R. Bras. Zootec., 53:e20230151, 2024
https://doi.org/10.37496/rbz5320230151

Ruminants
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
e-ISSN 1806-9290
www.rbz.org.br

Can dispersion methods affect the *in vitro* ruminal evaluation of substrates with different fermentabilities?

Franciele Caetano Sampaio[1], Juliana Maria Silva de Souza[1], Letícia Carolina Bortolanza Soares[1], André Soares de Oliveira[1], Dalton Henrique Pereira[1], Edenio Detmann[2], Thierry Ribeiro Tomich[3], Júlia Mara Campos de Souza[4], Erick Darlisson Batista[1,4*]

[1] Universidade Federal de Mato Grosso, Departamento de Zootecnia, Sinop, MT, Brasil.
[2] Universidade Federal de Viçosa, Departamento de Zootecnia, Viçosa, MG, Brasil.
[3] Embrapa Gado de Leite, Juiz de Fora, MG, Brasil.
[4] Universidade Federal de Lavras, Departamento de Zootecnia, Lavas, MG, Brasil.

***Corresponding author:**
erick.batista@ufla.br

Received: October 10, 2023
Accepted: April 17, 2024

How to cite: Sampaio, F. C.; Souza, J. M. S.; Soares, L. C. B.; Oliveira, A. S.; Pereira, D. H.; Detmann, E.; Tomich, T. R.; Souza, J. M. C. and Batista, E. D. 2024. Can dispersion methods affect the *in vitro* ruminal evaluation of substrates with different fermentabilities? Revista Brasileira de Zootecnia 53:e20230151. https://doi.org/10.37496/rbz5320230151

Editors:
Marcio de Souza Duarte
Gleise Medeiros da Silva

ABSTRACT - The objective of this study was to evaluate the *in vitro* fermentation products, digestibility, gas production (GP) kinetics, and enteric greenhouse emissions (CH_4 and CO_2) of substrates with different forage:concentrate ratios (100F, grass hay only; 100C, concentrate mixture only, and mixture, an equal proportion of them) within non-woven fabric (NWT; 100 g/m^2) or F57 (Ankom®) filter bags compared to directly dispersed in the medium (DIS), arranged in a 3 × 3 factorial arrangement. Substrates (0.5 g) were incubated using an Ankom^{RF} GP System. Gas samples were collected during 24 and 48 h of incubation. We observed substrate × dispersion method interactions on GP at 48 h (GP48) and on *in vitro* organic matter digestibility (IVOMD). The GP48 and IVOMD of the 100C substrate were greatest in DIS, intermediate in NWT, and least in F57. With mixture substrate, there were no differences in GP48 and IVOMD between DIS and NWT, but they were greater than in F57. The GP48 and IVOMD were greater in NWT than in DIS and F57 when 100F was incubated. There were no dispersion method × substrate interactions on molar proportions and total volatile fatty acids. With the increase in forage:concentrate ratio incubated, there was a linear decrease in CH_4 and CO_2 emission relative to organic matter digested. Overall, CH_4 and CO_2 emissions and digestibility were lower when substrates were incubated within filter bags. The noteworthy interaction between the incubation method and substrates indicates that the ranking of these variables for substrates with differing fermentabilities changes with the dispersion method employed.

Keywords: digestibility, filter bag, gas production, methane, rumen fermentation

1. Introduction

The *in vitro* fermentation technique has been routinely used during a long time to evaluate the nutritive values of feeds and additives (Tilley and Terry, 1963; Blümmel and Becker, 1997a; Bodas et al., 2008; Maccarana et al., 2016), especially regarding the influences on digestibility, gas production and composition, and volatile fatty acids profile (Blümmel et al., 1997b; Patra et al., 2010; Patra and Yu, 2012). Traditionally, *in vitro* incubation of substrates has been dispersed in the medium. However, incubation in filter bags also allows the measurement of *in vitro* digestibility and makes it easier due to less transfer of sample residues after incubation. The most common material used for these evaluations are F57 (Ankom®), which is used to analyze fiber content in feeds, but this has the inconvenience of being a high-cost material, making routine use in the laboratory unfeasible. As an alternative to this material, studies on the use of non-woven fabric (NWT; 100 g/m^2) have increased (Casali et al., 2009; Valente et al., 2015; Silva et al., 2017; Camacho et al., 2019).

However, studies investigating how NWT bags affect rumen *in vitro* fermentation and greenhouse gas production are lacking.

Considering the importance of greenhouse gases emission by ruminants, diet formulation with lower energetic loss during the digestive process and the dispersion method for *in vitro* fermentation studies, comparison between different filter bags, and those effects of *in vitro* ruminal fermentation have not been examined in a systematic fashion. We hypothesized that greenhouse gases yield, digestibility, and fermentation end-products in an *in vitro* system could be greater if substrates are incubated directly dispersed in the medium compared with when they are incubated within filter bags (F57 and NWT). Therefore, our objective was to investigate how different incubation methods, within bags and direct dispersion, affect the fermentation products made from substrates with different forage:concentrate ratios.

2. Material and Methods

The experiment was conducted in Lavras, MG, Brazil. All experimental procedures involving cattle were approved by the institutional Ethics Committee on Animal Use (case n° 23108.718652/2016-77).

2.1. Experimental design

The effects of substrate dispersion method, substrate type, and their interaction were evaluated in a 3 × 3 factorial arrangement with three dispersion methods (samples incubated directly dispersed in the medium, within F57 bags, and within non-woven bags, NWT) and three types of substrates with different forage:concentrate ratio (low-quality tropical grass hay only, 100F; a low-quality tropical grass hay and concentrate mixture in a 50:50 ratio, mixture; and concentrate mixture only, 100C, all on a dry matter (DM) basis). The three substrates were used to cover a large variability in chemical composition (low, average, and highly fermentable) and to generate different *in vitro* fermentation end-products in the bottle during incubation.

Low-quality tropical grass (*Brachiaria brizantha* cv. BRS Piatã) was collected from a dry season cutting of the forage available in a pasture located in the Midwest region of Brazil. The concentrate feed consisted (DM basis) of a mixture of ground corn (838 g/kg) and soybean meal (162 g/kg). Forage and concentrate were ground in a knife mill (Willey Type Mill, TE-650; Tecnal, Piracicaba, SP, Brazil) to pass through a 2-mm screen. After that, half of each ground sample was ground again to pass through a 1-mm screen. The samples ground through 1-mm sieves were analyzed for DM (dried overnight at 105 °C; method INCT-CA number G-003/1), ash (complete combustion in a muffle furnace at 600 °C for 4 h; method INCT-CA number M-001/1), nitrogen (Kjeldahl procedure; method INCT-CA number N-001/1), and neutral detergent fiber corrected for ash and protein (NDFap, using a heat-stable α-amylase, omitting sodium sulfite and correcting for residual ash and protein; method INCT-CA number F-002/1) by chemical analytical methods according to the standard analytical procedures of the Instituto Nacional de Ciência e Tecnologia de Ciência Animal (Detmann et al., 2012). Low-quality tropical grass hay and concentrate contained (g/kg DM): 926 and 894 g DM/kg, 952 and 980 organic matter (OM), 25 and 150 crude protein (CP), 678 and 143 NDFap, 12 and 625 starch, respectively.

2.2. Experimental procedures

Fresh rumen fluid (pH = 7.07 ± 0.144) was collected manually from the cranial, ventral, and caudal areas of two cannulated Holstein steers (442 ± 27.6 kg of body weight; housed at a metabolism barn) before morning feeding to obtain stable rumen microbial cultures. These two steers grazed Marandu palisade grass (*Brachiaria brizantha*) pasture and received 4 kg/day of commercial concentrate provided twice daily (09:30 and 17:30 h) to establish about 400 g concentrate kg^{-1} diet, on a DM basis (160 g CP/kg, based on ground corn, soybean meal, cottonseed cake, vitamin, and trace mineralized salts; Fortuna Nutrição Animal, Nova Canaã do Norte, MT, Brazil). The rumen fluid collected from

each of the steers was mixed in equal amounts, filtered through three layers of cheesecloth, and used as the inoculum in the *in vitro* rumen fermentation.

2.3. *In vitro* incubation

The *in vitro* batch fermentation was carried out using a commercial wireless gas production (GP) apparatus (Ankom^{RF} GP System, Ankom Technology®, Macedon, NY, USA) consisting of 24 bottles equipped with pressure sensors and wireless connection to a computer. Four incubation runs were conducted in four successive weeks. Each treatment (all combinations between the three dispersion methods and three substrates) plus blanks (bottles containing only the rumen fluid and the buffer solution) was run in duplicate across four successive runs (blocks). Samples of substrates milled through a 1-mm screen were weighed directly into bottles (310 mL), incubated within F57 filter bags (Ankom Technology Corp., Macedon, NY), or incubated using manufactured NWT filter bags (100 g/m^2) of 4.0 × 4.5 cm (Valente et al., 2011) that were sealed and placed into the bottles. The F57 and NWT filter bags presented 36 cm^2 of surface, and samples were put in the bags following the ratio of 20 mg DM/cm^2 of surface (Nocek, 1988). Both types of filter bags were previously washed with commercial neutral detergent, water, and acetone, and were dried in an oven at 55 °C before samples were weighed and sealed. This washing procedure was done to remove the surfactant added during the manufacturing process of the F57 bag, avoiding microbial digestion inhibition (Krizsan et al., 2012).

The buffer solution was prepared according to McDougall (1948), heated at 39 °C, purged continuously with nitrogen (N_2) for 30 min to maintain anaerobic conditions, and pH was adjusted to 6.8 with orthophosphoric acid (Carreño et al., 2015). The anaerobic buffered medium (40 mL) and the rumen inoculum (10 mL) were mixed (buffer:rumen fluid ratio of 4:1), dispensed into each bottle, pre-flushed with N_2 (Benedeti et al., 2018; Patra and Yu, 2013), and incubated for 48 h at 39 ± 0.5 °C. The bottles were shaken gently for proper mixing of feeds or bags with microbial cultures.

2.4. Sampling and measurements

The data acquisition software (Gas Pressure Monitor, Ankom technology, NY, USA) was set to monitor the cumulative pressure every 5 min, and data was recorded every 30 min for 48 h. Valves were set to automatically release the gas when the pressures reached 3.4 kPa (Tagliapietra et al., 2012). After correction for the blanks, the recorded cumulative gas pressure was converted to mL of gas produced under the manufacturer's instructions using Avogadro's law (gas volume (mL) = gas pressure × [V/(R×T)] × 22.4 × 1,000, in which V is headspace volume in the bottle in L = 0.26, R is the gas constant 8.314472 L kPa/K/mol, and T is the temperature in Kelvin).

Gas samples were drawn using a 10-mL gas-tight syringe from each bottle headspace at 24 and 48 h of incubation and stored in a 10-mL vacuum tube. Gas chromatograph (EZChrom Elite software interface, Model 7820A, Agilent Technologies Brazil, Barueri, SP, Brazil) was used for the quantification of CH_4 and CO_2. The gas chromatograph equipment was equipped with two six-way valves, one being used for the sampler system connected to a loop of 0.5 mL. Injector split-splitless type was used in split mode to 50:1 at 120 °C. The separation system consisted of two columns. The first column was a HP-Plot/Q 30 m × 0.530 mm × 40.0 mm (Agilent Technologies Brazil). The detection system comprised a thermal conductivity detector at 250 °C, with 25 mL H_2/min as flow reference. The second column was a Molesieve HP-30 m × 0.530 mm × 25.0 mm, using H_2 as carrier gas at a flow rate of 8.3 mL/min, with flame ionization detector at 270 °C and 15 mL/min H_2 flow rate, and 350 mL/min synthetic air flow. The gas chromatograph was also equipped with a methanizer maintained at 375 °C, which allows detecting very low concentrations of CO_2. The oven temperature was maintained at 55 °C. The calibration curves were performed with reference standards for CH_4 concentrations, as follows: 0 mL/L, 50.5 mL/L, 102 mL/L, 147 mL/L, and 201 mL/L; and for CO_2 as follows: 0 mL/L, 202 mL/L, 397 mL/L, 583 mL/L, and 799 mL/L.

After 48 h of incubation, fermentation was stopped by placing serum bottles in ice-water bath for 15 min. For the substrates that were dispersed directly into the buffered ruminal fluid, the contents of the bottles were filtered under vacuum through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100 to 160 µm). The fluid pH was measured with a pH meter (PG 2000 Portable pH meter; Gehaka, São Paulo, SP, Brazil) by submerging the probe approximately 3 cm in the fermentation media. An aliquot of fermentation media (1.2 mL) was combined with 0.4 mL of 250 g/L metaphosphoric acid and frozen for subsequent analysis of volatile fatty acids (VFA). Following, to estimate the apparently undigested DM residue, fermentation residues in grass crucibles and bags were oven-dried (55 °C/24 h and 105 °C/16 h, sequentially), placed in a desiccator, and weighed. The DM incubation residues in grass crucibles and filter bags were ashed at 600 °C for 4 h to estimate OM disappearance. Blank bags were also dried and ashed for the correct weight.

Sample VFA profile was obtained using a high-performance liquid chromatograph (Varian Pro Star 325 LC) equipped with an ultraviolet detector. The separation system consisted of a C18 Supelco reverse phase column (250.0 × 4.6 mm, 5 µm). The elution was carried out with an isocratic mobile phase consisting of an aqueous solution of metaphosphoric acid (HPO_3) (eluent A, pH adjusted to 2.5) and acetonitrile (eluent B). The gradient was constituted of 920 mL/L A and 80 mL/L B, with a flow of 1 mL/min and a running time of 8 min. The injection volume was 20 µL and detection at 210 nm. Quantification was obtained using a calibration curve with external standards.

2.5. Calculations

Gas production was calculated as the volume of gas (mL) produced after 24 h (GP24) and 48 h of incubation (GP48) divided by the amount of DM incubated (DMi, g). The fraction of *in vitro* DM (IVDMD) and *in vitro* OM (IVOMD) digestibilities for each bottle was calculated by subtracting the DM and OM residue weight (corrected for the blank) from the DM and OM substrate weight and dividing by the weight of substrate, respectively. The partitioning factor at 48 h of incubation (PF; a measure of fermentation efficiency) was calculated as the ratio of OM degradability *in vitro* (OMd, mg) to the volume (mL) of GP at 48 h (i.e., OMd/total GP), according to Blümmel et al. (1997b). The change of total VFA concentration in the medium during incubation was calculated by subtracting the total VFA concentration of the initial medium (rumen fluid and buffer) from the total VFA concentration in the medium after 48 h of incubation (ΔVFA). Apparent change of VFA per g OM incubated (ΔVFA/OMi) or g OM digested (ΔVFA/OMd) were calculated by multiplying total VFA by the volume of medium (50 mL) and dividing by the weight of substrate of OM incubated or OM digested, respectively. Acetic, propionic, and butyric acids were expressed as molar proportion relative to total VFA (mol/100 mol).

Because the present experiment was conducted without the use of tight bags for gas collection to measure the volume and concentration in vented gas, we used the following equation described by Cattani et al. (2016) to estimate methane production (MP, mL): $MP = HSCH_4 \times (260 + 0.0057 \times GP^2)$, in which HCH_4 (L/L) is the CH_4 proportion in the headspace and 260 (mL) is the total headspace volume. The same equation was also used to calculate CO_2 production, considering that CO_2 and CH_4 are in equilibrium in the headspace (i.e., the proportions of gases in the vented gas are the same as in the headspace), and the outflow of CO_2 and CH_4 produced is relative to their concentrations in the headspace (Ramin and Huhtanen, 2012). Methane and CO_2 yield were expressed relative to DM incubated and OM digested (mL/g). Proportion of CH_4 and CO_2 (mL/L) in the fermentation gas was calculated by multiplying the production of the relative greenhouse gas by 1,000 and dividing by GP.

To assess biological values, gas curves were fitted to a dual-pool logistic equation derived on the assumption that the rate of gas production is proportional to both the accumulated microbial mass and to the amount of digestible substrate remaining (Schofield et al., 1994). This equation is: $V = V_F / \{1 + \exp(2 + 4 \times (SR_F \times (Lag - t)))\} + V_S / \{1 + (\exp(2 + 4 \times (SR_S \times (Lag - Time)))\}$, in which V = gas volume produced up to the specific time t (mL); V_F and V_S = maximum gas volumes (mL) achieved from complete digestion of the first and second pool (i.e., faster- and slower-digesting fractions, respectively); SR_F and SR_S = specific rate of digestion (h^{-1}) for these fractions; and Lag = lag time (h).

2.6. Statistical analyses

All statistical analyses were performed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC), with the mean of the bottles within substrates in each incubation run (true replicate) as the experimental unit. Means for all variables were obtained using the LSMEANS statement. The model was as follows:

$$Y_{ijklm} = \mu + I_i + M_k + S_j + (M \times S)_{jk} + \varepsilon_{ijk}, \quad (1)$$

in which μ is the general mean, I_i is the random effect of incubation i, M_k is the fixed effect of dispersion method k, S_j is the fixed effect of substrate j, $(M \times S)_{jk}$ is the fixed effect of interaction between substrate type and dispersion method, and ε_{ijk} is the random error.

For GP, CH_4, and CO_2 proportion, emission, and yield, data were reported at 24 and 48 h of incubation. For total gas production over time data, logistic nonlinear functions for two pools and a discrete lag (Schofield et al., 1994) were adjusted for substrates (forage:concentrate ratio of 100:0, 50:50, and 0:100), as well as incubation methods, to compare possible differences in fermentation profiles. Treatment mean values were calculated using the LSMeans statement in SAS and, when the interaction between factors was significant (P<0.05), they were separated using PDIFF option with Tukey's adjustment. Orthogonal polynomial contrasts were used to examine linear and quadratic effects of increasing concentrations of forage when the effects of substrate were found significant (P<0.05). Pearson's correlation of variables at 48 h of fermentation among methods were analyzed using PROC CORR of SAS.

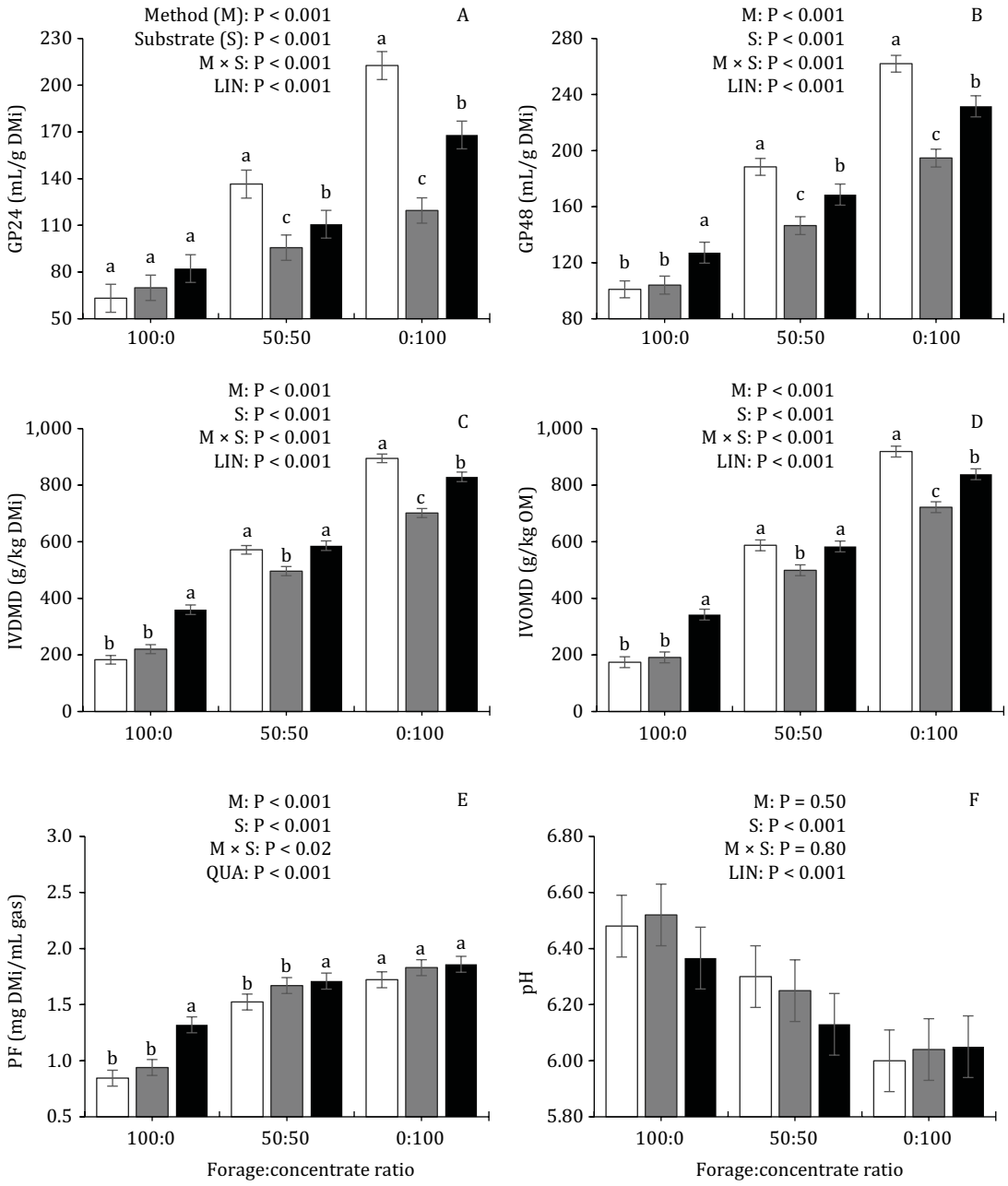
3. Results

3.1. Effects of dispersion method of substrates with different forage:concentrate ratios on *in vitro* rumen fermentation

There were interactions between different dispersion methods and substrate forage:concentrate ratio (P<0.02) on GP24, GP48, IVDMD, IVOMD, and PF (Figure 1, panel A–E), while no effect interaction was observed on pH (Figure 1F) (P = 0.80). There were no differences between methods (P≥0.08) for GP24 when the 100F ratio substrate was incubated (averaging, 71.6 mL/g DMi). The incubation of mixture and 100C substrates promoted greatest GP24 in DIS followed by NWT and F57 (P≤0.03). The method × substrate interaction showed similar effects for GP48 and GP24 (P≤0.04), except those when the 100F ratio substrate was incubated in NWT, which had greater gas production than in DIS and F57 (P<0.01).

The *in vitro* digestibilities (IVDMD and IVOMD) presented similar results (Figures 1C and D, respectively). The IVDMD and IVOMD were about 82 and 88% greater (P<0.001), respectively, when 100F substrate was incubated in NWT than in DIS and F57. With mixture substrate incubation, there were no differences for *in vitro* digestibilities between DIS and NWT (P≥0.58; on average, 578 and 585 g/kg for IVDMD and IVOMD, respectively), but they were greater than F57 (P<0.01; 494 and 499 g/kg, respectively). The *in vitro* digestibilities of concentrate substrate (100C) were enhanced in DIS, intermediate in NWT, and decreased in F57 (P<0.01). For IVOMD, these values were 918, 838, and 722 g/kg, respectively.

The PF was 46% greater in NWT method (P<0.05) than in DIS and F57, and both presented similar results (P = 0.26) when 100F substrate was incubated. When mixture substrate was incubated, PF was greater in NWT and smallest in DIS (P = 0.04; 1.70 and 1.52 mg OMd/mL, respectively), and F57 presented value similar to them (P≥0.08; 1.67 mg OMd/mL). With the concentrate incubation (100C), differences became less evident, without difference among methods (P≥0.11). The pH was affected only by substrate (P<0.001). As expected, increasing forage inclusion linearly increased pH from 6.00 to 6.52 (P<0.001).

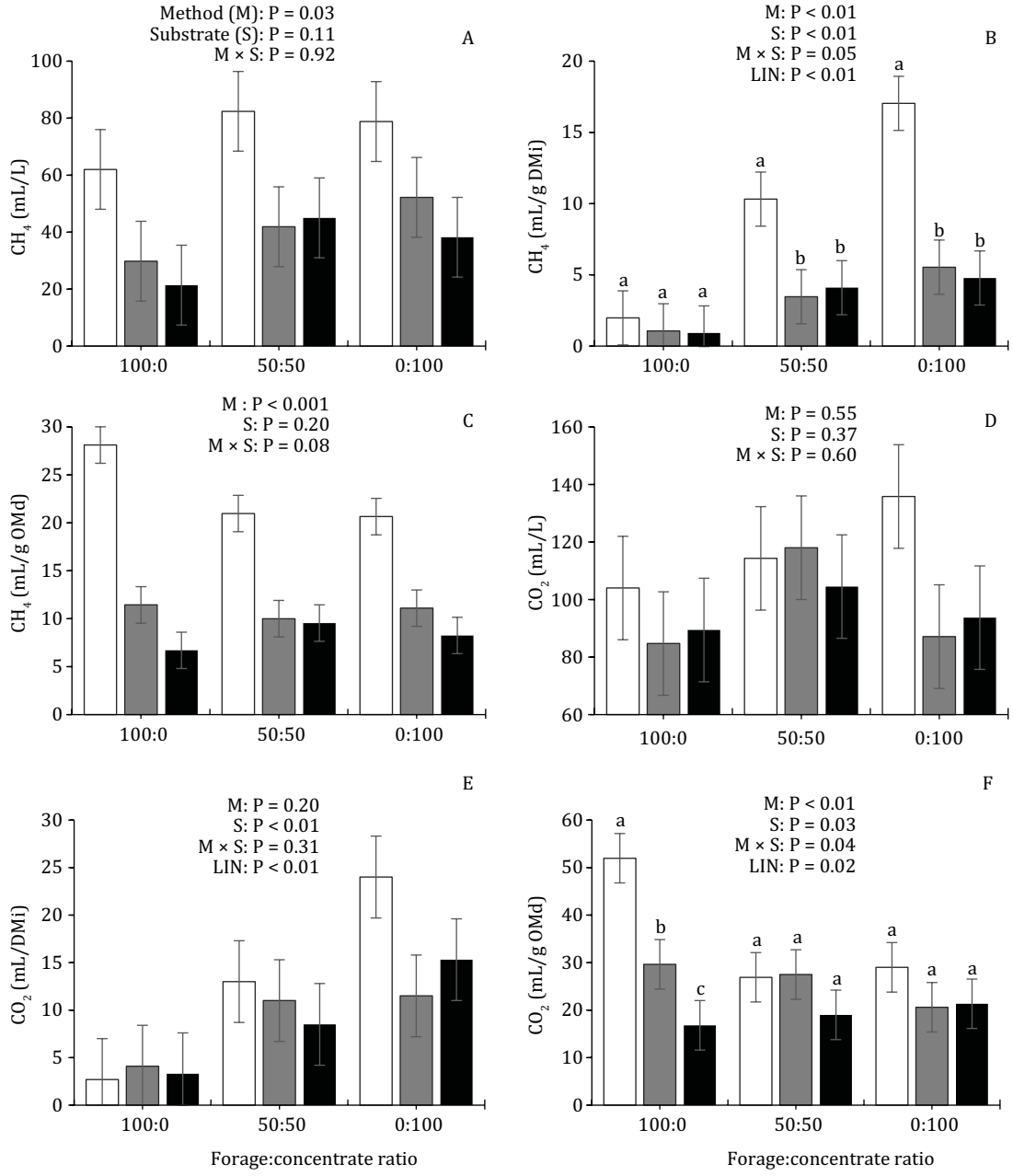


White, gray, and black bars correspond to samples that were incubated directly in the medium, within F57 and NWT bags, respectively. Error bars represent standard error of the mean. LIN and QUA represent linear and quadratic effects of forage:concentrate ratios, respectively.
For substrate dispersion method treatment effect, means within forage:concentrate ratios with different letters (a–c) differ (P<0.05).

Figure 1 - Effects of substrate dispersion method and substrate with different forage:concentrate ratios on total gas production (GP) at 24 h (A) and 48 h (B), *in vitro* dry matter and organic matter digestibility (IVDMD and IVOMD, respectively; C and D), partitioning factor (PF; E), and pH (F).

3.2. CO_2 and CH_4 emission at 24 and 48 h of incubation

A method × substrate interaction was found for CH_4 yield relative to DM incubated (DMi) (Figure 2B), but not for CH_4 proportion (Figure 2A) and yield relative to OMd (Figure 2C) at 24 h of incubation. There was no difference between methods in CH_4/DMi (P≥0.52) when 100F substrate was incubated. But for the other evaluated substrates, DIS produced about 2.4 times greater CH_4/DMi compared with filter bags (P<0.001). There were method effects on CH_4 proportion and CH_4/OMd (P≤0.03), with estimates about 67% greater when substrates were incubated in DIS than into filter bags

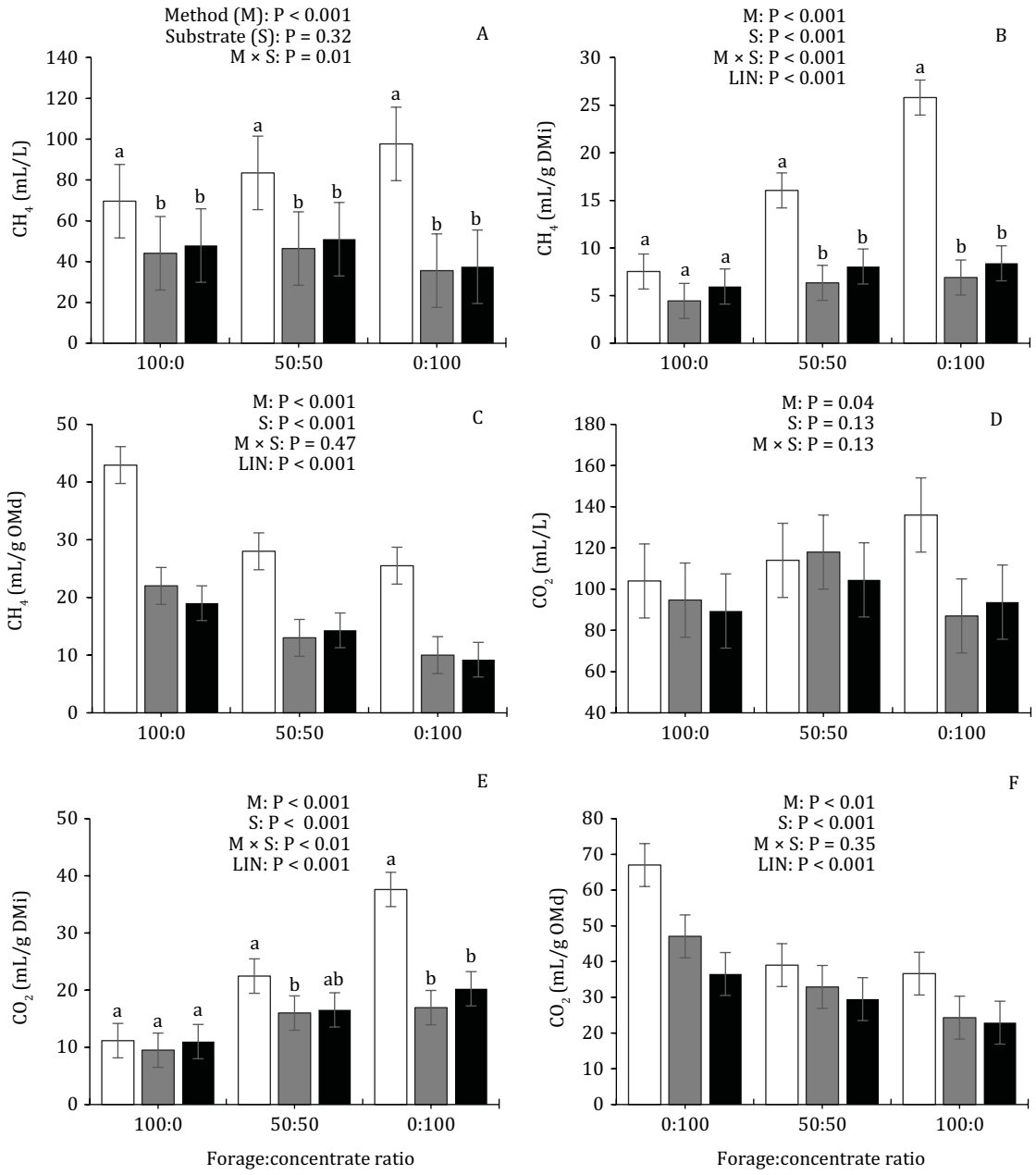


White, gray, and black bars correspond to samples that were incubated directly in the medium, within F57 and NWT bags, respectively. Error bars represent standard error of the mean. LIN represents linear effect of forage:concentrate ratios.
For substrate dispersion method treatment effect, means within forage:concentrate ratios with different letters (a–c) differ (P<0.05).

Figure 2 - Effects of substrate dispersion method and substrate with different forage:concentrate ratios on CH_4 (A, B, and C) and CO_2 (D, E, and F) output at 24 h of incubation.

(P≤0.01), but there were no differences between F57 and NWT bags (P≥0.06). Interactions were observed between the substrate dispersion method and forage:concentrate ratio of substrates with respect to CO_2 yield relative to OMd (P = 0.04), but not to CO_2 proportion (Figure 2C; P = 0.60), and CO_2 yield relative to DMi (Figure 2F; P = 0.31). When 100F substrate was incubated, CO_2 yield relative to OMd was greater in DIS, intermediate in F57, and lower in NWT method (P≤0.01). For the other substrates, methods provided similar results (P≥0.07). The CO_2 yield relative to DMi linearly increased when forage:concentrate ratio decreased (P<0.01).

At 48 h of incubation, there were interactions between substrates and dispersion method (P≤0.01) on CH_4 proportion (Figure 3A, mL/L) and yield relative to DM incubated (Figure 3B, mL/g DMi), but not on



White, gray, and black bars correspond to samples that were incubated directly in the medium, within F57 and NWT bags, respectively. Error bars represent standard error of the mean. LIN represents linear effect of forage:concentrate ratios.
For substrate dispersion method treatment effect, means within forage:concentrate ratios with different letters (a,b) differ (P<0.05).

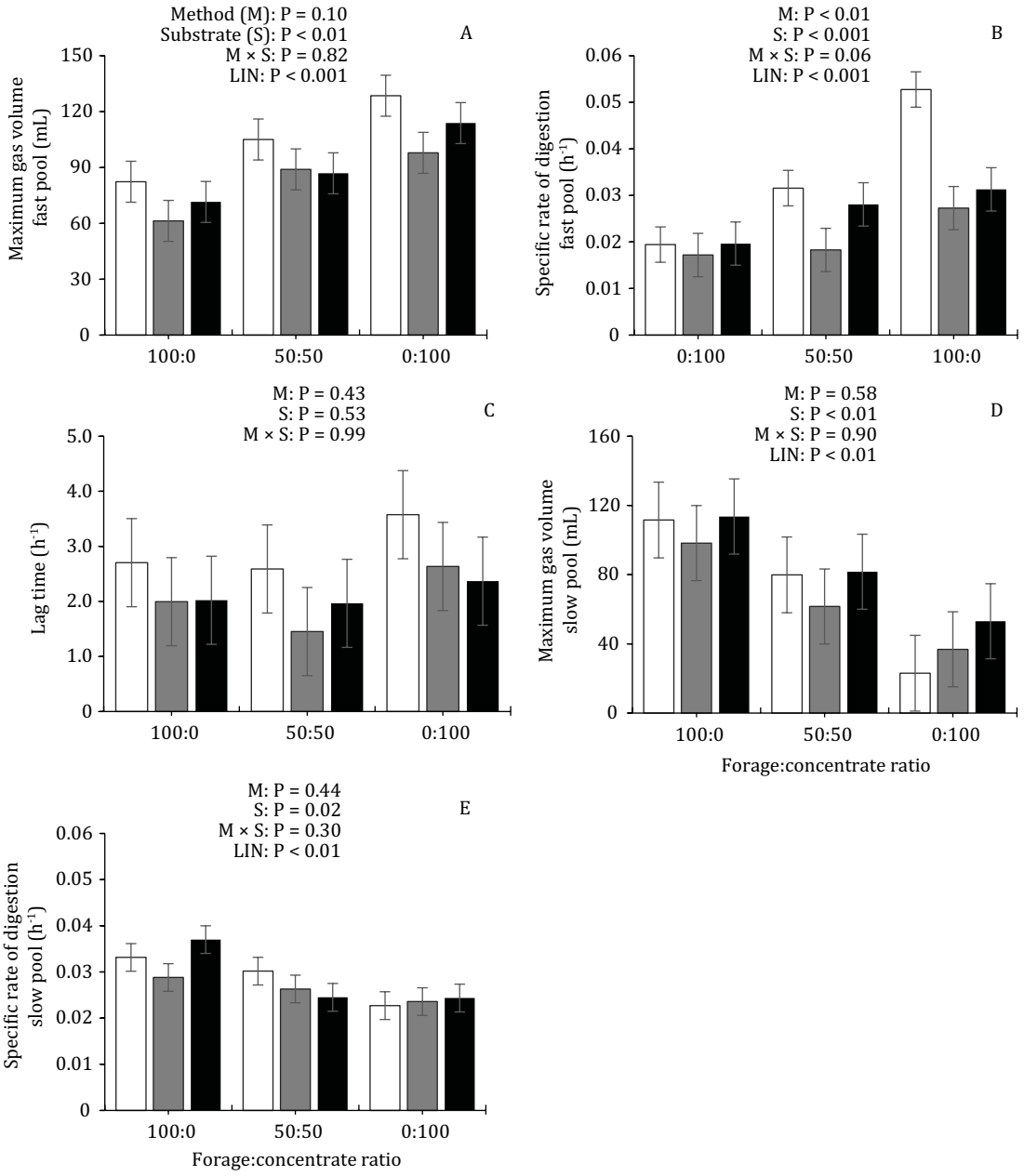
Figure 3 - Effects of substrate dispersion method and substrate with different forage:concentrate ratios on CH_4 (A, B, and C) and CO_2 output (D, E, and F) at 48 h of incubation.

yield relative to OM digested (Figure 3C, mL/g OMd; P = 0.47). The CH_4 proportion values were slightly altered (P<0.001) by the methods (DIS > filter bags). When 100F substrate was incubated, methods presented similar CH_4 yield relative to DMi (P≥0.12). However, differences between methods in CH_4 yield/DMi became more pronounced when substrate fermentability increased, in which DIS showed greater CH_4 yield/DMi than when incubated in the F57 and NWT bags (P<0.001; 13.2 vs. 8.9 mL/g DMi). There was method effect on CH_4 yield relative to OMd (P<0.001), with lower values found with the incubation substrates within bags than in DIS (14.6 vs. 32.2 mL/g OMd). Additionally, CH_4 yield/OMd linearly decreased in response to decrease of forage:concentrate ratio (P<0.001). Regarding CO_2 output at 48 h of incubation (Figure 3D–F), greater proportion and yield relative to OMd (P≤0.04) were noted for DIS compared with incubation within filter bags (118 vs 98 mL/L and 47.6 vs. 32.2 mL/g OMd, respectively). With decreasing forage:concentrate ratio of the substrates, CO_2 yield/OMd linearly decreased (P<0.001).

There was interaction (P<0.01) between substrate and dispersion method for CO_2 yield relative to DM incubated. The CO_2 yield/DMi did not differ (P≥0.60) between the three incubation methods when the substrate contained forage only, but it was 3.4 times greater in DIS than in filter bags when the substrate contained concentrate only (P<0.001). When the mixture substrate was incubated, CO_2 yield relative to DMi was greater in DIS than in F57 (P = 0.05), but NWT was similar to DIS and F57 (P≥0.07).

3.3. Kinetic parameters of gas production

There were no method × substrate interactions (P≥0.06) on kinetic variables of gas production (Figure 4A–E). The maximum gas volumes and specific rates of digestion for first and second pools were affected by substrates (P≤0.01), but in opposite ways. While maximum gas volumes and specific rates of digestion for first pool (fast degradation) increased linearly (P<0.01), these



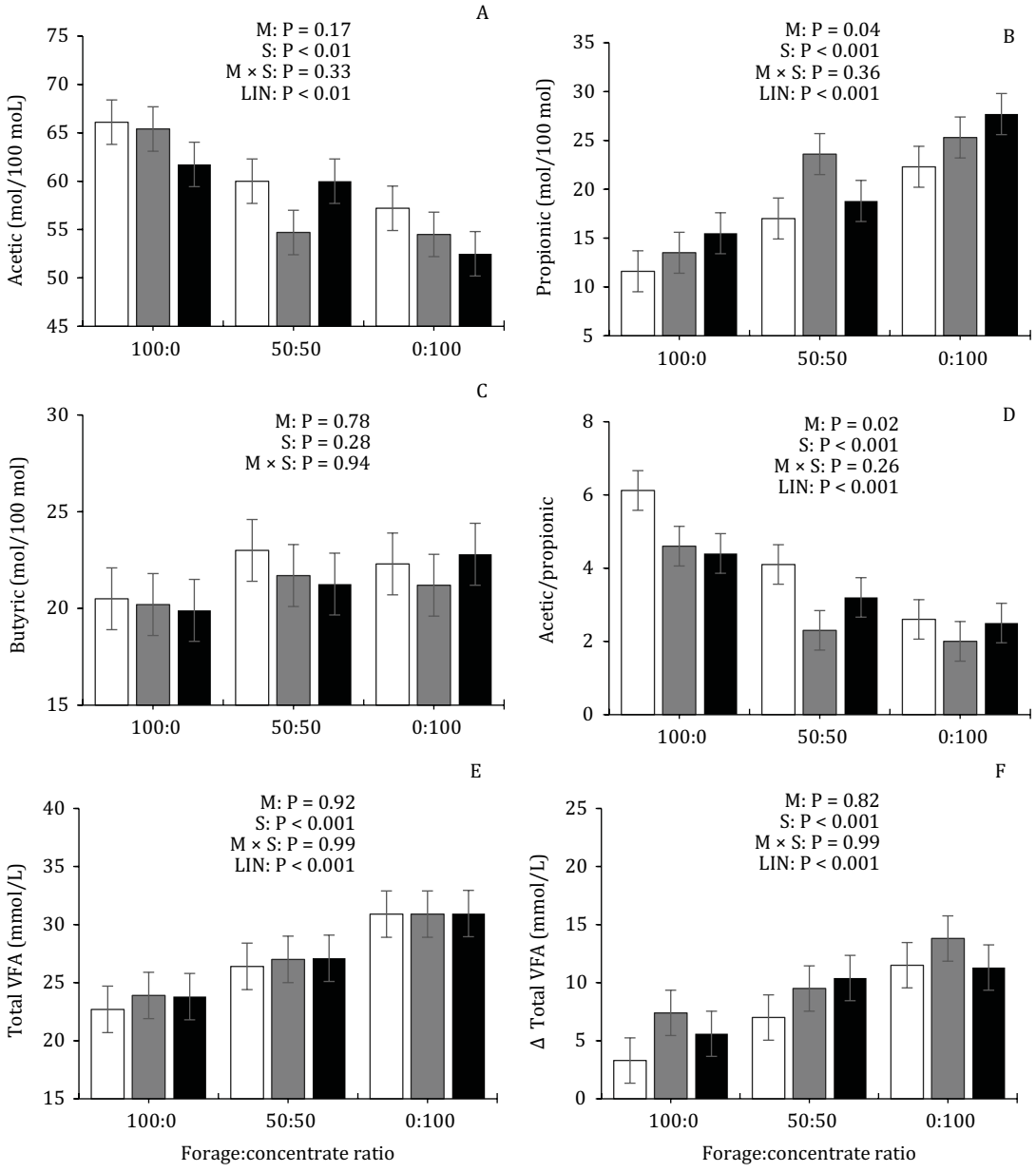
White, gray, and black bars correspond to samples that were incubated directly in the medium, within F57 and NWT bags, respectively. Error bars represent standard error of the mean. LIN represents linear effect of forage:concentrate ratios.

Figure 4 - Effects of substrate dispersion method and substrate with different forage:concentrate ratios on kinetic variables of gas production.

variables for second pool (slow degradation) depressed linearly (P<0.01) with each reduction in forage:concentrate ratio (i.e., increase of substrate fermentability). No effects were observed on the lag time (P≥0.43), averaging 2.37 h. Additionally, only specific rates of digestion for first pool differed between methods (P<0.01). The DIS had greatest specific rates of digestion for first pool than that into bags (0.035 vs. 0.024 h^{-1}; P≤0.02), but F57 was similar to NWT (P = 0.14).

3.4. Volatile fatty acids

There were no interactions between method × substrate (P>0.26) on molar proportions of individual VFA (i.e., acetic, propionic, and butyric), acetic:propionic ratio, total VFA, and Δ total VFA (total VFA post-incubation – total VFA pre-incubation; Figure 5, panel A–E). Dispersion methods had effects on molar proportion of propionic acid and acetic:propionic ratio (P≤0.04). The propionic acid

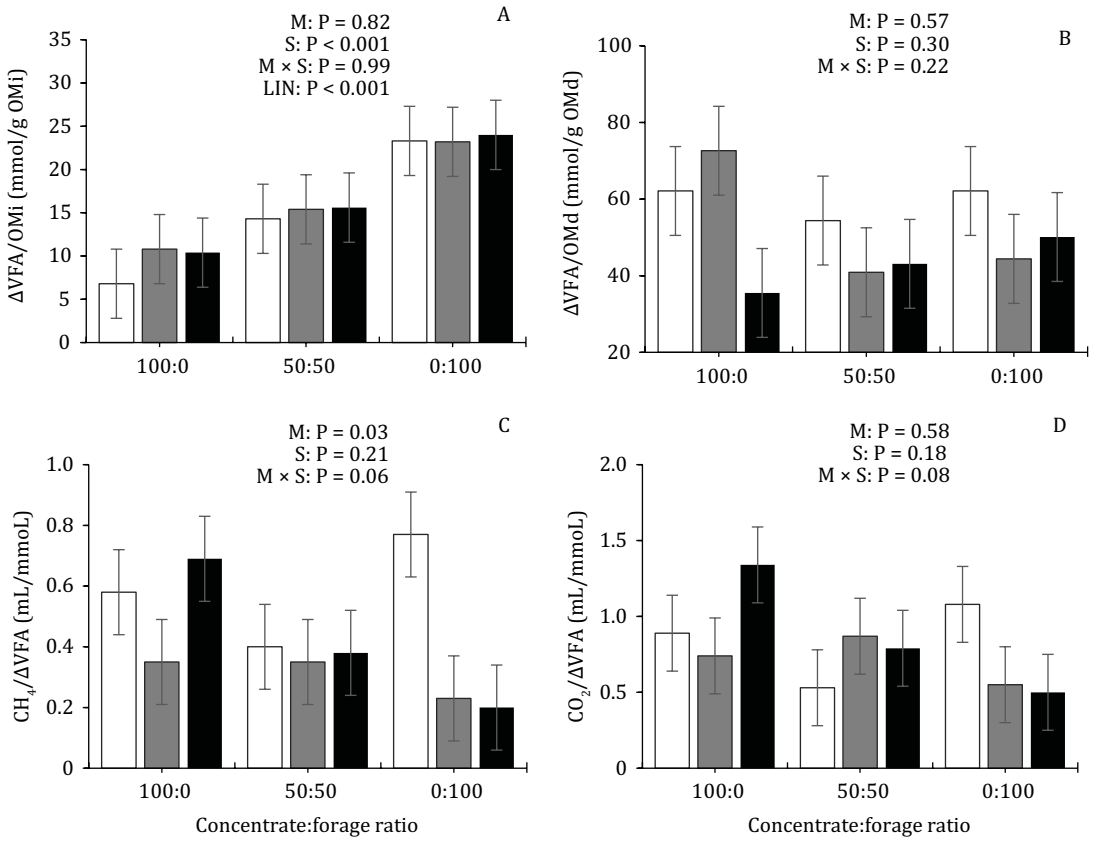


Δ Total VFA = change of total VFA concentration in the medium.
White, gray, and black bars correspond to samples that were incubated directly in the medium, within F57 and NWT bags, respectively. Error bars represent standard error of the mean. LIN represents linear effect of forage:concentrate ratios.

Figure 5 - Effects of substrate dispersion method and substrate with different forage:concentrate ratios on individual volatile fatty acid (VFA) proportions and total VFA concentration in fermentation medium.

(mol/100 mol) was greater (P≤0.03) when substrates were incubated within bags than in DIS (8.11 vs. 7.25 mol/100 mol), but no differences were found for F57 and NWT (P = 0.92). In an opposite way, acetic:propionic acid ratio showed lower values (P≤0.03) when substrates were incubated into bags than in DIS (3.20 vs. 4.21), but without differences between F57 and NWT (P = 0.57). Substrate affected (P<0.01) molar proportion of acetic and propionic acids, acetic:propionic acid ratios, total VFA, and ΔVFA in the medium. Acetic molar proportion and acetic:propionic ratio decreased linearly (P<0.01), while molar proportion of propionic acid, total VFA, and ΔVFA increased linearly (P<0.001) with each decrease in forage:concentrate ratio.

There were no interaction effects (P≥0.22) between method and substrate on ΔVFA expressed in relation to OM incubated or digested, as well as on CH_4 and CO_2 in relation to ΔVFA (P≥0.06) (Figure 6). The ΔVFA expressed in relation to OM incubated was substrate dependent (P<0.001), wherein decreasing the forage:concentrate ratio resulted in a positive linear response (P<0.001). The CH_4/ΔVFA was affected by the method (P = 0.03). The NWT presented similar CH_4/ΔVFA to DIS (P = 0.13), which was equal to F57 (P = 0.22).



ΔVFA/OMi = apparent total volatile fatty acids (VFA) output per g organic matter (OM) incubated; ΔVFA/OMd = apparent total VFA output per g OM digested; CH_4/ΔVFA = methane output/unit of total VFA output; and CO_2/ΔVFA = carbon dioxide output/unit of total VFA output.
White, gray, and black bars correspond to samples that were incubated directly in the medium, within F57 and NWT bags, respectively. Error bars represent standard error of the mean. LIN represents linear effect of forage:concentrate ratios.

Figure 6 - Effects of substrate dispersion method and substrate with different forage:concentrate ratios on kinetic variables of gas production.

3.5. Correlations between substrates directly dispersed and substrates within bags

The GP48, IVDMD, IVOMD, PF, molar proportion of propionic acid, acetic:propionic acid ratio, CH_4/OMd, and CO_2/OMd evaluated in DIS method were positively correlated (P<0.05), but molar proportion of butyric acid, ΔVFA, ΔVFA/OMi, CH_4 and CO_2 proportions, CH_4/ΔVFA, and CO_2/ΔVFA were not correlated (P>0.05) with these variables observed in either F57 and NWT (Table 1). The

Table 1 - Correlations between *in vitro* fermentation characteristics for substrates incubated directly dispersed in the medium and within filter bags

Item	F57	NWT
GP48	0.92***	0.90***
IVDMD	0.97***	0.98***
IVOMD	0.98***	0.99***
PF	0.92***	0.85***
pH	0.92***	0.52NS
Acetic acid	0.61*	0.34NS
Propionic acid	0.81**	0.85**
Butyric acid	0.30NS	–0.11NS
Acetic:propionic acid	0.87***	0.71*
ΔVFA	0.35NS	0.45NS
ΔVFA:OMi	0.35NS	0.45NS
ΔVFA:OMd	0.93***	–0.17NS
CH_4	0.58NS	0.32NS
CO_2	0.65NS	0.64NS
CH_4/DMi	0.51NS	0.78*
CO_2/DMi	0.45NS	0.79**
CH_4/OMd	0.73*	0.92**
CO_2/OMd	0.71*	0.84*
CH_4/ΔVFA	0.26NS	0.20NS
CO_2/ΔVFA	0.32NS	0.33NS

F57 - Ankom® filter bag; NWT - non-woven fabric; GP48 - gas production at 48 h; IVDMD - *in vitro* dry matter digestibility; IVOMD - *in vitro* organic matter digestibility; PF - partitioning factor; ΔVFA - total volatile fatty acids; OMi - organic matter incubated; OMd - organic matter digested; DMi - dry matter incubated; NS - not significant.
* P<0.05; ** P<0.01; *** P<0.001.

pH, acetic molar proportion, and ΔVFA/OMd for DIS were positively correlated (P<0.05) with these variables evaluated within F57 bags. Nevertheless, these latter variables were not correlated (P>0.05) when substrates were incubated within NWT bags, while CH_4/DMi, and CO_2/DMi were positively correlated with those evaluated by DIS.

4. Discussion

4.1. General perspective

Our objective was to examine the dispersion method effects in an *in vitro* gas production assay on estimates of enteric greenhouse gas emissions (CO_2 and CH_4), digestibility, gas production kinetic variables, and VFA profile from substrates with different fermentabilities (low, average, and high; i.e., 100F, mixture, and 100C, respectively). Traditionally, substrates in an *in vitro* rumen incubation are directly dispersed in the medium. In this current study, different textiles were utilized to make simultaneous determination of *in vitro* digestibility easier by avoiding transfers of sample residues after incubation when direct dispersion in the medium is used. Different forage:concentrate ratios and different dispersion methods were compared to identify differences in CO_2 and CH_4 output among them. Effects of these factors on gas production, degradability, greenhouse gas yield, and other fermentation characteristics were studied using three forage:concentrate ratio that varied widely in fermentability at 48 h of incubation (GP: 101 to 262 mL/g DMi; IVDMD: 183 to 899 g/kg; CH_4 yield: 9.2 to 42.9 mL/g OM digested; CO_2 yield: 22.9 to 67.0 mL/g OM digested; change of total VFA/g OM digested: 34.1 to 72.6 mmol/g).

After 48 h of fermentation, the measured GP of the various evaluated substrates ranged from 108 to 226 mL/g DM, suggesting that a steer consuming 10 kg/d DM might produce between 1,080 and 2,260 L/d of gas. The CH_4 and CO_2 yield from fermentation of these substrates ranged from 6.0 to 13.7 mL/g DM and 10.6 to 24.9 mL/g DM, respectively, suggesting that for a DM intake of 10 kg/d, a steer may produce 60 to 137 L/d of CH_4 and 106 to 249 L/d of CO_2.

4.2. Effects of methods

Incubating substrates within filter bags has been widely applied *in vitro* (Eun and Beauchemin, 2007; Krizsan et al., 2012; Tagliapietra et al., 2012; He et al., 2013; Ramin et al., 2013) and compared to dispersing the substrates into the medium. This approach has the advantage of being able to simultaneously determine *in vitro* digestibility without the need to capture residues after incubation. In addition, the filter bag system was found to be more advantageous than the conventional system, which uses crucibles, owing to its lower labor requirement and cost (Cherney, 2000), also avoiding losses of residues during the filtration process. However, Tagliapietra et al. (2012) reported lower IVDMD, while He et al. (2016) found greater IVDMD when feedstuffs were incubated in filter bags. In this sense, GP could be associated with these respective changes, as demonstrated by the similar results at this present study (Figures 1A–D).

The poor exchange of the fluid inside the bag with the medium promotes VFA accumulation, which may decrease the pH (Marinucci et al., 1992) and negatively affect the fibrolytic enzyme activities of microorganisms attached to substrate particles within the filter bags in an *in vitro* bottle (Krizsan et al., 2012). In fact, independent of the substrate type incubated, it resulted in an altered fermentation within bags with greater propionic acid produced and lower acetic:propionic ratio. Consequently, although overall pH and total VFA production of the medium at 48 h of incubation were not different, probably there was an inhibitory level of pH inside the bag associated with the physical constraint that could hinder the exchange of microorganisms, nutrients, buffer, and fermentation end-products through the small pores of the bags (Krizsan et al., 2012). These associated effects would explain the lower specific rate of digestion, lesser numeric gas volume of fast pool, and CH_4/ΔVFA verified for F57 and NWT bags compared with substrates dispersed in the *in vitro* medium in this study.

In general, substrates incubation within bags resulted in lower proportion of CO_2 and CH_4 in the fermentation gas, and yield relative to DMi or OMd at 48 h of incubation, but not relative to ΔVFA. The CO_2 and CH_4 emissions are calculated by multiplying GP by the respective gas proportion, and the division by incubated DM and digested OM only corrects for the incubated and fermentable amount of the substrate, respectively. In this sense, numerator variables such as GP (184 vs. 159 mL), CH_4 proportion (84 vs. 44 mL/L), and CO_2 proportion (118 vs. 98 mL/L at 48 h) were significantly greater when the substrates were dispersed into the medium than within bags. Nevertheless, denominator variables (DMi and OMd, which were 0.503 vs 0.505 g and 0.363 vs. 0.260 g, respectively) showed small difference, resulting in lower enteric greenhouse gas yield relative to DMi and OMd for the incubation within bags.

4.3. Effect of substrates

As expected, gas production, *in vitro* digestibilities, as well as enteric CO_2 and CH_4 proportion and production (mL/g DMi) increased linearly with the decreased proportion of F:C ratio of the substrates. On the other hand, pH, enteric CO_2 and CH_4 yield relative to OMd, and acetic:propionic ratio linearly decreased with the increase of F:C ratio. These results verified in CH_4 output agree with findings from *in vitro* studies comparing different substrates (Yan et al., 2000; Klevenhusen et al., 2008; Navarro-Villa et al., 2011). In general, there was an increase in CH_4 yield relative to substrate incubated as the quantity of starch in the diet increased; such an effect can be largely explained by the increasing amount of fermentable substrate with greater quality feeds. Otherwise, when the output is expressed on disappeared basis (i.e., DM or OM digested), CH_4 yield decreased as the quantity of fermentable substrate in the diet increased.

4.4. Effects of methods and substrates interactions

The incubation of low-fermentable substrate (100F) within filter bags showed differences for GP48 in *in vitro* digestibilities and PF, in which NWT presented higher values than when the substrates

were dispersed in bottles and within F57. We observed in dispersed method that some feed particles of the forage were floating on the top of *in vitro* medium and adhered to the side of the bottles. Consequently, this substrate would not come in direct contact with microorganisms, thus, negatively affecting digestibility and GP. Similar effects were described by He et al. (2016). The NWT and F57 bags were kept under the surface of the buffered rumen fluid, but only the first bag had greater digestibility, GP48, and PF (calculated from both variables) compared with the dispersed method. In that case, greater estimates only in NWT when 100F substrate was incubated was unexpected, because GP and digestibilities were not changed or lower for NWT compared with DIS when the other substrates were incubated.

The incubation of mixture and 100C (average- to high-fermentable substrate) into filter bags consistently decreased GP, IVDMD, and IVOMD as compared with direct dispersion in the medium, although NWT had greater values than F57 (Figures 1A–D). The small pore size of the bags could constrain the entry of some ruminal protozoa, resulting in reduced starch degradability (Mendoza et al., 2014). This lower feed digestibility may arise from the inability of microbes to readily gain access to substrates within the bags, lowering digestion (Krizsan et al., 2012). According to Valente et al. (2011), superficial structures of F57 and NWT are similar and both present irregular geometrical arrangements, being formed by deposition of synthetic fiber without weaving. From this physical consideration, it could be inferred that their porosities could affect mainly the inflow of microorganisms and outflow of fermentation products. Very low porosities can compromise the withdrawal of fermentation gases from inside the bags, which can restrict microbial access to the substrate (Udén and Van Soest, 1984). In fact, independent of substrate type, CO_2 and CH_4 yield relative to OMd (Figures 3 C and F) or ΔVFA at 48 h (Figures 6 C and D) were lower when feeds were incubated within bags than dispersed in the medium. However, NWT presented greater GP48 and digestibilities than F57, showing that there could be differences between fermentation within these different textiles.

Methane yield relative to OM digested (CH_4/OMd) is the preferred unit for expressing *in vitro* CH_4 output (Navarro-Villa et al., 2011; Yáñez-Ruiz et al., 2016). In this sense, we may also consider CO_2/OMd the better unit to express CO_2 output, as it is related with the amount of substrate degraded. The linear decrease in CO_2 and CH_4 yield, according to improvement in substrate fermentability, appeared to be associated with greater acetate:propionate ratio, although the extent of fermentation measured by ΔVFA/OMd was not affected. The CH_4 yield relative to OMi was greater when the substrates with average or high fermentation (mixture and 100C, respectively) were incubated directly dispersed in the medium compared with when they were in filter bags. Ramin et al. (2013) reported that the proportion of CH_4 (mL/L) was greater for substrates incubated within filter bags compared with feeds directly dispersed in the medium. On the other hand, in this study we observed a general lower CH_4 and CO_2 proportion to total gas at 24 and 48 h of incubation and positive correlation (r = 0.71 to 0.92) between enteric CH_4 and CO_2 yield relative OMd from substrates incubated within filter bags versus directly dispersed in the *in vitro* medium (Table 1). Ramin et al. (2013) attributed the greater CH_4 proportion to the difference in the extent of fermentation and fermentation pattern compared with the dispersion on the *in vitro* medium reflecting a biased measurement. Based on the weak relationship between enteric CH_4 (mL/g of DM) from substrates incubated in F57 bags versus directly dispersed in the medium, these authors concluded that it is not a relevant filter bag for ranking feedstuffs.

Several factors, including source and collection time of inoculum, inoculum size and preparation, apparatus design, incubation length, headspace gas pressure, and medium composition including buffer components, trace elements, nitrogen concentrations, and reducing agents (Cone et al., 1996; Rymer et al., 2005), have been evaluated for their effect on *in vitro* gas production. One of the main differences between this present study and that conducted by Ramin et al. (2013) could be the headspace gas composition, which was CO_2 in that study and N_2 in our experiment. In addition, we include the evaluation of NWT bags that has been used with success in some *in vitro* ruminal fermentation systems to evaluate digestibilities (Silva et al., 2017; Camacho et al., 2019).

A recent study reported that greater CO_2 concentration in the headspace would result in a greater dissolved CO_2 concentration in the media (Patra and Yu, 2013). Consequently, there is a greater CH_4 production corresponding to the CO_2 headspace. This might be attributed to the immediate and increased availability of CO_2 in the culture media as acceptor and H_2 (or formate) as electron donor for the methanogenesis pathway, associated with the increase in growth and activity of methanogen microorganisms. Furthermore, although CH_4 production was greater for CO_2 headspace than for N_2 headspace, total GP was lower for CO_2 than for N_2. These results could partially explain the differences found in this present study compared with Ramin et al. (2013). However, more studies are necessary to confirm this hypothesis, because we did not evaluate gas composition in headspace.

As CH_4 output generally decreases with the increasing amount of fermentable substrate found in higher-quality feeds (Klevenhusen et al., 2008; Navarro-Villa et al., 2011), we expected it when evaluating the three different substrates (i.e., 100F > mixture > 100C). Considering the enteric greenhouse yields relative to OMd for DIS method (24 and 48 h of incubation), they were greater for 100F than for mixture and 100C substrates, while these last two were similar. The enteric greenhouse yields relative to OMd evaluated in filter bags were positively correlated with those estimated at DIS method. However, when substrates were incubated within bags, the enteric greenhouse yields for the different substrates were similar at 24 h, but the results observed for DIS at 48 h of incubation was equal only to F57 (i.e., 100F > mixture = 100C substrate). However, CO_2 and CH_4 yield were, on average, 27 and 54% lower when substrates were incubated in F57 bags than in DIS. In this sense, we can infer that filter bags utilization in an *in vitro* system to rank CO_2 and CH_4 yield from feeds with different fermentabilities may be biased.

Considering that the feeds directly dispersed in the medium is the standard method of *in vitro* evaluation, we compared the relationships obtained with DIS with those obtained with substrates incubated within filter bags. In this way, considering the correlation coefficients, incubation into NWT bags showed more altered *in vitro* fermentation characteristics than that incubated within F57 bags. This can be justified by the absence of correlation for pH, acetic molar proportion, and ΔVFA/OMd between DIS and NWT methods (Table 1). The VFA profile and pH are important regarding the availability of H_2 for greenhouse production (Argyle and Baldwin, 1988; Bannink et al., 2006; Lana et al., 1998; Murphy et al., 1982). The greater propionic acid and lower acetic molar proportions are associated with lower H_2 production and thus lower CH_4 output (Demeyer and Van Nevel, 1975). However, absence of correlation between NWT and DIS methods for pH, acetic molar proportion, and ΔVFA/OMd suggests that the quantity of VFA produced from evaluated substrates with these methods, and thus the extent of fermentation, had no effect on CO_2 and CH_4 yield. This is supported by the positive correlation found in CO_2 and CH_4 yield (g/DMi and g/OMd), while ΔVFA/OMd was not correlated between DIS and NWT.

5. Conclusions

When substrates were incubated within filter bags instead of being directly dispersed in the medium, gas production, greenhouse gas emissions, and digestibility were all reduced. This suggests a reduction in substrate accessibility by the microorganisms, as well as a likely alteration in the microbial population and/or fermentation profile within the samples enclosed in filter bags. The noteworthy interaction between the incubation method and substrates indicates that the ranking of these variables for substrates with differing fermentabilities changes due to the dispersion method employed.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Sampaio, F. C.; Detmann, E. and Batista, E. D. **Data curation:** Souza, J. M. S. and Soares, L. C. B. **Formal analysis:** Sampaio, F. C. and Tomich, T. R. **Funding acquisition:** Batista, E. D. **Investigation:** Sampaio, F. C.; Souza, J. M. S. and Soares, L. C. B. **Methodology:** Sampaio, F. C.; Oliveira, A. S.; Detmann, E. and Batista, E. D. **Project administration:** Batista, E. D. **Supervision:** Pereira, D. H. and Batista, E. D. **Visualization:** Oliveira, A. S.; Pereira, D. H.; Detmann, E. and Souza, J. M. C. **Writing – original draft:** Sampaio, F. C.; Souza, J. M. S.; Soares, L. C. B.; Oliveira, A. S.; Pereira, D. H.; Detmann, E.; Tomich, T. R. and Batista, E. D. **Writing – review & editing:** Oliveira, A. S.; Detmann, E.; Souza, J. M. C. and Batista, E. D.

Acknowledgments

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant #430813/2018-0) for its financial support. We also thank Fortuna Nutrição Animal (Nova Canaã do Norte, Mato Grosso, Brazil) for support.

References

Argyle, J. L. and Baldwin, R. L. 1988. Modeling of rumen water kinetics and effects of rumen pH changes. Journal of Dairy Science 71:1178-1188. https://doi.org/10.3168/jds.S0022-0302(88)79672-1

Bannink, A.; Kogut, J.; Dijkstra, J.; France, J.; Kebreab, E.; Van Vuuren, A. M. and Tamminga, S. 2006. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. Journal of Theoretical Biology 238:36-51. https://doi.org/10.1016/j.jtbi.2005.05.026

Benedeti, P. D. B.; Fonseca, M. A.; Shenkoru, T.; Marcondes, M. I.; Paula, E. M.; Silva, L. G. and Faciola, A. P. 2018. Does partial replacement of corn with glycerin in beef cattle diets affect *in vitro* ruminal fermentation, gas production kinetic, and enteric greenhouse gas emissions? PLOS One 13:e0199577. https://doi.org/10.1371/journal.pone.0199577

Blümmel, M. and Becker, K. 1997a. The degradability characteristics of fifty-four roughages and roughage neutral-detergent fibres as described by *in vitro* gas production and their relationship to voluntary feed intake. British Journal of Nutrition 77:757-768.

Blümmel, M.; Makkar, H. P. S. and Becker, K. 1997b. *In vitro* gas production: A technique revisited. Journal of Animal Physiology and Animal Nutrition 77:24-34. https://doi.org/10.1111/j.1439-0396.1997.tb00734.x

Bodas, R.; López, S.; Fernández, M.; García-González, R.; Rodríguez, A. B.; Wallace, R. J. and González, J. S. 2008. *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. Animal Feed Science and Technology 145:245-258. https://doi.org/10.1016/j.anifeedsci.2007.04.015

Camacho, L. F.; Silva, T. E.; Palma, M. N. N.; Assunção, A. S.; Rodrigues, J. P.; Costa e Silva, L. F. and Detmann, E. 2019. Evaluation of buffer solutions and urea addition for estimating the *in vitro* digestibility of feeds. Journal of Animal Science 97:922-931. https://doi.org/10.1093/jas/sky464

Carreño, D.; Hervás, G.; Toral, P. G.; Belenguer, A. and Frutos, P. 2015. Ability of different types and doses of tannin extracts to modulate *in vitro* ruminal biohydrogenation in sheep. Animal Feed Science and Technology 202:42-51. https://doi.org/10.1016/j.anifeedsci.2015.02.003

Casali, A. O.; Detmann, E.; Valadares Filho, S. C.; Pereira, J. C.; Cunha, M.; Detmann, K. S. C. and Paulino, M. F. 2009. Estimação de teores de componentes fibrosos em alimentos para ruminantes em sacos de diferentes tecidos. Revista Brasileira de Zootecnia 38:130-138. https://doi.org/10.1590/S1516-35982009000100017

Cattani, M.; Maccarana, L.; Rossi, G.; Tagliapietra, F.; Schiavon, S. and Bailoni, L. 2016. Dose-response and inclusion effects of pure natural extracts and synthetic compounds on *in vitro* methane production. Animal Feed Science and Technology 218:100-109. https://doi.org/10.1016/j.anifeedsci.2016.05.014

Cherney, D. J. R. 2000. Characterization of forages by chemical analysis. p.281-300. In: Forage evaluation in ruminant nutrition. Givens, D. I.; Owen, E.; Axford, R. F. E. and Omed, H. M., eds. CAB International, Oxon, UK.

Cone, J. W.; van Gelder, A. H.; Visscher, G. J. W. and Oudshoorn, L. 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Animal Feed Science and Technology 61:113-128. https://doi.org/10.1016/0377-8401(96)00950-9

Demeyer, D. I. and Van Nevel, C. J. 1975. Methanogenesis: an integrated part of carbohydrate fermentation and its control. p.366-382. In: Digestion and metabolism in the ruminant. McDonald, I. W. and Warner, A. C. I., eds. The University of New England Publishing Unit, Armidale, Australia.

Detmann, E.; Valadares Filho, S. C.; Berchielli, T. T.; Cabral, L. S.; Ladeira, M. M.; Souza, M. A.; Queiroz, A. C.; Saliba, E. O. S.; Pina, D. S. and Azevedo, J. A. G. 2012. Métodos para análise de alimentos - INCT Ciência Animal. Suprema, Visconde do Rio Branco.

Eun, J. S. and Beauchemin, K. A. 2007. Enhancing *in vitro* degradation of alfalfa hay and corn silage using feed enzymes. Journal of Dairy Science 90:2839-2851. https://doi.org/10.3168/jds.2006-820

He, Z.; Ding, S.; Xu, L.; Beauchemin, K. A. and Yang, W. 2013. Using exogenous enzymes to increase the rumen degradability of wheat dried distillers grains with solubles. Archives of Animal Nutrition 67:381-392. https://doi.org/10.1080/1745039X.2013.840415

He, Z. X.; Zhao, Y. L.; McAllister, T. A. and Yang, W. Z. 2016. Effect of *in vitro* techniques and exogenous feed enzymes on feed digestion. Animal Feed Science and Technology 213:148-152. https://doi.org/10.1016/j.anifeedsci.2016.01.004

Klevenhusen, F.; Bernasconi, S. M.; Kreuzer, M. and Soliva, C. R. 2008. The methanogenic potential and C-isotope fractionation of different diet types represented by either C_3 or C_4 plants as evaluated *in vitro* and in dairy cows. Australian Journal of Experimental Agriculture 48:119-123. https://doi.org/10.1071/EA07240

Krizsan, S. J.; Jančík, F.; Ramin, M. and Huhtanen, P. 2012. Comparison of some aspects of the *in situ* and *in vitro* methods in evaluation of neutral detergent fiber digestion. Journal of Animal Science 91:838-847. https://doi.org/10.2527/jas.2012-5343

Lana, R. P.; Russell, J. B. and Van Amburgh, M. E. 1998. The role of pH in regulating ruminal methane and ammonia production. Journal of Animal Science 76:2190-2196. https://doi.org/10.2527/1998.7682190x

Maccarana, L.; Cattani, M.; Tagliapietra, F.; Bailoni, L. and Schiavon, S. 2016. Influence of main dietary chemical constituents on the *in vitro* gas and methane production in diets for dairy cows. Journal of Animal Science and Biotechnology 7:54. https://doi.org/10.1186/s40104-016-0109-5

Marinucci, M. T.; Dehority, B. A. and Loerch, S. C. 1992. *In vitro* and *in vivo* studies of factors affecting digestion of feeds in synthetic fiber bags. Journal of Animal Science 70:296-307. https://doi.org/10.2527/1992.701296x

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal 43:99-109. https://doi.org/10.1042/bj0430099

Mendoza, G. D.; Loera-Corral, O.; Plata-Pérez, F. X.; Hernández-García, P. A. and Ramírez-Mella, M. 2014. Considerations on the use of exogenous fibrolytic enzymes to improve forage utilization. The Scientific World Journal 2014:247437. https://doi.org/10.1155/2014/247437

Murphy, M. R.; Baldwin, R. L. and Koong, L. J. 1982. Estimation of stoichiometric parameters for rumen fermentation of roughage and concentrate diets. Journal of Animal Science 55:411-421. https://doi.org/10.2527/jas1982.552411x

Navarro-Villa, A.; O'Brien, M.; López, S.; Boland, T. M. and O'Kiely, P. 2011. Modifications of a gas production technique for assessing *in vitro* rumen methane production from feedstuffs. Animal Feed Science and Technology 166-167:163-174. https://doi.org/10.1016/j.anifeedsci.2011.04.064

Nocek, J. E. 1988. In situ and other methods to estimate ruminal protein and energy digestibility: a review. Journal of Dairy Science 71:2051-2069. https://doi.org/10.3168/jds.s0022-0302(88)79781-7

Patra, A. K.; Kamra, D. N. and Agarwal, N. 2010. Effects of extracts of spices on rumen methanogenesis, enzyme activities and fermentation of feeds *in vitro*. Journal of the Science of Food and Agriculture 90:511-520. https://doi.org/10.1002/jsfa.3849

Patra, A. K. and Yu, Z. 2012. Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. Applied and Environmental Microbiology 78:4271-4280. https://doi.org/10.1128/AEM.00309-12

Patra, A. K. and Yu, Z. 2013. Effects of gas composition in headspace and bicarbonate concentrations in media on gas and methane production, degradability, and rumen fermentation using *in vitro* gas production techniques. Journal of Dairy Science 96:4592-4600. https://doi.org/10.3168/jds.2013-6606

Ramin, M. and Huhtanen, P. 2012. Development of an *in vitro* method for determination of methane production kinetics using a fully automated *in vitro* gas system - A modelling approach. Animal Feed Science and Technology 174:190-200. https://doi.org/10.1016/j.anifeedsci.2012.03.008

Ramin, M.; Krizsan, S. J.; Jančík, F. and Huhtanen, P. 2013. Short communication: Measurements of methane emissions from feed samples in filter bags or dispersed in the medium in an *in vitro* gas production system. Journal of Dairy Science 96:4323-4332. https://doi.org/10.3168/jds.2013-6556

Rymer, C.; Huntington, J. A.; Williams, B. A. and Givens, D. I. 2005. *In vitro* cumulative gas production techniques: History, methodological considerations and challenges. Animal Feed Science and Technology 123-124:9-30. https://doi.org/10.1016/j.anifeedsci.2005.04.055

Schofield, P.; Pitt, R. E. and Pell, A. N. 1994. Kinetics of fiber digestion from *in vitro* gas production. Journal of Animal Science 72:2980-2991. https://doi.org/10.2527/1994.72112980x

Silva, T. E.; Detmann, E.; Camacho, L. F.; Saliba, E. O. S.; Palma, M. N. N. and Valadares Filho, S. C. 2017. Comparação de métodos *in vitro* para a quantificação da digestibilidade da matéria seca e da fibra em detergente neutro de forragens e concentrados. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 69:1635-1644. https://doi.org/10.1590/1678-4162-9096

Tagliapietra, F.; Cattani, M.; Hindrichsen, I. K.; Hansen, H. H.; Colombini, S.; Bailoni, L. and Schiavon, S. 2012. True dry matter digestibility of feeds evaluated *in situ* with different bags and *in vitro* using rumen fluid collected from intact donor cows. Animal Production Science 52:338-346. https://doi.org/10.1071/AN11206

Tilley, J. M. A. and Terry, R. A. 1963. A two-stage technique for the *in vitro* digestion of forage crops. Grass and Forage Science 18:104-111. https://doi.org/10.1111/j.1365-2494.1963.tb00335.x

Udén, P. and Van Soest, P. J. 1984. Investigations of the *in situ* bag technique and a comparison of the fermentation in heifers, sheep, ponies and rabbits. Journal of Animal Science 58:213-221. https://doi.org/10.2527/jas1984.581213x

Valente, T. N. P.; Detmann, E.; Queiroz, A. C.; Valadares Filho, S. C.; Gomes, D. I. and Figueiras, J. F. 2011. Evaluation of ruminal degradation profiles of forages using bags made from different textiles. Revista Brasileira de Zootecnia 40:2565-2573. https://doi.org/10.1590/S1516-35982011001100039

Valente, T. N. P.; Detmann, E. and Sampaio, C. B. 2015. Review: Recent advances in evaluation of bags made from different textiles used *in situ* ruminal degradation. Canadian Journal of Animal Science 95:493-498. https://doi.org/10.4141/cjas-2015-100

Yáñez-Ruiz, D. R.; Bannink, A.; Dijkstra, J.; Kebreab, E.; Morgavi, D. P.; O'Kiely, P.; Reynolds, C. K.; Schwarm, A.; Shingfield, K. J.; Yu, Z. and Hristov, A. N. 2016. Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants - a review. Animal Feed Science and Technology 216:1-18. https://doi.org/10.1016/j.anifeedsci.2016.03.016

Yan, T.; Agnew, R. E.; Gordon, F. J. and Porter, M. G. 2000. Prediction of methane energy output in dairy and beef cattle offered grass silage-based diets. Livestock Production Science 64:253-263. https://doi.org/10.1016/S0301-6226(99)00145-1