Methanogenic potential of commonly utilised South African subtropical and temperate grass species as influenced by nitrogen fertilisation

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Abstract

The aim of the study was to evaluate the effect of nitrogen (N) fertilisation on certain qualitative parameters and *in vitro* total gas and methane production of improved grass species commonly used as fodder species in South Africa. Treatments included seven grass species representing two photosynthetic pathways (C3 and C4) with three levels of N fertilisation (0, 50 and 100 kg N ha^{-1}). Plants were grown in a greenhouse and N was applied in a single application after a simulated defoliation. Sample material was harvested by hand after an 8 week regrowth period. Both grass species and rate of N fertiliser had effects ($P < 0.05$) on the nutritive value and *in vitro* organic matter digestibility of the selected species. No overall effect was found for N fertilisation on *in vitro* total gas or methane production in the trial. The crude protein concentration increased ($P < 0.05$) and the neutral detergent fibre concentration tended to decrease as the level of N fertilisation increased for both C3 and C4 species. Increasing the level of N fertiliser increased ($P < 0.05$) the methanogenic potential of *D. glomerata*, *F. arundinaceae* and *C. ciliaris* after the 24 hour incubation period but no significant effects were reported after the 48 hour incubation period.

Key words: nutritive value, digestibility, fermentation, C3 and C4 grass species

Introduction

Understanding the effect of forage quality on the production of anthropogenic greenhouse gases from livestock is important for the development of mitigation strategies for agricultural systems (Beauchemin *et al*. 2008; Bhatta *et al*. 2017). The livestock sector is a significant source of greenhouse gas (GHG) emissions in South Africa contributing 60% of total agricultural CO_2 equivalent emissions (Meissner *et al*. 2013). Beef cattle, sheep and privately owned game rely mainly on extensive forage-based production systems and accounts for 85% of total livestock methane (CH_4) emissions in South Africa (Du Toit *et al*. 2013a,b,c).

One of the main factors that contributes to the limited productivity of ruminant livestock in tropical and subtropical regions in developing countries is the poor nutritional conditions that are characterised by highly lignified, low digestible feed from poor quality, nitrogen (N) limited native rangeland and crop residues (Goel and Makkar, 2012). Meissner *et al*. (1999) categorised roughage quality according to the digestible organic matter (DOM) concentration as poor (<45%), low (45 – 55 %), medium (55 – 70%) and high (>70%). Improving forage quality offered to ruminants through forage specie selection, rangeland reinforcement through the introduction of more productive and nutritious species and improved rangeland management systems has the potential to reduce CH_4 emissions per unit animal product as a result of increased digestibility and reduced ruminal retention time of feed particles (Beauchemin *et al*. 2009; Banik *et al*. 2013). Benchaar *et al*. (2014) stated that a 15 % reduction in CH_4 emissions

could be possible by increasing the digestibility of forages and a 7% reduction through increasing voluntary feed intake of livestock.

The influence of N fertilisation on the improvement of forage quality and productivity has been investigated by several researchers (Valk *et al*. 1996; Rivera *et al*. 2017; Ullah *et al*. 2018). However, studies evaluating the effect of N fertilisation on the methanogenic potential (*in vitro* CH_4 gas/ Total *in vitro* gas production) of tropical and subtropical grass species are not readily available. Nitrogen fertilisation can influence the pattern and rate of degradation in the rumen of crude protein (CP) and neutral detergent fibre (NDF) (Valk *et al*. 1996) by increasing the concentration of neutral detergent insoluble nitrogen (NDIN) and altering the protein: carbohydrate ratio (Valk *et al*. 1996) which could influence the methanogenic potential of forages. Previous studies hypothesised that increased N fertilisation of forages would reduce fermentation gas production due to differences in the stoichiometry of fermentation of CP relative to the carbohydrate which will limit gas production and ruminal hydrogen (H^+) supply (Cone *et al*. 1999). Mathison *et al*. (1998) stated that increased nitrate levels in pastures can serve as an H^+ sink and reduce enteric methane production from ruminants. In a study conducted on perennial ryegrass cultivars Lovett *et al*. (2004) reported a decrease in total gas production (TGP) and CH_4 production with increasing N fertiliser application rates at short in vitro incubations.

In vitro techniques have been used by several researchers as a practical screening tool to predict plant digestibility, plant nutritive value, fermentation characteristics and methanogenic potential (CH_4:TGP) taking into consideration the complex interaction between rumen microbes and feed particles (Lovett *et al*. 2006; Durmic *et al*. 2010; Banik *et al*. 2013; Durmic *et al*. 2017). Variability in these traits among accepted improved pasture species would allow for the selection

of low methanogenic pastures that do not compromise animal productivity. This would improve the ability of producers to reduce CH_4 emissions from livestock, reducing the carbon foot print of production systems, allowing more efficient and climate friendly management without major changes in current production practices.

The aim of this study was to evaluate the influence of a range of N fertiliser application rates on the nutrient concentration, *in vitro* organic matter digestibility, *in vitro* total gas production and *in vitro* CH_4: TGP of commonly used improved sub-tropical (C4) and temperate (C3) grass species in South Africa.

Material and Methods

Study area description

The experiment was conducted in a glass greenhouse situated on the Hatfield experimental farm at the University of Pretoria, South Africa. Seven grass species of current economic importance in South Africa were investigated (Table 1) in two groups: four species in the C4 group and three species in the C3 group. The effects of three commonly used N fertilisation rates in South African pasture production systems were evaluated: 0, 50, and 100 kg N ha^{-1}. All treatments were replicated three times in a randomised complete block design. Seed were sourced from a commercial company and sown into 15L pots in a controlled environment where the temperature and humidity varied between 18 – 34 °C and 30 – 68 %, respectively. The pots were filled with 12 kg of an air dried and sieved potting soil mixture comprising of 20 % clay, 23 % silt and 57 % sand. Soil samples were analysed at a commercial accredited laboratory (Nvirotek laboratories, Unit 6, Nviro Business hub, Hartbeespoort, 0260, South Africa). Some physical and chemical

characteristics of the soil were: a bulk density of 1.1 g cm^{-3}; 300 mg kg^{-1} P (Bray I); 2554 mg kg^{-1} K; 556 mg kg^{-1} Na; 3650 mg kg^{-1} Ca; 616 mg kg^{-1} Mg; and a pH (KCl) of 5.63.

Ten seeds from each species were planted per pot and allowed to germinate. Once established, the seedlings were thinned out to three uniform seedlings per pot. All pots received a single dressing of N fertilizer, as limestone ammonium nitrate (28 %N) after the thinning process according to the experimental treatments. The pots were rotated once a week in the glasshouse to minimize the influence of environmental variation within the glasshouse. All pots were weighed and watered to 90% field capacity according to Pieterse *et al*. (1997). To prevent mineral loss all pots received a saucer and any leached water was returned to the pots an hour after watering. For the remainder of the trial period, the pots were weighed every three days and watered to 90% field capacity.

Samples for the analysis of nutritive value and *in vitro* fermentation were obtained from the second regrowth phase after an initial harvesting cycle. The initial growth period lasted for 6 weeks thereafter all pots were harvested and a soil core sample was taken from each pot using a thin polyvinyl chloride pipe and analysed to ensure all treatments had a similar soil nutrient composition before the N fertiliser treatment was applied per treatment as described for the second regrowth phase. Both harvest cycles were done by hand at 5cm above soil level. The second harvest period was done after an 8 week regrowth period when the C4 species started to flower and the C3 species were still vegetative. The harvested material was air dried and ground to pass through a 1.0 mm screen. Material was stored at room temperature (20 – 25 °C) in sealed containers for analysis.

Table 1. List of perennial grass species investigated including common and scientific names, cultivar, and photosynthetic pathway

Common name	Scientific name*	Cultivar	Photosynthetic pathway
Blue buffalo grass	*Cenchrus ciliaris*	cv. Molopo	C4
Rhodes Grass	*Chloris gayana*	cv. Katambora	C4
Smuts Finger grass	*Digitaria eriantha*	cv. Irene	C4
Buffalo grass	*Panicum maximum*	cv. Gatton	C4
Cocksfoot	*Dactylis glomerata*	cv. Cambria	C3
Tall fescue	*Festuca arundinaceae*	cv. Duramax	C3
Perennial ryegrass	*Lolium perenne*	cv. Halo	C3

Scientific name according to Gibbs Russel *et al*. (1991)

Nutritive value

Plant samples were analysed for dry matter (DM), organic matter (OM), CP, NDF, acid detergent fibre (ADF), acid detergent lignin (ADL), and *in vitro* organic matter digestibility (IVOMD) then metabolisable energy (ME) was estimated. The DM content was determined by drying samples for 24 h at 105 °C in a forced air oven after which the samples were weighted, then combusted at 450 °C for 8 h in a muffle furnace to determine the OM concentration (AOAC, 2000). Nitrogen concentration of samples were analysed by total combustion (AOAC, 2000) on a LECO FP-248 N and protein analyser (LECO Corporation, St Joseph, MI, USA). The NDF and ADF concentrations were determined using an ANKOM 200/220 Fibre Analyser (ANKOM Technology, Fairport, NY, USA) based on the methods described by Van Soest *et al*. (1991). Sodium sulphite and heat stable amylase were used in the analysis of NDF. The ADL concentration was determined according to Van Soest *et al*. (1991) through the solubilisation of

cellulose with sulfuric acid in the ADF residue. The fibre fractions were expressed inclusive of residual ash. Metabolisable energy (ME) was calculated from gross energy (GE) and IVOMD according to Minson (1990) and Robinson *et al*. (2004) as ME (MJ kg^{-1} DM) = 0.81[(GE x IVOMD) / 100].

In vitro digestibility, total gas, and methane production measurement

In vitro organic matter digestibility was determined using the Tilley and Terry method (Tilley and Terry, 1963) as modified by Engels and Van der Merwe (1967). Three rumen cannulated Döhne Merino wethers were used as rumen inoculum donors. The care, handling and maintenance of cannulated sheep were in accordance with animal welfare regulations of the animal ethics committee of the University of Pretoria (EC018-14). The donor sheep were fed a diet consisting of 50 % *Eragrostis curvula* hay and 50 % *Medicago sativa* hay. Rumen fluid was collected two hours after the morning feeding, pooled, and filtered through two layers of cheese cloth. The rumen fluid was stored in a pre-warmed insulated thermos flask pre-filled with CO_2.

Samples for gas analysis were incubated in triplicate according to the procedure described by Theodorou *et al*. (1994). Approximately 400 mg of dried plant sample was weighed into 120 ml serum bottles. Filtered rumen fluid (15 ml) was mixed with 30 ml an anaerobic buffer/ mineral solution prepared according to Goering and Van Soest (1988) with modifications suggested by Mould *et al*. (2005). After saturation with CO_2 the serum bottles were sealed with rubber stoppers and aluminum crimp seal caps. Possible gas build up was equalized by inserting a hypodermic needle through the rubber stopper for approximately 5 seconds. Thereafter the sample bottles were placed in an incubator at 39°C with a rotary shaker set at 120 rpm. The incubation and gas production measurements lasted for 48 hours, and all measurements were

corrected for blank gas production (gas production in buffered rumen fluid without sample). The system consisted of a digital data logger (tracker 220 series indicators, Omega Engineering, Inc., Laval, QC, Canada) connected to a pressure transducer (PX4200-015GI from Omega Engineering, Inc., Laval, QC, Canada). Gas pressure was measured at 0, 4, 12, 24, and 48 hour time intervals using the pressure transducer. After each pressure reading a small gas sample (2 ml) was taken from the headspace using a Hamilton gas tight syringe for immediate CH_4 analysis by gas chromatography (Agilent 490 Micro gas chromatograph). The gas chromatograph was equipped with a 10m stainless steel Porapak-Q column and a Thermal Conductivity Detector (TCD). The injector temperature and column temperature was set at 45°C and 50°C respectively with a 30 ms injection time and a static pressure of 80 kPa. Methane content (ml g^{-1} DM incubated) was calculated according to Banik *et al*. (2013).

Statistical analysis

The two groups of grass species (3 species in the C3 group and 4 in the C4 group) were analysed separately. The data were subjected to an analysis of variance (ANOVA) with 2 factors (specie and N application) and 3 block replications using the GLM procedure in SAS (SAS, 1999). The Shapiro-Wilk's test was performed on the standardized residuals to test for deviations from normality (Shapiro and Wilk, 1965). In cases where there were significant deviations from normality and it was due to skewness, outliers were removed until the distribution of the residuals were normal or symmetrical (Glass *et al*. 1972). Student's t-LSD (least significant difference) was calculated at a 5 % significance level to compare means of significant source effects.

Results

Forage quality

Both grass species and level of N fertilisation had effects ($P < 0.05$) on the nutritive value, *in vitro* OM digestibility and the *in vitro* gas production characteristics of the selected grass species (Tables 2 and 3). Interactions between grass species and N fertilisation level were significant for NDF and ADF concentrations (Table 2) and for IVOMD (Table 3) in C4 and C3 species respectively.

Table 2. Analysis of variance for forage quality factors (DM basis) for selected sub-tropical grass species

Parameters	Sp	N	Sp x N	R^2	CV	Mean
Ash (%)	<0.001	0.119	0.190	0.81	7.05	11.60
CP (%)	<0.001	<0.001	0.881	0.76	11.50	7.09
NDF (%)	<0.001	0.002	<0.001	0.96	1.63	64.94
ADF (%)	<0.001	<0.001	0.017	0.94	2.38	36.23
ADL (%)	<0.001	0.504	0.676	0.72	12.26	4.70
IVOMD (%)	<0.001	<0.001	0.065	0.94	4.54	58.21
ME (MJ/kg DM)	<0.001	0.046	0.086	0.75	8.23	7.67
TGP 24 h (ml/g DM)	0.002	0.816	0.443	0.55	12.63	96.70
CH_4 24 h (ml/g DM)	<0.001	0.459	0.318	0.69	12.99	4.16
TGP 48 h (ml/g DM)	0.019	0.560	0.424	0.48	5.56	150.34
CH_4 48 h (ml/g DM)	0.018	0.725	0.663	0.48	11.69	10.37

Sp: Specie; N: N kg/ha; Sp x N: Species x N kg/ha;

CP: Crude protein; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; IVOMD: *In vitro* organic matter digestibility; ME: Metabolisable energy; TGP: Total gas production; CH_4: *In vitro* methane production

A probability $P<0.05$ is considered as significant and $P<0.01$ as highly significant

Table 3. Analysis of variance for forage quality factors (DM basis) for selected temperate grass species

Parameters	Sp	N	Sp x N	R^2	CV	Mean
Ash (%)	<0.001	0.182	0.122	0.77	6.97	14.36
CP (%)	0.036	<0.001	0.379	0.90	8.16	9.87
NDF (%)	<0.001	0.126	0.315	0.75	3.30	54.66
ADF (%)	<0.001	0.408	0.095	0.87	4.26	31.40
ADL (%)	0.034	0.118	0.334	0.56	14.86	3.72
IVOMD (%)	<0.001	<0.001	<0.001	0.97	3.03	72.07
ME (MJ kg/DM)	0.007	0.460	0.846	0.58	12.12	11.67
TGP 24 h (ml/g DM)	<0.001	0.149	0.327	0.77	10.96	113.10
CH_4 24 h (ml/g DM)	<0.001	0.018	0.089	0.75	15.48	4.81
TGP 48 h (ml/g DM)	<0.001	0.367	0.404	0.77	7.64	158.79
CH_4 48 h (ml/g DM)	<0.001	0.163	0.406	0.87	9.30	9.60

Sp: Specie; N: N kg/ha; Sp x N: Species x N kg/ha; NS: Not significant

CP: Crude protein; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; IVOMD: *In vitro* organic matter digestibility; ME: Metabolisable energy; TGP: Total gas production; CH_4: *In vitro* methane production

A probability $P<0.05$ is considered as significant and $P<0.01$ as highly significant

The level of N fertilisation had no effect ($P > 0.05$) on the *in vitro* gas production parameters except for the 24 hour CH_4 production of C3 species (Table 3). *In vitro* gas production was not affected by interactions between species and the level of N fertilisation in either C3 or C4 species.

The nutritive analysis indicated that N fertilisation had an inconsistent effect on the ash and NDF concentrations of both C4 and C3 species. Increasing N decreased ($P < 0.05$) the ash concentration of *C. ciliaris* and *C. gayana* and the NDF concentration ($P < 0.05$) of *C. gayana* and *P. maximum* in the C4 species and decreased ($P < 0.05$) the ash and NDF concentrations of *D. glomerata* in the C3 species (Tables 4 and 5). Although no effect ($P > 0.05$) was shown in the

ADF and ADL concentrations of C4 and C3 species, except for the ADF concentration of *C. gayana* and *P. maximum* which decreased ($P < 0.05$), there was a tendency ($P < 0.10$) for the ADF concentration of C4 and C3 species to decrease with increased N level. The CP concentration increased ($P < 0.05$) with the level of N across all C4 and C3 species except for *D. eriantha*. *In vitro* organic matter digestibility increased in *C. ciliaris* and *D. eriantha* (Table 4) but decreased in *D. glomerata* (Table 5) as the level of N fertilisation was increased from 0 to 100 kg N ha^{-1}. Metabolisable energy concentration was not affected by the level of N fertilisation in any C4 or C3 grass species although between species differences were present across all N levels for C4 species and in the 100 kg N ha^{-1} treatment for C3 species.

In comparisons among C4 species, *C. ciliaris* presented with the lowest CP concentration ($P<0.05$) and the highest NDF, ADF and ADL concentrations ($P<0.05$) across all N treatments compared to *P. maximum* and *D. eriantha*. *Panicum maximum* had the highest IVOMD ($P<0.05$) compared to the other C4 species investigated at the 0 and 50 kg N ha^{-1} treatments as presented in Table 4.

The nutritive concentration from C3 species is presented in Table 5. There was less between species variation in the C3 species compared to the C4 species investigated in the trial. No differences were found for CP at the 0 and 50 kg N ha^{-1} treatments among C3 species but *L. perenne* had a higher CP concentration ($P < 0.05$) compared to *D. glomerata* and *F. arundinaceae* at the 100 kg N ha^{-1} treatment. *Dactylis glomerata* had a higher NDF concentration ($P < 0.05$) across the different N treatments compared to *F. arundinaceae* and *L. perenne*. Across all N treatments, *F. arundinaceae* had the lowest ADF ($P < 0.05$)

Table 4. Effect of nitrogen fertilisation on the chemical composition (% of DM) of improved sub-tropical C4 grass species commonly used in South Africa

	N (kg/ha)	Ash	CP	NDF	ADF	ADL	IVOMD	ME (MJ/kg DM)
C. ciliaris	**0**	10.58^{fg}	5.16^{f}	68.09^{bc}	40.49^{b}	5.78^{a}	60.15^{b}	8.26^{abc}
	50	9.66^{gh}	5.56^{ef}	73.04^{a}	42.82^{a}	5.74^{a}	58.14^{bc}	7.62^{bcde}
	100	8.95^{h}	6.58^{cde}	67.54^{bc}	40.70^{b}	5.89^{a}	66.29^{a}	8.73^{ab}
C. gayana	**0**	13.07^{ab}	6.00^{def}	69.33^{b}	35.53^{cd}	4.59^{bc}	55.27^{c}	7.05^{de}
	50	12.10^{abcde}	7.14^{bcd}	67.03^{c}	34.88^{de}	4.92^{ab}	53.76^{cd}	7.00^{de}
	100	11.44^{def}	7.66^{abc}	66.88^{c}	33.83^{ef}	4.30^{bc}	56.75^{bc}	7.98^{abcd}
D. eriantha	**0**	11.75^{bcdef}	7.16^{bcd}	59.75^{ef}	35.02^{de}	4.06^{bc}	42.28^{e}	5.61^{f}
	50	11.05^{ef}	8.37^{ab}	59.13^{f}	33.96^{ef}	4.49^{bc}	49.81^{d}	7.46^{cde}
	100	11.63^{cdef}	8.35^{ab}	60.17^{ef}	33.68^{ef}	4.36^{bc}	50.25^{d}	6.68^{ef}
P. maximum	**0**	12.67^{abcd}	6.77^{cde}	64.84^{d}	36.03^{cd}	3.73^{c}	66.02^{a}	8.31^{abc}
	50	12.97^{abc}	7.60^{abc}	64.64^{d}	36.71^{c}	4.04^{bc}	67.53^{a}	8.66^{ab}
	100	13.37^{a}	8.70^{a}	61.49^{e}	33.36^{f}	4.49^{bc}	70.23^{a}	8.79^{a}
LSD $_{p=0.05}$		1.386	1.380	1.832	1.492	0.975	4.683	1.147
MSE(df)		0.669 (22)	0.664 (22)	1.118 (21)	0.741 (21)	0.332 (22)	6.978 (20)	0.459 (22)

Values within a column with different superscripts differ significantly ($P < 0.05$)

LSD: Least significant difference; MSE: Mean square error; df: degrees of freedom; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; IVOMD: *In vitro* organic matter digestibility; ME: Metabolisable energy

concentrations and *L. perenne* had the highest IVOMD ($P < 0.05$) compared to other C3 species investigated (Table 5).

Table 5. Effect of nitrogen fertilisation on the chemical composition (% of DM) of improved temperate C3 grass species commonly used in South Africa

	N (kg/ha)	Ash	CP	NDF	ADF	ADL	IVOMD	ME (MJ/ kg DM)
D. glomerata	**0**	15.41^{ab}	8.12^{ef}	58.81^{a}	34.62^{a}	3.90^{abc}	53.30^{d}	10.40^{b}
	50	13.34^{c}	9.69^{d}	58.11^{a}	34.52^{a}	3.46^{bc}	59.66^{c}	10.75^{b}
	100	13.42^{c}	11.75^{b}	54.92^{b}	32.42^{ab}	4.38^{ab}	71.70^{b}	10.49^{b}
F. arundinaceae	**0**	13.76^{bc}	7.41^{f}	53.20^{bcd}	29.17^{c}	4.55^{a}	74.93^{b}	12.60^{ab}
	50	12.84^{c}	9.27^{de}	51.76^{cd}	26.93^{c}	3.48^{bc}	73.34^{b}	12.51^{ab}
	100	12.52^{c}	11.31^{bc}	51.26^{d}	27.73^{c}	3.88^{abc}	75.19^{b}	13.82^{a}
L. perenne	**0**	15.44^{ab}	7.85^{f}	54.48^{bc}	31.56^{b}	3.35^{c}	81.22^{a}	10.73^{b}
	50	16.50^{a}	10.13^{cd}	54.62^{bc}	33.03^{ab}	3.24^{c}	79.13^{a}	11.71^{ab}
	100	16.00^{a}	13.27^{a}	54.84^{bc}	32.64^{ab}	3.29^{c}	80.15^{a}	11.98^{ab}
LSD $_{p=0.05}$		1.732	1.394	3.118	2.314	0.958	3.787	2.447
MSE (df)		1.002 (16)	0.648 (16)	3.246 (16)	1.788 (16)	0.306 (16)	4.788 (16)	1.999 (16)

Values within a column with different superscripts differ significantly (P < 0.05)

LSD: Least significant difference; MSE: Mean square error; df: degrees of freedom; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; IVOMD: *In vitro* organic matter digestibility; ME: Metabolisable energy

Forage in vitro gas and methane production potential

The *in vitro* TGP, CH_4 and CH_4: TGP of the selected species is presented in Tables 6 and 7. There were no differences (P > 0.05) found within C4 species as the N fertilisation level increased (Table 6) for all *in vitro* parameters at either the 24 or 48-hour incubation periods,

except for *C. ciliaris* which showed an increase ($P < 0.05$) in CH_4: TGP in the 100 kg N ha^{-1} treatment at the 24 hour incubation interval.

Digitaria eriantha and *C. gayana* had the lowest ($P < 0.05$) *in vitro* TGP and CH_4 production, respectively, in the control treatment (0 kg N ha^{-1}) after the 24-hour incubation period for C4 grass species. No differences were found in either TGP or CH_4 production at the 50 kg N ha^{-1} treatment in either incubation periods except for *D. eriantha* which had a higher TGP at the 48 hour incubation compared to *C. gayana*. As the level of fertilisation increased to 100 kg N ha^{-1} *C. ciliaris* and *P. maximum* produced the highest *in vitro* CH_4 ($P < 0.05$) at the 24 hour incubation period compared to *C. gayana*.

When comparing among the C4 species in the different N treatments after the 48-hour incubation period (Table 6), *P. maximum* had the highest ($P < 0.05$) TGP in the 0 kg N ha^{-1} treatment. *Chloris gayana* had a lower ($P < 0.05$) TGP compared to *D. eriantha* and *P. maximum* at 50 kg N ha^{-1} but similar to *C. ciliaris*. No between species differences ($P > 0.05$) were reported for C4 species in the 100 kg N ha^{-1} treatment.

The *in vitro* TGP, CH_4, and CH_4: TGP for the C3 grass species evaluated are presented in Table 7. Increasing the level of N fertilisation increased *in vitro* CH_4 production ($P < 0.05$) at the 24 hour incubation period for both *D. glomerata* and *F. arundinaceae*, and at the 48 hour incubation period for *F. arundinaceae*. The level of fertilisation had no effect on the CH_4: TGP in the selected C3 grass species at 48 hours incubation but increased ($P < 0.05$) the CH_4: TGP of *D. glomerata* and *F. arundinaceae* as the level of N fertilisation was increased from 0 to 100 kg N ha^{-1} in the 24 hour incubation period. No effects were found on any *in vitro* gas production parameters for *L. perenne* among N treatments.

Table 6. The effect of nitrogen fertilisation on the *in vitro* total and methane gas production (ml/g DM) of improved sub-tropical C4 grass species commonly used in South Africa

		24 Hour		48 Hour		CH_4: TG	
	N (kg/ha)	**TG**	**CH_4**	**TG**	**CH_4**	**24 hour**	**48 Hour**
C. ciliaris	**0**	105.28^{abc}	4.68^{ab}	154.59^{abc}	11.00^{ab}	0.044^{b}	0.071^{ab}
	50	98.31^{abcde}	4.35^{bc}	149.05^{abc}	10.96^{ab}	0.045^{b}	0.074^{a}
	100	102.86^{abcd}	5.38^{a}	153.29^{abc}	10.90^{ab}	0.053^{a}	0.070^{abc}
C. gayana	**0**	85.18^{cde}	3.33^{d}	136.70^{bc}	9.52^{abc}	0.039^{b}	0.069^{abc}
	50	82.20^{de}	3.59^{cd}	135.43^{c}	9.82^{abc}	0.043^{b}	0.072^{ab}
	100	89.20^{bcde}	3.30^{d}	145.84^{abc}	9.06^{bc}	0.037^{b}	0.062^{c}
D. eriantha	**0**	78.89^{e}	3.50^{cd}	135.73^{c}	8.75^{c}	0.044^{b}	0.064^{bc}
	50	101.79^{abcd}	4.12^{bcd}	160.67^{a}	10.34^{abc}	0.044^{b}	0.064^{bc}
	100	91.19^{abcde}	4.07^{bcd}	151.88^{abc}	10.59^{abc}	0.044^{b}	0.069^{abc}
P. maximum	**0**	110.44^{a}	4.69^{ab}	162.21^{a}	11.32^{a}	0.043^{b}	0.069^{abc}
	50	106.05^{ab}	4.40^{bc}	157.73^{ab}	11.02^{ab}	0.042^{b}	0.070^{abc}
	100	109.03^{ab}	4.52^{ab}	161.02^{a}	11.18^{a}	0.041^{b}	0.069^{abc}
LSD $_{p=0.05}$		20.676	0.916	21.795	2.053	0.0074	0.0084
MSE (df)		149.096 (22)	0.292 (22)	165.67 (22)	1.469 (22)	<0.0001 (22)	<0.0001 (22)

Values within a column with different superscripts differ significantly ($P < 0.05$)

LSD: Least significant difference; MSE: Mean square error; df: Degrees of freedom; TG: Total gas

After 48 hours incubation *L. perenne* had higher ($P < 0.05$) CH_4: TGP compared to *D. glomerata* and *F. arundinaceae* in both 0 and 50 kg N ha^{-1} treatments and a higher CH_4: TGP in the 100 kg N ha^{-1} treatment when compared to *D. glomerata*. Although no differences among the C3 species were found at the 100 kg N ha^{-1} treatment after 24 hours of incubation, *F. arundinaceae* had a higher CH_4: TGP compared to *L. perenne* at the 50 kg N ha^{-1} treatment.

Table 7. The effect of nitrogen fertilisation on *in vitro* total and methane gas production (ml/g DM) of improved temperate C3 grass species commonly used in South Africa

		24 Hour		48 Hour		CH4: TG	
	N (kg/ha)	**TG**	**CH4**	TG	CH4	**24 hour**	**48 Hour**
D. glomerata	**0**	82.29^{e}	3.29^{c}	128.57^{c}	6.95^{d}	0.039^{bc}	0.054^{de}
	50	91.22^{de}	3.86^{bc}	133.86^{c}	6.94^{d}	0.041^{abc}	0.051^{e}
	100	105.67^{cd}	4.87^{b}	147.80^{bc}	8.03^{cd}	0.046^{a}	0.054^{de}
F. arundinaceae	**0**	122.12^{abc}	4.46^{bc}	168.84^{a}	9.46^{bc}	0.036^{c}	0.056^{cde}
	50	133.83^{ab}	6.18^{a}	171.91^{a}	10.05^{ab}	0.046^{a}	0.058^{cd}
	100	139.22^{a}	6.51^{a}	180.18^{a}	11.10^{a}	0.047^{a}	0.062^{bc}
L. perenne	**0**	114.78^{bc}	4.82^{b}	166.39^{ab}	11.42^{a}	0.042^{ab}	0069^{a}
	50	119.21^{abc}	4.74^{b}	170.69^{a}	11.33^{a}	0.040^{bc}	0.069^{a}
	100	109.59^{cd}	4.55^{bc}	160.90^{ab}	11.11^{a}	0.042^{abc}	0.067^{ab}
LSD $_{p=0.05}$		21.455	1.288	20.993	1.545	0.0061	0.0067
MSE (df)		153.642 (16)	0.554 (16)	147.096 (16)	0.797 (16)	<0.0001 (16)	<0.0001 (16)

Values within a column with different superscripts differ significantly ($P < 0.05$)

LSD: Least significant difference; MSE: Mean square error; df: Degrees of freedom; TG: Total gas

Discussion

The objective of the study was to elucidate the influence of N fertiliser application levels on the nutrient concentration, *in vitro* digestibility, *in vitro* TGP and CH_4 production of commonly used improved C4 and C3 grass species in South Africa.

Forage quality

Increasing the level of N fertilisation increased the CP concentration of both C4 and C3 grass species except for *D. eriantha*. These results agree with results reported by Morrison *et al*. (1980), Valk *et al*. (1996) and Warner *et al*. (2015). The CP concentration reported in the present trial for both C4 and C3 species are lower than previously reported values for similar species (Pieterse *et al*. 1997; Johnson *et al*. 2001; Taute *et al*. 2002; Navarro-Villa *et al*. 2012; Banik *et al*. 2013). This might be due to differences in the growth periods after N application between the studies as well as differences between growth phases harvested between the present study and previous reported studies. Wilman (1975) reported that the N concentration of pastures peak at 10 to 14 days after N application and thereafter decrease over time. The effect of the N application on forage CP concentration in the present study could have been reduced due to the 8 week regrowth period employed before harvesting. The age of forage, whether from initial or subsequent cuttings, has a negative effect on forage by increasing fibre components as well as decreasing digestibility and/or CP concentration (Salon and Cherney, 2000).

Increasing the level of N fertilisation decreased ($P < 0.05$) the NDF and ADF concentrations of *C. gayana* and *P. maximum* but it had no effect ($P > 0.05$) on the fibre fractions of *C. ciliaris* and *D. eriantha* (Table 4). Similarly, *D. glomerata* showed a decrease ($P < 0.05$) in NDF concentration with increasing level of N fertilisation (Table 5). The fibre fraction of *F. arundinaceae* showed a tendency to decrease with increasing N fertilisation but the level of fertilisation had no effect ($P > 0.05$) on the fibre fractions of *L. perenne*. The inconsistent influence of N fertilisation on NDF, ADF and ADL concentrations reported in Table 4 and 5 is similar to the findings of Minson (1990) and Valk *et al*. (1996) who reported that the physiological stage of development has a greater influence on the fibre fractions of forage

compared to the level of N fertilisation. A similar inconsistent effect of N on forage fibre fraction was reported by Peyraud and Astigarra (1998). These authors concluded that the nutrient composition response of forages to N fertilisation is species specific.

Increasing the level of N fertilisation increased the IVOMD of two of the C4 grass species, *C. ciliaris* and *D. eriantha* but no significant effect was found for *C. gayana* and *P. maximum*. Similarly, Johnson *et al*. (2001) reported an increase in IVOMD for star grass (*Cynodon nlemfuensis*) fertilised with increasing levels of N and Taute *et al*. (2002) reported no effect of N fertiliser on the IVOMD of *P. maximum*. The IVOMD of C3 species was not affected by the level of N fertilisation except for *D. glomerata* which showed a decrease as the level of N fertilisation increased. These results are similar to results reported by Valk *et al*. (1996) and Lovett *et al*. (2004) for *L. perenne*. The decrease in the digestibility of *D. glomerata* can be explained by a slight increase in the lignin concentration with increased N fertilisation (Table 5). Peyraud and Astigarra (1998) also reported that N fertilisation increased the tiller: leaf ratio of forages which could have a negative effect on the forage digestibility. Nitrogen fertilisation can however have an indirect positive effect on digestibility by enabling an earlier utilization of grass forage. A higher level of N application allows for grass to be harvested at an earlier physiological age due to an increased growth response and yield (Peyraud and Astigarraga, 1998). This could lead to an increased intake and production from livestock and thus a reduced methane intensity (CH_4 /unit product) of the pastures. Although, these aspects of N fertilisation were not explored in the present study.

Gas production

Methane production from forages depend both on the NDF concentration and forage digestibility, which are the two main drivers of H^+ production from carbohydrate fermentation in the rumen (Archimede *et al*. 2011). The gas production values reported in Table 6 are similar to gas production values reported by Gonzalez Ronquillo *et al*. (1998) for C4 grass species. In the present trial, *C. ciliaris* and *P. maximum* produced the highest average *in vitro* CH_4 values across all N treatments after the 24 and 48-hour incubation periods. This corresponds with a higher NDF concentration and IVOMD (Table 4) of these species compared to *D. eriantha* and *C. gayana*. These results correspond with results reported by Gemeda and Hassen (2014) and Doreau *et al*. (2016) who reported a positive correlation between CH_4 production, cell wall contents and IVOMD of forages. *Digitaria eriantha* had the lowest average *in vitro* CH_4 production after the 24 and 48 hour incubation periods and tended to have a lower CH_4: TGP compared to other C4 species (Table 6) in the present trial. This could be attributed to the lower IVOMD ($P < 0.05$) of *D. eriantha* (Table 4) which might have a negative effect on voluntary intake of the forage and subsequent animal production.

The increase ($P < 0.05$) in 24 hour CH_4 and CH_4: TGP of *D. glomerata* and *F. arundinaceae* corresponds with a significant increase in the CP concentration as the level of N fertilisation increased and a reduction in the fibre fraction of the species (Table 5). These results differ from data reported by Johnson and Johnson (1995) and Lovett *et al*. (2004) that indicated a decrease in CH_4 production when feed protein concentration increased. The increase in 24-hour gas production could have been due to changes in the degradability of the CP and fibre fractions due to an increase in N fertiliser as reported by Valk *et al*. (1996). Crude protein levels above the threshold of 70 g kg DM^{-1}, as reported in the present study, are considered to enhance

microbial multiplication in the rumen thus improving fermentation (Njidda and Nasiru, 2010). The negative correlation between NDF concentration and *in vitro* gas production reported by Njidda and Nasiru (2010) and Meale *et al*. (2012) was not observed in the present study. This might have been due to the relative high IVOMD of the species reported in Table 4 and 5. Increasing N fertilisation from 0 to 100 kg N ha^{-1} had no effect on the *in vitro* total gas and CH_4 production of *L. perenne* at both the 24 and 48 hour incubation periods. These results differ from results reported by Lovett *et al*. (2004) which showed a significant decrease in the *in vitro* gas and CH_4 production with increasing N application levels to *L. perenne*. These differences may have been due to differences in the physiological age of the forages between the two trials.

In the current study, *D. glomerata* emerged as the C3 species with the lowest CH_4: TGP after 48 hours of incubation compared to *F. arundinaceae* and *L. perenne*. However, while part of the reduced 48-hour CH_4: TGP could be attributed to a reduced CH_4 production; it may also be an indication of a reduced overall ruminal fermentation potential. *Dactylis glomerata* had the lowest ($P < 0.05$) TGP after the 48 hour incubation period. This reduced fermentation could be explained by the lower ($P < 0.05$) IVOMD of *D. glomerata* reported in Table 5. The lower IVOMD of *D. glomerata* in the present trial could negatively influence dry matter intake through a reduced ruminal clearance rate which can have negative implications for livestock productivity compared to *F. arundinaceae* and *L. perenne* (Banik *et al*. 2013).

Conclusion

This study demonstrated significant differences in nutrient composition, digestibility, and *in vitro* gas production characteristics among key South African improved pasture species. Although

increasing N fertilisation levels affected the nutrient composition of the pasture species, the effect on *in vitro* CH_4 production per unit of DM digested was limited. Between species differences were found for 24 hour *in vitro* CH_4 production in C4 and C3 species but these differences diminished at the 48 hour incubation period. The data suggests that reductions in enteric CH_4 production are unlikely to be achieved through increased N fertilisation levels alone. *Chloris gayana* and *D. glomerata* showed potential for a reduced CH_4 output form C4 and C3 grass species respectively, compared to the other species evaluated in the trial. However, these results are based on *in vitro* analysis and from hand harvested samples grown in a greenhouse. There is a need for further assessment of fermentation characteristics and management practices of these species at various stages of maturity and an *in vivo* evaluation is necessary before any specie can be promoted as a low methanogenic pasture.

Conflicts of interest

The authors declare no conflicts of interest.

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