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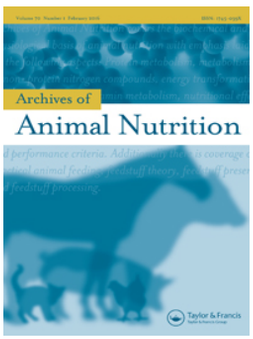

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Effect of different levels of concentrate on ruminal microorganisms and rumen fermentation in Nellore steers

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ABSTRACT

The aim of this study was to investigate the effect of different dietary levels of concentrate on feed intake, digestibility, ruminal fermentation and microbial population in steers. Eight Nellore steers fitted with ruminal cannulas were used in a double 4 × 4 Latin square design experiment. The dietary treatments consist of four different proportions of concentrate to roughage: 30:70, 40:60, 60:40 and 80:20% in the dry matter, resulting in Diets 30, 40, 60 and 80, respectively. The roughage was corn silage, and the concentrate was composed of corn, soybean meal and urea. Apparent digestibility of organic matter and crude protein showed a linear association with concentrate proportion ($p = 0.01$), but the increased concentrate levels did not affect the digestibility of fibre. The lowest ruminal pH-values were observed in animals fed with Diet 80, remaining below pH 6.0 from 6 h after feeding, while in the other diets, the ruminal pH was below 6.0 not before 12 h after feeding. After feeding Diet 80, the ammonia concentration in the rumen was significantly the highest. Higher dietary concentrate levels resulted in a linear increase of propionic acid concentrations, a linear reduction of the ratio acetic acid to propionic acid ($p < 0.01$) and a linear increased synthesis of microbial nitrogen ($p < 0.001$). The predicted production of methane was lower in diets with greater amounts of concentrate ($p = 0.032$). The population of methanogens, *R. flavefaciens* and *R. albus* decreased with higher concentrate levels, while the population of *S. ruminantium* increased ($p < 0.05$). The results indicate that greater amounts of concentrate do not decrease ruminal pH-values as much as expected and inhibit some cellulolytic bacteria without impairing the dry matter intake and fibre digestibility in Nellore steers.

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Bacteria; digestibility; fibre content; protozoa; rumen fermentation; steers; volatile fatty acids

1. Introduction

Ruminal microorganisms transform plant forage into high-quality foods through microbial fermentation, which also produces enteric methane as a natural by-product (Murray et al. 1976). Therefore, rumen fermentation has been greatly manipulated by

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increasing the amount of concentrate in the diet, with the aim of satisfying nutritional requirements, improving animal productivity and reducing the environmental impact. However, the use of diets with a high amount of concentrate may be related with metabolic disorders related to the low pH, high concentrations of volatile fatty acids (VFA) and high osmolality (Devries et al. 2014).

In addition, the grain-based concentrates may reduce the activity of fibrolytic microorganisms, which may cause a reduced digestibility, depressed nutrient intake and can lead to a decrease in the animal's performance (Archimède et al. 1997; González et al. 2012). However, in the rumen, the activity of consumers and producers of lactic acid increases because these microorganisms are not sensitive to lower pH and therefore can make use of greater substrate availability found in greater amounts of concentrates (Nagaraja and Titgemeyer 2007).

On the other hand, when ruminants fed with greater proportions of roughage this may limit the feed intake, reduces the energy efficiency and hence lesser the synthesis of microbial protein (Yang and Beauchemin 2006). Thereby, greater amounts of concentrate in the diet may improve the efficiency of microbial capture of ammonia in the rumen, thus bypassing the ammonia pool (Russell et al. 1983). Additionally, increasing the amount of concentrate in the diet has been used as a strategy to decrease methanogenesis per unit of feed intake, due to a shift in ruminal fermentation towards propionogenesis (Doreau et al. 2011).

In this study, the hypothesis was raised that greater amounts of concentrate in the diet can promote changes in the ruminal fermentation, resulting in a hostile environment for fibrolytic microorganisms, reducing the fibre breakdown and efficiency of use of the diets. The aim of this study was to investigate the effect of different dietary levels of concentrate on intake, digestibility, ruminal fermentation and microbial population in Nellore steers.

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 (protocol number 017621/11). The investigation was conducted at UNESP (Universidad Estadual Paulista), Faculdade de Ciências Agrárias e Veterinárias, Departamento de Zootecnia, Jaboticabal, São Paulo, Brazil.

2.1. *Animals and experimental diets*

Eight Nellore steers (initial body weight (BW) 308 ± 10.4 kg; final BW 389 ± 13.2 kg) fitted with silicone-type ruminal cannulas (10 cm i.d.) and duodenal T-type cannulas were used in a double 4 × 4 Latin square design experiment. The diets were formulated to provide a dry matter intake (DMI) of 1.6% of BW and were calculated according to AFRC (1993). The roughage was corn silage and the concentrate was composed of corn, soybean meal and urea (Table 1). The dietary treatments consist of four different proportions of concentrate to roughage (corn silage): 30:70, 40:60, 60:40 and 80:20%

Table 1. Dietary ingredients and chemical composition.

	Concentrate level of experimental diets			
	30% (Diet 30)	40% (Diet 40)	60% (Diet 60)	80% (Diet 80)
Ingredients [g/kg DM]				
Corn silage	700	600	400	200
Ground corn	232	324	488	694
Soybean meal	56	64	98	90
Urea	12	12	14	16
Chemical composition				
Dry matter (DM) [g/kg]	492	548	660	722
Organic matter [g/kg DM]	942	943	943	941
Crude protein (CP) [g/kg DM]	139	144	167	171
Ruminal degradable protein [% CP]	58.3	56.5	53.2	50.1
Ruminal non-degradable protein [% CP]	41.7	43.5	46.8	49.9
aNDFom[†] [g/kg DM]	451	404	311	216
ADFom[‡] [g/kg DM]	252	223	166	124
Hemicellulose [g/kg DM]	211	194	159	116
Lignin [g/kg DM]	28.2	25.3	19.3	13.5
Non-fibrous carbohydrates [g/kg DM]	357	399	473	568
Total carbohydrates [g/kg DM]	807	803	784	784
Starch [g/kg DM]	266	291	331	388
Ether extract [g/kg DM]	30.6	31.3	32.2	34.1
Gross energy [MJ/kg]	21.0	20.8	20.3	20.0

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

in the dietary dry matter (DM), resulting in Diets 30, 40, 60 and 80, respectively. The ingredients of the concentrate were ground in a hammer mill fitted with strainers and 2 mm sieves. Continuous homogenisation of the diets was performed in a horizontal mixer for 15 min.

For the feeding experiment, four consecutive 21-d periods were used. Each study period consisted of 14 d for adaptation, 6 d for collection of faeces and urine, and 1 d for sampling ruminal fluid for measurement of ruminal pH, ammonia N (NH_3-N) and VFA as well as bacteria and protozoa quantification.

Steers were kept in metabolism stalls equipped with individual feed bunks and water fountains during data collection. Animals were fed with corn silage, experimental concentrates and a mineral supplement (Bellnutri®, 100 g/animal; contained per kg supplement: calcium, 146 g; phosphorus, 40 g; sulphur, 40 g; sodium, 130 g; copper, 1.35 g; manganese, 1.04 g; zinc, 5 g; iodine, 100 mg; cobalt, 80 mg; selenium, 26 mg; fluorine max. 800 mg). The feed was offered as a total mixed ration two times a day at 06:00 h and 16:00 h.

Throughout the entire experimental period, the allowance was adjusted to allow refusals of approximately 100 g/kg in relation to 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.

2.2. *Data collection and sampling procedures*

Total faeces were collected for 6 d to estimate the digestibility of nutrients. At the end of each collection day, faeces were weighed and homogenised. At the end of each 21-d study period, a composite sample was made for each animal based on the pre-dried

weight of each sampling day. Total urine was collected for 6 d using funnel collectors attached to the animals. The funnel collector was connected to a polyethylene flexible tube, which directed the urine to containers containing 500 ml of H_2SO_4 (200 ml/l) to avoid loss of nitrogenous compounds. After each 24 h collection period, the total weight and volume of urine excreted were determined. Samples from the six sampling days were pooled and homogenised. A 10 ml subsample was diluted with 40 ml of 0.018 mM H_2SO_4.

Ruminal contents were sampled on day 5 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 the morning feeding and pH was measured after the contents were filtered. Two aliquots of 20 ml were stored at −10°C and later used to determine NH_3-N according to the methodology of Fenner (1965) adapted for use in Kjeldahl distillation, 2 ml of sample were used with 10 ml of distilled water and 5 ml of KOH 2N; this mix was recovered in 20 ml of boric acid to complete 50 ml to titrate in hydrochloric acid (0.05 N). 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) described by Chen and Gomes (1992) and it was calculated according to Pina et al. (2009).

To measured organic matter (OM) apparently digested in the rumen (OMADR), duodenal samples were collected over 2 d (day 4 and 5 of sample collection during the study period) at 6-h intervals. Collection during the second sampling day was delayed to ensure that every 3 h in a 24-h period was properly represented (Oliveira et al. 2007). Samples were kept at −10°C, and at the end of the period one sample was produced by pooling the samples for each animal in each period. Indigestible neutral detergent fibre (iNDF) was used as an indicator of daily DM flows in the duodenum (Harvatine and Allen 2006), obtained via an *in situ* methodology after 240 h (Casali et al. 2008) with incubated samples of feed offered, feed refusals, faeces and ground duodenal digesta (Wiley mill, 2 mm screen). The iNDF was analysed using an Ankom200 Fibre Analyser (Ankom Technology Fairport, NY, USA).

Feed offered, feed refusals and faeces samples were dried at 55°C for 72 h and ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass a 1 mm screen. Samples of corn silage, concentrates and feed refusals were analysed for DM (934.01), ash (942.05) and acid ether extract (954.02) according to AOAC (1990). The contents of ether extract were determined by extraction in ether (920.39). Nitrogen was determined using an LECO FP-528 nitrogen analyser (LECO Corp., St. Joseph, MI, USA). Gross energy was obtained by the combustion of samples in an adiabatic bomb calorimeter IKA model 2000 Basic.

Neutral detergent fibre (aNDFom) was determined using α-amylase and without the addition of sodium sulphite following Van Soest et al. (1991) and adapted for the Ankom200 Fiber Analyzer. Acid detergent fibre (ADF) was determined using the method described by Goering and Van Soest (1970) and adapted for the Ankom200 Fiber Analyzer. Acid detergent lignin (lignin) was determined by solubilisation of cellulose with sulphuric acid according to Van Soest and Robertson (1985). Starch was determined enzymatically according to Bach Knudsen et al. (1987).

Total carbohydrates and non-fibrous carbohydrates (NFC) were calculated values according to Sniffen et al. (1992). Ruminal degradable protein and ruminal non-degradable protein were calculated values according to Ørskov and McDonald (1979).

Measuring methane production in animals requires complex and often expensive equipment, and when cannulated cattle are used large animal numbers are needed to overcome the additional variability. Therefore, prediction equations are widely used to estimate methane emission. Some methane production was estimated by the equation of Ellis et al. (2007) derived from beef database models developed specifically to predict emissions. In this study, methane production [MJ/d] was predicted from the metabolisable energy intake (MEI), ADF and lignin:

$$\begin{aligned} CH_4\ [MJ/d] = 2.94\ (\pm 1.16) + 0.059\ (\pm 0.0201) \cdot MEI\ [MJ/d] + \ 1.44(\pm 0.331) \\ \cdot ADF\ [kg/d] - 4.16\ (\pm 1.93)\ Lignin\ [kg/d] \end{aligned}$$

This equation was selected because the level of CH_4 emissions caused by ruminal fermentation depends mainly on the composition and quantity of the diet (IPCC 1996), which was known in the present study. Furthermore, it has a low root mean square prediction error (RMSPE) (14.4%) and the R^2 value for this equation was 0.85. The CH_4 energy was converted to a mass value using the conversion factor of 0.02 kg/MJ (Brouwer 1965).

2.3. *Rumen bacteria and methanogens*

To determine the effect of diets on ruminal population, several ruminal bacteria were quantified by quantitative PCR. This included three bacteria species important for fibre degradation (*Ruminococcus albus, Fibrobacter succinogenes* and *Ruminococcus flavefaciens*), two ruminal bacteria important for starch digestion and lactic acid production (*Streptococcus bovis* and *Selenomonas ruminantium)* and rumen methanogens (*Archaea*).

Samples of ruminal contents were collected via cannula on day 16 of the trial period, before the morning feeding. Fifty grams of the rumen contents were weighed and immediately added to 50 ml phosphate saline buffer (pH 7.4), stirred vigorously for 3 min and then filtered with a mesh fabric (100 microns). The filtrate was subjected to centrifugation at 16,000 g for 10 min at 4°C. The supernatant was discarded and the remaining precipitate 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 precipitate was immediately stored at −20°C for a period of 2 months.

DNA extraction was conducted in samples of 250 mg using the extraction kit AxyPrep™ Bacterial Genomic DNA Miniprep (Axygen-Biosciences). The integrity and quantity of the DNA were 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.

For quantification of total bacteria and relative quantification of bacteria and methanogens, the technique used was relative qPCR. The primers used in this study are shown in Table 2. Four concentrations (200, 400, 600 and 800 nM) of forward and

Table 2. PCR primers used in this study for the quantification of specific rumen microbes by qPCR.

		Primers* (5′ to 3′)	Product size [bp]	Efficiency [%]
Total bacteria[†]	Forward	CGGCAACGACAACCC	130	100
	Reverse	CCATTGTAGCACCTGTGTAGCC		
Fibrobacter succinogenes[†]	Forward	GTTCGGAATTACTGGGCGTAAA	121	98
	Reverse	CGCCTGCCCCTGAACTATC		
Ruminococcus flavefaciens[†]	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	132	96
	Reverse	CGGTCTCTGTATGTTATGAGGTATTACC		
Ruminococcus albus[‡]	Forward	CCCTAAAAGCAGTCTTAGTTCG	175	96
	Reverse	CCTCCTTGCGGTTAGAACA		
Streptococcus bovis[§]	Forward	TTCCTAGAGATAGGAAGTTTCTTCGG	127	95
	Reverse	ATGATGGCAACTAACAATAGGGGT		
Selenomonas ruminantium[§]	Forward	GGCGGGAAGGCAAGTCAGTC	83	98
	Reverse	CCTCTCCTGCACTCAAGAAAGACAG		
Methanogens[♦]	Forward	TTCGGTGGATCDCARAGRGC	140	95
	Reverse	GBARGTCGWAWCCGTAGAATCC		

Notes: *Primers used for qPCR normalisation; [†]Denman and McSweeney (2006); [‡]Koike and Kobayash (2001); [§]Khafipour et al. (2009); [♦]Denman et al. (2007).

reverse primers were tested to determine minimum primer concentration giving the lowest threshold cycle (C_t) and to reduce non-specific amplification before starting the reaction.

The amplifications were performed in triplicate and negative controls were run in the assay, omitting the total DNA. Real-time PCR was performed with Applied Biosystems 7500 Real-time PCR System (Applied Biosystems). Rox was used as a passive reference dye. The qPCR reaction was carried out using 100 ng of total DNA in a reaction containing 6.25 μl of SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA), 400 or 600 nM of primer pair and H_2O to a final volume of 12.5 μl. Cycling conditions were 50°C for 2 min, 95°C for 10 min and 35 cycles with denaturation 95°C for 15 s, pairing 60°C for 60 s and extension 78°C for 1 min. After one cycle of amplification, a step was added to increase 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:

$$\text{Relative quantification} = 2^{-[C_t \text{ target} - C_t \text{ total bacteria}]}$$

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

2.4. *Rumen ciliate protozoa*

For the quantification and identification of rumen ciliate protozoa, samples of ruminal contents were collected via cannula on day 16 of the trial period after 3 h of morning feeding. Cell counts were obtained from rumen content aliquots that were preserved in formalin (a solution of equal parts water and 370 ml formaldehyde per l) according to D'Agosto and Carneiro (1999). Direct counts of protozoa were performed in a Sedgewick-Rafter counting chamber (Dehority 1984). The samples were diluted with 200 ml glycerol per l 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 formaldehyde per l) (D'Agosto and Carneiro 1999).

2.5. *Statistical analyses*

Feed intake, digestibility, microbial protein synthesis, methane predicted and protozoa quantification data were analysed as a double 4 × 4 Latin square using the PROC MIXED procedure of Statistical Analysis System software version 9.02 (SAS Institute, Cary, NC, USA). The fixed effect of the model consisted of the diet and random effects consisted of animals and periods.

The general mathematical model was represented as follows:

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

in which Y_{ijk} represents the observation on steers k receiving diet i at period j; α_i represents the fixed effect of the i-th diet, $i = 1, 2, \ldots, nt$; β_j represents the fixed effect of the j-th period, $j = 1, 2, \ldots, np$ and s_k represents the random effect of the k-th steers, $k = 1, 2, \ldots, nc$, with variance component σ^2_c. Ruminal pH, NH_3-N and VFA data were analysed as double 4 × 4 Latin square data with repeated ANOVA. The model included fixed effects of diet, time and the interaction. Random effects consisted of steers and periods. When the results of ANOVA were significant, for the analyses of feed intake, digestibility, protozoa population, pH, NH_3-N and VFA Tukey's test was carried out for comparison between treatments, time or the interaction diet × time, and the effects of diet were tested for linear or quadratic effects. Differences among means with $p < 0.05$ were considered as statistically significant differences.

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. The significance levels of $p < 0.05$ were accepted statistically significant differences.

3. Results

3.1. *Intake, digestibility and rumen fermentation*

The intake of crude protein (CP) showed a quadratic association with concentrate levels (Table 3; $p = 0.01$), where the lowest values were observed for animals fed Diet 40 ($p = 0.002$). Increasing the level of dietary concentrates resulted in a linear decreased intake of aNDFom ($p < 0.05$) and linear increased intake of NFC ($p = 0.040$).

However, the different proportions of concentrate did not affect digestibility of aNDFom. In all diets, aNDFom intake was below 1.2% of BW. Diet 30 showed the lowest apparent digestibility of DM, OM and CP. Apparent digestibility of OM and CP showed a linear association with the concentrate proportion ($p = 0.01$).

The lowest ruminal pH was observed in animals fed Diet 80, where the pH remained below 6.0 from 6 h after feeding until the end of measurements at 14 h after feeding. All

Table 3. Effect of different dietary levels of concentrate on intake and digestibility in Nellore steers.

	Concentrate level of experimental diets					*p*-Values[‡]	
	30% (Diet 30)	40% (Diet 40)	60% (Diet 60)	80% (Diet 80)	SEM*	Linear contrasts	Quadratic contrasts
Intake [kg/d]							
Dry matter	5.63	5.32	6.05	5.77	0.421	-[#]	-
Organic matter	5.14	4.75	5.77	4.37	0.462	-	-
Crude protein	0.82[ab]	0.79[b]	0.99[a]	0.96[ab]	0.078	-	<0.01
Ether extract	0.20	0.18	0.22	0.20	0.014	-	-
aNDFom[†]	2.40[a]	2.05[b]	1.89[b]	1.40[c]	0.159	<0.01	-
Non-fibrous carbohydrates	2.64[b]	2.59[b]	3.43[a]	3.35[a]	0.537	<0.01	-
Coefficients of total apparent digestibility							
Dry matter	0.70[b]	0.77[a]	0.80[a]	0.76[a]	0.016	-	<0.01
Organic matter	0.69[b]	0.78[a]	0.77[a]	0.77[a]	0.014	<0.01	-
Crude protein	0.61[c]	0.70[b]	0.74[ab]	0.80[a]	0.028	<0.01	-
aNDFom	0.66	0.63	0.63	0.64	0.022	-	-
Gross energy	0.84	0.82	0.86	0.85	0.029	-	-

Notes: *SEM, standard error of the mean; [‡]*p*-Values for non-significant contrasts ($p > 0.05$) are not reported; [†]aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; [#]-, not significant; [a,b]Means not sharing the same superscript are significantly different at $p < 0.05$.

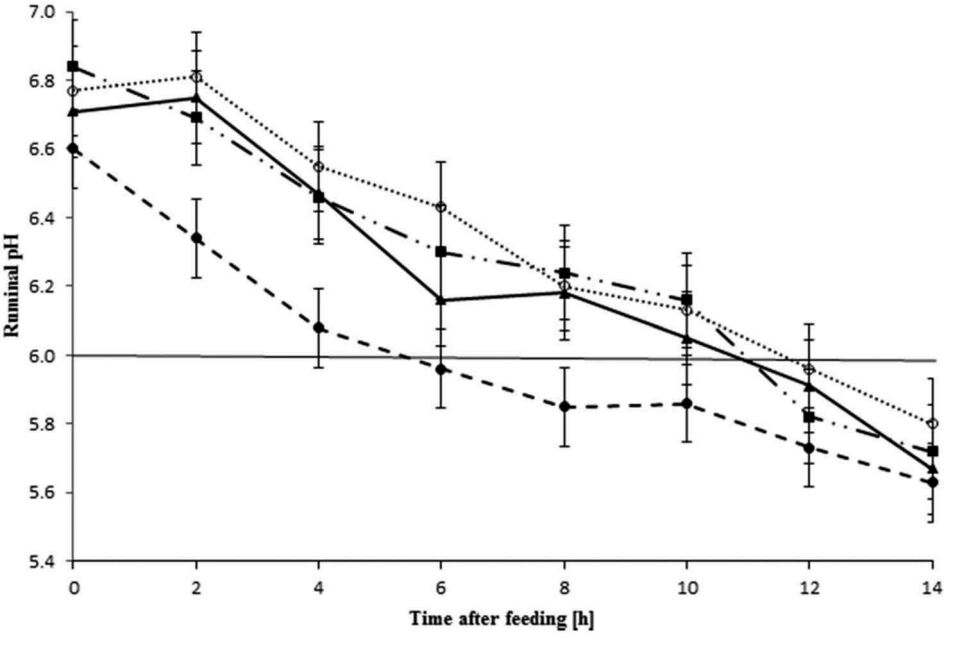

Figure 1. Ruminal pH in Nellore steers fed diets with different proportions of concentrate in the dietary dry matter: 30% (○); 40% (▲); 60% (■) or 80% (●). The critical point of ruminal pH for cellulolytic bacteria at pH 6.0 according to Russell and Dombrowski (1980) is indicated by a horizontal line. After the first feeding at 0 h, steers were fed again after 10 h. Error bars are standard errors of the mean.

other diets caused a pH-value > 6.0 until 10 h after feeding (Figure 1). The ruminal N-NH_3 concentration after feeding Diet 80 was the highest and differed significantly from all other groups (Table 4).

Table 4. Effect of different dietary levels of concentrate on rumen fermentation parameters, efficiency of microbial synthesis and predicted enteric methane in Nellore steers.

	Concentrate level of experimental diets					*p*-Values[◊]	
	30% (Diet 30)	40% (Diet 40)	60% (Diet 60)	80% (Diet 80)	SEM*	Diet	Time
pH	6.33[a]	6.23[a]	6.27[a]	6.00[b]	0.041	<0.01 (L[†])	<0.01(L)
N-NH_3 [mg/dl]	22.9[b]	21.1[bc]	18.4[c]	30.9[a]	3.82	<0.01 (Q[‡])	-[#]
Volatile fatty acids (VFA) [mmol/l]							
Total VFA	98.7	101.0	96.8	105.0	4.89	-	-
Acetic acid (C2)	67.5	68.3	64.6	64.9	3.59	-	-
Propionic acid (C3)	16.1[b]	17.2[b]	18.1[b]	23.6[a]	2.30	<0.01 (L)	-
iso-butyric acid (C4)	0.93	0.95	0.97	1.01	0.052	-	-
Butyric acid (C4)	9.93[ab]	11.0[a]	9.07[b]	11.7[a]	1.10	<0.01 (Q)	<0.01 (Q)
iso-valeric acid (C5)	2.89	3.40	3.03	3.09	0.221	-	-
Valeric acid (C5)	1.79	1.49	1.46	1.63	0.239	-	-
C2:C3	4.26	4.16	3.82	3.61	0.683	<0.01 (L)	-
Microbial N [g/d]	56.2[c]	71.8[b]	71.2[b]	85.9[a]	3.21	<0.01 (L)	-
Efficiency of microbial synthesis [g microbial N/kg OMADR[†]]	12.4	13.9	12.4	14.2	1.74	-	-
Predicted enteric methane [g CH_4/kg OMADR]	57.0[a]	56.1[a]	55.1[a]	49.5[b]	0.01	<0.05 (L)	-

Notes: *SEM, standard error of the mean; [◊]*p*-Values for non-significant contrasts ($p > 0.05$) are not reported; [†]L, linear contrast; [‡]Q, quadratic contrast; [#]-, not significant; [†]OMADR, organic matter apparently digested in the rumen; [a,b]Means not sharing the same superscripts are significantly different at $p < 0.05$.

The concentration of total rumen VFA, acetic acid, *iso*-valeric acid and valeric acid was not influenced by experimental feeding (Table 4). However, increasing proportions of concentrate in the diet resulted in a linear increased concentration of propionic acid and linear reduction of the ratio acetic acid: propionic acid (C2:C3) ($p < 0.01$).

With increasing amounts of concentrate, a linear increased synthesis of microbial nitrogen was observed (Table 4, $p < 0.001$). However, the efficiency of microbial synthesis did not differ among treatments. The predicted methane production showed a linear decrease with increasing the proportion of concentrate in the diet ($p = 0.032$).

3.2. *Changes in ruminal microorganism*

The profile of ruminal microbiota changes according to different diet compositions. Different proportions of concentrate can alter the rumen microbial population. In order to assess the treatment effect on the microbial population, the rumen microorganisms of animals fed Diets 30 and 80 were quantified. With increasing amounts of concentrate in the diet, the relative proportion of *R. flavefaciens* and *R. albus* decreased by 9 and 12 times, respectively ($p < 0.05$), but no difference was observed in the population of *F. succinogenes* (Table 5). Feeding Diet 80 was associated with an increased proportion of *S. ruminantium* (2 times, $p = 0.025$), while for *S. bovis* the numerical increase was not significant (Table 5). The proportion of methanogens decreased two times with increasing amounts of concentrate in the diet ($p < 0.05$).

The total number of protozoa was similar after feeding different proportions of concentrate (Table 6). Independent of the experimental diet, the genus *Entodinium* was most frequent in the rumen, representing 99.3% of all protozoa. The greatest diversity of genera of protozoa was observed in Diet 40, where nine genera were identified. The presence of the genera *Metadinium* and *Elytroplastron* was observed in

Table 5. Effect of different dietary levels of concentrate on ruminal bacteria and methanogens population of Nellore steers.[†]

	Concentrate level of experimental diets		SEM*	*p*-Value
	30% (Diet 30)	80% (Diet 80)		
Fibrobacter succinogenes	0.008	0.007	0.002	0.545
Ruminococcus flavefaciens	0.134^{a}	0.015^{b}	0.057	0.049
Ruminococcus albus	0.561^{a}	0.045^{b}	0.192	0.028
Selenomonas ruminantium	0.030^{b}	0.079^{a}	0.037	0.025
Streptococcus bovis	0.001	0.003	0.001	0.066
Methanogens	1.092^{a}	0.581^{b}	0.445	0.014

Notes: [†]Microbes measured as a proportion of total estimated rumen bacterial 16S ribosomal RNA gene (relative quantification = $2^{-(\text{Ct target-Ct total bacteria})}$); *SEM, standard error of the mean; [a,b]Means with different superscripts are differ significantly at $p < 0.05$.

Table 6. Effect of different dietary levels of concentrate on the population of rumen protozoa in Nellore steers.

	Concentrate level of experimental diets				SEM*	*p*-Values
Protozoa	30% (Diet 30)	40% (Diet 40)	60% (Diet 60)	80% (Diet 80)		(Diet effect)
Total protozoa [n · 10^6/ml]	2.31	1.69	2.11	2.14	0.128	-[#]
Entodinium [n · 10^6/ml]	2.30	1.66	2.11	2.13	0.109	-
Eudiplodinium [n · 10^4/ml]	0.28^{b}	0.64^{a}	0.04^{c}	0.08^{c}	0.091	<0.01 (L[†])
Metadinium [n · 10^4/ml]	0.44^{a}	0.20^{a}	0.00^{b}	0.00^{b}	0.003	<0.01 (L)
Eremoplastron [n · 10^4/ml]	0.00^{c}	1.84^{a}	0.08^{b}	0.16^{b}	0.108	<0.01 (Q[‡])
Diploplastron [n · 10^4/ml]	0.00^{c}	0.88^{a}	0.32^{b}	0.00^{c}	0.002	<0.01 (Q)
Elytroplastron [n · 10^4/ml]	0.04^{b}	0.16^{a}	0.00^{c}	0.00^{c}	0.001	<0.01 (Q)
Polyplastron [n · 10^4/ml]	0.00^{b}	0.02^{a}	0.00^{b}	0.00^{b}	0.001	<0.01 (Q)
Isotricha [n · 10^4/ml]	0.00^{b}	0.04^{a}	0.00^{b}	0.00^{b}	0.002	<0.01 (Q)

Notes: *SEM, standard error of the means; [#]-, not significant; [†]L, linear contrast; [‡]Q, quadratic contrast; [a,b]Means with different superscripts are significantly different at $p < 0.05$.

diets with lower proportions of concentrate (Diets 30 and 40); whereas the genera *Eremoplastron* and *Diploplastron* were observed in diets with medium and higher amounts of concentrate (Table 6).

4. Discussion

In this study, the effect of different dietary concentrate levels on microbial population and ruminal fermentation in Nellore steers were evaluated. It was found that greater amounts of concentrate altered some rumen microorganisms, however, did not affect fibre digestibility. Thus, the hypothesis that higher proportions of concentrate in the diet of Nellore steers may result in a hostile environment for fibrolytic rumen microorganisms reducing the fibre breakdown and the feed efficiency was not supported.

4.1. *Intake, digestibility and rumen fermentation*

It was expected that a higher dietary NDF concentration due to lower proportions of concentrate decreases the DMI of Nellore steers, due to distension in the gastrointestinal tract, which can limit the voluntary DMI in ruminants (Forbes 1996). However, in

this study diets with enlarged roughage level did not affect DMI; thus, bulk fill was not a limiting factor.

On the other hand, when forage is preserved as silage, the accumulation of fermentation end products can further limit feed intake (Huhtanen et al. 2007). Additionally, the intake is also associated with energy balance during longer periods of time and is related to the metabolic status of the animal (Carter and Grovum 1990). An increased amount of fermentable carbohydrate in the diet results in a higher fermentation rate, providing the animal with more energy for growth (Nagaraja and Titgemeyer 2007). The present results suggest that the lack of treatment effect on DMI results from the fact that the changes on VFA concentration were not sufficient to activate the satiety centre of the hypothalamus (Allen 2000). This is in line with previous investigations showing similar results for Nellore steers, which received the same increasing concentrate proportions (Ribeiro et al. 2015).

In this study, it was found that after feeding Diet 80, the ruminal pH was below 6.0 for a longer period. This was due to a higher NFC intake and the large amount of corn in that diet, which affects some microorganism in the rumen. The replacement of roughage by concentrate can reduce the mean particle size in the diet, increase the rate of degradation and usually results in a greater VFA production and molar proportion of propionate while reducing ruminal pH (Yang and Beauchemin 2006). Our results are consistent with other studies showing that higher proportions of NFC with lesser aNDFom in the diet of ruminants have been caused a low ruminal pH (Blanch et al. 2009).

Furthermore, it was observed that replacing roughage with concentrate increased the CP intake in Diet 80, an effect that can be explained by the higher CP content in this diet. The highest ruminal N-NH_3 concentration found in Diet 80 may be due to a greater level of urea in this diet and the highly digestible nature of its ingredients. In all diets tested, the N-NH_3 concentration remained above the optimal level (10 mg/dl) recommended and required for microbial growth in the rumen (Satter and Roffler 1975).

The similarity in the production of microbial N in Diets 40 and 60 is a reflection of similar intake and digestibility of nutrients and similar values of pH, VFA and NH_3-N in these diets, providing comparable substrates, rumen environment and fermentation characteristics that resulted in similar microbial growth. The further increased availability of NFC with greater amounts of concentrate optimised the synthesis of microbial cells, which was reflected in an extended synthesis of microbial N in Diet 80.

In the present study, an effect of different concentrate levels on the efficiency of microbial synthesis was not observed. It should be noted that besides the proportion of nutrients in the diet and greater sources of readily fermentable carbohydrates, other factors have been associated with the efficiency of microbial synthesis, such as the synchronisation of the degradation of foods that make up the diet and greater supply of ruminal degradable protein (Calsamiglia et al. 2010).

4.2. *Changes in ruminal microorganism*

Changes in the diet, such as the amount of fibre or starch, can affect the supply of substrates as well the microbial growth (González et al. 2012). The fact that after feeding Diet 80 the ruminal microorganisms remained for longer periods in an environment

with low pH (Figure 1) was the main factor responsible for decreasing the relative proportion of cellulolytic bacteria *R. albus* and *R. flavefaciens*. Our results are consistent with observations made earlier by Russell and Dombrowski (1980), reporting a high sensitivity of microorganisms to low pH when values remain below 6.0, which results in reduced activity.

Studies of Russell and Dombrowski (1980) and Fernando et al. (2010) indicated that *F. succinogenes* is highly sensitive to an acidic ruminal environment. However, this sensitivity was not observed in our study. Although the ruminal microorganisms remained for longer periods at lower pH after feeding Diet 80, the proportion of *F. succinogenes* did not change. Probably, this result can be attributed to the fact that *F. succinogenes* are gram-negative bacteria and have different cell membranes than *R. albus* and *R. flavefaciens*. In addition, some studies reported a tendency to increase the ruminal population of *Fibrobacter* spp. when animals were fed with silage (Ramirez et al. 2012).

Interestingly, compared with other fibrolytic species, such as *Ruminococcus* spp., *F. succinogenes* digest fibre faster and to a greater extent and even digests crystalline cellulose more actively (Kobayashi et al. 2008). The unique fibrolytic capacity of *F. succinogenes* probably explains why the fibre digestibility was not affected in our study.

However, recent studies showed that the fibre digestibility was reduced to a larger extent when the proportions of concentrate in the diet were increased (Ribeiro et al. 2015), especially when grain-based concentrates were fed (Archimède et al. 1997). It has to be emphasised that even though *R. albus, R. flavefaciens* and *F. succinogenes* are the most important microbes in ruminal fibre degradation, there are other bacterial populations not evaluated in this study, which are involved in the digestibility of the fibre. Additionally, a depression of fibre degradation in the rumen is balanced by an increased degradation in the hindgut (Metzler-Zebeli et al. 2013), and parameters other than these analysed here, such as the rate of passage and intake, also play a role in fibre digestibility.

Ruminal pH is a critical factor in the maintenance of ciliated protozoa in the rumen (Franzolin and Dehority 2010). However, after feeding Diet 80, the total number of protozoa was not changed, even longer periods at lower pH. The genus *Entodinium* is the group of ruminal protozoa most resistant to low pH and it is the most dominant fraction, accounting for 90–99% of the total population in cattle fed high-grain diets (Nagaraja and Lechtenberg 2007). This explains why the total number of protozoa in our study did not change after feeding different proportions of concentrate.

In Diets 60 and 80, lower concentrations of *Eudiplodinium* protozoa were found, which is most likely due to the lower aNDFom intake in case of these diets. The preference of this genus for swallowing cellulose particles and using this substrate in their metabolism (Naga and El-Shazly 1968) may have limited the activity of these microorganisms.

The genus *Diploplastron* was observed in diets with medium to higher proportions of concentrate (Diets 40 and 60), which can be explained by the fact that this genus of protozoa has the particularity to participate as a fibrolyitic and amylolytic microorganism in ruminal fermentation (Wereszka and Michałowski 2012).

The inclusion of concentrates into diets for ruminants is also a strategy to optimise the rumen environment and to reduce methane emissions by an increased propionogenesis (Doreau et al. 2011). In the present study, it was observed that an increased dietary concentrate level reduced the predicted enteric methane production and the population of methanogens. This might be explained by an increased propionogenesis observed for Diet 80, and because the propionate pathway acts as an H_2 sink, it should decrease the available H_2 to methanogens (Doreau et al. 2011).

Diets rich in NFC and with low fibre levels promote growth of amylolytic bacteria in the rumen. *S. ruminantium* is a species producing propionate through succinate decarboxylation (Stewart et al. 1997). This bacterium has the ability to utilise starch and sugars for growth. The highest concentration of propionic acid was found after feeding Diet 80 due to a higher concentration of starch in this diet. This is consistent with an increase in the relative proportion of *S. ruminantium* in the rumen. Similar to our results, the highest concentrations of propionic acid (Sutton et al. 2003) and increasing populations of *S. ruminantium* (Fernando et al. 2010) have been reported in the rumen of cattle fed high-grain diets. In addition, *S. ruminantium* has also the ability to utilise lactic acid, favouring the control of rumen pH (Russell and Baldwin 1978). Thus, the increase of its population in the rumen promoted an increasing use of fermentable substrates produced in the rumen after feeding Diet 80.

S. bovis is a facultative anaerobe known to predominate during lactic acidosis in ruminants fed high amounts of concentrate (González et al. 2012). Activity of *S. bovis* only increases at pH values below 5.75 (Russell and Dombrowski 1980). In our study, pH values above 5.75 were observed for all diets most times after feeding. As in our study, other studies did not find significant differences in *S. bovis* in ruminants fed high amounts of dietary concentrates (Fernando et al. 2010). Additionally, because of the fast rate of NFC fermentation in the rumen, growth of *S. bovis* was not observed when steers were adapted to diets rich in NFC (Nagaraja and Titgemeyer 2007).

In summary, this study suggests that diets with greater inclusion rates of concentrate can inhibit the growth of some cellulolytic bacteria without impairing the DMI and fibre digestibility in Nellore steers. In addition, higher amounts of concentrate in the diet result in a reduction of enteric methane production and the rumen population of methanogens through increasing the propionogenesis, which can reduce energy losses and the environmental impact.

Disclosure statement

No potential conflict of interest was reported by the authors.

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