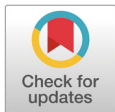


# Effects of nitrogen gas flushing in comparison with argon on rumen fermentation characteristics in *in vitro* studies

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## Competing interests

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## Abstract

In rumen *in vitro* experiments, although nitrogen gas (N<sub>2</sub>) flushing has been widely used, its effects on rumen fermentation characteristics are not clearly determined. The present study is the first to evaluate the effects of N<sub>2</sub> flushing on rumen fermentation characteristics in *in vitro* batch culture system by comparing with new applicable non-metabolizable gas: argon (Ar). The rumen fluid was taken from two Korean native heifers followed by incubation for 3, 9, 12, and 24 h with N<sub>2</sub> or Ar flushing. As a result, in all incubation time, N<sub>2</sub> flushing resulted in higher total gas production than Ar flushing ( $p < 0.01$ ). Additionally, in N<sub>2</sub> flushing group, ammonia nitrogen was increased ( $p < 0.01$ ). However, volatile fatty acids profiles and pH were not affected by the flushing gases ( $p > 0.05$ ). In conclusion, the present study demonstrated that N<sub>2</sub> flushing can influence the rumen nitrogen metabolism via increased ammonia nitrogen concentration and Ar flushing can be used as a new alternative flushing gas.

**Keywords:** Argon, Flushing gas, Headspace, Nitrogen, Rumen *in vitro*

## INTRODUCTION

In rumen fluid *in vitro* experiments, especially using batch culture, using flushing gas (headspace gas composition) in order to make an anaerobic condition in incubation bottle is a pivotal factor and thus should be chosen prudently [1]. However, the studies identifying the effects of flushing gases on rumen fermentation are limited. Nitrogen gas (N<sub>2</sub>) flushing is routinely used in rumen *in vitro* experiments [2–4]. It may affect rumen nitrogen metabolism as rumen microbes can utilize atmospheric N<sub>2</sub> [5–7] and thus may confound the obtained results. But the previous studies which investigated the effects of headspace N<sub>2</sub> with carbon dioxide (CO<sub>2</sub>) or dihydrogen on rumen fermentation characteristics in batch culture system found no changes in ammonia nitrogen (NH<sub>3</sub>-N) between the headspace gas compositions [8,9]. These results could be resulted from the gas used for the comparison. As CO<sub>2</sub> and H<sub>2</sub> concentration in the medium affects rumen fermentation thermodynamically [10], it is not proper to investigate the effects of N<sub>2</sub> by comparing its effects with CO<sub>2</sub> or H<sub>2</sub>. Conversely, if headspace N<sub>2</sub> could influence the rumen fermentation, then investigating the effects of CO<sub>2</sub> or H<sub>2</sub> by comparing its effects

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#### Availability of data and material

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

#### Authors' contributions

Conceptualization: Park KY, Lee HG.  
Data curation: Park KY.  
Formal analysis: Park KY.  
Methodology: Park KY.  
Software: Park KY.  
Validation: Lee HG.  
Investigation: Park KY.  
Writing - original draft: Park KY.  
Writing - review & editing: Park KY, Lee HG.

#### Ethics approval and consent to participate

All animal procedures were performed in accordance with the Institution of Animal Care and Use Committee (IACUC) at Konkuk University, Seoul (Approval no. KU19075).

with N<sub>2</sub> may not be relevant. Therefore, N<sub>2</sub> flushing effects should be demonstrated by comparing its effects with the non-metabolizable gas. In the present study, argon gas (Ar) was selected as an alternative gas because there is no possibility for Ar to influence the rumen nitrogen metabolism due to its extremely low biological availability and toxicity for microbes. Given the above, the objective of the present study was to investigate the effects of N<sub>2</sub> flushing on rumen fermentation kinetics by comparing its effects with Ar flushing using rumen *in vitro* batch culture system.

## MATERIALS AND METHODS

### Ruminal inoculum and diet

Two fistulated Korean native heifers fed a total mixed ration (TMR; Table 1) were used to obtain rumen fluid samples. The chemical analyses for the TMR were conducted in accordance with the AOAC [11], but the content of NDF was analyzed with a neutral detergent solution [12] containing sodium sulfite and a heat stable amylase. The rumen fluid was collected from ventral and dorsal sac of the heifers at 2 h after morning feeding and filtered through 4 layers of cheese cloth and then mixed in the same ratio. Afterwards, the mixed rumen fluid was transported to laboratory using preheated thermos bottles.

### *In vitro* incubation procedures

The TMR fed to the heifers was milled through a 1-mm screen and used as a substrate for the incubation. McDougall's buffer [13] which was heated at 39°C and purged continuously with CO<sub>2</sub> was mixed with the rumen fluid in a 3:1 (vol:vol) ratio. Next, 40 mL of the buffered rumen fluid was dispensed into 120 mL serum bottle filled with 0.4 ± 0.002 g of substrates. The ultra-high purity (99.999%) N<sub>2</sub> and Ar was flushed into headspace of the serum bottles, respectively. For each treatment, N<sub>2</sub> or Ar flushing, four replications were incubated at 3, 9, 12, and 24 h.

### Post-fermentation parameters analyses

Total gas production (TGP) was calculated from headspace gas pressure measured by a pressure transducer (Sun Bee Instrument Inc., Seoul, Korea) [14]. To measure the methane concentration, 0.3 mL of head space gas was collected by gas tight syringe (Gastight#1001; Hamilton Co., Reno, NV, USA) and then injected manually to a gas chromatograph (HP 6890 series GC system; Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a thermal conductivity detector and a capillary column (HP-PLOT/Q; Agilent Technologies Inc., Santa Clara, CA, USA). The temperatures of the inlet, oven and detector were 50°C, 50°C, and 250°C, respectively. The helium gas was used as carrier gas. The standard gas with known composition: H<sub>2</sub> 1.0%, CH<sub>4</sub> 10.1%, CO<sub>2</sub> 20.1% and N<sub>2</sub> 19.9% in He (MS Dong Min Specialty Gases, Inc., Pyeongtaek, Korea) was used to quantify CH<sub>4</sub> concentration.

After measuring the pH values using a digital pH meter (S20 SevenEasy pH; Mettler Toledo Co. Ltd., Greifensee, Switzerland), residual rumen fluid samples were stored at -20°C immediately for volatile fatty acids (VFA) and NH<sub>3</sub>-N analysis. After being thawed, 10 mL of sample was mixed with 1 mL of HgCl<sub>2</sub> 2% (wt/vol) solution and briefly centrifuged at 2,000 ×g for 10 min at 4°C

**Table 1. Chemical composition of the total mixed ration (TMR)**

	Moisture (%)	Composition (DM; %)				
		Crude protein	Crude fat	Crude ash	NDF	ADF
TMR	38.51	10.70	3.22	11.15	53.52	30.30

NDF, neutral detergent fiber; ADF, acid detergent fiber.

in order to remove feed particles. The supernatants were used for VFA and  $\text{NH}_3\text{-N}$  analysis.

To prepare samples for VFA analysis, 1.4 mL of the supernatants were mixed with 0.28 mL of 25% (wt/vol) *meta*-phosphoric acid and then centrifuged again at 20,000  $\times g$  for 20 min at 4°C. Next, a 1 mL of the supernatant was mixed with 50  $\mu\text{L}$  of 2% (wt/vol) pivalic acid as an internal standard. The gas chromatograph (HP 6890 series GC system; Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a flame ionization detector and a capillary column (DB-FFAP; Agilent Technologies Inc., Santa Clara, CA, USA) was used for measuring VFA profile. The temperatures of the inlet, oven and detector were set at 220°C, 100°C, and 250°C, respectively. Each sample for VFA analysis was duplicated.

For the  $\text{NH}_3\text{-N}$  analysis, the previously centrifuged samples were centrifuged again at 20,000 $\times g$  for 20 min at 4°C. The supernatants were used to determine the  $\text{NH}_3\text{-N}$  concentration by catalyzed indophenol reaction [15] using spectrophotometry (Synergy2; Biotek Instruments, Inc., Winooski, VT, USA). Each sample for the  $\text{NH}_3\text{-N}$  analysis was triplicated and measured 3 times (3  $\times$  3).

### Statistical analysis

Data were analyzed as a two-way ANOVA with source of flushing gases and time as separate factors using general linear model procedure of SAS software (SAS Institute, Cary, NC, USA). The significant differences were accepted if  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Gas and methane production

Nitrogen flushing resulted in higher TGP than Ar flushing ( $p < 0.01$ ; Table 2). This result could be attributed to the lower Henry's law constant of  $\text{N}_2$  than Ar [16]. Due to the lower Henry's law constant,  $\text{N}_2$  is less soluble in water than Ar, which resulted in higher TGP. As time does not affect the Henry's law constant, TGP did not show time gas interaction. Additionally, as VFA and methane production were not influenced by the flushing gases, the only assumption for the lower TGP in Ar flushed bottles could be the solubility differences between flushing gases.

### Ammonia nitrogen

Ammonia nitrogen was higher in  $\text{N}_2$  flushed group rather than Ar flushed group ( $p < 0.01$ ). If Ar flushing lowered  $\text{NH}_3\text{-N}$  concentration (e.g., by inhibiting amino acids biosynthesis), it should be accompanied with the different concentration of the branched chain fatty acids including iso-butyrate and iso-valerate which are used to synthesis valine and leucine [17,18]. However, these parameters were not influenced by the flushing gases which demonstrate that Ar flushing did not inhibit the rumen fermentation and thus can be used as an alternative flushing gas. On the other hand,  $\text{N}_2$  flushing enhanced  $\text{NH}_3\text{-N}$  which was partially due to the  $\text{N}_2$  fixation. Although  $\text{N}_2$  fixation in rumen is not quantitatively significant [5–7], the  $\text{N}_2$  fixation may be facilitated by flushed  $\text{N}_2$  in headspace which could result in the higher  $\text{NH}_3\text{-N}$  concentration. On the contrary, Patra and Yu [8] showed that the different headspace gas composition including  $\text{CO}_2$  and  $\text{N}_2$  did not affect the  $\text{NH}_3\text{-N}$  concentration in rumen *in vitro* fermentation. These contrasting results might arise from different experimental methods including diets, donor animals, rumen fluid sampling time, buffer [1,19,20] and, especially, the gas used for the comparison. In the study of Patra and Yu [8], the  $\text{CO}_2$  in headspace was used to compare its effects with the effects of  $\text{N}_2$  in headspace on rumen fermentation characteristics. As noted, if the flushed  $\text{CO}_2$  dissolved into rumen fluid and influenced  $\text{NH}_3\text{-N}$  concentration, then the different  $\text{NH}_3\text{-N}$  between  $\text{CO}_2$  and  $\text{N}_2$  flushing group could not be detected. For example, as Patra and Yu noted the increased methane production by  $\text{CO}_2$  flush-

**Table 2.** Over time impacts of N<sub>2</sub> and Ar flushing on rumen pH, TGP, NH<sub>3</sub>-N and VFA production

Items	3 h		9 h		12 h		24 h		RMSE	p-value <sup>1)</sup>	
	Ar	N <sub>2</sub>	Ar	N <sub>2</sub>	Ar	N <sub>2</sub>	Ar	N <sub>2</sub>		Gas	Time × Gas
pH	7.02	7.01	6.78	6.79	6.70	6.70	6.59	6.59	0.017	0.634	0.793
TGP (mL)	22.88	25.96	42.09	43.62	48.84	51.12	65.22	68.15	1.663	< .001	0.781
CH <sub>4</sub> (mL)	2.66	2.74	7.98	8.20	9.89	9.98	14.35	14.87	0.340	0.078	0.552
NH <sub>3</sub> -N (mg/dL)	8.24	10.66	9.38	9.88	10.56	12.30	18.96	24.65	1.897	0.001	0.081
TVFA (mM)	39.33	39.36	52.66	52.22	58.16	58.73	75.41	75.66	1.010	0.700	0.779
Acetate (mM)	25.51	25.55	34.80	34.43	38.34	38.84	49.50	49.72	0.702	0.574	0.751
Propionate (mM)	8.27	8.26	10.79	10.70	11.97	12.02	15.35	15.46	0.192	0.669	0.686
Butyrate (mM)	3.70	3.71	4.79	4.75	5.26	5.29	6.69	6.73	0.089	0.760	0.837
Iso-butyrate (mM)	0.41	0.40	0.51	0.49	0.52	0.53	0.90	0.85	0.025	0.100	0.166
Valerate (mM)	0.67	0.67	0.85	0.84	0.91	0.91	1.20	1.17	0.023	0.160	0.488
Iso-valerate (mM)	0.77	0.77	1.03	1.01	1.13	1.12	1.77	1.73	0.027	0.145	0.570
A:P ratio	3.09	3.09	3.22	3.22	3.21	3.23	3.22	3.22	0.019	0.456	0.396

<sup>1)</sup>p-value for time factor were < 0.001 in all items.

N<sub>2</sub>, nitrogen gas; Ar, argon gas; TGP, total gas production; NH<sub>3</sub>-N, ammonia nitrogen; VFA, volatile fatty acids; RMSE, root of mean square error; TVFA, total volatile fatty acids; sum of acetate propionate, butyrate, iso-butyrate, valerate and iso-valerate concentration (mM); A:P ratio, acetate and propionate ratio.

ing [8], CO<sub>2</sub> flushing could promote the activity of *Methanosarcina barkeri* and *Methanobacterium bryantii* which also can fix the atmospheric nitrogen [21]. However, it cannot be easily deemed that the nitrogen fixation was promoted simply due to the higher concentration of N<sub>2</sub> in headspace because the nitrogen fixation requires 16 ATPs [22] and only a few methanogens can fix the atmospheric nitrogen among the rumen microbes [21,23]. Nevertheless, increased NH<sub>3</sub>-N still indicates that N<sub>2</sub> flushing influences rumen N metabolism which should be considered when choosing a flushing gas. Therefore, further studies should demonstrate how rumen N metabolism was affected by N<sub>2</sub> flushing (e.g., acetylene reduction assay [24], detecting the nitrogenase activity and its gene expression).

### VFA production and pH

In the present study, VFA production and pH were not affected by the flushing gases. These results imply that the impacts of N<sub>2</sub> flushing on nitrogen metabolism did not dramatically change the overall rumen fermentation characteristics. Conversely, as VFA, methane and pH were not affected, this is the demonstration that Ar can be used as an alternative flushing gas to N<sub>2</sub>.

## CONCLUSION

It was demonstrated that Ar flushing can be used for rumen *in vitro* experiments as the volatile fatty acids and methane production were not influenced by the flushing gases. The increased ammonia nitrogen in N<sub>2</sub> flushed group demonstrated that N<sub>2</sub> can influence the rumen nitrogen metabolism which can mislead into confounding results. Further studies should be conducted to identify how N<sub>2</sub> flushing affect the rumen nitrogen metabolism and to reinvestigate the effects of CO<sub>2</sub> flushing on rumen fermentation by comparing it with Ar flushing.

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