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Effect of different genotypes of *Tithonia diversifolia* on fermentation of feed mixtures with *Urochloa brizantha* cv. Marandú

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Tithonia diversifolia (Mexican sunflower) is a shrub used for animal feed that has outstanding agronomic and chemical characteristics. Its potential to modify the dynamics of fermentation and improve the supply of nutrients to ruminants has received considerable attention. This study was designed to determine the effect of different genotypes of *T. diversifolia* on ruminal fermentation and degradation of dry matter (DM), concentration of volatile fatty acids, and production of methane (CH_4) when mixed with a low-quality tropical grass, *Urochloa brizantha* (palisade grass). In a randomised complete block design, mixtures of seven genotypes of *T. diversifolia* with *U. brizantha* cv. Marandú were evaluated by using the *in vitro* gas production technique. The effect of fertilisation was also evaluated for each genotype. Inclusion of *T. diversifolia* significantly ($P < 0.05$) increased the supply of nutrients and modified fermentation parameters. DM degradation of biomass after 72 h was greater in the presence of *T. diversifolia* than for feeds based only on *U. brizantha* (68.0% vs 63.4%; $P < 0.01$). CH_4 production was lower ($P < 0.05$) during fermentation with some *T. diversifolia* genotypes (25.3 vs 27.7 mg CH_4 g^{-1} incubated DM), and the acetic:propionic acid ratio was also lower. Fertilisation of *T. diversifolia* genotypes increased DM degradation, increased the content of certain nutrients (e.g. crude protein) and modified CH_4 production. Therefore, inclusion of *T. diversifolia* in mixtures based on low-quality tropical grasses such as *U. brizantha* increases the supply of nutrients (crude protein, minerals, energy) and can modify the products of enteric fermentation, with some genotypes decreasing enteric CH_4 emissions.

TOC Summary: *Tithonia diversifolia* is a shrub with outstanding agronomic and chemical characteristics for animal feed, and with wide genetic diversity. We evaluated genotypes for potential chemical differences and fermentative behaviour to identify potential improvements to the supply of nutrients in low-quality diets for ruminants. Regardless of genotype, *T. diversifolia* offers high amount of nutrients, which could increase animal productivity as well as mitigate CH_4 emissions, characteristics that will favour its introduction to ruminant systems.

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Running head: *T. diversifolia* and ruminal fermentation

Keywords: chemical composition, methane emission, Mexican sunflower, ruminal fermentation, volatile fatty acids.

Introduction

Under tropical and subtropical conditions, there are many shrub species with the ability to produce high amounts of highly nutritional biomass, constituting a reliable alternative for the development of highly productive and resource-efficient grazing systems (Rivera *et al.* 2018). Despite such richness, only some species are empirically used in cattle systems, limiting their true productive potential (Halmemies-Beauchet-Filleau *et al.* 2018). Identifying fodder species with the ability to produce adequate amounts of forage with high nutritional quality is a need that must be satisfied to generate efficient grazing systems in compliance with current environmental and productive requirements (Valenciaga *et al.* 2018).

In recent years, *Tithonia diversifolia* (Hemsl.) A.Gray (Mexican sunflower) has gained the attention of researchers due to its potential to increase rumen fermentative efficiency and degradation, as well as to modulate fermentation dynamics of feed offered under grazing conditions (Rivera *et al.* 2013; Terry *et al.* 2016; Ribeiro *et al.* 2016; Galindo-Blanco *et al.* 2018). In countries such as Mexico, Argentina, Brazil and Colombia, *T. diversifolia* is propagated by using asexual material from existing crops and wild plants; however, in recent years, there have been advances in seed propagation (Santos-Gally *et al.* 2020).

The benefits of *T. diversifolia* come from its superior nutritional value due to its high content of crude protein (CP), minerals and energy, low fibre content, the presence of beneficial phytochemical compounds such as phenolics, flavonoids and some essential oils, and its ability to adapt to different soil and climatic conditions including acidic and low-fertility soils (Chagas-Paula *et al.* 2012; Mauricio *et al.* 2017).

Some phytochemical compounds in *T. diversifolia* can decrease enteric methane (CH_4) production and modify gas production rates through inhibitory effects on specific groups of rumen microorganisms (Barahona *et al.* 2003; Delgado *et al.* 2012; Banik *et al.* 2013; Bhatta *et al.* 2013). Furthermore, the diversity of *T. diversifolia* in Latin America is very high (Rivera *et al.* 2019), and this shrub could represent part of a strategy to diversify nutrients available to ruminants and to modify rumen fermentation patterns, including enteric CH_4 emissions.

The addition of 30% *T. diversifolia* to a diet based on *Cynodon nlemfuensis* led to reduced CH_4 production in *in vitro* studies (Delgado *et al.* 2012), likely due to the presence of *T. diversifolia* secondary metabolites such as tannins, flavonoids, saponins and alkaloids that reduce rumen protozoan populations, which share a symbiotic relationship with ruminal CH_4-producing methanogens (Hook *et al.* 2010).

Rivera *et al.* (2019) identified different *T. diversifolia* genotypes with wide phenotypic diversity and adaptation to diverse agroecological conditions, and some of these genotypes had high nutritional quality. The present study evaluated whether these genotypes also differ in *in vitro* fermentation and degradation, the production of volatile fatty acids (VFAs) and the generation of CH_4 when included as a part of forages commonly offered to grazing ruminants in the lowland tropics.

Materials and methods

Plant materials and localisation

Seven of the best performing *T. diversifolia* genotypes identified by Rivera *et al.* (2017) of a group of 30 provenances were collected in experimental plots under lowland tropic conditions (Meta, Colombia: 3°47′21.43″N, 73°49′15.93″W; 530 m a.m.s.l.). The selected and collected genotypes presented outstanding performance in the production of biomass, number of stems and overall growth and exhibited genetic differences by cluster analysis (Shannon index 0.442 and Nei genetic diversity 0.2812). The experiment was a randomised complete block design in which half of the plots with the seven genotypes were fertilised, and the other half did not receive fertilisation; in total, 21 plots were fertilised and 21 plots had no fertiliser applied. Forage samples of the seven genotypes (leaves and thin stems <5 mm) with and without fertilisation (14 mixtures in total) were collected at a height of 83 cm and mixed individually with *Urochloa brizantha* (A.Rich.) R.D.Webster (palisade grass) cv. Marandú (CIAT 6780) at a rate of 25:75 of total dry matter (DM) (Molina *et al.* 2015). The different *T. diversifolia* samples were collected at 40 days of age (1-year-old crop and collection during the rainy–dry season), and *U. brizantha* was collected at 30 days of age. A grass-alone treatment (*U. brizantha* 100%) was used as a control, representing baseline forage. The level of fertilisation used was in accordance with the extraction of nutrients from 40-day-old *T. diversifolia* plants (Botero-Londoño *et al.* 2019). Fertilisation was with urea (46% N), diammonium phosphate (DAP) ($(NH_4)_2HPO_4$; 46% P_2O_5, 18% N)) and potassium chloride (KCl) at rates of 16.22, 2.15 and 4.89 g plant^{-1}, respectively.

In vitro *assessment of fermentation, production of CH_4 and VFA, and degradability of DM*

Production of CH_4, generation of short-chain VFAs, and degradability of DM of the different mixtures and the control were studied by using the *in vitro* gas production technique (Theodorou *et al.* 1994) following the recommendations of Yáñez-Ruiz *et al.* (2016). Pressure measurements of the gases were made 3, 6, 9, 12, 24, 36, 48 and 72 h after inoculation, and the total volume of gas production at each sampling point was determined via the equation developed by Rivera *et al.* (2015*a*): $y = 0.1312 + 4.9203x$, where x is pressure at each point (R^2 = 0.975). At eight time points post-inoculation, pressure measurements were taken of the gas

present in the headspace by using a pressure transducer (Sper Scientific, Scottsdale, AZ, USA) connected to a digital readout (Lutron Electronic Enterprise, Taiwan), a hypodermic needle, and a plastic syringe to measure and obtain the volume until the transducer reading reached zero.

Rumen liquid was collected from adult cattle grazing in *Urochloa* pasture, and the buffer solution was prepared as suggested by Brooks and Theodorou (1997). The mixtures were incubated only once, using three bottles per time-point for each treatment, as well as a control bottle with no forage added, allowing the value obtained to be corrected by the blank flask weight (i.e. bottle without forage sample but containing buffer solution, reducing agent, and ruminal fluid).

In vitro assessments of DM degradability (IVDMD), VFAs (acetate, propionate and butyrate) and CH_4 production were performed after 24 and 72 h of incubation. VFA determinations were made in the NUTRILAB-GRICA Laboratory of the University of Antioquia; filtered ruminal fluid (1 mL) was taken and added to 25% metaphosphoric acid (4 mL) to be stored at 4°C for further determination of VFAs. VFA concentration was assessed by high-performance liquid chromatography (20A Series; Shimadzu, Tokyo) using an Aminex HPX-87H column (300 mm by 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) with an ultraviolet/visible detector (UV/Vis, SPD-20AV) at 50°C and a wavelength of 210 nm; the mobile phase (0.005 M H_2SO_4) was used with a volume flow rate of 0.7 mL min^{-1}. The CH_4 concentration was quantified by using a gas chromatograph (GC-2014; Shimadzu) with the following specifications: packed 1/800 stainless steel columns HAYESEP T 80/100 mesh 1.0 m, HAYESEP D 80/100 4 m, 1.5 P–N, 0.7 m Shimalite Q 100/180; column temperature 80°C; detector temperature FID = 250°C, ECD 325°C; methanizer temperature 380°C; carrier gas nitrogen; column flow 30.83 mL min^{-1}; and injection volume managed by a loop with a capacity of 2 mL (International Centre for Tropical Agriculture, CIAT).

Chemical composition of assessed T. diversifolia *genotypes*

Plant materials were analysed for DM, CP, ash, ether extract, neutral detergent fibre (NDF), acid detergent fibre (ADF), calcium (Ca), phosphorus (P), total digestible nutrients, gross energy, net energy of lactation (NE_L), total phenols and total tannins. These assessments were determined by near infrared spectroscopy (NIRS) (Ariza-Nieto *et al.* 2018), and levels of Ca and P were determined by atomic absorption and UV-Vis spectrophotometry based on methods NTC 5151 (ICONTEC 2003) and 4981 (ICONTEC 2001), respectively (Animal Nutrition Laboratory of the Colombian Corporation for Agricultural Research, AGROSAVIA). Total phenols and tannins were quantified according to the Folin-Ciocalteu methodology (Animal Nutrition & Forages Quality Laboratory, CIAT).

Experimental design and statistical analyses

Gas production data were adjusted to the Gompertz nonlinear model (Lavrenčič *et al.* 1997) with the aim of performing a more accurate biological interpretation of the gas production results. Mixtures and control treatments were compared by means of a randomised complete block design with three replicates where blocks corresponded to sites from which the materials of *T. diversifolia* genotypes (experimental plots) were collected; Rivera (2020) planted *T. diversifolia* genotypes in blocks according to different chemical and physical soil conditions:

$$yklj = \mu + \alpha k + \gamma l + \xi kl + \beta j + \varrho klj$$

where μ is mean of the overall effect; αk is factor effect k (collected genotypes 1, 2, 3 … 7); γl is factor effect l (fertilisation level 1, 2); βj is block j effect; ξkl is factor interaction; ϱklj is random value, experimental error of experimental unit lkj; and $yklj$ is observation in the experimental unit of the variable to evaluate.

The incubations were performed during one run; the experimental units corresponded to the plots where the forages were collected (feeds); and each of the plots was represented by three bottles and a blank flask at each time-point in the laboratory. The fixed effects of the model were the mixtures (treatments/mixtures) evaluated and the incubation times, and the random effects corresponded to the plots where the forages were collected and the inoculums used in the incubation.

All analyses were performed using the RStudio tool and the *agricolae* and *easynls* libraries (R Foundation, Vienna). Differences between means were identified by Tukey's contrast test applied at a significance level of $P = 0.05$, and the normality (Shapiro–Wilk) and homogeneity (Levene) of variance and additivity (Tukey's test of additivity) were evaluated.

Results

Chemical composition

The chemical composition of the evaluated mixtures before fermentation is shown in Table 1. Compared with the grass-alone control treatment, inclusion of *T. diversifolia* resulted in significant ($P < 0.05$) differences in all variables except ether extract and gross energy; however, there were no significant differences among mixtures that included the different *T. diversifolia* genotypes. In general, including *T. diversifolia* decreased the DM and fibre content in the mixture and increased the content of minerals and NE_L ($P < 0.05$). Fertilisation of the seven genotypes of *T. diversifolia* increased ($P < 0.05$) CP and NE_L contents and decreased Ca compared with unfertilised plots. For total phenols and total tannins, no differences were observed among the genotypes, but there was an effect of fertilisation ($P < 0.001$).

In vitro *fermentation*

Inclusion of *T. diversifolia* modified some *in vitro* ruminal fermentation parameters (Table 2), with significant ($P < 0.05$) effects on the maximum rate of gas production (MRGP) and lag phase (LP) variables but not on timing at the inflection point (TIP) or gas at the inflection point. Overall, fertilisation of *T. diversifolia* significantly increased MRGP ($P = 0.004$) and reduced TIP ($P = 0.001$). Among the mixtures, genotypes 1, 3 and 7 presented lower MRGP values, and mixtures of genotypes 2 and 3 higher LP values (average of fertilised and unfertilised; $P = 0.007$).

Degradability of DM

There were no differences in IVDMD between the evaluated mixtures and the grass-alone control after 24 h (Table 3). By contrast, after 72 h of fermentation, mixtures that included *T. diversifolia* exhibited significantly ($P < 0.05$) higher IVDMD values than the grass-alone control treatment (68.0% vs 63.4%), with no significant differences in IVDMD among mixtures including *T. diversifolia*. Overall, fertilised *T. diversifolia* mixtures presented higher ($P < 0.01$) IVDMD than unfertilised mixtures.

VFA production

After 24 h of incubation, the *U. brizantha* control had a lower ($P = 0.0321$) propionic acid concentration than mixtures that included genotypes 5, 6 and 7 of *T. diversifolia* (average of fertilised and unfertilised). Genotypes 2 and 4 yielded greater amounts of acetate among mixtures. In turn, the acetate:propionate ratios of all mixtures were significantly ($P = 0.004$) different from the *U. brizantha* control (Table 4). Genotype 6 + *U. brizantha* produced the lowest values for total VFA. After 72 h, there were significant ($P < 0.05$) differences for all assessed parameters. Inclusion of *T. diversifolia* was associated with reduced concentrations of acetic acid and higher levels of propionic acid. Likewise, the acetate:propionate ratio was reduced in all *T. diversifolia* mixtures.

Methane production

Inclusion of *T. diversifolia* significantly favoured a decrease in CH_4 production compared with the *U. brizantha* control treatment, especially when CH_4 emissions were estimated per g DMD at 72 h (Table 5). On average, after 72 h of incubation, inclusion of *T. diversifolia* decreased the Ym units (CH_4 loss as a percentage of gross energy), and production of CH_4 (as mg per g incubated dry matter or DMD) by 0.99, 2.47 and 5.83, respectively. In addition, among *T. diversifolia* mixtures, those with genotypes 2, 4 and 7 had the lowest CH_4 production per g IDM ($P = 0.006$). The highest Ym was observed for the *U. brizantha* control and genotype 6 mixture.

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Discussion

Inclusion of *T. diversifolia* in tropical grass-based mixtures has been reported as a successful strategy for increasing nutrient supply, animal productivity, and stocking rate in tropical livestock systems (Rivera *et al.* 2015*b*; Ribeiro *et al.* 2016; Mejía-Díaz *et al.* 2017).

The results of this study demonstrated that replacing 25% (DM basis) of a tropical grass such as *Urochloa* sp. with *T. diversifolia* increased concentrations of CP, P, Ca and NE_L in the forage by 45%, 39%, 65% and 12%, respectively. Furthermore, it decreased NDF and ADF contents in the biomass by 15% and 19%, respectively (Table 1). This improved supply of nutrients because the lower fibre content favours DM degradability, which is beneficial for increased nutrient utilisation in ruminants and hence production (Rivera *et al.* 2015*b*; Mauricio *et al.* 2017).

Regardless of the *T. diversifolia* genotype used, addition of this species to conventional tropical grass increased the nutritional value and degradability of the forage. This was further improved by fertilisation, which had positive effects on some nutrient contents (CP, NE_L) and degradability parameters. The nutritional value of the genotypes evaluated in the present study was, in general, higher than that found by La O *et al.* (2012) for *T. diversifolia* ecotypes grown in Cuba. However, they identified an influence of the ecotypes on nutritional quality.

Galindo *et al.* (2011) and Galindo-Blanco *et al.* (2018) suggested that as a result of the phytochemical composition of *T. diversifolia*, its dietary inclusion in pasture-based mixtures modifies the rumen fermentation products, as observed in this evaluation. Changes in gas production might be due to a lower concentration of insoluble carbohydrates (Barahona and Sánchez 2005; Barahona *et al.* 2006) and a greater supply of nutrients, as well as optimisation of microbial fermentation when this shrub with a relatively high protein content is included in the feed mixture, as evident in the decrease of the LP in *T. diversifolia* mixtures (Table 2). The decreased contents of ADF and NDF in the *T. diversifolia* mixtures may have also contributed to the modification of the fermentation parameters.

According to Dhanoa *et al.* (2000) and Fondevilla and Barrios (2001), ruminal microorganisms and their enzymes first target fermentable carbohydrates, and then, through fibre colonisation and degradation, gas production increases. Inclusion of *T. diversifolia* improves the fermentative efficiency of tropical mixtures, improving their solubility in the rumen (i.e. LP). According to Gallego-Castro *et al.* (2016), soluble protein in *T. diversifolia* comprises up to 40% of total protein; in addition, *T. diversifolia* has high contents of total sugars (39.8%) and water-soluble carbohydrates (7.2%), which modify the fermentation dynamics, as shown by the lower LP values observed in the presence of *T. diversifolia*.

La O *et al.* (2012) reported differences in the fermentation and degradation parameters of nine ecotypes of *T. diversifolia* and identified high variability in parameters *a*, *b*, *a* + *b* and *c* of the Ørskov and McDonald (1979) model. In this study, although MRGP and LP showed differences among the mixtures that included *T. diversifolia*, these results are relevant because no differences were found among the *T. diversifolia* genotypes evaluated and the *U. brizantha* treatment alone. All the mixtures used showed better fermentative performance.

Inclusion of shrub species in forage for ruminant feed often leads to modifications in VFA production and reduced CH_4 emissions (Terry *et al.* 2016; Molina *et al.* 2016). Likewise, including these species in the forage can lead to increased DM intake (Cuartas *et al.* 2015; Gaviria-Uribe *et al.* 2015), as well as more efficient fermentation and changes in ruminal microbial populations (Terry *et al.* 2016; Mahecha-Ledesma *et al.* 2017).

In this study, including *T. diversifolia* in the forage mixture changed the total amount of VFAs, and some genotypes modified the proportion of acetate to propionate. These changes in VFA concentrations can be explained by the smaller proportion of structural carbohydrates (i.e. cellulose, hemicellulose) in the feed, which ferment slowly (Martin *et al.* 2008). An increased content of structural carbohydrates in the forage shifts VFA production towards acetate and to a lesser extent towards propionate and butyrate (Martin *et al.* 2008; Terry *et al.* 2016). Changes in the acetate:propionate ratio have been associated with changes in CH_4 production both *in vitro* (Holtshausen *et al.* 2009) and *in vivo* (Eugène *et al.* 2011), and changes in the (acetate + butyrate):propionate ratio have also been associated with *in vivo* CH_4 production (Danielsson *et al.* 2012), with the generation of H_2 in the rumen reduced and rumen energy use improved (Williams *et al.* 2019).

Inclusion of *T. diversifolia* genotypes decreased ADF and NDF contents in the mixtures, which may have modified VFA production. Fertilisation also affected these parameters and degradability, likely due to increases in the proportion of leaves and a reduction in lignified structures such as stems, that in turn increased the soluble fraction.

Finally, regarding CH_4 production, inclusion of *T. diversifolia* decreased the concentration of this gas compared with *U. brizantha* alone, but no significant differences were observed among genotypes (Table 5). Delgado *et al.* (2012) reported that *T. diversifolia* has methane-reducing properties when supplemented at 30% in a feed based on the forage grass *Cynodon nlemfuensis* and indicated that this was due to the secondary metabolites present in *T. diversifolia*, such as condensed tannins, essential oils and saponins. Chagas-Paula *et al.* (2012). Ejelonu *et al.* (2017) reported that *T. diversifolia* contains over 150 phytochemical compounds, particularly sesquiterpene lactones, diterpenes, flavonoids, tannins and saponins, that modify ruminal microbial populations without affecting the degradability or utilisation of nutrients in the feed.

Concentrations of total phenols and tannins differed between *T. diversifolia* and *U. brizantha*. Phytochemical compounds and low fibre content present in *T. diversifolia* may explain the reduction of CH_4 emissions in mixtures containing this species. The efficacy of tannins in modulating fermentation dynamics depends on their molecular weight (Foo *et al.* 1997). In a comparison of seven tropical legumes, Barahona *et al.* (2006) showed that the inhibitory effect of tannins on the activity of microbial cellulolytic and hemicellulolytic enzymes varied according to their response to changes in anthocyanidin content and molecular weight. This underlines the importance of conducting studies to improve understanding of the dietary effects of *T. diversifolia* tannins.

Studies have evaluated the effect of *T. diversifolia* on CH_4 emissions (Terry *et al.* 2016; Ribeiro *et al.* 2016), although the results are contradictory. According to the results of these studies, the effect of *T. diversifolia* on CH_4 emissions depends on the percentage inclusion in the mixture and the quality of the basal forage diet, and apparently, some ecotypes of this species offer more phytochemical compounds such as essential oils and polyphenols.

Low contents of ADF (<40%) and NDF (<50%), acceptable amounts of soluble carbohydrates (>12%), high degradability (>70%) and high contents of CP (>20%) appear to be the main features that decrease CH_4 at the ruminal level. Yan *et al.* (2006) reported that reducing the contents of NDF and ADF to 1% decreased the g CH_4 emissions per kg IDM by 2.01 and 2.26, respectively. Those authors also reported that for every 1% increase in protein content, emission of enteric CH_4 decreased by 6.22 L kg^{-1} DM consumed. Similarly, the consumption of grasses that are less lignified has a clear effect on ruminal digestibility and passage rate (O'Mara 2004). Thus, Blaxter and Clapperton (1965) reported that by decreasing the digestibility of forages from 75% to 55%, the emission of methane increases from 306 to 499 g day^{-1}. Lower fibre content and higher CP content could explain the decrease in CH_4 production found in this study with the inclusion of *T. diversifolia* in a basal diet of *U. brizantha* or another tropical pasture.

Finally, according to the results obtained, it is important to advance *in vivo* experiments using different levels of inclusion of *T. diversifolia* in the feed to analyse which chemical compound(s) from this species can significantly decrease CH_4 production.

Conclusions

We conclude that the use of *T. diversifolia* in lowland tropical mixtures improves the supply of nutrients, especially CP, energy and minerals, and decreases the dietary content of fibre regardless of the genotype used. Furthermore, owing to its chemical characteristics and nutrient content, *T. diversifolia* modifies fermentation parameters by increasing the production of propionic acid and the overall efficiency of the fermentative process and decreases the

acetate:propionate ratio, with some differences observed among genotypes. Finally, the inclusion of some *T. diversifolia* genotypes in low-quality feed mixtures can reduce CH_4 emissions under *in vitro* conditions.

Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Compliance with ethical standards

The work described herein was conducted using rumen fluid obtained from fistulated cattle maintained in accordance with the requirements of Colombian law No. 84/1989 and following protocol approved by the Ethics Committee of the Centro Para la Investigación en Sistemas Sostenibles de Producción Agropecuaria, CIPAV, assuring the welfare of animals used in the experiments.

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Table 1. Chemical composition of forage comprising *Urochloa brizantha* cv. Marandú alone and in 75:25 mixtures with individual genotypes of *Tithonia diversifolia* that were either not fertilised or fertilised (in parentheses)

DM, Dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; Ca, calcium; P, phosphorus; TDN, total digestible nutrients; GE, gross energy; NE_L, net energy of lactation. TP, total phenols; TT, total tannins; s.e.m., standard error of the mean. Where an effect is significant, parameter means followed by the same letter are not significantly different according to the Tukey test at $P = 0.05$

	T. diversifolia genotypes in mixtures with *U. brizantha*:							Fertilisation		*U. briz.* alone	*P*-value for effect of:		s.e.m.
	Gen. 1	Gen. 2	Gen. 3	Gen. 4	Gen. 5	Gen. 6	Gen. 7	Not fertilised	Fertilised		Forage type	Fert.	
DM (g 100 g^{-1})	20.65ab (19.97b)	20.23b (20.23b)	20.19b (20.01b)	20.32b (20.14b)	20.18b (20.11b)	20.23b (20.30b)	20.26b (20.14b)	20.41	20.25	22.11a	**0.028**	0.320	0.10
CP (g 100 g^{-1} DM)	16.50ab (17.87ab)	16.63ab (17.38ab)	16.64ab (17.57ab)	16.30b (17.44ab)	16.49ab (17.96a)	16.69ab (17.34ab)	17.21ab (18.03a)	16.62	17.66	11.81c	**<0.0001**	**<0.001**	0.23
Ash (g 100 g^{-1} DM)	10.31a (10.33a)	10.13a (10.42a)	10.40a (10.55a)	10.29a (10.26a)	10.60a (10.41a)	10.50a (10.34a)	10.37a (10.39a)	10.37	10.39	8.71b	**<0.0001**	0.787	0.07
EE (g 100 g^{-1} DM)	1.68 (1.65)	1.61 (1.63)	1.62 (1.67)	1.70 (1.70)	1.61 (1.62)	1.61 (1.63)	1.62 (1.65)	1.65	1.64	1.7	0.775	0.636	0.01
NDF (g 100 g^{-1} DM)	55.17b (55.30b)	55.20b (55.63b)	55.44b (55.73b)	55.55b (55.60b)	55.98b (55.19b)	55.70b (55.35b)	55.78b (55.29b)	55.55	55.44	63.73a	**0.0001**	0.545	0.37
ADF (g 100 g^{-1} DM)	38.24b (38.62b)	38.54b (38.89b)	38.41b (38.71b)	37.97b (38.46b)	38.50b (38.66b)	38.56b (38.55b)	38.68b (38.57b)	38.41	38.64	46.11a	**<0.0001**	0.064	0.32
Ca (g 100 g^{-1} DM)	0.76a (0.66ab)	0.82a (0.66ab)	0.81a (0.62ab)	0.78a (0.61ab)	0.76a (0.63ab)	0.77a (0.69a)	0.70a (0.64ab)	0.77	0.64	0.43b	**0.0001**	**<0.001**	0.02
P (g 100 g^{-1} DM)	0.25a (0.27a)	0.25a (0.28a)	0.25a (0.27a)	0.25a (0.26a)	0.27a (0.28a)	0.27a (0.27a)	0.27a (0.27a)	0.26	0.27	0.19b	**<0.0001**	0.382	0.13
TDN (g 100 g^{-1} DM)	59.00a (58.32a)	58.57a (58.02a)	58.76a (58.14a)	58.26a (57.98a)	59.06a (58.00a)	58.62a (58.14a)	59.12a (58.49a)	58.16	58.77	52.89b	**0.0014**	**0.007**	0.28
GE (MJ kg^{-1})	17.28 (17.36)	17.24 (17.32)	17.20 (17.32)	17.24 (17.32)	17.20 (17.32)	17.24 (17.32)	17.32 (17.36)	17.24	17.32	17.11	0.932	0.436	0.04
NE_L (MJ kg^{-1})	5.48ab (5.56a)	5.44b (5.52ab)	5.44b (5.52ab)	5.44b (5.48ab)	5.44b (5.56a)	5.44b (5.52a)	5.48ab (5.56a)	5.48	5.52	4.90c	**0.0003**	**0.034**	0.04
TP (g 100 g^{-1} DM)	1.98ab (1.46b)	2.19a (1.70b)	2.10ab (2.01ab)	2.11ab (1.76b)	2.03ab (1.76b)	1.74b (1.50b)	1.97ab (1.41b)	1.86	1.67	1.53b	**0.0406**	**<0.001**	0.11

TT (g 100 g^{-1} DM)	1.46a (0.67b)	1.81a (0.81a)	1.48a (1.09a)	1.57a (0.95a)	1.34a (0.87a)	1.32a (0.69b)	1.45a (0.67b)	1.49	0.82	0.65b	**0.0111**	<**0.001**	0.09

Table 2. Fermentation parameters according to the Gompertz model of forage comprising *Urochloa brizantha* cv. Marandú alone and in 75:25 mixtures with individual genotypes of *Tithonia diversifolia* that were either not fertilised or fertilised (in parentheses)

Gompertz model parameters: *a*, maximum gas production (mL); *b*, difference between initial gas and final gas at time *x*; *c*, specific gas accumulation rate; TIP, timing at the inflection point (h); GIP, gas volume at the inflection point (mL); MRGP, maximum rate of gas production (mL h^{-1}); LP, lag phase or microbial establishment (h). TIP = b/c; GIP = a/e; MRGP = $(a \times c)/e$; LP = $((b/c) - (1/c))$; where e is Euler's number (≈ 2.71828). Where an effect is significant, parameter means followed by the same letter are not significantly different according to the Tukey test at P = 0.05; s.e.m., standard error of the mean

	T. diversifolia genotypes in mixtures with *U. brizantha*:							*U. brizantha* alone	Fertilisation		*P*-value for effect of:		s.e.m.
	Gen. 1	Gen. 2	Gen. 3	Gen. 4	Gen. 5	Gen. 6	Gen. 7		Not fertilised	Fertilised	Forage type	Fertilisation	
a	195.1 (206.3)	211.5 (223.8)	210.9 (201.9)	210.1 (201.3)	212.9 (198.4)	228 (217.3)	222.7 (199.9)	225.1	214.5	207	0.201	0.238	2.5
b	1.30b (1.33b)	1.33b (1.39b)	1.36b (1.34b)	1.27b (1.33b)	1.36b (1.37b)	1.34b (1.33b)	1.32b (1.33b)	1.58a	1.33	1.34	**0.001**	0.249	0.01
c	0.10b (0.11ab)	0.10b (0.11ab)	0.09b (0.11ab)	0.10b (0.12a)	0.10b (0.12a)	0.09b (0.12a)	0.10b (0.11ab)	0.12a	0.1	0.11	0.752	**0.001**	0.002
TIP	13.48ab (12.53ab)	14.07a (13.28ab)	15.22a (12.63ab)	13.33ab (11.27b)	13.94ab (11.85ab)	14.96a (11.50b)	13.91ab (12.68ab)	13.46ab	14.1	12.3	0.241	**0.001**	0.31
GIP	71.76 (73.47)	77.81 (82.34)	77.57 (74.27)	77.31 (74.07)	78.31 (72.99)	83.87 (79.95)	81.92 (73.53)	82.8	78.6	76.5	0.294	0.238	0.92
MRGP	7.09b (8.32ab)	7.49ab (8.78ab)	7.13b (8.06ab)	7.49ab (8.80ab)	7.70ab (8.44ab)	7.72ab (9.23ab)	7.92ab (7.74ab)	9.74a	7.68	8.6	**0.023**	**0.004**	0.20
LP	3.15ab (3.08ab)	3.49ab (3.70ab)	4.10ab (3.18ab)	2.78b (2.78b)	3.63ab (3.13ab)	3.76ab (2.82b)	3.35ab (3.12ab)	4.93a	3.55	3.23	**0.042**	0.102	0.12

Table 3. *In vitro* ruminal dry matter degradability (%) of forage comprising *Urochloa brizantha* cv. Marandú alone and in 75:25 mixtures with individual genotypes of *Tithonia diversifolia* that were either not fertilised or fertilised (in parentheses)

Where an effect is significant, parameter means followed by the same letter are not significantly different according to the Tukey test at *P* = 0.05; s.e.m., standard error of the mean

T. diversifolia genotypes in mixtures with *U. brizantha*: Gen. 1	Gen. 2	Gen. 3	Gen. 4	Gen. 5	Gen. 6	Gen. 7	*U. brizantha* alone	Fertilisation: Not fertilised	Fertilised	*P*-value for effect of: Forage type	Fertilisation	s.e.m.
24 h of fermentation												
45.48abc (49.53abc)	42.25c (49.44abc)	45.04bc (48.89abc)	41.89dc (53.77ab)	45.71abc (54.11a)	45.21bc (52.44ab)	44.88bc (50.25abc)	45.76abc	44.6	51.1	**0.036**	**0.002**	0.91
72 h of fermentation												
67.43a (68.94a)	66.91a (69.76a)	67.37a (69.27a)	66.73ab (68.41a)	66.73ab (67.85a)	66.86ab (69.55a)	68.62a (68.90a)	63.38b	67.2	68.9	**<0.001**	**<0.001**	0.29

Table 4. Volatile fatty acid (VFA) production of forage comprising *Urochloa brizantha* cv. Marandú alone and in 75:25 mixtures with individual genotypes of *Tithonia diversifolia* that were either not fertilised or fertilised (in parentheses)

Individual VFAs are measured in mol 100 mol^{-1}; total VFAs in mmol L^{-1}. Where an effect is significant, parameter means followed by the same letter are not significantly different according to the Tukey test at *P* = 0.05; s.e.m., standard error of the mean

	T. diversifolia genotypes in mixtures with *U. brizantha*: Gen. 1	Gen. 2	Gen. 3	Gen. 4	Gen. 5	Gen. 6	Gen. 7	*U. briz.* alone	Fertilisation: Not fertilised	Fertilised	*P*-value for effect of: Forage type	Fert.	s.e.m.
24 h of fermentation													
Acetate (A)	67.96abcd (68.22abcd)	71.20ab (67.08bcd)	68.67abcd (67.36bcd)	67.28bcd (69.68abc)	69.53abc (63.75cd)	63.21d (65.74bcd)	67.76abcd (67.08bcd)	73.74a	68.3	67.4	**0.0018**	0.217	0.48
Propionate (P)	23.84abc (22.33abc)	20.49bc (24.50ab)	22.39abc (23.88abc)	23.32abc (22.80abc)	21.26abc (27.76a)	27.21a (25.23ab)	22.59abc (24.56ab)	17.62c	22.7	24.2	**0.0013**	0.079	0.59
Butyrate	8.20ab (9.45ab)	8.31ab (8.42ab)	8.94ab (8.76ab)	9.40ab (7.52b)	9.21ab (9.03ab)	9.58ab (9.03ab)	9.65a (8.65ab)	8.64ab	9.01	8.61	**0.0371**	0.074	0.17
A:P ratio	2.89bc (3.07bc)	3.51ab (2.75bc)	3.09bc (2.83bc)	2.89bc (3.07bc)	3.30abc (2.30c)	2.37c (2.66bc)	3.01bc (2.73bc)	4.19a	3.08	2.96	**0.0013**	0.073	0.08
Total VFAs	113.6b (81.0de)	105.5bc (104.2bc)	115.9b (97.7bcde)	85.0cde (143.5a)	99.2bcde (95.6bcde)	77.1e (84.9cde)	84.2cde (102.6bcd)	97.4ab	97.5	101.2	**0.045**	0.432	3.0

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					72 h of fermentation								
Acetate (A)	64.10def (68.91ab)	62.80ef (66.84bcde)	60.63f (68.31bc)	65.66bcde (65.78bcde)	66.22bcde (66.23bcde)	65.52bcde (64.67cde)	67.03bcd (66.41bcde)	72.89a	64.53	66.74	**<0.001**	**0.0192**	0.36
Propionate (P)	27.20abc (24.30c)	28.80ab (24.15cd)	30.44a (25.40bc)	27.25abc (27.52abc)	25.30bc (26.85abc)	26.66bc (26.98abc)	26.35bc (26.57bc)	20.48d	27.43	25.97	**<0.001**	0.112	0.28
Butyrate	8.80a (6.80d)	8.39ab (9.01a)	9.03a (6.29d)	7.10cd (6.69d)	8.48ab (6.93d)	7.82bc (8.35ab)	6.62d (7.02cd)	6.64d	8.03	7.3	**<0.001**	**0.006**	0.17
A:P ratio	2.36bcd (2.84b)	2.19cd (2.78b)	1.99d (2.69b)	2.42bcd (2.39bcd)	2.62bc (2.48bcd)	2.46bcd (2.40bcd)	2.55bc (2.51bc)	3.56a	2.37	2.58	**0.001**	**0.005**	0.06
Total VFAs	125.1f (150.7def)	179.5bcd (164.1cde)	157.7def (219.0a)	217.5a (224.2a)	144.1ef (167.0bcde)	171.5bcde (149.2def)	177.2bcde (197.5abc)	199.2ab	167.5	181.7	**<0.001**	**0.014**	5.2

Table 5. *In vitro* methane ruminal production of forage comprising *Urochloa brizantha* cv. Marandú alone and in 75:25 mixtures with individual genotypes of *Tithonia diversifolia* that were either not fertilised or fertilised (in parentheses)

Ym, CH_4 loss as a percentage of gross energy; IDM, incubated dry matter; DMD, DM degradation. Where an effect is significant, parameter means followed by the same letter are not significantly different according to the Tukey test at P = 0.05; s.e.m., standard error of the mean

	T. diversifolia genotypes in mixtures with *U. brizantha*:							*U. brizantha* alone	Fertilisation		*P*-value for effect of:		s.e.m.
	Gen. 1	Gen. 2	Gen. 3	Gen. 4	Gen. 5	Gen. 6	Gen. 7		Not fertilised	Fertilised	Forage type	Fert.	
					24 h of fermentation								
Ym (%)	3.51ab (3.20b)	3.44ab (3.19b)	3.27b (3.23b)	3.63ab (3.14b)	3.58ab (3.54ab)	3.66ab (3.29ab)	3.35b (3.34ab)	4.16a	3.49	3.28	**0.007**	**0.035**	0.09
CH_4 (mg g^{-1} IDM)	10.92b (9.92b)	10.74b (9.91b)	10.21b (10.02b)	11.31ab (9.72b)	11.19ab (11.01ab)	11.36ab (10.24b)	10.39b (10.38b)	12.54a	10.88	10.17	**0.030**	**0.029**	0.29
CH_4 (mg g^{-1} DMD)	21.71b (22.44ab)	23.44ab (21.62ab)	22.28b (20.62b)	22.83b (20.66b)	24.21ab (20.70b)	22.46b (21.89ab)	22.98b (20.56b)	26.24a	22.84	21.21	**0.034**	**0.005**	0.43
					72 h of fermentation								
Ym (%)	8.09b (8.28ab)	7.80b (8.47ab)	8.24b (8.10b)	8.00b (7.72b)	8.18b (8.35ab)	8.36ab (8.23b)	8.00b (7.99b)	9.19a	8.17	8.23	**0.001**	0.504	0.07
CH_4 (mg g^{-1} IDM)	25.77ab (25.16ab)	26.38ab (24.19b)	25.25ab (25.62ab)	24.01b (24.71b)	26.09ab (25.39ab)	25.57ab (26.04ab)	24.89b (24.85b)	27.70a	25.32	25.13	**0.015**	0.379	0.20
CH_4 (mg g^{-1} DMD)	38.65b (36.67b)	39.21ab (36.18b)	37.41b (38.05b)	35.95b (36.05b)	39.04ab (37.38b)	37.35b (38.97ab)	38.29b (35.49b)	43.27a	38.81	36.97	**0.002**	0.084	0.39