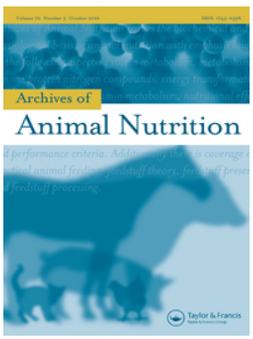

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Parameters of fermentation and rumen microbiota of Nellore steers fed with different proportions of concentrate in fresh sugarcane containing diets

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ABSTRACT

The objective of this study was to evaluate the effect of a fresh sugarcane-based diet and different roughage-to-concentrate ratios (70:30, 60:40, 40:60 and 20:80) on the rumen microbiota associated with rumen fermentation parameters and the intake and apparent digestibility of nutrients in Nellore steers. Eight rumen-cannulated Nellore steers (331 ± 8 kg BW) were distributed in a double 4 × 4 Latin square design balanced for the control of the residual effect. The ruminal pH decreased ($p < 0.01$) and the concentrations of N–NH_3, isovaleric and valeric acids increased linearly ($p < 0.05$) with an increase dietary concentrate level. Furthermore, an increased concentrate proportion reduced the population of *Fibrobacter succinogenes* and *Ruminococus flavefaciens* ($p < 0.01$) and increased the population of *Selenomonas ruminantium* and *Megasphaera elsdenii* ($p < 0.01$). The protozoa count revealed a predominance of the genus *Entodinium*. The synthesis of microbial N [g/d] and the efficiency of microbial synthesis [g of microbial N/kg of organic matter apparently digested in the rumen] increased as the proportion of concentrate was increased ($p < 0.05$). Therefore, it can be concluded that an increasing proportion of concentrate in sugarcane-containing diets enhances the synthesis of microbial protein and does not alter the fibre digestibility, although the population of fibre fermenting bacteria was reduced.

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1. Introduction

The maintenance of ruminal microbiota is of paramount importance to ruminants because the majority of the energy utilised in their biological processes originates from rumen fermentation products. Its composition may be influenced by the roughage-to-concentrate ratio of the diet (Schwartzkopf-Genswein et al. 2003). Thus, diets with a higher proportion of concentrate may stimulate microbial growth by increasing the availability of rumen-fermentable carbohydrates. However, high concentrate diets

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usually show fast rates of fermentation and production of volatile fatty acids (VFA), which may result in low rumen pH (<6.0) (Yang and Beauchemin 2009). Such conditions in the rumen may reduce the activity of fibrolytic microorganisms such as the bacteria *Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens* and increase the activity of lactate-producing bacteria (*Streptococcus bovis*) (Nagaraja and Titgemeyer 2007). In contrast, high-roughage diets may limit the intake and digestibility of nutrients (González et al. 2012), thereby restricting the availability of energy and ammonia in the rumen, which are nutritional factors that limit microbial growth (Clark et al. 1992). The intake and use of nutrients from roughage are influenced by the structure and chemical composition of the fibre fraction, which is a determinant of the quality of roughage (Tafaj et al. 2005) used for feeding animals in the feedlot.

In tropical and subtropical regions, among roughages utilised for feedlot feeding, like corn and sorghum silages, hay are fed most commonly because of its easy cultivation and high yields (Santos et al. 2011). Corn silage is the forage used most commonly in dairy cow diets in many countries. However, in regions of tropical and subtropical climate, sugarcane (*Saccharum officinarum* L.) is more productive than corn (Harris et al. 1983). Thus, an alternative roughage that can be used for cattle is freshly chopped sugarcane, which has a high biomass productivity, a maturity that coincides with the period of pasture scarcity, a high sucrose content and moderate levels of insoluble neutral detergent fibre (iNDF) (Lascano et al. 2012). In addition, the benefits of freshly chopped sugarcane as a fibre source for cattle are likely to be greater in Brazil than in the North America and Europe due to its availability and the low cost per unit of dry matter (DM) yield (Santos et al. 2011). Another advantage of diets with high sucrose content is the association with high concentrations of ammonia in rumen fluid which can promote growth of rumen microorganisms (Hall and Huntington 2008). Thus, the use of this roughage may be a strategy for maintaining a constant cattle production (Queiroz et al. 2012). However, as a basal feed for ruminants, freshly chopped sugarcane has nutritional limitations due to its low protein and mineral content and high levels of recalcitrant fibre (Queiroz et al. 2012). To date, no studies have examined the effect of an increased ratio of fresh sugarcane: concentrate on the population of ruminal microbes and its relationship with rumen-fermentation parameters in Nellore steers.

Therefore, it was hypothesised that the population of ruminal microorganisms could be affected by a fresh sugarcane-based diet with a higher proportion of concentrate, consequently promoting changes in the ruminal fermentation, intake, digestibility and efficiency of the supplied diet. Based on these assumptions, the objective of this study was to determine the effect of fresh sugarcane-based diets with different roughage-to-concentrate ratios on the population of rumen microorganisms, intake, digestibility and rumen fermentation.

2. Material and methods

The protocol used in this study was in accordance with the Brazilian College of Animal Experimentation (COBEA – Colégio Brasileiro de Experimentação Animal) guidelines and was approved by the Ethics, Bioethics, and Animal Welfare Committee (CEBEA – Comissão de Ética e Bem Estar Animal) of the FCAV–UNESP–Jaboticabal campus,

Brazil (017621/11). The material and methods were the same as in a previous study (Ribeiro et al. 2015), conducted simultaneously to evaluate the effect of diets with four different roughage (hay Tifton 85): concentrate ratios on intake, apparent total tract digestibility (ATTD), rumen fermentation and rumen microbiota of Nellore feedlot steers.

2.1. *Animals and experimental feeds*

Eight Nellore steers fitted with ruminal silicone-type cannulas (diameter 10 cm) and duodenal T-type cannulas were used in a double 4 × 4 Latin square design experiment. The steers were ruminally and duodenally cannulated at about 8 months of age and could recover for at least 8 weeks before the study. The mean initial and final body weight (BW) of steers was 318 ± 9 kg and 357 ± 12 kg, respectively. The experimental diets were formulated to provide a DM intake of 18.0 g/kg BW (daily 6.0 kg/animal), which were calculated using (AFRC 1993). The roughage was fresh sugarcane (*Saccharum officinarum* L., variety IAC-862480) and the concentrate was composed of corn, soybean meal and urea (Table 1).

The four dietary treatments were the following ratios of roughage to concentrate (on DM basis): 70:30, 60:40, 40:60 and 20:80 (Table 2).

The fresh sugarcane was chopped to a length of 2–3 cm. The ingredients of concentrate were ground with a hammer mill fitted with strainers and 2-mm sieves. Continuous homogenisation of the diets was performed in a horizontal mixer for 15 min. The animals were fed with fresh chopped sugarcane, experimental concentrates and a mineral supplement at 100 g/animal (BELLNUTRI®, containing per kg: 146 g calcium, 40 g phosphorus, 40 g sulphur, 130 g sodium, 1.35 g copper, 1.04 g manganese, 5 g zinc, 100 mg iodine, 80 mg cobalt, 26 mg selenium; max. 800 mg fluor) once a day at 06:00 h as total mixed ration (TMR). At 16:00 h the feed residues in the trough were mixed again to stimulate feed intake and to reduce the chances of fermentation. Throughout the entire experimental period, the allowance was adjusted to allow refusals of approximately 10% of the total amount consumed on the previous day. Feed refusals were collected and weighed before feeding and subsamples were obtained and frozen at −20°C.

For the feeding experiment, four consecutive 21-days periods were used. Each period consisted of 14 d adaptation, 5 d faeces and urine collection and 1 d sampling of

Table 1. Chemical composition of dietary ingredients.

Composition	Sugarcane	Ground corn	Soybean meal	Urea
Dry matter (DM) [g/kg]	276	885	866	990
Organic matter [g/kg DM]	964	965	924	–
Crude protein [g/kg DM]	29	91	518	2890
ADFom [g/kg DM]†	345	61	83	–
aNDFom [g/kg DM]‡	553	125	158	–
Lignin [g/kg DM]	46	8	2	–
Non fibrous carbohydrates [g/kg DM]	381	711	234	–
Ether extract [g/kg DM]	9	38	13	–
Gross energy [MJ/kg]	18.4	19.7	19.7	–

Notes: †ADFom, acid detergent fibre expressed exclusive of residual ash; ‡aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.

Table 2. Composition of the experimental diets containing different roughage (sugarcane)-to concentrate ratios.

	Roughage-to-concentrate ratios of the experimental diets			
	70:30	60:40	40:60	20:80
Ingredients [g/kg dry matter]				
Sugarcane	700	600	422	200
Ground corn	172	275	480	675
Soybean meal	116	113	84	110
Urea	12	12	14	15
Chemical composition				
Dry matter (DM) [g/kg]	478	540	627	763
Organic matter [g/kg DM]	956	946	949	947
Crude protein [g/kg DM]	130	155	139	162
ADFom [g/kg DM][†]	262	234	182	119
aNDFom [g/kg DM][‡]	427	385	307	211
NFC[§] [g/kg DM]	419	430	522	591
Gross energy [MJ/kg]	18.9	18.9	19.1	19.3

Notes: [†]ADFom, acid detergent fibre expressed exclusive of residual ash; [‡]aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; [§]NFC, non-fibrous carbohydrates, estimated as: NFC = 100 − (Crude protein + Ether extract + Ash + NDF).

ruminal fluid. The latter was collected for measurement of ruminal pH, ammonia N (NH_3–N), and VFA as well as bacteria and protozoa quantification.

2.2. *Data collection and sampling procedures*

Faeces were collected for 5 d to estimate the ATTD of dietary constituents. At the end of each collection day, faeces were weighed and homogenised. At the end of each period, for each animal a pooled sample was made based on the pre-dried weight of each sampling day. The total amount of urine was collected for 5 d using funnel collectors attached to the animals. The funnel collector was connected to a flexible tube of polyethylene, which directed the urine to vessels containing 200 ml 20% H_2SO_4 to keep the final urine pH below 3 to prevent bacterial destruction of nitrogenous compounds in urine. After each 24-h collection period, the total weight and volume of excreted urine were determined. A pooled sample was made from the five sampling days. The pooled sample was homogenised and a 10-ml subsample was diluted with 40 ml of 0.036 N H_2SO_4.

Duodenal samples were collected for 2 d (fourth and fifth data collection days during the study period) at 6 h intervals. The sample collection during the second day was delayed to ensure that every 3 h in a 24-h period was properly represented. Samples were kept at −10°C, and at the end of the period, one sample was made by pooling the samples of each animal in each period. The iNDF content was used as indicator for the daily DM flows at the duodenum as described by Ribeiro et al. (2015).

Ruminal contents were sampled at day five of each sampling period to determine the pH values and the concentrations of VFA and NH_3–N. Samples were taken at 0, 2, 4, 6, 8, 10, 12 and 14 h after feeding and pH was measured after the contents were filtered. Two 20 ml aliquots were stored at −10°C, which were later used to determine NH_3–N according to the methodology of Fenner (1965) adapted for use in Kjeldahl distillation. The VFA concentration was determined according to the methodology described by Palmquist and Conrad (1971).

Microbial protein synthesis was calculated via urinary total excretion of purine derivatives (allantoin + uric acid) according to the technique of Fujihara et al. (1987).

Samples of offered feed, feed refusals and faeces were dried at 55°C for 72 h and ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ) to pass a 1-mm screen. Samples of sugarcane, concentrates and feed refusals were analysed for DM (Method 934.01), ash (Method 942.05) and ether extract (EE) (Method 920.39) according to (AOAC 1990). The EE contents in faeces were also determined by Method 920.39. Nitrogen was determined using a LECO FP-528 nitrogen analyzer (LECO Corp., St. Joseph, MI).

Neutral detergent fibre was determined using α-amylase (aNDF) and without the addition of sodium sulphite following Van Soest et al. (1991) and adapted for the Ankom200 Fiber Analyzer (Ankom Technology, Fairport, NY). Acid detergent fibre (ADFom) was determined using the method described by Goering and Van Soest (1970) and adapted for the Ankom200 Fiber Analyzer (Ankom Technology, Fairport, NY). Acid detergent lignin was determined by solubilisation of cellulose with sulphuric acid according to Van Soest and Robertson (1985). Non-fibrous carbohydrates (NFC) were estimated as described by Ribeiro et al. (2015). Gross energy (GE) was obtained by the combustion of samples in an adiabatic bomb calorimeter IKA® Model 2000 Basic.

2.3. *Microbial population*

The quantification and identification of rumen bacteria was performed on the rumen fluid of animals consuming the most extreme diets, which contained fresh sugarcane-to-concentrate ratios of 70:30 and 20:80. Intake and ATTD were similar between diets with ratio of 60:40 and 40:60 and were therefore omitted from microbial population analysis. On day 16 of the trial periods, samples of ruminal contents were collected via cannulas before the morning feeding. Fifty grams of the rumen contents were immediately added to 50 ml of phosphate saline buffer (pH 7.4), stirred vigorously for 3 min and then filtered with a mesh fabric (100 µm). The filtrate was subjected to centrifugation at 16,000 *g* for 10 min at 4°C. The supernatant was discarded and the remaining pellet was resuspended in 4 ml of Tris-EDTA buffer (10X, pH 8.0). The resuspended content was centrifuged at 16,000 *g* for 10 min at 4°C, the supernatant was discarded, and the pellet was immediately stored frozen (−20°C) for a period of 3 months.

Extraction of DNA was conducted in sample of 250 mg using the extraction kit AxyPrep™ Bacterial Genomic DNA Miniprep (Axygen-Biosciences, Union City, USA). The integrity and quantity of the DNA was checked by electrophoresis on agarose gel (0.8%), and complementary DNA was assessed by spectrophotometry (Thermo Scientific NanoDrop™ 1000) for evaluation of its quality and quantity.

Rumen bacteria selected for qPCR quantification represent the most important ruminal microbial groups according to their metabolic activity. For example, fibrolytic (*R. albus, F. succinogenes*, and *R. flavefaciens*), amylolytic (*S. bovis* and *Selenomonas ruminantium*), lactate producers (*Lactobacillus* sp.), and lactate consumers (*M. elsdenii*) were represented (Belanche et al. 2012). The primers used in this study are shown in Table 3.

Four concentrations (200, 400, 600 and 800 nM) of forward and reverse primers were tested to determine minimum primer concentration giving the lowest threshold cycle (Ct) and to reduce non-specific amplification before starting the reaction. After the concentration of primers was chosen, it was validated with different concentrations

Table 3. Primers used in the present study.

		Primer* (5′ to 3′)	Product size [bp]	Efficiency of primer [%]
Fibrobacter succinogenes[†]	Forward	GTTCGGAATTACTGGGCGTAAA	121	98
	Reverse	CGCCTGCCCCTGAACTATC		
Ruminococcus flavefaciens[†]	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	132	96
	Reverse	CGGTCTCTGTATGTTATGAGGTATTACC		
Ruminococcus albus[‡]	Forward	CCCTAAAAGCAGTCTTAGTTCG	175	97
	Reverse	CCTCCTTGCGGTTAGAACA		
Streptococcus bovis [§]	Forward	TTCCTAGAGATAGGAAGTTTCTTCGG	127	95
	Reverse	ATGATGGCAACTAACAATAGGGGT		
Selenomonas ruminantium [§]	Forward	GGCGGGAAGGCAAGTCAGTC	83	98
	Reverse	CCTCTCCTGCACTCAAGAAAGACAG		
Lactobacillus sp.[§]	Forward	AGCAGTAGGGAATCTTCCA	61	95
	Reverse	ATTCCACCGCTACACATG		
Megasphaera elsdenii	Forward	GACCGAAACTGCGATGCTAGA	129	96
	Reverse	CGCCTCAGCGTCAGTTGTC		

Notes: *Primers used for qPCR normalisation; [†]Denman and McSweeney (2006); [‡]Mosoni et al. (2007); [§]Khafipour et al. (2009).

of DNA (150, 125, 100, 50 and 25 ng). The value of the slope was determined and efficiency of primers were calculated using the following the equation:

$$\text{Efficiency } [\%] = 10^{(-1/\text{slope})-1}$$

The amplifications were performed in triplicate along with a non-template control using a 7900 Real Time PCR System (Applied Biosystems, Foster City, California 94404, USA.). The qPCR reaction was carried out using 25 ng total DNA in a reaction containing 7.5 µl SYBR® Green PCR Master Mix (Bio-Rad, Hercules, California, USA), 10 pmol of primer pair and H_2O to a final volume of 25 µl. Cycling conditions were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 1 min and 78°C for 30 s. After one cycle of amplification, a step is added increasing the temperature from 60°C to 95°C to obtain a dissociation curve of the reaction products, used for analysing the specificity of amplification.

Relative quantification was used to determine species proportion. The results were expressed as a 16S rDNA ratio of general bacteria, following the equation:

$$\text{Relative quantification} = 2^{-(\text{Ct target} - \text{Ct total bacteria})},$$

where Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold.

Direct counts of protozoa were performed in a Sedgewick-Rafter counting chamber (Dehority et al. 1989). The samples were diluted with 20% glycerol and stained using Lugol's solution before counting the cells (D'Agosto and Carneiro 1999). Cell counts were obtained from rumen content aliquots that were preserved in formalin (a solution of equal parts water and 370 ml/l formaldehyde) (D'Agosto and Carneiro 1999).

2.4. *Statistical analyses*

The data of intake, ATTD, microbial protein synthesis and protozoa quantification were analysed as a double 4 × 4 Latin square using the PROC MIXED procedure of SAS

(Statistical Analysis System, version 9.2). The general mathematical model was represented as follows:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + s_k + \alpha\beta_{ij} + e_{ijk},$$

where Y_{ijk} is the observation on steers k given treatment i at period j; α_i is the fixed effect of the i-th treatment, $i = 1, 2, \ldots, nt$; β_j represents the fixed effect of the j-th period, $j = 1, 2, \ldots, np$; and s_k is the random effect of the k-th steers, $k = 1, 2, \ldots, nc$, with variance component σ^2_c (Tempelman 2004). In the statistical model normally and independently distributed errors were assumed. Ruminal pH, NH_3–N and VFA data were analysed as a double 4 × 4 Latin square data with repeated measures overtime using the PROC MIXED procedure of SAS (Statistical Analysis System, version 9.2), according to the following model:

$$Y_{ijkl} = \mu + S_i + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk} + H_l + \alpha H_{kl} + \omega_{ijkl},$$

where Y_{ij} is the dependent variable; μ is the overall mean; S_i is the effect of steer i; α_i is the i-th effect of the treatment or diet; β_j is the j-th effect of the period; $\alpha\beta_{ij}$ is the effect of the interaction between treatment i and period j; ε_{ijk} is the whole plot error; H_l is the effect of the sampling time l; αH_{kl} is the interaction between treatment k and sampling time l and ω_{ijkl} is the split plot error. The model included the fixed effect of treatment, time, treatment × time interaction, and random effects of animal and period. The structure of errors that best fitted the data according to the Bayesian information criterion (BIC) was used. The means were compared using the contrast option of the PROC MIXED of SAS and declared significant at $p < 0.05$ for linear or quadratic effects.

Relative quantities of 16S rRNA as determined from real-time PCR were analysed using PROC ANOM to determine significant differences in copy number between treatments for each targeted bacterial species. A significance level $p < 0.05$ was accepted as representing statistically significant differences.

3. Results

The intake of DM, organic matter (OM) and crude protein was not affected by the dietary concentrate level (Table 4). The increased dietary proportion of concentrate resulted in a linear increase of the ATTD of DM ($p < 0.01$) and OM, where the ATTD of OM displayed a quadratic behaviour ($p < 0.01$). However, the dietary treatments did not affect the ATTD of fibre or crude protein.

Higher dietary proportions of concentrate resulted in a linear decrease ($p < 0.01$) of the average pH value, and the opposite was found for the concentration of $N–NH_3$, which increased linearly with the dietary concentrate proportion ($p < 0.01$, Table 5). In animals fed a higher concentrate proportion of (20:80), ruminal pH remained below 6.0 already after 6 h of feeding, whereas in treatments 60:40 and 40:60 this occurred 10 h after feeding (Figure 1). On the other hand, when the sugarcane-to-concentrate ratio was 70:30, the mean pH values remained above 6, independent of time after feeding.

Table 4. Effect of four roughage (sugarcane)-to-concentrate ratios on feed intake and apparent total tract digestibility (ATTD) of nutrients after feeding to Nellore steers.

	Roughage-to-concentrate ratios of the experimental diets						Contrasts[†]	
	70:30	60:40	40:60	20:80	SEM	*p*-Value	Linear	Quadratic
Intake [kg/d]								
Dry matter	6.00	6.90	7.10	6.90	0.599	0.471	–	–
Organic matter	5.80	6.70	6.80	6.50	0.601	0.532	–	–
Crude protein	0.90	0.90	1.00	1.00	0.102	0.250	–	–
aNDFom [‡]	2.30	2.30	1.70	1.40	0.203	< 0.01	< 0.01	–
Non-fibrous carbohydrates	2.60	3.10	3.80	4.10	0.199	< 0.01	< 0.01	–
ATTD [%]								
Dry matter	70.9	70.9	80.1	81.1	7.80	< 0.01	< 0.01	–
Organic matter	69.9	70.8	79.8	81.8	2.50	< 0.01	–	< 0.01
Crude protein	69.6	63.4	72.9	74.8	3.10	0.123	–	–
aNDFom [‡]	58.0	52.5	62.4	65.4	3.30	0.145	–	–
Non-fibrous carbohydrates	71.7	79.6	79.5	70.0	7.10	0.073	–	–

Notes: [†]*p*-values > 0.05 are not reported; [‡]aNDF, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.

Table 5. Effect of different dietary roughage (sugarcane)-to-concentrate ratios on rumen parameters in Nellore steers.

	Roughage-to-concentrate ratios of experimental diets					*p*-Value	Contrast[†]	
	70:30	60:40	40:60	20:80	SEM	Diets	Diets	Time
N–NH_3 [mg/dl]	18.7	19.6	23.2	29.9	2.78	< 0.01	*L* (< 0.01)	*Q* (< 0.01)
pH	6.49	6.32	6.27	6.01	0.081	< 0.01	*L* (< 0.01)	*Q* (< 0.01)
Volatile fatty acids [mmol/l]								
Total	90.4	93.3	93.3	93.8	7.74	0.631	-	-
Acetic acid (C2)	55.1	57.9	57.2	56.9	4.16	0.724	-	-
Propionic acid (C3)	21.8	20.6	20.8	19.6	2.10	0.892	-	*L* (< 0.01)
Butyric acid (C4)	9.88	10.9	11.4	11.5	1.95	0.237	-	*L* (< 0.01)
Valeric acid	1.12	1.36	1.19	1.63	0.181	0.031	*L* (< 0.05)	*L* (< 0.05)
Isobutyric acid	0.88	0.86	1.08	1.34	0.171	0.981	-	-
Isovaleric acid	1.62	1.59	2.18	2.76	0.250	< 0.01	*L* (< 0.01)	*Q* (< 0.01)
C2:C3	3.06	2.97	2.86	2.76	0.232	0.741	-	*Q* (< 0.01)
Microbial N								
[g/d]	17.4	51.3	61.2	64.4	15.2	< 0.01	*L* (0.04)	-
[g/kg of OMADR[‡]]	4.9	12.8	11.8	15.6	3.7	0.032	*L* (0.01)	-

Notes: [†]Significant contrasts: *L* = linear, *Q* = quadratic; values for $p > 0.05$ are not reported; [‡]OMADR, organic matter apparently digested in the rumen.

No treatment effects were found for the concentration of total rumen VFA or acetic (C2), propionic (C3), butyric (C4) and isobutyric acid or on the C2:C3 ratio. However, the concentration of isovaleric acid ($p < 0.01$) and valeric acid ($p = 0.02$) was enhanced linearly with an increasing concentrate level (Table 5).

The population of fibrolytic bacteria was influenced by the dietary concentrate level (Table 6). The number of 16S copies of the species *F. succinogenes* and *R. flavefaciens* was significantly decreased in animals fed with a higher proportion of concentrate (20:80, Table 6). However, the species characterised as consumers of lactic acid (*S. ruminantium* and *M. elsdenii*) and producers of lactic acid (*Lactobacillus* sp.) increased ($p < 0.01$) when the animals received diets with more concentrates. The species *R. albus* and *St. bovis*, however, were not influenced by dietary treatments.

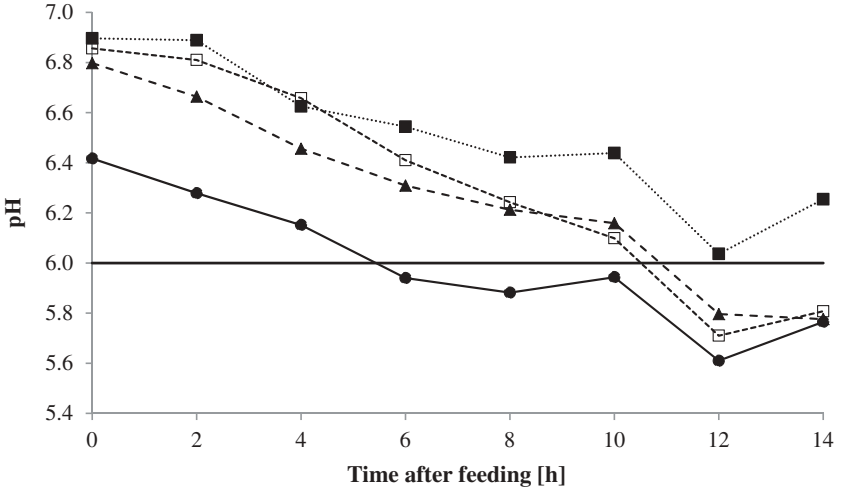

Figure 1. Rumen pH in Nellore steers fed with diets containing different fresh roughage (sugarcane)-to-concentrate ratios (■ 70:30; □ 60:40; ▲ 40:60; □20:80); horizontal line: critical pH according to Russell and Dombrowski (1980). Significant effects: diets ($p < 0.01$); interaction diets × time ($p < 0.05$) (standard error of means = 0.036).

Table 6. Effect of different roughage (sugarcane)-to-concentrate ratios on the proportion of ruminal bacteria [log copies/25 ng DNA].

	Roughage-to-concentrate ratios of the experimental diets			
	70:30	20:80	SEM	*p*-Value
Fibrobacter succinogenes	10.48	8.40	0.10	< 0.01
Ruminococcus flavefaciens	9.41	6.80	0.10	< 0.01
Ruminococcus albus	11.66	11.31	0.17	0.14
Selenomonas ruminantium	5.01	6.50	0.10	< 0.01
Streptococcus bovis	4.85	5.23	0.33	0.46
Megasphaera elsdenii	4.83	6.59	0.19	< 0.01
Lactobacillus sp.	4.15	6.47	0.13	< 0.01

The protozoan count revealed a predominance of the genus *Entodinium*, and the total population of protozoa and the genus *Eudiplodinium* decreased with an increased concentrate level in the diet ($p < 0.05$, Table 7). For the genus *Eremoplastron* the highest and lowest counts were measured at concentrate levels of 40% and 80%, respectively.

4. Discussion

In this study the effects of a fresh sugarcane-based diet and different roughage-to-concentrate ratios on the rumen fermentation of Nellore steers and their ruminal microorganism populations were evaluated. It was observed that an increased proportion of concentrate in a diet with fresh sugarcane changed the ruminal bacterial and protozoan populations and the fermentation parameters; however, ATTD of fibre and OM intake were not altered.

Table 7. Effect of different roughage (sugarcane)-to-concentrate ratios on the concentration of rumen protozoa in Nellore steers.

	Roughage-to-concentrate ratios of the experimental diets						Contrast[†]	
	70:30	60:40	40:60	20:80	SEM	*p*-Value	Linear	Quadratic
Total protozoa[‡] [10^6/ml]	21.27	21.67	18.78	17.48	1.39	0.01	< 0.001	–
Entodinium [10^6/ml]	7.26	7.19	7.24	7.26	0.10	0.83	–	–
Eudiplodinium [10^4/ml]	3.97	2.56	2.11	1.55	1.40	0.02	0.026	–
Metadinium [10^4/ml]	2.84	2.31	2.46	2.27	1.32	0.81	–	–
Eremoplastron [10^4/ml]	1.47	2.93	1.47	1.00	0.49	0.02	–	0.002
Dasytricha [10^4/ml]	1.95	2.51	1.49	1.93	0.62	0.72	–	–
Isotricha [10^4/ml]	1.00	1.49	1.87	1.00	0.38	0.30	–	–

Notes: [†]*p*-values > 0.05 are not reported; [‡]Include protozoa groups not shown in this table.

4.1. *Intake, digestibility and parameter fermentation*

The DM intake in ruminants is modulated by two factors: the rumen-fill effect and metabolic feedbacks that would be responsive to dietary energy (Carter and Grovum 1990). In this study, the intake of DM and OM were both unaffected by the tested diet, which demonstrates that none of the factors limited the intake. Fresh sugarcane as a source of roughage showed similar effects on intake as other roughages; for instance Tifton 85 hay (low sugar content) which was used in the same roughage proportions (70:30, 60:40, 40:60 and 80:20) by Ribeiro et al. (2015). On the other hand, the DM intake of the animals in this experiment was on average 11% higher than in the study with Tifton 85 hay (Ribeiro et al. 2015). This can be seen as a consequence of the amount of soluble carbohydrates (sucrose), which may result in higher intake due to the greater capacity for colonisation by fibre microorganisms.

The higher NFC intake caused by the increased proportion of concentrate also increased the ATTD of GE and DM. According to Forbes (1996), diets with higher levels of NFC are also better digested and contain more energy due to the higher degradation of these carbohydrates in the rumen, which promotes the accumulation of VFA, decreases the rumen pH, and reduces the aNDF intake, thereby reducing the rumination time required by the animals (Zebeli et al. 2006).

Regarding VFA, with an increased in NFC intake an enhanced ruminal concentration of propionic acid and consequently a reduction of the C2:C3 ratio was expected. However, this result did not occur, and the concentration of propionic acid observed in this study was different from that reported by Ribeiro et al. (2015), which was possibly due to the sugar content present in the fresh sugarcane increasing the propionate production (Sousa et al. 2014), similar to diets with high concentrate level. Another explanation for this result can be seen in the metabolism of valeric acid, which can be formed partly from the condensation and reduction of acetate and propionate (Prins 1977). This could have been the fate of a small proportion of VFA.

4.2. *Changes of ruminal microorganisms*

Changes in the diet, such as the amount of fibre, sugar and starch, can affect the ruminal ecosystem and the efficiency of ruminal fermentation. The population of the species *S. ruminantium* was increased at the highest dietary concentrate level. This

bacterial species plays a role in the production of propionic acid via succinate decarboxylation (Wolin and Miller 1997). Nevertheless, this alteration did not occur, which demonstrates that the roughage utilised (fresh sugarcane) was essential for the maintenance of the ruminal-fermentation conditions due to its high soluble-carbohydrate (sucrose) content. However, the increased concentration of valeric and isovaleric acid resulted by the raise of the population of *M. elsdenii*. This bacterium has the ability to ferment amino acids and produce valerate and isovalerate as fermentation products (Zebeli et al. 2012). On the other hand, the relative population of *S. bovis* was not influenced by the increased concentrate proportion, which was in contrast to the observations of Ribeiro et al. (2015). This effect was likely due to the high content of sucrose in the fresh sugarcane influencing the growth rate of *S. bovis* and rumen fermentation similarly to NFC, which was not observed when steers were fed with Tifton 85 hay.

The increased proportion of concentrate in the diet led to changes in the supply of substrates as well in the population of ruminal bacteria, which promoted an increase in the population of lactic acid bacteria (*Lactobacillus* sp.) at the expense of the fibrolytic bacteria (*F. succinogenes, R. flavefaciens*).

The growth of cellulolytic bacteria is favoured by higher concentrations of aNDF from roughage and by higher ruminal pH (Fernando et al. 2010). Some studies have shown that a ruminal pH above 6.2 is required for optimum growth of fibrolytic bacteria (Russell and Dombrowski 1980). In the present study, the ruminal pH was above 6.0 throughout the day with a higher proportion of fresh sugarcane in the diet (70%), and it was below 6.0 from 6 h after feeding with a higher concentrate level (80%), suggesting that pH was likely limiting for the optimal growth of fibrolytic bacteria.

Some authors have reported that the enzymatic activity of fibrolytic bacteria is inhibited when starch is present in the ruminal environment and that NFC-fermenting bacteria release inhibitory compounds of protein origin (bacteriocins), which restrain the activity of fibrolytic bacteria (Fernando et al. 1965). This assertion is corroborated by the results observed in this study because an increased proportion of concentrate in the diet increased the proportion of NFC and reduced the population of fibrolytic bacteria.

Despite the reduction of the fibrolytic bacteria, no significant differences were observed in the ATTD of aNDF. Some authors have reported that the three fibrolytic species studied in this experiment (*F. succinogenes, R. flavefaciens* and *R. albus*) are the most important in fibre degradation (Belanche et al. 2012). Furthermore, other nutritional factors are also responsible for fibre digestibility, such as dietary composition, feed intake, and feed passage rate.

The predominance of the genus *Entodinium* is consistent with the observations of other authors who investigated the populations of rumen protozoa in cattle under different feeding regimes, for example, diets with a higher proportion of sugarcane (Franzolin and Franzolin 2000).

When compared with the group receiving the diet with the lowest proportion of roughage (20:80), feeding the diet with the highest proportion of roughage (70:30) caused a 2.6-fold increase of the protozoa genus *Eudiplodinium*. This fact may be associated with its ability to use polysaccharides and soluble carbohydrates (Hungate

1966), insofar as diets with a higher proportion of fresh chopped sugarcane present higher levels of soluble carbohydrates (sucrose). However, the genus *Eremoplastron*, belonging to the subclass *Entodiniomorpha*, was found at higher levels in treatment 60:40 likely due to the ability of this genus to utilise cellulose in its metabolism (Williams 1986).

These results corroborate the findings of Calsamiglia et al. (2012), wherein an increased availability of highly rumen-fermentable carbohydrates determines the microbial growth rate and consequently improves the use of ruminal $N–NH_3$ when it is not limiting. This fact was observed also in our study in which $N–NH_3$ was not limiting because its concentration was higher than 5 mg/dl in all diets, which is the minimum concentration necessary (Satter and Roffler 1975). Therefore, the synthesis of microbial protein was not impaired.

The rumen microbiota can be manipulated according to the proportion of concentrate in the diet. However, when fresh sugarcane is used as roughage in diets with increasing proportions of concentrate, the synthesis of microbial protein can be optimised without changing the ATTD of fibre.

In conclusion, increasing the proportion of concentrate in diets containing fresh sugarcane promotes alterations in ruminal parameters, reduces populations of the bacteria *F. succinogenes* and *R. flavefaciens*, leaves fibre digestibility unaltered and increases the synthesis of microbial protein.

Disclosure statement

No potential conflict of interest was reported by the authors.

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