Tropentag 2015, Berlin, Germany
September 16-18, 2015

Conference on International Research on Food Security, Natural Resource Management and Rural Development
organised by the Humboldt-Universität zu Berlin and the Leibniz Centre for Agricultural Landscape Research (ZALF)

Faecal Inoculum as Alternative Microbial Source for *in vitro* Rumen Fermentation Techniques

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Introduction

In vitro techniques are simple method to determine feed value and most common source of microorganisms are fresh rumen liquor from fistulated animals. Rumen liquor obtained via esophagus is advantageous as it does not need surgical procedure in the animals, but the rumen liquor may be contaminated with saliva and not be representative from rumen (Cone et al., 2002). Whenever rumen liquor is obtained , from surgically modified animals there are many ethical and moral issues that have been raised (Mould et al., 2005).Because it is an invasive procedure and many countries restrict their use (Posada et al., 2012). The most common technique *in situ* is expensive, not suitable for routine analysis and is criticized for the use of surgically modified animals (Tagliapietra et al., 2012). Alternative sources of microorganisms have been studied, faecal inoculum from ruminants for use *in vitro* method to determine feed value. However, still there is no consensus between investigators about real potential of this source of microorganisms in the estimation of degradation and ruminal fermentation kinetics. The inexistence of a protocol that determine some aspects as obtain, species from donor, dilution rate and enrichment time (pre-incubation), and level of correlation of the assay with ruminal inoculum. However, there is need to use alternative inoculum due to the costly management of fistulated animals, surgical procedure is an highly invasive technique which many countries has restricted its use due to animal's welfare issues. The objective of the present study was to evaluate the degradability of dry matter (DDM), crude protein (DCP) and neutral detergent fiber (DNDF) of three substrates on *in vitro* and *in situ* assays using ruminal or faecal inoculum.

Material and Methods

Experiment was conducted at Laboratory of Animal Nutrition, CENA / USP - Brazil. Four adult fistulated Santa Ines animals were selected as inoculum donors of rumen liquor and fecal inoculum and animals were fed with 70:30 ratio of roughage:concentrate diet.
Substrates evaluated were: Maintenance Ration (MR) – diet formulated to feed sheep in maitenance composed 70% of Tifton 85 hay (Cynodon spp) and 30% concentrate (NRC, 2007); Tifton 85 Hay (TH) – substrate composed of 100% with Tifton 85 hay (Cynodon spp)

Concentrate LANA (CL) – Standard energy concentrate of LANA, composed of 70% ground corn, 28% soyben meal and 2% mineral salt. All substrates were ground in a Willey mill, with 2 mm screen sieve, for *in vitro* assay and chemical analysis.
Rumen digesta were obtained before morning feeding, from the rumen of the four fistulated sheeps. The rumen solid and liquid phase were collected separately from each animal and placed in prewarmed containers in anaerobic conditions. Equal volumes (50:50) of solid and liquid phases of rumen fluid from each animal were mixed in a blender for approximately 10 s and the rumen fluid was squeezed through two layers of gauze and combined among sheep. Every couple of animal formed a inoculum .The inoculum was maintained at 39°C and saturated with CO_2 to maintain anaerobic conditions until use (Maurice et al., 1999).
Fresh faeces samples was collected, about 50 or 100 g faeces, directly from the rectum of animals immediately after sampling of rumen fluid, and placed in plastic bags filled with CO_2, kept in styrofoam box. Every couple of animal formed a inoculum. The fresh faeces, 10 g, were diluted in 100 mL anaerobic buffer.
The fresh faeces (100 g) from all sheep were mixed in a blender for approximately 10 s, with half of the buffer solution (500 mL), then faecal inoculum was squeezed through two layers of gauze (first squeezed). The remaining solids were then remixed in a blender with more 500 mL of anaerobic buffer, was squeezed through two layers of gauze and homogenized with first squeezed. The faecal inoculum was maintained at 39°C and saturated with CO_2 to maintain anaerobic conditions until use (Maurice et al., 1999).
In situ degradation assay, samples were incubated in duplicate and due to the small volume of the rumen of sheep, incubation in the nylon bags was not done simultaneous, as recommended Huntington & Givens (1995). Bags were trapped in loops adapted to allow movement in the rumen, and these stoppers attached to the cannula through incubation.
In vitro degradation assay, nylon bags were incubated in the vessels of *in vitro* Daisy^{II} incubator (TECNAL model TE-150) containing faecal inoculum previously prepared, and was removed after 48 hours. Bags were also prepared to determine the washing loss of readily soluble material.
Nylon bags were used measuring 85 x 115 mm (35 µm porosity) with eight grams of sample (DM basis) previously ground in a Willey mill, with 2 mm screen sieve. After *in situ* and *in vitro* incubation, for every incubation times, nylon bags were quickly washed in water, placed in plastic bags and frozen until the end of incubation. Then, all bags were washed in a washing machine for three cycles of 10 minutes, as well as bags containing the same amount of sample to determine the loss of readily soluble material. After washing, bags were dried in an oven with forced air circulation at 60 ° C until constant weight.
Disappearance percentage of DM, CP and NDF were calculated in terms of weight difference between weighing before and after incubation *in situ* (rumen inoculum) and *in vitro* (faecal inoculum). The study was performed in a completely randomised design for 48h of incubation with a 3×2 factorial arrangement (feeds (100% concentrate, (70:30) maintenance ration and 100% Tifton hay) and two inoculants (rumen and faecal). Treatment means were compared by SNK test at 5% probability and correlation analysis was performed.

Results and Discussion

Disappearance percentage of DM, CP and NDF are present Table 1. There was interaction effect on substrate × inoculum for DDM ($P <0.01$), DCP ($P <0.01$) and DNDF ($P = 0.05$). The soluble fraction of the DM of maintenance ration, Tifton hay and concentrate were 23.91; 19:36 and 35.18%, respectively. On average, substrates incubated *in situ* resulted in greater potential degradation ($P <0.05$) compared to *in vitro* incubation for DDM (63.36×40.67), DCP (67.78×44.63) and DNDF (56.06×31.99) with regardless of incubation time.
These results differ from those obtained by Tufarelli et al., (2010), in this research no significant difference ($P> 0.05$) on dry matter digestibility (55.11x55.22), crude protein (56.68x56.79) and

neutral detergent fiber (35.98x36.06) of *Brachipodyum pinnatum* grass when incubated with rumen and fecal inoculum, respectively. These authors suggest as justification the high cell wall content, NDF and ADF. Structural carbohydrates are degraded more slowly, due to the structural conformation of cellulose and hemicellulose, and time 48 hours in this study was not sufficient to microbial attack from faecal inoculum was strongly evidenced.
Posada et al (2012) observed a lesser difference of dry matter digestibility between the rumen and fecal inoculum in time of 72 hours of incubation (0.0221 g/g incubated DM) compared to the time of 48 hours (0.0347 g/g incubated DM). These authors explain that the lower microbial density of faeces, affects the time of colonization of microorganisms.

Table 1 - Disappearance percentage of DM, CP and NDF on time 48 hours.

Substrate	Inoculum		CV (%)	P>F
	Ruminal	Faecal		
	DDM (%)			
Maintenance ration	57.30Ba*	39.10Bb	7.17	<0.0001
Tifton hay	44.62Ca	25.55Cb		
Concentrate	88.16Aa	57.36Ab		
	DCP (%)			
Maintenance ration	70.45Ba	64.93Ba	12.49	<0.0001
Tifton hay	47.52Ca	16.91Cb		
Concentrate	85.38Aa	52.04Ab		
	DNDF (%)			
Maintenance ration	52.12Ba	34.43Bb	8.66	0.0050
Tifton hay	27.98Ca	1.14Cb		
Concentrate	88.07Aa	60.39Ab		

*Values followed by the same lowercase letters (column) and capital (lines) do not differ by SNK test (P>0.05).

Among the three substrates tested, concentrate had higher DDM values, DCP and DNDF followed by maintenance ration and Tifton hay incubated *in situ* or *in vitro* respectively. The DCP did not differ (P <0.01) between sources of inoculum tested, but was the variable with the highest CV, on that basis, these values may be associated with poor uniformity of the material used. Laudadio et al (2009) found no differences in the digestibility of crude protein of *Artemisia campestris*, compared ruminal inoculum (43.2%) and fecal inoculum (42.5%) of sheep, however its CV was very low (1.22%). Results presented in this study are very scarce in the literature, especially if compared to the data *in vitro* from fecal inoculum versus data *in situ*.
However, the variation in results can be attributed to several factors, such as processing of the samples, differences in the chemical composition of feed, preparation of buffer solution, equipment handling and porosity of the filter bags (Laudadio et al, 2009;. Tufarelli et al., 2010).
Taking as reference the nutritional value of feeds tested and based on *in vitro* behavior of fecal inoculum, the concentrate showed the best results when incubated in the fecal inoculum, suggesting that for presenting high amounts of soluble carbohydrates associated with the lowest concentration of fibrous components (Wascheck et al., 2010). Another factor that is not directly associated with the source of inoculum would be the amount of sample incubated in nylon bags, which would be slightly above the contact area recommended by Huntington and Givens (1995), that would be 16 mg/cm^2. In this research, the surface area used was higher (40.92 mg/cm^2), so the very high value of surface area of the bag may have masked the true results using the faecal inoculum. Lattimer et al (2007) found higher rates (P <0.05) for *in vitro* digestibility of DM (55.90x52.23) and NDF (18.68x15.26) when used less amount of sample 0.250g (6.25 mg/cm^2) and 0,500g (12.5 mg/cm^2), respectively. These study suggest that less amount of incubated substrate would be equivalent to the amount of inoculum, resulting in higher degradation of

incubated material. The faecal microbial population differs from that of rumen fluid, as it has been influenced by gastric digestion and the contributions of the caecum to the microbial population.

Conclusions and Outlook

Our study concludes that although fecal inoculum showed lower degradability of feed constituents, it may be used as alternative microbial source for *in vitro* techniques.

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