In Vitro Rumen Degradability of Tropical Legumes and Their Secondary Metabolites Depends on Inoculum Source

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Abstract

In this study, the *in vitro* apparent rumen degradability of organic matter (ARDOM) and plant secondary metabolites (ARDPSM) of three tropical legumes (*Mucuna pruriens*, *Canavalia ensiformis* and *Leucaena leucocephala*) were assessed. For this, single end-point incubations (24 h) as well as kinetic assessments (0, 2, 4, 6, 8, 10, 12 and 24 h) were set up with different inocula sources, i.e. ruminal inocula from Belgian and Cuban sheep. L-mimosine, L-canavanine, Concanavaline A (Con A) and trypsin inhibitor (TI) were the plant secondary metabolites (PSM) targeted in this study. Bean and forage/bean meals of *M. pruriens* and *C. ensiformis* as well as their PSM were extensively degraded during 24 h incubation (0.70 and 0.78 g ARDOM/g of organic matter (OM), respectively and > 0.80 g/g for L-canavanine, > 0.76 TIU/TIU for TI and > 0.95 g/g for Con A, for both legumes). Forage meal of *L. leucocephala* was considerably less degraded, with apparent ruminal degradabilities of 0.20 and 0.35 g ARDOM/g OM after 24 h incubation with Belgian or Cuban sheep inoculum, respectively. This could - at least partially - be related to L-mimosine, present in *L. leucocephala*, which was hardly degraded in the Belgian incubation, while a more extensive ruminal breakdown was observed under the Cuban conditions (0.05 to 0.78 g ARDPSM/g PSM, respectively). The negative effect of L-mimosine on OM degradability was supported by *in vitro* incubations with straw and inoculum from Belgian sheep, as ruminal degradation of straw was 31% lower when L-mimosine was supplemented.

1. Introduction

The restricted availability of concentrates accompanied with limited availability of good quality forage mainly in the dry season hampers Cuban and generally tropical livestock production (Lima et al. 2011). Hence, tropical legumes are considered a valuable resource to enhance livestock productivity (Lima-Orozco et al. 2014, Mohamed et al. 2018, Soltan et al. 2017). However, they contain plant secondary metabolites (PSM) that may have negative effects on the rumen microbial community (Makkar et al. 2007b), cellular proliferation (Nguyen and Tawata 2016), organic matter (OM) degradability in the rumen, nutrient assimilation, animal performance and/or health (Janardhanan et al. 2003, Makkar, et al. 2007b).

Nevertheless, most of the studies towards anti-nutritional effects of tropical legumes have been performed in monogastric animals (Belmar et al. 2007, Siddhuraju et al. 1996), as ruminants are thought to tolerate these legumes to some extent due to degradation or inactivation of their PSM by ruminal microbiota, rendering the PSM anti-nutritive properties ineffective (Dominguez-Bello and Stewart 1990a, Hoffmann et al. 2003, Shelton et al. 2019). However, this implies that the extent of ruminal degradation of PSM might depend on the presence in the rumen microbial population of specialist microbes (Weimer 2015). As such, geographical differences in the rumen microbial ecosystem have been previously shown and related to the differences in the capacity of a ruminal breakdown of specific PSM [e.g. the L-mimosine breakdown by Hawaiian (Allison et al. 1990, Jones and Megarritty 1986) and Indian (Vaithiyanathan et al. 2005), but not by Australian ruminants (Jones and Megarritty 1986)].

Hence, we hypothesized that the apparent ruminal degradability of organic matter (ARDOM) and PSM (ARDPSM) of tropical legumes would depend on the source of ruminal inocula and the type of PSM. Accordingly, the aim of the current *in vitro* study was to concomitantly assess the apparent ruminal degradability of organic matter (ARDOM) and PSM (ARDPSM) of three tropical legumes (*M. pruriens*, *C. ensiformis*, and *L. leucocephala*) using different sources of ruminal inocula.

2. Material And Methods

2.1. Experimental site, legumes and sample collection

The three tropical legumes were sown in Santa Clara, in the center of Cuba (22°25'22.9"N, 80°03'13"W). *M. pruriens* was sown intercropped with elephant grass (*Pennisetum purpureum*) in a field with an area of 1 ha. *C. ensiformis* and *L. leucocephala* were sown in different plots (0.2 and 1 ha, respectively). Average precipitation, temperature and humidity during the cropping period were 47.5 ± 42.13 mm, 26.3 ± 2.02 °C and 82.5 ± 4.19 %, respectively. Crops were not fertilized nor irrigated. The forage of the three tropical legumes was harvested around noon at the end of the wet season (between 23/10/2014 and 27/10/2014). *M. pruriens* and *C. ensiformis* were harvested about 5 cm above the soil (at flowering stage), and *L. leucocephala* forage was cut from young branches (stems were removed to obtain only the twigs and leaves). Beans from *M. pruriens* and *C. ensiformis* were obtained from the same plots at the dry bean stage. Forage and beans were dried in a furnace at 65 °C for 72 hours. Dried material was ground and passed through a screen with mesh size of 0.1 cm to obtain both forage and bean meals. Bean meals of *M. pruriens* and *C. ensiformis* have been considered in the experiment focusing on PSM degradation kinetics as PSM particularly accumulate in beans, which consequently have a higher PSM concentration. Additionally, mixtures of beans and forage (1:1, DM basis) of either *C. ensiformis* or *M. pruriens* were prepared. This mixture is assumed to represent the maximum amount of beans which could be obtained in practice when concomitantly harvesting forage and beans at the hard dough stage of the beans (Díaz et al. 2003). *L. leucocephala* forage was used to prepare a forage meal. As such, three different types of meals were available for further testing, i.e. forage/bean (1:1) a meal consisting of beans and forage (1:1; w/w) (*C. ensiformis* and *M. pruriens*), forage meal (*L. leucocephala*) and bean meals (*C. ensiformis* and *M. pruriens*). These meals were stored in wide-mouth glass jars with a metal cap in a dry room at ambient temperature until use. A subsample of each different type of meal was transported separately at ambient temperature in a heat-sealed plastic bag to Belgium for analysis of PSM and to perform *in vitro* incubations.

2.2. Chemical analysis

2.2.1. Proximate chemical analysis.

Proximate chemical analysis of the beans, forage/bean (1:1) meal and forage meal (Table 1) was done according to AOAC (2005) (DM: dry matter - ID number 930.15; CP: crude protein – as $6.25 \times \text{N}$ - ID number 954.01); OM: organic matter (EC 2009) and fiber fractioning by Van Soest et al. (1991) (NDFom: neutral detergent fiber not assayed with a heat stable amylase and expressed exclusive of residual ash, ADFom:

acid detergent fiber expressed exclusive of residual ash, lignin (pm) was determined from the residue of the ADFom analysis with lignin being oxidized by permanganate). Hemicellulose was calculated as the difference of NDFom and ADFom, while cellulose was calculated as the difference of ADFom and lignin (pm).

2.2.2. Plant secondary metabolites.

Before and after the *in vitro* incubation with rumen fluid of either the bean meal, the forage/bean (1:1) meals or the forage meal, trypsin inhibitor (TI), L-canavanine, and L-mimosine were analyzed as described by [Makkar, et al. \(2007b\)](#), and Con A was determined by ELISA following the protocol provided by the manufacturer of the kit (Abbexa Ltd., Cambridge Science Park, Cambridge, CB4 0EY, UK). As such, the degradability of those PSM was assessed as follow:

$$\text{ARDPSM (g/g)} = \text{PSM content (after incubation)} / \text{PSM content (before incubation)} \quad [1]$$

with ARDPSM is the apparent degradability of the plant secondary metabolite (* g/g except for TI [trypsin inhibitor units (TIU)/TIU], and with PSM content expressed as ([TIU/kg DM] for TI and [g/kg DM] for L-canavanine, L-mimosine and Con A).

2.3. In vitro ruminal degradation and breakdown of PSM of three tropical legumes using different inoculum sources (Experiment 1)

2.3.1. Single end-point (24 h) in vitro rumen degradability and breakdown of PSM of the three tropical legumes using fresh inoculum from Belgian sheep (Experiment 1.1)

Incubations were carried out following the method described by [Vlaeminck et al. \(2014\)](#). Prior to the incubation period, rumen fluid was collected before feeding from three rumen fistulated crossbred sheep (Texel x Belgian milk sheep). After anaerobic transport to the laboratory, the rumen fluid of the three sheep was mixed and filtered through a sieve with a pore size of 1 mm under continuous CO₂ flushing where after it was kept anaerobically in a water bath at 39 °C to be transferred to the incubation flask after mixing anaerobically and CO₂ saturated (flushed overnight under CO₂ atmosphere) mixed bicarbonate/phosphate buffer at a rumen fluid/buffer ratio of 263.2 mL/L ([Castro-Montoya et al. 2015](#)). Each 120 mL incubation flask contained 250 mg of dry material of either forage/bean (1:1) meal of *C. ensiformis* or *M. pruriens* or forage meal of *L. leucocephala* (Figure 1A). Each treatment was incubated in triplicate at 39 °C in an incubator with intermittent shaking (Edmund Bühler GmbH SM – 30 Control, Germany) and all flasks were removed from the incubator after 24 h. At the end of the 24 h-incubation, all flasks were removed from the incubator, and the fermentation activity was stopped through transfer of the flask to an ice bath. After 5 min, the liquid content was sampled (2 mL) and acidified with 0.2 mL of formic acid that contained the internal standard (10 mg of 2-ethylbutyric acid/mL formic acid) for short chain fatty acids (SCFA) analysis. After 15 min of centrifugation (4 °C, 22,000 × g [Beckman Coulter, Avanti J-E model, CA, USA]), the supernatant was filtered and an aliquot was transferred into a 2 mL glass vial ([Castro-Montoya et al. 2012](#)). Samples were stored at 4 °C until analysis using a gas chromatograph

(HP 7890A, Agilent Technologies, Diegem, Belgium). The leftover was stored at -20° C until the measurement of the PSM as described before (section 2.2.2).

2.3.2. Single end-point (24 h) in vitro rumen degradability and breakdown of PSM of the three tropical legumes using fresh inoculum from Cuban sheep (Experiment 1.2)

This experiment was carried out to assess the concomitant ruminal degradation of OM and PSM using inoculum from another geographical origin. The incubations with inoculum from Cuban sheep was carried out in Santa Clara, Cuba (22°24'49"N 79°57'58"O). Rumen fluid was obtained before feeding by an esophageal tube from three Cuban Pelibuey sheep following the recommendation approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (approval number EC2015/12). Cuban sheep were grazing native grasses (*Andropogon* spp., *Cynodon nlemfluensis*, *Paspalum notatum*, *Panicum maximum*) and had free access to drinking water. Each 120 mL incubation flask contained 250 mg of dry material of either the forage/bean (1:1) meal of *C. ensiformis* or *M. pruriens* or the forage meal of *L. leucocephala* (Figure 1A). The preparation of the rumen fluid/buffer mixture and the 24 h incubation were as explained before (section 2.3.1). Each treatment was incubated in triplicate at 39 °C in an incubator (Mettler B40, Germany) additionally equipped with a shaker (Orbit™ Digital Shakers, USA) for intermittent shaking. All flasks were removed from the incubator after 24 h, and the fermentation activity was stopped through transfer of the flasks to an ice bath. After 5 min, samples were snap-frozen until the preparation for further analysis. Prior to shipment to Belgium, samples were thawed and prepared for analysis of SCFA as described before ([Castro-Montoya, et al. 2012](#)), stored at 4 °C and transported under cold conditions. The SCFA production was measured as explained in experiment 1.1, and the leftover was stored at -20° C until the measurement of the PSM as described before (section 2.2.2).

2.4. In vitro kinetics of ruminal degradation and breakdown of PSM from bean and forage meal of three tropical legumes using fresh inoculum from Belgian sheep (Experiment 2)

The kinetic study was used to assess the concomitant effect of PSM and OM degradation in time, using the plant parts with the highest PSM content (i.e. beans in the case of *C. ensiformis* and *M. pruriens*). Prior to the incubation period, rumen fluid was collected before feeding from three rumen fistulated crossbred sheep (Texel x Belgian milk sheep) and treated as explained previously (section 2.3.1). The 24 h *in vitro* rumen incubations, were carried out following the method described by [Vlaeminck, et al. \(2014\)](#). Each 120 mL incubation flask contained 250 mg of dry material of either bean meal of *C. ensiformis* or *M. pruriens* or forage meal of *L. leucocephala* (Figure 1B). All flasks were incubated in triplicate during 0, 2, 4, 6, 8, 10, 12 and 24 h at 39 °C in an incubator with intermittent shaking (Edmund Bühler GmbH SM – 30 Control, Germany). At the end of each incubation time, the fermentation was stopped and samples of rumen fluid were prepared for SCFA as described in experiment 1.1, and the leftover was stored at -20° C until the measurement of the PSM as described before (section 2.2.2).

2.5. Effect of L-mimosine on the ruminal degradation kinetics of straw using fresh inoculum from Belgian sheep (Experiment 3)

This experiment was carried out to assess whether L-mimosine directly affects ruminal degradability of fibrous rich feedstuffs. Prior to the incubation, rumen fluid was collected from three rumen fistulated crossbred sheep (Texel x Belgian milk sheep) as explained by [Vlaeminck, et al. \(2014\)](#). The 24 h *in vitro* rumen incubations, were carried out following the method described in the experiment 2, although the volume of the incubation flask (25 mL) as well as the amount of buffer, rumen fluid and substrate (52 mg DM of straw) was five times smaller. Incubations in such smaller volumes have been carried out before by our group and do not change the incubation outcome ([Debevere et al. 2020](#)). The smaller volume has been applied here to limit the amount of pure L-mimosine required. Straw was incubated with or without L-mimosine (purity $\geq 98\%$, Sigma-Aldrich Pty Ltd, St Louis, MO, USA) at the same concentration present in the *L. leucocephala* forage meal used in this study (Table 1). All flasks were incubated in triplicate during 0, 2, 6, 10 and 24 h at 39 °C in an incubator with intermittent shaking (Edmund Bühler GmbH SM – 30 Control, Germany). At the end of each incubation time, the fermentation was stopped and samples of rumen fluid were prepared for SCFA analysis as described in experiment 1.1.

2.6. Apparently rumen organic matter degradability (ARDOM)

ARDOM was calculated based on the stoichiometrical equation representing carbohydrate breakdown as:

$$\text{ARDOM (g/100g OM)} = (\text{acetate}/2 + \text{propionate}/2 + \text{butyrate} + \text{caproate}) \times 162 \quad [2]$$

with 162 as the assumed molecular weight of 1 mol of fermented carbohydrates; net production of acetate, propionate, butyrate and caproate expressed in mol/100 g OM of substrate added ([Demeyer 1991](#)). As compared with the stoichiometric relationship described previously, caproate was included in the equation based on the same stoichiometric principles of SCFA formation from the carbohydrate fermentation as outlined by [Demeyer \(1991\)](#).

2.7. Kinetic parameters and statistical analysis

All statistical analyses were performed with SPSS 21.0 ([SPSS. 2012](#)). Using nonlinear regression, the ARDOM and ARDPSM kinetics was estimated according to [Schofield \(2000\)](#) with some modifications:

$$\text{ARDOMt or ARDPSMt} = \text{Ass} / \{1 + \exp [2 - 4 \times kf \times (t - Ln)]\} \quad [3]$$

with ARDOMt or ARDPSMt (g/g except for TI [TIU/TIU]) ARDOM and ARDPSM at time (t); Ass, the asymptotic ARDOM or ARDPSM corresponding to maximum OM or PSM degradation, respectively (g/g except for TI [TIU/TIU], respectively); kf, the fractional ARDOM and ARDPSM rate (h^{-1}), t, the incubation time (h) and Ln, the lag time before degradation starts (h). A graph was plotted together with the observed values to show the trend of the degradation curve. Further, a One way ANOVA was applied to assess the differences between kinetic parameters within and between the tropical legumes. Differences were declared significant at $P < 0.05$ and a Tukey's test for multiple comparison was run.

Additionally, a One way ANOVA was applied: a) to assess the effects of the legume type on ARDOM and ARDPSM in the single end-point (24 h) *in vitro* rumen incubation using Belgian or Cuban sheep inocula; and b) to assess the effects of the addition of L-mimosine on ARDOM and SCFA production in 24 h *in vitro* ruminal fermentation of straw. Differences were declared significant at $P < 0.05$ and Tukey's test for multiple comparison was run.

3. Results

Different *in vitro* approaches were combined in 3 experiments (Figure 1) to assess whether ruminal degradation of three legume meals (i.e. *C. ensiformis*, *M. pruriens* and *L. leucocephala*) was influenced by the source of ruminal inoculum and whether ruminal OM and PSM degradation occurred concomitantly or independently. The latter was assessed from the results of the *in vitro* kinetic studies with either the legumes or with pure PSM under Belgian conditions. Further, the single-end-point 24 h batch incubation also was performed using Cuban sheep inoculum to verify the association of the PSM breakdown with the OM degradability using inoculum from another source. In case the results of both experiments suggest such an association, the direct effect of the PSM on the ARDOM kinetics was assessed using pure PSM added to a PSM-free fibrous substrate.

3.1. Experiment 1. In vitro ruminal degradation and breakdown of PSM of three tropical legumes using different inoculum sources

Experiment 1.1. Single end-point (24 h) *in vitro* rumen degradability and breakdown of PSM of three tropical legumes using fresh inoculum from Belgian sheep

The ARDOM and ARDPSM results of the three tropical legumes using ruminal inoculum from Belgian sheep are shown in the Table 2. The ARDOM and ARDPSM of the forage/bean (1:1) meal of *C. ensiformis* and *M. pruriens* were similar, with almost complete degradation of their PSM. Additionally, forage meal of *L. leucocephala* was the least ($P < 0.05$) degradable of the three legume meals (0.20 g ARDOM/g OM), while L-mimosine even was hardly degraded after 24 h (0.05 g/g). The fermentation pattern of the meals of the three tropical legumes differed. Fermentation of *L. leucocephala* resulted in higher molar acetate and propionate proportions, at the expense of molar proportions of butyrate and isoacids, valerate and caproate. On the contrary, the fermentation of *M. pruriens* resulted in higher proportions of butyrate and isoacids, valerate and caproate and was associated with lower molar acetate and propionate proportions. The fermentation pattern of *C. ensiformis* was intermediate.

Experiment 1.2. Single end-point (24 h) *in vitro* rumen degradability and breakdown of PSM of three tropical legumes using fresh inoculum from Cuban sheep

The ARDOM and ARDPSM of the forage/bean (1:1) meal of *C. ensiformis* and *M. pruriens* and the forage of *L. leucocephala* with Cuban sheep inoculum are presented in Table 3. Degradation of the *M. pruriens* forage/bean (1:1) meal was most extensive, while degradability of the forage meal of *L. leucocephala* was lowest, in agreement with the observations in experiment 1.1. In addition, as observed

in the experiment 1.1, the degradation of PSM in the forage/bean (1:1) meal of *C. ensiformis* and *M. pruriens* was almost complete. However, that was not the case for the PSM of the forage meal of *L. leucocephala*, although L-mimosine seemed to be more extensively degraded under the Cuban incubation conditions compared with the Belgian conditions in experiment 1.1. The fermentation patterns of the meals of the three tropical legumes differed with the exception of the molar propionate proportion ($P > 0.05$). Fermentation of *L. leucocephala* resulted in a greater molar acetate proportion, while the lowest molar butyrate proportion was observed in the fermentation of *C. ensiformis*. Further, the molar proportion of isoacids, valerate and caproate were lowest in the fermentation of *L. leucocephala*.

3.2. Experiment 2. In vitro kinetics of ruminal degradation and breakdown of PSM from bean and forage meal of three tropical legumes using fresh inoculum from Belgian sheep

The extensive ARDOM and ARDPSM of the forage/bean (1:1) meal of *C. ensiformis* and *M. pruriens* after 24h of incubation (experiment 1.1) does not necessarily imply similar kinetics of PSM and OM breakdown. Therefore, *in vitro* degradation kinetics were studied and presented in Table 4 and Figures 2, 3 and 4. As the PSM, quantified in this study, are enriched in the beans of *C. ensiformis* and *M. pruriens*, in this kinetic study, bean meals rather than forage/bean (1:1) meals were used for these two legumes. Within each legume meal, the kinetic parameters of the OM and PSM *in vitro* ruminal degradation were compared (Table 4). In the case of *L. leucocephala* forage meal the lag time and degradation rate of ARDOM and ARDPSM did not differ. On the contrary, for both other legumes the lag time and degradation rate of ARDOM and ARDPSM differed considerably. The apparent *in vitro* OM degradability of the bean meals from *C. ensiformis* and *M. pruriens* followed a similar and quite linear pattern reaching 0.84 and 0.75 g ARDOM/g OM after 24 h of incubation, respectively. The apparent *in vitro* OM degradability of *L. leucocephala* forage meal was considerably lower, reaching a maximum of 0.22 g ARDOM/g OM after 24 h. Lag time and degradation rate of ARDOM did not differ between legumes ($P > 0.05$). Degradation of TI from bean meals showed a lag time of about 6 h, but degradation increased sharply between 6 and 10 h of incubation and reached a plateau after 12 h of incubation (Figure 2 and 3). Strikingly, the TI degradation pattern of the forage meal of *L. leucocephala* forage differed compared to the other two legumes (Table 4) as no lag time was observed (Figure 4) and a maximum degradation of about 0.62 TIU/TIU was reached after 10 to 12 h of incubation. L-canavanine degradation did not differ between both bean meals ($P > 0.05$). L-canavanine was extensively degraded after 24 h (> 0.80 g/g) despite a lag time of 12 h in *C. ensiformis* versus the quite linear degradation kinetics in *M. pruriens*. In addition, Con A (only detected in *C. ensiformis* bean meal, Table 1) was fastly (0.70 g/g in the first 2 h of incubation) and extensively (0.95 g/g after 24 h) degraded (data not shown). In contrast, L-mimosine and its intermediates in the *L. leucocephala* forage meal incubation were hardly degraded (0.05 g/g) and the maximum degradation was already reached during the first 2 h of incubation (Figure 4).

3.3. Experiment 3. Effect of L-mimosine on the ruminal degradation kinetics of straw using fresh inoculum from Belgian sheep

In experiments 1 and 2, the extent of ruminal degradation of L-mimosine was considerably different between the Belgian and the Cuban set-up. Concomitantly, overall OM degradation of the legume containing L-mimosine, i.e. *L. leucocephala*, differed in these two incubations. Accordingly, it is unclear whether L-mimosine impaired overall OM degradation or vice versa, whether L-mimosine degradation was impaired because of limited degradation of the *L. leucocephala* forage meal. Hence, the effect of pure L-mimosine on the ruminal degradation of a fibrous feed resource was assessed. The kinetics of ARDOM of straw without and with L-mimosine are presented in Figure 5. The apparent ruminal OM degradability of straw was affected by the addition of L-mimosine. After 24 h of incubation, ARDOM was 1.45 times lower ($P < 0.001$) when L-mimosine was added.

4. Discussion

4.1. Characteristics of the tropical legume batches used in the current experiment

The CP content of the three tropical legumes investigated in the present study exceeded 200 g/kg DM, which is of interest to supplement the poor quality tropical forages with low CP contents (Ahmed et al. 2018, Njidda and Nasiru 2010). Nevertheless, *L. leucocephala* forage meal contained high amounts of fiber (mainly ADFom and lignin (pm)) which limits its metabolisable energy content (Du et al. 2016, Moore and Jung 2001). The PSM content of plants partially reflects their adaptation to their environment and defense against natural enemies, such as competitors, pathogens, and/or herbivores (Makkar et al. 2007a). As such, variation in PSM content may be related to different biotic and abiotic conditions, as well as plant genetics and phenology (Estell 2010, García et al. 2008, Soedarjo and Borthakur 1996). Compared to the bean and forage meals of the present study, others, e.g. Belmar, et al. (2007), reported higher concentrations in *C. ensiformis* bean meal of L-canavanine (50.8 mg/g DM), whereas Agbede and Aletor (2005) showed lower TI contents (2.1 TIU/g DM). Additionally, García, et al. (2008) and Honda and Borthakur (2019) reported a considerably lower content (20.4 - 69.5 g/kg DM) of L-mimosine than reported in the *L. leucocephala* forage meal of the present study. Literature on PSM in *M. pruriens* beans or forage/bean (1:1) meal are scarce which might be related to the reported absence of L-canavanine (Lackey 1977). 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 (2001).

4.2. Apparent degradation of three tropical legumes in relation to source of the ruminal inoculum and degradation of their PSM

In experiment 1, fresh ruminal inoculum was obtained from Belgian and Cuban sheep. Although the incubated substrate as well as the *in vitro* set-up was the same in Belgium and Cuba, the two incubations were not compared statistically, as they did not take place at the same time and under the same lab conditions. Also collection of the ruminal inoculum differed. Nevertheless, obvious inoculum-related differences in ARDOM within a legume source were interpreted as an indication of the a need for specific ruminal conditions to be fulfilled to ensure optimal ruminal degradation of this legume and their PSM.

Overall degradation (i.e. ARDOM) of the forage/bean (1:1) meal of *C. ensiformis* was not affected by the source of the inoculum. Additionally, both the Belgian as well as the Cuban sheep inoculum extensively degraded the PSM in this legume (TI and L-canavanine) during a 24h-incubation. Organic matter degradability was in line with formerly reported *in vitro* degradation rates (Ajayi et al. 2009), while the potential of the microbial population in the rumen to detoxify L-canavanine also has been suggested by Dominguez-Bello and Stewart (1990b). In line with *C. ensiformis*, the degradation of PSM in *M. pruriens* forage/bean (1:1) meal (TI and L-canavanine) was almost complete both during the 24 h-incubation performed in Belgium as well as in Cuba.

L. leucocephala forage meal was only poorly degraded in the current study. Low degradation rates obviously are related to the high fiber content and particularly a high amount of lignin (> 100 g/kg DM) and its detrimental effects on degradability (Du, et al. 2016). Nevertheless, values obtained under Belgian incubation conditions (0.20 and 0.22 after 24h incubation) were considerably lower than in other reports (Gaviria et al. 2015, Soltan, et al. 2017, Soltan et al. 2013). Furthermore, Cuban *in vitro* incubations showed a higher ARDOM (0.35) compared with Belgian incubations. This could be related to a better adaptation of the microbiome in the Cuban sheep inoculum to degrade fibrous feeds as shown by Cherney et al. (1993). Additionally, the extensive degradation of L-mimosine could have played a role in this case. Although concomitant occurrence of increased ARDOM and enhanced degradation of L-mimosine, does not proof a causal effect, results of experiment 3 showed supplementation of L-mimosine could significantly reduce *in vitro* ARDOM of a fibrous feedstuff. In line with this, others reported some anti-microbial effect of L-mimosine and DHP (Anitha et al. 2005, Rodgers et al. 2017), particularly towards fungi and Gram-positive bacteria, which might have impaired the degradability of this legume. Accordingly, the limited breakdown of L-mimosine in *L. leucocephala* during the Belgian 24 h-incubations may have reduced the overall OM degradability of this legume, while the increased ARDOM in the Cuban 24 h-incubations concomitantly occurred with a more extensive degradation of L-mimosine. This may be related to the presence of L-mimosine and DHP degrading ruminal bacteria (Galindo et al. 2009). In this respect, *Synergistes jonesii* is supposed to be the main ruminal bacteria responsible for the degradation of DHP (Allison, et al. 1990), although also other ruminal bacteria (e.g. *Streptococcus lutetiensis*, *Clostridium butyricum*, *Lactobacillus vitulinus*) are capable to degrade these metabolites (Derakhshani et al. 2016). Degradation of L-mimosine by ruminal fluid from sheep which grazed tropical pastures including *L. leucocephala* also could explain why Soltan, et al. (2017) did not observe any negative effect of L-mimosine on the OM degradation of lucerne (*Medicago sativa*) hay, while ruminal degradation of Bermuda grass (*Cynodon dactylon* L. Pers.; hybrid "Tifton-85") even increased by 10 % upon L-mimosine supplementation (11.6 mg/g DM).

The association between ARDOM and ARDPSM was further investigated in a kinetic assessment. For this, bean meals of *C. ensiformis* and *M. pruriens* were incubated, as particularly the beans of these legumes are rich in PSM. Organic matter breakdown started almost immediately, without major lag time in each of the tropical legumes, while the PSM degradation (Trypsin inhibitor in *C. ensiformis* and *M. pruriens*; and L-canavanine in *C. ensiformis*) was considerably delayed. Thus, inocula did not need to break down the plant secondary metabolites prior to the start of OM degradation. Trypsin inhibitor was

extensively degraded after 10 h of incubation for the three tropical legumes. Considering an average retention time in the rumen of about 12 – 14 h for legume beans (based on soybean data) and of about 16 – 20 h for forages (Gao et al. 2015), our data suggest that only a negligible amount of TI would bypass the rumen. As such, the trypsin inhibitor is not expected to reduce protein digestibility in the small intestine. In contrast to the other two legumes, for *L. leucocephala*, kinetics of ARDOM and apparently ruminal degradation of L-mimosine show a similar pattern (Table 4). As in single-end point incubations with ruminal inoculum of Belgian sheep, L-mimosine was hardly degraded in the kinetic study. This kinetic association could be partially due to the toxic effects of L-mimosine or its intermediates on the ruminal microbiome, as indicated by results of experiment 3 and suggested from literature (Ghosh and Samiran 2007, Rosenthal 1991).

5. Conclusion

C. ensiformis and *M. pruriens* (beans and forage/bean (1:1) meal), as well as their PSM (i.e. trypsin inhibitor and L-canavanine) were extensively degraded. Nevertheless, in some cases PSM degradation was characterized by a lag time, while this was not observed for OM degradation. On the other hand, *L. leucocephala* degradability could partially depend on the potential of the rumen microbes to degrade L-mimosine.

Abbreviations

ADFom, acid detergent fiber expressed exclusive of residual ash; ARDOM, apparent *in vitro* rumen degradability of organic matter; ARDSPM, apparent rumen degradability of plant secondary metabolites; Con A, concanavaline A; DM, dry matter; ha, hectare; ME, metabolisable energy; NDFom, neutral detergent fiber not assayed with a heat stable amylase and expressed exclusive of residual ash; NPAA, non-protein amino acids; OM, organic matter; CP, crude protein; PCA, proximate chemical analysis; PSM, plant secondary metabolites; SCFA, short chain fatty acids; TI, trypsin inhibitor.

Declarations

Author Contributions: Conceptualization, methodology, and supervision V. F., R. L-O., and E. A-O.; Formal analysis E. A-O.; Investigation E. A-O., P.Y. F-R., R. L-O., and B. R-B.; Writing - original draft E. A-O.; Writing - review & editing V. F., R. L-O.; project administration and funding acquisition V. F., R. L-O.

All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT (DAS)

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ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All experimental procedures with fistulated sheep were conducted and approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO) [approval number EC2014_222], in accordance with the European Directive (EU) No 241/2014.

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Tables

Table 1. Contents (Means ± SD) of organic matter (OM), crude protein (CP) and crude fiber (CF) as well as fiber fractions (g/kg DM) and plant secondary metabolites of forage, bean and forage/bean (1:1) meals of three tropical legumes (n=3).

Plant parts which meals originate from	Forage	Forage/bean (1:1) meal			Beans
Legumes	<i>L. leucocephala</i>	<i>C. ensiformis</i>	<i>M. pruriens</i>	<i>C. ensiformis</i>	<i>M. pruriens</i>
OM	939±1.0	946±0.5	937±0.2	963±2.0	945±2.0
CP	297±12.4	237±10.7	254±48.9	320±1.4	282±3.0
CF	259±1.7	115±23.3	119±1.7	90±0.3	70±0.0
Fiber fractions^a					
NDFom	582±28.1	302±61.1	337±5.2	365±0.7	296±2.4
ADFom	347±16.4	156±4.8	194±17.9	140±1.3	113±2.3
Hemicellulose	235±44.5	146±56.3	144±23.0	225±0.5	183±0.1
Cellulose	242±11.4	126±2.6	161±14.9	123±1.7	94±1.7
Lignin (pm)	105±5.0	30±2.2	33±3.0	17±0.1	19±0.6
Plant Secondary Metabolites (g/kg DM unless stated otherwise)^c					
TI	13.9±0.1	13.9±0.1	14.4±0.3	22.1±0.4	22.7±0.5
L-canavanine	nd ^b	17.8±0.6	19.1±0.0	31.2±1.0	33.4±0.1
L-mimosine	153±0.5	nd	nd	nd	nd
Con A	nd	nd	nd	8.4±0.5	nd

^a NDFom: Neutral detergent fiber, ADFom: acid detergent fiber; ^b nd: not determined; ^c TI: trypsin inhibitor, Con A: Concanavalin A.

Table 2. ARDOM (g/g OM), total SCFA (mmol/g OM), and fermentation pattern of mixed bean-forage meals of *C. ensiformis* and *M. pruriens* and forage meal of *L. leucocephala* and degradation of the main plant secondary metabolites (ARDPSM; g/g except for TI [TIU/TIU]) using rumen inoculum from Belgian sheep.

Items	Belgian sheep inoculum			SEM ²	P - value
	<i>C. ensiformis</i>	<i>M. pruriens</i>	<i>L. leucocephala</i>		
ARDOM	0.44 ^a	0.43 ^a	0.20 ^b	0.005	< 0.001
Total SCFA	5.06 ^a	4.93 ^a	2.38 ^b	0.055	<0.001
Fermentation pattern (mmol/mol SCFA ₄)					
Acetate	609 ^b	601 ^c	687 ^a	2.5	< 0.001
Propionate	251 ^b	214 ^c	260 ^a	1.4	<0.001
Butyrate	100 ^b	127 ^a	36 ^c	1.6	< 0.001
Iso+Val+Cap ¹	40 ^b	58 ^a	17 ^c	0.5	<0.001
ARDPSM					
Trypsin inhibitor	0.98 ^a	0.98 ^a	0.64 ^b	0.006	< 0.001
L-canavanine	0.92	0.92	-	0.004	0.557
L-mimosine	-	-	0.05	-	-

Different superscripts in the same row (^{a, b, c}) indicate significant differences (P<0.05) according to [Tukey \(1949\)](#). Abbreviations: ARDOM: apparent ruminal degradability of organic matter; SCFA: short chain fatty acids; ARDPSM: apparent ruminal degradability of plant secondary metabolites.

¹ Iso: isoacids (isobutyrate and isovalerate); Val: valerate; Cap: caproate.

² SEM: Standard error of the mean

Table 3. ARDOM (g/g OM), total SCFA (mmol/g OM), and fermentation pattern of mixed bean-forage meals of *C. ensiformis* and *M. pruriens* and forage meal of *L. leucocephala* and degradation of the main plant secondary metabolites (ARDPSM; g/g except for TI [TIU/TIU]) using rumen inoculum from Cuban sheep.

Items	Cuban sheep inoculum			SEM ²	P - value
	<i>C. ensiformis</i>	<i>M. pruriens</i>	<i>L. leucocephala</i>		
ARDOM	0.50 ^b	0.59 ^a	0.35 ^c	0.012	<0.001
Total SCFA	6.29 ^a	7.10 ^a	4.11 ^b	0.144	<0.001
Molar proportions of SCFA (mmol/mol SCFA _t):					
Acetate	652 ^b	609 ^c	682 ^a	2.6	< 0.001
Propionate	200	196	173	6.7	0.175
Butyrate	63 ^b	112 ^a	96 ^a	3.0	0.001
Iso+Val+Cap ¹	85 ^a	83 ^a	49 ^b	1.6	<0.001
ARDPSM					
Trypsin inhibitor	0.97 ^b	0.97 ^a	0.97 ^b	0.000	<0.001
L-canavanine	0.98	0.96	-	0.008	0.481
L-mimosine	-	-	0.78	-	-

Different superscripts in the same row (^{a, b, c}) indicate significant differences (P<0.05) according to [Tukey \(1949\)](#). Abbreviations: ARDOM: apparent ruminal degradability of organic matter; SCFA: short chain fatty acids; ARDPSM: apparent ruminal degradability of plant secondary metabolites.

¹ Iso: isoacids (isobutyrate and isovalerate); Val: valerate; Cap: caproate.

² SEM: Standard error of means

Table 4. Kinetic parameters of *in vitro* ruminal degradability of organic matter and plant secondary metabolites of bean meal from *C. ensiformis* and *M. pruriens*, and forage meal of *L. leucocephala* during a 24 h incubation period according to the model $ARDOM_t$ or $ARDPSM_t = Ass / \{1 + \exp[2 - 4 \times kf \times (t - Ln)]\}$.

Legumes		Ln (h) ¹	kf (h ⁻¹) ¹	Ass (g/g) ¹	R ²
Canavalia ensiformis	ARDOM	3.20 ^c	0.05 ^b	0.84 ^y	0.987
	Trypsin inhibitor	5.85 ^{b y}	0.42 ^{a y}	0.79 ^y	0.996
	L-canavanine	11.79 ^a	0.11 ^b	0.91	0.995
	SEM ²	0.472	0.034	0.048	
	P-value	< 0.001	< 0.001	0.114	
Mucuna pruriens	ARDOM	1.92 ^b	0.05 ^b	0.75 ^y	0.977
	Trypsin inhibitor	5.48 ^{a y}	0.37 ^{a y}	0.72 ^{xy}	0.996
	L-canavanine	0.00 ^b	0.05 ^b	0.90	0.949
	SEM	0.952	0.024	0.063	
	P-value	0.003	< 0.001	0.062	
Leucaena leucocephala	ARDOM	1.08	0.05	0.22 ^{b x}	0.962
	Trypsin inhibitor	-1.63 ^x	0.09 ^x	0.63 ^{a x}	0.880
	L-mimosine	1.21	1.46	0.05 ^c	0.997
	SEM	6.669	12.080	0.036	
	P-value	0.893	0.991	< 0.001	
Statistics: comparison of ARDOM and ARDPSM kinetics between legumes					
ARDOM		0.140	0.594	< 0.001	
Trypsin inhibitor		< 0.001	< 0.001	0.012	
L-canavanine		< 0.001	0.090	0.877	

Different superscripts (a, b, c) in the same column and within each tropical legume indicate significant differences (P < 0.05) according to [Tukey \(1949\)](#).

Different superscripts (^{x,y,z}) in the same column indicate significant differences ($P < 0.05$) of ARDOM or trypsin inhibitor kinetic parameters between the tropical legume according to [Tukey \(1949\)](#).

¹ Ln = lag time (h), kf = ARDOM and ARDPSM rate (h^{-1}) and Ass = asymptotic value, corresponding to the maximum degradation of OM and PSM (g/g except for TI [TIU/TIU]).

² SEM = Standard error of means.

Abbreviations: ARDOM: apparent ruminal degradability of organic matter; ARDPSM: apparent ruminal degradability of plant secondary metabolites.

Figures

Legend: or : inoculum freshly obtained from Belgian or Cuban sheep, in the incubation flasks indicated in purple; **Blue**: Buffer; **Green**: forage meal of *C. ensiformis*, *M. pruriens* or *L. leucocephala*; **Yellow**: bean meal of *C. ensiformis* or *M. pruriens*; **Dark Brown**: straw without L-mimosine; **Light Brown**: straw with L-mimosine.

Figure 1

Schematic representation of all in vitro ruminal incubations with the three tropical legumes. A) Experiment 1. In vitro assessment (24 h) of apparent ruminal degradation of OM (ARDOM) and plant secondary metabolites (ARDPSM) of forage/bean (1:1) meal (*C. ensiformis* or *M. pruriens*) or forage

meal of *L. leucocephala* using fresh inoculum from Belgian or Cuban sheep; B) Experiment 2. In vitro rumen kinetic assessment of ARDOM and ARDPSM of bean meal (*C. ensiformis* or *M. pruriens*) or forage meal of *L. leucocephala* using fresh inoculum from Belgian sheep; and C) Experiment 3. In vitro rumen kinetic assessment of ARDOM and ARDPSM of straw with or without L-mimosine using fresh inoculum from Belgian sheep.

Figure 2

Kinetics of apparent ruminal degradability of organic matter and plant secondary metabolites of bean meal from *Canavalia ensiformis*. All degradabilities are expressed as g/g, except for trypsin inhibitor, expressed in TIU/TIU, with TIU = trypsin inhibitor units.

Figure 3

Kinetics of apparent ruminal degradability of organic matter and plant secondary metabolites of bean meal from *Mucuna pruriens*. All degradabilities are expressed as g/g, except for trypsin inhibitor, expressed in TIU/TIU, with TIU = trypsin inhibitor units.

Figure 4

Kinetics of apparent ruminal degradability of organic matter and plant secondary metabolites of bean meal from *Leucaena leucocephala*. All degradabilities are expressed as g/g, except for trypsin inhibitor, expressed in TIU/TIU, with TIU = trypsin inhibitor units

Figure 5

Kinetics of apparent rumen degradability of organic matter (ARDOM) of straw with and without L-mimosine (One-way ANOVA test and Tukey's procedures for multiple comparison: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).