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Authors: D.F.A. Costa, S.P. Quigley, P. Isherwood, S.R. McLennan, X.Q. Sun, S.J. Gibbs, D.P. Poppi

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Small differences in biohydrogenation resulted from the similar retention times of fluid in the rumen of cattle grazing wet season C3 and C4 forage species

D. F. A. Costa[a,b,*], S. P. Quigley[a], P. Isherwood[a], S. R. McLennan[b], X.Q. Sun[c], S.J. Gibbs[d], D. P. Poppi[a]

[a]School of Agriculture and Food Sciences, The University of Queensland, Gatton, QLD, 4343, Australia
[b]Centre for Animal Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Dutton Park, QLD, 4102, Australia
[c]Northwest A&F University, Yangling, Shaanxi, 712100, China
[d]Lincoln University, Lincoln, Canterbury, 7647, New Zealand

[*] Corresponding author's current address: The University of Queensland, Gatton, QLD, 4343, Australia. Tel: +61 7 5460 1321; e-mail: diogo.costa@uq.edu.au

Research highlights

- An overview of the effects of forage type and season on retention time and the fatty acids profile of the rumen fluid of steers grazing a temperate grass, a range of tropical and subtropical grasses and a legume/grass mix;
- The native speargrass *Heteropogon contortus* in dry season had the lowest crude protein and digestibility, which was associated with a low concentration of volatile fatty acids, and longer retention time (20 h);
- The retention time of all wet season grasses, including the C3 ryegrass *Lolium perenne*, was similar, despite some small quantitative significant differences, and similar to legume/grass mix in the dry season;
- Total unsaturated fatty acids in rumen fluid was reduced markedly compared to the concentration of the forages with some small differences between forage species;
- It was concluded that retention time of wet season grasses was similar across all forage species (8-11 hrs) and would not result in different times for biohydrogenation within the rumen

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Abstract

The effects of forage type and season on retention time (RT) and the FA profile of the rumen fluid (RF) of steers grazing a range of grasses and a legume/grass mix were evaluated. Four rumen cannulated steers (790 ± 17 kg body weight (BW) grazed individual wet season pastures (herbage mass 2600-6200 kg DM^{-1}) of C3 ryegrass *Lolium perenne* and C4 grasses pangola *Digitaria eriantha*, signal grass *Brachiaria decumbens*, star grass *Cynodon dactylon*, kikuyu *Pennisetum clandestinum*, and speargrass *Heteropogon contortus* in both seasons, and a mixture of leucaena *Leucaena leucocephala* and green panic *Panicum maximum* in the dry season. Each grazing period consisted of at least 21d, followed by a 3d collection period. On d 22 CrEDTA was used to estimate RT (182mg Cr .100kg BW^{-1} via cannula) and RF samples collected at 0, 4, 8, 12, 16, 24, 28, 32 and 48h after dosing for Cr analysis. Diet crude protein (CP) and dry matter digestibility (DMD) were estimated by faecal NIRS. Concentration of NH_3N and volatile fatty acids (VFA) in RF was determined at 0, 8 and 16h. Dry season speargrass had the lowest CP (18 and 39 g/kg DM in plucked sample (PS) and estimated by fecal NIRS, respectively)

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and DMD (49%), which was associated with a low NH_3N (9 mg NH_3N /L) and VFA concentration (70 mM/L), and much longer RT of CrEDTA (20 h) than the other grasses. The RT of CrEDTA of the other wet season grasses was similar across all forage species (8-11 hrs). Total VFA was lowest for dry season grass, intermediate for grasses in the wet season and highest for animals grazing the legume/grass mix. Speargrass had the highest non-glucogenic:glucogenic VFA ratio. Ryegrass had higher CP (190 g/kg DM) and DMD (68%), but a similar NH_3N to kikuyu and leaucaena/grass mix (above 100 mg NH_3N /L). . Palmitic and stearic in RF were much higher than in plucked samples, but all grasses had similar total saturated FA in RF with a greater degree of saturation for ryegrass. A higher CLA *c*9,*t*11 in RF of steers grazing ryegrass most likely resulted from the linoleic content in the forage and the higher intake of ryegrass resulting in accumulation in RF. Total unsaturated FA (TUFA) content of RF was reduced markedly compared to forage samples with some small differences between species indicating an extensive biohydrogenation despite the grass type and season. It was concluded that RT of CrEDTA in the rumen of cattle grazing wet season grasses was similar across all forage species (8-11 hrs) and would not result in different times for biohydrogenation within the rumen.

Keywords: tropical grasses; rumen fluid; retention time; fatty acid; biohydrogenation; CLA isomers

Abbreviations: RT, retention time; FA, fatty acid; RF, rumen fluid; CP, crude protein; aNDFom, neutral detergent fibre; ADFom, acid detergent fibre; DM, dry matter; LW, live weight, LCFA, long chain FA; TSFA, total saturated FA; TUFA, total unsaturated FA; CLA, conjugated linoleic acid; TOBCFA, total odd branched-chain FA.

1. Introduction

Products derived from ruminants are of great importance from a human health perspective. The fatty acid (FA) content and profile of meat and milk have been extensively studied within temperate grazing and housed systems (Givens, 2005). Most forages have approximately 40 g lipid/kg DM (Jenkins, 1993), of which about 50% are FAs (Doreau and Ferlay, 1994), with the FA profile most

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influenced by vegetation stage and wilting or drying (Glasser et al., 2013). Grazing ruminants have higher concentrations of C18:3n-3 linolenic acid and CLA isomers in their fat tissues and milk fat than animals fed silages or concentrate rations (Chilliard et al., 2001; Noci et al., 2005; Elgersma et al., 2006). However, most work has been done with temperate grasses (Scollan et al., 2003; Noci et al., 2005) with few studies conducted on tropical C4 grasses, plants that cycle CO_2 into four-carbon sugar compounds to enter into the Calvin cycle (Taiz and Zeiger, 2002), such as conducted by O'Kelly and Spiers, (1991). The lipolysis by microbes within the rumen and the extent of biohydrogenation may vary depending on the retention time (RT) of fluid and particulate phases in the rumen (which are highly correlated) and the type of FA being hydrogenised which is a function of the plant species and degree of maturity of the plant. . In some cases, various FA isomers are produced as part of this process and some (e.g. *t*10 *c*12 CLA) can markedly inhibit FA synthesis in the mammary gland and adipose tissue in the body (Bauman et al., 2008; Smith et al., 2008). Therefore, gathering information about the FA profile and the RT in the rumen of cattle grazing various forages commonly used in the tropics and sub-tropics is important. We hypothesized that C4 grasses with an expected long RT of rumen fluid (RF) (Poppi et al., 1981a; Bowen et al., 2017) would lead to a much more extensive biohydrogenation than C3 grasses, plants that utilize the C3 carbon fixation pathway (Taiz and Zeiger, 2002), with the result of a greater degree of saturated FA and lower concentrations of CLA isomers.

2. Materials and methods

The experiment was conducted at the University of Queensland (Gatton, QLD), "Brian Pastures" Research Station (DEEDI; Gayndah, QLD) and Mt. Cotton Farm (Karremans; Mt Cotton, QLD). All procedures were conducted in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were reviewed and approved by the University of Queensland Animal Ethics Committee (SAS/281/10/MLA).

2.1. Animals, forages and experimental plan

Four 5-year-old rumen cannulated Brahman-cross steers [790 ± 17 kg body weight (BW) (mean ± SEM)] grazed paddocks that contained one of the following dominant pasture species: Ryegrass (*Lolium perenne*), pangola grass (*Digitaria eriantha*), signal grass (*Brachiaria decumbens*), black speargrass (*Heteropogon contortus*), star grass (Cynodon dactylon), kikuyu (*Pennisetum*

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clandestinum), and a paddock of a mixture of leucaena/green panic (*Leucaena leucocephala/Panicum maximum*) (Table 1). Speargrass was grazed at two different occasions, once during the wet season and once during the dry season. Each grazing experimental period consisted of a 21 d preliminary period followed by a 3 d collection period. Paddocks were grazed sequentially (Table 1).

Insert Table 1 here

2.2. Grazing management

The four steers grazed each forage for a minimum of 21 d prior to the commencement of the collection period to adapt to the forage and to avoid any carryover effects from the previous run. Sufficient biomass of each forage was available to ensure *ad libitum* intake by steers during the preliminary period and subsequent collection period (Table 1).

2.3. Sample collection

Rumen fluid, faeces and hand plucked forage samples were collected on d 22 to 24 of each grazing run. The four steers were walked to portable yards installed in each paddock for the collection of RF and faeces.

Samples of RF were collected to determine Cr, NH_3N and VFA concentration and the FA profile. A single dose of Cr-EDTA (approximately 65 mL/100 kg BW; 2.8 mg Cr/mL) was administered to three sites in the rumen (i.e. cranial, ventral and caudal sacs) of all steers via the cannula at approximately 0800 h. Rumen fluid samples were collected prior to dosing (0 h) and 4, 8, 12, 16, 24, 28, 32 and 48 h after dosing with the use of a sampling probe. Rumen fluid samples were stored on ice for approximately 15 min before collection of sub-samples for the following analysis: Cr concentration (10 mL RF); FA profile (10 mL RF), rumen NH_3N (8 mL RF + 1 mL 1 M H_2SO_4) and rumen VFA (3 mL RF (1 mL at 0, 8 and 16 h) + 1 mL 20% metaphosphoric acid + internal standard (i.e. 4 methyl n-valeric acid)). All samples were stored at -20°C until analysis. Samples collected at 0, 4, 8, 12, 16, 24, 28, 32 and 48 h were analysed for Cr concentration. Samples collected at 0, 8 and 16 h were analysed for FA profile and NH_3N concentration.

Faecal grab samples were collected from each steer on d 22 of each run. Samples were dried to a constant weight at 60°C prior to grinding and faecal NIRS analysis for estimation of DM digestibility. Hand plucked forage samples were collected at more than 20 sites within each paddock, providing a

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total of 300-500 g of fresh forage. The samples were stored at -20ºC prior to processing and subsequent chemical analyses.

2.4. Available pasture dry matter, morphological composition and sward height

The available pasture DM was estimated for each of the forages on d 22 of each run. Average sward height of the dominant forage species was determined by measuring the sward height at more than 40 sites across two transects spanning the entire paddock. Eight quadrats (0.5 m x 0.5 m), representative of the average sward height, were cut to 1 cm above ground level and bulked. Sub-samples were dried to a constant weight at 60ºC and average DM yield calculated. An additional sub-sample was separated into leaf, stem and dead material, with each of the components dried to a constant weight at 60ºC and DM proportion determined on most samples (Table 1).

2.5. Analytical procedures

Plucked samples of all forages and faeces were oven dried to a constant weight at 60ºC for chemical and NIRS analyses, respectively.

Dried forage samples were ground through a 1 mm screen (Retsch ZM 200; Haan, Germany) for chemical analysis. Residual moisture content of samples during chemical analysis was determined by drying samples at 105ºC for 24h. Organic matter content of samples was determined after incineration at 550ºC for 8 h in a muffle furnace (Modutemp Pty. Ltd.; Perth, WA, Australia) (AOAC, 1990).

Nitrogen content of all feeds was determined by the Kjeldahl method using a N analyser (Kjeltec, 8400 FOSS; Hillerod, North Zealand, Denmark), according to the manufacturers guidelines. A conversion factor of 6.25 was used to convert the total N to CP.

Total crude lipid (CL) content of samples was determined using an adaptation of the low-toxicity solvent method (Hara and Radin, 1978). Approximately 5 g of sample was mixed with 50 mL Chloroform:Methanol (2:1) and incubated at room temperature overnight. The sample was then filtered into a 100 mL volumetric cylinder through Whatman paper (12.5 cm, No. 1) and rinsed with Chloroform:Methanol (2:1) solution (between 10 to 20 mL). The filtered sample was mixed vigorously with 0.88% NaCl solution (at 5:1), and incubated at room temperature for 2 h, resulting in the separation of two distinct phases. The volume of the chloroform extract in the lower phase was recorded, and the

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upper phase was discarded. The chloroform extract (15 mL) was then transferred to a pre-weighed vial and evaporated to a constant weight at room temperature and CL weight determined.

Crude lipids (%) = (lipids in 15 mL/10) x (total volume/sample weight) x 100

Ash-free neutral detergent fibre assayed with a heat stable amylase (aNDFom) and ash-free acid detergent fibre (ADFom) were determined using an Ankom fibre digestion unit using procedures described by the manufacturer (Method 5 for ADF and Method 13 for NDF; Ankom Technology; Macedon, NY, USA).

The NH_3N concentration in RF was determined by titration with 0.01 M HCl using a TIM 840 Titration workstation manager (Radiometer Analytical SAS; Villeubanne, Cedex, France) after distillation (Büchi 321 distillation unit; Flawil, St Gallen, Switzerland) using sodium tetraborate to increase the pH. Ammonia concentration was then calculated after titration against a week HCl solution of known molarity.

Chromium concentration was determined in RF diluted (1 in 10) with distilled water, centrifuged at 4000 g for 5 min and then aspirated directly into the ICP (inductively coupled plasma spectrometer; Optima 7300 DV, PerkinElmer; Waltham, MA, USA). To overcome matrix effects, standards were prepared for samples in each run (i.e. forage type) by diluting known amounts of Cr with 0 h RF after bulking from all animals (i.e. each forage had a separate standard curve); this bulked fluid was also diluted (1 in 10) with distilled water and centrifuged at 4000 g for 5 min prior to use. In order to calculate RT, forage outflow rate (FOR) and rumen volume, the change in concentration of CrEDTA with time was measured. The slope (k) of Ln [Cr] against time is the fractional outflow rate (FOR, h^{-1}) and 1/k is the retention time (RT, h) of the marker in the rumen. The rumen volume, or pool size, was calculated by dividing the total amount of Chromium injected at time zero by the predicted concentration at time zero by extrapolating the regression of Ln [Cr] to time zero (Binnerts et al., 1968). In this approach, the limitations due to non-steady state conditions are recognised.

Fatty acid profiles were analysed using modifications of the method of Kramer et al. (1997). The RF and plucked forage samples were methylated as proposed by Sun and Gibbs (2012). Samples were weighed (0.05-0.1 g) into 5 ml tubes with 20 uL internal standard and 2.0 ml of 0.5 N NaOH in methanol and vortexed. The tubes were heated for 15min at 50°C on a heat block, and then cooled at ambient

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temperature. Two ml of 2% H_2SO_4 in methanol was added, and the tubes heated for 1hr at 50°C, then cooled at ambient temperature and vortexed. Heptane and deionised water were added at 1ml each, and the tubes centrifuged at 1500 g for 5 min. The upper heptane layer was transferred to a 2.0 ml micro-centrifuge tube, and 0.1 g of anhydrous sodium sulphate added to remove any residual water. Activated charcoal (0.01 g) was added, and the tubes kept for 1hr at ambient temperature, then centrifuged at 13000 rpm for 20 min and the upper heptane layer again transferred to a 0.2 ml vial and stored at -20°C until analysis. The gas chromatography (GC-HP6890; Hewlett Packard, Wilmington, DE) used a 25 m × 0.53 mm ×0.5 m BP21 column (SGE Australia Pty. Ltd., Australia). Initial temperature of the oven was 450°C, held for 4 min, ramp rate of 13°C /min to 175°C, held for 27 min, ramp rate of 4°C/min to 215°C, held for 35 minutes, bake-off at 250°C, for 5 min, with an equilibration time between runs of 5 min. The inlet split injection ratio was 1:15, the temperature 250°C. The flame ionisation detector was set at 250°C, and helium gas pressure on column and linear velocity at 16.7cm/s. The internal standard (heneicosanoic acid methyl ester)16:0 and the external standards (ME61, ME93, BR3, CLA c9,t11 and CLA t10,c12 methyl esters) used to identify peaks of C18s as described by Sun and Gibbs (2012) were all obtained from Larodan Fine Chemicals AB, (Malmo, Sweden). The CLA isomers without external standards were identified by order of elution with respect to those FA with external standards and interpolation from the peaks reported by Loor et al. (2004).

The concentration of VFAs present in RF were determined by gas chromatography (GC17, Shimadzu; Kyoto, Honshu, Japan) using a polar capillary column (ZB-FFAP, Phenomenex; Lane Cove, NSW, Australia) based on the methods of Cottyn and Boucque (1968), Ottenstein and Bartley (1971) and Playne (1985). A prepared multi-acid standard was mixed with the protein precipitant/internal standard and used to calibrate the gas chromatography. Samples were then analysed using the internal standardisation method for calibration.

Faecal NIRS analysis was conducted by Symbio Pty. Ltd. (Eight Mile Plains, QLD, Australia) on individual animal samples for leucaena and wet and dry season speargrass grazing samples, and were bulked for all four animals for the other pasture species evaluated. This relies on local regressions established in Australia (Coates, 2004).

2.6. Statistical analysis

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Because different animals grazed different pastures at different times it is not possible to estimate an animal effect for the whole experiment. Therefore, a one way model was used and analyses were carried out using the General Linear Model (GLM) procedure in the SAS statistical system (SAS® version 9.2©, 2008), adopting a 5% level to assess statistical significance in all cases. With this model any animal variance is included in the experimental error which will lead to any statistical test being conservative. This limitation in statistical analysis is recognised but provides a means of identifying major differences in the parameters between pasture types. Pastures needed to be grazed when they were at the right physiological stage of growth and so pastures were grazed and sampled according to that requirement.

The statistical analyses for FAs were carried out using the repeated measures procedure in General Linear Model of SPSS (SPSS for Windows, Version 17.0, SPSS Inc, Chicago, IL, USA), with time considered the "within subject factor", levels were n=5, and diet treatment (forage types) considered the "between subject factor", and SNK was used for treatment multiple comparisons.

3. Results

3.1. Forage quality

Leucaena had the highest CP concentration, followed by ryegrass, which was ten-fold higher than the value observed for the dry season speargrass (Table 2). Wet season speargrass had a higher CP concentration than the dry season speargrass but was still much lower than ryegrass and the other tropical forages. Ryegrass had the highest lipid concentration and green panic and dry season speargrass had the lowest lipid concentration and the highest ADFom concentration.

Insert Table 2 here

3.2. Fatty acid profile

Total odd and branched-chain FAs concentrations were low for all forages (Table 3). Ryegrass had the highest concentration of total unsaturated FAs (TUFA) and the sum of FAs with 18 C chains (TC18s), and the lowest concentration of total saturated FA (TSFA). The concentrations of TUFA in pangola and speargrass were the lowest (i.e. approximately 60% of total FA) and signal grass, star grass and kikuyu had intermediate values closer to 70%. Linolenic acid (C18:3n-3) was the most abundant FA in all forage types with the highest concentration in ryegrass and the lowest concentration in pangola

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grass. Palmitic (C16:0) and linoleic (C18:2n-6) acids were the second and third most abundant FA, with little difference between the forages.

Insert Table 3 here

3.3. Faecal near-infrared reflectance spectroscopy

Dietary CP in the diet, predicted using faecal NIRS, was highest for steers grazing ryegrass, followed by kikuyu, and lowest for dry season speargrass (Table 4). The dry matter digestibility (DMD) values predicted from faecal NIRS were approximately 60%, or higher, for all species except dry season speargrass which was less than 50%, which would account for the low metabolizable energy intake (MEI) prediction for steers grazing dry season speargrass (9.8 MJ/100 kg BW) compared with ryegrass (23.3 MJ/100 kg BW).

Insert Table 4 here

3.4. Rumen parameters

Steers grazing star grass had the greatest concentration of rumen NH_3N compared to all other forage treatments. There was no difference for rumen NH_3N between the wet and dry season speargrass and signal grass treatments. Wet and dry season speargrass and signal grass treatments had the lowest rumen NH_3N concentrations of the forage treatments at 29, 9 and 31 mg NH_3N /L, respectively and were significantly different to the ryegrass, pangola, kikuyu and the dry season leucaena/green panic treatments. Rumen NH_3N in the kikuyu and the dry season leucaena/green panic treatments were greater than the pangola grass, but were not different to the ryegrass treatment. In addition, there was no difference between the ryegrass and pangola grass treatments for rumen NH_3N concentrations (Table 5).

The analysis of VFAs in the RF of steers grazing signal grass and star grass generated abnormal peaks not easily described and so the results were discarded. Total VFA concentration in the RF was greater for the leuceana/green panic treatment compared to all other treatments. There was no difference between ryegrass, pangola, kikuyu and wet season speargrass for total VFA concentration. However, ryegrass, pangola, kikuyu and wet season speargrass had greater total VFA concentration in the rumen than the dry season spear grass treatment (Table 5). The proportion of acetic acid was higher in the RF of steers grazing speargrass in both wet and dry seasons, followed by pangola and leucaena with an

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intermediate value, with the exception of kikuyu, which resulted in a greater proportion of propionic and lower proportion of butyric acid in the RF. Speargrass, irrespective of season, had the highest non-glucogenic:glucogenic ratio of VFAs. Steers grazing ryegrass had greater proportions of the branched-chain VFAs isobutyric, isovaleric and valeric. Retention time of CrEDTA in the liquid phase in the RF of steers more than doubled for steers grazing the dry season speargrass when compared to most wet season grasses which were low and similar (20 hrs vs 8-11 hrs, Table 5). The retention time of CrEDTA for animals grazing leucaena was intermediate between those grazing dry season speargrass and the wet season grasses, but only significantly greater than ryegrass and pangola.

Insert Table 5 here

3.5. Fatty acids profile of rumen fluid

Steers grazing speargrass in the wet season had lower concentrations of total odd-branched FA (TOBCFA) in the RF, compared only to dry season forages. There were also lower concentrations of TSFA in the RF of steers grazing speargrass compared to the RF of steers grazing pangola grass, signal grass and star grass. The same trend was observed for TUFA in comparison with the fluid of steers grazing ryegrass, signal grass and kikuyu. The concentration of TC18s in the RF of steers grazing speargrass was significantly lower than in the RF of steers grazing ryegrass, pangola and signal grasses. No statistical differences in concentration of TOBCFAs were observed between steers grazing kikuyu, star grass, signal grass, pangola and ryegrass, but these were significantly different to the concentration in the RF of steers grazing speargrass. Similarly, no statistical differences in concentration of TUFA were observed between steers grazing kikuyu, signal grass and ryegrass, but these were significantly different to the concentration in the RF of steers grazing speargrass. The highest concentration of TUFA, found in the RF of steers grazing kikuyu, was also significantly higher than in the RF of steers grazing star grass. The concentration of TSFA in the RF of steers grazing kikuyu, speargrass and ryegrass were not different, but were significantly lower than the concentration in the RF of steers grazing pangola, signal and star grass. No statistical differences in concentration of TC18s were observed in the RF of steers grazing ryegrass, pangola and signal grass. The concentration of TC18s in the RF of steers grazing ryegrass was significantly greater than in the RF of steers grazing kikuyu and speargrass. Steers grazing speargrass had decreased concentrations of linolenic acid (C18:3n-3) in RF when compared to ryegrass,

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star grass and kikuyu grass treatments. However, there was no difference in the concentration of linolenic acid in the RF of steers grazing speargrass, signal grass or pangola grass pastures. In addition, there was no difference between the ryegrass, pangola grass, signal grass, star grass or kikuyu grass treatments for linolenic acid concentration in RF. Steers grazing ryegrass had lower concentrations of linoleic acid (C18:2n-6) in the RF compared to all C4 grass treatments. There was no difference between the C4 grass treatments for linoleic concentrations in the RF of steers.

Insert Table 6 here

The effect of sampling time was statistically significant (Table 7). However, no significant interactions were observed for pasture x sample time.

Insert Table 7 here

4. Discussion

Wet season C4 grasses had similar RT (8-11 hrs) to that of a temperate ryegrass (8 hrs) in cattle and the differences in biohydrogenation were minimal with little difference in the proportion of saturated FA and concentration of CLA isomers. Dry season C4 grass (speargrass) had a very long RT (20 hrs) and this was reflected in the FA profile and proportion of acetate within the rumen. The addition of a legume in the pasture had no major changes and the RT were similar to wet season grass. These results measure for the first time the RT of CrEDTA in the rumen when animals graze a variety of tropical grasses and may be compared to the only other study, that of Bowen et al (2017), who found RT of 9-10 h for C4 wet season tropical grasses typical of southern Queensland in Australia (black speargrass and forest bluegrass (*Bothriochloa bladhii*) in a native pasture, and the introduced pasture, creeping bluegrass (*Bothriochloa insculpta*)), and RT ranging from 8.5-12 h for tropical legumes *Lablab purpureus* and *Clitoria ternatea*, and 22 h for RT of the liquid phase (as measured by CrEDTA) in RF of cattle grazing the dry season tropical grass. These RTs are not much greater numerically to that found with ryegrass (10.8 h) (Bowen et al., 2017). The important parameter is the actual RTs, which are lower than those suggested from pen studies with tropical grass hays (16-19 h) (Poppi et al., 1981a). The statements made by Glasser et al (2013) that differences in FA profile between grass and legume species are lower than those induced by vegetation stage and wilting or drying apply to the results found here. The very low RT values recorded for the C4 pastures, which were similar to ryegrass suggested that the

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differences between temperate grasses and well managed C4 grasses are not as great as expected from pen studies using hays (Poppi et al., 1981a,b).

The FAs present in the diet of grazing cattle are usually derived from the FA present in a small fraction of lipids of the forages being grazed, or within protein supplements added to their diets. These FAs will undergo biohydrogenation within the rumen, affecting to some extent fat composition, excretion in milk or deposition in meat thereof. However, the effects of the FAs of the forages are usually disregarded (Chilliard et al., 2001). It is known that there are differences between the FA profile of grass fed animals and animals fed silage or concentrate-based diets (Bas and Morand-Fehr, 2000; Poulson et al., 2004; Noci et al., 2005), but there is little information in the literature, such as the work of O'Kelly and Spiers (1991), regarding differences in FA profile between animals fed different tropical grasses.. The present experiment was conducted to characterize the RT and FA profile in the RF of steers grazing a range of tropical and sub-tropical forages, and to compare these with a temperate forage (i.e. ryegrass) and a tropical legume and grass mixture.

Despite the difference in CP concentration and DMD between ryegrass and the tropical forages evaluated in this study, there was little difference in rumen NH_3N, VFA or RT, which was relatively similar between forage types. The tropical forages were all grazed in a growing vegetative state representative of wet season pastures when quality is highest and when animals deposit most weight and presumably fat. The exception to this was the grazing of dry season speargrass which resulted in significantly lower total VFA concentration and longer RT of liquid phase marker in the rumen and represents the lowest quality pasture in a seasonal grazing cycle, such as found in tropical regions of Australia (Winter et al., 1991). Bowen et al. (2017) found low efficiency of microbial synthesis in the rumen (i.e. <130 g per kg of digestible OM) in cattle grazing tropical pastures, but have emphasized that those were unfertilized grasses. There was a significantly higher rumen NH_3N concentration measured in RF of steers grazing star grass, compared to all the other forages partly a function of the CP content of the grass. The crude protein in tropical pastures fertilized with N during the rainy season increases (Johnson et al., 2001) above levels that are adequate for the rumen microbes.

The studied grass types had different lipid concentration and most likely a different lipid profile, with different proportions of galactolipids, phospholipids and triglycerides. Jenkins et al. (2008)

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reviewed the effects of plant lipases on breaking down of those lipids and stated that despite being important, most of the lipolysis preceding biohydrogenation can be done by microbial lipases. The latter authors emphasized the need for more research to further understand the interplay of different lipid fractions. Either way, the effects of biohydrogenation on FA profile in the rumen of steers grazing a range of forages in this study was relatively similar and indicated an extensive lipolysis preceding the saturation of FAs.

The FA profile in the rumen of steers grazing a range of forages in this study was relatively similar. Some differences were observed and these were generally associated with the speargrass diet in the wet and dry seasons, which had the lowest rumen NH_3N concentration and greater molar proportion of acetic acid, with little difference in total VFA and RT of liquid in the rumen in the wet season compared with the other forages from which rumen FA profiles were determined.

The TOBCFA concentration in the rumen of steers grazing speargrass was similar to that in RF of steers grazing dry season forages, and lower than in the rumen of steers grazing kikuyu in the wet season (with no differences between the other forages grazed). The odd and branched-chain FAs are almost exclusively microbial in origin (O'Kelly and Spears, 1991; Kim et al., 2005; Vlaeminck et al., 2006). A higher concentration of C15:0 anteiso was found in RF of steers grazing kikuyu. Vlaeminck et al. (2004) reported higher concentrations of the latter FA for acetate-producing bacteria which seems to be in contrast with the current experiment where C15:0 anteiso concentration was lower in RF of steers that had a higher proportion of acetic acid (i.e. steers grazing speargrass had a higher molar proportion of acetic acid in the rumen than steers grazing kikuyu) when total VFA concentration was relatively similar. Speargrass, irrespective of season, had the highest non-glucogenic:glucogenic ratio of VFA related to the high acetate concentration. The concentrations of the VFAs, isobutyric and isovaleric acids, were higher in the RF of steers grazing kikuyu and these short branched-chain acids are known precursors of branched-chain FAs (Kaneda, 1991), of which C15:0 anteiso is an example. Therefore, other factors resulted in different TOBCFA and based on the current data the difference in C15:0 anteiso between the two forages alone is not enough to speculate on bacterial species contribution. Kim et al, (2005) conducted two *in situ* experiments to examine the use of odd-chain FA profiles to study microbial colonization. The results observed in this experiment suggest that the use of individual odd

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and branched-chain FA or TOBCFA as microbial flow marker may lead to misinterpretations and false results.

The concentrations of TUFA in the RF of steers grazing ryegrass was amongst the highest during the wet season, whilst the lowest concentrations were observed in steers grazing speargrass, which reflects the differences observed between the forages themselves. Ryegrass has a very high concentration of linolenic acid compared to speargrass and this would have a marked effect on the concentration of FA in the RF. The estimated rumen volume was similar between these forage types in the wet and much lower for steers grazing speargrass in the dry season. The TUFA concentration in the RF of steers grazing speargrass in the dry season was higher than in RF of steers grazing the same grass in the wet season, which can be speculated to be because of a lower microbial activity and/or less biohydrogenation. The FA content of lipid from ruminants has a much higher participation of the geometric and positional isomers of linoleic acid, within the group of FAs called conjugated linoleic acid (CLA) (Schmid et al., 2006). The CLA represents the majority of the 18 C chain FAs, and the TC18s was highest in the rumen of steers grazing ryegrass and lowest in the rumen of steers grazing speargrass in the wet. The concentration of TC18s in RF was much lower than that measured in the forage material itself but a similar trend was evident, indicating that the FA profile of the basal forage will partially translate to FA of the milk and meat thereof. Linolenic acid (C18:3n-3) was the most abundant FA in all forages and that is in agreement with the literature for both temperate (Kalac and Samkova, 2010) and tropical grasses (O'Kelly and Reich, 1976), although a much higher concentration of C18:3n-3 was observed in ryegrass compared to the C4 grasses examined in this experiment, suggesting inherent differences between forages for this FA. Interestingly, the concentration of C18:2n-6 in RF from the forages was relatively consistent in RF of steers grazing all forages, suggesting that changes in the TC18s were due mostly to changes in C18:3n-3, with the exception of the dry season speargrass, which presented higher content of C18:2n-6 in the forage samples. A greater extent of biohydrogenation of C18:3n-3 in comparison to C18:2n-6 has been reported elsewhere (Doreau and Ferlay, 1994; Vlaeminck et al., 2006) and interestingly, the concentration of C18:2n-6 was higher in RF of steers grazing the dry season speargrass. Two other interesting findings, which could be linked, were the highest concentration of butyric acid coupled with reductions in linoleic concentration and a

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massive reduction in linolenic acid concentration in RF of steers grazing ryegrass. Linoleic acid hydrogenation leads to rumenic acid and Butyrivibrio bacteria are amongst the most important in this role whilst also producing butyric acid. However, not all butyrate producers formed rumenic acid (Jenkins et al., 2008). They observed a conversion of C18:3n-3 to 18:2 and 18:1 intermediates, and then to 18:0, stearic acid. Some of the microbes involved in those processes could potentially be butyrate producers, explaining the higher concentrations of butyrate in RF of steers grazing ryegrass, since other grass types had higher concentrations of linoleic acid.

The longer chain FAs C20:4n-6 (arachidonic) and C22:6n-3 (docosahexaenoic) and their precursors, C18:2n-6 (linoleic) and C18:3n-3 (linolenic) are physiologically important for humans (Koletzko et al., 1989). The levels of these in meat (Schmid et al., 2006) and milk (Scollan et al., 2003) have been used to denote a positive functional food end result.

Conjugated linoleic acid *c*9, *t*11 (i.e.C18:2n2) is the main isomer in forage based ruminant products (Kay et al., 2004; Schmid et al., 2006), including the adipose tissue of ruminants (where it accounts for 75-90% of the CLA isomers) (Bauman et al., 2008). The concentration of this isomer was significantly higher in the RF of steers grazing ryegrass, followed by pangola and lowest for other treatments. There are obvious differences in concentration of FAs in different forage species. The higher linolenic acid C18:3n-3 content of ryegrass would lead to an increased C18:1 *t*11 as observed in Noci et al, (2005), but in this study, the concentration of C18:1 *t*11 in the RF of steers grazing ryegrass was not significantly different to pangola, signal grass or kikuyu, being only higher than in the RF of steers grazing wet season speargrass and star grass. That difference is most likely related to the originally higher concentration of linolenic acid C18:3n-3 in the FA profile of ryegrass, only similar to the legume, in comparison to the latter grasses. The concentration of CLA *c*9, *t*11 ranged between approximately 0.2% of total FA for the majority of C4 grasses up to 0.6% of total FA for ryegrass. The levels of CLAs found here, mostly influenced by the basal diet, would contribute to the higher level expected in the meat of cattle grazing pastures (Schmid et al., 2006). A higher CLA *c*9,*t*11 in RF of steers grazing ryegrass most likely resulted from the linoleic content in the forage, considering the pathway to form this isomer (Jenkins et al., 2008), and the expected higher intake of ryegrass resulting in an accumulation in RF.

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A range of positive characteristics have been attributed to CLA isomers which can be related to human health. In the last decade, studies have focused on the role of specific isomers, in particular *t*10 *c*12 CLA, on lipid metabolism in the mammary gland and subcutaneous and intramuscular fat depots (Bauman et al., 2011; Smith et al., 2008) by a depression in *de novo* synthesis. The dose of *t*10 *c*12 CLA required to achieve 25% reduction in milk fat was 2.5 g/d (Bauman et al., 2008). The authors reported that doses 20 times greater would be required to inhibit fat synthesis in tissues. The extent of formation of this specific isomer within the rumen of steers in the current experiment was not identified due to the method utilized but at the concentration of CLA isomers present in the RF it is not expected that these levels would be reached to inhibit *de novo* synthesis.

Palmitic acid (C16:0) was the saturated FA present in the highest concentration in the forage material or in RF, for all the forages examined. This is in agreement with McDonald et al. (2002), who reported that palmitic acid was the most common saturated FA in plants. The concentration of palmitic acid was higher in the RF of steers compared with the forage material, and was reasonably consistent between forages, both in the plant material and in the rumen, although steers grazing speargrass did have significantly lower concentrations in the rumen than steers grazing signal grass and star grass, but no different to steers grazing ryegrass. The increased concentration of palmitic acid in the RF is due to hydrogenation of unsaturated hexadecanoic isomers and also due to microbial elongation of shorter chain FAs, such as butyric acid (Emmanuel, 1974). Bacteria seem to incorporate palmitic acid into their own FAs, whilst protozoa utilize butyric and acetic acid, which could be the reason why Or-Rashid et al. (2007) found higher concentrations of this FA in the lipids of protozoa and lower concentrations in bacteria. The concentration of the long chain FA (LCFA) identified, C20 up to C26, were relatively low for all forages and also in the RF of steers. The concentration of individual LCFA tended to be highest in the rumen of steers grazing pangola grass and lowest for steers grazing ryegrass, with the exception of C22:4 which was highest for ryegrass. The differences between forage types were small and are unlikely to be of little biological significance.

There was not a significant relationship with sampling time for all FAs, but in spite of that, it is evident that the adoption of an adequate sampling regimen is necessary in order to avoid misinterpretation of results. Sun and Gibbs (2012) observed extensive biohydrogenation of unsaturated

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FA over the grazing cycle for cattle grazing ryegrass pastures. The latter authors indicated that the most dramatic changes occurred in the evening and overnight, especially for odd- and branched-chain and TC18s FAs. In the current study, the concentrations of linolenic C18:3n-3 and linoleic C18:2n-6 acids responded quadratically, which is probably related to the feeding regimen of the animals (i.e. graze in the morning – high concentrations; lower throughout the day because of biohydrogenation and then higher again in the afternoon when animals return to graze). A quadratic relationship was also observed for TSFA, and most likely it would be due to a lower concentration when animals are grazing, but as biohydrogenation takes place, the concentration of saturated FAs increases and once again with grazing in the afternoon the total concentration decreases due to intake of grass containing high concentrations of unsaturated FAs. The important outcome of these relationships is that the differences found between some FAs demonstrate the necessity for adoption of an adequate sampling regimen which properly represents the whole day, instead of the use of a single sample randomly collected.

In summary, the extent of biohydrogenation of FA is significant in both tropical and temperate grasses. Small differences in FA present in the meat or milk could be expected due to the original FA profile of the basal forage. Most work found in the literature report the FA profile of milk fat and fat in the tissues of pasture fed animals, which had grazed temperate forages, to be a rich source of linolenic acid. In this work the concentration of linolenic acid in the RF of steers grazing ryegrass was significantly higher than in the rumen of steers grazing speargrass, but not significantly different when compared to other tropical forages during the wet season.

The concentration of rumen ammonia reflected the CP of the diet selected with the exception of signal grass. The rumen volumes estimated here have potentially large errors given the non-steady state conditions under which the CrEDTA marker is used but nevertheless they provide a comparative relative estimate. The values are comparable to the values derived by Poppi et al. (1981a) under steady state conditions and Bowen et al., (2017) under grazing conditions using a similar procedure. Visual observations, obtained when the cannula was opened for sampling, indicated that the rumens were quite full except for the dry season speargrass, which was also seen in the estimate by marker dilution. Thus, when tropical forages were grazed in a growing vegetative state, representative of high quality characteristic of the wet season, there were little differences in rumen NH_3N, VFA or RT between

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forage types. Furthermore, we can infer that during the wet season, a moment when animals deposit most weight and presumably fat, one could expect little differences in biohydrogenation between the different forages, considering that RT of liquid phase marker did not change much, as originally hypothesized.

5. Conclusions

Changes in retention time and fatty acid profile of RF are more likely to be influenced by major changes in forage quality rather than forage species *per se*. As long as tropical forages are managed to provide a similar quantity and quality of material for grazing, there are unlikely to be major shifts in retention time of material in the rumen. The majority of the differences in fatty acid profile measured during the wet season were minor and were between speargrass and the temperate C3 ryegrass, which were the most different in quality of the forages evaluated, with little difference between ryegrass and the other wet season C4 forages grazed in this experiment.

Declaration of interest

All authors of the work submitted under the title “Small differences in biohydrogenation resulted from the similar retention times of fluid in the rumen of cattle grazing wet season C3 and C4 forage species” declare having no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence in any aspect the submission of this manuscript.

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Table 1. Common and scientific names of pasture species grazed, average steer live weight (LW), estimated forage dry matter (DM) yield, canopy morphological composition (L:S:D)[1] and sward height (SH) when grazing was conducted and average rainfall for that month of the year

Common name	Scientific name	LW (kg)	DM on offer (t/ha)	L:S:D[1] (% DM)	SH[2] (cm)	Time grazed	Rain[3] (mm)
Ryegrass	*Lolium perenne*	791 ± 30	2.6	26:25:49	27	September 2010	72.6
Pangola grass	*Digitaria eriantha*	791 ± 26	5.3	20:23:57	29	October 2010	291.6
Signal grass	*Brachiaria decumbens*	nm[4]	2.9	23:21:56	21	November 2010	21.4
Speargrass (wet season)	*Heteropogon contortus*	815 ± 27	3.8	nm[4]	80	January 2011	230.6
Star grass	*Cynodon dactylon*	766 ± 13	11.6	33:25:42	40	February 2011	47.8
Kikuyu	*Pennisetum clandestinum*	799 ± 18	6.2	24:59:18	26	March 2011	106.8
Speargrass (dry season)	*Heteropogon contortus*	nm[4]	5.5	0:0:100	91	July 2011	87.6
Leucaena/ Green panic[5]	*Leucaena leucocephala/ Panicum maximum*	811 ± 29	5.2[6]	nm[4]	155[7]	September 2011	10.2

[1]L:S:D = Leaf:Stem:Dead material ratio; [2]SH = sward height; [3]Rain = rainfall; [4]nm = not measured; [5]Green panic was the dominant forage present between leucaena rows; [6]Measurement of grass + legume; [7]Average height of leucaena shrubs.

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Table 2. Chemical composition of bulked plucked samples of various grass types and leucaena leaves

Forage	Chemical composition (g/kg DM)				
	OM[1]	CP[2]	CL[3]	aNDFom[4]	ADFom[5]
Ryegrass	867	186	53	536	261
Pangola grass	923	102	33	680	340
Signal grass	906	83	42	597	248
Star grass	903	161	38	678	290
Kikuyu	889	147	34	637	297
Speargrass (wet season)	912	53	26	712	394
Speargrass (dry season)	882	18	16	752	455
Leucaena[6]	906	272	28	298	143
Green panic (dry season)	852	65	16	699	443

[1]Organic matter (OM); [2]crude protein (CP); [3]crude lipid (CL); [4]ash-free neutral detergent fibre assayed with a heat stable amylase (aNDFom); [5]ash-free acid detergent fibre (ADFom); [6]leucaena only leaves collected

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Table 3. Fatty acids in bulked plucked samples of ryegrass (Rye), pangola grass (Pang), signal grass (Sign), star grass (Star), kikuyu (Kik), speargrass (SG) in wet season and SG, leucaena leaves (Leu) and green panic (GP) in dry season[1]

Fatty acid	Rye	Pang	Sign	Star	Kik	SG wet	SG dry	Leu	GP	SEM
	% of total FA[2]									
C12:0	0.15	0.64	0.76	0.38	0.30	0.55	2.07	0.10	0.83	0.19
C14:0	0.28	0.00	0.77	0.44	0.40	0.67	2.22	0.33	0.93	0.20
C16:0	15.3	23.3	19.6	21.2	22.0	21.0	20.92	23.23	19.41	0.76
C16:1c9	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.43	0.83	0.10
C18:0	1.17	4.55	1.35	1.63	1.89	1.81	3.25	4.69	3.63	0.43
C18:1c9	2.02	9.18	2.09	1.51	2.36	3.80	9.43	1.85	6.35	1.01
C18:1c11	0.00	0.12	0.00	0.00	0.00	0.00	0.82	0.53	1.85	0.20
C18:2n-6	10.3	17.0	17.5	13.4	16.4	17.8	19.01	15.14	23.05	1.12
C18:3n-3	61.0	28.0	47.1	50.8	45.1	35.1	7.49	43.39	20.74	5.20
C20:0	0.23	1.67	0.53	0.40	0.46	0.78	3.89	0.76	2.35	0.03
C20:1c11	0.04	0.13	0.05	0.07	0.16	0.09	0.19	0.02	0.35	0.30
C22:0	0.36	1.47	0.54	0.39	0.55	0.57	2.42	0.70	2.89	0.08
C22:2c13,c16	0.10	0.29	0.50	0.27	0.17	0.26	0.84	0.12	0.72	0.07
C22:4	0.34	0.60	0.29	0.11	0.16	0.39	0.66	0.13	0.00	0.17
C24:0	0.33	1.44	0.94	0.62	0.99	0.98	0.00	0.00	0.00	0.16
C26:0	0.33	0.60	0.33	0.22	0.39	0.48	1.34	0.27	1.59	0.28
TOBCFA[3,7]	0.78	1.38	0.83	0.81	1.62	2.49	1.89	0.36	3.00	1.95
TSFA[4,7]	18.7	35.3	25.1	25.8	28.3	30.2	39.58	30.46	34.15	3.50
TUFA[5,7]	76.5	58.0	69.6	68.9	66.6	59.7	37.99	63.39	54.46	3.17
TC18s[6,7]	75.1	59.4	68.6	68.0	66.4	59.0	40.10	65.60	56.04	0.19

[1]Samples of forages were plucked samples collected across the paddocks and leaves combined from different rows of legume trees
[2]Identifiable and quantifiable fatty acids presented only.
[3]TOBCFA = total odd branched-chain fatty acids; [4]TSFA = total saturated fatty acids; [5]TUFA = total unsaturated fatty acids; [6]TC18s = total fatty acids containing 18 carbon chains.
[7]Total of individual fatty acids listed in the table plus other identifiable fatty acids

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Table 4. Crude protein, dry matter digestibility, metabolizable energy intake in the diet estimated by faecal NIRS

Forage	CP[1]	DMD[2]	MEI[3]
	(g/kg DM)	(%)	(MJ/100 kg LW)
Ryegrass	200	68.1	23.3
Pangola grass	126	63.2	19.8
Signal grass	128	59.7	17.4
Star grass	138	59.3	17.0
Kikuyu	176	62.4	19.2
Speargrass (wet season)	99	59.2	16.9
Speargrass (dry season)	39	48.8	9.8
Leucaena/ Green panic (dry season)	135	58.1	16.2

[1]Crude protein (CP); [2]dry matter digestibility (DMD); [3]metabolizable energy intake (MEI);
All components estimated with the use of faecal near-infrared reflectance spectroscopy.

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Table 5 Rumen NH_3N concentration, and the concentration and molar proportion of volatile fatty acid (VFA), retention time (RT) and fractional outflow rate (FOR) of liquid phase marker (CrEDTA) in the rumen and estimated rumen volume of steers grazing various forages

Parameter	Ryegrass	Pangola	Signal grass	Star grass	Kikuyu	Speargrass (wet)	Speargrass (dry)	Leucaena /Green panic (dry)	SEM
NH_3N (mg/L)	113^{bc}	86^{b}	31^{a}	215^{d}	130^{c}	29^{a}	9^{a}	147^{c}	12.2
Total VFA (mM/L)	94.9^{b}	95.6^{b}	na[1]	na[1]	93.1^{b}	96.2^{b}	69.9^{a}	114.2^{c}	3.0
Acetic (% total VFA)	62.5^{a}	70.0^{b}	na[1]	na[1]	65.6^{a}	78.1^{c}	79.9^{c}	72.9^{b}	1.4
Propionic (% total VFA)	19.2^{b}	14.3^{a}	na[1]	na[1]	26.5^{c}	11.5^{a}	11.5^{a}	13.8^{a}	1.1
Butyric (% total VFA)	13.7^{d}	12.4^{d}	na[1]	na[1]	4.5^{a}	8.4^{b}	7.1^{b}	10.3^{c}	0.7
Isobutyric (% total VFA)	1.4^{d}	1.0^{b}	na[1]	na[1]	1.2^{c}	0.8^{a}	0.7^{a}	1.0^{b}	0.1
Valeric (% total VFA)	2.1^{d}	1.3^{c}	na[1]	na[1]	1.2^{c}	0.8^{b}	0.6^{a}	1.2^{c}	0.1
Isovaleric (% total VFA)	1.2^{d}	0.8^{c}	na[1]	na[1]	0.8^{a}	0.5^{b}	0.4^{b}	0.9^{c}	0.1
NGVFA:GVFA	4.8^{b}	6.6^{c}	na[1]	na[1]	2.8^{a}	8.3^{d}	8.2^{d}	6.8^{c}	0.4
RT (h)	8.3^{a}	8.3^{a}	10.1^{ab}	9.8^{ab}	10.1^{ab}	11.3^{ab}	19.8^{c}	13.4^{b}	0.7
FOR (%/h)	12.3^{c}	12.5^{c}	10.0^{bc}	10.4^{bc}	10.0^{bc}	9.2^{bc}	5.1^{a}	7.6^{b}	0.5
Rumen volume (L)	50.6^{bc}	41.9^{b}	46.7^{b}	42.2^{b}	61.4^{c}	51.3^{bc}	24.1^{a}	96.6^{d}	3.8
Rumen volume (ml/kg liveweight)	64^{bc}	53^{b}	62^{bc}	54^{b}	80^{c}	62^{bc}	25^{a}	114^{d}	4.8

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Values are means with standard error of the mean (SEM); Different alphabetical superscripts across the rows indicate significant difference between treatments (P<0.05); [1]na = not analysed; [2]NGFA:GFA = ratio of non glucogenic to glucogenic volatile fatty acids [*e.g.* (Acetic + 2 Butyric)/Propionic].

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Table 6 Fatty acid (FA) profile in the rumen fluid of steers grazing ryegrass (Rye), pangola grass (Pang), signal grass (Sign), speargrass (SG), star grass (Star) and kikuyu (Kik) during the wet season (wet)[1] and SG and leucaena (Leu) green panic (GP) mix in the dry season (dry) [1]

Fatty acid	Rye	Pang	Sign	Star	Kik	SG		Leu/GP	SEM
	wet	wet	wet	wet	wet	wet	dry	dry	
	% of total FA[2]								
C12:0	0.61[a]	1.19[b]	1.31[b]	1.11[b]	0.71[a]	1.32[b]	1.46[c]	1.10[b]	0.10
C12:1	0.32[a]	0.44[a]	0.38[a]	0.39[a]	0.41[a]	0.64[b]	0.68[b]	0.63[b]	0.04
C13:1	1.18	1.60	1.33	1.85	1.48	1.67	0.77	0.93	0.12
C14:0	3.42[ab]	3.85[b]	3.94[b]	3.83[b]	3.12[a]	4.52[c]	4.41[c]	3.36[ab]	0.15
C15:0anteiso	7.55[ac]	7.68[bc]	8.81[cd]	8.77[cd]	10.10[d]	6.28[b]	3.83[a]	5.61[ab]	0.63
C15:0	3.76[b]	3.61[b]	3.71[b]	4.97[bc]	5.82[c]	3.51[c]	2.70[a]	2.90[a]	0.32
C15:1	1.77	2.09	1.50	1.87	1.35	1.78	1.37	1.38	0.09
C16:0	30.55[bc]	31.33[c]	34.16[d]	34.00[d]	32.32[cd]	29.10[ab]	24.50[a]	33.73[d]	1.01
C16:1c7	0.72[de]	0.48[ab]	0.40[a]	0.66[cd]	0.85[e]	0.59[bc]	0.58[bc]	0.66[cd]	0.04
C16:1c9	0.16[a]	0.16[a]	0.19[a]	0.29[c]	0.24[b]	0.15[a]	nd[3]	nd[3]	0.02
C17:0iso	0.69	0.70	0.61	0.65	0.48	0.74	1.59	0.82	0.11
C17:0anteiso	1.28[c]	1.33[c]	1.46[c]	1.13[bc]	0.59[a]	0.88[ab]	1.49 [c]	1.34 [c]	0.10
C17:0	1.16[ab]	2.02[c]	1.20[ab]	1.08[ab]	0.58[a]	1.57[bc]	1.19[ab]	0.88[ab]	0.13
C18:0	7.40[b]	9.09[c]	6.52[b]	7.38[b]	3.35[a]	7.11[b]	8.16[bc]	6.75[b]	0.52
C18:1t10+t11	4.21[c]	3.02[bc]	3.40[bc]	1.84[b]	4.43[c]	1.74[b]	0.71[a]	2.26[bc]	0.40
C18:1c9	3.04[bc]	2.50[ab]	3.44[c]	2.17[a]	2.86[ab]	2.27[a]	9.69[e]	6.22[d]	0.82
C18:1c11	0.97[a]	1.09[a]	1.04[a]	1.66[b]	1.04[a]	0.85[a]	1.29[ab]	2.14[c]	0.14
C18:1c12	0.32[b]	0.34[b]	0.27[ab]	0.32[b]	0.16[a]	0.14[a]	nd	nd	0.03
C18:2n-6	3.91[a]	5.19[b]	5.59[b]	5.54[b]	5.30[b]	5.56[b]	17.17[e]	10.54[d]	1.38
C18:3n-3	5.49[c]	4.47[bc]	4.31[bc]	4.90[c]	5.42[c]	3.50[b]	1.53[a]	3.28[b]	0.41
CLAc9,t11	0.63[c]	0.49[b]	0.22[a]	0.17[a]	0.19[a]	0.28[a]	0.39[ab]	0.13[a]	0.05
C20:0	0.42[a]	0.80[c]	0.60[b]	0.48[a]	0.61[b]	0.53[ab]	1.10 [d]	0.62[b]	0.07
C20:1c11	0.09[b]	0.06[a]	0.07[a]	0.07[a]	0.17[c]	0.17[c]	0.00 [a]	0.25[d]	0.03
C22:0	0.38[a]	0.79[c]	0.48[b]	0.32[a]	0.37[a]	0.36[a]	nd	nd	0.06
C22:2c13,c16	0.54[ab]	0.32[a]	0.71[b]	0.54[ab]	0.98[c]	0.41[a]	nd	nd	0.08
C22:4	1.01[d]	0.66[c]	0.44[b]	0.19[a]	0.49[b]	0.31[ab]	1.30[e]	0.27 [ab]	0.12
C24:0	0.33[a]	0.56[b]	0.52[b]	0.33[a]	0.46[b]	0.50[b]	0.54[b]	0.28[a]	0.03
C26:0	0.31	0.32	0.20	0.12	0.26	0.23	0.00	0.02	0.04
TOBCFA[4,8]	16.91[bc]	17.90[bc]	17.95[bc]	19.17[bc]	19.81[c]	15.16[ab]	12.15[a]	13.46[a]	0.85
TSFA[5,8]	58.54[a]	63.68[b]	64.16[b]	64.83[b]	59.65[a]	57.05[a]	56.85[a]	63.07[b]	1.03
TUFA[6,8]	29.72[bc]	27.19[ab]	28.16[bc]	27.08[ab]	31.49[c]	23.76[a]	37.25[c]	32.30 [c]	1.28
TC18s[7,8]	29.95[c]	28.57[bc]	27.85[bc]	25.49[ab]	25.11[ab]	22.49[a]	39.16[e]	33.45[d]	1.65

Different alphabetical superscripts across the rows indicate significant difference between treatments ($P<0.05$).

[1]Arithmetic average between samples of rumen fluid of steers grazing different forage types collected in five different times within 16 h period.

[2]Identifiable and quantifiable fatty acids presented only.

[3]nd = non detected; [4]TOBCFA = total odd branched-chain fatty acids; [5]TSFA = total saturated fatty acids; [6]TUFA = total unsaturated fatty acids; [7]TC18s = total fatty acids containing 18 carbon chains; [8]Total of individual fatty acids listed in the table plus other identifiable fatty acids.

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Table 7 The statistical relationship in change in fatty acid concentration within a 16 h period[1]

Fatty acid[2]	Linear	Quadratic	Time P value
C12:0	0.034	1.576	< 0.001
C14:0	0.786	0.000	0.014
C15:1	1.384	<0.001	<0.001
C16:0	0.006	0.136	0.017
C16:1c7	<0.001	0.634	<0.001
C17:0iso	0.010	<0.001	0.001
C17:0anteiso	<0.001	<0.001	<0.001
C17:0	<0.001	0.339	<0.001
C18:0	0.037	0.798	0.049
C18:2n-6	0.011	0.001	0.001
C18:3n-3	0.032	<0.001	0.006
TSFA[3,4]	0.465	<0.001	0.006

[1]Rumen fluid samples were collected 0, 4, 8, 12 and 16 h after dosing with Cr-EDTA.
[2]Only those FA with a significant time effect on concentration are presented.
[3]TSFA = total saturated fatty acids.
[4]Total of individual, identifiable saturated fatty acids present in the rumen fluid samples.

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