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PASTURE AND FORAGE UTILIZATION

In-vitro assessment for ensilabillity of *Tithonia diversifolia* alone or with *Pennisetum purpureum* using epiphytic lactic acid bacteria strains as inocula

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ABSTRACT. It is expected that the availability of forage for animals in the tropics will fluctuate in the future due to climate change. Ensiling of tropical forages constitutes a strategy to cope with food scarcity during long dry seasons. *Tithonia diversifolia* (TD), is a plant belonging to family Asteraceae, with a wide adaptation in tropical areas. A Rostock Fermentation Test (RFT) was performed to compare the ensilabillity potential among four epiphytic lactic acid bacteria (LAB) strains isolated from TD. Further, two experiments were designed 1) to evaluate the acidification potential of the best strain from the preliminary test (T735), and 2) to determine the ensilability of different levels of inclusion in the TD / PP mixture. For the first experiment, T735 and a blend of lactic acid-fermenting bacteria (BLB) decreased the pH rapidly. In experiment 2, different PP/TD ratios (0/100; 33/67; 67/33; 100/0) were ensiling, all inoculated with T735, (BLB) and their mixture. Increasing of grass PP in the mixtures brought about a fast drop in pH, facilitating TD ensilabillity. The use of T735 improved acidification and fermentation parameters of TD/PP silage.

Keywords: tropical forages; inoculum; acidification; wild sunflower.

Avaliação *in vitro* para ensilabilidade de *Tithonia diversifolia* sozinha ou com *Pennisetum purpureum* utilizando cepas de bactérias epífitas ácido lácticas como inóculos

RESUMO. Espera-se que a disponibilidade de forragem para animais nos trópicos flutue no futuro devido a mudanças climáticas. Silagem de forragem tropical é uma estratégia para lidar com a escassez de alimentos durante estações secas. *Tithonia diversifolia* (TD), é uma planta pertencente à família Asteraceae, rica em proteínas com ampla adaptação em zonas tropicais. Um teste de fermentação de Rostock (RFT) foi realizado para comparar a adequação potencial para silagem de quatro cepas isoladas de TD de bactérias epífitas ácido láticas (BAL). Assim, dois experimentos foram projetados: 1) avaliar o potencial de acidificação da melhor estirpe identificada em um teste preliminar (T735) e 2) determinar a capacidade de ensaio de diferentes níveis de inclusão na mistura TD/PP. Para o primeiro experimento, o T735 e uma mistura de fermentação BAL (BLB) diminuíram o pH mais rapidamente do que o controle. No experimento 2, diferentes proporções de PP/TD (0/100; 33/67; 67/33; 100/0) foram ensiladas, todas inoculadas com T735, (BLB) e sua mistura. O aumento da inclusão de PP nas misturas provocou uma rápida queda no pH, facilitando a ensilabilidade TD. O uso de T735 melhorou os parâmetros de acidificação e fermentação da silagem TD/PP.

Palavras-chave: forragens tropicais; acidificação; botão dourado.

Introduction

Conservation strategies of forage such as ensiling are topics of interest for animal scientists in tropical countries who aim to cope with seasonal forage scarcity. In particular, there is a demand to ensure food availability throughout the year and fill the gaps in times of food shortage for animal maintenance and production (Martens, Hoedtke, Avila, Heinritz, & Zeyner, 2014). Local forages are the feed base for herbivore production in tropical America. Forages can contribute to a sustainable food system if their nutritional value is properly preserved. Furthermore, local vegetation resources help to diminish the use of external inputs and create a more resilient agriculture (Rao et al., 2015; Ribeiro et al., 2016).

Tithonia diversifolia (TD) is a bush originated from Mexico and widely distributed throughout the

humid and sub-humid tropics of Central and South America, Asia and Africa (Jama et al., 2000; Holguín, Ortiz-Grisalez, Velasco-Navia, & Mora-Delgado, 2015; Ribeiro et al., 2016). Ensilability of leguminous and non-leguminous forbs can be limited by the high buffering capacity (Lević, Prodanović, & Sredanović, 2005), organic acids (Lukač, Kramberger, Meglič, & Verbič, 2012), low water soluble carbohydrates (WSC) concentrations and high content of secondary compounds (Martens et al., 2014). Thus, the ratio of WSC to buffering capacity (BC) for TD, is presumably low for proper ensiling. However, it has been documented that ensiling commonly reduces antinutritional compounds such as tannins, oxalic acid and trypsin inhibitory activity (Lukač et al., 2012; Martens, Tiemann, Bindelle, Peters, & Lascano, 2012; Titterton & Bareeba, 2001) have shown that blending grasses and legumes can improve ensilabillity (pH between 3.7 – 4.5; NH3-N < 12% of total N).TD is a non-legume forage with a high protein value (20% in DM) on average (La et al., 2012). Therefore, it is necessary to develop strategies to compensate for TD's buffering capacity (Nhan, Hon, & Preston, 2011) and for low WSC when ensiling. The Rostock Fermentation Test (RFT) is a rapid *in vitro* test in an aqueous solution that allows a quick evaluation of the ensilabillity of forages, it was used by Pieper, Hoedtke, Wensch-Dorendorf, Korn, Wolf, & Zeyner (2016) as a method for assessing the ensiling potential of herbage. The pH is used as an indicator for the degree of lactic acid fermentation that has taken place (Hoedtke & Zeyner, 2011).

This study was realized to evaluate the fermentative ability of LAB strains isolated from *Tithonia diversifolia* (TD) compared to other local and commercial additives to favor acidification of TD forage for ensiling. Likewise, this study investigates whether the inclusion of *Pennisetum purpureum* cv. king grass (PP) can improve the ensilabillity of TD.

Material and methods

Forages and experimental design

TD forage was harvested (10 cm above-ground biomass including leaves and stems) at the pre-flowering stage in February 2013 at the experimental farm from the National University of Colombia, Palmira (1.000 m.a.s.l. 24°C, annual precipitation 1,020 mm and relative humidity 72%). PP was harvested at vegetative state (10 cm above ground level) at the same time and location. The Rostock Fermentation Test (RFT) was developed to quickly evaluate the *in vitro* ensilabillity, with pH measured at 0, 20, 28, 44 and 48h along the incubation period (37°C).

Bacterial strains determination was performed at the Clinical Laboratory of Tolima University as follow: an aliquot of 50 g of TD forage was taken, to which 450 mL of buffered peptone water (1/10) were added. Then, serial dilutions (10^{-2}, 10^{-3}, 10^{-4}…10^{-7}) were performed. Dilutions were cultivated in MRS agar by the pour plate method. Epiphytic lactic acid bacteria (LAB) from TD were cultivated on MRS agar (Petri dishes) and incubated at 37°C for 72 hours under anaerobic conditions.

Colonies were counted and single colonies were isolated for their further use as an inoculum. The criteria to select the LAB's strains were in base of their ability to growth on Rogosa agar and their ability to produce lactic acid (3M Petrifilm AC) according to Nero et al. (2006). Further, the API 50 CH test kit and the API CHL medium (bioMérieux Vitek Inc.) were used to determine the strains of each *LAB* isolate by characterizing the ability to ferment 49 carbohydrates. The culture dilutions were then loaded to the API 50CH test strips following the manufacturer's protocol. Species identification was read in the software API web (BioMérieux, Inc. 2009). To label the four isolates, a "T" (from Tithonia) was used followed by a consecutive number, according to the internal serial number of the LAB collection of the International Center for Tropical Agriculture (CIAT): T732, T733, T734 and T735.

A preliminary assay (RFT) was performed to compare ensilabillity among the four epiphytic bacteria strains isolated from TD to acidify TD forage. They were tested against a blend of lactic acid-fermenting bacteria (BLB) based on *Streptococcus faecium* (*CNCM I-3236*), *L. plantarum* (CNCM I-3235), *Pediococcus acidilactici* (CNCM I-3237) and *L. salivarius* (CNCM I-3238) and a control of TD with sucrose (TD+S) in triplicate. The registration numbers belong to the National Collection of Microorganisms Cultures – CNCM.

In the first experiment, five treatments in three replicates were tested on TD forage. The best epiphytic strain, isolated from TD (T735) was tested against two LAB from the CIAT bacterial collections (C726 = *L. plantarum*, C727 = *L. pentosus,* both isolated from Flemingia macrophylla, a blend of lactic acid-fermenting bacteria (BLB) and a control (TD+S). A sample of 50 g of forage was minced in a food processor (Power Pro II FP1510) and placed into an autoclave beaker (500 mL) containing 200 mL of a solution with sucrose (0.5% w v^{-1}).

All treatments were enriched with sucrose (S) as the energy source. Sucrose was used to ensure that the availability of WSC for microbial activity would not be limiting. A sterilized stirring rod was used to homogenize the preparation. Later, each inoculant was applied to the medium in three replicates (0.1 ml of an inoculum $4x10^9$ cfu mL^{-1} previously cultivated in 10 ml MRS broth at 37°C for 24h). The preparations were covered with a sterile plastic lid and incubated at 37°C for 48h. The pH was measured using a pH meter (Mettler Toledo, o SevenGo, with pH electrode InLab@ 41356/2mat) at 0, 20, 28, 44 and 48h, disinfecting the electrode with 70% ethanol before each measurement. The fermentation coefficient (FC) was estimated following the formula of Weissbach, Schmidt, and Hein (1967):

$$FC = DM(g100g - 1) + 8WSC/BC.$$

In experiment 2, the effects of including the grass (PP) with TD on the ensilabillity was evaluated, using the inoculants T735, (BLB) and their combination (T735+BLB*)* plus a control. All treatments (in triplicate) on the (RFT) essay were enriched with sucrose at 2 % of fresh matter.

Chemical analysis

The nutritive value analyzes were performed according to the Association Official Analytical Chemist (AOAC, 2010) – Method 930.15, NFTA Method 2.1.4 standards. For determination of dry matter, we used the Method 973.18, NFTA Method 4.1. For that, 1 g of the fodder of freeze-dried material was weighed and dried for 3h at 105°C in a cabinet dryer. The dried sample was collected in a desiccators and weighed again after cooling down. The sample for dry matter was then incinerated for 5h at 600°C in a muffle furnace to determine the crude ash. For *in vitro* digestibility of dry matter and crude protein (CP) was determined by the Method 984.13.

The method of Tilley and Terry (1963) modified by Moore (1970) was used for the analysis of the cell wall components, as neutral detergent fiber (NDF) and for Acid Detergent Fiber (FDA), Van Soest, Robertson, and Lewis (1991) was used. The determination of total water soluble carbohydrates was done by anthrone method, following the protocol of Herrera Flores, Ortíz, Delgad, Galleros, and Alberto (2014). Buffering capacity (BC) was determined using the method of Weissbach (1967).

Statistical analyses

In experiment 1, the general effect of inoculant treatments was assessed using the model:

$$Yi = \mu + \alpha i + \varepsilon i$$

where Y= is the target variable, μ = is the overall mean, α = inoculant, ε = random experimental error). Analysis of variance was performed by the GLM procedure, Duncan mean comparisons ($p \leq 0.05$), using SAS 9.2 (Statistical Analysis System [SAS], 2006).

In experiment 2, we used a split-plot design with factorial treatment structure, where the first factor was the inoculant used and the second factor was the inclusion level of PP in the mixtures:

$$Yij = \mu + Ii + PPj + Ii * PPij + \varepsilon ij$$

where Y= is the target variable, μ = is the overall mean, I = inoculant [control, T735; a blend of lactic acid-fermenting bacteria (BLB); T735 x (BLB)], PP = proportion of grass in the silage [(0:100, 33:67, 67:33; and 100:0 (FM weight)] and ε= random experimental error. Analysis of variance was performed and statistical differences were detected by Duncan mean comparisons ($p < 0.05$) using Infostat (Di Rienzo et al., 2008).

Results and discussion

The data from table 1 show the chemical composition of *Tithonia diversifolia* and *Pennisetum purpureum* used in the study. TD confirmed its role as source of protein, while the grass was the source of fiber i.e. a higher percentage of dNDF and ADF in PP *vs.* a higher protein content in TD was statistically observed.

Table 1. Chemical composition (DM base) of *Tithonia diversifolia* and *Pennisetum purpureum* evaluated in the Rostock Fermentation Test.

Parameter	Unit	*T. diversifolia*	*P. purpureum*
OM	(g kg^{-1})	870.9±0.4[a]	860.5±0.5[a]
DM	(g kg^{-1})	280.0±1.4[a]	170.2±1.4[b]
Ash	(g kg^{-1})	120.1±0.4[a]	130.5±0.1[b]
CP	(g kg^{-1})	160.9±0.1[a]	50.0±0.2[b]
NDF	(g kg^{-1})	410.6±1.1[a]	630.5±0.3[a]
dNDF	(g kg^{-1})	270.1±2.1[a]	330.9±2.1b
ADF	(g kg^{-1})	280.1±1.4[a]	400.9 ±1.6[b]
WSC	(g kg^{-1})	230.4±0.6[a]	210.2±1.9[a]
BC	(g lactic acid 100 g^{-1} DM)	8.4±0.6[a]	3.1±0.2[a]
WSC/BC	-	2.80±0.2[a]	6.70±1.1[b]
IVDDM	%	67.0±1.3[a]	66.0±0.3[a]
FC	-	49.6	71.2

Different letters in the same row mean significant differences between species ($p < 0.05$). OM=Organic matter; DM= Dry matter; CP=Crude protein; NDF= neutral detergent fiber; dNDF= digestible neutral detergent fiber; ADF=acid detergent fiber, WSC=Water soluble carbohydrates WSC; BC=Buffering capacity; IVDDM= *in vitro* digestibility of dry matter; ± standard deviation; FC= fermentation coefficient. FC = DM (g 100 g^{-1}) + 8 WSC/BC. *Author's personal copy.*

Water soluble carbohydrates (WSC) was slightly higher in TD, but without statistical differences; IVDDM in both forages were similar. However, the WSC/BC ratio confirmed the higher ensilabillity of the grass, derived from its smaller buffering capacity, *i.e.*, the high buffering capacity of TD limits its ensilabillity.

Preliminary Rostock fermentation test (RFT)

The cultured epiphytic bacteria strains isolated from TD were identified as *Lactobacillus paracasei* (T735) and *L. plantarum* (T732, T733, T734), respectively. In this assessment, neither the control nor any of the epiphytic LAB strains tested, achieved a pH below 4.0 at 20 hours. However, T735 strain (*L. paracasei*) showed the best acidification potential among the native isolates after 48h (Table 2). Therefore, this was selected for further evaluations in experiment 1 and 2. The rest of the bacterial strains were discarded from further evaluations.

Table 2. Acidification potential of epiphytic strains isolated from *Tithonia diversifolia* (TD) against a blend of lactic acid-fermenting bacteria (BLB)* inoculum enriched with sucrose *(S)* in a preliminary test.

	Control (n=3)	T732 (n=3)	T733 (n=3)	T734 (n=3)	T735 (n=3)	BLB (n=3)	P-Value
20h	5.5±0.1^{b}	5.3±0.2^{b}	5.3± 0.1^{b}	5.4±0.2^{b}	5.3±0.1^{b}	4.1±0.0^{a}	0.0001
48h	5.8±0.5^{b}	4.6±0.2^{b}	4.6±0.6^{ab}	5.1±0.5^{ab}	4.5±0.4^{ab}	4.0±0.0 ^{a}	0.3348

Different letters in the same row mean significant differences between treatments ($p < 0.05$). Control = TD + S without inoculum; T732= TD + *S* + T732 (*L. plantarum*); T733= TD + *S* + T733 (*L. plantarum*), T734 = TD + *S* + T734 (*L. plantarum*); T735= TD + *S* + T735 (*Lactobacillus paracasei*). (BLB) = blend based on *Streptococcus faecium* (CNCM I-3236), *L. plantarum* (CNCM I-3235), *Pediococcus acidilactici* (CNCM I-3237) and *L. salivarius* (CNCM I-3238).

Experiment 1

The T735 (epiphytic bacteria from TD and C727 (from CIAT's LAB collection) inocula lowered the silage pH ($p < 0.05$) in comparison to the control (TD without inoculum) after 20h onwards. In line with these results, a difference was found between T735 and C727 to (BLB) from 28 to 48h of incubation. BLB and control treatments showed the worst pH development during the RFT. The pH measurement with T735 was maintained at 3.6 at the end of the assay, presenting the lowest acidity, however, was not different statistically from treatments inoculated with C726 and C727 (Table 3).

Experiment 2

Within the first 20h, blends with a high grass proportion, resulted in rapid acidification. The pH value ranged from > 6 at 0 hours, to 3.6-3.8 in 100% PP, compared to 4.6-4.9 in 100% (TD) (Table 4). The data suggest that acidification is faster as the proportion of the grass increases ($p = 0.01$). The T735 strain *per se* was successful in reducing pH. The enrichment of TD with BLB did not provide any additional advantage compared to TD alone. There was no independent effect of treatments PP x Inoculant ($p = 0.44$) at any time.

Table 3. Development of pH during the Rostock Fermentation Test (RFT) for *Tithonia diversifolia* (TD) using different epiphytic lactic acid bacteria strains enriched with sucrose (S) in experiment 1.

Treatment	pH				
	0h	20h	28h	44h	48h
TD + S (control)	6.9±0.1	4.1±0.0^{b}	4.3±0.0^{b}	3.9±0.1^{b}	3.8±0.1^{c}
TD + S + C726	7.1±0.0	3.9±0.0^{ab}	3.7±0.0^{a}	3.7±0.0^{a}	3.7±0.0^{ab}
TD + S + C727	7.1±0.0	3.9±0.0^{ab}	3.8±0.0^{a}	3.7±0.0^{a}	3.7±0.0^{a}
TD + S + T735	6.9±0.1	3.8±0.1^{a}	3.7±0.0^{a}	3.6±0.0^{a}	3.6±0.0^{a}
TD + S + BLB	7.0±0.1	4.1±0.0^{b}	4.2±0.0^{b}	3.9±0.1^{b}	3.8±0.1b^{c}

Different letters in the same column mean significant differences between treatments ($p < 0.05$). (BLB) = blend of lactic acid-fermenting bacteria based on *Streptococcus faecium* (CNCM I-3236), *L. plantarum* (CNCM I-3235), *Pediococcus acidilactici* (CNCM I-3237) and *L. salivarius* (CNCM I-3238).

T. diversifolia is a shrub belonging to family Asteraceae, which produce forage recognized as a source of protein (Fasuyi & Ibitayo, 2011; Orozco et al., 2009). However, in this study we found that can be also an important source of WSC comparable to PP. The analysis of fiber fractions indicates larger values in PP biomass than TD. These findings take relevance since FDN and FDA parameters are closely related to animal performance (e.g. milk yield and live weight gain), because influence directly animal intake and digestibility of forages (La et al., 2012). The evaluation of the *in vitro* digestibility of dry matter (IVDDM) of TD was lower than the (La et al., 2012) reported values, but are at the level of other tropical legumes (Heinritz, Martens, Avila, & Hoedtke, 2012).

On the other hand, PP presented a similar IVDDMD (66%) values to other tropical grasses (Martens et al., 2012). Therefore, blending both forages will not only benefit acidification and fermentation parameters for silage making, but also the digestibility and the intake of silage by animals, probably resulting in improved productivity.

Epiphytic LAB strains isolated from tropical silages are promising candidates to be used as silage additives (Heinritz et al., 2012). However, it is important to identify strains with the capacity to favor lactic fermentation (homofermentative strains) in the target forage. Here, the employment of 735 LAB (*L. paracasei*) isolated from TD, improved lactic acid fermentation of TD and PP mixtures, followed by more acidic pH values.

Table 4. Development of pH during the Rostock Fermentation Test for *Tithonia diversifolia* alone or with *Pennisetum purpureum* using different lactic acid bacteria strains in experiment 2.

Aditive	TD/PP	pH				
		0h	20h	28h	44h	48h
Control	0/100	5.9 ± 0.0^{a}	3.8 ± 0.0^{ab}	3.8 ± 0.0^{abc}	4.0 ± 0.3^{abcde}	3.7 ± 0.0^{abc}
Control	33/67	6.5 ± 0.0^{b}	4.3 ± 0.0^{cdef}	4.1 ± 0.1^{bcd}	3.7 ± 0.0^{abc}	3.6 ± 0.0^{ab}
Control	67/33	6.8 ± 0.1^{cde}	4.7 ± 0.0^{fgh}	4.7 ± 0.0^{ef}	4.2 ± 0.1^{bcdf}	4.2 ± 0.1^{cdef}
Control	100/0	7.1 ± 0.0^{e}	4.9 ± 0.1^{h}	5.0 ± 0.1^{f}	4.7 ± 0.1^{f}	4.6 ± 0.1^{fgh}
T735	0/100	6.0 ± 0.0^{a}	3.7 ± 0.1^{a}	3.7 ± 0.1^{ab}	3.4 ± 0.1^{a}	3.4 ± 0.1^{a}
T735	33/67	6.6 ± 0.0^{c}	3.9 ± 0.0^{abc}	3.8 ± 0.1^{abc}	3.6 ± 0.1^{ab}	3.6 ± 0.1^{ab}
T735	67/33	6.8 ± 0.1^{cde}	4.2 ± 0.1^{cde}	4.1 ± 0.1^{cd}	3.9 ± 0.1^{abcd}	3.9 ± 0.1^{bcd}
T735	100/0	7.1 ± 0.0^{e}	4.6 ± 0.1^{efgh}	4.4 ± 0.1^{de}	4.3 ± 0.2^{cdef}	4.3 ± 0.2^{defg}
BLB	0/100	5.9 ± 0.1^{a}	3.7 ± 0.2^{ab}	3.7 ± 0.3^{abc}	3.9 ± 0.3^{abcd}	3.7 ± 0.3^{abc}
BLB	33/67	6.6 ± 0.1c	4.1 ± 0.1^{bcd}	4.1 ± 0.0^{cd}	3.9 ± 0.3^{abcd}	4.2 ± 0.1^{cde}
BLB	67/33	6.8 ± 0.0^{cde}	4.4 ± 0.3^{defg}	4.4 ± 0.3^{de}	4.4 ± 0.2^{def}	4.4 ± 0.2^{efgh}
BLB	100/0	7.1 ± 0.0^{e}	4.8 ± 0.3^{h}	4.8 ± 0.3^{ef}	4.6 ± 0.4^{ef}	4.8 ± 0.2^{h}
T735+ BLB	0/100	6.2 ± 0.4^{a}	3.6 ± 0.0^{a}	3.5 ± 0.0^{a}	3.4 ± 0.1^{a}	3.5 ± 0.1^{ab}
T735+ BLB	33/67	6.6 ± 0.0^{cd}	$3.9 \pm 0.^{abc}$	3.8 ± 0.1^{abc}	3.7 ± 0.2^{abc}	3.8 ± 0.2^{abc}
T735+ BLB	67/33	6.9 ± 0.0^{de}	4.4 ± 0.3^{defg}	4.3 ± 0.3^{de}	4.3 ± 0.3^{bcdf}	4.3 ± 0.3^{defg}
T735+ BLB	100/0	7.0 ± 0.2^{e}	4.7 ± 0.1^{gh}	4.7 ± 0.2^{ef}	4.7 ± 0.2^{f}	4.7 ± 0.2^{gh}

Different letters in the same column mean significant differences between treatments ($p < 0.05$). BLB = blend of lactic acid-fermenting bacteria based on *Streptococcus faecium* (CNCM I-3236), *L. plantarum* (CNCM I-3235), *Pediococcus acidilactici* (CNCM I-3237) and *L. salivarius* (CNCM I-3238).

Acidification of the medium below pH 4 is important because it reduces proteolysis from enzymatic activity and chemical hydrolysis (Rooke & Hatfield, 2003). Furthermore, the acidic medium inhibits the growth of enterobacteria, which are undesirable for the hygienic quality of silage (Buxton, Mertens, & Fisher, 1996). It is important to notice that *L. paracasei* is a recognized strain to improve the ensiling process and it is considered safe by the European Food Safety Authority (EFSA, 2013).

For forage ensilabillity, buffering capacity (BC) is a critical parameter to achieve good silage quality. It is accepted that BC is high for high-protein feeds and legumes whereas it is low for energetic feeds and low-intermediate BC for low-protein feeds and grass forages (Lević et al., 2005). Therefore, BC seems to be the determinant factor to limit the ensilabillity of TD as the WSC content in the present study were statistically similar to PP (234 *vs.* 212g kg^{-1} for TD and PP, respectively). These results indicate that the epiphytic strain T735 was potentially efficient to induce a pH drop and to increase lactic fermentation in blends with high proportion of PP (Table 3). This suggests that acidification is easiest when blends contain a high proportion of PP.

The increase of grass in the mixtures probably reduced the buffering capacity of TD. Martens et al., (2014) demonstrated that the use of readily available carbohydrates in combination with selected lactic acid bacteria strains could improve the fermentation of tropical forages. Thus, the combination of LAB and sucrose generally bring the fastest and most effective reductions of pH in most grasses (Hoedtke & Zeyner, 2011). In the present study, the epiphytic strain of TD (T735) in the forage enriched with sucrose was potentially efficient to induce a pH drop and to increase lactic fermentation in those blends (Table 3).

In line with these results, the ratio WSC/BC of TD indicates its low suitability for ensiling. On the contrary, PP grass and high grass proportion of blends were much more suitable for ensiling due to their favorable WSC/BC ratio, meanwhile, the higher this relationship is, the greater the Ensilability (Heinritz et al., 2012). The fermentation coefficient (FC), which indicates the high ensilabillity of both TD and PP, demonstrated a significant advantage for the grass in comparison to TD (49.6 and 71.2 for TD and PP, respectively). Weissbach et al. (1967) suggests that if the FC is below 35, the material is difficult for ensiling. Heinritz, et al. (2012) found a satisfactory FC in Mulato II (*Brachiaria ruziziensis* x *B. brizantha* x *B. decumbens*) grass (FC = 52), with better results with the addition of LAB (FC = 65), contrasting to the low FC average for tropical forage legumes (FC = 39). This finding helps to explain why pH values are lower in mixtures with a higher percentage of PP. Therefore, the pH of grass blends (PP plus TD) was lower than in silage of TD alone (Table 4). Favoring high WSC/BC ratio either by the increasing WSC content or by reducing BC would benefit the ensilabillity of TD.

Conclusion

T. diversifolia (TD) ensilabillity can be improved by the addition of LAB epiphytic bacteria. Mixtures of TD with *P. purpureum* (PP) improve ensilabillity of TD. Larger amounts of PP facilitate the ensilabillity of TD. In further studies, TD and PP will be ensiled on a larger scale with different proportions and different LAB inocula to verify the findings from the *in-vitro* experiments.

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References

Association of Official Analytical Chemist [AOAC]. (2010). *Official Methods of Analysis* (18th ed.). Gaitherburg, MD: AOAC International.

Buxton, D. R., Mertens, D. R., & Fisher, D. S. (1996). Forage quality and ruminant utilization. *Cool-Season Forage Grasses, 1*, 229-266.

Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., Gonzalez, L., Tablada, M., & Robledo, C. W. (2008). *InfoStat, versión 2008*. Córdoba, AR: Grupo InfoStat, FCA, Universidad Nacional de Córdoba.

European Food Safety Authority [EFSA]. (2013). Scientific opinion on the safety and efficacy of *Lactobacillus paracasei* (NCIMB 30151) as a silage additive for all animal species. *EFESA Journal, 11*(5), 3611. doi: 10.2903/j.efsa.2013.3219

Fasuyi, A. O. & Ibitayo, F.J. (2011). Nitrogen balance and morphometric traits of weanling pigs fed graded levels of wild sunflower (*Tithonia diversifolia*) leaf meal. *African Journal of Food, Agriculture, Nutrition and Development*, *11*(5), 1-17. doi: 10.4314/ajfand.v11i5.70441

Heinritz, S. N., Martens, S. D., Avila, P., & Hoedtke, S. (2012). The effect of inoculant and sucrose addition on the silage quality of tropical forage legumes with varying ensilability. *Animal Feed Science and Technology, 174*(3-4), 201-210. doi: 10.1016/j.anifeedsci.2012.03.017

Herrera Flores, T. S., Ortíz, C. J., Delgad, A. A., Galleros, A., & Alberto, J. (2014). Contenido de osmoprotectores, ácido ascórbico y ascorbato peroxidasa en hojas de frijol sometidas a estrés por sequía. *Revista Mexicana de Ciencias Agrícolas, 5*(5), 859-870.

Hoedtke, S., & Zeyner, A. (2011). Comparative evaluation of laboratory-scale silages using standard glass jar silages or vacuum-packed model silages. *Journal of the Science of Food and Agriculture, 91*(5), 841-849.

Holguín, V. A., Ortiz-Grisalez, S., Velasco-Navia, A., & Mora-Delgado, J. (2015). Multi-criteria evaluation of 44 introductions of *Tithonia diversifolia* (Hemsl.) A. Gray in Candelaria, Valle del Cauca. *Revista de la Facultad de Medicina Veterinaria y de Zootecnia, 62*, 57-72.

Jama, B., Palm, C. A., Buresh, R. J., Niang, A., Gachengo, C., Nziguheba, G., & Amadalo, B. (2000). *Tithonia diversifolia* as a green manure for soil fertility improvement in western Kenya: a review. *Agroforestry Systems, 49*(2), 201-221.

La, O., Castillo, Y., Ruiz, O., Estrada, A., Rios, F., Bernal, H., ... Castro, B. I. (2012). Chemical composition, in situ rumen degradability, and *in vitro* digestibility of *Tithonia diversifolia* ecotypes of interest for ruminant feeding. *Cuban Journal of Agricultural Science, 46*(1), 47-53.

Lević, J., Prodanović, O., & Sredanović, S. (2005). Understanding the buffering capacity in feedstuffs. *Biotechnology in Animal Husbandry, 21*(5-6), 309-313.

Lukač, B., Kramberger, B., Meglič, V., & Verbič, J. (2012). Importance of non-leguminous forbs in animal nutrition and their ensiling properties: a review. *Zemdirbyste-Agriculture, 99*(1), 3-8.

Martens, S. D., Hoedtke, S., Avila, P., Heinritz, S. N., & Zeyner, A. (2014). Effect of ensiling treatment on secondary compounds and amino acid profile of tropical forage legumes, and implications for their pig feeding potential. *Journal of the Science of Food and Agriculture, 94*(6), 1107-1115.

Martens, S. D., Tiemann, T. T., Bindelle, J., Peters, M., & Lascano, C. E. (2012). Alternative plant protein sources for pigs and chickens in the tropics–nutritional value and constraints: a review. *Journal of Agriculture and Rural Development in the Tropics and Subtropics, 113*(2), 101-123.

Moore, J. E. (1970). Procedures for the two-stage in vitro digestion of for ages. In L. E. Harris, *Nutrition research techniques for domestic and wild animals* (Vol. 1). Logan, UT: Utah State University.

Nero, L. A., Beloti, V., de Aguiar Ferreira Barros, M., Ortolani, M. B. T., Tamanini, R., Melo, F., & Gombossy, B. R. (2006). Comparison of Petrifilm aerobic count plates and de Man–Rogosa–Sharpe agar for enumeration of lactic acid bacteria. *Journal of Rapid Methods & Automation in Microbiology, 14*(3), 249-257.

Nhan, N. T., Hon, N. V., & Preston, T. R. (2011). Studies on ensiling of *Tithonia diversifolia* and Taro (*Colocasia esculenta*) and feeding the silage to fattening pigs as partial replacement of a basal diet of rice bran, broken rice, soybean meal and fish meal. *Livestock Research for Rural Development, 23*(5), Art. 105.

Orozco, A., Ruíz, O., Rodríguez, C., Valenciaga, D., Gutiérrez, E., Arzola, C., ... Castillo, Y. (2009). Effect of the combination of *Tithonia diversifolia* and (PP) VC. Cuba CT-115 in the kinetics and gas production *in vitro*. *Revista Cubana Ciencia Agrícola, 43*(2), 149-152.

Pieper, B., Hoedtke, S., Wensch-Dorendorf, M., Korn, U., Wolf, P., & Zeyner, A. (2016). Validation of the Rostock fermentation test as an in vitro method to estimate ensilability of forages using glass jar model silages as a basis for comparison. *Grass and Forage Science, 72*(3), 568-580.

Rao, I., Peters, M., Castro, A., Schultze-Kraft, R., White, D., Fisher, ... Rudel, T. (2015). LivestockPlus: sustainable intensification of tropical forage-based systems for improving livelihood and environmental

benefits. *Tropical Grasslands - Forrajes Tropicales, 3*(2), 59-82.

Ribeiro, R. S., Terry, S. A., Sacramento, J. P., Silveira, S. R., Bento, C. B. P., Silva, E. F. d., ... Chaves, A. V. (2016). *Tithonia diversifolia* as a supplementary feed for dairy cows. *PLoS One*, 11, e0165751.

Rooke, J. A., & Hatfield, R. D. (2013). *Biochemistry of Ensiling*. Lincon, NE: U.S. Department of Agriculture - Agricultural Research Service.

Statistical Analysis System [SAS]. (2006). *SAS/STAT User Guide, Version 9.2*. Cary, NC: SAS Institute Inc.

Tilley, J. M. A., & Terry, R. A. (1963). A two□stage technique for the in vitro digestion of forage crops. *Grass and Forage Science, 18*(2), 104-111.

Titterton, M., & Bareeba, F. (2001). Ensilaje de gramíneas y leguminosas en los trópicos. In L.'t Mannetje (Ed.), *Uso del ensilaje em el trópico privilegiando opciones para pequeños campesinos* (p. 43-53). Roma, IT: FAO.

Van Soest, P. J. & Robertson, J. B. (1985). *Analysis of forage and fibrous foods. A laboratory manual for animal science* (Vol. 613*)*. Ithaca, NY: Cornell University.

Weissbach, F. (1967). The determination of the buffer capacity of the forage plants and their importance for the assessment of fermentability. *Akademie der Landwirtschaftswissenschaften, 92*, 211-220.

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