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Impacts of rumen fluid, refrigerated or reconstituted from a refrigerated pellet, on gas production measured at 24h of fermentation

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ABSTRACT

Rumen fluid is used as fresh inoculum for gas production fermentations to predict the nutritional value of feeds and rations for ruminants. However, collection of rumen fluid from animal donors is invasive, expensive, time consuming and results in fluids of variable quality. The general aim was to identify a procedure to manipulate rumen inoculum in order to facilitate its storage and transfer between laboratories. This strategy would also limit fluid collections from animals. Two experiments were completed based on gas production from graduated 100 mL glass syringe with five feeds as substrates.

In experiment 1, the gas production and some fermentation parameters of fresh rumen fluids were compared with those preserved at 4 °C for 24, 48, 72 and 96 h. Refrigeration did not modify concentration of volatile fatty acids and pH, but ammonia in liquids refrigerated for 48–96 h was higher ($P < 0.05$) compared to fresh. In contrast, rumen fluid refrigeration for 24, 48 or 72 h did not depress gas production at 24 h, but it was lower at 96 h. In experiment 2, the rumen fluid was centrifugated at 13,000 x *g* and sedimented material (*i.e.*, pellet) was refrigerated for 48 h at 4 °C. The asymptote of gas production kinetics from rumen fluid regenerated from the pellet was 8 % lower ($P < 0.05$) than that from fresh. However for 24 h gas production, the correlation between fresh liquid and pellet inoculum, calculated for five ingredients, was high ($R^2 = 0.94$). Results support the use of rumen fluid preserved by refrigeration for up to 72 h, and rumen fluid reconstituted from refrigerated pellet, as an alternative to fresh. This would reduce the need for laboratories to maintain animal donors and/or frequently collect rumen fluid.

1. Introduction

Rumen fluid is used as fresh inoculum for *in vitro* fermentations to predict the nutritional value of feeds and rations for ruminants. Gas production is a common *in vitro* test in many research centers and commercial laboratories because it is inexpensive, rapid and easy to reproduce with manual or automated systems (Menke and Steingass, 1988).

However, collection of rumen fluid requires animals that are cannulated or intubated with esophageal tubes. Rumen cannulated animals often require complex procedures to obtain official permissions, can have high maintenance costs, while the suction of rumen fluid through esophageal probe needs immobilized animals and is unsuitable for routine sampling. An alternative is to collect rumen fluid immediately after slaughter, but the prior feed composition the animals is often unknown, and it requires access to a

Abbreviations: DM, dry matter; VFA, volatile fatty acids

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slaughterhouse located near the laboratory.

Storing rumen fluid while maintaining viability of the microbial activity would allow the same inoculum to be used for several *in vitro* runs within a laboratory with likely advantages in reproducibility. Alternatively, it would be possible to concentrate rumen fluid collection in specialized centers and transfer inoculum to laboratories in the area. In addition, reduction in volume by separating a fraction containing most of the bacteria in the rumen fluid would facilitate storage and transportation.

Unfortunately, research efforts aimed at preserving rumen fluid for long periods by freezing or freeze drying have not produced satisfactory results in terms of maintaining its original fermentation characteristics (Robinson et al., 1999; Belanche et al., 2018; Spanghero et al., 2019). However, refrigeration has been demonstrated (Robinson et al., 1999; Spanghero et al., 2019) to be suitable for storing rumen fluid for up to 48 h.

Our general aim was to study a procedure to manipulate rumen inoculum in order to facilitate its storage and transfer between laboratories before using as inoculum for the gas production technique. Specific aims were to test (i) rumen fluids refrigerated in a 24 h progressive steps storage period and (ii) rumen pellets, obtained by centrifugation of the fresh rumen fluids, refrigerated and then reconstituted.

2. Materials and methods

The study is comprised of two *in vitro* rumen fermentation experiments with gas production measured from graduated 100 mL glass syringes (Menke and Steingass, 1988).

2.1. In vitro experiments

Rumen fluid was collected at a slaughterhouse from culled dairy cows previously fed under controlled conditions (*i.e.*, animals fed a production diet based on corn silage, slaughtered in good health, transported from farms located near the slaughterhouse). The inoculum was delivered to our laboratory within 30 min from slaughter in airtight glass-bottles refluxed with carbon dioxide and maintained at 39 °C.

In experiment 1, a total of about 5 L of rumen fluid was collected from 4 cows, mixed and then divided into 5 equal aliquots. The first fraction was used immediately for gas production while the other 4 fractions were stored in a refrigerator at 4 °C in closed bottles flushed with CO_2. The aliquots were stored for 24, 48, 72 or 96 h and then were warmed at 39 °C, sampled for pH, ammonia and volatile fatty acid (VFA) analysis and used as inoculum for gas production fermentations. The experiment was repeated 3 times, thereby using 12 cows.

In experiment 2, rumen fluids were collected from 3 cows (1 L per cow) and divided into 2 equal aliquots. A first aliquot from each cow was used immediately for gas production and the other was weighed and centrifuged at 13,000 × *g* for 30 min at 4 °C. The supernatant was discharged, the residual pellet weighed to calculate pellet yield and then refrigerated at 4 °C in centrifuge bottles flushed with CO_2. After 48 h the three pellets were reconstituted to a volume equal to that of the original rumen fluids using warm (39 °C) McDougall's buffer, with 5 *g*/L of added maltose (Luchini et al., 1996) and were used as fermentation inoculum. The experiment was repeated 2 times, thereby using 6 cows.

In both experiments, the *in vitro* evaluations were completed on corn grain meal, soybean meal, wheat grain meal, dehydrated alfalfa meal and a total mixed ration (TMR). Samples of TMR were dried at 60 °C for 48 h and all feed samples were milled through a 1 mm sieve. Approximately 220 mg of each dried sample was weighed into three graduated 100 mL glass syringes, which were filled with 30 mL of dilute rumen fluid (Menke and Steingass, 1988). Syringes were placed vertically in a water bath at 39 °C with three syringes without substrate as blanks. Gas production was measured at 24 h in experiment 1 and at 0, 1, 2, 4, 6, 8, 24 and 48 h after incubation in experiment 2. Syringes were manually agitated every 2 h until 8 h of incubation, and then at 24 h.

In both experiments, we compared the gas production of different aliquots of the same rumen fluid in subsequent incubation sessions (*e.g.* fresh, refrigerated at 24, refrigerated at 48 h,…). To account for the incubation session effect, a standard rumen fluid was included in each incubation session within each fermentation run. The fluid had been collected from 4 cows, slaughtered in the same conditions as described above, and divided into 40 mL aliquots and frozen at −80 °C. Two syringes containing 220 mg of corn meal incubated with frozen-thawed inoculum (at 39 °C for about 2 h and kept at the same temperature for another 2 h) were included in each incubation session within each fermentation.

2.2. Chemical analysis

The dry matter (DM) content of the feeds was determined by heating at 105 °C for 3 h. Samples of rumen fluids (10 mL) for ammonia analysis were preserved at -20 °C. Before analysis, samples were thawed and ammonia was measured by an ammonia electrode (Ammonia Gas Sensing Combination Electrode, ©Hach Company, Loveland, CO, USA). For VFA analysis the rumen fluids (10 mL) were acidified with H_2SO_4 0.1 N and stored at -20 °C. Samples were then thawed at room temperature, centrifuged at 20,000 x *g* for 30 min at 20 °C and the supernatant was filtered using polypore 0.45 µm filters (Agilent Technologies, Milano, Italia). Filtrate was injected into a high-performance liquid chromatograph (Perkin-Elmer, Norwalk, CN, USA), set to 220 nm according Martillotti and Puppo (1985). The pH of rumen fluids was measured using a glass electrode connected to a pH meter (GPL 22, Crison Instruments, S.P.A., Barcelona, Spain).

2.3. Statistical analyses

In both experiments the fermentation runs were completed in sequenced periods (weeks), and replicates among runs were the statistical unit.

In experiment 1, gas production data after 24 h of fermentation were analyzed as a factorial randomized complete block (fermentation run) design with repeated measures (*e.g.*, measured on the same fresh and refrigerated rumen fluid) as:

$$y_{ijk} = \mu + \alpha_i + \beta j + \gamma_k + (\beta\gamma)_{ik} + \varepsilon_{ijk}$$

where: μ is the overall mean, α_i is the random effect (block) of the fermentation trial (i = 1,3); βj is the effect of the feed (j = 1,5); γ_k is the repeated measures on the same rumen fluid (fresh and refrigerated for 24, 48, 72 and 96 h) and ε_{ijk} is the random error. Data of pH, ammonia and VFA contents were similarly analyzed without the β (feed substrate) effect.

In experiment 2, cumulative gas production (y, mL/g DM) produced at time t (h) was recorded from each syringe and data were fitted to the exponential model with a lag phase as:

$$y = B\ (1 - \exp^{-k*(t+L)}),$$

where: B (mL/g DM) is the asymptotic gas volume and k (mL/h) is a constant rate and L is the discrete lag phase (h).

The asymptotic gas volume, the constant rate of gas production and the estimated gas yield at 24 h of incubation (y) were statistically analyzed as a randomized complete block (fermentation run) factorial design with repeated measures (*e.g.*, fresh and pelleted from the same rumen fluid) as:

$$y_{ijkl} = \mu + \alpha_i + \beta_{j(i)} + \gamma_k + \delta_l + (\gamma\delta)_{kl} + \varepsilon_{ijkl}$$

where: μ is the overall mean, α_i is the random effect (block) of the fermentation run (i = 1,2); $\beta_{j(i)}$ is the random effect of the j th rumen fluid of the ith fermentation run (j = 1, 3; i = 1, 2); γ_k is the repeated measure (fresh or rumen fluid regenerated from the pellet) on the same rumen fluid (k = 1,2); δ_l is the fixed effect of feed (l = 1,5), and ε_{ijkl} is the random error.

The estimated gas yield at 24 h of incubation obtained with fresh rumen fluid (y) and with the refrigerated rumen pellet (x) were regressed according to the linear mixed model:

$$Y_{ij} = \beta_0 + \beta_1 X_{ij} + s_i + e_{ij}$$

where: Y_{ij} is gas production from the fresh rumen fluid observed for feed j in the fermentation of the rumen fluid i; β_0 is the overall intercept across the rumen fluids (fixed effect), β_1 is the overall regression coefficient for the linear effect of x (fixed effects), X_{ij} is the gas production from the refrigerated bacterial pellet for the ith rumen fluid of the jth feed (i = 1, 6; j = 1, 5), s_i is the random effect of type of rumen fluid i, approximately normal (0, σ2 s), and e_{ij} is the residual error, also approximately normal. Adjusted values for the rumen fluid effect, calculated according to St-Pierre (2001), were used to generate a two-dimensional graph.

For all statistical analyses significance level (P) were $P < 0.05$ and $P < 0.01$ which represented different, or highly different, values respectively.

3. Results

The fermentation of the standard rumen fluid gave similar gas yields among incubation sessions in both experiments (variations less than 5%, not shown) and therefore data were not corrected.

3.1. Experiment 1- cooling rumen fluid for up to 96 h

Refrigeration did not modify total VFA yield, VFA proportions and pH (Table 1), but increased ($P < 0.05$) the ammonia level in rumen fluids refrigerated for 48, 72 and 96 h compared to fresh (36.6–44.7 *vs* 23.4 mg/dL). Gas yields from rumen fluid refrigerated through 72 h did not differ from fresh inoculum, but there was a decrease ($P < 0.01$) when rumen fluid was used after 96 h of refrigeration (Table 2).

3.2. Experiment 2 – cooling rumen fluid solids for 48 h

The six rumen fluids had (not shown in tables) different physic-chemical characteristics (pH 6.1 $\pm$ 0.4, ammonia 22.9 $\pm$ 13.7 mg/dL; total VFA 111.7 $\pm$ 13.1 mmol/L) and the pellet yield was 151 $\pm$ 42 g / Kg of the initial rumen fluid weight.

There was an interaction between inoculum × feed substrate ($P < 0.01$) for the rate of gas production, but the impact of the interaction to the mean square of the model was much lower than that of the corresponding main effects. Therefore, only the results of the main effects of the model are shown and discussed. Maximum gas yield (asymptote) was higher with fresh inoculum compared to that obtained from the pellet (286 *vs* 263 mL/g DM, $P < 0.01$) whereas the rate of degradation showed the opposite pattern (0.072 and 0.093 mL/h for fresh inoculum and pellet, respectively, $P < 0.01$). In addition, the reconstituted liquid had a longer lag phase compared to fresh (0.70 *vs* 0.14 h, $P < 0.01$). Gas production at 24 h was similar for fresh and pellet inoculum and in Fig. 1 the linear regression between gas measured at 24 h of fermentation from fresh and pellet inoculum (adjusted for the rumen fluid effect) is shown ($R^2 = 0.94$) Fig. 1.

Table 1
Ammonia, VFA contents, pH and gas production (at 24 h) from five feed substrates using fresh and refrigerated rumen fluid at 4 °C for 24, 48, 72 and 96 h.

	Inoculum						
	Fresh	Refrigerated					
		24 h	48 h	72 h	96 h	SEM	P[a]
pH	5.8	5.7	6.0	5.8	5.9	0.096	0.49
NH_3 (mg/dL)[b]	23.4^{B}	33.6^{AB}	39.3^{A}	44.7^{A}	36.6^{A}	3.580	0.04
Total VFA (mmol/L)	122.1	125.5	131.6	129.4	125.6	6.050	0.82
VFA (mol/100 mol)							
Acetate	73.2	72.5	70.9	71.3	72.4	1.049	0.54
Propionate	13.2	12.9	13.3	12.9	13.0	0.279	0.83
Butyrate	9.4	9.7	10.0	10.3	9.5	0.421	0.60
iso-butyrate	0.9	2.2	2.7	2.7	2.1	1.017	0.68
iso-valerate	1.8	1.8	1.9	1.9	1.8	1.295	0.96
Valerate	1.4	0.9	1.2	1.0	1.2	0.132	0.12
A:P	5.6	5.6	5.3	5.5	5.6	0.140	0.69
Gas 24 h (mL/g DM)							
Feeds[c]							
Corn	310	304	301	293	234		
Soy meal, extracted	219	213	199	203	135		
Wheat bran	217	221	212	209	148		
Alfalfa meal	169	146	147	150	91		
Total mixed ration	228	214	210	211	164		
Mean[b]	229^{A}	219^{A}	214^{A}	213^{A}	154^{B}	7.2	< 0.01

[a] P: probability.
[b] Means with different superscript are statistically different.
[c] The "feed x inoculum" interaction was not significant (*i.e.*, $P > 0.05$) and the "feed" effect was significant ($P < 0.01$).

4. Discussion

The concentration of fermentation metabolites in rumen fluid was not changed by refrigeration, apart from an increase in ammonia concentration. Cold shock response is a complex reaction by which bacterial groups adapt to cold temperatures. This includes several metabolic processes, such as modification in membrane lipids (Mansilla and de Mendoza, 2019) and synthesis of proteins able to favor sugar metabolism (Phadtare and Inouye, 2008). It is likely that the higher ammonia levels after refrigeration was due to increased protein metabolism due to cold. Phadtare and Inouye (2008) suggested that a refrigeration phase could improve the subsequent viability of cells due to the protective effect of the cold shock proteins produced. If so, a practical implication in the field of rumen inoculum preservation could be the investigation of optimal pre-cooling protocols prior to full refrigeration.

4.1. Cooling rumen fluid for up to 96 h

Despite the similarity of fermentative metabolites with 96 h of cooling, there was a decline in 24 h gas production at 96 h of refrigeration. However, these results were not unexpected based on Robinson et al. (1999) who found a stable NDF degradability of fresh and 48 h refrigerated (4 °C) rumen fluid. Another experiment based on a gas test (Hervás et al., 2005) reported that chilling inoculum for 6 h did not affect *in vitro* gas production, but 24 h of refrigeration reduced fermentation rate. Also, Prates et al. (2010) and Spanghero et al. (2019) maintained rumen fermentability of fresch rumen fluid, measured in terms of gas production, when it was preserved for about 4 h at 4 − 6 °C.

4.2. Cooling rumen fluid solids for 48 h

Centrifugation is widely used to separate rumen microbes from liquids and feed particulates in rumen fluid. Unfortunately, centrifugation protocols (*e.g.* centrifugal force, time, temperature) are not standardized. Important references in this field include Hsu and Fahey (1990) and Luchini et al. (1996), who tested three centrifugation speeds (5, 10 and 26 × 10^3 x *g* and 5, 17 and 30 × 10^3 x *g*, respectively) for 20 or 30 min. As both groups reported no differences in the amount of ruminal bacteria separated by different centrifugations speeds, we used an intermediate value of 13 × 10^3 × *g*. Hall and Hatfield (2015) also used this centrifugation speed in preparing a rumen pellet to study the glycogen recovery from rumen microbes, as well Martinez-Fernandez et al. (2019) in comparing methods to isolate rumen bacteria. In our study, the yield of wet pellet was variable (*i.e.*, 151 $\pm$ 42g/Kg of the initial rumen fluid volume), likely reflective of differences in the particulate density of the rumen fluid collected, despite using a common filtration procedure. Variations in pellet yield will be reflected in reconstituted rumen fluid making utilization of blanks (*i.e.*, fermentation from bottles containing only fermentation fluid) required.

Maximum potential gas production (*i.e.*, asymptote) with fresh rumen inoculum was very close to those reported by Getachew

Table 2
Parameters of the kinetics of gas production (asymptote, lag and rate of gas production) and estimated gas yield at 24 h of fermentation of five feed (F) substrates using fresh rumen fluid and the reconstituted refrigerated pellet at 4 °C for 48 h (least square means).

	Inoculum (I)			Significance, P[b]		
	Fresh	Pellet	SEM[a]	F × I[c]	I	F
Asymptote (mL/g DM)						
Corn	421	360				
Soy meal, extracted	257	244				
Wheat bran	247	248				
Alfalfa meal	210	200				
Total mixed ration	295	262				
Mean	286	263	5.6	ns	< 0.01	< 0.01
Lag (h)						
Corn	0.59	1.16				
Soy meal, extracted	0.02	0.71				
Wheat bran	0.00	0.06				
Alfalfa meal	0.01	0.69				
Total mixed ration	0.06	0.89				
Mean	0.14	0.70	0.13	ns	< 0.01	< 0.01
Rate (mL/h)						
Corn	0.053	0.075				
Soy meal, extracted	0.087	0.144				
Wheat bran	0.079	0.083				
Alfalfa meal	0.078	0.084				
Total mixed ration	0.059	0.077				
Mean	0.072	0.092	0.0067	< 0.01	< 0.01	< 0.01
Gas 24 h (mL/g DM)						
Corn	307	302				
Soy meal, extracted	234	235				
Wheat bran	205	213				
Alfalfa meal	174	173				
Total mixed ration	220	222				
Mean	228	229	9.0	ns	ns	< 0.01

[a] SEM standard error of the inoculum means.
[b] P: probability; ns: not significant.
[c] Interaction "feed x inoculum".

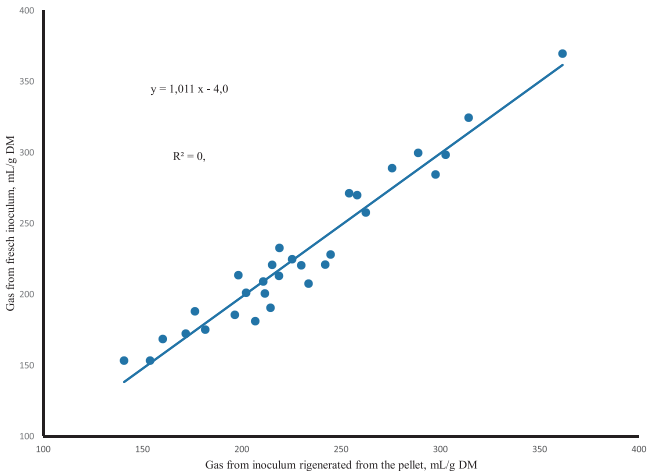

Fig. 1. Linear regression between gas yield at 24 h of fermentation obtained from fresh rumen inoculum (y, adjusted for rumen fluid effect, experiment 2) and from inoculum reconstituted from the refrigerated (at 4 °C for 48 h) rumen pellet (x).

et al. (2004) for corn, soya meal and alfalfa hay (394, 253 and 211 mL/g of DM) and by Gallo et al. (2016) with corn meal samples having different particle sizes (334–367 mL/g of OM) using the same syringe methodology. While our study did not assess feed differences, we did observe that reconstituted rumen fluid from refrigerated pellet caused a depression of asymptotic gas production (-10/11 % for corn and TMR, -5% for soya and hay), even though values of 24 h gas yield did not differ.

4.3. Gas production at 24h

Gas production at 24 h data between fresh and refrigerated/regenerated fluids was similar despite the minimal survival of protozoa at low temperatures (De la Fuente et al., 2006; Takizawa et al., 2019). Similar reductions are probable for bacterial strains with limited tolerance to low temperatures. However, as gas production and other *in vitro* tests require surplus fermentation capacity (*e.g.*, at a high ratio of inoculum/substrate, 50 mL/g incubated DM), the loss in microbiota function due to refrigeration/regeneration appears not to be substantial. This is relevant, from a practical point of view, because gas yield measured at 24 h of incubation by the syringe apparatus is the only gas test output that has been systematically used in predictive equations of the metabolizable energy (ME) content of ruminant feeds (Menke and Steingass, 1988). It has also been positively evaluated in terms of precision (Getachew et al., 2002), accuracy of the calculated ME (Robinson et al., 2004), additivity of values of single feeds in compound feeds (Grubješić et al., 2020) and it is used in ME predictions of ruminant feeds and rations (Getachew et al., 2004; Spanghero et al., 2017).

5. Conclusion

Present results show that rumen fluid preserved by refrigeration (at 4 °C until 72 h) or reconstituted from a refrigerated bacterial pellet (at 4 °C for 48 h), does not depress 24 h gas production. Therefore, refrigerated rumen fluid (or pellet) is an alternative to fresh fluid and would aid in the supply and transport of rumen fluid between laboratories, as well as the potential use of the same inoculum in repeated fermentation runs within the laboratory. This would reduce the need for laboratories to maintain animal donors and/or reduce the frequency for the collecting rumen fluid.

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CRediT authorship contribution statement

Carla Fabro: Data curation, Formal analysis. **Chiara Sarnataro:** Data curation, Formal analysis, Methodology. **Mauro Spanghero:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper

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