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Non-ruminants

Equine fecal inoculum optimization in *in vitro* fermentation assays of dehydrated roughage

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ABSTRACT - This study evaluated the influence of coastcross hay substrate hydration and equine fecal inoculum dilution on the parameters of fermentation and microbiology in *in vitro* essays. A 2 × 2 factorial block design was used. The first factor was hydration of the coastcross hay substrate 12 h before incubation or at the time of incubation, and the second factor was the dilution of fecal inoculum with a nutrient solution in a weight: weight ratio of 1:1 or 1:3. Degradation of the dry matter (DM), organic matter (OM), and neutral detergent fiber (NDF) were evaluated at 24, 48, and 72 h. Microorganisms were counted 24 h after inoculation. The ammoniacal nitrogen concentration (NH_3-N), pH, and cumulative gas production were measured up to 72 h and adjusted by the non-linear Gompertz regression model. Hydration of substrate and time of incubation increased nutrient degradation of coastcross hay, as well as the final volume of gases and the concentration of *Streptococcus* spp. The 1:3 dilution increased the final pH and *Streptococcus* spp. concentration. The hydration of substrate did not have any effect on NH_3-N, *Lactobacillus* spp., cellulolytic, and total anaerobic bacteria concentrations. In addition, no effect of hydration was observed on the fermentation rate and the maximum fermentation time on the model used. The fermentation profile of the grass substrate is not affected by dilution, and, therefore, horse feces can be used as a source of inoculum in *in vitro* fermentation trials. Hydration increases the gas volumes and the nutrient degradation of grass hay, renders the lag phase time insignificant and, therefore, can be irrelevant in terms of fermentation model settings.

Key Words: degradation rate, gas production, microbiology, modeling, roughage

Introduction

The *in vitro* gas production technique is important to better understand horse nutrition, in which the diet effects on the activity of intestinal microbiota can be evaluated, and the nutrient degradation can be kinetically quantified without the need of fistulated animals (Murray et al., 2014), making it an important alternative to *in vivo* studies. Equine feces are easy to obtain and are mildly invasive.

However, some studies indicate that feces may present less fermentative capacity than other equine inoculum sources (Murray et al., 2014). There are differences in fecal inoculation procedures in the *in vitro* tests: feces dilution (Elghandour et al., 2014) and substrate hydration (Rymer et al., 1999). The lack of standardization of fecal inoculum preparation generates differences in results and makes comparisons difficult, impairing the technique.

Mathematical modelling assists in the interpretation of the *in vitro* fermentation kinetics studies, which provides a dynamic approach applied to the model parameters (Mould et al., 2005). The mathematical models for gas production applied to equine studies were originally from research on ruminants (Groot et al., 1996).

There are anatomical differences between ruminant and equine species; therefore, some essential characteristics of the substrate that will be fermented must be considered (Sunvold et al., 1995), such as the substrate already pre-digested and hydrated when the first contact with the microorganisms occurs in the cecum. Such conditions play an important role in the substrate recognition

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time, consequently, on the estimates of fermentation model parameters that will be influenced. There is no standardized protocol for the use of equine feces as a source of inoculum in gas production trials, and there is also a lack of fermentative studies with microbiological evaluation using fecal inoculum.

The objective of this study was to evaluate the influence of hay hydration and the equine feces dilution on *in vitro* fermentation parameters and microbial population.

Material and Methods

The experiment was carried out in Seropédica, Rio de Janeiro, Brazil (22°45'52.7" S, 43°41'22.4" W, and 33 m altitude). Research on animals was conducted according to the institutional committee on animal use registered under case no. 2015/002590.

The feces from three stallions of Mangalarga Marchador breed were used to provide the fecal samples, and the coastcross hay provided for feeding the animals was used as a substrate in the *in vitro* fermentation essays throughout the experimental period.

A 2 × 2 factorial block design was used. The first factor was hydration of the substrate 12 h before incubation and at the time of incubation. The second factor was dilution of the feces (inoculum) with a nutrient solution in a weight:weight ratio of 1:1 or 1:3. The cumulative gas production and nutrient degradation were analyzed in a randomized block design with repeated measures over time. The test was repeated for five consecutive weeks, each week being an experimental block.

The horses with an average body weight of 400 kg, which had been previously dewormed with ivermectin (Eqvalan Golden®, Merial), were kept in individual stalls with a feeder and water *ad libitum* and exercised three times a week for 30 min. The diet of the horses was composed of coastcross hay (*Cynodon* spp. cv coastcross), mineral salt (Essencefós®, Presence), water *ad libitum*, and concentrate meal (Table 1) provided at 07:00 and 16:00 h. The average dry matter intake of the diet was 26 g kg^{-1} of liveweight in the ratio of 66:34 of roughage:concentrate.

The coastcross hay and concentrate meal samples were milled to 1-mm sieve in a Willey mill. The analysis performed were of dry matter, crude protein, and ash (AOAC, 1995), and ether extract and crude energy (Silva and Queiroz, 2006). The neutral detergent fiber (NDF) (Van Soest et al., 1991) and lignin were analyzed by oxidation of lignin with permanganate (Van Soest and Robertson, 1980).

The gas production incubations were perfomed as follows: feces were collected simultaneously from the rectum of horses at 09:00 h, two hours after the meal. The fecal samples were kept closed in preheated thermal bottles until processing in the laboratory. The time elapsed between the collection of feces and preparation of the inoculum in the laboratory was 20 min.

The fermentation test protocol required the insertion of 1 g of hay milled to 2 mm into 160 mL flasks, plus 90 mL of nutrient solution (Theodorou et al., 1994). The nutrient solution was added 12 h before and at the moment of inoculation, constituting, therefore, the procedures adopted to perform the hydration treatment. The flasks that had been hydrated for 12 h were kept under refrigeration at 4 °C and, 2 h before inoculation, they were heated in a water bath at 39 °C.

The procedude adopted to prepare the fecal inoculum were: the fecal samples from three animals were proportionally mixed, then divided into two fractions – the first was diluted with nutrient solution at a ratio of 1:1 (w:w) and the second diluted with nutrient solution at the ratio of 1:3 (w:w). The diluted feces were then shaken for 1 min in a blender, after which they were placed in a water bath at 39 °C under constant spraying with CO_2 (Desrousseaux et al., 2012). After 1 h, the feces diluted with nutritive solution of each treatment were filtered through a 45-µm nylon cloth to obtain two different inoculums (1:1 and 1:3). Finally, 10 mL of inoculum were added to bottles already prepared, and correspondent to the treatment described above, and sealed with rubber stoppers.

Fourteen flasks (replicates) were incubated for each treatment. After inoculation, the flasks were kept in a water bath at 39 °C with constant stirring. The gas production was measured manually by means of a pressure transducer

Table 1 - Chemical composition (g kg^{-1} DM) of concentrate (ground meal) and coastcross hay

Nutrient	DM	MM	EE	CP	NDFom	ADF	Cel	Hem	Lig	NFC
Concentrate	885	109	47	231	165	73	50	92	21	456
Coastcross hay	888	84	25	139	737	359	283	38	53	15

DM - dry matter; MM - mineral matter; EE - ether extract; ADF - acid detergent fiber; Cel - cellulose; Hem - hemicellulose; Lig - lignin determined by oxidation of lignin with permanganate; NFC - non-fibrous carbohydrates (= 100 – (CP + EE + NDFom + MM); CP - crude protein; NDFom - neutral detergent fiber not assayed with a heat stable amylase and expressed exclusive of residual ash.

(LOGGER AG100, Universal Datalogger) at times: 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 21, 24, 27, 30, 36, 42, 48, 54, 60, 66, and 72 h, making a total of 23 readings. After each reading, accumulated gases were released and flasks were shaken. Pressure in psi was converted to volume per gram of dry matter using the specific equation for experimental conditions of gas production:

$$\hat{y} = -0.07 + 3.79x + 0.077x^2,$$

in which each psi corresponds to 3.80 mL (Martins, 2012).

The dry matter degradation (DMD), organic matter degradation (OMD), and neutral detergent fiber degradation expressed exclusive of residual ash (NDFD) were determined by the difference in nutrient weight present in the substrate before incubation and after 24, 48, and 72 h of fermentation. After 72 h of incubation, pH (pH 300; Analyzer®) was recorded and NH_3-N ammonia nitrogen (mg/dL) was evaluated after centrifugation at 10,000 *g* for 10 min at 4 °C. Thereafter, 5 mL of the supernatant were pipetted and subjected to distillation and titration according to the micro-Kjeldahl method (Fenner, 1965; Souza et al., 2013).

Microorganisms were quantified 24 h after inoculation. The concentrations of total viable anaerobic bacteria were determined with complete modified agar medium (Leedle and Hespell, 1980) after 48 h of incubation at 39 °C by the most-probable-number at 10^{-6}, 10^{-7}, and 10^{-8} dilutions. The concentrations of cellulolytic bacteria were determined by the most-probable-number at 10^{-3}, 10^{-4}, and 10^{-5} dilutions with modified (Halliwell and Bryant, 1963) liquid culture medium (Julliand et al., 1999) with readings taken after 15 days incubation at 39 °C. The concentrations of *Lactobacillus* spp. and *Streptococcus* spp. were determined by counting colony forming units in Petri dishes prepared with MRS *Lactobacillus* base agar medium (200019, Biolog, Italy) and Azide Bile Esculine agar (06105-500g, Sigma-Aldrich, Buchs, Switzerland), respectively, in dilutions of 10^{-4}, 10^{-5}, and 10^{-6} after 48 h of incubation at 39 °C in anaerobic jars.

The cumulative production of gases was adjusted using the nonlinear Gompertz equation (Winsor, 1932):

$$Vf = Vm\, e^{-e^{-b\,(t\,-\,t^*)}}$$

in which *Vf* = final volume of gases (mL/g DM), estimated as a function of *Vm*; *Vm* = volume of gases at time t (mL/g DM); *b* = fermentation rate (mL/hour); *t** = time (hours) when fermentation rate is maximum; and *t* = time (hours). The estimated equation was used to calculate the fermentation rates (mL/g DM) as a function of time (t) by derivation of the Gompertz equation:

$$dWt'/dt = Wmbe^{\,-b\,(t\,-\,t^*)\,-e^{-b\,(t\,-\,t^*)}}$$

The NLIN procedure of the Statistical Analysis System, version 9.2 was used for parameter estimation. The parameter estimates were obtained by a modified iterative Gauss-Newton method developed by Hartley (1961) for nonlinear models. The distribution of the studentized residuals, used to detect discrepant points, in general, is considered as a marginally discrepant observation if $|Rti^*|>3$ (Lemonte, 2008). The parameters of the models were compared using the likelihood ratio test (Regazzi, 2003).

The results from DMD, OMD, NDFD, pH, NH_3-N, and the bacteriological count (logarithmic basis) were evaluated for the homoscedasticity of variances and normality. When the assumptions were met, they were subjected to ANOVA (α = 0.05) and compared by the Fisher test (α = 0.05). The analyses were carried out with the SISVAR statistical package (Ferreira, 2011).

Results

There was no interaction between the inoculum dilution and substrate hydration in nutrient degradation (P>0.05), but there was an effect of substrate hydration on DMD (P<0.05), OMD (P<0.05), and NDFD (P<0.05), and time (P<0.001) (Table 2).

After adjusting the model, no effect of inoculum dilution on the parameters of the model was observed (P>0.05). However, the substrate hydration produced a significant effect on the final gas volume (P<0.001), but not on fermentation rate and time of maximum fermentation between both treatments of 12 h hydration before incubation or at the time of incubation (Table 3).

There was an effect of dilution factor on pH at the end of the fermentation (P<0.001), but no effects of hydration or interaction on pH were observed (P>0.05) (Table 4). However, there was no effect of treatments on NH_3-N concentration of the fermentation residue after 72 h of incubation.

There was no effect of substrate hydration or inoculum dilution (P>0.05) on cellulolytic and total anaerobic *Lactobacillus* spp. bacteria concentration after 24 h of fermentation (Table 5). However, there was an effect of substrate hydration (P<0.05) and inoculum dilution (P<0.05) on the concentration of *Streptococcus* spp., but without significant interaction effect, with greater concentrations of microorganisms observed with the hydration starting at the moment of inoculation and with dilution 1:3.

Discussion

No correction for soluble fraction on degradation of DM, NDF, and OM were performed. Pre-hydration of the substrate increased DMD, and, even disregarding the mineral fraction, this procedure increased OMD by approximately 10.2%. Considering the total degradable substrate until 72 h, the degradation extend reached 62.5 and 56.7% for DMD and OMD, respectively, after 24 h, showing that there was greater activity of fecal microorganisms at the beginning of the *in vitro* tests with the grass substrate.

In addition, there was an increase of NDFD when the substrate was pre-hydrated; consequently, different degradation profiles were observed for the treatment 0 h compared to 12 h of hydration. The relative difference observed between treatments at 24 h of degradation was approximately 30%, reducing the observed difference a

Table 2 - Nutrient degradation (mg g^{-1}) of coastcross hay hydrated for 12 h or immediately before inoculation with feces diluted in the ratios 1:1 and 1:3 up to 72 h (n = 60)

Nutrient degradation		Time (h)					
		24	48	72	Mean[5]		
Dry matter[1]	Hydration						
	12 h	302	389	469	387a		
	0 h	268	360	442	356b		
	Dilution						
	1:1	283	380	465	376		
	1:3	286	368	446	367		
	Time mean[4]	285C	374B	456A			
	Hydration (H)	Dilution (D)	Time (T)	H × D	T × H	T × D	T × H × D
P-value	0.008	0.321	0.000	0.452	0.849	0.314	0.662
SEM	6.08	6.08	2.78	8.61	7.32	7.32	10.35
Organic matter[2]	Hydration						
	12 h	257	345	433	345a		
	0 h	218	318	404	313b		
	Dilution						
	1:1	236	339	430	335		
	1:3	239	324	408	324		
	Time mean[4]	237C	332B	419A			
	Hydration	Dilution	Time	H × D	T × H	T × D	T × H × D
P-value	0.009	0.265	0.000	0.620	0.741	0.293	0.635
SEM	6.67	6.67	3.57	9.43	8.19	8.19	11.58
Neutral detergent fiber[3]	Hydration						
	12 h	171	289	386	282a		
	0 h	120	240	366	242b		
	Dilution						
	1:1	145	278	390	271		
	1:3	146	261	363	253		
	Time mean[4]	146C	264B	376A			
	Hydration	Dilution	Time	H × D	T × H	T × D	T × H × D
P-value	0.025	0.269	0.000	0.540	0.311	0.327	0.528
SEM	10.38	10.38	7.47	14.68	10.95	10.95	15.49

SEM - standard error of the mean; CV - coefficient of variation; H × D - hydration and dilution interaction; T × H - time and hydration interaction; T × D - time and dilution interaction; T × H × D - time, hydration, and dilution interaction.

[1] CV%, 3.36.

[2] CV%, 4.86.

[3] CV%, 12.76.

[4] Means with different letters within the row differ significantly (P<0.05) by the Fisher test.

[5] Means with different letters within the column differ significantly (P<0.05) by the Fisher test.

Table 3 - Parameters of the fermentation model with coastcross hay hydrated for 12 and 0 h before inoculation (mean±SEM)

Parameter	Hydration		P-value
	12 h	0 h	
Final volume of gases (mL g^{-1} DM)	52.6±1.9	39.1±1.4	0.000
Fermentation rate (mL h^{-1})	0.142±0.0143	0.139±0.0142	0.897
Maximum fermentation time (h)	7.04±0.47	8.29±0.48	0.060

SEM - standard error of the mean; DM - dry matter.

Table 4 - Mean values of pH from the residual solution and ammoniacal nitrogen concentration (mg dL^{-1}) after 72 h of coastcross hay fermentation under different hydration and dilution (n = 20)

	Hydration			
	Dilution	12 h	0 h	Mean[3]
pH[1]	1:1	6.53	6.55	6.54b
	1:3	6.62	6.65	6.64a
	Mean	6.58	6.60	
	Hydration (H)	Dilution (D)	H × D	
P-value	0.060	0.000	0.685	
SEM	0.008	0.008	0.012	
NH_3-N[2]	1:1	2.90	2.13	2.51
	1:3	2.70	2.27	2.48
	Mean	2.80	2.20	
	Hydration	Dilution	H × D	
P-value	0.272	0.955	0.750	
SEM	0.368	0.368	0.521	

H × D - hydration and dilution interaction; SEM - standard error of the mean; CV - coefficient of variation.
[1] CV%, 0.41.
[2] CV%, 46.60.
[3] Means with different letters differ significantly (P<0.05) by the Fisher test.

Table 5 - Concentration of *Lactobacillus* spp., *Streptococcus* spp., cellulolytic bacteria, and total anaerobes (log_{10} cfu mL^{-1}) of the residual solution after 24 h fermentation of coastcross hay subjected to different hydration and dilution (n = 20)

	Hydration			
	Dilution	12 h	0 h	Mean
Lactobacillus spp.	1:1	8.17	8.14	8.16
	1:3	8.05	8.14	8.10
	Mean	8.11	8.14	
	Hydration (H)	Dilution (D)	H × D	
P-value	0.617	0.375	0.347	
SEM	0.0461	0.0461	0.0651	
Streptococcus spp.[1]	1:1	7.61	7.90	7.76a
	1:3	7.93	7.97	7.95b
	Mean	7.77a	7.94b	
	Hydration	Dilution	H × D	
P-value	0.025	0.010	0.073	
SEM	0.0458	0.0458	0.0648	
Cellulolytic bacteria	1:1	3.78	3.72	3.75
	1:3	4.08	3.97	4.02
	Mean	3.93	3.84	
	Hydration	Dilution	H × D	
P-value	0.682	0.203	0.900	
SEM	0.1425	0.1425	0.2016	
Total anaerobes	1:1	6.53	6.67	6.60
	1:3	6.45	6.44	6.44
	Mean	6.49	6.56	
	Hydration	Dilution	H × D	
P-value	0.583	0.188	0.514	
SEM	0.0800	0.0800	0.1132	

H × D - hydration and dilution interaction; SEM - standard error of the mean.
[1] Means with different letters within the row or column differ significantly (P<0.05) by the Fisher test.

long time; as observed at the end of 72 h, this difference was reduced to 5.2%. According to Julliand et al. (2001), microorganisms that degrade the plant cell wall are considered when quantifying cellulolytic bacteria. Although no difference was observed in the populations at 24 h, maximum fermentation occurred between 7 and 8 h after incubation, indicating that this period is the best moment to observe differences in the cellulolytic community. At this time, gas production includes methane and CO_2, which are derived from the substrate fermentation (Coles et al., 2005).

Hydration of the substrate speeds up the fermentation process at the initial incubation times; however, at this stage, only the gas production variable had been recorded. Even the fermentation prolonged up to 72h, nutrient degradation and final volume of gases were not compensated when equine feces were used as inoculum. According to Coles et al. (2005), the goal of an *in vitro* study is to represent *in vivo* events, adapting the physiological conditions of the species under study, such as pH, temperature, incubation time, and so on.

Several studies with equine species were performed using *in vivo* or *in situ* methodologies to better understand the digestion process, which invariably involves kinetics of passage and nutrient degradation. Therefore, we have used the kinetic of passage (Van Weyenberg et al., 2006; Silva et al., 2014) and nutrient degradation information (Hyslop, 2006; Silva et al., 2010) to support the interpretations of the present study. Additionally, due to limitation to obtain fistulated animals for *in vitro* fermentation studies, a limiting factor nowadays, we had to use the information currently existing in the literature.

Therefore, Van Weyenberg et al. (2006) described values of mean retention time (MRT) in horses fed different feedstuffs no longer than 48 h. Verifying the *in situ* degradation of nutrients of fibrous feed, Hyslop (2006) used the incubation up to 48 h as the maximum time for cecal incubation, and recently, Silva et al. (2014) fed fistulated horses coastcross hay with different particle size and observed values of 36.7 h of MRT. Consequently, the incubation time in *in vitro* fermentation essays with equine fecal inoculum should not exceed 48 h.

According to Silva et al. (2010), who evaluated the *in situ* cecal degradation of nutrients in fistulated horses, a value of 34.3% NDFD of coastcross hay (*Cynodon* spp. cv coastcross) was observed after 48 h of incubation, which was greater than that observed *in vitro* in this study. The nutrient degradation depends on the fermentative activity and rate of digesta passage, which provides a constant supply of nutrients to microorganisms, maintaining the

degradation activity of the bacteria on the fibrous substrate; this is a limiting element in *in vitro* techniques.

Total gas production is the sum of H_2, CO_2, and CH_4 production along with *in vitro* incubation, and these gases are used as fermentative activity indicators in the hindgut of horses (Elghandour et al., 2016; 2018). Additionally, by quantifying the total gas production, it is possible to estimate the metabolizable energy and *in vitro* digestibility of organic matter (Menke et al., 1979) as well as the short-chain fatty acid concentration (Getachew et al., 2002). However, the procedures described above were developed with ruminal fluid studies but have been recently used in *in vitro* studies in equine nutrition (Elghandour et al., 2016; 2017 and 2018).

Rymer et al. (1999) identified that the gas production of the fibrous substrate increased after 6 h of hydration; however, 12 h were used in the present study to facilitate the semi-automatic experimental procedure.

The kinetics of gas production model developed for ruminant nutrition presents a sigmoid profile characterized by a lag phase followed by the exponential growth phase that slows down and reaches the asymptotic phase (Rymer et al., 2005; Vieira et al., 2008). As the fermentation profile of coastcross hay hydrated for 12 h presented a short lag phase and was not detectable by the model, the authors suggested that an exponential model that disregards the lag phase would be more adequate for equine studies, even using equine feces as inoculum.

According to Rymer et al. (1999), 6 h of hydration were sufficient to provide the soluble fraction from substrate into the medium, which does not change along the *in vitro* study period prior inoculation. Immediately after inoculation, the soluble nutrients are rapidly consumed; after that, the insoluble and potentially fermentable fraction of substrate will predominate. However, it needs to have been previously hydrated for the colonization of microorganisms (Groot et al., 1996). The initial development of the microorganisms present in the feces and the adhesion to the insoluble particles are time-dependent. Therefore, the hydration of coastcross hay facilitated adhesion to the substrate, optimizing the fermentation process. Murray et al. (2006) observed that lag phase time differences would occur depending on the chemical characteristics of the substrate, so that fibrous feedstuffs will present lag phases greater than the soluble substrates. However, they did not mention the hydration procedure on their protocol.

Coastcross is a C_4 grass widely used in the tropics to feed horses and has a high lignification of the plant cell wall (Van Soest, 1994). Therefore, the 12-h hydration was fundamental to reduce the lag phase to 0.28 h, allowing rapid access to the substrate by the fecal microbial community. Although we did not use the lag phase information for statistical comparisons, it was determinant in the choice and adjustment of the model. In addition, it made the *in vitro* procedures similar to what occurs naturally *in vivo* in the equine species.

The inoculum from equine feces may present a fermentation pattern similar to cecal inoculum, but it differs when compared to that of the colon (Murray et al., 2014). However, Julliand and Grimm (2016) reported that the fecal bacterial population was similar to that seen in the dorsal colon and lower colon. Thus, the production of gases may be more associated with the ability of the inoculum microorganisms to ferment a particular substrate than its concentration. In the present study, the same composite sample of feces diluted in 1:1 or 1:3 (weight:weight) was used, and the gas production appeared to be more influenced by the physical characteristics of the substrate than by the concentration of the fecal microorganisms. In addition, there was no difference in the concentration of microorganisms from the fecal inoculum of horses when collected at different times throughout the day; however, the gas production differed according to the substrates used (Desrousseaux et al., 2012). Therefore, the dilution of feces in the proportions used in the present *in vitro* fermentation assay can be used without affecting fermentation parameters of hay grasses and some microorganisms. In addition, the use of feces for the inoculum avoids the use of surgically modified or euthanized animals for collection of inoculum (Murray et al., 2014).

Cone et al. (1997) incubated glucose and observed an alteration in the fermentation profile after 45 h, suggesting microbial turnover on *in vitro* fermentation systems. Additionally, these authors reported that the microbial protein was greater between 5 and 10 h of incubation, which coincided with the total intake of glucose, and the microbial protein was reduced after a prolonged period of fermentation with the formation of NH_3. This suggests that the microbial nutrients were recycled, i.e., the development of new cells using dead cells as a nutrient source.

The microbial population present in the cecum uses non-protein N to generate volatile fatty acids (VFA) in detriment of population growth as a metabolic strategy (Santos et al., 2012). However, the quantification and identification of groups that would benefit from this nitrogen source were not carried out (Santos et al., 2012). The concentration of NH_3-N in *in vitro* fermentation assays is inversely proportional to the concentration of microbial protein (Cone et al., 1997). In the present study,

the NH_3-N values observed after 72 h were greater than those observed by Santos et al. (2012), possibly due to the greater true protein fermentation present in the substrate.

The reduction of pH in *in vitro* studies is an indication of the accumulation of final fermentation products, such as VFA and lactate (De Fombelle et al., 2001; Coles et al., 2005) or the saturation of buffering agents in the medium. At the 1:3 dilution, there was a greater proportion of buffering agents from the nutrient solution in relation to the total number of microorganisms incubated. Therefore, for the fermentation products, the buffer was more effective as it left the solution less acidic compared to the 1:1 dilution. However, pH remained within the normal fermentation range (from 7.2 to 6.2) of large intestine for healthy horses (Clarke et al., 1990). The 1:3 dilution may be a strategy for *in vitro* studies in which there is an limited amount of feces to prepare the inoculum, such as in studies with new-born foals (Silva, 2017)[1].

Although the pH was within clinical normality, the concentration of the genus *Streptococcus* spp., which is related to metabolism of rapidly fermenting carbohydrates, may have interfered with acidification (Julliand et al., 2001). The reduction of these nutrients caused by greater concentrations of microorganisms of the diluted 1:1 inoculum may have affected their growth. Besides, the hydration of the substrate also had an inhibitory effect on the growth of these microorganisms. Although the genus *Streptococcus* spp. is associated with the development of laminitis, they have a proteolytic and degradative activity of mono-, di-, and oligosaccharides of plant origin providing rapid access to these nutrient sources without relying on other microorganisms (Jans et al., 2015). However, a high concentration of *Streptococcus* leads to a reduction in the medium pH, which may compromise the development of other important microorganisms in the fermentation of fibrous carbohydrates. Therefore, high concentrations of this microorganism are not desirable. Thus, treatments with lower dilution and greater hydration could offer a more balanced environment among the important bacterial populations.

The concentrations of *Streptococcus* observed in the present study were similar to those observed by Julliand et al. (2001) of 7.64 $\log_{10}$ cfu/mL in the colon of fistulated horses fed a diet composed of 50% hay and 50% barley. When the diet ratio was changed to 70% hay and 30% barley, similar to the ratio of 66:34 (roughage:concentrate) used in the present study, the authors observed concentrations of 6.84 $\log_{10}$ cfu/mL in the colon. The concentrations of cellulolytic bacteria observed in the present study resemble that quantified by Julliand et al. (2001) of 4.78 $\log_{10}$ cfu/mL in the cecum and colon of equines fed different proportions of barley, and this community is correlated with fiber utilization efficiency (Drogoul et al., 2001). *In vitro* studies have shown the concentration of cellulolytic bacteria of 5.11 $\log_{10}$ cfu/mL fecal inoculum from animals fed meadow hay and commercial concentrate (60:40) (Desrousseaux et al., 2012).

Assuming that the fecal inoculum was diluted three times as much in 1:3, consequently, there was an initial incubation of smaller amounts of microorganisms when compared with the population present in the 1:1 dilution, which suggests that there was a slower development of the bacterial population until 24 h when the 1:1 dilution was used. As a result, the microorganisms of the 1:3 inoculum developed rapidly due to the greater amount of nutrients available from the nutrient solution (Santos et al., 2011).

According to Bueno et al. (2005), the cellulolytic bacteria adhere to the solid particles of the inoculum and, therefore, with an increased solid phase in the incubation, are able to reduce the lag phase period of the fibrous substrates. In the present study, adjustments were made to the inoculum preparation protocol: feces were kept diluted in nutrient solution for 1 h in a water bath at 39 °C, under constant agitation and sprayed with CO_2. This process aimed to increase the activity of cellulolytic bacteria present in the fecal inoculum.

Total anaerobes and *Lactobacillus* spp. values observed in the present study showed the same dilution factor as the concentrations of microorganisms observed by Julliand et al. (2001) and De Fombelle et al. (2001) in the colon of horses fed a diet with the same concentrate:roughage ratios as the horses of the present study.

The peak microbial growth was observed at approximately 10 h after incubation, when the cumulative gas production and nutrient degradation data were associated. However, the quantification of the microorganisms occurred at 24 h, which was after the point of maximum bacterial development. Thus, in *in vitro* studies with fecal inoculum, the microbial community should be quantified in the exponential growth phase.

Conclusions

Dilution does not alter the fermentation profile of the roughage substrate. Therefore, this procedure does not limit the comparison between studies and allows the use of equine

[1] Silva, V. P. 2017. Personal communication. Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brasil.

feces as source of inoculum for *in vitro* fermentation tests. The hydration of the substrate increases the fermentation process; thus, the lag phase time becomes insignificant and, therefore, can be irrelevant in terms of fermentation model settings. In addition, it increases the extent of substrate fermentation and nutrient degradation. Hydration of the substrate should be considered in *in vitro* fermentation test protocols for dehydrated roughage feedstuffs.

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