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Effect of the method of preservation on the chemical composition and in vitro fermentation characteristics in two legumes rich in condensed tannins

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Highlights

- The fractions of condensed tannins (CT) were more affected in silage than in hay
- Hay produced more gas and similar methane than fresh sainfoin.
- Hay produced less gas and methane than fresh sulla.
- Silage produced the least gas and methane in both legumes.
- The method of preservation affected individual VFA in both legumes.

Abstract

The objective of the present study was to evaluate the effects of preservation (hay and silage) on the chemical composition and the *in vitro* fermentation characteristics in comparison with fresh forage in two legumes rich in condensed tannins (CT). Sainfoin (*Onobrychis viciifolia*) was collected at the late bloom stage and sulla (*Hedysarum coronarium*) at the early bloom stage. In each forage, a part was immediately freeze-dried, a part was dried at ambient temperature to obtain hay, and another part was ensiled in vacuum-packages for 82 days. An

in vitro assay to study the fermentation was carried out with an Ankom system during 72 h. In both forages, the silages had different contents of polyphenols and condensed tannins fractions than fresh forage and hay ($P<0.05$). Sainfoin hay only had greater content of fibre-bound CT fraction (FBCT) than fresh sainfoin, whereas sulla hay had greater protein-bound CT and FBCT contents than fresh sulla ($P<0.05$). Sainfoin silage had lower gas and methane production than hay and fresh forage ($P<0.001$), whereas fresh sulla had greater gas and methane production, followed by hay and silage, which produced the least ($P<0.05$). In both legumes, hay had lower *in vitro* organic matter digestibility (IVOMD) than silage and fresh forage ($P<0.001$). The method of preservation affected the total production of volatile fatty acids (VFA) only in sulla ($P<0.05$). Most of the proportions of individual VFA were affected by the method of preservation in both legumes ($P<0.001$). In conclusion, polyphenols content, total content and fractions of CT were more affected in the silages than in the hays when compared to fresh forages. However, according to gas and methane production and IVOMD, silage may have greater feed nutritive value than hay although further studies on animal performance are warranted before recommendation.

Abbreviations: *A*, potential gas production; ADFom, acid detergent fibre exclusive of residual ash; *c*, rate of gas production; C_2:C_3, acetic/propionic acid ratio; CH_4, methane; CP, crude protein; CT, condensed tannins; DM, dry matter; ECT, extractable condensed tannin; FBCT, fibre bound condensed tannin; GC, gas chromatograph; IVOMD, *in vitro* organic matter digestibility; lignin (sa), lignin determined by solubilisation of cellulose with sulphuric acid; NDFom, neutral detergent fibre exclusive of residual ash; NH_3-N, ammonia; OM, organic matter; P, cumulative gas production; PBCT, protein bound condensed tannin; TCT, total condensed tannin; VFA, volatile fatty acids.

Keywords

Sainfoin; sulla; condensed tannins; gas production; hay; silage.

1. Introduction.

The interest in sainfoin (*Onobrychis viciifolia)* and sulla (*Hedysarum coronarium),* which are two perennial legumes extensively used in the Mediterranean area, has increased due to their content of condensed tannins (CT), which have a positive effect on the reduction of methane production in the rumen (Waghorn and Clark, 2006; Bodas et al., 2012; Hatew et al., 2016). Both legumes have high productive capacity (15 and 25 t DM/ ha, respectively), high nutritional value and medium to high CT content (50-80 g/kg DM) (Waghorn et al., 1998). However, these legumes have differences in the fractions of extractable CT (ECT), protein bound-CT (PBCT) and fibre bound-CT (FBCT), which influence the bioactivity of the CT (Jonker and Yu, 2017).

It is advisable to preserve these forages to optimize their use for animal feeding because two-thirds of the annual production of these forages is obtained in the first spring cut (Delgado et al., 2008). Preservation can increase the fibrous fraction, reduce the crude protein (CP) (Dentinho et al., 2006; Aufrère et al., 2008) and change the fractions of CT (Wang et al., 2015; Huang et al., 2016). Nevertheless, the extent of the effects is dependent on the method of preservation, silage or hay and the legume species (Scharenberg et al., 2007a).

Condensed tannins reduce microbial action, gas and methane (CH_4) production and ammonia (NH_3-N) content (Frutos et al., 2004; Ramírez-Restrepo and Barry, 2005; Jonker and Yu, 2017), depending on the species of CT-containing legume (Huyen et al., 2016; Grosse-Brinkhaus et al., 2017). To the best of our knowledge, the effects of preservation on ruminal

fermentation have been scarcely studied in sainfoin (Scharenberg et al., 2007b; Wang et al., 2015) and sulla (Dentinho et al., 2006).

The hypothesis of the experiment was that the type of preservation affects the presence of CT and the fermentation in these two species, however, the extent of the effect is unknown. The aim of this study was to assess the effect of the method of preservation (hay *vs*. silage) on the chemical composition and *in vitro* fermentation characteristics and compare them with fresh forage in two CT-containing legume species: sainfoin (*Onobrychis viciifolia)* and sulla (*Hedysarum coronarium*).

2. Materials and methods.

2.1. Animal and diets

2.1.1. Experimental design.

The current experiment evaluated a single factor with multiple levels (fresh forage, hay and silage) in two CT-containing legumes, sainfoin and sulla. The forages are analysed separately because they differ in the phenological stage at sampling (see below).

2.1.2. Forages

Two legumes were grown at CITA Research Institute at Zaragoza (41° 42′ N, 0° 49′ W, altitude 216 m a.s.l., annual mean temperature of 15 ± 7.3 °C and annual average rainfall of 296 ± 78 mm, Ebro Valley, north-eastern Spain). The silt-loam soil at the site had pH 8.1 and 1.81% organic matter (OM) and contained 16% clay, 53.5% silt and 30.5% sand. No mineral fertilizer was applied. Sainfoin (*Onobrychis viciifolia* cv *Reznos)* and sulla (*Hedysarum coronarium* cv *Carmen)* were sown each in a paddock in autumn 2013 at seeding rates of 100 kg/ha and 20 kg/ha, respectively. Each paddock was divided in 3 plots of 50 x 30 m. During spring, irrigation was applied every 15-21 days.

2.1.3. Harvest and preservation of the forages.

Samples of sainfoin were collected the 7th May 2015 at the late bloom stage, and samples of sulla were collected the 21st April 2015 at the early bloom stage. Ten samples were obtained from 0.25 m^2 areas randomly allocated in each plot. Then, the 10 samples from each plot were mixed homogenously and divided into three samples for fresh forage, hay and silage.

The samples destined to make hay were extended in 10-cm elevated "mosquito" nets for 16 days. The samples were naturally sun-dried, however, they were dried indoors under unfavourable climatic conditions. The samples destined to make silage were wilted one day, chopped (3-5 cm) and vacuum-ensiled in plastic bags (2 kg/bag). The plastic bags were protected from light and were kept at room temperature.

Part of the samples of fresh forage, hay and silage were dried in an oven at 60 °C for 48 h and the other part was immediately freeze-dried. Part of the oven- and the freze-dried samples was ground and sieved through a 1 mm screen (Rotary Mill, ZM200 Retsch, Germany) whereas another part through a 0.2 mm screen. All the samples were stored in total darkness at – 20 °C temperature until further analyses.

2.1.4. Animals and sampling of ruminal digesta

All the procedures used in the experiment were carried out in accordance with the Spanish guidelines for experimental animal protection (RD 53/2013) with the approval of the Institutional Animal Care and Use Committee of the Research Centre (Procedure number 2011-05).

Four Rasa Aragonesa wethers (65 ± 2.1 kg body weight) fitted with rumen cannula were used as donors of ruminal contents. The animals were housed in individual pens (150 cm x 150 cm) with free access to water and a mineral-vitamin mixture. Wethers were fed a 70:30 mixed diet of alfalfa hay:barley grain at energy maintenance levels, distributed in two equal meals at 8:00 h and 13:00 h. The diet had 913, 168, 426 and 269 g/kg of organic matter (OM), crude protein (CP), neutral detergent fibre (NDFom) and acid detergent fibre (ADFom),

respectively. The day of the assay, before the morning feeding, ruminal digesta from each wether was collected into a pre-warmed (39 °C) insulated thermos and transported to the laboratory, which was located next to the animal facilities. Rumen digesta was individually strained through four layers of cheesecloth and homogenized. Rumen fluid from the four wethers was mixed, and a buffer solution was added based on the protocol of Menke and Steingass (1988) in a proportion of 1:2 (v/v).

2.1.5. In vitro gas production technique and sampling

Gas production was determined with the Ankom system (Ankom Technology Corporation, Fairport, NY, USA), which consists of 310-mL capacity bottles fitted with pressure and temperature sensors. The freeze-dried samples (500 mg) were incubated with 60 mL of buffered solution:rumen fluid (2:1 v/v) in a water bath at 39 °C for 72 h. Three runs were conducted on three separate days, and each sample was incubated in duplicate in each run. Two bottles without substrate were used as negative controls (blanks). Gas production was recorded for 72 h and was corrected with the blanks.

After 72 h of incubation, the bottles were placed for 5-10 minutes in ice to stop fermentation. Then the bottles were tempered at room temperature for 10-15 minutes. A sample of gas was collected from each bottle at atmospheric pressure with a syringe attached to a manometer into a Vacutainer® tube. The tubes were conserved at 4 °C until CH_4 determination. At the end of gas sampling, the pH of the fermentation liquid was measured immediately with a microPH 2002 (Crison Instruments S.A., Barcelona, Spain). To determine the ammonia (NH_3-N) content in the fermentation fluid, 2.5 mL of liquid was mixed with HCl 0.1 N in a proportion of 1:1 (v/v). For volatile fatty acid (VFA) determination, 0.5 mL of liquid was added to 0.5 mL of deproteinizing solution [5 mL of 85% (v/v) ortho-phosphoric acid and 0.125 mL of 4-methylvaleric acid (Sigma Aldrich, St.Louis, MO, USA) as internal standard,

dissolved in 250 mL of distilled water] and 1 mL of distilled water. Tubes with samples of NH_3-N and VFA were stored at -20 °C until future analyses.

2.2. Analytical methods

2.2.1. Chemical composition

All the analyses of the chemical composition were run in duplicate. The dry matter (DM) (index no. 934.01) and ash (index no. 942.05) contents of the feedstuffs were determined in oven-dried samples according to the AOAC methods (AOAC, 2000). The content of CP (Nitrogen x 6.25) was determined following the Dumas Procedure (index no.968.06) using a nitrogen analyser (Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain) (AOAC, 2000). Neutral detergent fibre (NDFom), acid detergent fibre (ADFom), and acid detergent lignin (lignin (sa)) contents were determined according to the method described by Van Soest et al. (1991) using the Ankom 200/220 fibre analyser (Ankom Technology Corporation). The NDFom from forages was assayed with a heat stable amylase. The lignin (sa) was analysed on ADFom residues by solubilization of cellulose with sulphuric acid. All values were corrected for ash-free content.

The total polyphenols were extracted in freeze-dried samples according to the method described by Makkar (2000) and quantified following the method of Julkunen-Tiitto (1985) using the tannic acid as standard. Samples and standard calibration were measured with a Heλios β spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA) at 725 nm, and polyphenol contents were expressed as tannic acid equivalents.

The extractable CT (ECT), protein-bound CT (PBCT) and fibre-bound CT (FBCT) were extracted in freeze-dried samples and fractioned following the method of Terrill et al. (1992), and quantified by the colorimetric HCl-butanol method described by Grabber et al. (2013). The standard used for the quantification of the samples was extracted and purified from sainfoin using the method described by Wolfe et al. (2008). Finally, samples and standard

calibration were measured with the Heλios β spectrophotometer at 550 nm and CT concentrations were expressed as sainfoin CT equivalents, because they have the same main polymer (prodelphinidin).

2.2.2. Determination of parameters of the in vitro gas production technique

To study the kinetics of fermentation, gas production was recorded hourly for 72 h using the Ankom system. The gas produced in batch cultures was adjusted to the model described by France et al. (2000):

$$P = A \times (1 - e^{-ct})$$

where P is the cumulative gas production (mL) at time (h), *A* is the potential of gas production (mL), and *c* is the rate of gas production (h^{-1}).

Methane was determined by an HP-4890 (Agilent, USA) gas chromatograph (GC) equipped with a flame ionization detector (FID) and a TG-BOND Q+ capillary column (30 m x 0.32 mm id x 10 µm film thickness, Thermo Scientific, USA) using helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature was maintained at 100 °C (isothermal programme). The splitless injection volume was 200 µL. Methane identification was based on the retention time compared with the standard. The analysis time was 3 min (included equilibration time). Methane concentration was calculated from the peak concentration:area ratio, using the peak area generated from standard gas as the reference (CH_4; 99.995% purity [C45], Carburos Metalicos, Spain).

The *in vitro* organic matter degradability (IVOMD), after 72 h of incubation, was estimated by filtering the content of the bottle through a pre-weighed bag (50 µm; Ankom Technology Corporation). The bags with sample were washed twice with distilled water and were dried at 103 °C for 48 h to a constant weight to obtain the dry matter content. After 48 h, the bag content was weighed, and the sample was placed in a muffle at 550 °C to obtain the ashes.

The organic matter of bag content was obtained as DM-ashes, and the IVOMD was calculated.

The content of NH_3-N in the ruminal fluid was assessed using the Berthelot reaction (Chaney and Marbach, 1962). Ammonia was determined by the colorimetric method, and the chromophore was measured at 625 nm in an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

The concentrations of VFA: acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids were determined using a Bruker Scion 460 GC (Bruker, USA) equipped with CP-8400 autosampler, FID and a BR-SWax capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness, Bruker, USA) using helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature programme was 100 °C, followed by a 6 °C/min increase to 160 °C. The injection volume was 1 µl at a split ratio of 1:50. The VFA were identified based on retention time comparisons with commercially available standards of acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and 4-methyl-valeric acids of ≥ 99% purity (Sigma-Aldrich).

2.3. Calculations and statistical analyses

Data were analysed using SAS statistical software (SAS V.9.3). The fermentation kinetic parameters (*A* and c) were estimated through a non-linear regression model using the SAS NLIN programme. The statistical analyses were run separately for each legume. The chemical composition and the contents of polyphenols and CT were tested by analyses of variance using the GLM procedure, considering the method of preservation (fresh, hay and silage) as a fixed effect. The following model was used:

$$Y_i = \mu + T_i + e_i$$

where Y_i is the chemical composition parameter, μ is the overall mean, T_i is the effect of preservation method and e is the error term.

The fermentation parameters (pH, gas, CH_4, NH_3-N, VFA, and IVOMD) and the fermentation kinetic parameters (*A* and c) were analysed with mixed models considering the method of preservation as fixed effect and the run as random effect according to:

$$Y_{ij} = \mu + T_i + R_j + e_{ij}$$

where Y_{ij} is the *in vitro* fermentation parameter, μ is the overall mean, T_i is the effect of preservation method, R_j is the effect of run and e is the error term.

The LSMeans and their associated standard errors and differences in between means were obtained. Pearson correlation coefficients between variables were calculated for each forage using the CORR procedure of SAS. The effects were considered significant at a probability value of $P<0.05$.

3. Results

The forages were harvested in the optimum phenological stage for farmers, which differs between sainfoin and sulla. The results of sainfoin and sulla were not statistically compared because the phenological stage could be a confounding effect. Then, the results are presented separately for each forage.

3.1. Sainfoin

The method of preservation affected all the parameters of the chemical composition ($P<0.05$), except for the ash content ($P>0.05$) (Table 1). Fresh sainfoin had greater CP ($P<0.05$) and lower fibre contents ($P<0.01$) than both preserved forages, which only presented differences in lignin content ($P<0.05$). Regarding total polyphenols, TCT and CT-fractions, fresh forage and hay only differed in FBCT content ($P<0.01$). In contrast, silage presented lower total polyphenols ($P<0.001$), TCT ($P<0.05$) and ECT ($P<0.001$) contents and greater PBCT and FBCT ($P<0.001$) contents than fresh forage and hay.

The parameters obtained in the *in vitro* fermentation are shown in Table 2, and the fermentation kinetics during the incubation is represented in Figure 1. Fresh forage had

greater *A* and *c* than both preserved forages ($P<0.001$). Total gas production was greater in hay, intermediate in fresh forage and lower in silage ($P<0.05$). The CH_4 production was greater in fresh forage and hay than that in silage ($P<0.01$). The IVOMD was greater in fresh forage and silage than that in hay ($P<0.001$). The production of CH_4 was negatively correlated with the contents of PBCT and FBCT (r = -0.76 and r = -0.75, $P<0.05$, respectively) and positively correlated with ECT content (r = 0.72, $P<0.05$).

The method of preservation did not affect the NH_3-N content and total VFA production ($P>0.05$), but affected most of the proportions of the individual VFA ($P<0.05$). Fresh forage and hay had greater acetic acid and lower propionic acid proportions and, consequently, greater C_2:C_3 ratios than silage ($P<0.001$). Fresh forage had greater butyric acid and lower branched-chain VFA acid proportions than both preserved forages ($P<0.001$). Hay had higher proportion of iso-butyric and lower butyric than silage ($P<0.05$). Fresh forage had a greater CH_4/VFA ratio than silage ($P<0.05$), presenting hay an intermediate ratio ($P>0.05$).

3.2. Sulla

The method of preservation greatly affected the chemical composition, total polyphenols and CT contents in sulla (Table 3). Fresh forage had greater CP content and lower fibre content than both preserved forages ($P<0.001$), which presented differences in fibre contents ($P<0.001$). Regarding the content of total polyphenols, fresh forage had the greatest content, the hay intermediate and silage had the lowest content ($P<0.001$). Fresh forage and hay had different contents of PBCT ($P<0.05$) and FBCT ($P<0.001$). Silage presented the lowest contents of TCT ($P<0.01$) and ECT ($P<0.001$), greatest FBCT ($P<0.001$) and intermediate content of PBCT ($P>0.05$) compared with fresh forage and hay.

The parameters obtained in the *in vitro* fermentation are shown in Table 4, and the fermentation kinetics during the incubation is shown in Figure 2. Fresh forage had greater total gas and CH_4 production, *A*, *c* and IVOMD than both preserved forages ($P<0.001$). Hay

produced more gas and CH_4 and had greater *A* but lower IVOMD than silage ($P<0.05$) with similar c ($P>0.05$). Gas production was correlated with the contents of ECT (r = 0.80, $P<0.05$) and FBCT (r = -0.82, $P<0.01$), whereas CH_4 production was correlated with the contents of TCT (r = 0.70, $P<0.05$), ECT (r = 0.89, $P<0.01$) and FBCT (r = -0.90, $P<0.001$). The method of preservation affected the total VFA ($P<0.05$) and their proportions (Table 4; $P<0.001$). The fresh forage and hay had greater total VFA and acetic acid proportions than the silage ($P<0.05$). Fresh forage had the greatest propionic acid proportion, hay intermediate and silage the lowest ($P<0.01$). Silage had greater proportions of butyric, valeric and iso-valeric acids than fresh forage and hay, which differed in the proportions of butyric and valeric acids ($P<0.001$). The fresh forage presented the greatest CH_4/VFA ratio, the hay intermediate ratio, and the silage the lowest CH_4/VFA ratio ($P<0.001$).

4. Discussion.

4.1 Chemical composition and secondary compounds.

The reduction of CP content might be associated with proteolysis, mainly during the drying process (Foster et al., 2011), as extreme care was taken to avoid losses of leaves during the preparation of the samples. The different responses of NDFom and ADFom contents to hay- and ensiling of the sainfoin and sulla could be partially ascribed to the different proportions of leaves and stems of both legumes (Waghorn et al., 1998; Borreani et al., 2003) and the different phenological stages of the legumes at harvest (Guglielmelli et al., 2011; Theodoridou et al., 2011a).

The decreased polyphenols and TCT contents due to ensiling could be due to a rupture of plant cells during chopping and crushing before ensiling, as reported in purple prairie clover *(Dalea purpurea)* by Huang et al. (2016). In that sense, hay and silage had similar TCT content when sainfoin was not chopped before ensiling (Scharenberg et al., 2007a; Scharenberg et al., 2007b). In the present study, the similar TCT contents of hay and fresh

forage in both legumes agrees with the results reported from *Dalea purpurea* (Huang et al., 2016), but is contrary to the reduction of TCT content observed in sainfoin by Aufrère et al. (2008), who ascribed this result to losses of leaves and/or sun exposure.

The noticeable reduction of ECT content of silage but the similar content of hay compared to fresh forage in both legumes agrees with the results reported in sainfoin (Wang et al., 2015), *Dalea purpurea* (Huang et al., 2016) and sulla (Minnee et al., 2002). In silage, the reduction of ECT content is associated with an increase in the binding of CT to protein and/or fibre, mainly due to the reactions between the polyphenolic compounds and other plant molecules with the disruption of plant cells (Wang et al., 2015). The fractions of CT had similar behaviour in both legume forages studied, except for PBCT in silage, which increased drastically in sainfoin but not in sulla. The increase in PBCT in hay with respect to fresh forage, although only numerical in sainfoin, agrees with the results reported from *Dalea Purpurea* (Huang et al., 2016) but disagrees with the drastic decrease observed when sainfoin was dried with air forced at 30 °C (Scharenberg et al., 2007a). The effect of preservation on CT fractions seems to be affected by several factors, such as the drying conditions (under warmed forced air), the phenological stage, the variety (Scharenberg et al., 2007a; Lorenz et al., 2010; Li et al., 2014; Huang et al., 2016) or the analytical procedure and the standards used to determine PBCT and FBCT contents (Makkar, 2003; Frutos et al., 2004).

4.2 Production of gas and methane as well as IVOMD.

The effect of the preservation, which was greater for silage than for hay, on the gas and CH_4 production and on the IVOMD can be related to the change in fibre contents and CT fractions. According to Chaudhry and Khan (2012), the high content of fibre inhibits the proliferation of the rumen ciliates or protozoa, decreasing the methanogenic activity. However, the content of fibre in the current experiment was not that high. Then, the PBCT and FBCT contents could be a more plausible explanation of the changes observed as these fractions protect the protein

and fibre from attack by microorganisms interfering with the proteolytic and fibrolytic activity of bacteria (McSweeney et al., 2001; Bodas et al., 2012). Regarding the kinetics of fermentation, both methods of preservation reduced the *A* and *c* in both legume species, although the magnitude of the reduction depends on the fibre content and the fractions of CTs of each forage species (Kaplan, 2011; Saminathan et al., 2015). The reduction of *A* and *c* in preserved forages could be due to a decrease of non-structural carbohydrates, more rapidly fermentable than fibre, as consequence of the plant metabolic processes registered during preservation (Romero-Huelva et al., 2013).

It is well known that hays have lower IVOMD than fresh forages related to high fibre contents (Bal et al., 2006; Kaplan, 2011), as was observed in the present study. Silage had lower IVOMD than fresh forage in sulla, which could be related to the more acute increase in the FBCT fraction in sulla silage. The OM degraded in the rumen has a double fate: gas and VFA production and microbial biomass synthesis (Mauricio et al., 2001); thus, the decrease in gas production in both silages is advantageous to reduce the energy losses. Therefore, the higher IVOMD in silages than in hays, producing less gas and CH_4, may indicate that the degradation of OM was destined for microbial mass synthesis. However, the reduction of total gas and CH_4 production was associated with the reduction in IVOMD in hay in comparison with fresh forage.

4.3 Fermentation end-products

In the current experiment, hay and silage in both legumes had similar CP and similar NH_3-N content and total VFA production as that reported when dried and silage sainfoin was fed to lambs (Scharenberg et al., 2007b). It could have been expected that the fresh and preserved forages that had different chemical compositions and CT contents and fractions had different NH_3-H contents and total VFA production. The reasons for these results remain unclear. On the one hand, the amplitude of the effect of CT on N degradation depends on the forage

species, CT content and structure (Aufrère et al., 2008). The contents of CT and the fractions studied in the current experiment did not explain the similar NH_3-H content. Other characteristics, such as the chemical characteristics of CT (molecular weight, degree of polymerisation, prodelphinidin/procyanidin ratio, cis/trans ratio…) or CP degradability and N linked to fibre, should be studied to elucidate the reasons for the absence of differences. Huang et al. (2016) observed greater CP degradation in silage than in hay or in the freeze-dried forage during the first hours of fermentation; however, at 72 h, the degradation was similar, which agrees with the results in the present study.

The effect of preservation on the proportions of VFA varied according to the legume species studied due to the different structural carbohydrate contents and to the concentration, structure and characteristics of CTs (Gray and Pilgrim, 1952; Theodoridou et al., 2011b). In sainfoin, the increase in propionic acid of silage that concomitantly decreases H_2 availability for methanogens and reduces the CH_4 production compared to hay and fresh forage (Tavendale et al., 2005). In contrast, in sulla both preserved forages presented a reduction of the propionic acid proportion and an increase in the C_2:C_3 ratio with respect to fresh sulla, as a result of different ruminal metabolism not associated with a reduction of CH_4 production (Getachew et al., 1998; Tavendale et al., 2005). These different effects depending on the type of forage might be due to the fibre content rather than the CT fractions because the slow fermentation of structural carbohydrates in hay yields low propionic acid (Getachew et al., 1998).

The reduction of the proportions of branched-chain VFA in the presence of CT can be due to an increase in the utilization of branched-chain VFA for microbial protein synthesis (Martínez et al., 2006) and/or to the reduction of protein degradation in the rumen (Getachew et al., 2008; Hassanat and Benchaar, 2013). Studies on the effect of CT on individual proportions of VFA are not conclusive. The presence of CT in the sainfoin reduced iso-valeric and iso-butyric acid proportions (Guglielmelli et al., 2011; Calabrò et al., 2012) or had no effect on

the proportions of branched-chain VFA (Scharenberg et al., 2007b). This discrepancy and the lack of association between the proportion of branched-chain VFA and the content of CT might be closely related to the type and degree of polymerization of CT, as reported by Hatew et al. (2016).

The CH_4/VFA_{total} ratio was used to compare methanogenesis and VFA production as reported by Hassanat and Benchaar (2013). The reduced ratio together with the high IVOMD and the low CH_4 production in silages with respect to fresh forages might indicate a more efficient microbial fermentation (Guglielmelli et al., 2011). The reduction of the CH_4/VFA_{total} ratio in both preservation processes compared with the fresh forage in sulla, which might be associated with the high FBCT content because methane is mainly generated from structural carbohydrates, but the degradation by the ruminal microbes can be reduced when the fibre fraction is linked to CT.

5. Conclusions

The polyphenols, TCT content and CT-fractions were more affected in the silage than in the hay compared to the fresh forage in both legumes. The intensity of the effect of preservation on the parameters of *in vitro* fermentation depends on the type of preservation and the legume species. The silage produced less gas and methane than the fresh forage in both legumes, whereas the hay only produced less gas and methane than the fresh forage in sulla. Sainfoin silage presented a shift towards propionate proportion, reducing the $C_2:C_3$ ratio in comparison with hay and fresh forage. Ensiling might be the best preservation method in both forages considering the gas and methane production, CH_4/AGV_{total} ratio and IVOMD.

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from mjoy@cita-aragon.es

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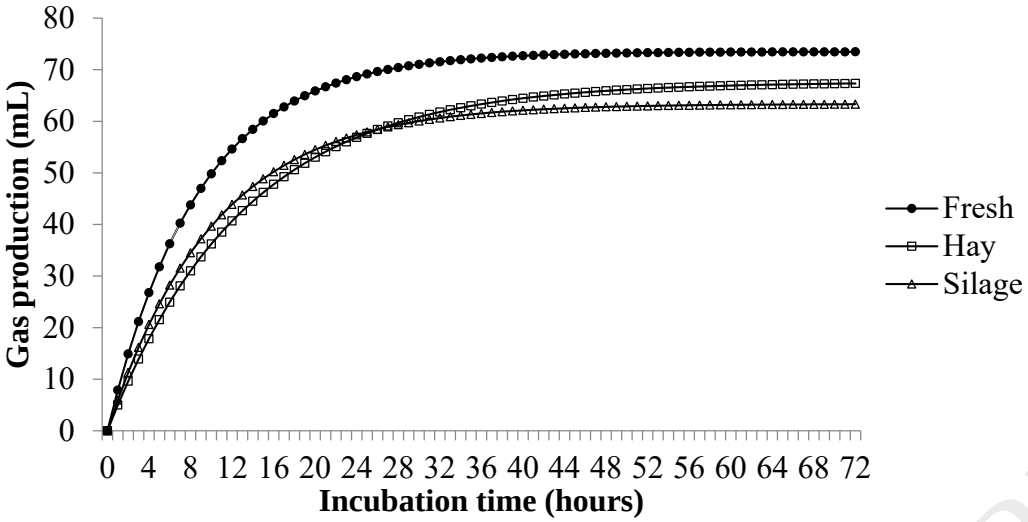

Figure 1. Effect of the conservation method on the fermentation kinetics during incubation of Sainfoin.

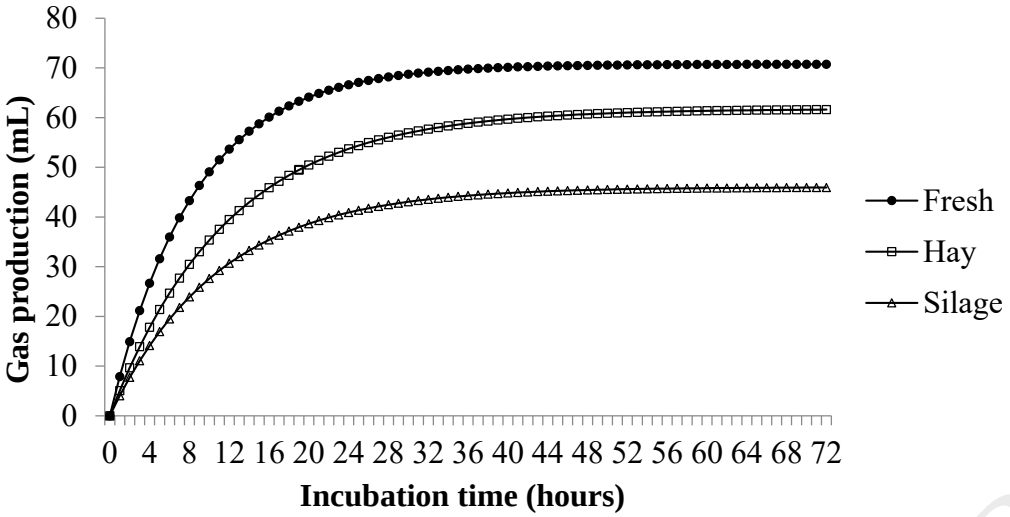

Figure 2. Effect of the conservation method on the fermentation kinetics during incubation of of Sulla.

Table 1. Effect of the preservation method on the chemical composition, polyphenols and condensed tannins (CT) of Sainfoin.

Parameters	Fresh	Hay	Silage	s.e.m.[1]	P-value
Dry Matter (DM) (g/kg)	220[c]	898[a]	293[b]	7.78	0.001
Ash (g/kg DM)	90	80	86	2.7	0.40
Crude Protein (CP) (g/kg DM)	182[a]	170[b]	168[b]	1.79	0.03
NDFom (g/kg DM)	422[b]	471[a]	456[a]	3.4	0.003
ADFom (g/kg DM)	278[b]	338[a]	332[a]	3.5	0.001
Lignin (sa) (g/kg DM)	70[c]	94[b]	107[a]	1.0	0.001
Total polyphenols (eq-g tannic acid/kg DM)	44.6[a]	47.1[a]	34.1[b]	0.53	0.001
Condensed tannins					
Total (eq-g CT/kg DM)	38.6[a]	42.2[a]	33.2[b]	0.91	0.02
Extractable (eq-g CT/kg DM)	31.6[a]	32.6[a]	8.2[b]	0.88	0.001
Protein-bound (eq-g CT/kg DM)	5.3[b]	7.3[b]	17.0[a]	0.46	0.001
Fiber-bound (eq-g CT/kg DM)	1.8[c]	2.3[b]	7.9[a]	0.04	0.001

Within a parameter, means with different letter differ at $P<0.05$

[1]standard error mean.

Table 2. Effect of the preservation on the pH, production of gas and methane (CH_4), the kinetics of fermentation, *in vitro* organic matter digestibility (IVOMD), ammonia (NH_3-H) and volatile fatty acids (VFA) after 72 h of incubation of Sainfoin.

Parameters	Fresh	Hay	Silage	s.e.m.[1]	P-value
pH	6.6[b]	6.64[a]	6.65[a]	0.034	0.01
Total gas production (mL/g dOM[2])	192[b]	205[a]	177[c]	13.5	0.001
Potential gas production (*A*) (mL)	73[a]	68[b]	63[c]	2.8	0.001
Rate of gas production (*c*) (h^{-1})	0.11[a]	0.08[c]	0.10[b]	0.010	0.001
Total CH_4 production (mL/g dOM)	51[a]	50[a]	45[b]	3.7	0.01
CH_4/gas (mL/L)	209[a]	191[b]	196[b]	9.2	0.001
IVOMD (g/kg)	797[a]	717[b]	785[a]	22.1	0.001
NH_3-H (mg/L)	160	178	159	28.2	0.28
Total VFA (mmol/L)	84	78	82	6.0	0.19
Acetic acid (C_2) (mmol/mol)	664[a]	667[a]	656[b]	4.6	0.001
Propionic acid (C_3) (mmol/mol)	155[b]	154[b]	167[a]	2.9	0.001
Butyric acid (mmol/mol)	111[a]	100[c]	104[b]	2.7	0.001
Valeric acid (mmol/mol)	16	17	17	1.2	0.15
Iso-butyric acid (mmol/mol)	18[c]	25[a]	19[b]	0.6	0.001
Iso-valeric acid (mmol/mol)	36[b]	37[a]	37[a]	0.9	0.03
C_2:C_3 ratio (mol/mol)	4.3[a]	4.4[a]	3.9[b]	0.11	0.001
CH_4/VFAtotal (mL/mmol)	3.7[a]	3.5[ab]	3.3[b]	0.34	0.05

Within a parameter, means with different letter differ at $P<0.05$

[1]standard error mean; [2] degraded organic matter.

Table 3. Effect of preservation on chemical composition, polyphenols and condensed tannins (CT) of Sulla.

Parameters	Fresh	Hay	Silage	s.e.m.[1]	P-value
Dry Matter (DM) (g/kg)	130^{c}	839^{a}	144^{b}	0.6	0.001
Ash (g/kg DM)	127^{c}	145^{b}	176^{a}	0.3	0.001
Crude Protein (CP) (g/kg DM)	220^{a}	199^{b}	192^{b}	1.3	0.005
NDFom (g/kg DM)	347^{c}	409^{b}	483^{a}	1.5	0.001
ADFom (g/kg DM)	248^{c}	310^{b}	368^{a}	0.7	0.001
Lignin (sa) (g/kg DM)	54^{c}	90^{b}	122^{a}	0.8	0.001
Total polyphenols (eq-g tannic acid/kg DM)	33.7^{a}	28.4^{b}	20.5^{c}	0.44	0.001
Condensed tannins (CT)					
Total (eq-g CT/kg DM)	30.5^{a}	32.8^{a}	23.2^{b}	0.61	0.002
Extractable (eq-g CT/kg DM)	21.6^{a}	20.0^{a}	2.1^{b}	0.39	0.001
Protein-bound (eq-g CT/kg DM)	6.6^{b}	9.1^{a}	7.9^{ab}	0.26	0.02
Fiber-bound (eq-g CT/kg DM)	2.3^{c}	3.7^{b}	13.3^{a}	0.17	0.001

Means with a,b or c letter differ at $P<0.05$.

[1]standard error mean.

Table 4. Effect of the preservation on the pH, production of gas and methane (CH_4), the kinetics of fermentation, *in vitro* organic matter digestibility (IVOMD), ammonia (NH_3-H) and volatile fatty acids (VFA) after 72 h of incubation of Sulla.

	Fresh	Hay	Silage	s.e.m[1]	P-value
pH	6.64[b]	6.68[a]	6.70[a]	0.032	0.002
Total gas production (mL/g dOM[2])	193[a]	161[b]	140[c]	11.5	0.001
Potential gas production (*A*) (mL)	71[a]	62[b]	46[c]	2.8	0.001
Rate of gas production (*c*) (h^{-1})	0.12[a]	0.09[b]	0.09[b]	0.011	0.001
Total CH_4 production (mL/g dOM)	46[a]	41[b]	34[c]	3.2	0.001
CH_4/gas (mL/L)	188[a]	188[a]	172[b]	12.3	0.001
IVOMD (g/kg)	853[a]	788[c]	822[b]	18.3	0.001
NH_3-H (mg/L)	177	171	165	25.6	0.64
Total VFA (mmol/L)	83[a]	82[a]	78[b]	3.6	0.02
Acetic acid (C_2) (mmol/mol)	651[a]	658[a]	646[b]	8.0	0.02
Propionic acid (C_3) (mmol/mol)	159[a]	154[b]	139[c]	4.0	0.001
Butyric acid (mmol/mol)	116[b]	104[c]	129[a]	3.3	0.001
Valeric acid (mmol/mol)	18[c]	19[b]	22[a]	1.3	0.001
Iso-butyric acid (mmol/mol)	19[c]	27[a]	22[b]	0.8	0.001
Iso-valeric acid (mmol/mol)	38[b]	39[b]	41[a]	1.9	0.001
C_2:C_3 ratio (mol/mol)	4.1[c]	4.3[b]	4.7[a]	0.16	0.001
CH_4/VFAtotal (mL/mmol)	3.4[a]	2.8[b]	2.4[c]	0.26	0.001

Within a parameter, means with different a, b or c superscript differ at $P<0.05$

[1]standard error mean; [2] degraded organic matter.