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Combined Ensiling of Tropical Beans and Sugarcane Stalks: Effects on Their Secondary Metabolites

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Abstract: In this study, the effect of mixed silage on the chemical composition, ensilability, in vitro cellulase digestibility and some of their plant secondary metabolites (PSM) were assessed. The lab-scale silage mixes were made in triplicate from sugarcane (*Saccharum* spp.) stalk and *Canavalia ensiformis* or *Mucuna pruriens* beans (Santa Clara, Cuba). Sugarcane stalks and beans were mixed at a ratio of 40:60 (on DM basis), respectively, in combination with a mixture of microbial inoculants (*Lactobacillus acidophilus*, and two *Kluyveromyces fragilis* strains, 4×10^5 colony forming units (CFU)/g of fresh matter). Before and after ensiling, the chemical composition, ensilability and contents of some PSM (trypsin inhibitor, L-canavanine, total tannins, L-dopa and phytic acid) were determined. A complete factorial design to assess the effect of the ensiling time (30, 60 or 90 days), the legume type (LT) and their interactions on the assessed parameters were performed. A better silage fermentation quality of the *M. pruriens*–sugarcane mixed material [e.g., lower ($p < 0.05$) pH and ammonia N content and higher ($p < 0.05$) concentration of lactic acid] was observed as compared with the *C. ensiformis*–sugarcane mixed material. The ensiling process reduced ($p < 0.001$) the studied PSM, being higher ($p < 0.001$) in the *M. pruriens*–sugarcane silages than in the *C. ensiformis*–sugarcane silages [trypsin inhibitor (86 vs. 76%); L-canavanine (70 vs. 53%); total tannins (54 vs. 50%); L-Dopa (89 vs. 86%)], except for phytic acid (65 vs. 68%), respectively]. It was concluded that ensiling enhances the quality of the feeds by predigestive reduction of the amount of secondary plant metabolites.

Keywords: ensilability; rumen degradability; *Canavalia ensiformis*; *Mucuna pruriens*; plant secondary metabolites

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1. Introduction

Most tropical areas suffer food deficits, especially during the dry season. An efficient use of available food resources during the rainy season or at the beginning of the dry season can increase animal production [1,2]. A constant availability of good quality food throughout the year is required and constitutes the biggest challenge for the development of tropical livestock production [3,4]. The high costs of producing and importing raw materials for concentrates lead to a growing demand for cheaper national production, especially in underdeveloped or developing countries such as Cuba [5–7]. In that respect, the conservation (e.g., ensiling) of forage with high protein contents (e.g., *Dichrostachys cinerea*, *Tithonia diversifolia*) combined with forage with high fiber contents (e.g., *Saccharum officinarum* L.) and additives (e.g., molasses and/or microorganisms) plays a significant role in the search for different alternative feeds for ruminants [3,4].

This line of work justifies the use of alternative vegetable protein sources such as *Canavalia ensiformis* (L.) DC and *Mucuna pruriens* (L.) DC beans, which through their high beans yield [2–4 t/ha [2,8]] and crude protein content [280–340 g/kg of dry matter (DM)] might be a good feed source for ruminants [2,9]. Although these legumes are important sources of dietary protein for animals, their acceptability and utilization has been limited due to some secondary metabolites (PSM) [10,11]. Previous works have demonstrated the potential effect of the ensiling process on the reduction of some PSM. Kalač, et al. [12] observed a significant reduction in the saponins content in alfalfa after ensiling. In other work, Abbasi, et al. [13] observed a reduction of the phenolic compounds contents, but no effects of the ensiling process or additives on the oxalic acid contents were observed. However, Fan et al. [14] showed that polyphenols, flavonoids and terpenoids in the stems and leaves of the artichoke (*Cynara scolymus* L.) were preserved well in silage.

Obviously, the extent of PSM disappearance may depend on the silage quality. However, legumes cannot be easily ensiled alone due to their low content of water-soluble carbohydrates (WSC) which are essential for a successful silage [15,16]. Accordingly, from their treatments with soybean forage and molasses, Lima, et al. [17] modelled that at least 40 g WSC/kg of fresh matter (FM) were needed without inoculant (equivalent to 134 g WSC/kg DM) and provided by molasses, to ensure a good silage quality. Inclusion of 35.3% (DM basis) of sugar cane stalks, containing 380 g WSC/kg DM, in a silage mixture could provide an equivalent amount of 134 g WSC/kg DM. As such, a silage mixture with 40% of sugarcane stalks could provide enough WSC (71.8 g/kg FM or 152 g WSC/kg DM) to conserve a mixed silage with 204 g CP/kg DM. However, it is unclear whether the ensiling time affects the decrease in some of the PSM in ensiled legumes and its relation with the silage degradability, as reports on the effects of silage on the PSM breakdown are scarce.

Hence, the present work aimed to assess the effect of the combined ensiling of *C. ensiformis* or *M. pruriens* beans with sugarcane stalks on the ensilability, dry matter (DM) and organic matter (OM) digestibility, and some plant secondary metabolites contained in these legumes. Accordingly, the following specific goals were formulated: (i) to quantify the nutritional value and ensilability of *C. ensiformis* or *M. pruriens* beans mixed with sugarcane stalks before and after ensiling (30, 60 and 90 d); (ii) to determine the content of plant secondary metabolites (trypsin inhibitor, L-dopa, phenols and L-canavanine) of *C. ensiformis* or *M. pruriens* beans mixed with sugarcane stalks before and after ensiling (30, 60 and 90 d); and (iii) to assess the cellulase organic matter digestibility in dry matter (CDOMD) of *C. ensiformis* or *M. pruriens* beans mixed with sugarcane stalks before and after ensiling (30, 60 and 90 d).

2. Materials and Methods

2.1. Plant Materials

Two tropical legume species, *C. ensiformis* (Ce) and *M. pruriens* (Mp), with the former having a higher grain yield as well as a lower tannin content than the latter [18,19], and one sugarcane crop (*Sacharum* spp.) cultivar B80-250 [20] were sown at a research farm (22°43′ N, 79°90′ W, 100 masl) of the Centro de Investigaciones Agropecuarias (CIAP) at Universidad Central "Marta Abreu" de Las Villas (UCLV), Santa Clara, in the center of Cuba. The sugarcane was sown two years (November 2014) before the legumes and harvested 12 months (November 2015) after sowing to guarantee the maturity of this crop at the next harvesting time (18 November 2016). The legumes species were cropped with 70 cm row spacing at a density of 3.8 plants/m^2 and sown on 29 April 2016 to guarantee the hard grain stage at sugarcane harvest. Each crop was sown in triplicate on separate fields (250 m^2/parcel), but with similar soil type (brown calcareous inceptisols [21]) and fertility. The precipitation, temperature and humidity during the cropping period were 190 ± 22 mm, 28.3 ± 0.3 °C and 80 ± 2.1% (average ± SD). Crops were not fertilized or irrigated. Harvesting was scheduled around noon as it took place during the beginning of the dry season. Sugarcane was harvested approximately 5 cm above the soil and the top was removed at the level of the lowest leaf. After harvesting, the beans (all beans were

sun-dried for one day before being chemically analyzed) were ground to pass a 5 mm screen and sugarcane stalks were chopped to a particle size of 2 cm and ensiled immediately.

2.2. Procedure to Prepare Laboratory Silage

The materials to be ensiled were chopped, homogenized and packed in laboratory silos (1.3 L of volume) following the methodology proposed by Lima-Orozco and González Díaz [22]. In brief, two treatments were performed, mixing 400 g of dry matter (DM) sugarcane stalks (SS) and 600 g DM of *C. ensiformis* or *M. pruriens* beans with BIOPRANAL at 4×10^5 colony forming units (CFU)/g of fresh matter [a mixture of microbial inoculants [23], containing *Lactobacillus acidophilus*, *Kluyveromyces fragilis* L/12-8-1 (L/1930) and *Kluyveromyces fragilis* (L-4 UCLV)]. The plant material and additive were homogenized before packing and compacted by hand (helped with a stick) assuring air removal and to obtain a density of 850 kg of FM/m^3. Twenty-four silos (twelve per treatment) were established. The laboratory silos were covered and placed in the dark at room temperature (average ± SD: 28 ± 3 °C) until their opening 30, 60 or 90 days later (each time four silos per treatment were opened).

2.3. Sampling and Chemical Analysis of Fresh and Ensiled Forage

Fresh forage: after chopping and thorough mixing, 350 g of fresh material was dried at 65 °C for 72 h in triplicate. Both pure plant materials as well as their combinations were dried, after which the material was ground to pass a 1 mm screen and stored in glass bottles at room temperature (average ± SD: 28 ± 3 °C) until chemical analysis.

Ensiled forage: after 30, 60 or 90 days of ensiling, and immediately at the opening, a subsample was collected to determine the silage pH, ammonia, lactic acid, short chain fatty acids (SCFA) and alcohol. Another subsample was dried (65 °C for 72 h) and stored for proximate chemical analysis. A silage extract was prepared, stored and analyzed (pH, lactate and alcohol) as described by Lima et al. [24]. Ammonia was determined prior to acidification according to Broderick and Kang [25]. For SCFA, acidified extracts (2 mL of orthophosphoric acid per 100 mL of extract) were centrifuged (15 min at 31,000× *g*, Beckman J2-HS, Palo Alto, CA, USA) and analysis was performed on the supernatant through gas chromatography [26].

2.4. Proximate Chemical Analysis

Samples were assayed in duplicate for the proximate chemical fractions: DM (ID 930.15), nitrogen (N; ID 979.09) converted to crude protein (CP = N × 6.25), ether extract (EE; ID 920.39), crude fiber (CF; ID 962.09) [27] and organic matter (OM) [28] and the starch according to the enzymatic method proposed by Al-Rabadi et al. [29]. Neutral detergent fiber (NDFom) not assayed with a heat stable amylase, was expressed exclusive of residual ash [30]. Acid detergent fiber (ADFom) was expressed exclusive of residual ash [30]. Lignin and cellulose were determined according to Van Soest et al. [30], with lignin being oxidized by permanganate. Silage dry matter was corrected for SCFA, lactic acid, alcohol and ammonia losses according to Lima et al. [17], depending on the silage pH.

2.5. Plant Secondary Metabolites

The contents of the PSM (trypsin inhibitor [TI]; L-canavanine, total tannins, and phytic acid) were determined by standardized methods at Ghent University (Belgium) and UCLV (Cuba) following the protocols described by Makkar et al. [31], while L-Dopa were determined following the protocol described by Doss et al. [32].

- Trypsin inhibitor

Fifteen mL of 0.01 M NaOH was added to 1 g of sample and kept on a homogenizer (Ultra-Turrax, IKA Werke GmbH and Co. KG, Staufen, Germany) at 20,000 rpm for 2 min at 0 °C and pH of 9.5 to 9.8. Then, samples were centrifuged (at 3000× *g* for 10 min) and the suspension was stored at −20 °C until analysis. Two ml of fresh sample were combined with 2 mL of trypsin solution and placed in a water bath at 37 °C. Two

mL of trypsin solution (0.02 mg/mL in 0.01 M HCl) were added to two of the three aliquots, and the vials were incubated (10 min, 37 °C). Then, 5 mL of substrate solution [40 mg BAPNA/mL dissolved in dimethyl sulfoxide, freshly diluted 1/100 in TRIS buffer (0.05 M, pH 8.2) containing 0.02 M $CaCl_2$, previously warmed at 37 °C] was added to each sample tube. A control in which the sample was replaced by assay buffer was run with each set of samples. The reaction was stopped by addition of 1 mL acetic acid (30%, v/v), and centrifuged (at 3000× g for 10 min at room temperature) and the supernatant was transferred to a cuvette. Absorbance was read at 410 nm by a UV-VIS spectrophotometer (Genesys 6, Thermo Electron Corporation, Waltham, MA, USA). The third aliquot that did not receive trypsin solution served as blank and was used to correct for intrinsic color of the samples. The net absorbance was related to the uninhibited control and expressed as relative inhibition in percentage, or as total activity in trypsin units inhibited per ml sample. One trypsin inhibitor unit (TIU) is defined as the number of trypsin units inhibited by 1 mL of the extract and expressed in TIU/kg DM.

- L-canavanine

Finely ground 5 g of plant sample was de-fatted in a Soxhlet extractor with hexane for 5 h and air-dried. Afterwards, 25 mL of 0.1 M HCl was added, and the mixture was stirred using a magnetic stirrer for 6 h at room temperature. Samples were centrifuged at 4000× g for 20 min. The supernatant was saved, and the extraction was repeated using 20 mL of 0.1 M HCl (steps 2, 3 and 4). Then, both supernatants were combined, and the pH was adjusted to 7.0 with 0.2 M NaOH solution and rinsed to a final volume of 100 mL using 0.2 M phosphate buffer, pH 7.0. Samples were stored at −20 °C until analysis. Then, 1 mL of the plant extract was placed in a 10-mL volumetric flask, mixed with 6.5 mL of phosphate buffer, 1 mL of 1% potassium persulfate and 0.5 mL of 1% aqueous photoactivated pentacyanoaminoferate solution (PCAF) and rinsed to a volume up to 10 mL with distilled water. Later, the solution was mixed thoroughly and after 15 min the absorbance read at 520 to 530 nm against a blank (in this case the phosphate buffer replaced the canavanine solution) in a UV-VIS spectrophotometer (Genesys 6, Thermo Electron Corporation, USA). The concentration of canavanine in the sample was determined using a calibration curve and the results were expressed as g L-canavanine/100 g DM.

- Total tannins

Finely ground plant samples (leaves, stem, beans) were placed in a glass beaker of approximately 25-mL capacity mixed with 10 mL of aqueous acetone (70%) suspended in an ultrasonic water bath at 300 W for 20 min at room temperature. The content of the beaker was then transferred to centrifuge tubes and centrifuged (at approximately 3000× g at 4 °C during 10 min). The supernatant was then collected and kept on ice until analysis. Suitable aliquots of the above sample extract were added in test tubes and rinsed to 500 µL with distilled water. Then 250 µL of Folin–Ciocalteu reagent (1 N) and 1.25 mL of the sodium carbonate solution (20%) were added and vortexed. Afterwards, the absorbance was measured at 725 nm after incubation for 40 min under dark conditions using a UV-VIS spectrophotometer (Genesys 6, Thermo Electron Corporation, USA). The amount of total phenols was expressed as tannic acid equivalents which was calculated from a calibration curve. The results were expressed as total phenolics as g eq. tannic acid/kg DM.

Afterward, 100 mg polyvinylpolypyrrolidone was weighed in each test tube and mixed with 1.0 mL distilled water and 1.0 mL of the sample extract. Then, the test tubes were vortexed and kept at 4 °C for 15 min, vortexed again, and centrifuged at 3000× g for 10 min. The phenolic content of the supernatant was measured as mentioned above which represented the non-tannin phenolics, expressed on a dry matter basis. From the above results, the tannin content of the sample was calculated as follows:

$$\text{Total phenolics (\%)} - \text{Non-tannin phenolics (\%)} = \text{Total Tannins (\%)} \quad (1)$$

The results were expressed as g eq. tannic acid/kg DM.

- Phytic acid

Briefly, 5 g of plant materials were extracted with 100 mL of 3.5% HCl for 1 h at room temperature using a magnetic stirrer. Then, the contents were centrifuged at 3000× *g* during 10 min at room temperature and the supernatant collected. Afterward, an aliquot was diluted by adding between 1 mL and 5 mL of the supernatant to 25 mL with distilled water and passed through an AG1 X8 chloride anion exchange (200–400 µm mesh) column (0.5 g). Inorganic phosphorus and other interfering compounds were eluted with 15 mL of 0.1 M NaCl, and subsequently the phytate was eluted with 15 mL of 0.7 M NaCl. Three mL of the above-eluted sample was separated in a test tube and mixed with 1 mL of the Wade reagent, vortexed and centrifuged at 3000× *g* for 10 min. The absorbance value was measured at 500 nm against a calibration curve in an UV-vis spectrophotometer (Genesys 6, Thermo Electron Corporation, USA), and the results were expressed as g phytic acid/kg DM.

- L-Dopa

The L-dopa content was quantified in 1 g of seed flour, which was mixed with 5 mL of 0.1 N HCL in a test tube and kept in a boiling water bath for 5 min. After cooling, 5 mL of ethanol was added and shaken for 10 min. Then, the contents were centrifuged at 5000× *g* for 10 min and the supernatant was collected and adjusted to a known volume. From this extract, the L-dopa content was determined by measuring the ultraviolet light absorption at 282 nm in a UV-VIS spectrophotometer (Genesys 6, Thermo Electron Corporation, USA) and the concentration was calculated from a calibration curve using L-dopa (Sigma-Aldrich, Saint Louis, MO, USA) as a standard, and expressed as g L-Dopa/kg DM.

2.6. Calculations

Water-soluble carbohydrates (WSC): The water-soluble carbohydrates (g/kg DM) were estimated from the chemical composition as follows:

$$\text{WSC} = 1000 - \text{ash} - \text{CP} - \text{EE} - \text{NDF} - \text{St} \quad (2)$$

where CP is crude protein; EE, ether extract; NDF, neutral detergent fiber; and St, starch (g/kg DM).

2.7. In Vitro Cellulase Digestibility of Organic Matter

The in vitro cellulase digestible OM of each silage mix (*C. ensiformis*–sugarcane stalks or *M. pruriens*–sugarcane stalks) at each ensiling time (30, 60 or 90 d) was assessed following the cellulase technique according to De Boever et al. [33] as follows: an air-dried sample (ground to pass a 1-mm screen) of 0.3 g was weighed to the nearest mg into a sintered glass crucible (capacity 50 mL, porosity 1), provided with a screw cap at the bottom end. Each treatment was evaluated in triplicate. Thirty ml of the pre-heated pepsin–HCl solution was added. After closing the top with plastic film, the crucibles were placed in an incubator at 40 °C for 24 h. The contents were stirred after approximately 5 h. The crucibles were transferred into a water bath at 80 °C for exactly 45 min. After filtration, the contents were rinsed with warm water. Thirty ml of the pre-heated cellulase–buffer mixture was added to the crucibles (bottom closed) and after closing the top, returned to the incubator for a further 24 h at 40 °C, and stirred after about 5 h. The residue, after filtration and washing, was dried overnight in an oven at 103 °C, cooled and weighed. It was then ignited in a muffle furnace for 1.5 h at 550 °C and again cooled and reweighed. Subtracting the percentage of indigestible organic matter from 100 provides the cellulase digestible organic matter (CDOM). The cellulase digestible organic matter in the dry matter (CDOMD) was calculated by multiplying the CDOM by the content of organic matter in the dry matter. The metabolisable energy (ME) was estimated based on CDOM as recommended by De Boever et al. [33], where:

$$\text{ME} = 5.99 + 0.0696 * \text{CDOM} + 0.0218 * \text{fat} - 0.151 * \text{ash} \quad (3)$$

2.8. Statistical Analysis

The model (GLM, univariate [34]) to assess the effect of the ensiling time and legume type on the proximate chemical analysis, ensilability and PSM contents of the mixed silages was as follows:

$$Y_{ijk} = \mu + Lt_i + \text{ensiling time}_j + (\text{Lt ensiling time})_{ij} + \varepsilon_{ijk} \quad (4)$$

where $Lt_{i = 1–2}$ is the legume type (*C. ensiformis* vs. *M. pruriens*); ensiling $\text{time}_{j = 1–4}$, not ensiled vs. 30 vs. 60 vs. 90; $Lt_i \times \text{ensiling time}_j$, the interaction between the main factors as well as the residual error ε. Further, a post-hoc Tukey test [35] was performed when $p < 0.05$. Additionally, a one-way ANOVA was applied to: a) assess the effects of the legume type on the chemical composition, ensilability, CDOMD, and ME at different ensiling times, and b) to assess the effects of the ensiling time on the chemical composition, ensilability, CDOMD, and ME within each mixed silage with a legume. Differences were declared significant at $p < 0.05$, and Tukey's test [35] for multiple comparisons was run.

3. Results

3.1. Chemical Characteristics and Plant Secondary Metabolites (PSM) of Fresh and Ensiled Feed

The chemical composition of the fresh material (at harvest) used in the experiment is shown in Table 1. As expected, both beans showed high CP contents, while the sugarcane stalks showed a high fiber content, reflected in a low ME content. None of the PSM assessed in the present study were detected in the sugarcane stalks (Table 1), while *C. ensiformis* showed 2.5 times more L-canavanine, and 8.5 and 1.9 times fewer total tannins and L-dopa than *M. pruriens*, respectively. However, phytic acid and TI showed similar contents in both beans.

Table 1. Mean (± standard deviation) of chemical composition and some plant secondary metabolites of bean and forage meals of two tropical legumes and sugarcane stalks, respectively (n = 3).

Parameters	*Canavalia ensiformis*	*Mucuna pruriens*	*Saccharum officinarum*
Proximate chemical analysis (g/kg DM unless stated otherwise)			
Dry Matter (g/kg FM) †	870 (±2)	873 (±2)	280 (±2)
Organic Matter	963 (±2)	945 (±2)	950 (±2)
Crude Protein	320 (±2)	282 (±3)	30 (±2)
Crude Fiber	90.0 (±0.2)	70.0 (±0.4)	280.0 (±0.7)
Ether Extract	29.0 (±0.5)	42.0 (±1.0)	nd ‡
Starch	365 (±1)	298 (±3.0)	nd
Calculated WSC [1]	20 (±2)	127 (±7)	380 (±1)
Fiber fractions			
Neutral Detergent Fiber	229 (±1)	196 (±2)	540 (±1)
Acid Detergent Fiber	140 (±1)	113 (±2)	340 (±2)
Cellulose	123 (±2)	94 (±2)	270 (±2)
Lignin	17.0 (±0.1)	19.0 (±0.6)	70.0 (±0.3)
Plant secondary metabolites (g/kg DM unless stated otherwise)			
Trypsin inhibitor (TIU/g DM)	220.9 (±0.3)	227.1 (±0.3)	nd
L-canavanine	41.1 (±1.3)	16.3 (±2.1)	nd
Total tannins	9.1 (±0.2)	77.4 (±0.1)	nd
L-dopa	25.5 (±0.9)	49.9 (±0.2)	nd
Phytic acid	18.4 (±0.3)	19.3 (±0.2)	nd

† FM: fresh matter; ‡ nd: not detected. [1] calculated WSC using Equation (2).

The chemical composition before and after ensiling of the mixed silage of *C. ensiformis*–sugarcane or *M. pruriens*–sugarcane is presented in Table 2. The ensiling time affected ($p < 0.05$) the contents of DM, OM, CF and NDF. For DM and OM, relevant differences were limited to changes between fresh and ensiled material. No biologically relevant differences were observed between 30, 60 and 90 days of ensiling ($p > 0.05$) in any of the silage mixes (*C. ensiformis*–sugarcane or *M. pruriens*–sugarcane). Although significant,

changes in CF and NDF between fresh and ensiled material or due to ensiling time (30, 60 vs. 90 days) occurred, differences were of no biological relevance. On the other hand, all the parameters (proximate chemical analysis, Van Soest fiber fractions and starch) were influenced by legume type ($p < 0.05$), provoked by differences between the fresh material of both legume beans. Furthermore, no interaction effects were observed in the proximate chemical compounds, starch, or the Van Soest fiber fractions (Table 2).

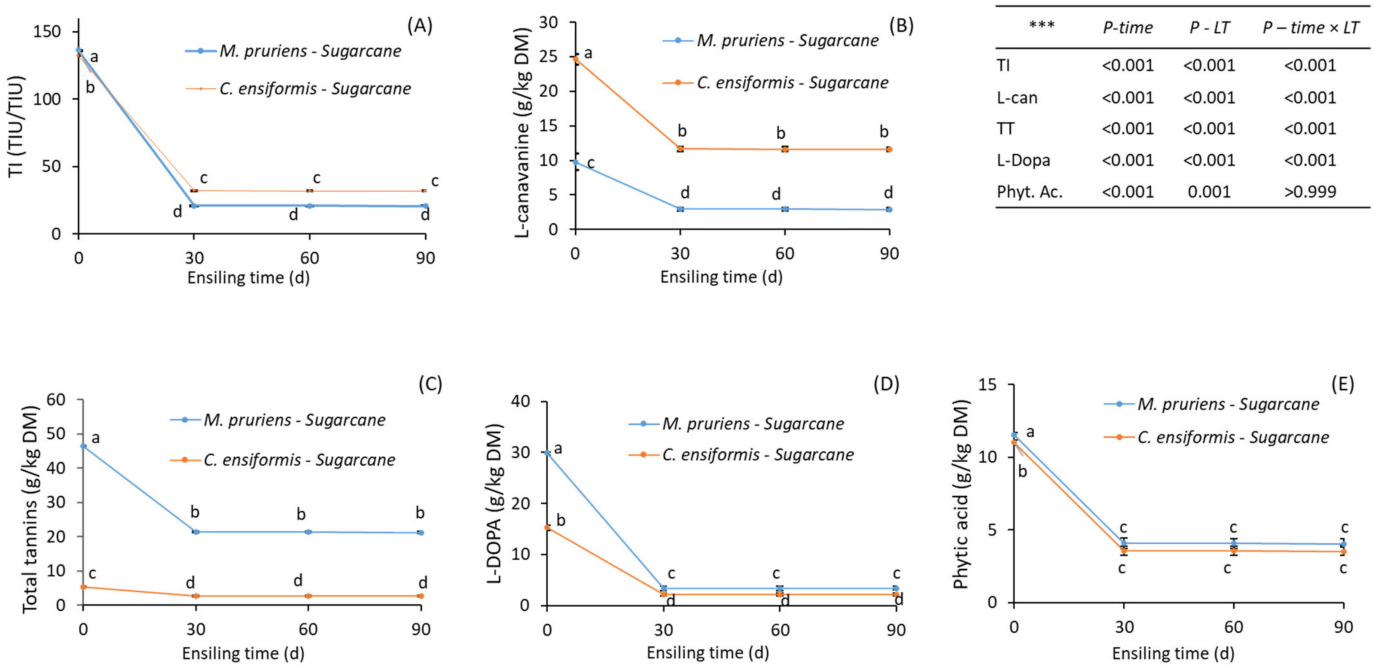
Table 2. Mean (±standard deviation) of chemical composition and some plant secondary metabolites of mixed *C. ensiformis* (Ce) or *M. pruriens* (Mp) grains with sugarcane stalks (SS) fresh or ensiled for 30, 60 or 90 days ($n = 3$).

Parameters	Ensiling Time (Days)								*p*-Value †		
	0		30		60		90				
	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ensiling Time	LT	Ensiling Time × LT
Proximate chemical analysis (g/kg DM unless stated otherwise)											
Dry Matter (g/kg FM ‡)	472 (±1) ^{x}	473 (±0) ^{x}	437 (±1) ^{y}	438 (±1) ^{y}	435 (±2) ^{by}	438 (±2) ^{ay}	434 (±2) ^{y}	436 (±2) ^{y}	<0.001	0.014	0.789
Organic Matter	965 (±1) ^{x}	964 (±1) ^{x}	958 (±1) ^{y}	960 (±1) ^{xy}	955 (±2) ^{yz}	958 (±2) ^{y}	952 (±3) ^{z}	955 (±3) ^{y}	<0.001	0.050	0.378
Crude Protein	205 (±2) ^{a}	182 (±2) ^{b}	204 (±2) ^{a}	181 (±2) ^{b}	203 (±2) ^{a}	181 (±2) ^{b}	203 (±2) ^{a}	180 (±2) ^{b}	0.397	<0.001	0.992
Crude Fiber	167 (±1) ^{ax}	121 (±0) ^{bx}	166 (±1) ^{ay}	120 (±0) ^{by}	166 (±1) ^{ay}	120 (±0) ^{byz}	165 (±1) ^{ay}	120 (±0) ^{bz}	0.040	<0.001	0.774
Ether Extract	16.1 (±1.0) ^{b}	25.4 (±0.6) ^{a}	16.0 (±1.0) ^{b}	25.3 (±0.6) ^{a}	16.0 (±1.0) ^{b}	25.2 (±0.6) ^{a}	15.9 (±1.0) ^{b}	25.1 (±0.7) ^{a}	0.984	<0.001	0.984
Starch	217 (±1) ^{a}	180 (±2) ^{b}	215 (±1) ^{a}	179 (±2) ^{b}	214 (±1) ^{a}	178 (±2) ^{b}	214 (±2) ^{a}	178 (±2) ^{b}	0.098	<0.001	0.983
Fiber fractions											
Neutral Detergent Fiber	352 (±1) ^{ax}	330 (±1) ^{bx}	350 (±1) ^{axy}	329 (±1) ^{by}	349 (±2) ^{axy}	328 (±2) ^{by}	348 (±2) ^{ay}	327 (±2) ^{by}	0.006	<0.001	0.943
Acid Detergent Fiber	231 (±2) ^{a}	165 (±1) ^{b}	229 (±2) ^{a}	164 (±1) ^{b}	228 (±2) ^{a}	164 (±1) ^{b}	228 (±2) ^{a}	163 (±1) ^{b}	0.145	<0.001	0.835
Cellulose	183 (±2) ^{a}	132 (±1) ^{b}	182 (±2) ^{a}	132 (±1) ^{b}	181 (±2) ^{a}	132 (±1) ^{b}	181 (±2) ^{a}	131 (±1) ^{b}	0.491	<0.001	0.916
Lignin	38.6 (±0.5) ^{a}	31.3 (±0.4) ^{b}	38.3 (±0.5) ^{a}	31.2 (±0.4) ^{b}	38.1 (±0.5) ^{a}	31.1 (±0.4) ^{b}	38.1 (±0.6) ^{a}	31.0 (±0.4) ^{b}	0.533	<0.001	0.954
Plant secondary metabolites (% disappeared, expressed relative to the original amount)											
Trypsin Inhibitor			75.8 (±0.25) ^{b}	84.7 (±0.2) ^{a}	75.9 (±0.3) ^{b}	84.7 (±0.2) ^{a}	75.9 (±0.3) ^{b}	84.8 (±0.3) ^{a}	0.744	<0.001	0.976
L-canavanine			52.7 (±1.98) ^{b}	69.7 (±4.9) ^{a}	52.8 (±1.9) ^{b}	69.8 (±4.9) ^{a}	52.9 (±1.9) ^{b}	69.9 (±4.9) ^{a}	0.995	<0.001	0.999
Total tannins			49.6 (±1.28) ^{b}	53.9 (±0.3) ^{a}	49.7 (±1.4) ^{b}	54.0 (±0.3) ^{a}	49.9 (±1.3) ^{b}	54.1 (±0.3) ^{a}	0.886	<0.001	0.998
L-dopa			85.7 (±1.60) ^{b}	88.7 (±1.4) ^{a}	85.7 (±1.6) ^{b}	88.7 (±1.4) ^{a}	85.8 (±1.6) ^{b}	88.8 (±1.4) ^{a}	0.996	0.001	>0.999
Phytic acid			67.6 (±2.30) ^{a}	64.7 (±2.6) ^{b}	67.7 (±2.3) ^{a}	64.8 (±2.6) ^{b}	67.8 (±2.2) ^{a}	64.9 (±2.7) ^{b}	0.989	0.025	>0.999

Means with different superscript (^{a,b}) in the same row show statistical differences between the tropical legume inside of each ensiling time to $p < 0.05$ [35]. Different superscripts (^{x,y,z}) in the same row and within each tropical legume indicate significant differences ($p < 0.05$) according to Tukey [35]. † refers to the statistical significance according to the general linear model of SPSS [34]; *LT*: legume type. ‡ FM: fresh matter. Plant secondary metabolites (g/kg DM unless stated otherwise) at 0 d (TI [TIU/kg DM], L-canavanine, Total tannins, L-dopa, and Phytic acid: *C. ensiformis*–sugarcane silage: 132.6, 24.6, 5.4, 15.3, and 11.0 respectively; *M. pruriens*–sugarcane silage: 136.2, 9.8, 46.4, 29.9, and 11.6, respectively.

Although the PSM content of the fresh and ensiled material differed for all PSM studied ($P_{LT} \leq 0.001$ for all PSM), the process of ensiling reduced ($p < 0.001$) the content of all PSM studied (Table 2), as compared with the fresh material, while ensiling for more than 30 days did not further reduce any of the PSM in either of the two mixed legume silages (Figure 1). However, for most of the PSM studied (except for phytic acid) an interaction effect between

legume type and ensiling time was observed, which was related to differences in the PSM concentrations of the fresh beans (0 days of ensiling) and PSM levels reached after 30 days of ensiling. As such, a steeper decrease between 0 and 30 days of ensiling was observed for trypsin inhibitor (Figure 1A), total tannins (Figure 1C) and L-Dopa (Figure 1D) of mixed silages of sugarcane and *M. pruriens* than mixed *C. ensiformis*–sugarcane silages. On the other hand, canavanine decreased more strongly in the mixed *C. ensiformis*–sugarcane silages than in the mixed *M. pruriens*–sugarcane silages (Figure 1B). Finally, changes due to ensiling were equal for phytic acid (Figure 1E) in the mixed silages of both legume beans ($P_{interaction} > 0.999$).



***	*P-time*	*P - LT*	*P – time × LT*
TI	<0.001	<0.001	<0.001
L-can	<0.001	<0.001	<0.001
TT	<0.001	<0.001	<0.001
L-Dopa	<0.001	<0.001	<0.001
Phyt. Ac.	<0.001	0.001	>0.999

Figure 1. Effect of ensiling process on PSM concentrations (**A**) TI (trypsin inhibitor), (**B**) L-canavanine, (**C**) Total tannins, (**D**) L-dopa, and (**E**) Phytic acid] of mixed *C. ensiformis*–sugarcane and *M. pruriens*–sugarcane silage. *** upper right table: L-can (L-canavanine), TT (total tannins), Phyt. ac. (Phytic acid), *P*—time (*p*-value of the ensiling time), *P*—LT (*p*-value of the legume type), and *P*—time × LT (*p*-value of the interaction of ensiling time × legume type). Different superscript (^{a,b,c,d}) show statistical differences to $p < 0.05$ [35].

Ensilability

The ensiling time and legume type showed a significant ($p < 0.01$) effect on the silage pH. The mixed silage of *M. pruriens*–sugarcane showed a lower pH value after 60 days, decreasing to approximately 4.1 and remaining stable till 90 days of ensiling. The legume type affected all the fermentation parameters ($p < 0.01$), regardless of the ensiling time (except for pH, where an interaction effect was observed). In addition, *M. pruriens*–sugarcane silage presented a higher ($p < 0.001$) lactic acid concentration (Table 3), and higher ($p < 0.01$) lactic acid in the total fatty acids (L/TFa ratio). Furthermore, the ensiling time only affected ($p < 0.001$) the pH and ethanol content.

Table 3. Mean (±standard deviation) of ensiling fermentation characteristics of mixed *C. ensiformis* (Ce) or *M. pruriens* (Mp) beans meal with sugarcane stalks (SS) ensiled for 30, 60 or 90 days (n = 3).

Parameters §	Ensiling Time (Days)						*p*-Value ‡		
	30		60		90				
	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ensiling Time	LT	Ensiling Time × LT
pH	4.41 (±0.14) ^{bc}	4.43 (±0.10) ^{c}	4.26 (±0.04) ^{b}	4.08 (±0.06) ^{a}	4.29 (±0.02) ^{bc}	4.10 (±0.07) ^{a}	<0.001	<0.001	0.005
NH_3-N/N (g/100 g)	5.91 (±0.03) ^{i}	5.30 (±0.04) ^{j}	5.92 (±0.03) ^{i}	5.30 (±0.05) ^{j}	5.92 (±0.03) ^{i}	5.30 (±0.05) ^{j}	0.995	<0.001	0.956
Lactic acid (mg/g FM)	31.4 (±0.2) ^{j}	33.1 (±0.3) ^{i}	31.3 (±0.2) ^{j}	33.0 (±0.3) ^{i}	31.2 (±0.2) ^{j}	32.9 (±0.3) ^{i}	0.281	<0.001	0.999
Acetic acid (mg/g FM)	27.0 (±0.2) ^{i}	21.3 (±0.1) ^{j}	26.9 (±0.1) ^{i}	21.2 (±0.1) ^{j}	26.8 (±0.2) ^{i}	21.2 (±0.1) ^{j}	0.075	<0.001	0.962
Ethanol (mg/g FM)	7.26 (±0.27) ^{i}	6.33 (±0.04) ^{jy}	7.40 (±0.16) ^{i}	6.46 (±0.10) ^{jy}	7.57 (±0.17) ^{i}	6.60 (±0.11) ^{x}	<0.001	<0.001	0.968
L/Fa	0.54 (±0.23) ^{j}	0.61 (±0.29) ^{i}	0.54 (±0.23) ^{j}	0.61 (±0.29) ^{i}	0.54 (±0.23) ^{j}	0.61 (±0.29) ^{i}	>0.999	0.001	>0.999

Means with different superscript (^{a,b,c}) in the same row show statistical differences to $p < 0.05$ [35]. Means with different superscript (^{i,j}) in the same row show statistical differences between the tropical legume inside of each ensiling time to $p < 0.05$ [35]. Different superscripts (^{x,y}) in the same row and within each tropical legume indicate significant differences ($p < 0.05$) according to Tukey [35]. § NH_3-N/N: ammonia nitrogen/total nitrogen ratio; FM: fresh matter; L/Fa: lactic acid/total fermentation acids ratio; the propionic, butyric, isobutyric, valeric, isovaleric and caproic acids were not detected. ‡ refers to the statistical significance according to the general linear model of SPSS [34]; LT: legume type.

3.2. In Vitro Cellulase Digestibility of Organic Matter

No interaction effect ($p > 0.05$) was observed (Table 4) for either the CDOMD or ME. The *C. ensiformis*-containing silage showed higher cellulase digestibility and ME ($p < 0.01$) as compared with the silage of *M. pruriens* with sugarcane at the different ensiling times, these seeming to depend on the differences shown in the starting materials. The ensiling time affected ($p < 0.05$) the digestibility and energy, especially when the silage was compared with the material before ensiling (0 days). However, after 30 days of ensiling, this effect was not observed ($p > 0.05$) in either silage mix (*C. ensiformis*–sugarcane or *M. pruriens*–sugarcane) for CDOMD. Furthermore, the highest ($p > 0.01$) CDOM and ME values were obtained at 30 days as compared to 90 days of ensiling.

Table 4. Mean (±standard deviation) of in vitro cellulase organic matter (CDOMD) digestibility and metabolizable energy (ME) of mixed *C. ensiformis* (Ce) or *M. pruriens* (Mp) bean meals with sugarcane stalks (SS) fresh or ensiled for 30, 60 or 90 days (n = 3).

Parameters	Ensiling Time (Days)								*p* Value ‡		
	0		30		60		90				
	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ce-SS	Mp-SS	Ensiling Time	LT	Ensiling Time × LT
CDOMD (g/kg DM)	767 (±1) ^{ax}	742 (±2) ^{b}	764 (±1) ^{ay}	740 (±2) ^{b}	763 (±1) ^{ayz}	739 (±2) ^{b}	762 (±1) ^{az}	738 (±1) ^{b}	<0.001	0.001	0.827
ME (MJ/kg DM)	11.1 (±0.0) ^{ax}	11.0 (±0.0) ^{bx}	11.0 (±0.0) ^{ay}	10.9 (±0.0) ^{by}	10.9 (±0.0) ^{yz}	10.8 (±0.0) ^{yz}	10.8 (±0.0) ^{z}	10.8 (±0.0) ^{z}	<0.001	<0.001	0.446

Means with different superscript (^{a,b}) in the same row show statistical differences between the tropical legume inside of each ensiling time to $p < 0.05$ [35]. Different superscripts (^{x,y,z}) in the same row and within each tropical legume indicate significant differences ($p < 0.05$) according to Tukey [35]. ‡ refers to the statistical significance according to the general linear model of SPSS [34]; LT: legume type.

4. Discussion

4.1. Effect of Ensiling Process on the Ensilability

To assess the silage fermentation quality, it is essential to use the individual proportion of each fermentation acid, where lactic acid plays the most important role [36]. This importance is attributed to its lower dissociation constant (Ka = 3.86), being the major

acid responsible for the decreasing of pH [37]. Indeed, in the present study, the high lactic acid content associated with the low ammonia and alcohol content were proof of good fermentation and silage quality, which is in line with former reports [7,24]. However, the presence of a high concentration of lactic acid is not enough to avoid the aerobic spoilage caused by aerobic yeast and moulds [38,39]. In that respect, acetic acid produced by heterofermentative bacteria is known to play an important role in the aerobic stability of silage [40]. The *M. pruriens*-containing silage showed a better fermentation quality as compared with the *C. ensiformis*–sugarcane silage [lower ($p < 0.001$) pH, ethanol concentration, and ammonia N content]. Furthermore, the lower ammoniacal nitrogen (NH_3-N) content in the *M. pruriens*–sugarcane silage mixes ($p < 0.05$) could be due to the differences in the CP content in the raw materials. Additionally, the ensiling time only affected ($p < 0.001$) the pH and ethanol content, although both were within the target parameters (pH < 4.5 and ethanol < 10 mg/g FM) for all timepoints.

4.2. Effect of Ensiling Process on the Chemical Composition and Digestibility

As expected, both beans showed the highest CP and energy contents, while the sugarcane stalks had the highest fiber content (Table 1). The CP content of both legumes exceeded 250 g/kg DM, which is of interest to supplement poor quality tropical forage with low CP contents [41]. The *C. ensiformis* beans meal has a low content of water-soluble carbohydrates [WSC 20.0], an aspect that limits their ensiling [15]. The high WSC content in *M. pruriens* is quite unrealistic, which may be related to an underestimation of the starch content of *M. pruriens* [42,43]. The low sugar content of legume beans together with the high buffering capacity of their proteins and minerals prevent a rapid drop in pH, which leads to the poor preservation of the material [10]. Nevertheless, the sugarcane stalks contained high amounts of fiber (mainly ADF and lignin (pm)), which limits their metabolizable energy content [44]. The use of sugarcane stalks in mixed silage, despite the high content of structural carbohydrates, is supported by their high content of water-soluble carbohydrates (380 g/kg DM), which contribute to the development of lactic acid bacteria responsible for the reduction of pH and the conservation of the material in the silo [6,7].

In addition, the *C. ensiformis*–containing silage showed higher ($p < 0.01$) digestibility than the *M. pruriens*–sugarcane silage at the different ensiling times, which is in line with the earlier study by Lima-Orozco et al. [7]. Additionally, the digestibility of both silages was higher than those presented by Lima-Orozco et al. [7] of *C. ensiformis* or *M. pruriens* mixed silage with sorghum (*Sorghum bicolor* (L.) Moench) under similar ensiling conditions, but using the in sacco DM degradability technique with the ruminal fluid of sheep.

Furthermore, the OM digestibility and ME content of both types of mixed silage were similar and slightly higher, respectively, than reported in maize silage by De Boever et al. [33]. The highest values of digestibility of DM and OM, and ME were obtained at 30 days of ensiling, and are in correspondence with the best ensiling time (fermentation parameters and silage quality). These correspond with the results reported by McDonald et al. [45] and Lima-Orozco et al. [7]. Although the ensiling process induced changes in the chemical composition and cellulase digestible organic matter of both mixed silages, differences are minor and biologically non-relevant.

4.3. Effect of Ensiling Process on the PSM Concentrations

Variation in the PSM content in tropical legumes may be related to their adaptation to the environment and defense against natural enemies, such as competitors, pathogens, and/or herbivores [46]. As such, these may be related to different biotic and abiotic conditions, as well as plant genetics and phenology [47]. Compared to the beans meal of the present study, others, e.g., Agbede and Aletor [48], showed lower TI contents (2.1 TIU/g DM), whereas Belmar et al. [49] reported higher concentrations in *C. ensiformis* beans meal of L-canavanine (50.8 mg/g DM). Furthermore, compared to the beans meal of the current experiment, others, e.g., Janardhanan et al. [50] reported higher L-dopa (56.0–69.8 g/kg DM), but lower phytic acid, and tannins contents (4.8–7.0 and 0.3–0.6 g/kg DM,

respectively) in *M. pruriens*, while Doss et al. [32] showed lower tannins and L-dopa content (0.8–0.9 and 17 g/kg DM, respectively) in *C. ensiformis*. Literature on PSM in *M. pruriens* beans meal is scarce, which might be related to the reported absence of L-canavanine [51]. However, in the present study, the content of L-canavanine in *M. pruriens* was similar to that in *C. ensiformis*, which is in line with the low concentration of L-canavanine in *Mucuna* spp. reported by Udoh and Ekpenyong [52].

Apart from the preservation function, ensiling has other benefits, as fermentation is reported to reduce the concentration of several PSM [22,53]. The PSM quantified in both types of silage assessed in the current study were reduced to acceptable levels for feeding ruminants (Tables 1–3), which is in line with former reports [e.g., tannins: 4.86–18.50 g/kg DM [13,54]; phytic acid: 2.85–5.81 g/kg DM [55]; L-dopa: 11–16 g/kg DM [43], L-canavanine: 7.5 g/kg DM [56]]. Likewise, other investigations under other experimental conditions have shown the potential of good silage as a method to reduce TI [53,57], saponins [12], swainsonine [58] and cyanogenic glycoside [59].

In the current experiment, the PSM reduction mainly occured in the active phase of the ensiling rather than during the stabilized phase. In the active or fermentation phase, silage becomes anaerobic, and the lactic acid bacteria become the predominant population, producing lactic acid and other acids (e.g., acetic acid) with the concomitant pH dropping [60]. As such, analysis during the first 30 days should be done to assess the kinetic of PSM disappearance and their relationship with the lactic acid bacteria proliferation, lactic and acetic acids production, and the decreasing pH. Overall, the ensiling process resulted in approximately a 50% reduction of the tannins content in both silages. Tannins disappearance during ensiling could be associated to the presence of strains of fungi [e.g., *Aspergillus* spp. and *Penicillium* spp. [61]] and bacteria [e.g., *Bacillus* spp. [62], *Pediococcus* spp. and *Lactobacillus* spp. [13,63]]. These fungal and bacterial genera produce tannase, an enzyme that hydrolyses the ester and depsidic bonds of hydrolysable tannins and other more complex tannins [62,64]. Other nutrients in beans may also be changed by the presence of enzymes and acids secreted by these microorganisms, such as deglycosylation reactions to generate isoflavone aglycones [65,66]. Lactic acid bacteria not only produce tannases, but also phytases, which can degrade phytic acid. Furthermore, the disappearance of phytic acid is pH dependent, with an optimal pH range between 4.0 and 6.0 for phytase activation [63,67]. As such, the quick reduction of pH near 4.4 during the fermentation phase might activate the microbial phytase to reduce the phytic acid content by more than 60%. Additionally, El-Shinnawy et al. [68] reported that ensiling with multiple strains of *L. bucheri*, *L. plantarum* and *Enterococcus faecium* reduced the tannins, phytic acid and trypsin inhibitor contents. As such, the external microbial inoculant which was added at the start of the ensiling process could have also contributed to the PSM disappearance in the current study. Furthermore, Tao et al. [58] reported that all of the most representative strains of *Lactobacillus* were able to reduce the swainsonine content, a PSM present in a mixed silage of corn with *Oxytropis glabra*.

5. Conclusions

Silage mixes containing *M. pruriens* were better than those containing *C. ensiformis*, which seemed to depend on the differences shown in the starting materials. Further, the combination of sugarcane stalks with legume beans resulted in silage with good quality. Indeed, changes occurred in the chemical composition and digestibility of both mixed silage during the ensiling process, but they are minor and biologically not-relevant. Nevertheless, after 30 days of ensiling, the silage enhanced the quality of the mixed forage by pre-digestive reduction of their secondary plant metabolite contents. In this respect, mixed forage from protein-rich plants and fibrous by-products can be ensiled and incorporated as a substitute for concentrate in tropical ruminant feeding systems.

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Abbreviations

ADF: acid detergent fiber; CDOM: cellulase digestible organic matter; CDOMD: cellulase organic matter digestibility in the dry matter; Ce: *Canavalia ensiformis*; CF: crude fiber; CFU: colony forming units; CIAP: Centro de Investigaciones Agropecuarias; CP: crude protein; DE: digestible energy; DM: dry matter; EE: ether extract; FM: fresh matter; GE: gross energy; L-can: L-canavanine; LT: legume type; ME: metabolizable energy; Mp: *Mucuna pruriens*; nd: non determined; NDF: neutral detergent fiber; OM: organic matter; PCAF: photoactivated pentacyanoaminoferate solution; Phyt. Ac.: Phytic acid; PSM: plant secondary metabolites; SCFA: short chain fatty acids; SS: sugarcane stalks; St: starch; TI: trypsin inhibitor; TIU: trypsin inhibition units; TT: total tannins; UCLV: Universidad Central Marta Abreu de Las Villas; WSC: water-soluble carbohydrates.

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