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ANIMAL PRODUCTION

Rumen fermentation of feed samples incubated in filter bags made from different textiles or dispersed in the medium using an *in vitro* gas production system

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ABSTRACT: The effect of different feed substrates incubated in filter bags [no bag (NB); Ankom® F57, 25 µm pore size (25AN); polyester, 45 µm pore size (45PB); polyester, 67 µm pore size (67PB)] or dispersed in the medium on gas production, digestion and rumen fermentation was evaluated using an *in vitro* gas production system. Filter bags reduced ($P < 0.01$) gas production but increased ($P < 0.01$) the *in vitro* dry matter digestibility (45PB and 67PB). Additionally, the use of filter bags with smaller pore size, reduced total volatile fatty acid ($P < 0.01$), but had minimal effects on acetate, propionate, and butyrate concentration. Our research suggested that using filter bags with a pore size of 67 µ would reduce some negative effects of incubating feed substrate dispersed in the medium or in filter bags with smaller pore size. However, the use of bags with a larger pore size might allow the wash out of small feed particles with consequent overestimation of digestibility.
Key words: incubation, digestibility, kinetics, volatile fatty acids.

Fermentação ruminal de amostras de alimento incubadas em sacos de filtro feitos de diferentes tecidos ou dispersos no meio usando um sistema de produção de gás *in vitro*

RESUMO: O efeito de diferentes substratos alimentares incubados em sacos de filtro [sem saco (NB); Ankom® F57, tamanho de poro de 25 µm (25AN); poliéster, tamanho de poro de 45 µm (45PB); poliéster, tamanho de poro de 67 µm (67PB)] ou disperso no meio na produção de gás, digestão e fermentação ruminal foi avaliada usando um sistema de produção de gás *in vitro*. As bolsas de filtro reduziram ($P < 0,01$) a produção de gases, mas aumentaram ($P < 0,01$) a digestibilidade da matéria seca *in vitro* (45PB e 67PB). Adicionalmente, o uso de bolsas de filtro com tamanho de poro menor reduziu o ácido graxo volátil total ($P < 0,01$), mas teve efeitos mínimos na concentração de acetato, propionato e butirato. Nosso trabalho sugere que usar sacos de filtro com tamanho de poro de 67 µ reduziria alguns efeitos negativos da incubação de substrato de alimentação disperso no meio ou em sacos de filtro com tamanho de poro menor. No entanto, o uso de sacolas com poros maiores pode permitir a eliminação de pequenas partículas de ração com consequente superestimação da digestibilidade.
Palavras-chave: incubação, digestibilidade, cinética, ácidos graxos voláteis.

The *in vitro* gas production technique, allows the evaluation of the rate and extent of fermentation of feeds for ruminants and has become an alternative to overcome the cost and animal welfare considerations of *in vivo* trials (AMANZOUGARENE & FONDEVILA, 2020). Filter bags have been used for several years to estimate rumen degradation of feeds (*in situ* and *in vitro*). According to VALENTE et al. (2015), filter bags must have a porosity that prevents the washout of undigested particles, while allowing the inflow of rumen fluid and the outflow of fermentation products to ensure that feed fermentation inside the bags is like that observed in the rumen environment. The incubation of feed dispersed in the rumen medium or inside commercial filter bags (Ankom Technology, Macedon, NY, USA) has been widely used for *in vitro* fermentation using the gas production technique (YANG, 2017), while the use of non-commercial bags (nylon or polyester) is not common.

The cost of commercial bags (Ankom F57) ranges from 0.98 to 1.28 United States dollars per bag depending on the number of bags purchased. For this reason, some laboratories have adopted the use of less expensive, non-commercial filter bags to reduce their costs of analyses (ADESOGAN, 2005).

While incubating feeds in filter bags makes simultaneous determination of digestibility easier by applying fewer transfers of sample residues after incubation (RAMIN et al., 2013), more information regarding the consistency,

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accuracy, precision of estimates, analytical costs, and operational facilities of different types of bags is needed (VALENTE et al., 2015).

An *in vitro* study comparing feed substrates incubated in commercial filter bags or weighed as loose powder into medium bottles concluded that the use of filter bags inhibited rumen fermentation, as indicated by reduction in digestibility and gas production (SCHLAU et al., 2021). Additionally, rumen digestibility using nylon (50 µm), F57 (Ankom®), and non-woven textile (100 g/m^2) bags was evaluated *in situ* (VALENTE et al., 2015). In that experiment, feed digestibility was overestimated for nylon bags, possibly due to significant loss of particles associated to its porosity (VALENTE et al., 2015).

Considering that non-commercial bags can be used as an alternative for *in vitro* rumen fermentation of feed substrates, it is important to verify that using them does not negatively affect rumen fermentation variables as compared to other options. To our knowledge, a simultaneous comparison among feed samples incubated using less expensive non-commercial filter bags, commercial filter bags or directly dispersed in the medium has not been reported for the *in vitro* gas production technique.

We hypothesized that *in vitro* feed degradation and rumen fermentation variables could be negatively affected when feed samples are incubated within filter bags. Additionally, the use of filter bags with bigger pore size, may reduce some negative effects on rumen fermentation. Thus, the the present study evaluated the rumen fermentation of feed samples incubated in filter bags made from different textiles or dispersed in the medium using an *in vitro* gas production system.

A completely randomized block design with a factorial arrangement of treatments, including four types of filter bags [no bag (NB); Ankom® F57, 25 µm pore size (25AN); polyester, 45 µm pore size (45PB); polyester, 67 µm pore size (67PB)] and three substrates (corn grain, alfalfa hay and corn stover), was used. Ankom® F57 bags were purchased from ANKOM Technology (ANKOM Technology, Fairport, New York, USA). Polyester (Sefar SA de CV, México) bags (4 × 5 cm) were made using heat-sealing (Uline, 20" H-1252, Mexico). Feed substrates were analyzed based on dry matter (DM; method 934.01) content according to (AOAC, 2005) for organic matter (OM; method 942.05) and crude protein (CP; method 2001.11). For neutral detergent fiber (NDF) the methodology of VAN SOEST et al. (1991) was used. The chemical composition of substrates (g/kg DM) was as follows: corn grain (OM = 986.4, CP = 72.2, NDF = 75.0), alfalfa hay (OM = 895.7; CP = 173.0, NDF = 393.1), and corn stover (OM = 939.1, CP = 60.9, NDF = 585.9).

The *in vitro* gas production technique described by MAURICIO et al. (1999) was used. Briefly, dried substrates, ground to pass through a 1 mm screen (Wiley Mill; Arthur Thomas Co., Philadelphia, PA, USA), were weighed (0.5 g) in triplicate into 125 mL glass bottles or weighed into acetone-washed filter bags, heat sealed and deposited in glass bottles. Three bottles containing inoculum without substrate were prepared for each type of bag and used as blank controls. Rumen liquid from three rumen-cannulated sheep consuming an 80:20 forage to concentrate diet was used as inuculum. About 45 mL of pre-warmed buffer medium (GOERING & VAN SOEST, 1970) and 15 mL of rumen liquid were added to the glass bottles while flushed with O_2-free CO_2. Bottles were sealed with rubber stoppers, and aluminum crimp caps, deposited in an oscillating water bath (Thermo Scientific, Precision TM TSSWB27, Newington, NH, USA) set to 50 rpm, and kept at 39 °C for 48 h.

Gas pressure (Gp) inside the bottles was measured at different time points (3, 6, 9, 12, 18, 24, 36, and 48 h) using a digital manometer (Traceable®, Fisher Scientific, USA). Following Gp reading, the headspace gas was removed until the Gp reading was equal to zero. Gas pressure readings were converted to gas volume using an equation developed under our lab conditions: gas volume = 7.0245 × Gp – 1.0849. Gas volume data at each time point, corrected for the gas released from the blanks, were used for calculation of gas production kinetics parameters. Gas production kinetics parameters (maximum volume of gas produced after 48 h of incubation, Vmax48; lag phase, L; gas production rate, S), were obtained using the logistic model of (PITT et al., 1999).

After 48 h incubation, bottles were placed in an ice bath for 10 minutes to stop fermentation. Subsequently, bottles were opened, and pH was immediately measured (pH Tester 30 Double Fuction®). A sample of the incubation liquid (5 mL) was mixed with 1 mL of metaphosphoric acid (25% w/v) and stored at -20 °C until volatile fatty acids (VFA) analysis. Concentration of VFA was quantified using gas chromatography (Perking Elmer, AutoSystem XL Model, USA).

Residuals of substrates incubated with no bags were vacuum filtered using filter paper (Whatman grade 41). Filter bags were washed with tap water. Both, filter paper and filter bags with residuals were dried at 55 °C for 48 h. Dry matter

disappearance (IVDMD) was determined through the mass difference between time 0 and 48 h. The assay was replicated in three independent runs.

Data were analyzed as a completely randomized block design with factorial arrangements of treatments using the MIXED procedure of SAS (SAS Institute, Inc., Cary). Run was used as blocking criteria. The model included type of bag, substrate, and type of bag × substrate interaction as fixed effects with run as a random effect. When a single factor or the interaction between factors was significant, means were compared using the Tukey test. Differences between treatments were declared significant at P≤0.05.

Bag × substrate interactions were observed for gas production kinetic parameters such as Vmax48 and S (P < 0.01; Table 1). The highest Vmax48 (P < 0.01) was observed when corn grain was incubated dispersed in the medium with no bag. Conversely, the lowest Vmax48 (P < 0.01) was observed when corn stover was incubated using 25AN or polyester filter bags. The rate of gas production was higher (P < 0.01) when corn grain and 67PB were used, and

Table 1 - Gas production kinetics and dry matter disappearance (IVDMD) of corn grain, alfalfa hay, and corn stover contained in different types of filter bags after 48 h of *in vitro* incubation.

Item	Vmax48, mL g^{-1}	S, h^{-1}	L, h	IVDMD g/100 g
----------Bag----------				
NB	116.3	0.042	3.93a	64.74
25AN	98.7	0.041	2.88b	63.85
45PB	106.7	0.045	3.68a	70.47
67PB	107.4	0.044	3.99a	71.35
SEM	6.38	0.003	0.211	1.17
----------Substrate----------				
Corn grain	153.3	0.049	5.57a	85.59
Alfalfa hay	91.3	0.043	4.11b	64.93
Corn stover	77.3	0.038	1.18c	52.28
SEM	6.34	0.003	0.183	1.08
----------Bag × Substrate----------				
----------NB----------				
Corn grain	166.0a	0.051b	5.57	80.39b
Alfalfa hay	97.4c	0.043c	4.72	59.47de
Corn stover	85.5cd	0.035d	1.52	54.35de
----------25AN----------				
Corn grain	138.9b	0.038cd	4.99	83.19ab
Alfalfa hay	89.0cd	0.045c	3.16	61.13d
Corn stover	68.3d	0.041c	0.5	47.22e
----------45PB----------				
Corn grain	150.9b	0.051ab	5.79	89.32a
Alfalfa hay	91.7c	0.046bc	3.78	68.52cd
Corn stover	77.5d	0.039cd	1.46	53.56e
----------67PB----------				
Corn grain	157.3ab	0.056a	5.95	89.46a
Alfalfa hay	87.0cd	0.040cd	4.79	70.58c
Corn stover	77.9d	0.036d	1.23	54.01de
SEM	6.71	0.003	0.366	1.74
----------*P-value*----------				
Bag	<0.01	0.06	<0.01	<0.01
Substrate	<0.01	<0.01	<0.01	<0.01
Bag × Substrate	<0.01	<0.01	0.53	<0.01

Means within a column, and within a category, with different letters differ significantly (P < 0.05).
Vmax48 = maximum volume of gas produced after 48 h of incubation; S = rate of gas production; L = lag phase; IVDMD = dry matter disappearance; NB = No bag; 25AN = Ankom® F57, 25 µm pore size; 45PB = polyester bag, 45 µm pore size; 67PB = polyester bag, 67 µm pore size; SEM=Standard error of the mean.

lower (P < 0.01) when corn stover was incubated dispersed in the medium. Lag phase was reduced (P < 0.01) when 25AN filter bag was used. Additionally, lag phase was higher (P < 0.01) for corn grain, followed by alfalfa hay and corn stover. When corn stover was incubated using 25AN filter bags, IVDMD was significantly reduced (P < 0.01). However, when corn grain was incubated in polyester bags (45PB and 67PB), IVDMD was increased (P < 0.01).

For rumen fermentation variables (Table 2), total VFA concentration was higher (P < 0.01) for corn grain incubated with no bag. Likewise, acetate molar proportion was higher (P < 0.01) for feed samples incubated using 45PB. Additionally, acetate molar proportion was higher for alfalfa hay and lower for corn grain (P < 0.01). Propionate was only affected by the type of substrate (P < 0.01), where corn grain had the highest and alfalfa hay the lowest molar proportion respectively. Consequently, a higher (P < 0.01) acetate to propionate ratio for alfalfa and a lower acetate to propionate ratio for corn grain (P < 0.01) was observed. Butyrate was only affected by

Table 2 - Volatile fatty acids and pH of corn grain, alfalfa hay, and corn stover contained in different types of filter bags after 48 h of *in vitro* incubation.

Item	Total VFA, mM	Acetate, mol/100 mol	Propionate, mol/100 mol	Butyrate, mol/100 mol	A:P	pH
----------Bag----------						
NB	84.72	52.03b	22.94	13.91	2.31	6.53
25AN	75.98	52.21b	23.34	14.86	2.34	6.54
45PB	77.26	57.45a	22.98	12.40	2.63	6.55
67PB	76.05	55.34ab	23.82	12.77	2.40	6.54
SEM	3.06	1.15	0.90	0.73	0.11	0.02
----------Substrate----------						
Corn grain	92.2	44.40c	26.98a	18.58a	1.66c	6.35b
Alfalfa hay	75.2	61.08a	20.41c	9.99b	3.026a	6.65a
Corn stover	68.2	57.28b	22.42b	11.88b	2.58b	6.62a
SEM	2.65	1.07	0.85	0.64	0.13	0.02
----------Bag × Substrate----------						
----------NB----------						
Corn grain	113.55a	42.61	24.89	19.26	1.72	6.34
Alfalfa hay	72.23b	57.59	20.99	10.92	2.75	6.64
Corn stover	68.39b	55.89	22.95	11.53	2.46	6.60
----------25AN----------						
Corn grain	81.69b	40.75	28.00	20.57	1.46	6.33
Alfalfa hay	81.11b	60.41	20.68	10.55	2.93	6.65
Corn stover	65.15b	55.46	21.33	13.47	2.63	6.63
----------45PB----------						
Corn grain	89.27ab	48.66	27.86	16.89	1.75	6.37
Alfalfa hay	73.74b	63.1	19.51	9.61	3.32	6.64
Corn stover	68.75b	60.6	21.57	10.70	2.81	6.63
----------67PB----------						
Corn grain	84.1b	45.6	27.19	17.62	1.68	6.36
Alfalfa hay	73.61b	63.22	20.48	8.87	3.09	6.67
Corn stover	70.44b	57.18	23.80	11.83	2.43	6.60
SEM	5.20	1.68	1.18	1.25	0.19	0.03
----------*P-value*----------						
Bag	0.155	<0.01	0.64	0.11	0.07	0.81
Substrate	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Bag × Substrate	0.02	0.51	0.15	0.88	0.48	0.90

Means within a column, and within a category, with different letters differ significantly (P < 0.05).
VFA = Volatile Fatty Acid; A:P = Acetate to propionate ratio; NB = No bag; 25AN = Ankom® F57, 25 µm pore size; 45PB = polyester bag, 45 µm pore size; 67PB = polyester bag, 67 µm pore size; SEM=Standard error of the mean.

type of substrate ($P < 0.01$) where corn grain showed the highest molar proportion. Mean pH was lower ($P < 0.01$) for corn (6.35) compared with alfalfa hay (6.65) or corn stover (6.62).

Filter bags can act as a physical barrier to feed fermentation, restricting microbial digestion and the free movement of fermentation end products (RAMIN et al., 2013). Thus, the higher volume of gas produced for corn grain (a highly digestible fed ingredient) incubated dispersed in the medium was expected. Moreover, the lower volume of gas produced when using 25AN filter bags as compared to polyester bags (45PB and 67PB), might be related to the lower theoretical pore size (25 µ) reported for 25AN, since bags with a smaller pore size would limit feed digestion and lower microbial activity within the filter bags (RAMIN et al., 2013). Previously, the National Research Council (NRC, 2001) recommended bags with a pore size between 40 and 60 µ for *in situ* determination; and therefore, using a smaller pore size could also have a negative effect on feed digestion for *in vitro* incubations. Conversely, the higher rate of gas production for corn grain incubated using 67PB, may be associated to the larger pore size, which allows for a more efficient movement of rumen liquid, feed particles, microbes, and end-products of fermentation across the bag.

The lower IVDMD observed for NB, could be related to the process of filtration of feed residuals, as the filter paper (Whatman grade 41) used for this treatment, had a smaller pore size (20 µm) compared to polyester bags and can withhold more undigested feed samples. The same reasoning applies to the lower IVDMD observed for 25AN. Conversely, there is a possibility that a greater pore size in polyester bags allowed small feed particles to be washout with consequent overestimation of IVDMD (VALENTE et al., 2015).

The higher total VFA concentration for corn grain incubated dispersed in the medium, agrees with the higher volume of gas produced observed in this study. As expected, rumen fermentation moved to a higher molar proportion of acetate and a lower molar proportion of propionate when forages such as alfalfa hay and corn straw were incubated.

Incubating feed substrate freely dispersed in the medium reduced *in vitro* dry matter digestibility, possibly as a result of a subsequent filtering process with filter paper with a reduced pore size. Conversely, the use of filter bags reduced gas production and total volatile fatty acid concentration, particularly when using bags with smaller pore size. Nevertheless, the effects of filter bags on acetate, propionate, and butyrate concentration were minimal. Therefore, the use of filter bags with larger pore size (e. g. 67 µ) would reduce some negative effects associated with the incubation of feed substrate dispersed in the medium or in filter bags with smaller pore size when using the *in vitro* gas production technique. However, a larger pore size might allow small feed particles to be washed out from the bags with consequent overestimation of digestibility.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was approved by an Institutional Committee for the Care and Use of Experimental Animals, according to the Official Mexican Standard NOM-062-ZOO-1999 under protocol SICUAE.MC-2021/2-6.

DECLARATION OF CONFLICT OF INTEREST

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

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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