Comparison of three artificial rumen systems for rumen microbiome modeling

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Abstract

Background

The rumen contains a complex mixture of microbes, which are crucial for ruminant health and feed fermentation. During the fermentation process some of the feed-derived carbon becomes carbon dioxide and methane, which are released into the atmosphere where they act as greenhouse gases and contribute to climate change. There is growing interest in reducing the loss of feed-derived carbon and making it available to the animal, improving animal productivity, while also reducing the carbon footprint of the ruminant industry. To this end, artificial rumen systems (ARS) have been used for evaluating novel feed additives for their effect on the rumen microbiome and rumen function prior to conducting resource intensive animal trials. Whereas ARS are capable of predicting the response of the rumen and its microbiome, it is unclear how accurately different in vitro systems simulate the natural system and how results compare between the artificial systems that are being employed. Here we evaluated physical, chemical and microbiome metrics of three ARS over five days and compared them to those metrics in the in vivo rumen.

Results

Over a 48 hrs sampling period, the batch style platform (Ankom) was able to replicate pH, volatile fatty acid profile, and bacterial and fungal microbiome of the in vivo rumen, but its accuracy of mimicking in vivo metrics dropped significantly beyond 48 hrs. In contrast, the semi-continuous RUSITEC models, RUSITEC PP and RUSITEC prime, were able to mimic the volatile fatty acid profile and microbiota of the in vivo rumen for up to 120 hrs of rumen simulation. Comparison of gas production across vessel types demonstrated that the semi-continuous RUSITEC platforms display less variability among vessel replicates and time compared to the Ankom system.

Conclusions

In this study, we found that three widely used ARS were able to simulate the rumen ecosystem adequately for the first 48 hrs, with predictions from the more advanced semi-continuous ARS being more accurate when simulations extended over 48 hrs. Findings of this study will help to select the appropriate in vitro system for evaluating the response of the complex rumen microbiome to feed additives. Further work is necessary to improve the capabilities of these platforms and to standardize the methodology for large-scale application.

Background

Ruminant animals, such as cattle, sheep and goats, are an important source of inexpensive high-quality protein, and they will be crucial for providing food safety to the growing global population.
managed correctly ruminants can utilize land unsuitable for row crop agriculture [1], decrease risk for 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$_4$). Enteric CH$_4$ is a byproduct of the complex microbial community associated with the rumen, where it facilitates the conversion of complex plant carbohydrates, otherwise inaccessible to the ruminant animal, to metabolic intermediates, such as volatile fatty acid (VFAs) and hydrogen (H$_2$) [6, 7]. Whereas VFAs and H$_2$ can be utilized further by some rumen microbes or the host animal, CH$_4$ is an end product that is released into the atmosphere where it acts as a greenhouse gas with a global warming potential 28 times more potent than that of carbon dioxide (CO$_2$) on a 100 year time scale [8]. The search for potent feed additives that redirect feed-derived carbon away from CH$_4$, a process that has been estimated to account for a loss of feed-energy up to 12% [9], and towards animal protein has always been of interest to the ruminant industry. A successful strategy to routing feed-derived carbon would increase the overall profitability of the livestock operation and reduce the environmental footprint of the ruminant industry. Artificial (in vitro) rumen systems have been used for many years to screen potential feed additives and feed formulation for their impact on the rumen ecosystem and the host animal [10–13]. Despite the value of these in vitro systems there are significant challenges associated with results from these artificial rumen systems (ARS) and the extent to which the obtained results can be compared and applied to predict in vivo response due to the variability that has been observed across the different ARS studies. This variability can be caused by the different nature of the employed ARS (e.g., continuous, semi-continuous, batch fermentation systems), operational differences, and diet of the animal; but even less obvious differences (e.g., time of sampling, rumen sampling site, handling of inoculum) can have profound implication on the observed microbial response and subsequently on how well the in vitro response will mimic the in vivo response [14, 15]. To determine how accurate widely used in vitro techniques (i.e., continuous and semi-continuous systems), capture major physical, chemical and microbial parameters of the rumen ecosystem, we monitored pH, conductivity, volatile acid profiles as well as the archaeal, bacterial and fungal communities in the rumen and three different types of in vitro systems. To complement the microbial profiles, we also monitored the production of enteric CH$_4$ and CO$_2$ production in the different in vitro systems. To our knowledge this is the first study that provides insight into the temporal succession of the bacterial and fungal population in the rumen and different in vitro systems that have been inoculated with rumen content.

Diets and feed supplements such as ionophores, biologically active compounds sourced from plants, dietary lipids, and exogenous enzymes have been tested using engineered (in vitro) models capable of simulating the rumen environment prior to being tested in vivo [16, 17] and such in vitro models have been used extensively in ruminant research in the fields of ruminant microbiology and nutrition over several decades. Previously, in vitro rumen models have been used to test simple parameters such as feed degradation during incubation. Recently, more advanced systems are now capable of measuring gas (i.e., total gas, CH$_4$, CO$_2$, and H$_2$) production throughout the incubation period and have become useful
tools to better understand the microbial ecology of the rumen [18, 19]. Engineered rumen models involve inoculating a vessel with rumen fluid and solids from one or more donor animals, basal feed, artificial saliva buffer, and the substrate under investigation. Two main types of ARS include batch and semi-continuous systems, each aiming to maintain anaerobic conditions throughout the incubation period to create an environment comparable to the rumen, and capable of sustaining the microbial populations typically found within it. Batch systems are composed of vessels filled with rumen-derived content and that are either placed in a water bath or surrounded with a heating jacket to maintain an operating temperature of 39°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 [20]. These batch systems are usually run for a relatively short incubation period, typically between 2 and 48 hrs. More complex semi-continuous ARS, such as the rumen simulation technique (RUSITEC), have a continuous influx of saliva buffer and effluent tubing, allowing for experimental conditions up to multiple weeks [21]. Semi-continuous systems are also engineered to allow for repeated feeding throughout incubation and simulate the addition of saliva in the animal, making these systems useful models to gain insight into the long-term effects of diets and potential methane mitigating substrates on rumen function.

In this study, 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 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 pH, gas production, VFA production, and microbiome assemblage were evaluated. The experimental outline is shown in Fig. 1. Understanding both the limitations and the advantages of each system is necessary to improve such artificial models, increase the reliability of each, as well as optimize their usage for testing the effects of potential methane-mitigating substrates for use in ruminant agriculture.

Results

General Parameters

The pH and conductance of the fluid samples from the in vivo sample and the in vitro vessels were measured every 24 hrs for a total incubation time of 120 hrs (Fig. 2). Whereas the pH in vivo remained steady at a mean of 7.08 (SD ± 0.11) throughout the experiment, the pH value within the Ankom systems declined steadily from 6.3 to 5.4 (average SD ± 0.05) over the incubation period. Both semi-continuous systems, on the other hand, maintained a consistent pH (Fig. 2A). Redox potential was measured every 24 hrs. In vivo redox potential remained negative, ranging between −13 mV and −1 mV, while the redox potential for all in vitro measurements was in the positive range (p < 0.0001) (Fig. 2B). The Ankom system displayed the most divergence from in vivo (-9.4 mV; SD ± 6.84), with a vessel average of 74.6 mV (SD ± 3.10) over the total incubation period. The RUSITEC systems both maintained redox potentials closer to the in vivo samples, 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.
Gas Production

Carbon dioxide (CO$_2$) and methane (CH$_4$) were measured for each in vitro vessel at 24 hrs intervals over a total of 120 hrs (Fig. 3). Ankom systems displayed a notable level of variation for both CO$_2$ and CH$_4$ production throughout the entire incubation period (Fig. 3A & 3B). Gas production within the Ankom vessels differed significantly from the production in the RUSITEC vessels, with differences after 24 hrs and 72 hrs for CO$_2$ and after 24 hrs for CH$_4$ ($p < 0.05$). 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 hrs the standard deviation for the Ankom vessel CO$_2$ production was 11.892 mL/g*dry matter, as compared to 2.048 mL/g*dry matter for the RUSITEC PP and 1.047 mL/g*dry matter for the RUSITEC prime. On average, deviation for gas concentrations of both CO$_2$ and CH$_4$ within each platforms’ replicates was lowest amongst the RUSITEC prime vessels. Both RUSITEC systems displayed an increase in CO$_2$ output between 48 hrs and 120 hrs, following consistent production between the initiation of the experiment (0 hrs) and 48 hrs. A drop in CH$_4$ production was observed for both RUSITEC systems after 48 hrs with consistent, but low, levels of CH$_4$ after 72, 96 and 120 hrs.

VFA Comparison

Volatile fatty acids (VFAs) profiles were analyzed for each in vitro vessel and the in vivo cow sample at 24 hrs intervals over 120 hrs (Fig. 4). Total VFA concentration was transiently significant ($p < 0.05$) between the rumen and the Ankom system at 24, 48, and 120 hrs (Fig. 4A). 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 hrs. 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, when they reached equilibrium after 72 hrs (Fig. 4A). Acetic acid concentrations followed a similar trend as total VFA concentrations over the entire experiment, with a steady increase in the Ankom units, and stabilization after 72 hrs in both RUSITEC systems. Significant differences between in vivo samples and all three in vitro platforms were detected 24 hrs after initiation of rumen fermentation, with $p < 0.05$, $p < 0.005$, $p < 0.05$ for Ankom, RUSITEC PP, RUSITEC prime respectively. Importantly, differences were no longer significant after 48 hrs (Fig. 4B). Isovaleric acid and propionic acid production within the Ankom reactors differed significantly from in vivo production after 72, 96 and 120 hrs (Fig. 4E & 4F). Isobutyric acid and valeric acid production was significantly different only at 24 and 48 hrs (Fig. 4D & 4G). In summary, both of the RUSITEC platforms maintained VFA profiles over the 120 hrs incubation period, with in vitro VFA profiles consistent with profiles of the rumen samples.

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. After quality filtering, pairing and removal of chimeric sequences, an average of 78.48% (SD ± 3.78) of reads remained per sample (Supplemental Table S1). The in vivo rumen bacterial community was stable across the 120 hrs study period and was
primarily comprised of Firmicutes (33.59% SD ± 3.79) and Bacteroidota (52.98% SD ± 3.50) (Table 1, Fig. 5A). The Ankom vessels maintained similar bacterial profiles at 24 and 48 hrs, but members belonging to Firmicutes dominated the community at 72 hrs and beyond, with contributing > 90% of the prokaryotic reads at 120 hrs (Table 1, Fig. 5A). Both RUSITEC systems were capable of maintaining a more natural microbiome composition. In the RUSITEC PP vessels at 24 hrs, Bacteroidota made up an average 54.37% (SD ± 2.54) of the total abundance. After 120 hrs, 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) Bacteroidota abundance and mean 23.89% (SD ± 3.29) Firmicutes relative abundance at 24 hrs. After 120 hrs 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, Fig. 5A). Community diversity analysis, using Faith's Phylogenetic Diversity and Pielou's Evenness [22], revealed that the community profile and temporal community succession in the RUSITEC systems is more similar to the one observed in the natural rumen system than those observed in the Ankom platform (Fig. 5B & Fig. 5C; Supplemental Table S2). Though both RUSITEC systems reflect in vivo bacterial diversity succession over time fairly well, the RUSITEC prime system was superior since it also displayed less inter-vessel variation than reactor vessels of the RUSITEC PP system.

**ITS Sequencing Results**

A total of 5,278,972 total 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 S3). As with the prokaryotic population, the in vivo rumen fungal community composition remained consistent over 120 hrs (Fig. 6A). 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 5 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. Comparative analysis revealed that all three in vitro platforms had similar fungal profiles and captured the native rumen mycobiome adequately at 24 hrs. However, after 24 hrs 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 shifted significantly 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 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 hrs. Thereafter, 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 (Fig. 6B & Fig. 6C; Supplemental Table S4).

**Discussion**
Although all three ARS were suitable to mimic rumen conditions for up to 48 hrs, only the two RUSITEC platforms maintained representative rumen conditions for in vitro rumen fermentation for extended periods. Superior performance, including less variability between the individual vessels, of the RUSITEC platforms was likely due the semi-continuous nature of these more complex rumen modeling platforms.

Other studies have tested various feed additives using a semi-continuous culture system with in vitro results being later confirmed in vivo. Roque et al. (2018) used the polypropylene RUSITEC vessels to test the effects of 5% DM Asparagopsis taxiformis on in vitro rumen fermentation and subsequent in vivo experiments showed similar rumen responses [23, 24]. 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 [25] were supported in vivo [26]. 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, the Ankom system has also been used successfully to predict changes in enteric methane production [13]. In combination with results presented here, this suggests that the Ankom system might be a more economical alternative and an adequate solution for efficient functional response studies, specifically to identify most valuable feed additives before moving into RUSITEC or in vivo systems. For more mechanistic studies, especially when conducted over extended periods, RUSITEC systems seems 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 RUSTEC screens might be the most efficient and economical solution prior to performing resource intensive animal trials. It is necessary to recognize that the use of such artificial models is not standardized across laboratories, making study comparisons challenging. Despite utilizing the same artificial system, there often are differences in the set-up of these in vitro platforms [16, 27]. 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 a 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.

**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 most in vivo metrics of interest were replicated in the different ARS, replication of the in vivo redox potential was not achieved in either of the engineered systems. Specifically, the redox potential of
the rumen samples was negative throughout the experiment while in vitro redox potential was consistently positive, with redox from the RUSITEC systems being lower than values from the Ankom vessels. Although none of the engineered systems was 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 +100mV [28]. Since the tested ARS were not equipped with real-time $E_{h}$ probes and acquisition of metrics as well as feeding of the reactors required brief opening of the units, it cannot be excluded that some of the increased in $E_{h}$ value might have been caused by the handling of the ARS and some of the sampling protocols. 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 application of those advanced approaches might be suitable when required resources are available.

**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 hrs 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., RUSITEC system) remain and in the Ankom vessels over time. With this stagnation in mind, it is not surprising to see significant changes resulting from product accumulation. Accumulation of VFAs, one of the major microbial products in the rumen, is very likely responsible for the decline of the pH value observed after 48 hrs. More importantly, accumulation of VFAs resembles ruminal acidosis, which can have negative effects on an animal’s ability to absorb nutrients [29]. Unless questions related to ruminal dysfunction are of particular interest to a study, utilization of Ankom units after 48 hrs appears inadequate for accurate rumen function simulation.

The Ankom system also displayed a higher inter-reactor/vessel variability compared to the semi-continuous RUSITEC platforms, which suggests that 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 to the complex and costly RUSITEC systems, the Ankom system remains a good option for the initial evaluation of potential feed additives.

**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 to 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 of mimicking peristaltic rumen movement, instead of the usually observed stir-mixing of the reactor content. The continued movement and mixing most likely enables equal access to the feed surface and therefore nutrient
accessibility, while at the same time removal of toxic byproduct 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.

### 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 to 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 hrs.

### Conclusions

The performance of three artificial rumen models were compared to 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). Results of this work suggest that the widely used RUSITEC approach is well suited to generate in vitro rumen conditions with potential of further improvement by adapting the RUSITEC prime. Both semi-continuous rumen fermentation systems reached equilibration after 48 hrs and are better equipped for long-term rumen studies compared to the Ankom batch system, which appeared to undergo drastic shifts in its in vitro rumen microbiome after 48 hrs of operation, making them ideal options for initial in vitro rumen assays and to identify the most promising compounds to then be studied in one of the RUSITEC systems. Whereas 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.

### Methods
MATERIALS AND METHODS

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 composed of 46.7% wheat hay, 30% alfalfa hay, 20% almond hulls, and 3.3% mineral pellet (Supplemental Table S5). 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. Four liters of rumen fluid and 90 grams of rumen solids were collected prior to morning feeding on the first day of the experiment. Approximately 100 mL of rumen fluid was collected every morning in 24 hrs increments for five days from the same animal for comparative VFA and microbiome analysis with the in vitro and in vivo system. Rumen fluid collection was performed using a perforated PVC pipe, 500 mL syringe, and Tygon tubing (Saint-Gobain North America, PA, USA). Rumen fluid was immediately strained through a colander into two 4L pre-warmed vacuum insulated containers. Redox potential values and pH of the collected rumen content were recorded at the dairy, then containers were transported to the laboratory.

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 S5). 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 hrs using a Model 10 Quincy Lab Oven (Quincy Lab Inc, Chicago, IL, USA). Feed was stored in airtight containers at 4°C until use.

In vitro Rumen Systems

Three in vitro models (Fig. 7) simulating the rumen were set-up and run in parallel for 120 hrs in the laboratory. The first model was the Ankom RF Gas Production System (Ankom Technology RF Gas Production System, Macedon, NY) in a 300 mL vessel (Fig. 7A). The second was a semi-continuous fermentation system, using 1L Polypropylene (PP) vessels (Fig. 7B), based on the rumen simulation technique (RUSITEC) developed by Czerkawski and Breckenridge [21]. The third was a semi-continuous fermentation system, also based on the RUSITEC, in which the 1L PP vessels were replaced with vessels manufactured from tri-clamp food-grade stainless steel (Fig. 7C).

Experimental Design

Rumen fluid collected from the animal was used to inoculate all vessels of each model. Experiments in the Ankom-based systems and both RUSITEC-based systems were run in triplicate (Fig. 1).
Ankom Gas Production System

Each 300 mL vessel contained 150 mL of rumen fluid and 50 mL of an artificial saliva buffer [30] 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 gases. Vessel content was kept at average rumen temperature by placing the vessels in a circulating water bath at 39°C.

RUSITEC, Polypropylene (PP) Vessels

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 were connected to a peristaltic pump administering 0.39 mL/min of artificial saliva buffer to each vessel 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 39°C water bath and contents were mixed continuously via piston agitation to simulate rumen conditions.

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.

Sample Collection

pH, and conductivity measurements were taken every 24 hrs. Vessels were fed every 24 hrs and each feed bag remained in the designated vessel for a total of 48 hrs 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 hrs increments post-feeding. 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 N₂ gas to maintain anaerobic conditions. Gas bags were also collected every 24 hrs for total gas production analysis, and CH₄ and CO₂ concentrations. Gas volumes were measured by manual expulsion of each bag through a flow meter (MGC-1 V3.3, Ritter, Bochum, Germany).

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, 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 1µL sample was injected through Split/Splitless Injectors (SSL), with an 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) fitted with a 3′ × 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 30mL/min. The FID was held at 300°C. A 1mL 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).

**DNA Extractions**

DNA extractions were performed with 300uL 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), then DNA was stored at -20°C until PCR amplification and sequencing.

**PCR Amplification, Library Preparation, and Sequencing**

Sequencing was done 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 [31]. DNA sequence data was 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 [31, 32]. 515F: AATGATAC-GCGACCAGATCTACACGCTAXAXAXAXAXXTATGGTAATTGTGTGAYCGCMCCGCAGTAA; 806R: CAAGCAGAGACGCAGATATGCAGATACGACTACNVGGGTWTCTAAT. The forward amplification primer contained a twelve base barcode sequence to support pooling of up to 2,167 different samples in each lane [31, 32]. Each 25 µL PCR reaction contains 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 (2x concentration, 1x final, (Quanta Bio, Beverly, MA, USA), 1 µL Golay barcode tagged Forward Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. The conditions for PCR are as follows: 94°C for 3 minutes to denature the DNA, with 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; with a final extension of 10 min at 72°C to ensure complete amplification. Amplicons are then quantified using PicoGreen (Invitrogen, Waltham, MA, USA) and a plate reader (Infinite® 200 PRO, Tecan). Once quantified, volumes of each of the products are pooled into a single tube so that each amplicon is represented in equimolar amounts. This pool is then
cleaned up using AMPure XP Beads (Beckman Coulter, Brea, CA, USA), and then quantified using a fluorometer (Qubit, Invitrogen, Waltham, Massachusetts). After quantification, the molarity of the pool is 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 sequencing on the Illumina MiSeq (Illumina, San Diego, CA, USA). Amplicons are sequenced on a 251bp x 12bp x 251bp MiSeq run using customized sequencing primers and procedures [31]. Genomic DNA was amplified using an ITS barcoded primer set, adapted for the Illumina HiSeq2000 and MiSeq [33]. ITS1f:

AATGATACGGCGACCACCGAGATCTACACGGCTTGGTCATTTAGAGGAAGTAA; ITS2:
CAAGCAGAAGACGGCATACGAGATNNNNNNNNCGGCTGCTTCATCGATGC. The reverse amplification primer also contained a twelve base barcode sequence that supports pooling of up to 2,167 different samples in each lane [31, 32]. Each 25 µL PCR reaction contains 9.5 µL of MO BIO PCR Water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2x concentration, 1x 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 are also follows: 94°C for 3 minutes to denature the DNA, with 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; 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 are pooled into a single tube so that each amplicon is represented equally. This pool is 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 is 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 2x251bp sequencing on the Illumina MiSeq.

**Microbiome Analysis**

Qiime2 2022.2 [34] was used to process the raw reads, assign taxonomy, and perform diversity analyses. Reads were demultiplexed and quality filtered using the q2-demux plugin [31]. DADA2 [35], along with R 4.1.3 [36], was used to denoise the quality filtered sequences and pair the 16S reads. Internal transcribed spacer sequences were ran unpaired with the forward reads only. Denoised sequences were aligned and sorted into amplicon sequence variants (ASVs) using MAFFT Fast Tree [37] and assigned taxonomy using SciKit-learn [38]. Bacterial 16S sequences were classified using the SILVA 138.1 database at 99% sequence identity [39] and fungal ITS sequences were classified using the UNITE V8.3 database at 99% sequence identity [40]. Faith’s PD and Pielou Evenness diversity analyses were calculated using the q2-diversity plugin [41] and the MAFFT Fast Tree output.

**Abbreviations**

3-NOP
3-Nitrooxypropanol
ARS
artificial rumen system
ASV
amplicon sequence variants
CH₄
methane
CO₂
carbon dioxide
RUSITEC
rumen simulation technique
RUSITEC PP
rumen simulation technique polypropelene vessels
RUSITEC prime
rumen simulation technique stainless steel vessels
VFA
volatile fatty acid

Declarations

Ethics approval and consent to participate

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

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests

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Authors contributions:

Designed the experiment: CS, MG, CB and MH; Performed the experiments: CS, MG, YP, CB and MH; Generated and analyzed the microbiome data: CS, MG, YP, and MH. Generated and analyzed GC data: CS, MG, YP, PP and MH; Wrote the paper: CS, MG, YP, PP, CB and MH.
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References


**Tables**

Table 1 and 2 are available in the Supplementary Files section.

**Figures**
Figure 1

Experimental Outline. Rumen fluid (RF) and solid (RS) were collected from a fistulated Holstein cow (A), 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 (B). Rumen content/buffer/feed mix was performed in triplicates at 37°C for a total of 120 hrs. Aliquots for subsequent data collection and analyses were collected from the individual vessels and the donor animal every 24 hrs (C).
Physical fermentation parameters. Content of the *in vitro* rumen systems and the cow's rumen were collected in 24 hrs intervals and pH (**A**) and conductivity (**B**) of was determined. Statistical significance: **** = p<0.00005.

Gas production profiles. Methane (CH\textsubscript{4}) (**A**) and carbon dioxide (CO\textsubscript{2}) (**B**) production during *in vitro* fermentation was determined in 24 hrs intervals by gas chromatography. Statistical significance: * = p<0.05.
Figure 4

Volatile fatty acid profiles. Total volatile fatty acid (VFA) 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 hrs over a total period of 120 hrs. Statistical significance: * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$, **** = $p<0.00005$. 
Figure 5

**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”.
Figure 6

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”.
Figure 7

*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).

**Supplementary Files**

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