

The Effect of Sources of Rumen Fluid and Dissolved Oxygen Concentration on Rumen Fermentation Parameters, Methane Gas Emissions, and Microbiota Diversity Changes in Laboratory Conditions

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Abstract

This study assessed the impact of rumen fluid source, basal diet, and dissolved oxygen content in rumen liquor on *in vitro* rumen fermentation, ruminal gas production, and changes in ruminal microbiota diversity. Two sets of *in vitro* experiments were conducted. The first study examined the effects of sources of individual or equally pooled rumen liquors (Exp. 1) on ruminal pH, greenhouse gas (GHG) emissions, and microbiota changes. The second part of the experiment (Exp. 2) focused on assessing how different levels of dissolved oxygen (O₂) and different diets (grain-based versus forage-based) influence *in vitro* digestibility (IVDMD), methane (CH₄) and other gas emissions, rumen fermentation, and microbial populations. All gases were collected using an ANKOM Gas Production System to analyze CH₄, nitrous oxide (N₂O), and hydrogen sulfide (H₂S). Triplicate qPCR analyses were used to evaluate microbial diversity in the rumen of cannulated steers. Incubations with mixed rumen fluid (RF) produced more gas ($P < 0.01$) than those with RF from individual donor steers. Consequently, CH₄ and H₂S production, Firmicutes (F), Bacteroidetes (B), F/B ratio, and total methanogens were at intermediate levels with mixed RF compared to individual donor steer samples. The dissolved O₂ treatment did not affect the average *in vitro* dry matter disappearance rate (IVDMD), with no significant diet × treatment interactions. Increasing O₂ supply reduced ($P < 0.05$) total gas (mL/24h), CH₄ (mL/24h), and H₂S (mL/24h) production in the forage-based diet, with no significant diet × treatment interaction ($P > 0.05$).

The findings indicated that RF obtained from a single animal was prone to error due to host-animal variation and suggested that mixed RF from multiple donor animals can be used in an *in vitro* study. Dissolved O₂ levels in the rumen and diets significantly influenced rumen gas production, resulting in different fermentation end products, microbiome diversity, and total methanogen abundance.

Keywords

Bacteroidetes, Firmicutes, Methanogens, Methane, Oxygen

1. Introduction

The effect of rumen fluid donors on *in vitro* rumen fermentation associated with microbiota is significant. Different rumen fluid donors and diets can influence the fermentation process, the resulting fermentation end products, and microbiome diversity [1]. Since *in vivo* measurement of nutrient digestion in the rumen requires ruminally cannulated animals, which are invasive, costly, labor-intensive, and prone to errors due to host-animal variation, alternative techniques have been developed. These include *in vitro* procedures for estimating ruminal fermentation and nutrient digestion of potential feeds or feed additives [2]-[4]. *In vitro* methods using rumen fluid from animals as an inoculum have been widely used for years to evaluate dietary feed ingredients [5] [6]. However, few studies have examined the effects of one or more donor steers on *in vitro* gas production, rumen fermentation profiles, changes in bacterial diversity, and greenhouse gas (GHG) emissions. Furthermore, research using rumen-fistulated animals has shown no significant differences between cannulated and non-cannulated cows in dry matter intake (DMI), volatile fatty acid (VFA) profiles, milk production, milk composition, or enteric methane (CH₄) emissions. However, it was noted that the CH₄ yield and intensity decreased [7] [8], suggesting that trace levels of oxygen (<20 µM O₂) may inhibit CH₄ emissions by suppressing the viability of methanogens. Nonetheless, no *in vitro* or *in vivo* studies have confirmed this hypothesis. Therefore, we hypothesize that using an elevated dissolved O₂-water supply during *in vitro* fermentation could inhibit and alter methanogenesis and alter the bacterial community.

Collecting rumen liquor from multiple donor animals is common for *in vitro* studies [9] [10]; however, to our knowledge, no studies have directly examined how one or more donor steers, about dissolved oxygen (O₂) levels in the rumen fluid (RF), affect *in vitro* rumen fermentation profiles, gas production, changes in rumen bacteria, and greenhouse gas (GHG) emissions. Although it is well established that rumen microbes are strictly anaerobic, and only particular species can survive for some time when exposed to more than a few parts per million (ppm) of O₂ [11], specific information on how dissolved O₂ levels in the rumen liquor influence ruminal dry matter (DM) digestibility (DMD), GHG emissions [CH₄,

nitrous oxide (N₂O)], and microbial dynamics during *in vitro* incubation appears to be lacking. Additionally, while the O₂ sensitivity of methanogen growth is well known [12] [13], the presence of O₂ and its impact on methanogenesis in rumen liquor have been overlooked, and no published data exist on attempts to measure its concentration.

It has been suggested that O₂ may enter the rumen with food, saliva, or by diffusion from the blood [14]. Typically, a small proportion of O₂ (0.5% - 6.5% by volume) is found in rumen gases [15] [16]. The rumen contains facultatively anaerobic bacteria that can tolerate or utilize O₂ [11]. For example, coliform bacteria, which are facultative anaerobes, have been isolated from the rumen [17]. It has been reported that methanogenesis and growth of methanogens (*Methanococcus voltae*, *M. vanniellii*, *Methanosarcina*, and *Methanobacterium ruminantium*) were inhibited after short-term (10 min) exposure to O₂ (up to 5 KPa O₂) [12] [18] [19]. However, recent research shows that some methanogens, such as *M. bryantii*, are O₂-stable and can survive exposure to air for several hours or more without losing viability [20]. Typically, up to 500 nmol/L O₂ has been found in the rumen of cows, sheep, and goats [16]. An O₂-affinity study [13] reported that rumen ciliates like *Polyplastron multivesiculatum* and *Eudiplodinium maggii* could tolerate O₂ concentrations of up to 5 to 10 µM, with apparent half-saturation constants (K_m values) of 1.0, 1.7, and 5.2 µM O₂, respectively. Rumen ciliate species such as *Dasytricha ruminantium* and *Isotricha prosoma*, along with rumen fungus *Neocallimastix patriciarum*, also possess hydrogenosomes related to O₂ utilization [21]-[24]. However, exposure to liquid-phase O₂ concentrations above 10 µM causes rapid inactivation of respiration, and an excess of 14 µM leads to 50% inhibition of H₂ production in the hydrogenosome-containing rumen fungus *Neocallimastix patriciarum* [24]. Some rumen bacteria, such as *Bacteroides ruminicola*, contain CO₂-binding cytochromes, and O₂-consuming substrates have been isolated from rumen microorganisms, such as *Butyrivibrio fibrisolvens*. The current study aims to evaluate: 1) the potential effects of a single or pooled ruminal inoculum and dissolved oxygen (O₂) concentrations on gas production and GHG emissions over a 24-hour incubation, and 2) how various levels of dissolved O₂ and different dietary contrasts influence *in vitro* digestibility, CH₄ and other gas emissions, fermentation rate, and microbial changes.

2. Materials and Methods

The experiment was conducted at the Caprine Research and Education Unit at Tuskegee University. The Tuskegee University Animal Care and Use Committee approved the animal care, handling, and sampling procedures for this experiment (#T02-2002-1).

2.1. Experimental Design

Two sets of *in vitro* experiments were conducted. An initial study evaluated the effects of individual or equally pooled rumen liquors and dissolved oxygen (O₂)

concentration on ruminal pH, gas production, CH₄ production, and changes in microbiota. The second objective involved experiments to assess the effects of various levels of dissolved O₂ concentration [control (0 ppm), 0.1 ppm, and 0.2 ppm], and two contrast diets (grain-based vs. forage-based diets) on the *in vitro* digestibility, CH₄ and other gas emissions, rumen fermentation rate, and microbial changes.

2.2. Rumen Fluid Donor Animals

This experiment was conducted to compare the utility of using ruminal populations collected separately from six individual steers with that of a mixture prepared from those six steers. Six individual rumen-cannulated steers were selected as donors (2 years of age, Black Angus; *Bos taurus*) based on similar body weight (BW; 795.0 ± 23.76 kg). Rumen fluid was collected from each of the six steers 2 h after feeding. Animals had been receiving a total mixed ration (TMR) diet based on steam-flaked corn (52%), WDGS (20%), wet corn gluten (10%), cottonseed meal (10%), chopped corn stalks (7%), and mineral and vitamin premix (1%).

Ruminal contents from individual steers were placed into pre-warmed insulated thermal bottles, transported to the laboratory, homogenized, filtered through 4 layers of cheesecloth, and used as an *in vitro* inoculum. Once filtered, an equal volume of each liquor collected was sampled and equally pooled with the others to obtain the seventh treatment (e.g., pooled rumen fluid). Rumen fluid was maintained in a water bath at 39°C under CO₂-saturated conditions until inoculation. The duplicated anaerobic condition was maintained by continuously flushing the bottle headspace with CO₂.

2.3. In Vitro Experiment

All gases were collected using an ANKOM Gas Production system for analyses of CH₄, nitrous oxide (N₂O), and hydrogen sulfide (H₂S). For *in vitro* incubations, substrates of mixed diets containing steam-flaked corn (62.5%), WDGS (30%), cotton-seed meal (7.0%), and urea (0.5% as-fed) were placed in 250 mL ANKOM (ANKOM Technology Corp. Macedon, NY) sample bottles containing 6.0 g of substrate, 50 mL of the individual or pooled ruminal fluid (pH 5.8) and either 40 mL artificial saliva (pH 6.8, as fed) or 40 mL of different levels of dissolved O₂-contained artificial saliva for 24 h incubation (pH 6.8); [25] [26]. It has been reported that over the range of substrate concentrations (0.2 - 2.0 g/100mL buffer solution) used, the quantity of substrate added to the bottle had no significant effect on the lag time and fractional rates of gas production, except the total amount of gas production [27].

2.3.1. Experiment 1

A set of *in vitro* incubations was conducted to evaluate the effects of individual or equally pooled rumen liquors and dissolved oxygen (O₂) concentration on ruminal pH, gas production, CH₄ production, and changes in microbiota. For *in vitro* incubations, 2.5 g of feedlot ration diets (% as-fed; **Table 1**) were placed in 250 mL

ANKOM (ANKOM Technology Corp. Macedon, NY) sample bottles containing either 40 mL of pooled ruminal fluid (pH 5.61) and or 40 mL of individually collected rumen fluid incubated with 160 mL artificial saliva (pH 6.8; Min *et al.*, 2005). Each incubation was done in three replications. The bottle was flushed with CO₂ gas before being capped with an ANKOM Gas Production module. Dissolved oxygen (O₂) concentration was measured before the experiment commenced (Day 0) and after completion (Day 30). An initial ruminal pH was measured prior to the start of rumen incubation. Bottles were flushed with CO₂ gas before being capped with an ANKOM Gas Production module. One-liter FlexFoil PLUS sample bags (SKC Inc., Eighty-Four, PA) were connected to the modules. Bottles were placed in a shaking incubator at 39°C—bottles containing only inoculum and buffer served as blanks.

Table 1. Ingredients and nutrient composition (% DM) of the experimental diets tested *in vitro*.

Item ¹	Diet (% as-is)				
	SFC	Alfalfa	Grain-based	Forage-based	SEM
Ingredients					
Steam-flaked corn	-	-	65.0	19.0	
WDGS	-	-	10.0	10.0	
Cottonseed meal	-	-	5.5	5.5	
Alfalfa, ground	-	-	19.0	65.0	
Urea	-	-	0.5	0.5	
Chemical composition					
DM	90.3	91.0	94.9	94.6	0.04
CP	9.6	24.4	17.2	18.9	0.81
ADF	3.9	29.7	7.2	21.1	0.82
NDF	9.2	37.3	15.2	35.3	2.27
Lignin	1.4	6.8	2.7	11.1	0.25
NFC	76.7	23.0	48.6	24.5	0.11
Starch	76.0	0.7	31.4	10.1	0.33
CF	3.2	2.2	4.7	4.6	0.25
Ash	1.5	13.2	14.3	16.7	0.28
TDN	71.7	59.0	69.0	59.9	1.68

SFC = steam-flaked corn, WDGS = wet distiller's grains, soluble, DM = dry matter, CP = crude protein, ADF = acid detergent fiber, NDF = neutral detergent fiber, NFC = non-fiber carbohydrate (100-CP-NDF-CF-ash), CF = crude fat, TDN = total digestible nutrient [86.2 – (0.513 × % NDF) × 0.88].

After 24 hours of incubation, two 1.0 mL samples were taken from each sample bag for gas analysis. Gas sample bags were closed and separated from the module, and gas concentrations (CH₄ and N₂O) were then determined using an SRI gas

chromatograph equipped with a mechanized electron capture detector (ECD) and flame ionization detector (FID) (SRI Instruments, Torrance, CA). Analyzers were calibrated with GHG gases of known concentrations. Hydrogen sulfide (H₂S) production was measured with a gas monitor (GX2012 model, RIKIN KEIKI Co., Ltd., Tokyo, Japan). Specific gas concentrations were plotted against total gas production to estimate total gas emission per hour or gas produced on a DM basis.

2.3.2. Experiment 2

The second part of the experiment (Exp. 2) focused on assessing how different levels of dissolved oxygen (O₂) and different diets (grain-based versus forage-based) influence *in vitro* digestibility (IVDMD), methane (CH₄) and other gas emissions, rumen fermentation, and microbial populations. Triplicate *in vitro* gas production and 16S pyrosequencing analyses were conducted. All other *in vitro* fermentation conditions were similar to those described in Exp. 1. Anaerobic conditions were maintained by continuous flushing of the bottle headspace with CO₂.

For the different dissolved O₂ concentration test, O₂ gas was prepared as a pre-made stock solution with different dissolved O₂ levels in a buffer solution, bubbled at 2.0 psi, and then added to each fermenter. The final dissolved O₂ concentrations in the fermenters were set to 0.0 (control), 0.1 (3.13), and 0.2 (6.25) ppm (μM/L), respectively. Cumulative ruminal gas production was recorded every 30 min for 24 h [28] via the ANKOM system, which records pressure buildup in individual vessels. Cumulative ruminal gas productions were recorded for 24 h because ruminal CO₂ and CH₄ productions were generally produced with short intervals throughout 15 to 24 h after feeding (alfalfa hay and grain diets) [29].

2.4. Laboratory Analysis

Dairy One Forage Testing Laboratory (Ithaca, NY) analyzed the nutrient and mineral content of ingredients and dry matter. Analytical DM concentrations of diet samples were determined by oven-drying at 105°C for 24 h [30] [31]. Ash, ether extract, and minerals were analyzed according to AOAC methods [30] [31]. Concentrations of N were determined using an organic elemental analyzer (Flash 2000; CE Elantech Inc., Lakewood, NJ, USA; AOAC, 1998). Concentrations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were sequentially determined using an ANKOM200/220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to the company's methodology, based on [32]. Sodium sulfite was used in the procedure for NDF determination and pre-treated with heat-stable amylase (Type XI-A from *Bacillus subtilis*; Sigma-Aldrich Corporation, St. Louis, MO, USA). Total digestible nutrient (TDN) concentrations were calculated based on % NDF content [TDN = 86.2 – (% NDF × 0.513) × 0.88]; [30]. The ether extract was measured [31] using a fat analyzer (XT20, ANKOM Technology).

Following 24 h of fermentation, 1 mL samples from each incubator were transferred into 5 mL Falcon tubes and stored at –80°C for future volatile fatty acids (VFA) analysis. Fermentation products (alcohols, VFAs, and aromatic ring compounds) in frozen samples were analyzed by gas chromatography (Hewlett-Packard 6890, Ag-

ilent Technologies, Palo Alto, CA) as previously described [31]. L-Lactate was analyzed using an autoanalyzer (Model 2700, Yellow Springs Instrument, Yellow Springs, OH). Additionally, two 10 mL samples were collected from each sample bag for analysis. Gas sample bags were sealed and separated from the module. Then the gas concentrations of CH₄ and N₂O were determined using an SRI gas chromatograph equipped with an electron capture detector (ECD) and a flame ionization detector (FID) (SRI Instruments, Torrance, CA; Bucha *et al.*, 2025). The analyzers were calibrated using a known gas concentration. Hydrogen sulfide (H₂S) production was measured using a gas monitor (GX2012 model, RIKIN KEIKI Co. Ltd., Tokyo, Japan). Specific gas concentrations were plotted against total gas production to estimate overall gas emissions per hour or gas produced on a dry matter basis.

2.5. Genomic DNA Extraction and Quantitative PCR

Triplicate qPCR analyses were conducted to quantify the diversities of three major bacterial phyla: Bacteroidetes, Firmicutes, and total methanogens (Table 2). By necessity, this required an examination of bacterial phylum (as measured by Firmicutes and Bacteroidetes) and total methanogen changes associated with CH₄ emission and digestibility. One mL aliquots of rumen fluid samples were centrifuged at 17,000× *g* for 1 min. According to the manufacturer's protocol, DNA was extracted from 100 mg of the collected pellets using the Quick-DNA Fungal/Bacterial Mini-prep Kit (ZymoResearch, Irvine, CA). DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA) using a Synergy 2 Microplate Reader (BioTek Instruments, Winooski, VT). Samples were diluted to a final concentration of 5 ng·μL⁻¹. Quantitative PCR was performed using primers specific to Firmicutes, Bacteroidetes, and total methanogens, as described by Min *et al.* [25].

Table 2. Primers were used for qPCR of rumen microorganisms in the current study.

Target	Primer/probe name	App. Size Product (bp)	Annealing Temp. °C	Reference
Bacteroidetes				
Bact 934F	GGARCATGTGGTTTAATTCGATGAT	126	60	Guo <i>et al.</i> , 2008
Bact 1060 R	AGCTGACGACAACCATGCAG			
Firmicutes				
Firm 934R	GGAGYATGTGGTTTAATTCGAAGCA	160	60	Guo <i>et al.</i> , 2008
Firm 1060R	AGCTGACGACAACCATGCAC			
Total methanogens				
UniMet-1F	CCGGAGATGGAACCTGAG AC	160	60	Zhou <i>et al.</i> , 2009
UniMet-1R	CGGTCTTGCCAGCTCTTATTC			

2.6. Data Analysis and Interpretation

The statistical model included the fixed effects of animal, dietary treatment, and their interaction. All the data in the Tables were analyzed by the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) using the following statistical model:

$$Y_{ijk} = \mu + T_i + Y_j + TY_{ij} + e_{ij}$$

where Y_{ijk} = observation; μ = overall mean for each parameter; T_i = effect of diet; Y_j = effect of host animal; TY_{ij} = interaction between treatments; and e_{ij} = random error, used to test diet, host animal (Exp. 1), and diet $\times$ dissolved O_2 levels (Exp. 2) interactions. Animals were the experimental unit (Exp. 1) and were treated as a random effect. Treatment means were separated by the least significant differences when the diet $\times$ dissolved O_2 levels interaction was significant ($P < 0.05$). Significance levels were predetermined at $P < 0.05$; trends were determined at $0.05 < P < 0.10$.

3. Results and Discussion

Chemical Composition of Diets

The chemical composition of the dietary components and treatments is presented in **Table 1**. Of the primary ingredients used in this study, steam-flaked corn had higher starch contents (76.0% DM) than alfalfa hay (0.7% DM). However, steam-flaked corn had a lower NDF (9.2%) and ADF (3.9% DM) compared to alfalfa forage (37.3% and 29.7% DM, respectively). In grain-based diet, DM, CP, ADF, NDF, lignin, NFC, starch, and TDN contents were 94.9%, 17.2%, 7.2%, 15.2%, 2.7%, 48.6%, 31.4%, and 69.0% DM, while forage-based diets for DM, CP, ADF, NDF, lignin, NFC, starch, and TDN contents were 94.6%, 18.9%, 21.1%, 35.3%, 11.1%, 24.5%, 10.1%, and 59.9% DM, respectively (**Table 1**). Therefore, the nutrient contents of the experimental diets increased for CP, ADF, NDF, and lignin with increasing alfalfa inclusion in the forage-based diet. Across the experimental grain-based diets, NFC, starch, and TDN increased as steam-flaked corn content increased.

3.1. Experiment 1

Individual animal variations

The effect of single or multiple liquor-donor steers on rumen bacterial phylum, methanogens populations, and dissolved oxygen (O_2) concentration is presented in **Table 3**.

The study by Brulc *et al.* [33] revealed significant differences in microbiome composition among different animals. The same authors reported that one steer had a markedly different microbiome composition from the other, despite similar housing and dietary conditions and without apparent differences in the animals' physiology. These results are consistent with the present study. In the present study, the bacterial population of Firmicutes (23.3×10^{-8}) and Bacteroidetes (19.5×10^{-8}) phyla was greater for steer tag # 9 than for other steers' rumen fluid. However, total methanogen archaea populations in the rumen were most heightened composition for steer tag # 18 (41.5×10^{-5}), intermediate for steers # 19 (30.3×10^{-5}) and # 7 (26.8×10^{-5}), and lowest methanogens for steers # 6 (9.8×10^{-5}), #9 (4.3), #13 (6.6), and mixed rumen fluid (13.4×10^{-5}) when animals received the similar feedlot diets. Likewise, Durso *et al.* [34] reported significant animal-to-animal variation in microbial communities when typical feedlot diets were fed,

variation that could not be attributed to breed, gender, diet, age, or weather among beef cattle. Several factors affect rumen microbial activity [35], including collection time [36] [37], donor animals, diet type [38], and the final dilution rate with buffer [39] [40]. As a result, the variation in the rumen microbiome due to inoculum source is ultimately attributed to variation in rumen gas production and rumen fermentation profiles. Therefore, collecting rumen inoculum from several animals is often recommended, and combining it can reduce variation [38].

Table 3. The effect of single or multiple liquor-donor steers on rumen bacterial phylum, methanogens populations, and dissolved oxygen (O₂) concentration.

Item	Source of rumen fluid (RF)								P-value
	Mixed RF	Steer 6	Steer 7	Steer 9	Steer 13	Steer 18	Steer 19	SEM	
Initial BW, kg	-	784.9	824.5	897.1	752.3	688.3	823.1	23.76	-
Rumen bacterial phylum and total methanogens									
Firmicutes (F: 1×10^{-8})	12.5 ^b	12.8 ^b	14.3 ^b	23.3 ^a	15.1 ^b	13.2 ^b	12.1 ^b	1.45	0.01
Bacteroidetes (F: 1×10^{-8})	9.3 ^b	11.7 ^b	11.5 ^b	19.5 ^a	7.7 ^c	14.3 ^b	9.5 ^b	1.44	0.01
F/B ratio	1.34 ^{ab}	1.09 ^b	1.24 ^b	1.19 ^b	1.96 ^a	0.92 ^b	1.27 ^{ab}	0.04	0.01
Total methanogen (1×10^{-5})	13.4 ^c	9.8 ^d	26.8 ^b	4.3 ^c	6.6 ^c	41.5 ^a	30.3 ^b	1.34	0.001
Dissolved O ₂ concentration ³									
Day 0 ppm	0.39 ^{cd}	0.25 ^d	0.54 ^c	0.63 ^b	0.85 ^a	0.34 ^{cd}	0.34 ^{cd}	0.039	0.01
μM/L	12.19 ^{cd}	7.81 ^d	16.88 ^c	20.0 ^b	25.56 ^a	10.63 ^{cd}	10.63 ^{cd}	1.04	0.01
Day 30 ppm	0.15	0.06 ^c	0.08 ^c	0.26 ^b	0.33 ^b	0.36 ^{ab}	0.54 ^a	0.030	0.01
μM/L	4.69	1.88 ^c	2.50 ^c	8.13 ^b	10.31 ^b	11.25 ^{ab}	16.88 ^a	0.946	0.01
Average ppm	0.27 ^c	0.16 ^c	0.31 ^b	0.45 ^a	0.59 ^a	0.35 ^b	0.44 ^a	0.024	0.01
μM/L	8.44 ^c	4.85 ^c	9.69 ^b	14.07 ^a	17.93 ^a	10.94 ^b	13.76 ^a	0.790	0.01

^{a-d}numbers in a row with superscript letters are significantly different ($P < 0.05$).

Oxygen exists in the rumen of cattle, both in the liquid phase [12] and in the gas phase [41] [42]. Interestingly, concentrations of O₂ and N₂ in rumen gas increased during feeding, while concentrations of CO₂ and CH₄ decreased in cannulated sheep fed 100% hay and/or 20%:80% corn-flaked hay diets [41]. The present study showed that dissolved O₂ concentrations in rumen liquors on day 0 were 0.25 (7.8), 0.54 (16.4), 0.15 (4.7), 0.63 (19.7), 0.85 (26.6), 0.34 (10.6), and 0.39 (12.2) ppm in cannulated steers #6, #7, #9, #13, #18, #19, and mixed rumen fluid (Table 3), respectively, which is similar to other data [16]. These results indicate that dissolved O₂ concentrations in the rumen of cannulated steers were measured (on day 0 and day 30) and varied (SD = 0.21) among animals. It has been reported that a rumen cannula may permit outside air into the rumen headspace [43].

In addition, high levels of dissolved O₂ concentration in the rumen fluid in steers # 9 and #13 on day 0 (Table 3) were relatively lower levels of the total methanogen population (4.3 and 6.6×10^{-5}) compared to other lower O₂-groups (9.8

to 41.5×10^{-5}), indicating that short- or long-term exposures of O_2 through rumen cannula could be inhibited methanogenesis activity [16]. These results are consistent with other data [16] [44]. Their research indicates that ruminal methanogenesis was totally and irreversibly inhibited after short-term exposure (about 10 min) to 5 KPa (37.5 mmHg or 0.05 atm) [12]. Previous studies have shown that CH_4 production is dependent on dissolved O_2 concentrations in ruminal fluid [12] [45] [46], reflecting the high sensitivity of methanogenic bacteria to O_2 . However, the protozoal and bacterial populations had equivalent rates of O_2 utilization at both the lower detectable level of O_2 within the rumen ($0.25 \mu M$) and at the concentration range attained immediately before feeding in sheep, goats, and cattle (1.0 to $7.0 \mu M$; [13] [16] [47]). These results could also be related to the fundamental differences among individual animals [48] [49]. Methanogenic archaea comprise a distinct cluster of strictly anaerobic microorganisms that obtain energy for growth by reducing CO_2 or other simple compounds (e.g., acetate, formate, methanol, and alkylamines) to CH_4 [20]. It has been reported that rumen cannulation did not affect dry matter intake (DMI), milk production, milk composition, or CH_4 emissions (g/d per cow); however, CH_4 yield (g/kg per DMI) and intensity (g/kg per energy corrected milk) were decreased [8], indicating that trace amount of dissolved O_2 concentration in the rumen may alter ruminal CH_4 production and selected methanogen populations. Thus, it is possible to hypothesize that trace amounts of dissolved O_2 in the rumen fluid could lead to greater anti-methanogenic activity and warrant further study. However, some studies show interrelationships between individual animals' genetics and their microbiome, as well as the heritability of some rumen microbiome constituents [50] [51].

Oxygen can come into the rumen 1) with food and drink, 2) with saliva, or 3) from the blood and rumen tissues [45] [52]. In the present study, dissolved O_2 concentration in the grain-based, forage-based diets and control blank (rumen fluid only without diet) fermenters decreased ($P < 0.01$) with increasing incubation time (0 h vs. 24 h; **Figure 1(a)**), while ruminal pH increased with increasing incubation time (**Figure 1(b)**): forage-based diet and blank significantly increased ($P < 0.001$) the ruminal pH with incubation time (**Figure 1(b)**). These results are consistent with other studies in sheep, goats, and cattle [12] [47]. Although the rumen has been frequently regarded as an anaerobic atmosphere [11] [53], direct measurement of dissolved ruminal gases in rumen-cannulated sheep, goats, and cattle has shown that dissolved O_2 is present in ruminal fluid at concentrations of $0.4 \mu M/L$ in goats, $0.5 - 1.0 \mu M/L$ in sheep, and $4.5 - 7.0 \mu M/L$ in cattle. However, these O_2 intensities were undetectable ($<0.25 \mu M$) 15 - 30 min after post-feeding in the rumen of goats [16] [47]. This could elucidate that the O_2 ingested with the food is promptly utilized by the facultatively aerobic organisms present in the rumen [12]. Some microbes seek O_2 concentration in the following ranges: *Bacillus subtilis*, an obligate aerobe, $200 \mu M$; *Escherichia coli*, a facultative anaerobe, $50 \mu M$; *Azospirillum brasilense*, a microaerophile, $4 \mu M$; and *Desulfovibrio vul-*

gari, an aerotolerant anaerobe, 0.4 μM [54]-[56]. In addition, Hillman *et al.* (1989) [47] reported that the presence of rumen protozoa decreases the O_2 sensitivity of CH_4 production by suspensions of a cultured methanogenic bacterium *Methanosarcina barkeri*. This could be explained by the fact that rumen protozoa and some facultative aerobic bacteria play a role in ruminal O_2 utilization, in addition to producing fermentation end products (e.g., VFAs) utilized by the ruminant and H_2 utilized by methanogenic bacteria [47]. In addition, some rumen microbes may have an energy-sensing mechanism, such as an aerotaxi system [57]. Aerotaxi is a response to changes in respiratory electron flow that result from an increase or decrease in O_2 concentration [58] [59]. This is the basis for electron acceptor taxis [57]. Optimal electron transport requires an adequate source of electron donors (NADH or FADH_2) and O_2 or an alternative electron acceptor to serve as a sink for electrons [60]. In the presence of O_2 , alternative electron acceptors such

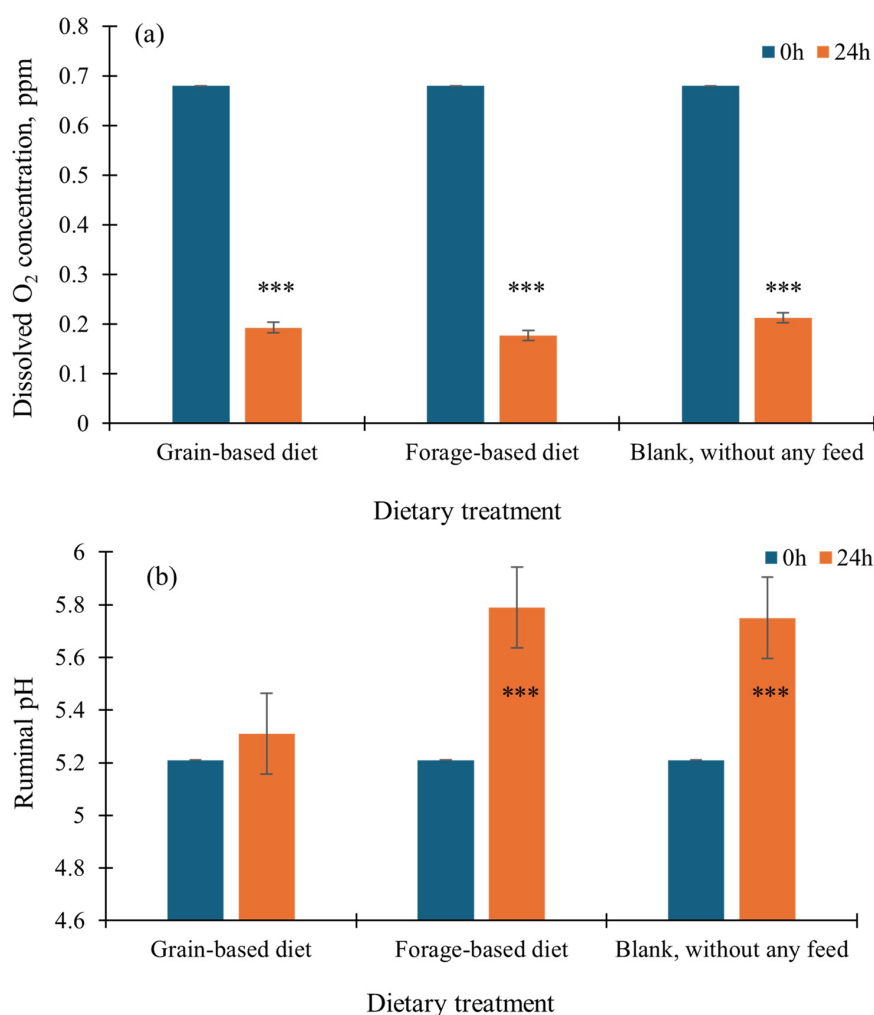


Figure 1. Dissolved O_2 concentration (a) and ruminal pH (b) among grain-based, forage-based diets, and blank (only rumen fluid without dietary supplementation) incubated with pooled rumen fluid (pH 5.2) at the beginning (0 h) and 24 h. Asterisks (***) indicate significance at $P < 0.001$.

as fumarate and nitrate support electron flow [57]. Further research is necessary to determine the specific contributions of bacteria, protozoa, and methanogens, which play a crucial role in maintaining the rumen under near-anoxic conditions.

The effect of the source of rumen fluid on *in vitro* gas production was extensively discussed [61] and frequently the primary source of variation both within and among donor animals [3] [4] was studied to study the fermentation kinetics of ruminant feeds. In the present study, cumulative *in vitro* gas, total gas production, and bacterial phylum changes as a function of animal variation among individual cannulated donor steers are presented in Table 4 and Figure 2. In the current study, incubations with pooled rumen fluid resulted in improved ($P < 0.01$) *in vitro* gas production (Figure 2 and Table 4), CH_4 , and N_2O emissions ($P < 0.001$; Table 4) compared with rumen fluid collected from individual steers. In this experiment, significant differences were observed in the gas production profiles among the donor animals' rumen fluid sources. Part of this variation is likely due to factors such as host animals, as while the animals were fed the same diet throughout the experiment, considerable day-to-day variation has been observed in the rumen metabolism profiles of sheep [7]. However, CH_4 and H_2S production, Firmicutes (F), Bacteroidetes (B), F/B ratio, and total methanogens (Table 4) were intermediate values for the pooled rumen fluid than for the individual steer rumen fluid samples. Results indicated that variation in rumen gas production due to inoculum source is ultimately attributed to differences in microbial population profiles in the rumen.

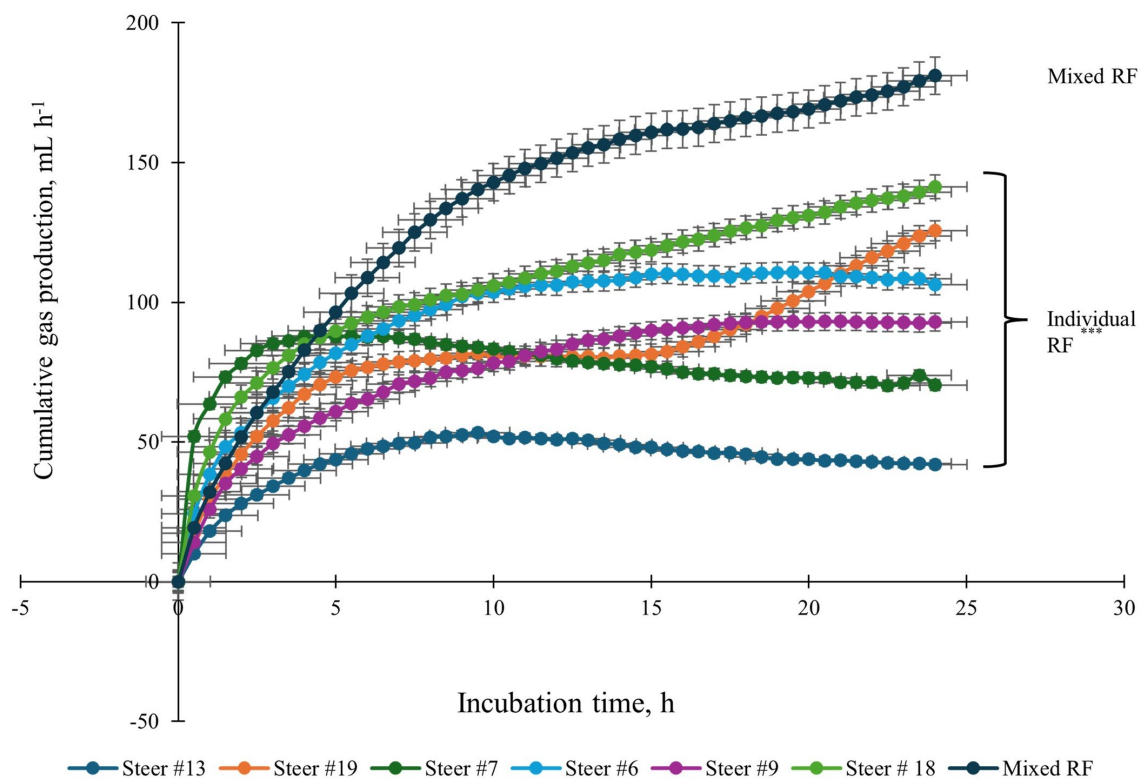


Figure 2. Cumulative *in vitro* gas production using combined rumen fluid (RF) or single RF-donor steers (Experiment 1: Individual RF versus pooled RF; $n = 2$). Asterisks (***) indicate significance at $P < 0.001$.

Table 4. The effect of the source of rumen fluid on *in vitro* gases emissions.

Item	Source of rumen fluid (RF) ¹							SEM	<i>P</i> -value
	Mixed RF	Steer 6	Steer 7	Steer 9	Steer 13	Steer 18	Steer 19		
<i>In vitro</i> gas production (n =3)									
Total gas production ¹	181.0 ^a	106.2 ^c	70.3 ^d	92.9 ^c	41.9 ^d	141.3 ^b	125.6 ^b	14.93	0.001
CH ₄ , mL 24 h ⁻¹	0.97 ^a	0.32 ^b	0.43 ^b	0.41 ^b	0.39 ^b	0.94 ^a	0.87 ^a	0.052	0.001
N ₂ O ppm 24 h ⁻¹	3.7 ^a	– ²	– ²	0.12 ^c	0.46 ^b	0.20 ^c	0.69 ^b	0.069	0.001
H ₂ S ppm 24 h ⁻¹	5.0 ^b	41.8 ^a	19.6 ^{ab}	3.5 ^b	3.2 ^b	2.9 ^b	12.5 ^b	5.69	0.01

¹All the gas production was substituted by blank gas production with rumen fluid and buffer solutions without substrates (total gas = 6.48 ± 0.34; n = 3). Inoculum for *in vitro* gas production was used on day 0 only. ²Lost gas samples. ^{a-d}numbers in a row with superscript letters are significantly different ($P < 0.05$).

3.2. Experiment 2

In vitro ruminal gas production

In the present study, pooled rumen fluid produced more consistent CH₄ gas and microbial diversity results than individual rumen fluid donors. Therefore, combined rumen fluid was chosen as the inoculum source in experiment 2 based on these preliminary data. The current study evaluated the effects of dissolved O₂ supply associated with two contrasts, grain-based and forage-based dietary supplementations, on *in vitro* DMD, cumulative gas, and GHG emissions over a 24-hour incubation (**Figure 3; Table 5**). There was no dissolved O₂ treatment effect on average IVDMD, with no diet x treatment interactions, but IVDMD was lower ($P < 0.01$) for forage-based diets than for grain-based diets (41.8% vs. 52.6%, respectively). Czerkawski and Breckenridge [45] reported that neither the disappearance rate of sugar nor the digestibility of organic matter and the production of volatile fatty acids (VFA) were affected by the addition of O₂ at the three different incubation times.

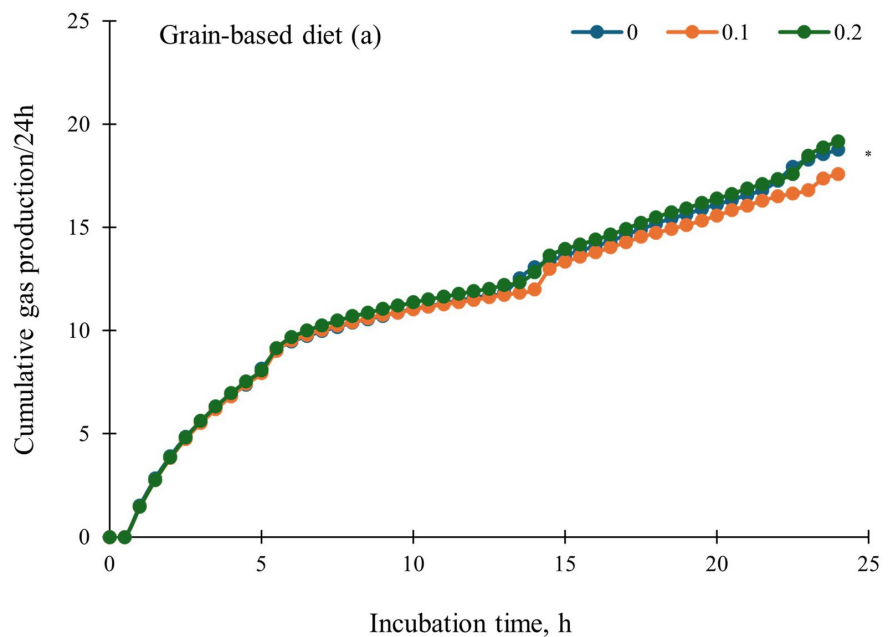
In the present study (**Table 5**), overall mean values of total ruminal gas (18.7 vs. 18.6 mL/23h) and CH₄ (3.9 vs. 3.8 mL/h) were similar between grain-based and forage-based diets, while H₂S (0.34 vs. 0.93 mL/d) gas production was higher ($P < 0.05$) for the forage-based diet than for the grain-based diet, respectively. However, increasing O₂ supply in the diet decreased ($P < 0.05$) total gas (mL/24h), CH₄ (mL/24h), and H₂S (mL/24h) production in the forage-based diet, with no diet × treatment interaction ($P > 0.05$). In addition, cumulative *in vitro* gas production (**Figure 3**) was generally lower ($P < 0.05 - 0.01$) in the O₂-contained water supply group, particularly in the forage-based diet compared to the grain-based diet (**Figure 3**), similar to what was reported by Czerkawski and Breckenridge [45]. Czerkawski and Breckenridge [45] reported that the 15 mL of O₂ infusion into an *in vitro* rumen fermenter had little or no effect on CH₄ production, but the addition of 26 mL of O₂ supply resulted in progressive inhibition of CH₄ production and accumulation of hydrogen (H₂). Similar results regarding the effects of O₂ on CH₄ production have been reported [16] [62]. This agrees with the data from Scott *et al.* [12], who reported that increasing the partial pressure of O₂ in the gas phase decreased methanogenesis. This decreased

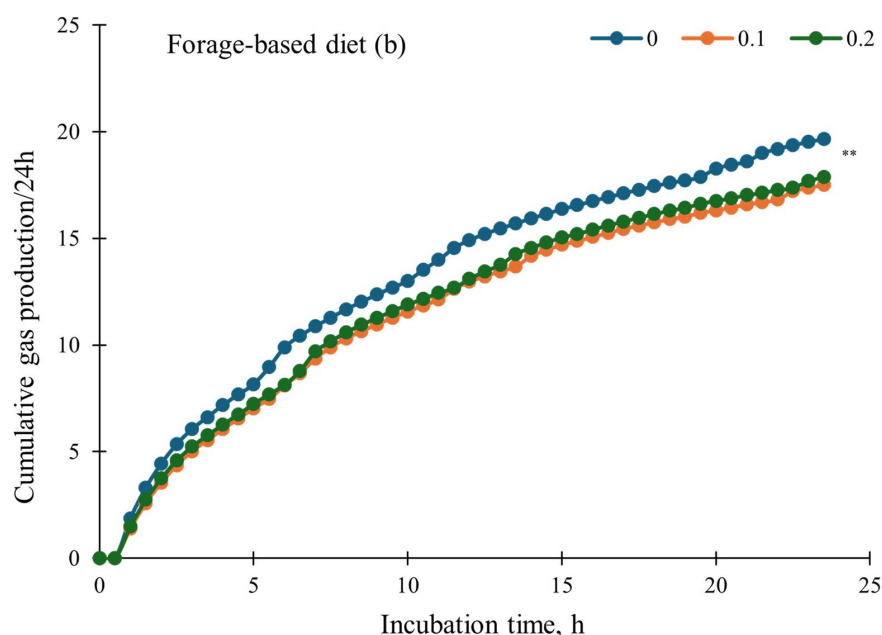
methanogenesis is partially reversible even after exposure to 2.5 kPa O₂ (6.25 mmHg O₂); 5 kPa O₂ (15.0 mmHg O₂) results in a significant, total interruption of CH₄ production [12]. Results indicated that trace levels of O₂ concentration, ranging from 2.5 to 5.0 kPa [12], in the rumen could reduce CH₄ emissions from ruminants without affecting IVDMD and warrant further study.

Table 5. The effect of oxygenated water supply and different basal diets on *in vitro* ruminal gas production and microbiota changes in either grain-based (GBD) or forage-based diets (FBD) supplementation¹.

Diet/O ₂ , ppm	Dietary treatment						Average		SEM	Diet	Tret	Diet × Tret
	Grain-based diet			Forage-based diet			Grain	Forage				
	Control 0 ppm	0.1 ppm	0.2 ppm	Control 0 ppm	0.1 ppm	0.2 ppm						
IVDMD, %	53.9	53.1	50.8	41.2	42.8	41.1	52.6 ^a	41.7 ^b	0.63	0.01	0.71	0.68
Ruminal gas production												
Total gas production, 24 h	18.9	17.7	19.4	19.9 ^A	17.7 ^B	18.2 ^{AB}	18.7	18.6	0.30	0.60	0.03	0.25
CH ₄ , mL/24h	4.1	3.6	3.9	4.4 ^A	3.4 ^B	3.5 ^B	3.9	3.8	0.14	0.99	0.05	0.68
H ₂ S, mL/24h	0.34	0.26	0.43	1.2 ^A	0.7 ^B	0.9 ^{AB}	0.34 ^b	0.93 ^a	0.08	0.01	0.05	0.52
Rumen bacterial phylum and total methanogens												
Firmicutes (F: 1 × 10 ⁻⁸)	13.1 ^c	18.1 ^b	20.6 ^a	28.1 ^A	25.0 ^B	23.7 ^B	17.3 ^b	25.6 ^a	0.73	0.01	0.50	0.01
Bacteroidetes (B: 1 × 10 ⁻⁸)	15.1 ^a	12.4 ^b	11.3 ^b	14.7 ^B	18.0 ^{AB}	19.2 ^A	12.9 ^b	17.3 ^a	0.12	0.01	0.01	0.01
F/B ratio	0.9 ^b	1.0 ^{ab}	1.1 ^a	1.9 ^B	2.0 ^{AB}	2.1 ^A	1.00 ^b	2.00 ^a	0.05	0.01	0.01	0.01
Total methanogen (1 × 10 ⁻⁵)	5.7 ^b	8.9 ^a	8.3 ^{ab}	21.0 ^B	24.3 ^A	21.2 ^B	7.6 ^b	22.2 ^a	0.73	0.001	0.001	0.001

¹40 mL of buffer solutions containing different levels of dissolved O₂ (40 mL) and 50 mL of rumen fluid were added. Diet = grain-based vs. forage-based diets. IVDMD = *in vitro* dry matter digestibility. ^{a,b,c} and ^{A,B,C} numbers in a row with superscript letters are significantly different ($P < 0.05$) for grain-based and forage-based diets, respectively.





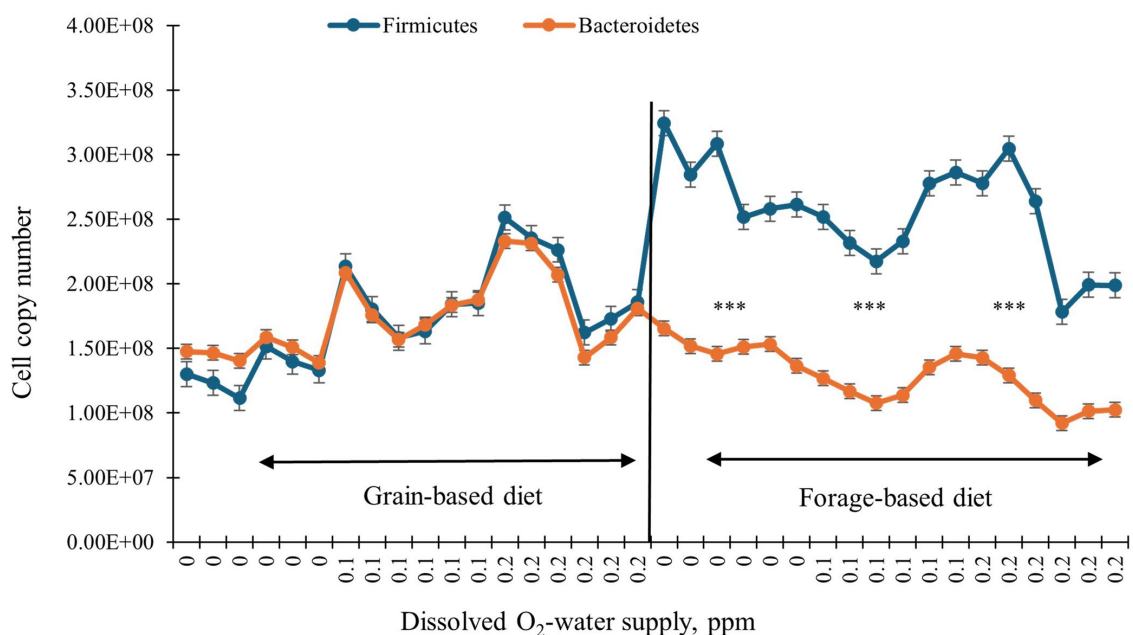
Asterisks (*) and (**) denote significance at $P < 0.05$ and $P < 0.01$, respectively.

Figure 3. Cumulative *in vitro* gas production ($n = 2$) with various levels of dissolved O₂-concentration. Dissolved O₂ concentration was 0, 0.1, and 0.2 ppm, either grain-based (a) or forage-based (b) diets, supplemented with mixed rumen fluid for 24 h.

3.3. Rumen Microbial Community Changes

The modification of the rumen microbiota community due to dietary changes is of considerable interest, as it increases rumen energy supply and improves feed efficiency and animal performance [63]–[65]. In addition, the rumen microbiota is a complex, highly diverse community in a dynamic rumen environment, shaped by host physiology. We investigated how rumen O₂ concentration affects bacterial microbiota composition and total methanogen abundance. We also assessed whether two contrasting diets (grain-based vs. forage-based) on rumen bacteria (Firmicutes vs. Bacteroidetes) and total methanogens changes (Table 5 and Figure 4). An average phylum population was lower ($P < 0.01$; average 15.1%) for Bacteroidetes than for Firmicutes (average 21.5%) for both forage-based and grain-based diets, with diet $\times$ treatment interactions ($P < 0.01$), indicating that increased O₂ concentration and dietary ration may alter the composition of the rumen microbial composition. However, direct evidence for the role of the rumen microbiota in regulating O₂ levels remains lacking. In the present study, there were increased ($P < 0.01$) F/B ratios with a forage-based diet (2.0) compared to a grain-based diet (1.0; Table 5), similar to the earlier reports where a shift from grain to fiber-rich diets led to an increase in the F/B ratios [66]–[68]. During transition periods when ruminants are moved from a primarily forage-based diet to a high-grain diet, significant alterations in the microbiome's relative abundances are observed. The shift from a forage-based diet to a high-grain diet (80% grain) led to numerous changes in the microbiome, including alterations in the F/B ratio, decreased abundance of cellulolytic bacteria such as *Fibrobacter* spp., and in-

creased abundance of *Prevotella* spp., among others [67]. These changes have also been demonstrated in other studies [69]–[71], which support the results of the present study. The ratio of F/B has been connected with other phenotypical features, namely, in adipose metabolism [66] [72]–[74]. In addition, the abundances of the Bacteroidetes and Proteobacteria phyla increased as the grain-based diet became a more significant percentage of the diet, which may be more efficient at fermenting the soluble carbohydrates existing in grain-based diets [67]. They may also be tolerant of the lower pH that occurs when larger amounts of VFA and lactic acid are produced. It has been reported that the proportion of Bacteroidetes decreased drastically when animals were switched from a diet containing a low level of fat to a high level of a plant-based diet [74], even though Bacteroidetes can utilize starch, xylan, pectin, galactomannan, and arabinogalactan (Martine *et al.*, 2011).



Solutions: 40 mL of dissolved O₂-water, 40 mL buffer solution, and 50 ML of rumen fluid were added. Dissolved O₂ concentrations in the *in vitro* rumen incubation were 0.0 (control), 0.1, and 0.2 ppm. Asterisks (***) indicate significance at $P < 0.001$.

Figure 4. Effects of a level of dissolved O₂ concentration on *in vitro* rumen bacterial community changes in either grain-based or forage-based diets supplemented using an RT-qPCR.

Methane production is a subject of significant attention worldwide due to its role in GHG emissions. Numerous sources of CH₄ emissions exist, including wetlands, energy sectors, biomass burning, landfills, and ruminant animals, among which enteric fermentation is the most significant [75] [76]. Increasing dissolved O₂-supply decreased ($P < 0.05$) CH₄ emissions, but quadratic increased total methanogens population when addition of O₂ solution at 0.1 ppm/mL of O₂, with diets × treatment ($P < 0.001$) interactions (Table 5), suggesting that small amounts of oxygen (<0.1 μM/L) addition primarily transit increased total meth-

anogen populations when forage-based diet was suspended in the rumen incubation compared to the grain-based diet. However, increased O₂ supply to 0.2 µM/L was unaffected by the total Methanogen population concentration, consistent with previous data [45] [77]. Tholen *et al.* [77] reported that the addition of a specific O₂ flux of 0.45 µM (mg dry weight)/h did not influence the methanogenesis of the cell suspensions, while higher O₂ flux of 1.35 µM (mg dry weight)/h, CH₄ production caused a reversible decrease in methanogenesis, which was accompanied by a reversible, partial conversion of coenzyme F₄₂₀ to factor F₃₉₀. The same authors reported that *Methanobrevibacter cuticularis* could simultaneously produce CH₄ and consume O₂. Seedorf *et al.* [78] showed that O₂ consumption in *Methanobrevibacter arboriphilus* is mediated by F₄₂₀H₂ oxidase (FprA), an A-type flavoprotein that catalyzes the reduction of O₂ to H₂O. In the presence of O₂, F₄₂₀H₂ is thought to be oxidized by O₂ more rapidly than F₄₂₀ is reduced by H₂, leading to an accumulation of the oxidized form [78]. Consequently, Czerkawski and Breckenridge [52] reported that the 15 mL of O₂ solution (9% initial concentration of O₂ in stock solution) infusion into an *in vitro* rumen fermenter had little or no effect on CH₄ production, but the addition of 26 mL of O₂ supply resulted in progressive inhibition of CH₄ production and accumulation of H₂. It has been reported that CH₄ production was positively correlated with *Methanobrevibacter thaueri*, *Methanobrevibacter millerae*, and *Methanobrevibacter smithii* compared with *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae*, whereas no relationship was found between CH₄ production and total number of methanogens in dairy cattle fed grain plus forage mixed (500:500 g/kg DMI) diets [79]. Likewise, *Methanobacterium wolfeii*, *Methanomicrobia barkeri*, and *Methanobacterium formicicum* are highly resistant to acute O₂ exposure (2 h), as reported [80]. Therefore, the assumption that methanogens are completely metabolically inactive in the presence of O₂ [81] [82] must be reconsidered [77].

In the present study, the average methanogen population was greater ($P < 0.001$; 22.2 vs. 7.6×10^{-5}) for a forage-based diet than for a grain-based diet. Results reveal that an increase in the forage-based diet increased the total methanogen populations (Table 5). The results are similar to previous studies in dairy cattle, where a similar increase in the methanogen population was observed with a roughage-rich diet [79] [83]. This suggests that increasing dietary concentrate levels decreased the ruminal archaeal population [83] [84] and CH₄ production [79]. It has been reported that as the proportion of concentrate increased in diets, the relative abundance of cellulolytic bacteria (e.g., *Fibrobacter* and *Saccharofermentans*) and ciliates protozoa (*Ostracodinium* and *Polyplastron*) linearly decreased, but linearly increased the relative abundance of *Entodinium* ciliates, which is a non-fibrous carbohydrate degrader [83] [84]. Therefore, the observed associative effect of diets and a high level of dissolved O₂ concentration in the rumen on the suppression of methanogenesis was primarily due to their anti-methanogenic activity and, secondarily, to their modification of microbial community structures (e.g., Firmicu-

tes) and rumen fermentation profiles.

3.4. Oxygen Levels and Rumen Fermentation Profiles

Table 6. The effect of dissolved oxygenated water supply and different basal diets on *in vitro* ruminal gas production either grain-based (GBD) or forage-based diets (FBD) supplementation¹.

Diet/O ₂ , ppm	Volatile fatty acids, mM						Average		SEM	Diet	Tret	Diet × Tret
	Grain-based diet			Forage-based diet			Grain	Forage				
	Control 0 ppm	0.1 ppm	0.2 ppm	Control 0 ppm	0.1 ppm	0.2 ppm						
Propanol	0.54	0.99	1.10	0.10	0.01	0.01	0.88 ^a	0.04 ^b	0.216	0.001	0.54	0.34
Butanol	0.16	0.14	0.08	0.02	0.03	0.03	0.12 ^a	0.03 ^b	0.03	0.01	0.54	0.35
Pentanol	0.06	0.02	0.05	0.02	0.02	0.01	0.04 ^a	0.01 ^b	0.009	0.01	0.20	0.19
Acetate	64.8 ^c	58.8 ^b	69.2 ^a	75.5	76.3	76.2	64.3 ^b	76.0 ^a	1.28	0.001	0.02	0.01
Propionate	47.3 ^a	43.0 ^b	50.3 ^a	30.8	31.3	31.2	46.9 ^a	31.1 ^b	1.027	0.001	0.03	0.03
Iso-butyrate	1.0	1.0	1.1	1.9	1.9	1.2	1.03 ^b	1.67 ^a	0.04	0.01	0.38	0.68
Butyrate	10.3	9.6	11.1	12.7	12.3	12.6	10.3 ^b	12.5 ^a	0.25	0.001	0.04	0.11
Iso-valerate	1.3	1.2	1.4	3.7 ^A	3.5 ^B	3.6 ^{AB}	1.3 ^b	3.6 ^a	0.06	0.001	0.10	0.30
Valerate	8.6	8.1	8.7	7.5	7.3	7.2	8.4 ^a	7.3 ^b	0.22	0.001	0.29	0.39
Iso-hexanoate	0.26	0.29	0.29	0.12	0.01	0.0	0.28 ^a	0.04 ^b	0.07	0.01	0.79	0.55
Hexanoate	5.4	3.6	3.0	1.7	1.7	0.0	4.0	1.1	1.72	0.08	0.53	0.88
Heptanoate	0.48	0.45	0.55	0.38	0.35	0.34	0.50 ^a	0.36 ^b	0.043	0.01	0.59	0.43
Octanoate	0.77	0.68	0.77	0.48	0.0	0.86	0.74	0.45	0.206	0.13	0.14	0.25
Phenol	0.05	0.04	0.05	0.04	0.03	0.04	0.05 ^a	0.03 ^b	0.006	0.04	0.69	0.83
p-cresol	0.23	0.21	0.24	0.28	0.26	0.26	0.23	0.27	0.02	0.06	0.57	0.87
4-Ethylphenol	0.10	0.12	0.15	0.37	0.40	0.39	0.13 ^b	0.39 ^a	0.021	0.001	0.29	0.75
Indole	0.03	0.04	0.03	0.03	0.06	0.06	0.03	0.05	0.010	0.08	0.35	0.21
Skatole	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.006	0.22	0.38	0.56
Benzoate	0.04	0.04	0.03	0.06	0.10	0.09	0.04 ^b	0.08 ^a	0.011	0.01	0.35	0.40
Phenylacetate	0.24	0.25	0.26	0.85	0.84	0.82	0.25 ^b	0.84 ^a	0.016	0.001	0.98	0.29
Phenylpropionate	0.28	0.29	0.33	0.91	0.92	0.91	0.30 ^b	0.91 ^a	0.015	0.01	0.61	0.22
A/P ratio	1.36	1.36	1.37	2.5	2.4	2.4	1.37 ^b	2.45 ^a	0.02	0.001	0.92	0.96

¹40 mL of different levels of dissolved O₂-containing buffer solution and 50 mL of rumen fluid were added. Diet = grain-based vs. forage-based diets. ^{a,b,c} and ^{A,B,C} numbers in a row with superscript letters are significantly different ($P < 0.05$) for grain-based and forage-based diets, respectively.

It has been reported that as the level of dietary concentrate increased from 20 to 80% DM, the acetate: propionate (A/P) ratio and the proportion of acetate decreased linearly [83], which supports the results of the present study (Table 6). Decreased A/P ratio (1.37 vs. 2.45) was observed when increasing the grain-based diet, suggesting that the grain-based diet increased feed efficiency (as measured

by A/P ratio). Concerning the effect of a grain-based diet on the A/P ratio, the results of the current study were comparable to those of Min *et al.* [10], who found that A/P ratios for a grain-based diet were lower compared to those for a forage-based diet. Similar results were observed in sheep fed [85]. Among volatile fatty acids (VFA), propionate was the major VFA contributing to glucogenesis [63]. Therefore, the higher ratio of ruminal propionate fed a high-concentrate diet could be beneficial to the energy metabolism, even though the detection of physiological functions of ruminal pH should be warranted. Lower A/P ratios are associated with higher average daily gain [86]. Diets with a high proportion of grain have been shown to decrease CH₄ production (g/kg DMI) per unit of DMI, which has been linked to enhanced growth of starch-fermenting bacteria, resulting in lower A/P ratios and H₂ production and thus reduced CH₄ production [87] [88]. In addition, the increased proportion of propionate could be associated with a decrease in rumen pH, which has been suggested to slow methanogen activity [89] [90]. In contrast, diets with a high proportion of forage have been shown to increase CH₄ production (g/kg DMI); [6]. This is most likely due to the fermentation of plant cell wall carbohydrates, increasing A/P ratios compared with the fermentation of soluble carbohydrates [6] [91]. However, there is very limited research available on the effect of dissolved O₂ concentration in the rumen, particularly in steers, on rumen fermentation rate from cattle. In the presence of O₂, the average concentration of propanil, pentanol, propionate, valerate, iso-hexanoate, heptanoate, and phenol decreased ($P < 0.05 - 0.01$), whereas acetate, iso-butyrate, butyrate, iso-valerate, 4-ethylphenol, benzoate, phenylacetate, phenylpropionate, and A/P ratio increased with increasing forage diet (Table 6). In the presence of O₂, quadratic responses with O₂ by diets ($P < 0.03 - 0.01$) for acetate and propionate production suggested that it may be possible to have a minimum of O₂ concentration in the rumen to alter rumen fermentation and suppress CH₄ losses.

4. Conclusion

The findings indicated that rumen fluid obtained from a single donor animal was prone to error due to host-animal variation and suggested that mixed RF from multiple donor animals can be used in an *in vitro* study. Dissolved O₂ levels in the rumen and diets potentially influenced ruminal gas production, resulting in different fermentation end products, some bacterial diversity, and methanogen abundance. Furthermore, studies investigating the associations among CH₄ emissions, rumen methanogen abundance, rumen fermentation profiles, and feed efficiency are needed to develop more specific nutritional management strategies that reduce CH₄ emissions without compromising animal health, growth, and productivity.

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Supplementary Material

Not applicable.

Data Availability

Upon reasonable request, the datasets of this study can be made available from the corresponding author.

Ethics Approval

Tuskegee University. Tuskegee University Animal Care and Use Committee approved animal care, handling, and sampling procedures for this experiment (#T02-2002-1).

Declaration of Generative

No AI tools were used in this article.

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Conflicts of Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the content of this paper.

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