0.58	0.19 ± 0.09	0.94 ± 0.45

Table 1. *Cont.*

	Desulfobacterota	*Elusimicrobiota*	*Synergistota*	*Planctomycetota*	*Bdellovibrionota*	*Unassigned*	*WPS-2*	*SAR324_clade(Marine_group_B)*	*Campilobacterota*	*Armatimonadota*	**<0.1%**
Cow-24 h [1]	0.18	0.21	0.01	0.08	0.01	0.18	0.00	0.00	0.01	0.00	0.01
Cow-48 h [1]	0.25	0.12	0.02	0.06	0.01	0.09	0.01	0.00	0.00	0.00	0.00
Cow-72 h [1]	0.37	0.16	0.03	0.08	0.02	0.13	0.00	0.01	0.00	0.01	0.00
Cow-96 h [1]	0.41	0.17	0.03	0.10	0.01	0.17	0.00	0.02	0.00	0.00	0.00
Cow-120 h [1]	0.22	0.32	0.04	0.07	0.01	0.14	0.00	0.01	0.01	0.00	0.00
Cow-Total [2]	0.29 ± 0.1	0.19 ± 0.08	0.03 ± 0.01	0.08 ± 0.02	0.01 ± 0	0.14 ± 0.04	0 ± 0	0.01 ± 0.01	0 ± 0	0 ± 0	0 ± 0
Ankom-24 h	0.13 ± 0.03	0.28 ± 0.03	0.03 ± 0.01	0.05 ± 0.03	0.02 ± 0.02	0.11 ± 0.02	0 ± 0.01	0.02 ± 0	0 ± 0	0 ± 0	0 ± 1
Ankom-48 h	0.29 ± 0.12	0.24 ± 0.06	0.05 ± 0.01	0.11 ± 0.02	0.01 ± 0	0.08 ± 0.01	0 ± 0	0.04 ± 0.01	0 ± 0	0.01 ± 0	0 ± 0
Ankom-72 h	0.37 ± 0.17	0.14 ± 0.01	0.08 ± 0.03	0.05 ± 0.02	0 ± 0	0.04 ± 0.02	0 ± 0	0.02 ± 0	0 ± 0	0.01 ± 0	0 ± 1
Ankom-96 h	0.29 ± 0.22	0.06 ± 0.03	0.07 ± 0.04	0.06 ± 0.01	0 ± 0	0.04 ± 0.01	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0.01	0 ± 0
Ankom-120 h	0.12 ± 0.05	0.03 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0 ± 0	0.02 ± 0.02	0 ± 0	0 ± 0.01	0 ± 0	0 ± 0	0 ± 0
Ankom-Total [2]	0.26 ± 0.18	0.08 ± 0.05	0.06 ± 0.04	0.04 ± 0.02	0 ± 0	0.03 ± 0.02	0 ± 0	0.01 ± 0.01	0 ± 0	0.01 ± 0.01	0 ± 0
RUSITEC PP-24 h	0.4 ± 0.12	0.27 ± 0.21	0.06 ± 0.01	0.16 ± 0.04	0.17 ± 0.07	0.18 ± 0.03	0 ± 0.01	0.06 ± 0.02	0.01 ± 0	0.01 ± 0.01	0.01 ± 0.01
RUSITEC PP-48 h	0.57 ± 0.28	0.65 ± 0.07	0.23 ± 0.07	0.18 ± 0.05	0.14 ± 0.05	0.25 ± 0.03	0.01 ± 0.01	0.06 ± 0.02	0.02 ± 0	0.01 ± 0.01	0.01 ± 0.01
RUSITEC PP-72 h	0.27 ± 0.1	0.35 ± 0.22	0.16 ± 0.1	0.16 ± 0.1	0.04 ± 0.03	0.25 ± 0.24	0.06 ± 0.07	0.04 ± 0.01	0.02 ± 0	0 ± 0.01	0.01 ± 0.01
RUSITEC PP-96 h	0.16 ± 0.05	0.21 ± 0.1	0.07 ± 0.03	0.11 ± 0.04	0.01 ± 0	0.15 ± 0.15	0.09 ± 0.05	0.02 ± 0.02	0.01 ± 0.01	0 ± 0	0 ± 0
RUSITEC PP-120 h	0.23 ± 0.08	0.32 ± 0.18	0.08 ± 0.02	0.13 ± 0.02	0.01 ± 0	0.16 ± 0.06	0.12 ± 0.04	0.02 ± 0	0.02 ± 0	0 ± 0	0 ± 0
RUSITEC PP-Total [2]	0.33 ± 0.2	0.36 ± 0.21	0.12 ± 0.09	0.15 ± 0.06	0.08 ± 0.08	0.2 ± 0.12	0.05 ± 0.06	0.04 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
RUSITEC Prime-24 h	0.25 ± 0.07	0.4 ± 0.07	0.05 ± 0	0.13 ± 0.01	0.21 ± 0.08	0.15 ± 0.02	0.01 ± 0.01	0.05 ± 0.01	0.01 ± 0	0.01 ± 0.01	0 ± 0
RUSITEC Prime-48 h	0.35 ± 0.12	0.58 ± 0.05	0.17 ± 0.04	0.18 ± 0.03	0.19 ± 0.06	0.19 ± 0.03	0.01 ± 0	0.06 ± 0.01	0.05 ± 0.01	0.01 ± 0	0 ± 0
RUSITEC Prime-72 h	0.23 ± 0.16	0.42 ± 0.13	0.17 ± 0.09	0.22 ± 0.08	0.07 ± 0.06	0.13 ± 0.05	0.01 ± 0.02	0.04 ± 0.02	0.04 ± 0.01	0.01 ± 0	0 ± 0
RUSITEC Prime-96 h	0.39 ± 0.15	0.23 ± 0.1	0.18 ± 0.06	0.32 ± 0.11	0.07 ± 0.01	0.14 ± 0.02	0.11 ± 0.07	0.05 ± 0.01	0.09 ± 0.02	0.01 ± 0.01	0 ± 0
RUSITEC Prime-120 h	0.42 ± 0.2	0.25 ± 0.1	0.12 ± 0.06	0.24 ± 0.08	0.02 ± 0.02	0.1 ± 0.02	0.11 ± 0.02	0.05 ± 0.03	0.11 ± 0.03	0 ± 0	0 ± 0
RUSITEC Prime-Total [2]	0.33 ± 0.15	0.38 ± 0.15	0.14 ± 0.07	0.22 ± 0.09	0.11 ± 0.09	0.14 ± 0.04	0.05 ± 0.06	0.05 ± 0.02	0.06 ± 0.04	0.01 ± 0	0 ± 0

[1] No standard deviation (SD), since sample was collected from a single cow (n = 1). [2] Mean and SD of all relative abundances (%) collected over 120 h.

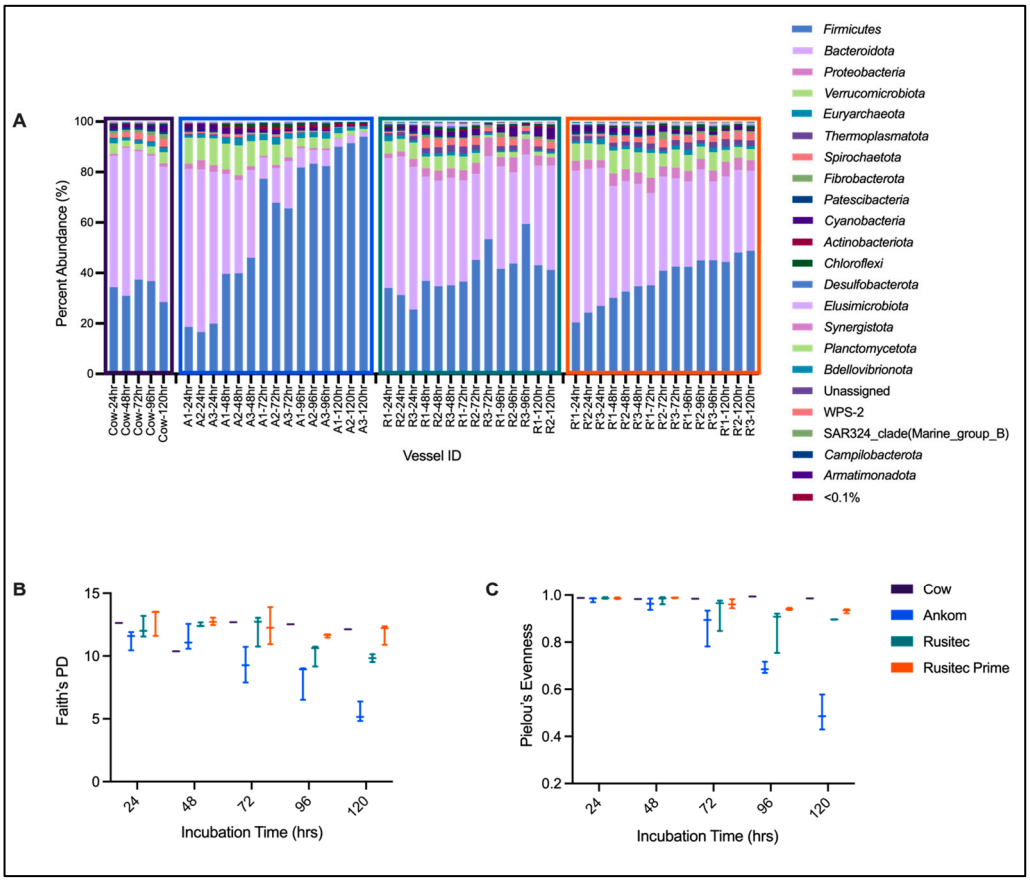

Figure 6. Temporal succession of the prokaryotic population during in vivo and in vitro fermentation. Relative abundance of archaeal and bacterial phyla was determined in vivo and in vitro via sequencing of the V4 region of 16S rRNA gene (**A**). Alpha diversity was assessed via Faith's PD index (**B**). Evenness of the prokaryotic communities was evaluated using Pielou's evenness index (**C**). In vitro platforms were operated in technical triplicates. Ankom vessels are indicated by "A1", "A2", and "A3", RUSITEC PP vessels by "R1", "R2", and "R3", and RUSITEC prime vessels by "R'1", "R'2", and "R'3".

Both the RUSITEC systems were capable of maintaining a more natural microbiome composition than the Ankom systems over time. In the RUSITEC PP vessels at 24 h, Bacteroidota made up an average 54.37% (SD ± 2.54) of the total abundance. After 120 h, both Bacteroidota and Firmicutes recruited ~40% of the prokaryotic reads (SD ± 1.28 and SD ± 1.29, respectively) generated from the RUSITEC PP vessels. The RUSITEC prime vessels had a mean 57.27% (SD ± 2.73) and 23.89% (SD ± 3.29) of Bacteroidota and Firmicutes relative abundance, respectively, at 24 h. After 120 h of in vitro fermentation, Bacteroidota and Firmicutes accounted for 32.75% (SD ± 1.08) and 47.1% (SD ± 2.37) of the reads in the RUSITEC prime vessels, respectively (Table 1, Figure 6A). The community diversity analysis, using Faith's Phylogenetic Diversity and Pielou's evenness [37], revealed that the community profile and temporal community succession in the RUSITEC systems are similar to the ones observed in the natural rumen system (Figure 6B,C; Supplemental Table S7). Though both RUSITEC systems reflect the in vivo bacterial diversity succession over time fairly well, the RUSITEC prime system was superior, since it also displayed less inter-vessel variation than the reactor vessels of the RUSITEC PP system.

3.4. ITS Sequencing Results

A total of 5,278,972 reads were generated from 48 samples, with a mean of 109,979 (SD ± 67,450) reads and a minimum read count of 17,240 per sample. After quality filtering and pairing, an average of 92.14% (SD 3.33) of the reads remained for subsequent analysis

(Supplemental Table S8). As with the prokaryotic population, the in vivo fungal rumen community composition remained consistent over 120 h (Figure 7A). Neocallimastigomycota was the most abundant fungal phyla, recruiting on average 94.9% (SD ± 5.65) of the reads. The remaining reads (~5%) were assigned to five different phyla (i.e., Basidiomycota, Ascomycota, Mucoromycota, Rozellamycota, and Mortierellomycota), with <1% of the reads belonging to fungal taxa that are unclassified as of today.

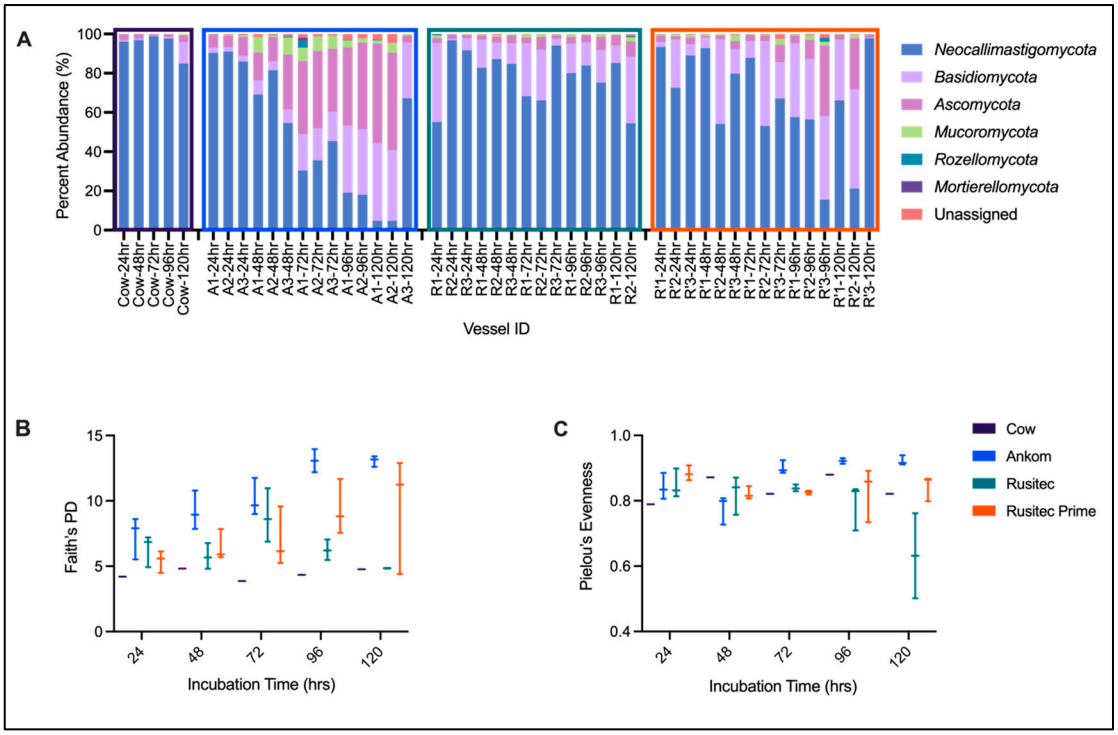

Figure 7. Temporal succession of the fungal population during in vivo and in vitro fermentation. Relative abundance was determined in vivo and in vitro via sequencing of the ITS1 region (**A**). Alpha diversity was assessed via Faith's PD index (**B**). Evenness of the fungal population was evaluated using Pielou's evenness index (**C**). In vitro platforms were operated in technical triplicates. Ankom vessels are indicated by "A1", "A2", and "A3", RUSITEC PP vessels by "R1", "R2", and "R3", and RUSITEC prime vessels by "R'1", "R'2", and "R'3". Table 1: Mean and standard deviation of relative abundances of prokaryotic phyla.

A comparative analysis revealed that all three in vitro platforms had similar fungal profiles and captured the native rumen mycobiome adequately at 24 h. However, after 24 h, the mycobiome profile in the Ankom reactors started to diverge from the in vivo community. At the termination of the experiment, the mycobiome of the Ankom units had drastically shifted with an average of 34.69% (SD ± 5.61) and 34.69% (SD ± 27.05) of the reads recruited by Basidiomycota and Ascomycota, respectively, although the variation between the different vessels was high (Table 2). Both RUSITEC and RUSITEC prime systems were able to maintain a stable mycobiome dominated by Neocallimastigomycota over 72 h. Thereafter, the relative abundance of Basidiomycota as well as Ascomycota increased in the RUSITEC and RUSITEC prime reactors (Table 2). The mycobiome within the Ankom vessels displayed a sharp and continuing shift in alpha diversity over the duration of the experiment, while the mycobiome in the RUSITEC systems maintained a more consistent alpha diversity and evenness (Figure 7B,C; Supplemental Table S9). A detailed breakdown of the fungal population during in vivo and in vitro fermentation and its temporal succession is summarized in Table 2.

Table 2. Mean and standard deviation of relative abundances (%) of fungal phyla.

	Neocallimastigomycota	*Basidiomycota*	*Ascomycota*	*Mucoromycota*	*Rozellomycota*	*Mortierellomycota*	**Unassigned**
Cow-24 h [1]	96.11250628	0.772045634	2.648585301	0.150678051	0.004305087	0.203774126	0.108105522
Cow-48 h [1]	96.84371793	0.84187354	2.098238412	0.106597651	0.007437045	0.046605485	0.055529939
Cow-72 h [1]	98.86820007	0.245738198	0.742363393	0.092678406	0.031828947	0	0.019190983
Cow-96 h [1]	97.73148245	0.797102957	1.307831589	0.104892922	0	0	0.058690087
Cow-120 h [1]	84.96034062	10.95354187	3.323833396	0.683308725	0.020602273	0	0.058373107
Cow-Total [2]	94.9 ± 5.65	2.72 ± 4.61	2.02 ± 1.03	0.23 ± 0.26	0.01 ± 0.01	0.05 ± 0.09	0.06 ± 0.03
Ankom-24 h	89.12 ± 2.78	2.56 ± 0.35	7.23 ± 2.1	0.61 ± 0.35	0.08 ± 0.08	0 ± 0	0.4 ± 0.1
Ankom-48 h	68.48 ± 13.45	6.19 ± 1.4	18.2 ± 8.58	5.85 ± 4.33	0.16 ± 0.2	0 ± 0	1.12 ± 0.49
Ankom-72 h	37.14 ± 7.59	16.63 ± 1.76	36.24 ± 3.78	7 ± 0.35	1.18 ± 2.03	0.5 ± 0.87	1.3 ± 0.61
Ankom-96 h	18.62 ± 0.76	33.75 ± 0.55	42.03 ± 3.04	2.92 ± 0.92	0.12 ± 0.17	0 ± 0	2.56 ± 0.65
Ankom-120 h	25.56 ± 36.1	34.69 ± 5.61	34.69 ± 27.05	2.42 ± 2.3	0.09 ± 0.16	0 ± 0	2.55 ± 2.21
Ankom-Total [2]	49.87 ± 31.59	17.7 ± 13.99	26.65 ± 17.42	3.82 ± 3.17	0.34 ± 0.92	0.11 ± 0.4	1.52 ± 1.27
RUSITEC PP-24 h	81.2 ± 22.71	15.77 ± 21.41	2.12 ± 0.46	0.66 ± 0.73	0.18 ± 0.28	0 ± 0	0.07 ± 0.04
RUSITEC PP-48 h	84.93 ± 2.24	11.03 ± 3.05	3.09 ± 0.98	0.67 ± 0.2	0.01 ± 0.02	0 ± 0	0.28 ± 0.21
RUSITEC PP-72 h	76.16 ± 15.56	18.73 ± 13.27	3.89 ± 2.43	1.06 ± 0.62	0 ± 0	0 ± 0	0.17 ± 0.03
RUSITEC PP-96 h	79.74 ± 4.39	14.45 ± 2.39	4.66 ± 1.83	0.96 ± 0.38	0.04 ± 0.07	0 ± 0	0.15 ± 0.04
RUSITEC PP-120 h	69.8 ± 21.73	21.52 ± 17.63	6.5 ± 1.86	1.31 ± 1.21	0.27 ± 0.38	0 ± 0	0.6 ± 0.65
RUSITEC PP-Total [2]	78.98 ± 13.45	15.93 ± 11.69	3.88 ± 1.98	0.9 ± 0.58	0.09 ± 0.19	0 ± 0	0.23 ± 0.26
RUSITEC Prime-24 h	84.98 ± 10.96	10.97 ± 11.84	2.82 ± 0.97	0.98 ± 0.4	0.06 ± 0.03	0 ± 0	0.19 ± 0.1
RUSITEC Prime-48 h	75.58 ± 19.68	20.38 ± 20.12	2.27 ± 1.75	1.41 ± 1.33	0 ± 0	0 ± 0	0.35 ± 0.22
RUSITEC Prime-72 h	69.38 ± 17.51	23.48 ± 17.89	4.93 ± 3.36	1.26 ± 1.49	0 ± 0	0 ± 0	0.95 ± 1.4
RUSITEC Prime-96 h	43.21 ± 23.97	37.08 ± 5.96	16.51 ± 17.07	1.57 ± 0.89	0.67 ± 1.16	0 ± 0	0.95 ± 0.91
RUSITEC Prime-120 h	61.71 ± 38.46	27.37 ± 25.34	10.02 ± 13.82	0.73 ± 0.9	0 ± 0	0 ± 0	0.17 ± 0.16
RUSITEC Prime-Total [2]	66.97 ± 24.96	23.86 ± 17.28	7.31 ± 10.09	1.19 ± 0.96	0.15 ± 0.52	0 ± 0	0.52 ± 0.74

[1] No SD, since sample was collected from a single cow (n = 1). [2] Mean and SD of all relative abundances (%) collected over 120 h.

4. Discussion

All three ARSs tested in this study were suitable for mimicking rumen conditions for up to 48 h. Once rumen fermentation exceeded 48 h, the two RUSITEC systems maintained more representative rumen conditions during in vitro rumen fermentation. The more consistent performance over a longer timescale of the RUSITEC platforms, including less variability between the individual vessels, was likely due to the semi-continuous nature of these more complex rumen modeling platforms. Whereas our results confirm that the batch-style Ankom units are less suitable for maintaining rumen conditions over an extended time, a known limitation of this system [22], our results also provide strong evidence that Ankom units are an economic option for short-term rumen simulations, including of the rumen mycobiome, of up to 48 h.

Multitier approaches using a combination of various in vitro systems prior to in vivo trials have been widely used. For example, Roque et al. (2018) used the RUSITEC PP system to test the effects of 5% DM *Asparagopsis taxiformis* on in vitro rumen fermentation and subsequently showed a similar rumen response in vivo [38,39]. More recently, the effect of 3-Nitrooxypropanol (3-NOP) was explored in relation to changing concentrate ratios of feed rations for dairy cattle, and the RUSITEC results [40] were supported in vivo [41]. Whereas these in vitro/in vivo study pairs support our hypothesis that RUSITEC systems can be used to adequately mimic in vivo rumen fermentation and its response to feed additives in long-term studies, the Ankom system has also been used successfully to predict changes in the enteric methane production [11]. In combination with the results presented here, this suggests that the Ankom system might be a more economical alternative and an adequate solution for short-term and efficient functional response studies, specifically to identify the most valuable feed additives before moving on to RUSITEC or in vivo systems.

For more mechanistic studies, especially when conducted over extended periods, RUSITEC systems seem to be the in vitro system of choice. Considering the increased complexity and costs associated with RUSITEC systems, initial Ankom-based screening efforts followed by RUSITEC screens might be the most efficient and economical solution prior to performing resource-intensive animal trials. It should be noted that regardless of which in vitro platform is used, rumen fluid from multiple donor animals should be collected and mixed as the initial inoculum [42,43]. Just as in vitro modeling has shortcomings, so too does sourcing from a single animal. This study utilized only a single donor cow, as the ultimate goal was to evaluate the ability of these vessels to emulate a single condition over time, but a single donor does not produce results that are applicable to a greater population of ruminants.

Not performed in this study, but of importance to long-term RUSITEC work, is the inclusion of an adaptation period. To test all platforms and in vivo samples in conjunction, the short-term use of the ANKOM dictated that the experimental set-up did not include this separate adaptation period for the RUSITECs. The adaptation periods for semi-continuous fermenters range from 1 to 7 days [12,16,44]. Given the results obtained in this study, the inclusion of an adaptation period in long-term studies is a necessary step for reproducibility, but the diversion from the in vivo sample over time must also be considered. RUSITECs and other comparable platforms may stabilize after an equilibration period, but ultimate findings must be viewed from the perspective that in vitro platforms are not able to wholly reproduce the ruminant conditions, particularly when it comes to the mycobiome.

It is necessary to recognize that the use of such artificial models currently is not standardized across laboratories, making study comparisons challenging. Despite utilizing the same artificial system, there are often differences in the set-up of these in vitro platforms [12,45]. Those differences included the amount of rumen solids incubated, the rate of saliva buffer entering the system, the initial volume of rumen fluid added, and the inclusion of an adaptation period. Future work is needed to determine the set-up that is most appropriate for reliably depicting in vivo fermentation. Apart from comparing artificial rumen models and bringing to light improvements that need to be made in these systems, this project aimed to encourage the standardization of their usage to facilitate

better comparisons. Understanding the advantages and limitations of each platform is essential in deciding when and how to implement such devices in future studies.

4.1. In Vitro Rumen Modeling

The three in vitro platforms evaluated in this work performed well at the onset of the incubation period but started to deviate from the in vivo rumen conditions over the duration of the experiment. Conditions that were well mimicked in vitro included gas production, VFA profiles, and microbiome/mycobiome assemblage. Unlike the RUSITECs, the Ankom vessels quickly deviated from the in vivo sample with respect to gas production, VFA profiles, and the microbial composition.

Whereas many in vivo metrics were captured reasonably well by the different ARSs, a good replication of the in vivo pH and redox potential was not achieved in any of the engineered systems. Specifically, the redox potential of the rumen samples was negative throughout the experiment, while in vitro, the redox potential was consistently positive, with redox from the RUSITEC systems being lower than the values from the Ankom vessels. Although none of the engineered systems were capable of maintaining a negative redox potential during sample acquisition, the engineered rumen systems are still suitable for anaerobic fermentation, since their E_h remained below +100 mV [46]. Since the tested ARSs were not equipped with real-time E_h probes and acquisition of metrics, and since the feeding of the reactors required a brief opening of the units, the possibility that some of the increase in E_h value might have been caused by the handling of the ARS and some of the sampling protocols cannot be excluded. More stringent feeding and sampling strategies that limit the introduction of oxygen, such as placing the entire reactor systems in anaerobic chambers, may minimize these differences in future in vitro studies, and the application of those advanced approaches might be suitable when the required resources are available and only a limited number of reactors have to be handled.

Similar to the potential redox deviation, the rumen fluid temperature within the vessels varied and diverged from the rumen fluid temperature (~39 °C) measured in vivo. The temperature is critical for microbial processes and can impact a variety of rumen microbiome functions and, therefore, also the parameters measured during this study [47]. A common problem when conducting in vitro rumen assays is maintaining a consistent temperature over time, which is emphasized by the fact that a reduced temperature was measured consistently across all three different ARSs employed in this study. It is feasible to hypothesize that some of the differences between the in vivo and in vitro rumen function is driven by the lower temperature in the ARSs, and improved insulation of the individual vessels as well as the overall system might enhance our ability to accurately mimic rumen function and response in vivo.

4.2. Platform Comparison

The batch fermentation (i.e., Ankom) system seemed to be capable of reliably capturing rumen conditions (i.e., pH, temperature, and VFA profiles) over the initial 48 h of the experiment, and it is therefore not surprising that the prokaryotic and fungal population profiles of the Ankom units were consistent with those observed in vivo. Due to the nature of batch systems and the lack of internal mixing of the reactor content, products that would be removed or buffered in a continuous system (e.g., a RUSITEC system) remain in the Ankom vessels over time. With this stagnation in mind, it is not surprising to see significant changes resulting from product accumulation. The accumulation of VFAs, one of the major microbial products in the rumen, is very likely responsible for the decline in the pH value observed after 48 h. More importantly, the accumulation of VFAs resembles ruminal acidosis, which can have negative effects on an animal's ability to absorb nutrients [48]. Unless questions related to ruminal dysfunction are of particular interest to a study, the utilization of Ankom units after 48 h appears inadequate for accurate rumen function simulation.

The Ankom system also displayed a higher inter-reactor/-vessel variability compared with the semi-continuous RUSITEC platforms, which suggests that a higher number of replicate reactors should be employed for Ankom-based in vitro screening efforts to achieve statistically robust data. Considering the low cost of these systems as well their relatively simple operation, when compared with the complex and costly RUSITEC systems, the Ankom system remains a good option for the initial evaluation of potential feed additives.

4.3. Selecting Semi-Continuous Modeling Platforms

In contrast to the batch-style Ankom system, the semi-continuous RUSITEC platforms fared better in maintaining an in vivo-like rumen environment, which is not surprising considering that these systems have some features that render them more rumen-like than simple batch reactors. One of the features that distinguishes the RUSITEC system from off-the-shelf reactors is their ability to mimic the peristaltic rumen movement, instead of the usually observed stir-mixing of the reactor content. The continued movement and mixing most likely enables an equal access to the feed surface and therefore nutrient accessibility, while at the same time, the removal of toxic byproducts is facilitated. Artificial saliva buffer added at a steady rate stabilized the pH of the in vitro rumen system. The addition of buffer in combination with the continuous removal of "spent" rumen content allowed both RUSITEC systems to circumvent conditions that lead to an unsuitable environment for a healthy rumen microbiome. Therefore, RUSITEC platforms appear better suited for longer periods of modeling and predicting rumen function and response than more simplistic batch reactors.

4.4. Primed for Rumen Modeling

The novel stainless steel RUSITEC prime model we developed as part of this study is made of food-quality stainless steel. It includes quick disconnect fittings and features a tri-clamp-based construction with 3D-printed inner vessels to hold rumen solids and feed while moving them vertically through the stainless steel reactor for maximal contact with the rumen fluid. The advanced RUSITEC reactors have demonstrated their potential for extended periods of in vivo-like in vitro rumen simulation. The RUSITEC prime vessels displayed similar trends to the polypropylene RUSITEC vessels of the RUSITEC PP system, currently in use in many other laboratories, and they therefore represent a valuable baseline for standardization across different laboratories. The RUSITEC prime vessels, made mostly from off-the-shelf parts, displayed less variability compared with its counterpart, yielding more consistent measurements. Moreover, the stainless steel vessels of the RUSITEC prime platform are autoclavable, facilitating sterilization, which is essential to avoid any potential cross-over contamination of these systems between experiments. Developing the RUSITEC prime model with tri-clamp fittings makes options for system modularity and additions cheaper and easier to obtain due to the large commercial infrastructure, including brewing and distilling, that rely on this format. Further studies are needed to evaluate how well RUSITEC prime vessels maintain the microbiome over periods exceeding 120 h.

5. Conclusions

The performance of three artificial rumen models were compared with the rumen of a dairy cow to determine the efficiency of each in vitro platform in maintaining an environment capable of supporting a natural rumen microbiota and its corresponding function (i.e., VFA and greenhouse gas production). The results of this work suggest that the widely used RUSITEC approach is well suited to the simulation of the rumen ecosystem for microbiome studies, and that further modifications of the customizable RUSITEC prime could further improve this established technique towards even more in vivo-like rumen conditions. Both semi-continuous rumen fermentation systems reached equilibration after 48 h and are better equipped for long-term rumen studies compared with the Ankom batch system. The Ankom vessels appeared to undergo drastic shifts in their in vitro rumen microbiome after 48 h of operation, making them ideal options for initial in vitro rumen

assays and for identifying the most promising compounds to then be studied in one of the RUSITEC systems. Although neither of the two semi-continuous systems are flawless, they represent a great opportunity to develop complex and standardized approaches to replicate and study rumen conditions in the laboratory, therefore reducing the risk of performing unnecessary and costly animal trials. Finally, we want to note that no in vitro system will replace the animal, and animal trials will continue to be crucial. The value of the different in vitro rumen systems is to minimize the number of animal trials that are performed and therefore to reduce costs and, more importantly, improve animal welfare and well-being.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9110953/s1, Supplemental Figure S1: Bray-Curtis Emperor plot with 16S reads; Figure S2: Weighted Unifrac Emperor plot with 16S reads; Figure S3: Bray-Curtis Emperor plot with ITS reads; Figure S4: Weighted Unifrac Emperor plot with ITS reads. Plot generated using q2-diversity QIIME2 plugin and QIIME View for visualization. Supplemental Table S1: Total Mixed Ration Ingredient Breakdown; Table S2: Physical parameters of the cows' rumen and the in vitro rumen system vessels; Table S3: Temperature in Celsius; Table S4: Gas production profiles; Table S5: Volatile fatty acids profiles; Table S6: Quality Filtering Results for 16S rRNA Bacterial Sequences; Table S7 Diversity Metrics for 16S rRNA Sequencing Results; Table S8: Quality Filtering Results for ITS rRNA Fungal Sequences; Table S9: Diversity Metrics for ITS rRNA Sequencing Results.

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Institutional Review Board Statement: Rumen content collection was performed in accordance with the Institution of Animal Care and Use Committee (IACUC) at the University of California, Davis under protocol number 21117.

Data Availability Statement: Sequence data generated during this study are available through NCBI's Sequence Read Archive under the BioProject ID PRJNA863571. The bash code for the processing and analysis of sequencing data is available at GitHub. All other data are included in this published article and its supplementary information files.

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References

1. Wang, T.; Jin, H.; Kreuter, U.; Teague, R. Expanding grass-based agriculture on marginal land in the U.S. Great Plains: The role of management intensive grazing. *Land Use Policy* **2021**, *104*, 105155. [CrossRef]
2. Huntsinger, L.; Barry, S. Grazing in California's Mediterranean Multi-Firescapes. *Front. Sustain. Food Syst.* **2021**, *5*, 715366. [CrossRef]
3. Gennet, S.; Spotswood, E.; Hammond, M.; Bartolome, J.W. Livestock grazing supports native plants and songbirds in a California annual grassland. *PLoS ONE* **2017**, *12*, e0176367. [CrossRef]
4. Oltjen, J.W.; Beckett, J.L. Role of ruminant livestock in sustainable agricultural systems. *J. Anim. Sci.* **1996**, *74*, 1406–1409. [CrossRef] [PubMed]

5. Barral-Martinez, M.; Fraga-Corral, M.; Garcia-Perez, P.; Simal-Gandara, J.; Prieto, M.A. Almond By-Products: Valorization for Sustainability and Competitiveness of the Industry. *Food* **2021**, *10*, 1793. [CrossRef]
6. Klop, G.; Schuppen, S.v.L.-V.; Pellikaan, W.F.; Hendriks, W.H.; Bannink, A.; Dijkstra, J. Changes in in vitro gas and methane production from rumen fluid from dairy cows during adaptation to feed additives in vivo. *Animal* **2017**, *11*, 591–599. [CrossRef]
7. Wang, Y.; Janssen, P.H.; Lynch, T.A.; van Brunt, B.; Pacheco, D. A mechanistic model of hydrogen-methanogen dynamics in the rumen. *J. Theor. Biol.* **2016**, *393*, 75–81. [CrossRef]
8. Greening, C.; Geier, R.; Wang, C.; Woods, L.C.; Morales, S.E.; McDonald, M.J.; Rushton-Green, R.; Morgan, X.C.; Koike, S.; Leahy, S.C.; et al. Diverse hydrogen production and consumption pathways influence methane production in ruminants. *ISME J.* **2019**, *13*, 2617–2632. [CrossRef]
9. Pachauri, R.K.; Allen, M.R.; Barros, V.R.; Broome, J.; Cramer, W.; Christ, R.; Church, J.A.; Clarke, L.; Dahe, Q.; Dasgupta, P.; et al. Climate Change 2014: Synthesis Report. In *Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Pachauri, L.M.R., Ed.; IPCC: Geneva, Switzerland, 2014; p. 151.
10. Lan, W.; Yang, C. Ruminal methane production: Associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Sci. Total Environ.* **2019**, *654*, 1270–1283. [CrossRef]
11. Brooke, C.G.; Roque, B.M.; Shaw, C.; Najafi, N.; Gonzalez, M.; Pfefferlen, A.; De Anda, V.; Ginsburg, D.W.; Harden, M.C.; Nuzhdin, S.V.; et al. Methane Reduction Potential of Two Pacific Coast Macroalgae During in vitro Ruminant Fermentation. *Front. Mar. Sci.* **2020**, *7*, 561. [CrossRef]
12. Roque, B.M.; Brooke, C.G.; Ladau, J.; Polley, T.; Marsh, L.J.; Najafi, N.; Pandey, P.; Singh, L.; Kinley, R.; Salwen, J.K.; et al. Effect of the macroalgae Asparagopsis taxiformis on methane production and rumen microbiome assemblage. *Anim. Microbiome* **2019**, *1*, 3.
13. Calsamiglia, S.; Busquet, M.; Cardozo, P.; Castillejos, L.; Ferret, A. Invited review: Essential oils as modifiers of rumen microbial fermentation. *J. Dairy Sci.* **2007**, *90*, 2580–2595. [CrossRef] [PubMed]
14. Durmic, Z.; Busquet, M.; Cardozo, P.; Castillejos, L.; Ferret, A. In vitro screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation. *J. Sci. Food Agric.* **2014**, *94*, 1191–1196. [CrossRef] [PubMed]
15. Fouts, J.Q.; Honan, M.C.; Roque, B.M.; Tricarico, J.M.; Krebab, E. Board Invited Review: Enteric methane mitigation interventions. *Transl. Anim. Sci.* **2022**, *6*, txac041. [CrossRef]
16. Wetzels, S.U.; Eger, M.; Burmester, M.; Kreienbrock, L.; Abdulmawjood, A.; Pinior, B.; Wagner, M.; Breves, G.; Mann, E. 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* **2018**, *13*, e0192256. [CrossRef] [PubMed]
17. Garcia-Rodriguez, J.; Saro, C.; Mateos, I.; González, J.S.; Carro, M.D.; Ranilla, M.J. Effects of Replacing Extruded Maize by Dried Citrus Pulp in a Mixed Diet on Ruminal Fermentation, Methane Production, and Microbial Populations in Rusitec Fermenters. *Animals* **2020**, *10*, 1316. [CrossRef]
18. Min, B.R.; Castleberry, L.; Allen, H.; Parker, D.; Waldrip, H.; Brauer, D.; Willis, W. Associative effects of wet distiller's grains plus solubles and tannin-rich peanut skin supplementation on in vitro rumen fermentation, greenhouse gas emissions, and microbial changes. *J. Anim. Sci.* **2019**, *97*, 4668–4681. [CrossRef]
19. Czerkawski, J.W.; Breckenridge, G. Design and development of a long-term rumen simulation technique (Rusitec). *Br. J. Nutr.* **1977**, *38*, 371–384. [CrossRef]
20. Belanche, A.; Palma-Hidalgo, J.M.; Nejjam, I.; Serrano, R.; Jiménez, E.; Martín-García, I.; Yáñez-Ruiz, D.R. In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum. *J. Sci. Food Agric.* **2019**, *99*, 163–172. [CrossRef]
21. Metzler-Zebeli, B.U.; Scherr, C.; Sallaku, E.; Drochner, W.; Zebeli, Q. Evaluation of associative effects of total mixed ration for dairy cattle using in vitro gas production and different rumen inocula. *J. Sci. Food Agric.* **2012**, *92*, 2479–2485. [CrossRef]
22. Vinyard, J.R.; Faciola, A.P. Unraveling the pros and cons of various in vitro methodologies for ruminant nutrition: A review. *Transl. Anim. Sci.* **2022**, *6*, txac130. [CrossRef]
23. Oeztuerk, H.; Schroeder, B.; Beyerbach, M.; Breves, G. Influence of living and autoclaved yeasts of Saccharomyces boulardii on in vitro ruminal microbial metabolism. *J. Dairy Sci.* **2005**, *88*, 2594–2600. [CrossRef] [PubMed]
24. Motulsky, H.J.; Brown, R.E. Detecting outliers when fitting data with nonlinear regression—A new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinform.* **2006**, *7*, 123. [CrossRef] [PubMed]
25. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S.M.; Betley, J.; Fraser, L.; Bauer, M.; et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **2012**, *6*, 1621–1624. [CrossRef] [PubMed]
26. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Lozupone, C.A.; Turnbaugh, P.J.; Fierer, N.; Knight, R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. USA* **2011**, *108* (Suppl. S1), 4516–4522. [CrossRef] [PubMed]
27. Smith, D.P.; Peay, K.G. Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. *PLoS ONE* **2014**, *9*, e90234. [CrossRef]
28. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]

29. Callahan, B.J.; Mcmurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
30. R Core Team. *R: A Language and Environment for Statistical Computing*; R Core Team: Vienna, Austria, 2021.
31. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [CrossRef]
32. Pedregosa, F.; Varoquaux, G.; Gramfort, A.; Michel, V.; Thirion, B.; Grisel, O.; Blondel, M.; Prettenhofer, P.; Weiss, R.; Dubourg, V.; et al. Scikit-learn: Machine learning in Python. *J. Mach. Learn Res.* **2011**, *12*, 2825–2830.
33. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef] [PubMed]
34. Koljalg, U.; Nilsson, H.R.; Schigel, D.; Tedersoo, L.; Larsson, K.-H.; May, T.W.; Taylor, A.F.S.; Jeppesen, T.S.; Frøslev, T.G.; Lindahl, B.D.; et al. The Taxon Hypothesis Paradigm-On the Unambiguous Detection and Communication of Taxa. *Microorganisms* **2020**, *8*, 1910. [CrossRef]
35. Lozupone, C.; Knight, R. UniFrac: A new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **2005**, *71*, 8228–8235. [CrossRef] [PubMed]
36. Bokulich, N.A.; Kaehler, B.D.; Rideout, J.R.; Dillon, M.; Bolyen, E.; Knight, R.; Huttley, G.A.; Gregory Caporaso, J. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **2018**, *6*, 90. [CrossRef] [PubMed]
37. Chao, A.; Chiu, C.-H.; Jost, L. Phylogenetic Diversity Measures and Their Decomposition: A Framework Based on Hill Numbers. In *Biodiversity Conservation and Phylogenetic Systematics: Preserving Our Evolutionary Heritage in an Extinction Crisis*; Pellens, R., Grandcolas, P., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 141–172.
38. Roque, B.M.; Venegas, M.; Kinley, R.D.; de Nys, R.; Duarte, T.L.; Yang, X.; Kebreab, E. Red seaweed (Asparagopsis taxiformis) supplementation reduces enteric methane by over 80 percent in beef steers. *PLoS ONE* **2021**, *16*, e0247820. [CrossRef]
39. Roque, B.M.; Salwen, J.K.; Kinley, R.; Kebreab, E. Inclusion of Asparagopsis armata in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. *J. Clean. Prod.* **2019**, *234*, 132–138. [CrossRef]
40. Schilde, M.; von Soosten, D.; Hüther, L.; Kersten, S.; Meyer, U.; Zeyner, A.; Dänicke, S. Dose—Response Effects of 3-Nitrooxypropanol Combined with Low- and High-Concentrate Feed Proportions in the Dairy Cow Ration on Fermentation Parameters in a Rumen Simulation Technique. *Animals* **2021**, *11*, 1784. [CrossRef]
41. Van Gastelen, S.; Dijkstra, J.; Binnendijk, G.; Duval, S.M.; Heck, J.M.; Kindermann, M.; Zandstra, T.; Bannink, A. 3-Nitrooxypropanol decreases methane emissions and increases hydrogen emissions of early lactation dairy cows, with associated changes in nutrient digestibility and energy metabolism. *J. Dairy Sci.* **2020**, *103*, 8074–8093. [CrossRef]
42. Zhao, W.; Abdelsattar, M.M.; Wang, X.; Zhang, N.; Chai, J. In Vitro Modulation of Rumen Fermentation by Microbiota from the Recombination of Rumen Fluid and Solid Phases. *Microbiol. Spectr.* **2022**, *11*, e0338722. [CrossRef]
43. Brewster, A.N.; Pless, L.A.; McLean, D.J.; A Armstrong, S. Time of rumen fluid collection relative to feeding alters in vitro fermentation gas parameters. *Transl. Anim. Sci.* **2018**, *2* (Suppl. S1), S97. [CrossRef]
44. Wenner, B.A.; Kesselring, E.; Antal, L.; Henthorne, T.; Carpenter, A. Dual-flow continuous culture fermentor system updated to decrease variance of estimates of digestibility of neutral detergent fiber. *Appl. Anim. Sci.* **2021**, *37*, 445–450. [CrossRef]
45. Teoh, R.; Caro, E.; Holman, D.B.; Joseph, S.; Meale, S.J.; Chaves, A.V. Effects of Hardwood Biochar on Methane Production, Fermentation Characteristics, and the Rumen Microbiota Using Rumen Simulation. *Front. Microbiol.* **2019**, *10*, 1534. [CrossRef] [PubMed]
46. Huang, Y.; Marden, J.P.; Julien, C.; Bayourthe, C. Redox potential: An intrinsic parameter of the rumen environment. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 393–402. [CrossRef] [PubMed]
47. Kimura, A.; Sato, S.; Kato, T.; Ikuta, K.; Yamagishi, N.; Okada, K.; Mizuguchi, H.; Ito, K. Relationship between pH and temperature in the ruminal fluid of cows, based on a radio-transmission pH-measurement system. *J. Vet. Med. Sci.* **2012**, *74*, 1023–1028. [CrossRef]
48. Goto, H.; Qadis, A.Q.; Kim, Y.-H.; Ikuta, K.; Ichijo, T.; Sato, S. Effects of a bacterial probiotic on ruminal pH and volatile fatty acids during subacute ruminal acidosis (SARA) in cattle. *J. Vet. Med. Sci.* **2016**, *78*, 1595–1600. [CrossRef]