Technical note: In vitro total gas and methane production measurements from closed or vented rumen batch culture systems

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ABSTRACT

This study compared measured gas production (GP) and computed CH₄ production values provided by closed or vented bottles connected to gas collection bags. Two forages and 3 concentrates were incubated. Two incubations were conducted, where the 5 feeds were tested in 3 replicates in closed or vented bottles, plus 4 blanks, for a total of 64 bottles. Half of the bottles were not vented, and the others were vented at a fixed pressure (6.8 kPa) and gas was collected into one gas collection bag connected to each bottle. Each bottle (317 mL) was filled with 0.4000 ± 0.0010 g of feed sample and 60 mL of buffered rumen fluid (headspace volume = 257 mL) and incubated at 39.0°C for 24 h. At 24 h, gas samples were collected from the headspace of closed bottles or from headspace and bags of vented bottles and analyzed for CH₄ concentration. Volumes of GP at 24 h were corrected for the gas dissolved in the fermentation fluid, according to Henry's law of gas solubility. Methane concentration (mL/100 mL of GP) was measured and CH₄ production (mL/g of incubated DM) was computed using corrected or uncorrected GP values. Data were analyzed for the effect of venting technique (T), feed (F), interaction between venting technique and feed (T × F), and incubation run as a random factor. Closed bottles provided lower uncorrected GP (−18%) compared with vented bottles, especially for concentrates. Correction for dissolved gas reduced but did not remove differences between techniques, and closed bottles (+25 mL of gas/g of incubated DM) had a greater magnitude of variation than did vented bottles (+1 mL of gas/g of incubated DM). Feeds differed in uncorrected and corrected GP, but the ranking was the same for the 2 techniques. The T × F interaction influenced uncorrected GP values, but this effect disappeared after correction. Closed bottles provided uncorrected CH₄ concentrations 23% greater

than that of vented bottles. Correction reduced but did not remove this difference. Methane concentration was influenced by feed but not by the T × F interaction. Corrected CH₄ production was influenced by feed, but not by venting technique or the T × F interaction. Closed bottles provide good measurements of CH₄ production but not of GP. Venting of bottles at low pressure permits a reliable evaluation of total GP and CH₄ production.

Key words: in vitro gas production, methane, venting technique, rumen fermentation

Technical Note

Several batch culture systems are available to measure gas production (GP) during in vitro rumen fermentation. Many of these systems are equipped with devices for gas venting at fixed interval times (Theodorou et al., 1994) or at a fixed pressure (Cone et al., 1996; Davies et al., 2000; Calabrò et al., 2005; Tagliapietra et al., 2010). Gas venting is recommended to avoid pressure conditions that cause a partial dissolution of CO₂ in the fermentation fluid, with a consequent underestimation of total GP (Tagliapietra et al., 2010) and possible disturbance of microbial activity (Theodorou et al., 1994) and thereby CH₄ production. Vented GP systems are less frequently used to evaluate the composition of gas produced, because the vented gas must be collected in bags that are leak proof and are not permeable to CO₂ or CH₄. Closed GP systems are more commonly used for measurements of CH₄ production. With a closed system, the gas is not vented and remains in the bottle headspace until the time of collection for analysis (Pell and Schofield, 1993; Getachew et al., 2005; Pellikaan et al., 2011). However, the gas pressure generated in the headspace will cause a partial dissolving of CO₂ in the fermentation fluid that can alter the GP composition, so that a correction for solubilized CO₂ is required. The difference in CO₂ concentration in the headspace and the amount dissolved in the fluid can, in turn, alter the CH₄ concentration in the collected gas (Patra and Yu, 2013). The objective of the current study was to

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compare, also in term of repeatability, the effects of 2 systems of gas collection, one based on closed fermentation bottles and one based on vented bottles connected to tight plastic bags, on total GP, gas composition and CH_4 production computed from GP and gas composition measures.

Two forages (meadow hay and ryegrass hay) and 3 concentrates (corn grain, dry sugar beet pulp, and expeller flaxseed) were incubated. The 5 feeds were selected to cover a large variability in chemical composition and to generate different pressure conditions in the bottle headspace during incubation. All feeds were provided by a dairy farm located in the province of Brescia (Italy). About 1 kg of each feed was ground by a hammer mill with a screen size of 1 mm. From each feed, 15 subsamples were prepared: 12 samples were used for the incubations (6 for each run) and the remaining 3 for chemical analysis. Feeds were analyzed in 3 replicates for proximate composition (AOAC International, 2012). The NDF, inclusive of residual ash, was determined with α -amylase and sodium sulfite (Mertens, 2002) using the Ankom²²⁰ Fiber Analyzer (Ankom Technology, Macedon, NY). The ADF, inclusive of residual ash, and sulfuric acid lignin [$\text{lignin}_{(\text{sa})}$] contents were sequentially determined according to Robertson and Van Soest (1981). Chemical composition of the 5 feeds is reported in Table 1.

Incubations were conducted following the procedures detailed by Tagliapietra et al. (2012), according to a factorial design where the 5 feeds were simultaneously tested using the 2 techniques, closed or vented bottles, in each of 2 consecutive incubations. In each incubation, each feed was tested with 3 replications (using 3 bottles) plus 2 blanks containing only the buffered rumen fluid, for a total of 32 bottles. Rumen fluid was collected using an esophageal probe (Tagliapietra et al., 2012) from 3 intact, dry Holstein cows fed hay ad libitum and 2.5 kg/d of concentrates (0.5 kg of dry sugar beet pulp, 1 kg of corn grain, and 1 kg of sunflower meal) for 3 wk preceding fluid collection. Differences between fluid collected with the probe instead of fluid collected from fistulated cows were not considered rel-

evant for the comparative purposes of this work. The rumen fluid collected from the 3 cows was poured into 2 thermal flasks preheated to $39 \pm 0.5^\circ\text{C}$ and immediately transferred to the laboratory. The rumen fluid collected from the 3 cows was mixed, filtered through 3 layers of cheesecloth to eliminate feed particles, and then mixed with the buffer mineral solution in a 1:2 ratio (Menke and Steingass, 1988). All operations were conducted under anaerobic conditions by flushing with CO_2 , and the time required for all operations was less than 30 min. Each bottle (317 mL) was filled with 0.4000 ± 0.0010 g of feed sample and 60 mL of buffered rumen fluid (Menke and Steingass, 1988), leaving a corresponding headspace volume of 257 mL. Bottles, placed into an incubator at $39.0 \pm 0.5^\circ\text{C}$ for 24 h, were not agitated during incubation. All bottles used in this experiment were from the A#^{RF} GP System (Ankom Technology), which was successfully tested for absence of gas pressure variation (70 kPa) over a period of 7 d. For the purpose of the current study, half of the bottles were not vented during incubation, to simulate the closed system technique, and the others were connected with gas-proof Tygon tubing (Saint-Gobain Performance Plastics, Paris, France) to 1-L gas sample bags (standard FlexFoil, SKC Inc., Pennsylvania, PA) and automatically vented by an open-closed valve (model LHLX0502100BC, The Lee Co., Essex, CT) when headspace pressure reached 6.8 kPa. This pressure corresponded to 17 mL for a headspace volume of 257 mL. All gas sample bags were previously vacuum-sealed to eliminate possible traces of air. The closed and vented bottles were equipped with pressure sensors (pressure range: -69 to +3,447 kPa; resolution: 0.27 kPa; accuracy: ± 0.01 of measured value) that measured the pressure inside the bottles every 1 min. At the end of incubation (24 h), gas was collected with a 10-mL, gas-tight syringe (Artsana S.p.A., Como, Italy) from the headspace of each closed bottle, and from the headspace and gas-tight plastic bag for each vented bottle. At sampling, the syringe was flushed to ensure the collection of a homogeneous sample, which was immediately injected into a 9-mL Vacuette (Greiner Bio-One

Table 1. Dry matter (g/kg) contents and chemical composition (g/kg of DM) of the feeds ($n = 3$)

Feed	DM	NDF	ADF	Lignin _(sa) ¹	CP	Ether extract	Ash	NSC ²
Corn grain	900	106	23	— ³	93	37	15	749
Dry sugar beet pulp	935	443	250	29	95	60	51	351
Flaxseed expeller	923	260	118	40	375	91	59	215
Meadow hay	893	435	305	60	90	15	86	374
Ryegrass hay	888	591	347	30	149	15	111	134

¹Lignin_(sa) = sulfuric acid lignin.

²NSC = nonstructural carbohydrates, computed as $(100 - \text{NDF} - \text{CP} - \text{ether extract} - \text{ash})$.

³Amount not measurable.

GmbH, Kremsmünster, Austria). From each Vacuette, an aliquot of 2.7 μL of gas was extracted with a gas-tight syringe (1701N, Hamilton, Bonaduz, Switzerland) and immediately analyzed for CH_4 concentration by GC with flame ionization detection (8000 TOP, Thermo Quest, Rodano, Milano, Italy) using a 30-m stainless steel column (GS-CarbonPLOT, Agilent Technologies, Milano, Italy) and hydrogen as carrier gas (flow rate: 1.8 mL/min; isothermal oven temperature: 150°C). A 6-point standard curve was generated by mixing known volumes of CH_4 (99.5% pure, SAPIO s.r.l., Monza, Italy) with known volumes of air using the same graduated gas-tight syringe (1701N, Hamilton). The 6 gas mixtures used for calibration contained 10, 15, 20, 25, 50, and 100 mL of CH_4/L . The calibration regression had an $R^2 > 0.99$.

Cumulative pressure values were calculated from measured pressure changes at given times, converted to units of volume (GP, mL) using the ideal gas law, and expressed per gram of incubated DM. Bottles with only rumen fluid and buffer (blanks) produced minimal amounts of gas (on average 7.2 ± 2.2 mL) and therefore were not used in further calculations. The GP volumes were corrected or not for the amount of dissolved gas computed according to Henry's law, from total gas pressure and CO_2 solubility, as described by Pell and Schofield (1993). Corrections for solubilized CO_2 are hereafter called adjustments. The total CH_4 production achieved from closed bottles was computed according to Lopez et al. (2007) as follows: CH_4 concentration in the headspace $\times$ [headspace volume (257 mL) + total GP volume (mL)]. Methane production from vented bottles was computed as CH_4 concentration in the headspace $\times$ headspace volume (257 mL) + CH_4 concentration in the gas bag $\times$ total GP volume (mL). The CH_4 concentration and calculated production were both computed using adjusted and unadjusted total GP volumes.

Data were analyzed using PROC MIXED of SAS Institute (2007) with a model that included, as fixed factors, the venting technique (**T**; 1 df), the feed (**F**; 4 df), the interaction between venting technique and feed (**T** $\times$ **F**; 4 df), and as a random variable the incubation run (1 df). A model considering all variables as random was used to estimate the variance components. The repeatability, defined as the value below which the absolute difference between 2 single measures obtained with the same technique and under the same conditions (same incubation run, same feed) is expected with a 95% probability (IOS, 1994), was computed as $2\sqrt{2\sigma_e^2}$, where σ_e^2 is the residual variance.

Closed bottles produced lower (approximately -18% ; $P < 0.001$) amounts of unadjusted GP compared with vented bottles (Table 2). The differences were particu-

larly pronounced for highly fermentable substrates such as corn grain (-56 mL of gas/g of incubated DM; $P < 0.001$) and dry sugar beet pulp (-54 mL of gas/g of incubated DM; $P < 0.001$), whereas smaller, but still significant, differences were found for flaxseed expeller (-26 mL of gas/g of incubated DM; $P < 0.001$), meadow hay (-26 mL of gas/g of incubated DM; $P < 0.001$), and ryegrass hay (-17 mL of gas/g of incubated DM; $P = 0.005$; data not shown). Values of repeatability were 5.9 and 8.9 mL of gas/g of incubated DM for closed and vented bottles, respectively. The **T** $\times$ **F** interaction ($P < 0.001$) was found to influence the unadjusted values of GP, but this interaction effect was not significant when GP values were adjusted for dissolved gas ($P = 0.25$). After adjustment for dissolved gas, the GP values provided by the 2 techniques continued to be different ($P < 0.001$), even if this difference was numerically smaller. The magnitude of such adjustment was notable for closed bottles where a considerable increase of GP values (on average $+25$ mL of gas/g of incubated DM) was found, whereas the adjustment value was negligible for vented bottles (on average $+1$ mL of gas/g of incubated DM; Figure 1a and b). Feeds differed for unadjusted and adjusted GP ($P < 0.001$), even though the ranking for GP was unaffected by venting. Values of repeatability for adjusted GP were 6.3 and 9.0 mL of gas/g of incubated DM for closed and vented bottles, respectively. The 2 techniques provided different ($P < 0.001$) unadjusted CH_4 concentration (mL/100 mL of GP), with values 23% greater for closed compared with vented bottles. The adjustment for dissolved GP reduced but did not completely remove this difference ($P = 0.002$). Methane concentration was influenced by feed ($P = 0.005$), but not by the **T** $\times$ **F** interaction ($P = 0.57$). Unadjusted or adjusted CH_4 production (mL/ of incubated DM) was influenced by feed ($P < 0.001$) but not by the venting technique or the **T** $\times$ **F** interaction.

Results of GP and gas composition obtained from different researchers in vitro are often difficult to compare because of the influence of several confounding sources of variation. Among these, the major sources are represented by different operative conditions, such as the procedures of rumen fluid collection and treatment (Cornou et al., 2013), the type of buffer used (Patra and Yu, 2013), the ratios among feed sample size, fermentation fluid, and headspace volume (Ramin and Huhtanen, 2012), and the type of GP equipment, such as syringes or bottles that can be closed or vented at fixed times or at fixed pressure (Gierus et al., 2008; Tagliapietra et al., 2010; Pirondini et al., 2012). The magnitude of the difference of unadjusted gas composition between closed and vented bottles found in this experiment indicates that GP equipment would affect

Table 2. Effect of gas collection technique, feed, and their interaction on the unadjusted or adjusted measures of gas production (GP, mL/g of incubated DM), CH₄ concentration (mL/100 mL of GP), and CH₄ production (mL/g of incubated DM)

Item	GP		CH ₄ concentration		CH ₄ production	
	Unadjusted	Adjusted ¹	Unadjusted GP	Adjusted GP ¹	Unadjusted GP	Adjusted GP ¹
Technique						
Closed bottles	168	193	14.2	12.4	23.0	23.6
Vented bottles	204	205	11.5	11.2	22.9	23.0
SEM	7.3	7.7	0.63	0.23	0.43	0.44
Feed						
Corn grain	287	306	11.7	10.8	32.3	33.0
Dry sugar beet pulp	254	271	12.6	11.6	31.2	31.9
Flaxseed expeller	154	165	14.1	13.0	21.5	21.8
Meadow hay	137	147	12.8	11.7	17.1	17.3
Ryegrass hay	98	106	13.1	12.0	12.6	12.7
SEM	7.7	8.0	0.71	0.38	0.70	0.71
<i>P</i> -value						
Technique (T)	<0.001	<0.001	<0.001	0.002	0.88	0.38
Feed (F)	<0.001	<0.001	0.006	0.005	<0.001	<0.001
T × F	<0.001	0.25	0.55	0.57	0.83	0.79
Repeatability ²						
Closed bottles	5.9	6.3	2.38	2.22	1.91	1.93
Vented bottles	8.9	9.0	1.88	1.84	1.80	1.81

¹Values of GP adjusted by adding the amount of dissolved gas computed from total gas pressure and CO₂ solubility, according to Henry's law (Pell and Schofield, 1993).

²Repeatability, defined as the value below which the absolute difference between 2 single measures obtained with the same technique and under the same conditions (same incubation run, same feed) is expected with a 95% probability, was computed as $2\sqrt{2\sigma_e^2}$, where σ_e^2 is the residual variance (IOS, 1994).

the measured gas composition equally or more than that attributable to differences among diets or feeds. One major aspect that differentiates various GP systems concerns the different pressures generated within the fermentation units and the way by which such pressures are controlled. Despite the popularity of the various automated GP systems in use and the great effort of research done in this field, these aspects have been poorly investigated (Ramin and Huhtanen, 2012). Results of the current study show that closed bottles produced a notable underestimation of actual GP. The magnitude of this effect was small in the first phases of incubation (<6 h), when the headspace pressure was low, but it progressively increased at later incubation times as the headspace pressure increased (Figure 1). Moreover, the magnitude of this effect mainly depends on the ratios among headspace volume, fermentation fluid, and sample size. In the current experiment, with 0.5 g of feed sample, the ratio between the volume of headspace and that of fermentation fluid was 4.3, a value greater than those frequently found in literature. This suggests that a greater underestimation of GP would be expected when smaller ratios between headspace and fermentation fluid are adopted, particularly when feeds are incubated for long times. In the vented bottles, the kinetics of GP were not influenced by the adjustment for dissolved CO₂, as venting occurred when the 6.8 kPa threshold pressure was reached. On average, valve opening occurred 2 to 3 times for hays,

4 times for flaxseed, and 7 to 8 times for corn grain and sugar beet pulp. To overcome GP underestimation due to the dissolved CO₂, Pell and Schofield (1993) proposed that GP measures may be adjusted for dissolved CO₂, according to Henry's law, which considers the CO₂ solubility in saline solution, which may not be the same in buffered rumen fluid. In support of the results of Tagliapietra et al. (2010), our results confirm that this adjustment is not sufficient, particularly in closed systems, for a complete accounting of dissolved CO₂ in the buffered rumen fluid. A further adjustment is needed, either to compensate for more dissolved CO₂ than Henry's law allows or to account for differences caused by secondary interference of dissolved CO₂ on rumen fermentation. This implies that caution must be made in evaluating data of gas production and composition obtained from closed bottles, as an overestimation of CH₄ concentration and an underestimation of actual GP would be expected, even when an adjustment for dissolved CO₂, using Henry's law, is applied. In contrast, for bottles vented at low fixed pressures, the adjustment factor for dissolved CO₂ at the end of fermentation is small and results in minimal changes in the measures of GP and CH₄ concentration, suggesting that equipment operating at low pressure will provide a reliable measure of both total gas and CH₄ production. The main reason why closed bottles are preferred for measurements of CH₄ production is that all the CH₄ produced remains in the bottle headspace

Figure 1. Gas production measurements (a) unadjusted, or (b) adjusted for dissolved CO_2 in closed (dotted lines) or vented bottles (solid lines) where, in 2 incubation runs, 5 feeds were incubated in 3 replications with buffered rumen fluid for 24 h.

(often small) without the need for the connected bag. This saves space in the incubator equipment, allowing an increased number of replications, and avoids the risk of undesired gas loss. In the current experiment, irrespective of the GP adjustment, closed and vented bottles provided similar measures of CH_4 production, but only when these measures were expressed per unit of incubated DM. This was expected because, in contrast to CO_2 , small or negligible amounts of CH_4 are dissolved in the fermentation fluid of closed bottles because of the very low CH_4 solubility in water (0.024

g/L at 20°C and 101.3 kPa of pressure). At the same time, the lack of difference in CH_4 production using the different gas collection techniques shows that gas losses from vented bottles connected to gas-tight bags used in this experiment are negligible or very low. The small or absent difference in CH_4 production between closed and vented bottles may also be due to the fact that care was taken to ensure that the internal pressure in the closed bottles would not reach 45 kPa after 24 h of fermentation. This value is lower than the threshold of 48 kPa above which microbial activity can be disturbed (Theodorou et al., 1994). This was done by considering the ratios between the volume of fermentation fluid, the bottle headspace, the amount of feed incubated, and the expected GP at 24 h for each feed. The maximum headspace pressure found for corn samples after 24 h of incubation was, on average, 39 kPa. However, Patra and Yu (2013) demonstrated that headspace gas composition and bicarbonate concentration in the medium can affect CH_4 production and other characteristics of *in vitro* rumen fermentation. They proposed that a greater amount of dissolved CO_2 would stimulate CH_4 production. Reliable measurements of *in vitro* total GP and CH_4 would be particularly useful to explore effects of vitamins, additives, or bioactive molecules on rumen fermentation and microbial activity (Cattani et al., 2012; Hansen et al., 2012; Tagliapietra et al., 2013).

We conclude that closed systems permit reliable and comparable measurements of CH_4 production per unit of incubated substrate but not reliable measurements of GP, particularly in those cases where high gas pressures are generated from feed degradation. In these cases, GP could be indirectly computed from stoichiometric relations based on VFA production after *in vitro* fermentation, as described by Blümmel et al. (1997), but this requires time and additional analysis and costs, and uncertainty remains due to the possible influence of dissolved CO_2 on CH_4 production. Use of systems based on automated venting of gas at a low pressure threshold permits a reliable evaluation of both total GP and CH_4 , with benefits in terms of less labor being required to control the fermentation process with respect to other semiautomated venting systems.

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