Ciência Rural, Santa Maria, v.50:8, e20190486, 2020 http://doi.org/10.1590/0103-8478cr20190486

ISSNe 1678-4596
ANIMAL PRODUCTION

Pre-incubation of ruminal inocula to assess *in vitro* gas production and digestibility

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ABSTRACT: *In vitro gas production techniques represent a valuable tool to describe the kinetics of ruminal degradation of food. However, the ruminal liquor used as a microbial inoculum has been a great source of variation and error. A standardization of this factor should contribute to assure the independence of food fermentation parameters from those of the inocula. In this research it was hypothesized that a controlled pre-incubation treatment of ruminal liquor could contribute to stabilize and homogenize the undigested residues of blanks and as a consequence, of the production of residual cumulative gas production (CGP). A pre-incubation (i.e. previous real incubation) of rumen inocula was developed with a simple substrate similar to the diet offered to donors at 1% w/v for 0, 1, 2 and 4 h (Control, Prei-1, Prei-2 and Prei-4 treatments respectively). Once the pre-incubation hours were completed, they were incubated with contrasting substrates and without substrate (i.e. blanks) in order to evaluate the CGP, in vitro digestibility of the DM and fermentation products. Although, the fermentative activity of the pre-incubated inoculums worked satisfactorily in the in vitro system, contrary to what was speculated, residues of the pre-incubation increased the variability and heterogeneity of variances among blanks. Consequently, it was concluded that the pre-incubations did not work to generate more homogeneous and less variable ruminal liquor for the in vitro gas production system.*
Key words: *Inoculum preparation, rumen fluid, blanks variability.*

Pré-incubação de inóculo ruminal para avaliação da produção de gases e digestibilidade *in vitro*

RESUMO: *Técnicas de produção de gás in vitro representam uma ferramenta valiosa para descrever a cinética de degradação ruminal dos alimentos. No entanto, o líquido ruminal utilizado como inóculo microbiano tem sido uma grande fonte de variação e erro. A padronização deste fator deve contribuir para garantir a independência dos parâmetros de fermentação dos alimentos a partir dos inóculos. Neste trabalho, hipotetizou-se que um tratamento controlado de pré-incubação do líquido ruminal poderia contribuir para estabilizar e homogeneizar os resíduos não digeridos dos brancos e, como conseqüência, da produção de produção cumulativa de gás residual (CGP). Uma pré-incubação (ou seja, incubação real prévia) dos inóculos do rúmen foi desenvolvida com um substrato simples semelhante à dieta oferecida aos doadores a 1% p/v por 0, 1, 2 e 4 h (Controle, Prei-1, Pré- 2 e Prei-4 tratamentos respectivamente). Uma vez completadas as horas de pré-incubação, elas foram incubadas com substratos contrastantes e sem substrato (ou seja, brancos) para avaliar o CGP, a digestibilidade in vitro da MS e os produtos de fermentação. Embora a atividade fermentativa dos inóculos pré-incubados tenha funcionado satisfatoriamente no sistema in vitro, ao contrário do que foi especulado, os resíduos da pré-incubação aumentaram a variabilidade e heterogeneidade das variâncias entre os brancos. Consequentemente, concluiu-se que as pré-incubações não funcionaram para gerar um líquido ruminal mais homogêneo e menos variável para o sistema de produção de gás in vitro.*
Palavras-chave: *preparação do inóculo, fluído ruminal, variabilidade dos brancos.*

In vitro gas production techniques (ivGPT, MENKE & STEINGASS, 1988; THEODOROU et al., 1994) are widely employed to offer a repetitive, economical and easily applicable laboratory technique to estimate *in vitro* dry matter digestibility (ivDMD), based on the correspondence between dry matter degradability and cumulative gas production (CGP, MOULD et al., 2005). While these techniques are used and accepted worldwide, the inoculum has been considered the main source of variation (RYMER et al., 2005; WILLIAMS, 2000; YANG, 2017).

The husbandry conditions of experimental animals (RYMER et al., 2005) and their diet (BOGUHN et al., 2013) are parameters which require more control if we look for standard conditions in the system. Thus, the ideal rumen fluid should provide a diverse (i.e. capable of supplying representatives of the different microorganisms groups), healthy and active microbial starter capable of developing and imitating the rumen digestion process. Consequently, and in order to get comparable results (i.e. among laboratories and runs), blank bottles are run simultaneously (i.e. fermentation bottles with rumen fluid and buffer without substrate). Even though blanks have been analyzed in the past as correctors of CGP (ARAUJO

Received 06.28.19 Approved 04.25.20 Returned by the author 05.29.20
CR-2019-0486.R1

et al., 2011; CARRO et al., 2005), there is scarce information about the control of variability and the fermentative capacity thereof.

Throughout this paper it was hypothesized that a pre-incubation treatment (Pre-i) could contribute to the stabilization of the non-digested residues (responsible of the variability of blank bottles); and consequently, to the residual CGP. The objective of this study was to obtain a ruminal inoculum capable of performing *in vitro* incubations that could minimize the variability of blanks and improve reproducibility.

Rumen fluid (ca. liquid: solid fraction, 50: 50) from two male adult sheep with permanent rumen cannulas, fed on a standard diet (ca. alfalfa hay: corn grain, 70: 30), was collected in the morning (i.e. 12 h fasting). Pre-incubations were conducted into two liters dark plastic bottles which had a perforated cap to allow continuous CO_2 influx. The inocula were pre-incubated with carbonate-bicarbonate buffer (1:10 ratio, MENKE & STEINGASS, 1988) and a substrate (alfalfa hay: corn grain, 70:30, similar to the donors diet at 1% w/v, dried and milled= 1 mm) for 0, 1, 2 and 4 hours respectively (i.e. Control, Prei-1, Prei-2 and Prei-4), so that each treatment initial incubation time was different. Immediately before the incubation of bottles, pre-incubated inocula were strained through four layers of cheesecloth into a flask with O_2 free headspace and an aliquot was assessed for pH (Hanna® HI-9025) and ammonia N concentration ($N\text{-}NH_3$, Uremia Kit, Wiener®). Immediately after filtered, 4 ml of ruminal inocula were added with 38 ml of the buffer (1:10 ratio) to each bottle (100 ml of capacity), and were kept in a thermostatic bath at 39°C for 24 h. Each treatment was incubated in triplicate with two substrates (0.250 ± 0.025 g DM, dried and milled= 1 mm), a commercial dairy concentrate (CON) and alfalfa hay (Organic matter [OM]= 925 and 872, neutral detergent fiber with α-amylase [$aNDF_{OM}$]= 340 and 596, acid detergent fiber [ADF_{OM}]= 124 and 388, lignin= 32 and 113 and crude protein [CP]= 185 and 193 g/100 g DM, for CON and Hay respectively) and they were run together with five blanks per treatment (analytical replicates). *In vitro* CGP was performed according to THEODOROU et al. (1994). Pressure (T443A, Bailey and Mackey Ltd, UK) was measured at 1, 2, 4, 6, 8, 12, 16 and 24 h. The gross CGP (GCGP) was corrected by the CGP of blanks to generate net CGP (NCGP).

After 24 h, pH was measured in each bottle, aliquots were taken to assess volatile fatty acids (VFA; stabilized with 25% orthophosphoric acid; 1:5, acid: sample) and $N\text{-}NH_3$ (stabilized with 0.02 N sulfuric acid, 1:1, acid: sample) and the fermentation residues were filtered through fiber filter bags (ANKOM® #F57, ANKOM technology, NY, USA) to calculate the ivDMD after being treated with neutral detergent solution according to VAN SOEST et al. (1966) as follows,

$$ivDMD = (100 - aNDF_{OM}\ \text{residue}) \times 100 / (\text{DM incubated}) \quad (1)$$

Samples were prepared by drying (65ºC, 48h) and milling (1 mm; Willey mill), then they were analyzed for DM (105 °C during4 h), ash (AOAC, 1990, #942.05), CP (total N × 6.25, by Kjeldahl, AOAC, 1990, #976.05) with a Pro-Nitro® (Selecta J.P., Barcelona, Spain) and ether extract (Soxhlet apparatus with petroleum ether, AOAC, 1990, #920.39), $aNDF_{OM}$ and ADF_{OM} were reported ash-free according to VAN SOEST et al. (1991) with an ANKOM® equipment (Model 220, ANKOM™, NY, USA). Lignin content was obtained from sulfuric acid (AOAC, 1990, #973.18D). The VFA were analyzed with a gas chromatograph (Nukol capillary column [30 m × 0.32 mm i.d.× 0.25 µm film thickness]; Perkin Elmer - Elite FFAP; Part. N9316354. Carrier: Hydrogen. Column flow: 2.4 mL/min. Standar: volatile acid mix Supelco [Cat. No. 46975-U]).

A Complete Block Design (block≡ run) with a factorial arrangement (i.e. four treatments and two substrates) was used and represented by the following model:

$$Y_{ijkl} = \alpha_i + \beta_j + \gamma_k + (\alpha \times \gamma)_{ik} + \varepsilon_{ijkl}, \quad (2)$$

Where, Y_{ijk} is the measured parameter of the ijk treatment, α_i the effect of substrates (i= 2), β_J the effect of block or run (j= 3), γ_k the effect of treatment (k= 4), $(\alpha \times \gamma)_{ik}$ the interaction of substrate and treatment and ε_{ijkl} is the residual error term.

The CGP was adjusted to a non-linear model (i.e. $CGP=A + B \times (1 - e^{(-c\times t)})$) using the NLIN procedure of SAS (2002). Data of VFA, $N\text{-}NH_3$ and pH as well as CGP fitting parameters driven from different Pre-i were compared against the Control through Dunnett's test, through Proc Mixed of SAS (2002), with Kenward-Roger approximation. Differences were declared significant when $P<0.05$. Regression analysis was performed using the REG procedure (SAS, 2002) and correlations between the values with and without blank corrections through the Proc CORR (SAS, 2002). Differences among treatments variances in blanks were tested by Bartlett's test.

It is desirable that the CGP of blanks constitute a small proportion of the total gas produced

by substrates incubations, with low variability among replicates. In this study, it was reported that par A (fraction rapidly disappeared) of fermentation kinetic model in blanks was not different from zero for any treatment (SEM= 0.127; Table 1) and that replicates contributed to 33% of their total variability. However, PAYNE et al. (2002) reported that the collection of rumen fluid with 4 or 8 h fasting reduced CGP variability between runs compared with an inoculum extracted with a 12 h fasting (i.e. Control inoculum used here), generating greater reproducibility. The Pre-incubated inocula tested here were incubated at 1, 2 and 4 h after providing substrate to the bacterial population similar to the inocula obtained post-intake, but in a more controlled environment than the rumen itself. Moreover, initial inoculum characteristics were similar for pre-incubated rumen fluids of blanks (pH= 7.0, SEM= 0.13; P>0.05; $N\text{-}NH_3$= 38 mg/L; SEM= 3.1; P>0.05).

The CGP of blanks during the first hour of incubation (CGP_{t1}) did not show differences among treatments (P>0.05), being the total variation mostly described by the period and it was also shown that CGP_{t1} differed from 0 (Control and Prei-1, P<0.09; Prei-2 and Prei-4, P<0.05). Parameter A of the model accounted for less than 3% to the total gas produced in 24 h (A + B), but both model predictions as well as actual measurements increased sharply within the first hour of fermentation reaching to 19 to 37%; consequently, the rate of CGP (Par c) was high to accommodate to this fact. This fast increment in CGP should be as result of residual gas dissolved in the original inoculum (CORNOU et al., 2013) and fermentable leftovers escaping the filtering

Table 1 - Cumulative gas production and volatile fatty acids contents of rumen fluid pre-incubation treatments of blanks for 0, 1, 2 and 4 h (Control, Prei-1, Prei-2 and Prei-4 respectively).

Variable[a]	Treatments				SEM[b]	Sign[c]	Distribution of variability[d] (%)		Heterogeneity of variance[e]
	Control n=3	Prei-1 n=3	Prei-2 n=3	Prei-4 n=3			Run	Res	
Cumulative gas production; CGP = $A+B\times(1-e^{(-c\times time)})$									
A (ml)	0.19	0.05	0.02	0.33	0.127	ns	67	33	*
B (ml)	7.3a	16.2b	14.2a	11.9a	2.37	*	53	47	ns
c (h^{-1})	0.44	0.21	0.24	0.28	0.054	ns	73	27	*
Cumulative gas production (ml)									
t1	2.9	2.7	3.4	3.7	0.88	ns	79	21	*
t1-t24	4.7a	12.7b	10.7b	8.6a	1.53	*	56	44	ns
t24	7.9a	15.9b	14.0b	12.3a	2.22	*	68	32	ns
Volatile fatty acids (VFA, mmol/l)									
Totals	27.0a	43.6b	52.8b	52.2b	3.10	*	90	10	*
Acetic acid	14.9a	30.0b	27.8b	30.3b	2.52	*	79	21	ns
Propionic acid	5.3a	12.9b	13.3b	13.6b	1.67	*	98	2	*
Butiric acid	3.1	3.5	3.7	4.8	1.19	ns	57	43	*
Valeric acid	3.6	3.6	3.6	3.6	3.01	ns	99	1	*
Acetic/Propionic	3.2	2.4	2.5	2.3	0.54	ns	97	3	*
Ac+Pr/VFA totals (%)	78	87	85	84	8.9	ns	97	3	*

[a]Parameters: A: Fraction rapidly disappeared; B: Fraction disappeared at a constant fractional rate per unit time and c: Rate of B. t1, CGP to hour 1; t1-t24, CGP from hour 1 to hour 24; t24, CGP at 24 h. Acetic/Propionic, Rate between acetic and propionic acids; Ac+Pr/AGV totals, Proportion of acetic acid + propionic acid in total VFA concentration. [b] Standard error of the mean, n=3, correspond to 3 independent runs. [c] Sign, Significance; ns, non significative; *, P<0.05. Comparisons according to Dunnett (Control vs Prei-1, Prei-2 and Prei-4), different letters in the same line indicated P<0.05. [d] Run (i.e. variation among inocula); Res, Residual of the error (i.e. variation among replicates/bottle). [e] Assessed by Bartlett's test; ns, not significant (P>0.05); *, P<0.05.

process. Considering the small magnitude of these contributions, it is not surprising the heterogeneity of variances observed among treatments (Table 1). Furthermore, GIERUS et al. (2008) reported that the effect of runs increased the variability in CGP of corrected blanks, which would suggest that the pre-incubation of inocula does not imply a uniform CGP_{t1} or Par A. The CGP after the first hour of incubation (CGP_{t1-t24}) and during the complete incubation (CGP_{t24}) were significantly (P<0.05) affected by Prei-1 and Prei-2. Conversely, Par B (fraction disappeared at a constant fractional rate) showed an increment in the Prei-1, which could be explained by the increased microbial activity in pre-incubated treatments as a result of the extra substrate offered (LUTAKOME et al., 2017) even though the inocula maintained the fermentative characteristics and normal buffer capacity (ARAUJO et al., 2011). Likewise, for Par B a linear correspondence was reported with CGP_{t24} ($CGPt_{24}$ (ml) = 0.98 (SE= 0.018) × B + 0.27 (SE= 0.243), r^2= 0.98).

In order to generate trustable data there must exist a balance between inocula microbial activity and their reproducibility among different laboratories and times (CORNOU et al., 2013), in this sense CGP of blanks must be significantly different from zero (as an expresion of microbial activity), and should present low variability among replicates within incubation batchs while representing a low proportion of CGP of the assessed samples. In this sense, our preincubation treatments almost doubled Control CGP at 24 h (15.9, 14.0, 12.3 and 7.9, ml, for Prei-1, Prei-2, Prei-4 and Control) reflecting a media environment more favorable for microbial fermentation and reaching close to the minimum CGP for blanks recommended by NAGADI et al. (1999; i.e. 15.5 ml in 24 h).

The increase of VFA concentration on Pre-i in ca. 94% (P<0.05; Table 1) could been explained by acetic and propionic acids (84% of total VFA in all treatments; P>0.05). This increase was observed due to the incorporation of additional substrate in Pre-i inocula. Even though acetic + propionic acids increased in Pre-i, no statistical differences were reported in the proportion of these acids in totals VFA, as well as in acetic/propionic ratio, valeric and butyric acids concentration (P>0.05; Table 1). In general (except for butyric acid), replicates contributed scarcely to total variation (i.e. 1 to 21% of distribution of variability). For all VFA (except for acetic acid) variance treatments were heterogeneous. These changes in VFA concentrations could induce a change in the $N\text{-}NH_3$ concentration; however, no differences were reported (Great mean= 51.3 mg/L; SEM= 8.02; P>0.05) possibly due to the fact that the dependence of this parameter on the diet protein level is much stronger (OUDA & NSAHLAI, 2009). Conversely, pH near to neutral (Great mean= 7.3; SEM= 0.07; P>0.05) could maintain the viability of the microbial population (BUENO et al., 2005).

Analysis of NCGP (after correction with blank) parameters showed differences associated with the treatments on Par B (Prei-1 was 99% higher than Control, P<0.05; Table 2), but no differences were reported in Par A and Par c (P>0.05). The variability of Par A was largely explained by internal variability (62% of total), and for Par B, there was large variability among bottles (95% of total variation) despite large differences of Prei-2 and Prei-4 compared to Control. Replicates accounted for the highest percentage of variability of kinetic parameters.

These results are contrary to what GETACHEW et al. (2002) found in an inter-laboratory assay or those reported by KEIM et al. (2017) in different *in vitro* systems wherein most variation came from laboratory source or *in vitro* system assay.

When analyzing the ivDMD, there were differences between substrates (P<0.05, Table 2) but the Pre-i did not produce changes (P>0.05), and $N\text{-}NH_3$ concentration was similar and coherent with literature (LORENZ et al., 2011). The fermentation parameters of CON (758 g/kg of ivDMD and 347 ml/g DM of NCGP) were similar to a fermentation of a concentrate (mixture of cereal grains, dried beet pulp and soybean hulls) reported by GIERUS et al. (2008; i.e. 720 g/kg of ivDMD and 328 ml/g DM of NCGP assessed by Hohenheim gas test).

Volatile fatty acids profiles did not have differences among treatments (P>0.05, Table 2). However, assessment of substrates effects on VFA production showed a noticeable increment (P<0.05) for CON regarding Hay. Moreover, Hay showed an increased acetic/propionic ratio (Hay= 2.8; CON= 2.1; P<0.05). Likewise, GETACHEW et al. (2005) tested eight different commercial dairy concentrates and even though VFA profiles were lower than those reported here, the relationship between acetic/propionic was similar (i.e. 2.4 and 2.1, for the 8 rations and CON, respectively). This similar behavior of Pre-i and Control on fermentation kinetics, ivDMD and ruminal environment were coherent with the characteristics of a normal inoculum (DIJKSTRA et al., 2005).

Table 2 - Parameters of the kinetic of net cumulative gas production (with blanks correction), in vitro dry matter digestibility (ivDMD), pH, ammonia-N (N-NH_3) and volatile fatty acids of rumen fluid pre-incubated for 0, 1, 2 and 4 h (Control, Prei-1, Prei-2 and Prei-4, respectively) with 2 substrates incubated for 24 h.

Parameters[a]	Treatments				SEM[b]	Sign[c]	Substrate[d]		SEM	Sign	Distribution of variability[e] (%)	
	Control n=6	Prei-1 n=6	Prei-2 n=6	Prei-4 n=6			CON n=12	Hay n=12			Run	Res
----Net cumulative gas production (NCGP, A+B×(1-e$^{(-c\times time)}$))----												
A (ml)	-4.7	-6.9	-4.7	-2.4	1.00	ns	-6.9	-2.4	0.66	***	38	62
B (ml)	205a	408b	286a	313a	27.5	*	347	259	17.8	**	5	95
c (h^{-1})	0.03	0.02	0.03	0.02	0.006	ns	0.03	0.01	0.004	**	41	59
----Characterization of rumen environment----												
ivDMD (g/kg DM)	662	632	643	652	6.7	ns	758	536	104.9	***	36	64
pH	7.2a	7.1b	7.1b	7.1b	0.04	***	7.0	7.2	0.03	***	66	34
N-NH3 (mg/l)	50.9	53.9	43.8	45.8	5.69	ns	46.7	50.5	3.56	ns	13	87
----Volatile fatty acids (VFA, mmol/l))----												
Totals	29.8	24.9	31.9	31.2	3.92	ns	39.7	19.2	2.41	***	41	59
Acetic acid	17.7	14.4	17.9	18.9	3.84	ns	21.1	13.4	3.08	***	58	42
Propionic acid	7.9	6.8	8.5	8.3	0.89	ns	11.6	4.1	0.62	***	30	70
Butyric acid	3.9	2.5	3.2	2.8	0.48	ns	4.9a	1.2	0.33	***	59	41

[a] Parameters: A: Fraction rapidly disappeared; B: Fraction disappeared at a constant fractional rate per unit time and c: Rate of B.[b] Standard error of the mean. [c] Sign; Significance; ns, not significant (P>0.05); *, P<0.05; **, P<0.01; ***, P<0.001. Comparisons according to Dunnett (Control vs PreI-1, Prei-2 and Prei-4), different letters in the same line indicated P<0.05 for treatments or substrates. [d] CON, commercial concentrate for dairy cows; Hay, alfalfa hay. [e] Run (i.e. variation among inocula); Res, Residual of the error (i.e. variation among replicates/bottle).

Analysis of correlation between NCGP (after correction with blank) and GCGP (without correction) parameters obtained from a non-linear modeling CGP of full database showed that only Par A (r= 0.83) and B (r= 0.52) were significant (P<0.001). For each treatment, the analysis showed a significant relationship between the Par A of corrected and uncorrected models (r, 0.71, 0.68, 0.64 and 0.93 for Control, Prei-1, Prei-2 y Prei-4 respectively; P<0.05). On the contrary, Par B and Par c did not show a similar behavior (P>0.05) which indicated that blanks had a significant effect on the estimation of these parameters and only in the first hours of fermentation, blanks correction would not affect the NCGP of substrates (JUDD & KOHN, 2018).

Conversely, the Pre-i generated a greater variability and heterogeneity of variances, visible in a poor correlation between the corrected and uncorrected CGP. The largest proportion of total gas produced by blanks is released in the early hours of incubation (ARAUJO et al., 2011), and it is unusual to find a minor influence of the correction in Par A in contrast to Par B and Par c (Figure 1).

Although the objective of this research was to generate a more stable and robust inoculum, it was reported that Pre-i generated greater variability due to a poor correlation between corrected and uncorrected CGP. Furthermore, alterations in the production and fermentation rate possibly produced due to the extra substrate offered in the pre-incubations could have generated a greater and less controlled microbial

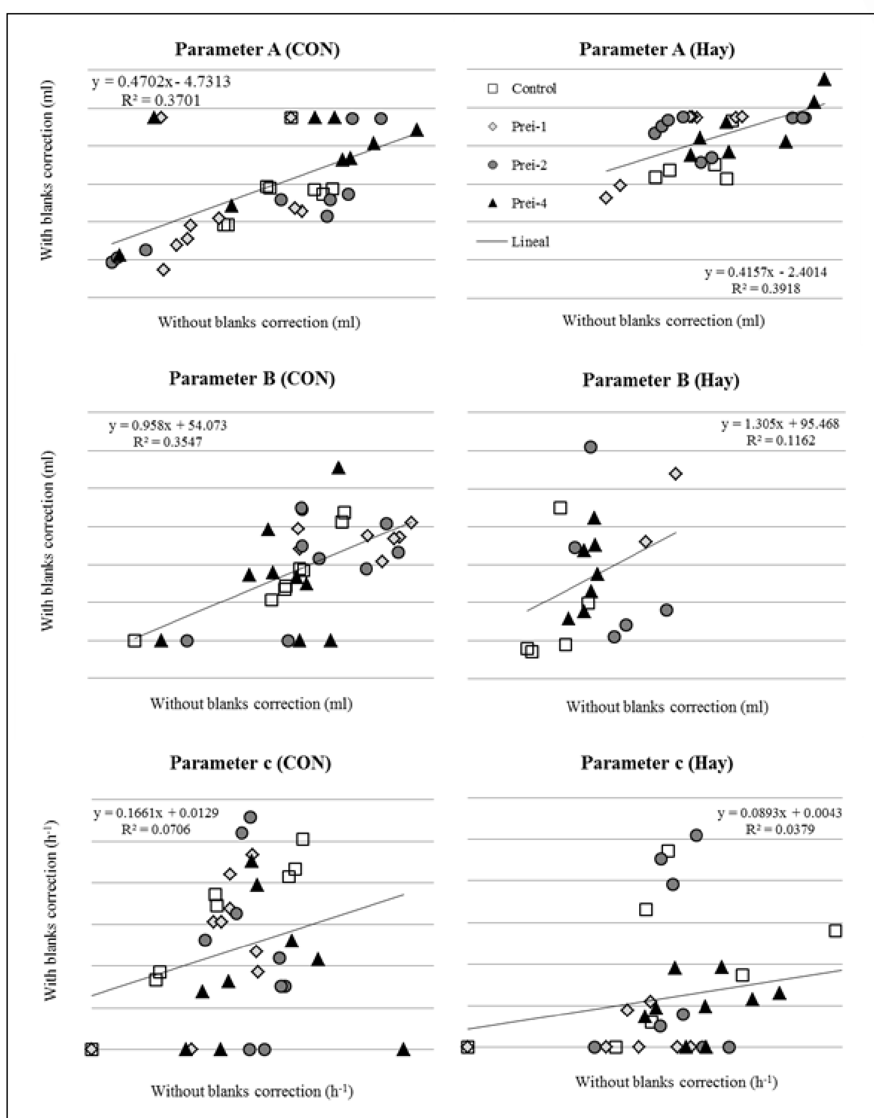

Figure 1 - Correlation between corrected and uncorrected values with blank for cumulative gas production parameters (CGP = A + B × (1-e$^{(c \times time)}$)), obtained after 24 h of fermentation of two substrates, i.e. commercial dairy concentrate (CON) and alfalfa hay incubated in ruminal liquor pre-incubated for 0, 1, 2 and 4 h (Control, Prei-1, Prei-2and Prei-4, respectively).

activity. The authors believe that further studies are necessary to assess the possibility of extending the pre-incubatory times, but always considering the control of production of fermentation products.

ACKNOWLEDGEMENTS

The authors acknowledge the University of Buenos Aires for the financial support (UBACyT 569-11, entitled "Metanogénesis y eficiencia de digestión ruminal de forrajeras templadas y megatérmicas"). The doctoral grant for J.M. Cantet was provided by the University of Buenos Aires and by the National Scientific and Technical Research Council (CONICET, Argentina).

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

We authors of the article CR-2019-0486 entitled "Pre-incubation of ruminal inocula to assess *in vitro* gas production and digestibility" declared, for all due purposes, the project that gave rise to the present data of the same has not been submitted for evaluation to the Ethics Committee of the Faculty of Agronomy, University of Buenos Aires, Argentina, but we are aware of the content of the Brazilian resolutions of the National Council for Control of Animal Experimentation – CONCEA. Thus, the authors assume full responsibility for the presented data and are available for possible questions, should they be required by the competent authorities.

DECLARATION OF CONFLICT OF INTERESTS

We have no conflict of interest to declare. The founding sponsors had no role in the design of the study; in the analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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