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Goats fed with non-protein nitrogen: ruminal bacterial community and ruminal fermentation, intake, digestibility and nitrogen balance

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Abstract

The current study assessed ruminal fermentation parameters and bacterial community, nutrient intake, nutrient digestibility and nitrogen balance of goats fed diets containing buffel grass hay and ruminal ammonia nitrogen ($N\text{-}NH_3$). Five rumen-cannulated mixed-breed castrated adult goats (45 ± 2.3 kg) were used in a 5 × 5 Latin square design represented by five $N\text{-}NH_3$ levels (3.43, 9.95, 17.2, 23.0 and 33.7 mg/dl). Control animals were fed hay exclusively. Other treatments were represented by ruminal infusion composed of a mixture containing urea, ammonium sulphate and casein. The increasing $N\text{-}NH_3$ concentrations did not affect rumen fluid pH, which averaged 6.43. Rumen ammoniacal nitrogen increased linearly in response to $N\text{-}NH_3$. Volatile fatty acids were not affected by increasing $N\text{-}NH_3$ concentrations. A higher abundance of *Ruminococcaceae* (*Ruminococcus* 1, *Ruminococcaceae* UCG-014 and *Ruminococcaceae* NK4A214 group) was observed in the rumen of goats infused with higher concentrations of $N\text{-}NH_3$ (17.2 and 33.7 mg/dl $N\text{-}NH_3$). There was a quadratic effect ($P < 0.050$) of $N\text{-}NH_3$ levels on neutral detergent fibre intake with maximum values estimated at 13.7 mg/dl $N\text{-}NH_3$. Nutrient intake, nitrogen excretion and nitrogen balance presented a positive linear effect ($P < 0.050$). In conclusion, 3.43 mg/dl of $N\text{-}NH_3$ is the minimum level to maintain microbial activity, whereas the recommended level to optimize the microbial community is 14.5 mg/dl of $N\text{-}NH_3$ in the rumen of goats fed buffel grass.

Introduction

Most ruminal bacterial species are known to be able to utilize ammonia for the synthesis of nitrogenous compounds (Jin *et al*., 2018). Likewise, ammonia is also essential for the growth of fibrous carbohydrate-degrading bacteria. Cellulose and hemicellulose fermentation is increased when the growth of these microorganisms is stimulated, which results in the production of short-chain fatty acids, used as an energy source by ruminants (Lu *et al*., 2019).

Ruminants fed with poor-quality pasture, when nutritionally supplemented with nitrogen, show an accelerated growth of fibrolytic bacteria, improving fibre degradation (Figueiras *et al*., 2010). Therefore, herbage digestibility is also enhanced, which translates into increased energy uptake.

Tropical forages present low quality due to low protein and high fibre content. The limited nitrogen content impairs the use of fibre carbohydrates by ruminal microorganisms and, consequently, animal performance is reduced (Paulino *et al*., 2008). In the specific case of the xerophilous forage buffel grass, cuttings or grazing is followed by fast growth, finishing its regrowth cycle (Santana Neto *et al*., 2019). This fast growth results in a low lignin synthesis; therefore, neutral detergent fibre is low in lignin and fibre digestibility can be high even though the protein content is low (Pinho *et al*., 2013).

Several studies have been performed to identify the ruminal ammonia concentration necessary to optimize the fermentation of fibrous carbohydrates since the study of Satter and Slyter (1974). Despite all the information available, there is not a consensus on an ideal concentration (Leng, 1990; Detmann *et al*., 2009; Sampaio *et al*., 2009; Khattab *et al*., 2013; Batista *et al*., 2016). Discrepancies between suggested values may be related to divergent factors between studies, e.g. ruminant species, environmental conditions and diet variations in terms of sources of fibrous carbohydrates and other nutrients (Hobson and Stewart, 1997; Kamra, 2005; Shi *et al*., 2008). Thus, studies about ruminal microorganisms from a given ruminant species may not present the same results when repeated with other ruminant species.

Salah *et al*. (2014) reported that the digestible crude protein requirements are higher in lambs (3.36 g/kg $LW^{0.75}$) when compared to cattle (2.81 g/kg $LW^{0.75}$) and goats (2.38 g/kg

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$LW^{0.75}$). Thus, goats are supposedly more susceptible to the excess of nitrogen in the diets and are affected by ruminal and metabolic alkalosis. The excretion of excessive ammonia is metabolically expensive, because a lot of energy (12 kcal/g de nitrogen) is used to maintain the ideal body nitrogen content (Santos *et al.*, 2014). Furthermore, the excessive protein supplementation increases animal feeding costs and soil contamination due to urinary and faecal nitrogen elimination (Pacheco and Waghorn, 2008).

Determining the ideal concentration of ammonia to optimize buffel grass digestion is of great importance, especially for dairy goat farming in semi-arid regions, where this grass has been widely used. However, there is no information on the putative effects of different concentrations of non-protein nitrogen (NPN) on the caprine rumen microbiome and on buffel grass fibre digestibility. Defining which bacterial populations are inhibited or stimulated by different ammonia contents would allow us to better understand the fermentation of fibre carbohydrates in the rumen and it will be possible to manipulate correctly the rumen microorganisms in order to increase fibre digestion and microbial synthesis, resulting in better animal performance.

Therefore, the present study investigated fermentation parameters, the ruminal bacterial communities, nutrient intake, nutrient digestibility and nitrogen balance of goats fed buffel grass-based diets supplemented with different concentrations of ruminal ammonia originated from NPN.

Material and methods

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee at the Federal University of Paraíba – UFPB, in Brazil, protocol no. 0209/14).

Local considerations for carrying out the experiment

The experiment was carried out from July to October 2016 in the Small Ruminant Research Center (UPPR) at the Animal Science Section of the Center for Agrarian Sciences, Federal University of Paraíba, in the municipality of Areia, PB, Brazil.

Animals and experimental design

The experiment involved five castrated rumen-cannulated mixed-breed adult goats, with an average weight of 45 ± 2.3 kg and prophylactically treated against endo- and ectoparasites. Goats were housed individually in cement-floored pens (2 m^2/stall) equipped with individual food and water troughs. Feed, water and mineral salt were provided *ad libitum*.

Ruminal parameters were assessed according to a 5 × 5 Latin square experimental design, consisting of five periods, five treatments and five animals. Each period (19 days) was divided into adaptation to the diet (14 days) and data collection (5 days).

Food management and experimental diets

Different levels of NPN in the goat diets were tested. The animals were fed a low-protein, deferred buffel grass diet that was supplemented with five protein levels (Table 1). Control treatment consisted exclusively of roughage, whereas the other treatments included ruminal infusion of a nitrogen supplement to increase dietary CP by 1.94, 3.89, 5.83 and 7.77%. The ruminal infusion resulted in five levels of ruminal ammonia nitrogen ($N\text{-}NH_3$; 3.43, 9.95, 17.2, 23.0 and 33.7 mg/dl $N\text{-}NH_3$). The supplement comprised urea, ammonium sulphate and casein at the ratio of 75.00:8.33:16.67. Ammonium sulphate was used to provide sulphur (S), whereas casein was included as a source of branched-chain fatty acids; both ingredients were used to ensure favourable conditions for ruminal fermentation (Table 1).

Table 1. Chemical composition of forage and supplement components based on dry matter (DM)

Item	Buffel grass hay (g/kg of DM)	Urea	Casein	Ammonium sulphate
DM[a]	840.90	995.40	900.00	977.30
Ash	78.60	4.60	27.60	22.70
Crude protein	55.00	2637.70	889.70	1426.00
Ether extract	11.10	–	3.20	–
[a]NDFom-NDF[b]	698.40	–	–	–
NFC[c]	156.90	–	–	–
Lignin (sa)[d]	41.30	–	–	–
NDIP[e]	12.03	–	–	–
ADIP[f]	7.01	–	–	–
Total carbohydrate	855.30	–	–	–

[a]Based on natural matter.
[b]Neutral detergent fibre assayed a heat-stable amylase and expressed exclusive of residual ash.
[c]Non-fibrous carbohydrate.
[d]Lignin determined by solubilization of cellulose with sulphuric acid.
[e]Neutral detergent indigestible protein.
[f]Acid detergent indigestible protein.

Buffel grass was harvested from a pasture that had not been grazed for 90 days. The grass was harvested at 10 cm above soil level using a backpack mower. The material was then baled, transported to and stored in a shed where the experiment took place.

Ground hay was supplied twice daily, at 08:00 h and 16:00 h, in two equal portions. Orts were weighed daily, and the supplied amount was adjusted according to the intake of the previous day so as to allow 10% orts. The supplement was fractioned and administered directly into the rumen of the animals at the time the roughage was supplied. The amount of supplement infused daily was calculated considering the roughage intake of the previous day.

Intake and digestibility of nutrients

Nutrient intake was estimated using the difference between the averages of total nutrient in the offered diet and in the remaining food. Samples of hay, supplement and leftovers were taken to perform the chemical analysis.

The digestibility trial was carried out by total faeces collection. Between days 11 and 13 of each period, faeces were collected twice per day in bags fitted to the animals, weighed and kept at −15°C. Samples of diet, leftovers and faeces of each animal in each period were pooled and homogenized. One composite sample per animal was pre-dried in a forced circulation drying oven at 60°C for 72 h

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to be used in chemical analyses: dry matter (DM), crude protein (CP), ether extract (EE) and neutral detergent fibre (NDF). Digestibility was calculated using the equations described by Berchielli *et al.* (2006).

Analysis of purine derivatives

Spot urine samples were collected at 4 h after the first feed delivery during spontaneous urination, using collection bags (adapted 65 mm colostomy bags) attached to the animals. The urine sample from each animal was filtered and 10 ml aliquots were taken, immediately diluted in 40 ml of 0.036 N sulfuric acid (Valadares *et al.*, 1999) and frozen for later analysis.

Urinary levels of allantoin, xanthine and hypoxanthine were determined according to Chen and Gomes (1992). Uric acid and urea concentrations in the urine were measured using commercial kits (Bioclin®, Bio 380 and Bio 800, Belo Horizonte, Brazil).

To determine the total purine derivatives, the allantoin, uric acid, xanthine and hypoxanthine contents were summed. The amount of absorbed microbial purines (mmol/day) was estimated from the excretion of total purine derivatives (mmol/day), using the equations proposed by Chen and Gomes (1992).

Analysis of chemical composition

Diet samples were pre-dried, ground and homogenized for chemical analyses, which were performed in duplicate following the Association of Official Analytical Chemists (AOAC 2012), for DM (method 934.01), CP (method 954.01), EE (method 920.39), ash (method 942.05) and lignin (method 973.18). The methodology described by Van Soest *et al.* (1991) was used to determine NDF and acid detergent fibre (ADF) contents, using an ANKOM fibre analyser (ANKOM200 – ANKOM Technology Corporation, Fairport, New York, USA). NDF and ADF contents were corrected for ash and protein, with their residues incinerated in a muffle furnace at 600°C for 4 h. Protein was corrected using neutral (NDIP) and acid (ADIP) detergent insoluble protein. The NDIP and ADIP were determined according to Mertens (2002).

Total (TC) and non-fibrous carbohydrates (NFC) were calculated using the following equations proposed by Sniffen *et al.* (1992):

$$TC = 100 - (\%CP + \%EE + \%ash)$$

$$NFC = 100 - (\%CP + \%NDFap + \%EE + \%ash)$$

Ruminal fluid collection for analysis of fatty acids, pH and ammonia nitrogen

Ruminal fluid samples were collected via ruminal cannula on the 15th day of each experimental period at 04:00 h, 08:00 h, 12:00 h, 16:00 h, 20:00 h and 24:00 h. The pH, amoniacal nitrogen ($N\text{-}NH_3$) and volatile fatty acid (VFA) concentrations were determined in the rumen fluid.

Rumen fluid samples were harvested 4 h after the morning feed for VFA analysis and microbial DNA extraction. The samples were filtered through gauze and placed in 1.5 ml microcentrifuge tubes. Each variable was analysed in duplicate.

Rumen fluid pH was measured in microtubes immediately after collection using a digital pH potentiometer (Schott®Handylab, Mainz, Germany). For the subsequent analysis of $N\text{-}NH_3$, rumen fluid samples were centrifuged in microtubes at 12 000 rpm for 10 min and the supernatant was transferred to a new microtube and frozen. Ammoniacal nitrogen concentrations were determined by a colorimetric method (Chaney and Marbach, 1962).

To determine VFAs, a 2.0 ml sample of growth medium was placed in microtubes and centrifuged at 5200 ***g*** for 10 min. The supernatant was then frozen for VFA analysis, performed by means of high-performance liquid chromatography (Shimadzu model SPD-10a VP, Dallas, Texas, USA) coupled to an ultraviolet detector at the wavelength of 210 nm. VFA concentrations were analysed using a 30 cm × 4.5 mm diameter commercial column (HPX-87H, Biorad, Hercules, CA, USA) under the following conditions: 0.8 ml/min column flow, 74 kgf pressure, 0.05 MM sulphuric acid as mobile water phase and 20 µl injection volume.

The test results for each time (04:00 h, 08:00 h, 12:00 h, 16:00 h, 20:00 h and 24:00 h) were used to calculate the average ruminal pH, $N\text{-}NH_3$ and VFA of the treatments.

Analysis of the bacterial community of ruminal fluid by sequencing 16s rRNA marker genes (metataxonomics) using high-throughput sequencing

Bacterial community analyses by 16S ribosomal RNA sequencing were carried out for three treatments (3.43, 17.2 and 13.27% CP) to characterize the lowest, intermediate and highest levels of nitrogen supplement inclusion. DNA was extracted from the rumen fluid samples using a commercial kit (Power Soil DNA Isolation kit, MoBio, Carlsbad, CA, USA), following the manufacturer's instructions. The V3–V4 region of the 16S rRNA gene was amplified by PCR (95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min), using the 16S Amplicon PCR Forward Primer = 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG 3′ and the 16S Amplicon PCR Reverse Primer = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGGACTACHVGGGTATCTAAC 3′. Reactions were carried out in triplicate, in a final volume of 25 µl containing 12.5 µl 2 × KAPA HiFi HotStart Ready Mix, 5 µl of each primer and 2.5 ng of DNA template. The purified PCR products were quantified by fluorometry using Qubit 3.0 (Life Technologies, Mississauga, Toronto, Canada).

The library was prepared using the kit Nextera XT sample Prep (Illumina, San Diego, CA, USA). Subsequently, DNA fragments were purified using the Agencourt AMPure XP reagent (Beckman, Brea, CA, USA). After purification, the library was analysed in a capillary electrophoresis system (Fragment Analyzer, Agilent Technologies, Santa Clara, USA). Paired-end sequencing was performed on MiSeq (Illumina) using a 2 × 250 bp V2 kit (Illumina) according to the manufacturer's instructions.

Sequencing data analysis

Raw, demultiplexed and paired sequences were processed using QIIME 2 v.19.7 (Bolyen *et al.*, 2019). Paired-end reads (200–500 bp) with a minimum Phred score of 20 were joined and dereplicated using VSEARCH (Rognes *et al.*, 2016). Chimeric sequences were removed using UCHIME (Edgar *et al.*, 2011). Clusterization was performed by the *de novo* approach with 99% similarity between the centroid groups for Operational Taxonomic Units (OTUs) classification. The number of sequences per sample was normalized at 14 900 reads for α and

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β ecological diversity analyses. Taxonomic classifications were performed using a Naïve Bayes classifier on the SILVA v. 132 trained database with 99% for the V3–V4 region (Quast *et al.*, 2013).

The phylogenetic tree was constructed in FastTree2 (Price *et al.*, 2010) by means of Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh *et al.*, 2002). Visualizations of taxonomical composition (relative abundance and α diversity) were performed using the Phyloseq v.1.8.2 package (McMurdie and Holmes, 2013) in R software v.3.5.7.

The α diversity was assessed using the ecological indices Chao1 and Shannon, which estimate richness and evenness, respectively. For β diversity analysis, the distance matrix across the samples was constructed using the unweighted qualitative metric Unifrac (Lozupone and Knight, 2005) and visualized by principal coordinate analysis.

Differential bacterial abundance across the treatments was assessed by Linear Discriminant Analysis Effect Size (LEfSe) according to Segata *et al.* (2011).

Statistical analyses

Ruminal parameters, nutrient intake, nutrient digestibility and nitrogen balance data were subjected to variance (ANOVA) and regression analyses, using the GLM and REG procedures of the Statistical Analysis System software (SAS, 2002), and a probability level of 5%. A Latin square experimental design (5 × 5) was used, consisting of five periods, five treatments and five animals, and the following statistical model:

$$Y_{ijk} = \mu + T_i + P_j + A_k + \varepsilon_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, T_i is the effect of treatment i ($i = 1, 2, 3, 4$ or 5), P_j is the effect of period j ($j = 1, 2, 3, 4$ or 5), A_k is the effect of animal k ($k = 1, 2, 3, 4$ or 5), and ε_{ijk} is the experimental error.

The α diversity indices were evaluated by the paired Kruskal–Wallis test, whereas dissimilarity between treatments was investigated by the permutational multivariate method (PERMANOVA) using QIIME 2 v.19.7 (Bolyen *et al.*, 2019).

Results

Dry matter intake (g/day and g/100 g live weight), digestibility of DM and NDF, faecal nitrogen and microbial protein were not affected by nitrogen infusion, with mean values of 862 g/day, 1.83 g/100 g live weight, 0.74, 0.78, 1.91 and 87.6 g/day, respectively (Table 2). There was a quadratic effect of N-NH_3 levels on NDF ($P = 0.035$) intake; maximum NDF intake estimated by the model was 681 g/day NDF, which corresponded to the 13.7 mg/dl N-NH_3 level. There was a linear increase in CP intake ($P < 0.001$) and EE intake ($P = 0.004$), as well as CP digestibility ($P = 0.010$) and EE digestibility ($P = 0.038$).

There was a linear increase in nitrogen intake ($P < 0.001$), urinary nitrogen excretion ($P = 0.029$) and nitrogen balance ($P < 0.001$) as a function of N-NH_3 levels (Table 2).

Rumen fluid pH was not affected by N-NH_3, and the overall mean was 6.43 ($P = 0.872$), as presented in Table 2.

Rumen ammoniacal nitrogen (N-NH_3) increased linearly ($P = 0.011$) in response to N-NH_3 infusion (Table 2). Ruminal acetate ($P = 0.785$), propionate ($P = 0.895$), butyrate ($P = 0.827$) and total VFA ($P = 0.828$) were not affected by infusion; mean values were 21.6, 3.85, 3.23 and 28.7 mmol/l, respectively (Table 2).

A total of 39 336 different OTUs were identified after clusterization, with joined sequences ranging from 222 to 491 bp (mean = 448.94 bp). There was no difference between treatments ($P > 0.050$) regarding the α diversity indices Chao1 and Shannon (Figs 1(*a*) and (*b*)). There was a high similarity in the microbial composition between the three N-NH_3 (Fig. 2) and no significant differences ($P > 0.050$) were observed in β diversity between treatments.

The detailed composition of the bacterial communities is shown in Fig. 3. *Firmicutes*, *Bacteroidetes*, *Patescibacteria*, *Proteobacteria*, *Spirochaetes* and *Fibrobacteres* were the most abundant phyla in all treatments. At the family level (Fig. 3(*b*)), *Ruminococcacea* abundance increased progressively (27.0, 31.4 and 33.8%) with increasing ammoniacal nitrogen (3.43, 17.2 and 33.7 mg/dl N-NH_3, respectively). By contrast, a lower abundance (28.9%) of *Lachnospiraceae* was observed in animals on 33.7 mg/dl N-NH_3 compared with those animals on 3.43 mg/dl N-NH_3 (31.5%) and 17.2 mg/dl N-NH_3 (32.0%).

It is noteworthy that the bacterial composition of the rumen of goats with 17.2 and 33.7 mg/dl N-NH_3 was quite similar. Minor OTU shifts at the genus level can be observed in the rumen of goats with 3.43 mg/dl N-NH_3 compared with the other two treatments. A higher abundance of *Ruminococcaceae* (Ruminococcus 1, *Ruminococcaceae* UCG-014 and *Ruminococcaceae* NK4A214 group) was observed in the rumen of goats infused with higher concentrations of N-NH3 (17.2 and 33.7 mg/dl N-NH3). On the other hand, animals from these groups presented lower ruminal abundance of *Prevotelacea* and *Treponema* (Fig. 3(*c*)).

Discussion

The increase in the ammoniacal nitrogen (N-NH_3) provided by NPN infusion corroborates a previous *in vitro* study which showed a linear increase of N-NH_3 in a medium containing buffel grass and different concentrations of nitrogen ammonia (Santana Neto *et al.*, 2019). Interestingly, no changes in other ruminal parameters were observed in our study (pH, acetate, propionate, butyrate and total VFA). VFA production and NDF digestibility even in non-supplemented animals (Table 2) suggest that the microorganisms in the caprine rumen are able to grow and maintain fermentation and metabolic processes when fed buffel grass only. This is corroborated by the fact that *in vitro* fibre degradation of buffel grass and ruminal microorganism growth were shown to occur even in the absence of NPN supplementation, when N-NH_3 concentration was only 1.63 mg/dl (Santana Neto *et al.*, 2019). Stefański *et al.* (2020) also observed that when ruminal ammonia content is low, ruminal bacteria are more efficient in the use of extracellular nitrogen, and that the fermentation and synthesis processes are maintained.

On the other hand, the results presented herein conflict with the concept that minimum ruminal ammonia nitrogen is necessary for microbial growth (Sampaio *et al.*, 2009; Khattab *et al.*, 2013; Batista *et al.*, 2016), since the levels provided by buffel grass alone are lower than reported previously. According to Satter and Slyter (1974), the low N-NH_3 concentration in forages with <7% CP limits microbial growth. Sampaio *et al.* (2009) reported that the minimum CP content (%DM) for rumen microorganisms to exhibit minimal fibre-degradation ability is 7%, which corresponds to a minimum ammoniacal nitrogen concentration of 6.24 mg/dl in the rumen. This minimum CP content is

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Table 2. Mean values of ruminal ammoniacal nitrogen (N-NH_3), pH, volatile fatty acids (VFA) ruminal fluid, intake, digestibility and nitrogen balance of goats with infusion of N-NH_3 in the rumen

	Ruminal N-NH_3 (mg/dl)[1]						*P*-value[2]	
Variables	3.43	9.95	17.2	23.0	33.7	S.E.M.[3]	*L*	*Q*
Dry matter intake								
g/day	855	915	916	874	749	108.1	0.092	0.057
g/100 g LW[4]	1.72	1.89	1.92	1.91	1.70	0.245	0.814	0.070
Intake (g/day)								
CP[5]	51.1	56.4	90.8	100.0	105.6	10.83	<0.001[a]	0.159
EE[6]	11.9	12.1	14.2	16.9	15.6	2.18	0.004[b]	0.238
NDF[7]	646	652	681	644	546	67.8	0.030	0.035[c]
Digestibility								
DM[8]	0.74	0.74	0.77	0.77	0.75	0.007	0.814	0.515
CP	0.71	0.81	0.85	0.86	0.89	0.009	0.010[d]	0.250
EE	0.64	0.72	0.79	0.79	0.82	0.012	0.038[e]	0.358
NDF	0.76	0.77	0.79	0.80	0.78	0.072	0.558	0.522
Nitrogen balance (g/day)								
N intake	8.18	9.05	14.5	16.0	16.9	1.70	<0.001[f]	0.096
Faecal N	2.03	1.89	1.98	1.97	1.68	0.257	0.757	0.389
Urinary N	0.43	0.75	0.82	1.01	1.01	0.012	0.029[g]	0.307
NB[9]	5.50	6.08	11.7	13.0	14.2	1.55	<0.001[h]	0.106
MP[10]	83.3	94.4	92.5	90.0	77.6	8.31	0.556	0.175
N-NH_3, mg/dl	3.24	9.09	15.55	20.47	29.96	2.730	0.011[i]	0.273
pH	6.51	6.42	6.36	6.43	6.42	0.091	0.396	0.872
Volatile fatty acids (VFA, mmol/l)								
Acetate	21.9	19.4	20.9	21.9	23.7	2.34	0.308	0.785
Propionate	3.71	3.70	3.79	3.80	4.26	0.444	0.171	0.895
Butyrate	2.86	3.26	3.78	2.93	3.32	0.601	0.776	0.827
Total VFA	28.5	26.4	28.4	28.7	31.3	2.85	0.282	0.663

[1] Levels of infused ruminal ammoniacal nitrogen; [2]*L*: linear; *Q*: quadratic; [3]Standard error of the mean; [4]Live weight; [5]Crude protein; [6]Ether extract; [7]Neutral detergent fibre; [8]Dry Matter; [9]Nitrogen balance; [10]Microbial protein; [a]$\hat{Y} = 45.5 + 2.02x$ ($R^2 = 0.87$); [b]$\hat{Y} = 11.4 + 0.15x$ ($R^2 = 0.71$); [c]$\hat{Y} = 613 + 8.67x - 0.31 \times ^2$ ($R^2 = 0.96$); [d]$\hat{Y} = 73.2 + 0.55x$ ($R^2 = 0.84$); [e]$\hat{Y} = 65.5 + 0.57x$ ($R^2 = 0.84$); [f]$\hat{Y} = 5.60 + 2.44x$ ($R^2 = 0.91$); [g]$\hat{Y} = 0.002 + 0.001x$ ($R^2 = 0.66$) and [h]$\hat{Y} = 2.80 + 2.44x$ ($R^2 = 0.91$); [i]$\hat{Y} = 0.285 + 0.881x$ ($R^2 = 0.99$).

based on previous studies that determined minimal concentrations of ruminal N-NH_3 for fibre degradation to occur, e.g. 4–5 mg/dl (Satter and Slyter, 1974) and 10 mg/dl (Van Soest, 1994).

Other studies have focused on the definition of the optimal ruminal N-NH_3 concentration for NDF intake and fermentation. Leng (1990) observed maximum intake and ruminal degradation of NDF with 10–20 mg/dl of N-NH_3. Detmann *et al.* (2009) indicated 8–15 mg/dl as optimum ruminal N-NH_3 for fibre intake and degradation. Pires *et al.* (2016) reported a 20% increase in the NDF degradation rate at 15 mg/dl N-NH_3 in the rumen fluid, compared to the treatment without N-NH_3. Santana *et al.* (2019) also observed that an N-NH_3 concentration of 17.76 mg/dl maximized NDF degradation from buffel grass by goat rumen microorganisms.

In the present study, the lowest N-NH_3 in the rumen was 3.24 mg/dl, corresponding to the diet buffel grass only (5.5% CP) or 3.43 mg/dl N-NH_3 (Table 2), which is lower than the minimum values recommended by other authors for fibre degradation (Table 2). On the other hand, 9.95 mg/dl N-NH_3 (9.4%CP) and 23.0 mg/dl N-NH_3 (11.3%CP) provided ammoniacal nitrogen concentrations between 9.09 and 20.47 mg/dl (Table 2) in the rumen; maximum fibre degradation is supposed to occur within this range, according to the above-mentioned authors. Interestingly, these different N-NH_3 levels did not lead to significant shifts in the ruminal microbial populations (Figs 1–3).

The phyla found in the rumen fluid (Fig. 3(*a*)) comprised bacterial organisms capable of degrading fibrous carbohydrates (Gradel and Dehority, 1972; Dehority, 1973; Dušková and Marounek, 2001; Oliveira *et al.*, 2007). *Firmicutes* and *Bacteroidetes* were the most abundant phyla in the goat rumen regardless of the diets (Fig. 3(*a*)), corroborating previous studies that report these as the dominating phyla in the microbiota of all herbivorous animals (Wang *et al.*, 2016; O'Donnell *et al.*, 2017).

Ruminococcus, *Prevotella* and *Butyrivibrio* are among the important carbohydrate-degrading bacteria in the rumen (O'Donnell *et al.*, 2017). In the present study, these genera were

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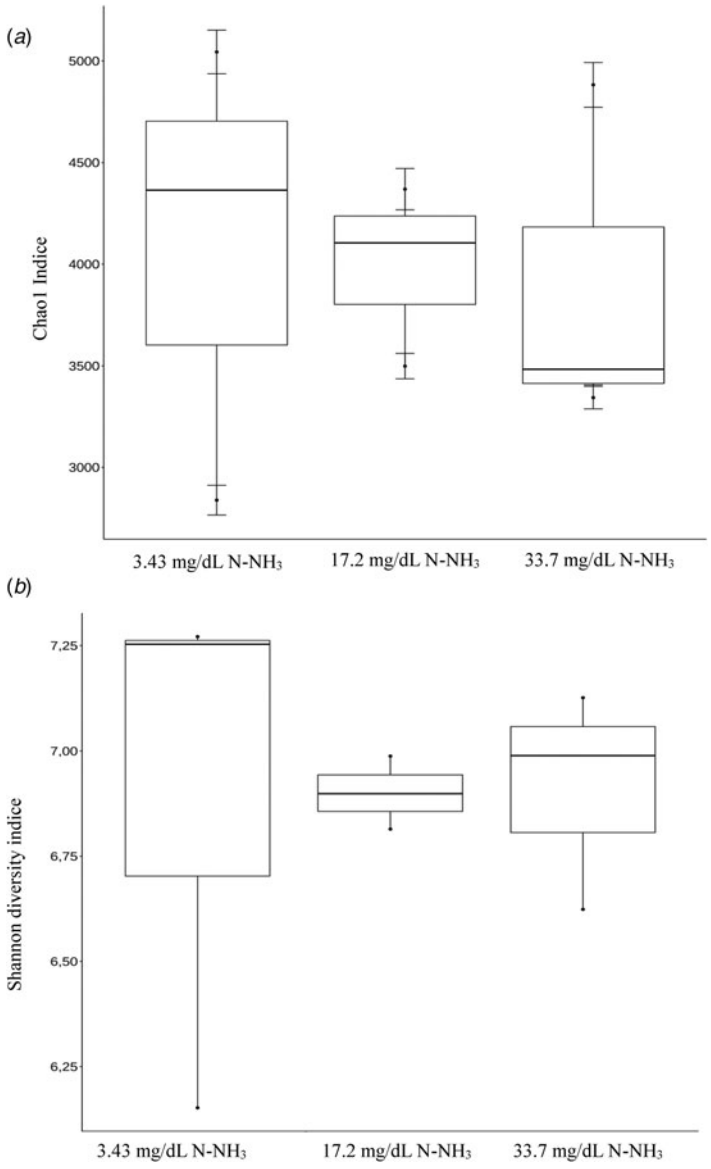

Fig. 1. Chao1 (*a*) and Shannon (*b*) diversity indices for the ruminal bacterial communities of goats fed diets supplemented with three levels of ruminal ammoniacal nitrogen (N-NH_3; 3.43, 17.2 and 33.7 mg/dl N-NH_3).

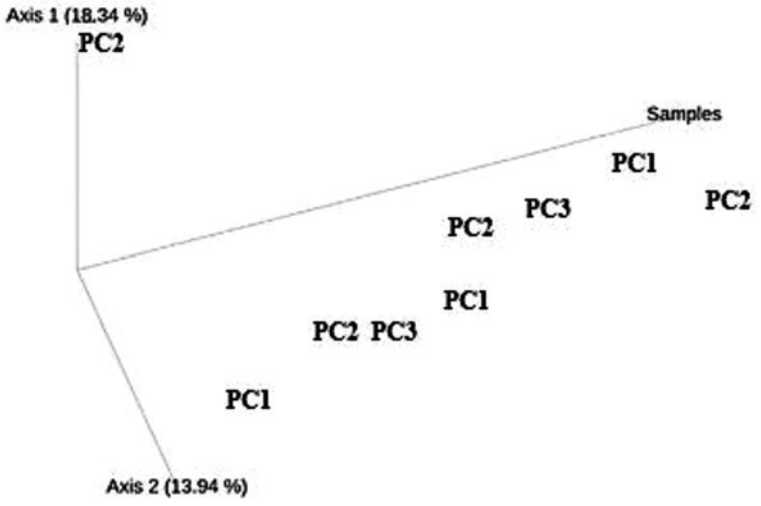

Fig. 2. Principal coordinate analysis (PCoA) plot based on the Unifrac unweighted distance matrix generated from rarefied taxon abundances and depicting β diversity patterns of the ruminal bacterial communities of goats fed diets supplemented with different levels of ruminal ammoniacal nitrogen – N-NH_3 (PC1 = 3.43 mg/dl N-NH_3; PC2 = 17.2 mg/dl N-NH_3; and PC3 = 33.7 mg/dl N-NH_3).

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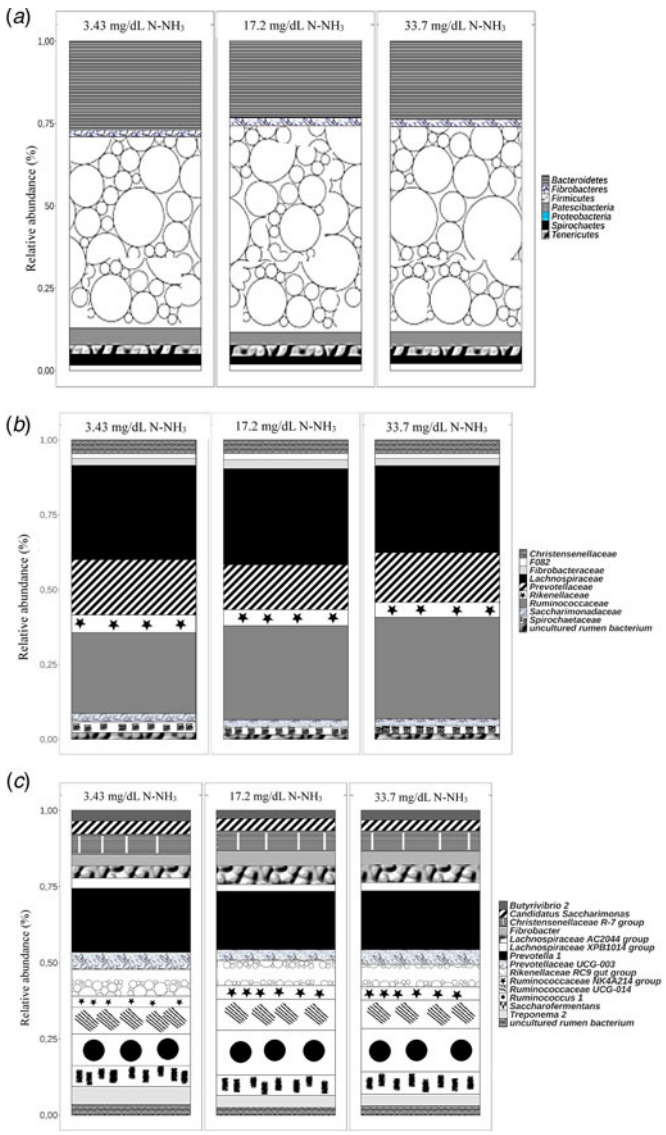

Fig. 3. Relative abundances at the phylum (*a*), family (*b*) and genus (*c*) taxonomic levels of bacterial populations in the rumen of goats fed diets supplemented with three levels of ruminal ammoniacal nitrogen (N-NH_3; 3.43, 17.2, and 33.7 mg/dl N-NH_3).

abundantly found in the rumen of goats fed diets supplemented with different concentrations of N-NH_3. The microbial composition analysis corroborates previous reports indicating *Ruminococcus* as one of the most abundant microbial genera in the goat rumen (Ferreira *et al.*, 2017). As expected for herbivorous species, *Prevotella* was also highly abundant (Fig. 3(*b*)).

Compared to those of sheep and cattle, the rumen microbial population of goats is more specialized in fermenting fibrous carbohydrates (Ferreira *et al.*, 2017). This is supported by the high abundance of *Ruminococcaceae* in the rumen of animals under all NPN supplementation levels reported herein (Fig. 3(*b*)). Unlike the present study, the phylum *Lachnospiraceae* was not detected in goats, only in sheep and cattle (Ferreira *et al.*, 2017). This is possibly due to differences in the composition of fibrous carbohydrates present in the diet used to feed the animals in these studies. Different from other grasses, buffel grass has a low lignin content (4.13%) (Table 1) because of its rapid growth and flowering after the rainy period. Indeed, several studies have shown that lignin is not synthesized in large amounts during its regrowth (Moreira *et al.*, 2007; Perazzo *et al.*, 2017; Pereira *et al.*, 2018).

Lignin is a phenolic compound of the plant cell wall that is toxic to various fibrolytic bacteria, such as the family *Lachnospiraceae* (Paciullo, 2002; Santana and Cavali, 2006; Lage, 2009; Ferreira *et al.*, 2017). Therefore, the low lignin contents of the diet could have favoured *Lachnospiraceae* growth in the animals of the current study. Lignin also negatively affects the digestion of the feed protein and can thereby inhibit the proliferation of ammonia-producing bacteria (Flythe and Kagan, 2010). In the present study, even the control diet presented high nutrient digestibility (Table 2), probably because of the low lignin contents of buffel grass hay.

Prevotella are known to be ammonia-producing bacteria (Cherdthong and Wanapat, 2010), and the high abundance of *Prevotella* in all treatments (Fig. 3(*b*)) might be related to a high proteolytic activity through deamination of the protein from buffel grass, which served as a nitrogen source for the growth of other microorganisms.

The higher abundance ($P < 0.050$) of *Lachnobacterium* in the rumen of goats indicates that the highest ammoniacal nitrogen (33.7 mg/dl N-NH_3) did not compromise the ruminal microbiome as these organisms are involved in both hemicellulolytic and cellulolytic metabolisms in herbivores (Witzig *et al.*, 2010). There is a high diversity of acetate-producing bacteria in the rumen, including the fibrous carbohydrate-fermenting *Lachnospiraceae* species (Gagen *et al.*, 2015).

According to Cherdthong and Wanapat (2010), at high N-NH_3 concentrations, ruminal microorganisms utilize the dehydrogenase glutamate pathway to incorporate nitrogen for microbial growth. However, at low concentrations of N-NH_3 in the rumen, nitrogen is incorporated in two steps and both require ATP: glutamine synthetase and glutamate synthase (Cherdthong and Wanapat, 2010). Thus, rumen bacteria do not cease to incorporate nitrogen when it is present in small amounts in the rumen; rather, there is a shift in metabolism to a pathway that requires more energy. Since energy availability was similar between NPN supplementation levels tested in the present study (Table 1), ruminal N-NH_3 concentration did not interfere with microbial growth and, consequently, with the profile of the ruminal bacterial community. The lack of a significant effect of NPN supplementation on VFA concentration and pH (Table 2) and the high nutrient digestibility even in low ruminal ammonia concentrations are not only indicative of a lack of significant changes in microbial growth, but also that energy availability in the rumen remained the same.

It is important to point out that the crude protein intake of animals submitted to 3.43 and 9.95 mg/dl N-NH_3 levels (Table 2) was lower than the crude protein requirement (66 g/day) for goats with an average body weight of 45 kg preconized by NRC (2007). However, the animals had a positive nitrogen balance showing that levels between 3.43 and 9.95 mg/dl N-NH_3 were enough to maintain both the microbial growth and ruminal substrate degradation (Table 2). According to Michalski *et al.* (2012), goats are more efficient than cattle in recycling nitrogen.

The positive nitrogen balance (Table 2) shows the importance of recycling urea in order to maintain minimum ruminal nitrogen to meet the demands of rumen microorganisms. The positive nitrogen balance in all N-NH_3 contents indicates nitrogen retention by the animals independent of supplementation levels.

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According to Zeoula *et al.* (2006), nitrogen balance is obtained by difference between ingested and excreted nitrogen. This value can be positive, negative or null. Knowing these values, it is possible to determine the protein metabolism, and if there was gain or loss of protein (Ladeira *et al.*, 2002). Therefore, it is suggested that the protein maintenance requirements of goats were fulfilled in the current study, because nitrogen balance was positive in all ruminal N-NH_3 levels (Table 2). Nitrogen retention is only possible when available protein is higher than that required for maintenance. It is noteworthy that ruminal N-NH_3 was lower in the present study than those observed for cattle by Detmann *et al.* (2009).

On the other hand, excessive ruminal N-NH_3 levels result in free blood nitrogen and increase the metabolic activity of the liver as a means to recycle urea in order to maintain homeostasis. As a result, an energy deficit may occur (Noro and Wittwer, 2012) and animal performance can be compromised (Detmann *et al.*, 2007). Hence, ruminal ammoniacal nitrogen presented a positive effect up to 14.5 mg/dl N-NH_3, since nutrient intake reduced in higher levels. Based on these results, buffel grass use by goats is optimized at 14.5 mg/dl N-NH_3. Detmann *et al.* (2009) reported similar results in beef cattle fed *Brachiaria brizantha* (15 mg/dl N-NH_3).

Therefore, the optimum level for goats fed buffel grass is 14.5 mg/dl N-NH_3, whereas 3.43 mg/dl N-NH_3 is enough to maintain microbial activity and fibre degradation.

Based on the observed results, it can be assumed that carbohydrate-fermenting ruminal microorganisms are able to grow in ammoniacal nitrogen concentrations lower than 5.00 mg/dl, without affecting microbial populations in the goat rumen. On the other hand, it is important to point out the role of the energetic source that provides the carbon skeleton for protein microbial synthesis. In the current study, the low lignin content of buffel grass and the consequent high NDF digestibility could explain the observed results. Furthermore, it is necessary to increase ruminal ammoniacal nitrogen up to 14.5 mg/dl to optimize DM and NDF intake. This concentration is close to that recommended for cattle (Leng, 1990; Detmann *et al.*, 2009; Pires *et al.*, 2016) and can be supplied by NPN sources.

Further research on the use of forages with low lignin contents (e.g. buffel grass), as well the use of true protein sources in goat feeding is warranted, in order to better elucidate the nitrogen utilization process of fibrous-carbohydrate-fermenting bacteria in the goat rumen. Moreover, the high stability of the caprine ruminal microbiota under different ruminal nitrogen concentrations must be further investigated.

Conclusion

The minimum ammoniacal nitrogen to maintain microbial activity in the goat rumen is 3.43 mg/dl and the recommended level to optimize the microbial community and buffel grass utilization is 14.5 mg/dl of ruminal ammoniacal nitrogen in goats.

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Conflict of interest. None.

Ethical standards. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee at the Federal University of Paraíba – UFPB, in Brazil, approval no. 0209/14).

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