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Article

In vitro methane production and fermentative parameters of wild sunflower and elephant grass silage mixtures, either inoculated or not with epiphytic lactic acid bacteria strains

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Abstract:

The present investigation was carried out to determine the extent of the incorporation of *Tithonia diversifolia* (TD) and the possibility of blending it with *Pennisetum purpureum* (PP) to obtain the maximum benefit for ensilability and for animal nutrition. Silage mixtures of wild sunflower (TD) and elephant grass (PP) were evaluated based on chemical composition,

quantification of gas production, methane release and fermentation parameters. The silage blends were arranged in four *T. diversifolia* / *P. purpureum* proportions, namely: 100/0; 67/33; 33/67; and 0/100 (fresh weight). Silages with higher proportions of *T. diversifolia* increased crude protein content, *in vitro* digestibility while decreasing NDF and ADF fractions ($P<0.05$). High amounts of *T. diversifolia* showed the lowest gas production values (160.2 ml), while treatments with higher grass inclusion produced a greater amount of gas up to 194.5 ml. Methane production was higher by increasing the proportion of *P. purpureum* into the silage blends. The silage inoculum did not have any impact on *in vitro* gas production ($P<0.05$). Also, higher proportions of *T. diversifolia* reduced acidification process while *P. purpureum* inclusion facilitated lower pH values. Lactic acid bacteria inoculum tended to decrease pH of silages but no clear effects on silage temperature were observed. Silages with high proportions of *T. diversifolia* (67 % of inclusion) would be more palatable for animals and might also translate into larger animal performance due to greater protein supply and better digestibility than silages with larger proportion of *P. purpureum* (67 and 100 % of inclusion).

Key words: Gas production, Inoculum, Ruminal fermentation, Mexican sunflower, Tropical forage.

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Introduction

The current growing competition between human food production and animal feed resources calls for new animal feed alternatives that do not compromise human food supply. There is a special interest in research on non-conventional forage species that can be offered as hay or silage all year round, particularly at times of feed scarcity as in case of drought or floods[1,2,3]. *Tithonia diversifolia* (TD) is a non-leguminous species that is widely distributed in humid and sub-humid areas of America, Africa, Asia, and in regions close to the tropical and subtropical belts[4,5]. It has been described as a multi-purpose shrub with considerable potential for animal production due to its valuable source of protein and high palatability[6,7]. Also, low quality tropical forages constitute some of the main factors that limit the development of livestock production systems due to poor animal performance. This is the reason for which high-quality excessive forage produced during the rainy season should be

preserved as silage[2,8]. Considering that TD shows high natural distribution in tropical countries, this underutilized plant can contribute to livestock production if its foliage is conserved as silage[5,9,10].

Among tropical forages, some legumes and non-leguminous forbs as TD show better nutritional values than grasses, thus including TD in animal diets favors crude protein content, reduced fiber values and enhance digestibility of feeds[7]. Despite the high protein content of TD ranging from 10.3 to 25.6 %[4,5], only a few silage production studies have been conducted to investigate the ensilability potential of TD. Therefore, it is necessary to determine the extent of incorporation of TD and the possibility of blending it with grass to obtain the maximum benefit for animal nutrition and for farmer's households.

Appropriate ensiling procedure means that lactic acid fermentation is produced in the absence of oxygen. A rapid acidification normally occurs when a sufficient amount of lactic acid is produced by lactic acid bacteria (LAB) present on the plant surfaces[9,11]. In this sense, it is important to improve lactic acidification for achieving a successful fermentation process. This effect can be facilitated by inoculating LAB[12,13]. Selected homofermentative lactic acid bacteria have been traditionally developed in temperate countries to favor lactic acidification and to lower pH of silages[8,9,11]. However, most of the commercially available strains up to date have a poor performance when they are inoculated to tropical silages[12]. Thus, selected epiphytic bacteria seem to be a good alternative for improving fermentative parameters of tropical forages during the ensiling process. Research works have studied epiphytic LAB strains isolated from tropical forage species as promising candidates that could be used as silage additives to overcome the limitations of commercial inoculants and to minimize the nutritional value losses of silage[11,12,14]. However, methane emissions studies dealing with silages and LAB additives are also scarce[15].

On the other hand, one of the commonly accepted methods for determining animal feed quality is the gas production technique. The *in vitro* gas production methodology determines the extent and kinetics of feed degradation based on the released gas volume, both directly as a fermentation product and indirectly from neutralization of the ruminal fluid[16,17,18]. In this respect, it has been established that accumulated enteric CH_4 production in ruminants increases with fermentation time, but also decreases the energy utilization efficiency and contributes to the global greenhouse gas effect[19,20]. Methane is produced under anaerobic conditions by rumen microorganisms called methanogenic archaea, which gain energy by reducing CO_2 with H_2 to form CH_4[19,21]. Methane production depends primarily upon the quantity and quality of forages consumed by animals[22,23,24]. Thus, the mitigation of methane production from ruminants could be achieved by altering their diet[21,25]. Previous studies have suggested that increased forage quality decreases the CH_4 proportion in the emitted gas, which means reduced emissions per unit of weight gain or per unit of animal product produced as milk or meat due to improvement of animal productivity[22,26,27]. Therefore, the

aim of this study was to evaluate the effect of four different inclusion levels of *T. diversifolia* (100, 67, 33 and cero %) in mixture with *P. purpureum* as well as the enrichment or not with an epiphytic or commercial LAB strain. Nutritional quality (DM, CP, NDF, ADF, IVDMD and ash), methane release and ensilability of silages parameters were assessed.

Material and methods

Plant material

Samples of TD forage were harvested (40 cm above-ground biomass, including leaves and stems) at pre-flowering stage in February 2014 and 60 d of age. The dry matter of TD at harvest was 17.6 %. The cultivars were located at the experimental farm of the Universidad Nacional de Colombia in Palmira, at 1,000 m asl, 24°C, annual precipitation 1,020 mm, and relative humidity 72 %. Complementary, *P. purpureum* (PP) was harvested at vegetative stage, 10 cm above ground level and 75 d of age at the same time and location. Dry matter of PP at that age averaged 18.7 %. Plant biomass of TD and PP was collected by hand and was moved to the lab to elaborate forty-eight micro-silos mixtures of one kilogram each. After that, vegetation samples were mechanically chopped into sections of a particle size ranging from two to three cm using an electrical grass chopper (7.5 hp, 1400 rpm; Gaitan).

Silage preparation

Forage material of TD and PP were wilted to 30 % and 35 % dry matter before ensiling. The different silage mixtures were made as follows: TD and PP were arranged in four different proportions. Proportion 1: 100/0; Proportion 2: 67/33; Proportion 3: 33/67; and Proportion 4: 0/100, FM basis. Later, every proportion was either inoculated or not: 1) control sample (no inoculum); 2) epiphytic lactic acid bacteria (LAB) strain T735; and 3) SIL-ALL®4x4, resulting in 12 treatments in total. T-735 is an epiphytic LAB strain (*Lactobacilus paracasei*) isolated from TD tissue surface and has been tested in previous studies as silage inoculant[9,10]. SIL-ALL®4x4 is a commercial inoculum blend of *Streptococcus faecium*; *L. plantarum*; *Pediococcus acidilactici* and *L. salivarius*. The bacterial inoculum was diluted to apply 1 ml per kg of fresh forage. Each inoculant was previously cultivated as follows: 0.1 ml of an inoculum 4x109 CFU ml-^{1} cultivated into 10 ml MRS broth at 37 °C for 24 h. The

forage mixtures of 1,000 g each, were packed in vacuum-packed plastic bags (18.5 cm × 29 cm; Quart micro channel vacuum sealer bags, model No. 30-0101-W, 0.9 L, China) as Rostock Model Silages guidelines[28,29] TD and PP forage mixtures were compressed by hand. Later micro-silos were air evacuated and heat-sealed to assure hermeticity. A vacuum sealer (Westonbrand PRO 2300 stainless steel, Vista, CA, USA) was used to provide the anaerobic conditions to facilitate lactic fermentation and silage acidification. Micro-silos were wrapped up using adhesive tape to avoid deformations of micro-silos because of bloating. Later the polyethylene bags were punctured using disinfected injection needle. Every wrapped bag was placed immediately into a second bag (26 cm × 39 cm; micro channel vaccum sealer bags, model No. 30-0102-W, China) that was air-evacuated under the same conditions. Two additional replicates of samples (~250 g) were filled in smaller bags to be opened after three days of ensiling at 25 °C storage temperature for the determination of DM. Later, micro-silos were stored in darkness for 90 d at room temperature of about 25 °C. At d 90, the bags were opened and pH and temperature were measured. pH was measured using a pH meter (Mettler Toledo, o SevenGo, with pH electrode InLab@ 41356/2mat), disinfecting the electrode with 70% ethanol before each record. Temperature was measured using a digital indoor–outdoor min–max thermometer. A sensor was introduced into the silage material while a second one was used to record the ambient temperature simultaneously. Further, samples were lyophilized and ground in a Thomas Wiley mill laboratory mill rotor model 4 provided with a 1.0 mm mesh screen to perform the test gas. Thus, 12 treatments in total were analyzed, with four replicates, resulting in 48 micro-silos as a hole.

Nutritional value

A set of silage samples was analyzed at the Forage Quality Laboratory at CIAT. The content of dry matter (DM) was determined using a forced air oven at 65 °C until constant weight during 72 h. The following determinations were also made: i) crude protein (CP) by the Micro-Kjeldahl method; ii) insoluble fiber in neutral and acid detergent (NDF and ADF) following the sequence described by Van Soest *et al*[30]; iii) and ether extract (EE) by soxhlet extraction described by Palmquist and Jenkins[31]. Tilley and Terry[18] methodology modified by Moore[32] was use to determinate digestibility. The ash content was determined by direct incineration of the dried material in a muffle furnace at 500 °C as per the AOAC official method[33]. The analysis was done in the twelve treatments (with the four corresponding repetitions) in triplicate.

In vitro gas production

The fresh forage underwent a drying process in a conventional oven at a temperature of 65 °C for 72 h. It was then processed in a model 4 Thomas Wiley Laboratory Mill with a 1.0 mm mesh sieve. The ruminal fluid was obtained from two young cannulated Brahman bulls pasturing *Cynodon plestotachyus* (star grass) and supplemented mineralized salt *ad libitum*. The cannulated bulls did not have access to feed for an hour before starting the collection of ruminal fluid. Collection of ruminal samples was carried out by hand. Then, it was squeezed through a piece of gauze to extract the solid rumen contents, stored in 2.0 L thermos, and preserved in hot water at 39 °C for about 10 min, to be sent to the laboratory. Then, the ruminal fluid was liquefied for 20 sec and refiltered before transferring it into Erlenmeyer flasks. The ruminal liquor was then saturated with CO_2.

Following Theodorou *et al*[17] a digestion medium with some modifications was prepared. Incubation was performed in flasks with a capacity of 160 ml each. To this end, one dried and ground gram of sample was weighed, and 85 ml of digestion medium gassed with CO_2 were added. Later, four ml of reducing agent were added (prepared at the time of use by mixing cysteine-HCl (625 mg), 1M NaOH (4 ml) and $Na_2S \bullet 9H_2O$ (625 mg) in 100 ml distilled water); the rubber lids were placed and secured with metal seals. Lastly, the bottles were cooled in the refrigerator to 4 °C for 24 h. After this period elapsed, the bottles were removed from the refrigerator and placed into a water bath at 39 °C. When the incubation system was at temperature equilibrium, inoculation to each bottle was conducted using 10 ml of ruminal liquor. Together with the samples, six bottles containing only the "digestion medium" were inoculated. These were considered "blanks", which were used to correct gas production caused by the inoculum fermentation and the medium. To this end, a pressure transducer (Sper Scientific®, USA) connected to a digital reader and a three-way valve were used. The first way was connected to a 22G needle (25 mm x 0.7 mm); the second to the transducer, and the third to a 60 ml syringe. The latter made it possible to measure the volume.

Prior to beginning the incubation and fermentation process, all bottles were reset to zero psi, removing any volume produced in the upper section of each bottle. Volume and pressure measurements were performed at 3, 6, 9, 12, 24, 33, 48, 60, 72, 96, 120, and 144 h. After each reading, the bottles were shaken and incubated again in a water bath at 39 °C. On completion of the incubation period, the content of each flask was filtered (filter paper; 5 µm pore size) using a vacuum pump and dried in an oven at 104 °C overnight. Degraded dry matter was calculated as the difference between the sample weight at the beginning of incubation and the weight of the residue on the crucible at the end of incubation. The samples were done in quadruplicate.

Methane release

The concentration of CH_4 in the gas produced was determined at the Greenhouse Gas Laboratory at the International Center for Tropical Agriculture (CIAT) using a Shimadzu GC-2014 gas chromatograph (Shimadzu®, Japan) with a flame ionization detector (FID) at a temperature of 250 °C and an electron capture detector at 325 °C under the following conditions: oven at 80 °C and columns Shimadzu 4mH-D 80/100.07m S-Q and 1.5 P-N. The direct injector operated at room temperature. The carrier gas was nitrogen and column flow rate was 30.83 ml/min. The injection volume was handled by a loop with a capacity of 2 ml. The samples were done in quadruplicate.

Regression model

Different non-linear models were tested and the Gomperztz model was the one that presented the best goodness of fit determined by the Bayesian information criterion (BIC) and Akaike information criterion (AIC) statistics. Thus, a Gompertz equation[34,35,36] was used to model gas accumulation from different mixtures used in the silages, where parameters α, β, and γ, were estimated by non-linear regression analysis using the Infostat software. The following equation (Eq. 1) was employed: $Y = \alpha*\exp(-\beta*\exp(-\beta*\gamma))$; Where: Y is equal to the cumulative production of gas at time x, $\alpha > 0$ is the maximum gas production; parameter $\beta > 0$ is the difference between the initial gas and the gas at time x, and parameter $\gamma > 0$ describes the specific gas accumulation rate. The practical application of this model requires translating parameters α, β, γ into their biological relevance. For the purposes of this study, the parameters are: time to point of inflection (HIP, hours), gas inflection point (GIP ml), maximum gas production rate (MRGP, ml/h), and lag phase (LP or microbial accommodation h). To estimate the biological parameters, the following formulas were used Eq. 2: $HIP = \alpha/\gamma$; Eq. 3: $GIP = \alpha/e$; Eq. 4: $MRGP = (\alpha*\gamma)/e$; and Eq. 5: $FL = ((\beta/\gamma) - (1/\gamma))$; where e is Euler's number, which equals approximately 2.718281828459.

Statistical analysis

The gas production values were analyzed with the InfoStat program, using the subroutine "Estimation of General and Mixed Linear Models", assuming heterogeneous variances[37]. For the general model applied to the factors studied, the proportion *T. diversifolia / P.*

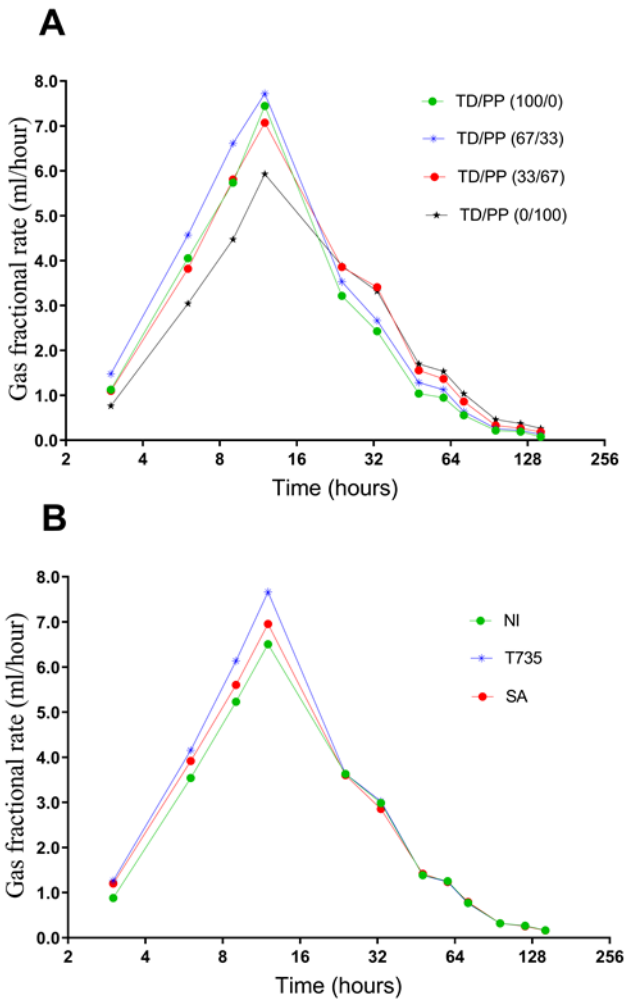
purpureum - (TD/PP) in the diet and inoculum were the predictor variables (factors). It was used a two-factor experimental design, where the first factor was the inclusion level of TD in the silages, and the second the type of inoculant used: $Y_{ij}= \mu + PTD_i + I_j + PTD_{ij}$ x I + ε ; whereas Y= is the target variable; μ is the overall mean; PTD = proportion of TD in the silage (100, 67, 33 and cero %); I= inoculant (control; T735; SIL-ALL®4x4) and ε= the random experimental error. In the present experiment, twelve treatments in total were evaluated. To perform the statistical analysis, the n value employed was 156, while twelve iterations were used. In turn, different analysis of the variance corresponding to each sampling hour was carried out to show the statistical differences between treatments, and statistical differences were detected by Duncan mean comparisons ($\alpha<0.05$).

Results

The results showed that as the level of inclusion of PP in the silage mixtures increased, lower levels of protein ($P<0.05$) were obtained (Table 1). This effect was opposite for TD; i.e., larger TD inclusion yielded higher CP contents in the silage mixtures. In 100 and 67 % of *T. diversifolia* inclusion, CP was superior in silages without inoculum (18.1 and 15.1 %) than both inoculated silages. However, no effects of inoculum on silages with larger inclusion of PP were observed. The values of CP on average for 100, 67, 33 and cero % of TD inclusion were 16.9, 13.4, 8.7 and 5.2 %, respectively. Depicting a negative trend as the inclusion of TD diminished. In contrast, (NDF and ADF) fibers have an opposite response. When TD was ensiled alone; the values were lowest: 36.1 and 25.2 % for NDF and ADF, respectively. However, when TD ensiled at 67 % (41.9 and 29.0), 33 % (44.7 and 33.9) and cero % (58.0 and 38.5) of inclusion, the values of both NDF and ADF increased. Also, the data shows that the inclusion of TD significantly improves IVDMD in comparison to silage with PP at 100 %; e.g TD ensiled alone (67.2 %) was more digestible than PP ensiled alone (63.6 %).

The highest rates of fractionated gas (ml/h) from the ensiled mixtures occurred between 9 h and 12 h. Most gas was produced by the 67:33 TD/PP ensiled mixture at 12 h (Figure 1A). Although there were no significant differences with respect to silages prepared exclusively from TD (100:00 TD/PP) and from silages prepared with high proportion of grass (33:67 TD/PP), but there were differences ($P<0.05$) respect to silages prepared exclusively from PP (00:100 TD/PP). Gas production after 12 h dropped significantly from the previous peak with gas production between 3.2 ml/h and 3.9 ml/h, which means a 50 % drop. The largest effect of the interaction between treatments and inocula on gas production (ml) per hour was observed during the first hours of fermentation (Figure 1B). No great differences were observed after 32 h of observation for the fractionated gas production.

Figure 1: Gas fractional rate of silage mixtures of *Tithonia diversifolia/Pennisetum purpureum* either enriched or non-enriched with lactic acid bacteria strains

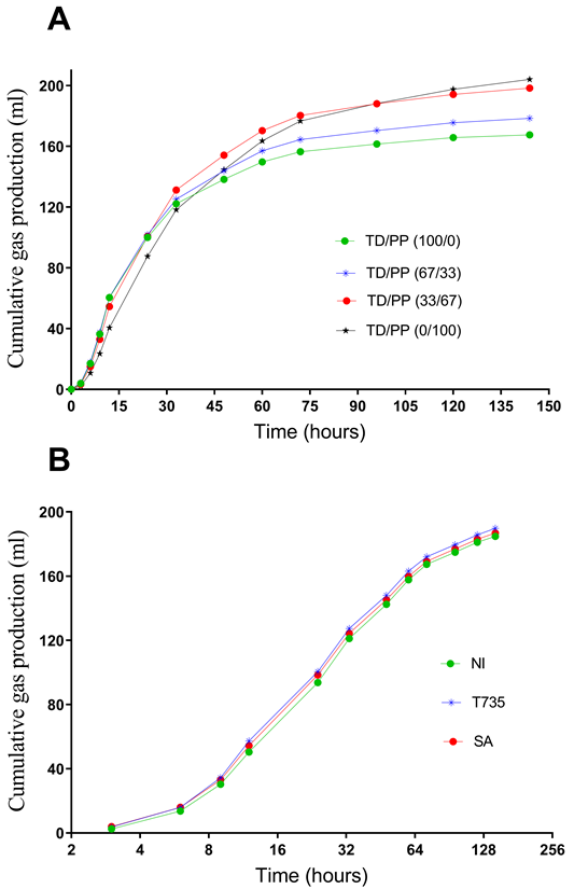


A= effect of the treatment. B= effect of the inoculum. TD/PP= proportions of *Tithonia diversifolia/Pennisetum purpureum* [Fresh matter (FM) base]. NI= no inoculum. T-735= is an epiphytic lactic acid bacteria strain. SA= Sil-all is a commercial inoculum blend of lactic acid bacteria.

The results showed lower gas production accumulated at 144 h in silages prepared exclusively from TD (160 ml) compared to the other treatments (Figure 2A). An increased gas production was found in silages prepared exclusively from grass (194 ml). The results of the interaction between the mixture (TD/PP) and inoculum indicate that, regardless of LAB inoculation, silages prepared with a higher inclusion of TD produced a lesser amount of gas, which means that a larger rate of inclusion of PP in the ensiling process increases gas production. The Gompertz equation results indicate that the highest gas production rates were the following: silages prepared exclusively from grass+Silall; silages prepared exclusively from grass+T735; and silages prepared exclusively from grass without inoculum, respectively (Table 2). On the contrary, lower values were reported for the treatments with

larger proportions of TD [TD/PP: 100/0 and 67/33 % of inclusion, respectively]. An analysis of the accumulative gas revealed that the α parameter, which represents the maximum gas production rate was higher in silages prepared with high proportion of grass and silages prepared exclusively from grass (194.5 and 189.7 mL) where the proportion of grass was the highest. Ensiled mixtures showed increases in the cumulative gas production per gram of dry matter over time. The same occurred with the GIP parameter, where silages prepared with high proportion of grass and silages prepared exclusively from grass (67 and 100 inclusion of PP) showed higher values than the silages prepared exclusively from TD (TD/PP: 100/0) and low proportions of grass (TD/PP: 67/33) (P<0.05). Silages prepared exclusively from grass had the longest time of inflection point (HIP) close to 64 h and the same trend for microbial colonization time (LP; 44.8) with a statistically significant delay versus the rest of the treatments.

Figure 2: Cummulative gas production of silages mixture of *Tithonia diversifolia/Pennisetum purpureum* either enriched or non-enriched with lactic acid bacteria strains



A= effect of the treatment. B= effect of the inoculum. DDM: Degraded dry matter. TD/PP= proportions of *Tithonia diversifolia/Pennisetum purpureum* [Fresh matter (FM) base]. NI= no inoculum. T-735= is an epiphytic lactic acid bacteria strain. SA= Sil-all is a commercial inoculum blend of lactic acid bacteria.

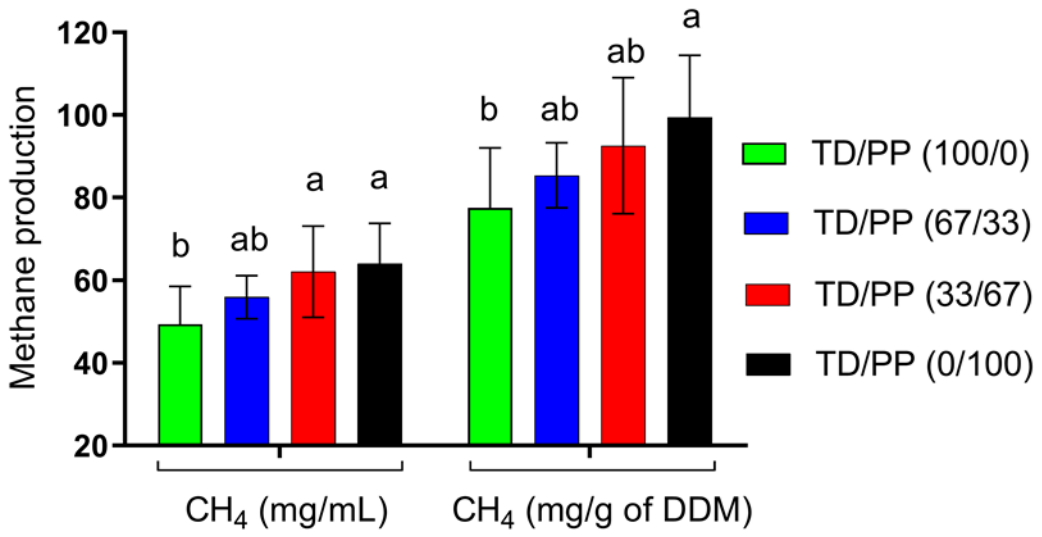
Table 2: Parameters of the Gompertz model for the production of gas observed at different levels of inclusion of *Tithonia diversifolia* (TD) and *Pennisetum purpureum* (PP) silage mixtures either enriched or non-enriched with lactic acid bacteria strains

Inclusion	α	β	γ	HPI (h)	GIP (mL)	MRGP (mL/h)	LP (h)
TD/PP: 100/0	160.2±1.62^{c}	3.09±0.17^{c}	0.08±0.0^{a}	39.08^{c}	58.93^{c}	4.67^{a}	26.43^{c}
TD/PP: 67/33	170.11±1.75^{b}	3.03±0.15^{c}	0.07±0.0^{b}	42.35^{c}	62.58^{b}	4.48^{a}	28.36^{c}
TD/PP: 33/67	189.65±1.19^{a}	3.20±0.09^{b}	0.06±0.0^{c}	51.36^{b}	69.77^{a}	4.36^{a}	35.29^{b}
TD/PP: 0/100	194.45±1.33^{a}	3.34±0.10^{a}	0.05±0.0d	63.96^{a}	71.53^{a}	3.76^{b}	44.79^{a}

TD/PP = proportions of *Tithonia diversifolia/Pennisetum purpureum* [Fresh matter (FM) base]. α= is the maximum gas production volume; β= is the difference between the initial gas and the gas at time x; γ= describes the specific gas accumulation rate. HPI= time to point of inflection; GIP= gas inflection point; MRGP= maximum gas production rate. LP= lag phase (LP or microbial accommodation).

Analyses were performed of the net production of CH_4 from the gas generated at 72 h of incubation of 1 g of silage (Figure 3). The DM degradation ranged between 63.7 % and 64.4 %. It must be noted that at 60 h after start of incubation and close to the inflection point (HIP), approximately from 80 to 88 % of the total gas produced during the experiment was obtained. A lesser amount of methane was produced in silages prepared exclusively from TD (TD/PP: 100/0) in relation to silages prepared with high proportion of grass and silages prepared exclusively from grass (67 and 100 inclusion of PP), but was no significant different from silages prepared with low proportions of grass (TD/PP: 67/33). Larger methane release was observed as the proportion of PP in the blend increased while the lowest methane production was found in silages containing the largest share of TD (100/0 of TD/PP).

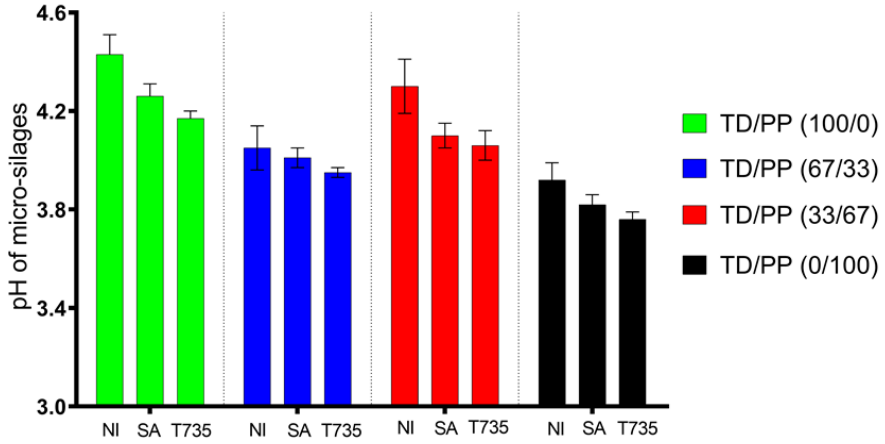
Figure 3: Methane production per gram of dry matter silage (at 70 h) incubated at different levels of inclusion of *Tithonia diversifolia/Pennisetum purpureum*



TD/PP= proportions of *Tithonia diversifolia/Pennisetum purpureum.* [Fresh matter (FM) base]. DDM: Degraded dry matter. Different letters in the same column mean significant differences between treatments (P<0.05).

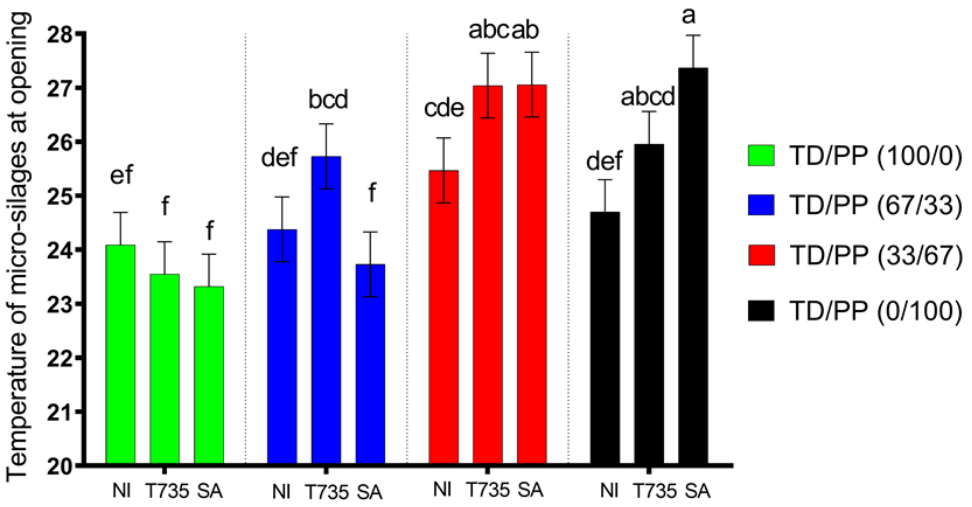
Also, the findings of the experiment indicate the pH tended to diminish as the proportion of PP in the mixtures increased, but no statistical differences were observed (Figure 4). Somehow, the LAB addition decreased the acidification of the silages. Though the extent of the acidification was maximum whit the addition of T735 strain followed by Silall and the control treatment, no statistical differences were obtained. An opposite trend was observed in the temperature parameter, which increased as the proportion of PP in the blends increased, except for silages prepared exclusively from TD (TD/PP: 100/0). Also, no clear effect of the inoculum was observed on silage temperature, however, T735 and Silall, were more effective as the share of PP increased in the silage mixtures (Figure 5; P<0.05).

Figure 4: pH at opening of *Tithonia diversifolia/Pennisetum purpureum* silages either enriched or non-enriched with lactic acid bacteria strains



TD/PP= proportions of *Tithonia diversifolia/Pennisetum purpureum* [Fresh matter (FM) base]. NI= no inoculum. SA= Sil-all is a commercial inoculum blend of lactic acid bacteria. T-735= is an epiphytic lactic acid bacteria strain.

Figure 5: Temperature at opening of *Tithonia diversifolia/Pennisetum purpureum* silages either enriched or non-enriched with lactic acid bacteria strains



NI= no inoculum. SA= Sil-all is a commercial inoculum blend of lactic acid bacteria. T-735= is an epiphytic lactic acid bacteria strain.

Discussion

Distinct proportions of *Tithonia diversifolia* (TD) and *Pennisetum purpureum* (PP) were tested to obtain the maximum benefit for silage making and for animal nutrition. Also, as divergent shares of forages in the silage mixtures determine the neutral detergent fiber (NDF) and crude protein (CP) content, which further shift the methane release parameters. In the present study, increasing the proportion of PP in the silage mixtures decreased the CP and the IVDMD while increasing the NDF and ADF values. This results are in accordance with other studies were the gramineous forages are a source of readily available energy as fermentative carbohydrates but with lower contents of protein ranging from 5 to 11 g/100 g[9,38]. As NDF and ADF traits are close related to forage intake and forage digestibility, these findings might have further implications on animal's performance; i.e. silages with high proportions of TD would be more palatable for animals and might also translate into larger animal performance due to greater protein supply and better digestibility than silages with larger proportion of PP. Therefore, it should be prudent to corroborate these findings on *in vivo* models.

Also, during the ruminal fermentation process, the plant material is colonized by ruminal microorganisms causing different degradation rates depending on the concentration of structural carbohydrates. It is recommended that at the beginning of the ensiling process, should not be a limitation of carbohydrates to initiate the fermentation process; as in the case of silages with high proportion of PP. When acidification takes place, there is a depletion of substrates for LAB metabolism and stabilization phase of silage starts[8]. Despite, a rapid acidification of silages is highly desirable and it is promoted by using high shares of grasses in the silage mixtures. The protein content is another parameter that deserve a special attention because most of the conserved materials for animal feeding show low values of this component[6]. Here, this problem is clearly overcome with the use of TD in the blends. The most significant effects were observed when TD was included in larger proportions (TD/PP: 100/0 and 67/33), as this forage is a source of crude protein in the evaluated silages. However, this effect is negligible on treatments with larger proportion of grass in the silage mixtures (TD/PP: 33/67 and 0/100). This finding might be useful to discriminate the use the LAB for silage acidification. High proportion of PP in silage mixtures might not require LAB addition to produce favorable conditions to facilitate lactic acid fermentation and to preserve moderate content of crude protein. On the contrary, forage blends, which need to preserve a considerable crude protein content, should be highly desirable to use LAB to prevent proteolysis.

During fermentation, ruminal microorganisms and their enzymes first attack easily fermentable carbohydrates. Thus, gas production after the first 12 h was significantly reduced in relation to the first hours after ensiling. It is evident that in the early hours of fermentation a portion of the substrate containing soluble sugars ferments immediately; soluble sugars, however, generally represent only a small portion of potentially digestible materials[2]. After that, with colonization of fiber by cellulolytic bacteria and their degradation, an increase of gas production is achieved. In the derivation of the Gompertz equations, an increase of gas production was observed over time. The behavior of the cumulative gas production was characterized by an increase in the exposure time of the plant material from silages to microorganism attack. Bezabih *et al*[23] suggest that this increase can be interpreted as an increase in microbial activity per unit of feed, but it does not involve any assumptions on the constancy of microbial growth yield. The substrate degradation rate reduction is possibly related to the higher amount of cell wall in silage after the inflection point (HIP), further decreasing the fractional growth rate and consequently reducing microbial yield. In the maximum gas production rate parameter no significant differences were observed among silages prepared exclusively from TD (TD/PP: 100/0), silages prepared with low proportions of grass (TD/PP: 67/33), and silages prepared with high proportion of grass (TD/PP: 0/100). However, differences were significant compared to silages prepared exclusively from grass, which is presumably related to the higher digestibility of the proteinaceous material[24] represented in TD. Besides, lower gas production is related to propionic fermentation as in the case of silage prepared exclusively from TD. To build a propionic acid molecule it is necessary H_2. In comparison with propionic acid, acetate and butyric acid production release H_2[16]. Therefore, larger proportion of PP in the silage mixtures would lead to butyric and acetic fermentation which is associated to higher CO_2 and methane production. This would have direct implication on animal performance, since propionic fermentation would promote better animal performance due to better efficiency on energy utilization of feeds.

It is highly advisable to link the *in vitro* findings of methane emissions of this study to aim high animal productivity while mitigating methane production. In contrast to the proportion of TD and PP in the mixtures that modified methane release and fermentation indicators, LAB additives did not change these parameters. A reason for this observation, is that LAB has the major impact at the beginning of the fermentation process with an important impact on readily digestible carbohydrates to facilitate silage acidification, but this effect stopped during the stabilization phase of silage with no further effects on other chemical components as NDF or ADF; thus null impact on methane release was observed[8]. This finding is in line with the results of Bureenok *et al*[11] whom reported no reduction of NDF content in PP silages treated with LAB additives with respect to untreated silages. Additionally, such authors indicated that addition of a rich source of readily fermentable carbohydrates modified NDF content by a dilution effect and by means of improving lactic fermentation[11].

It is important to note that microbial growth yield vary with factors such as microbial population, pH, and availability of N substrates[13]. It is clear that these factors can change over the incubation period. The findings indicate that in only-grass silage, methane production is high but may diminish as the inclusion of TD increases. This is consistent with data reported by La O *et al*[38] who in fresh forage TD/PP mixtures found a declining methane production (166.5, 150.3, and 84.2 mL/g) by adding TD in the blends (15, 30, and 100 %, respectively). Clearly, the values obtained from this study were higher (up to 204 mL/g) than those obtained by the above authors. Forages with a high cell wall content represent low quality feed, thus, depending on quality and diet composition, about from 2 to 12 % of feed gross energy (GE) could be emitted in the form of CH_4[16,21]. The use of gramineous forages as PP seems to increase the release of methane. Therefore, the benefits for animal production from TD seems to be the nitrogenous content and larger extent of digestibility. However, this should be balanced due to the buffer capacity as acidification is lower in silages with larger proportion of gramineous species that would compromise ensilability of forages[10,12]. Thus, the use of high proportions of TD in the silage mixtures would increase buffer capacity[12]. This effect would be maximized if high protein content is observed and buffering ability of the amino groups is activated. An associated factor that should be taken into account is that buffer capacity is improved with high amounts of ammonia (NH_3) due to deamination of protein. Additionally, high fiber content of forages has been also related to high buffer capacity, since the ability of fiber components to exchange cation during ensiling process may affect forage buffer capacity. Also, high temperature of silages at opening is related to microbial activity. This effect was more evident in silages with superior shares of PP in the silage mixtures. Increase of the temperature might further impact aerobic stability of silages after exposure of preserved material to oxygen during the opening of the silo[1,8].

Conclusions and implications

Silages with higher proportions of *Tithonia diversifolia* increased crude protein content, *in vitro* digestibility while decreasing NDF and ADF fractions. Low amounts of TD showed the lowest gas production values, while treatments with higher grass inclusion produced a greater amount of gas. Methane production was higher by increasing the proportion of *Pennisetum purpureum* into the silage blends. The silage inoculum did not have any impact on *in vitro* gas production. Higher proportions of TD decreased acidification process while PP inclusion facilitated lower pH values. Lactic acid bacteria inoculum tended to decrease pH of silages but no clear effects on temperature were observed. Silages with high proportions of *T. diversifolia* (67 % of inclusion) would be more palatable for animals and might also translate into larger animal performance due to greater protein supply and better digestibility than

silages with larger proportion of *P. purpureum* (67 and 100 % of inclusion). Therefore to corroborate should be prudent these findings on *in vivo* models.

Conflict of interest

The authors declare that they have no competing interests.

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Table 1: Nutritive value of *Tithonia diversifolia* (TD) and *Pennisetum purpureum* (PP) silage mixtures either enriched or non-enriched with lactic acid bacteria strains

Treatments	Inoculum	ADM (%±EE)	CP (%±EE)	NDF (%±EE)	ADF (%±EE)	IVDMD (%±EE)	ASH (%±EE)
TD/PP: 100/0	T-735	88.0±0.3^{d}	16.8±0.4^{b}	34.9±0.7^{f}	23.1±0.7^{e}	68.9±0.6 ^{a}	13.2±0.2^{de}
	Silall	88.3±0.3^{cd}	15.9±0.4^{bc}	34.7±0.7^{f}	24.4±0.7^{e}	68.8±0.6 ^{a}	13.3±0.2^{bcde}
	Control	88.9±0.3^{c}	18.1±0.4^{a}	38.6±0.7^{e}	28.1±0.7^{d}	63.9±0.6 ^{d}	14.1±0.2^{a}
	Average	88.4±0.2^{b}	16.9±0.3^{d}	36.1±0.4^{a}	25.24±0.7^{a}	67.2±0.3^{b}	13.5±0.1^{ab}
TD/PP: 67/33	T-735	87.9±0.3^{de}	12.7±0.4^{d}	40.8±0.7^{d}	28.3±0.7^{cd}	68.3±0.6^{ab}	13.2±0.2^{cde}
	Silall	87.2±0.3^{e}	12.3±0.4^{d}	41.7±0.7^{cd}	28.7±0.7^{cd}	66.6±0.6 ^{c}	12.9±0.2^{e}
	Control	87.8±0.3^{de}	15.1±0.4^{c}	43.3±0.7^{c}	30.0±0.7^{c}	66.7±0.6 ^{c}	13.6±0.2^{abcd}
	Average	87.6±0.2 ^{a}	13.4±0.3^{c}	41.9±0.4^{b}	29.0±0.4^{b}	67.2±0.3^{b}	13.2±1.2^{a}
TD/PP: 33/67	T-735	88.5±0.3^{cd}	8.6±0.4^{e}	49.1±0.7^{b}	33.4±0.7^{b}	65.8±0.6^{c}	13.9±0.2^{a}
	Silall	89.1±0.3^{bc}	9.0±0.4^{e}	41.7±0.7^{cd}	34.0±0.7^{b}	67.1±0.6^{bc}	12.9±0.2^{e}
	Control	88.4±0.3^{cd}	8.4±0.4^{e}	43.3±0.7^{c}	34.4±0.7^{b}	67.4±0.6^{abc}	13.6±0.2 ^{abcd}
	Average	88.7±0.2^{b}	8.7±0.3^{b}	44.7±0.4^{c}	33.9±0.4^{c}	66.8±0.3^{b}	13.5±0.1^{ab}
TD/PP: 0/100	T-735	89.9±0.3^{ab}	5.3±0.4f	57.3±0.7^{a}	38.1±0.7^{a}	64.0±0.6^{d}	13.7±0.2^{ab}
	Silall	89.8±0.3^{ab}	5.1±0.4f	57.9±0.7^{a}	38.3±0.7^{a}	63.0±0.6^{d}	14.0±0.2^{a}
	Control	90.6±0.3^{a}	5.1±0.4f	58.9±0.7^{a}	39.1±0.7^{a}	63.8±0.6^{d}	13.7±0.2^{abc}
	Average	90.1±0.2^{c}	5.2±0.3^{a}	58.0±0.4^{d}	38.5±0.4^{d}	63.6±0.3^{a}	13.8±0.1^{b}

TD/PP = proportions of *Tithonia diversifolia/Pennisetum purpureum* [Fresh matter (FM) base]. ADM: analytical dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; IVDMD; *In vitro* dry mater digestibility. T-735= is an epiphytic lactic acid bacteria strain. Sil-all is a commercial inoculum blend of lactic acid bacteria.