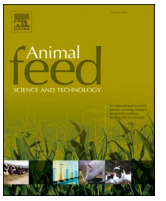
Animal Feed Science and Technology 304 (2023) 115740

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



The effect of sainfoin (*Onobrychis viciifolia* Scop.) drying temperature and pelleting on *in vitro* fermentation characteristics using equine faecal inocula

W.F. Pellikaan *, A.N.W.M. klein Breteler, A.F.B. van der Poel

Animal Nutrition Group, Wageningen University & Research, P.O. Box 338, 6700 AH Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Horse
In vitro gas production
Condensed tannin
Fibre physical effectiveness
Reactive lysine

ABSTRACT

This study was conducted to determine the effects of drying temperature and pelleting of the tanniniferous forage legume sainfoin (*Onobrychis viciifolia* Scop.) on the *in vitro* fermentation characteristics by equine faecal inocula. The sainfoin used for the experiment was air dried on the land and oven dried at 30, 70 and 130 °C for 2.5 h. Part of the dried sainfoin samples was left as dried hay, another part was pelleted, resulting in 6 different samples. The hay and pellets were tested for particle size distribution and pellets were tested for their pellet quality in terms of hardness, durability and bulk density. The gas production technique was used to simulate fermentation and faecal samples of eight randomly selected horses were used to create four independent inocula mixtures that were examined in two subsequent gas production runs. Per inocula mixture, the hay and pellet samples were incubated in triplicate as is, or in combination with polyethylene glycol to inactivate the tannins. Cumulative gas production, a proxy for the organic matter (OM) fermentation, was measured for 72 h, whereafter fermentation fluids were sampled and analysed for volatile fatty acids (VFA) and pH. The results of the pellet quality measurements showed that the particle sizes of the sainfoin particles in the pellets were decreased by a higher drying temperature or by the pelleting process, indicating a loss of fibre physical effectiveness, a factor that may be indicative for chewing activity and hence saliva production. The gas production results showed a significant ($P < 0.05$) effect of drying temperature and the addition of polyethylene glycol on the cumulative gas production, the VFA production and the percentage of branched chain VFA produced. Hay dried at 130 °C had a lower ($P < 0.05$) gas production compared to hay dried at 30 and 70 °C, which indicates a lower OM fermentability. The addition of polyethylene glycol increased the gas and total VFA production, which indicates that tannins in sainfoin decrease the production of gas and VFA in hay pellets compared to the hay product. The pelleting process had no effect on the fermentation characteristics, but pelleting conditions strongly decreased the level of the amino acid lysine and its reactivity. The results indicate the increase in drying temperature to cause a decrease in the OM hindgut fermentation and the total and reactive lysine contents of sainfoin. The positive effects of the tannins (a higher

Abbreviations: A, asymptotic gas production (mL/g OM); B, switching characteristic; BCP, branched chain volatile fatty acid production; C, halftime (h) of gas production; DM, dry matter; F, form (hay versus hay pellet); GMD, geometric mean diameter; GP, cumulative gas production (mL/g OM); GSD, geometric standard deviation; HAc, acetic acid; HBu, butyric acid; HPr, propionic acid; OM, organic matter; PEG, polyethylene glycol; Rmax, maximal rate of gas production per hour (mL/h); SEM, standard error of the mean; T, drying temperature; Tmax, time at which Rmax occurs (h); VFA, volatile fatty acid(s).

* Corresponding author.
E-mail address: wilbert.pellikaan@wur.nl (W.F. Pellikaan).

https://doi.org/10.1016/j.anifeedsci.2023.115740
Received 26 April 2023; Received in revised form 14 July 2023; Accepted 18 July 2023
Available online 26 July 2023

proportion of propionic acid relative to acetic acid and a lower protein fermentation) remain, despite of technological treatments.

1. Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume, which can be used by horses for grazing or can be used for making hay or silage. It has some interesting characteristic constituents such as tannins and polyphenols, which have been shown to have anthelmintic effects in horses (Collas et al., 2018) and ruminants (Barrau et al., 2005), to increase protein utilization in ruminants and has the potential to contribute to the reduction of greenhouse gasses and prevents ruminal bloat (McMahon et al., 1999; Mueller-Harvey et al., 2019; Rufino-Moya et al., 2019). The condensed tannins (proanthocyanidins) in sainfoin bind to plant protein during drying, ensiling and in the animal itself (Mueller-Harvey, 2006) and this effect may also account for the horse.

Horses are monogastric hindgut fermenters and are adapted to continuous feeding of high fibrous diets (Al Jassim and Andrews, 2009; Dicks et al., 2014) for the optimal functioning of their gastrointestinal tract. In the hindgut, a large variety of microbiota is present that includes cellulolytic, hemicellulolytic, amylolytic, proteolytic and lactate fermenting bacteria, anaerobic fungi, methanogen population and protozoa (Costa et al., 2015; Edwards et al., 2020; Julliand and Grimm, 2016). The feed is fermented for 21–27 h in the hindgut (Hansen et al., 2020; Miyaji et al., 2008) and in a well-balanced situation the pH in the caecum and colon ranges between 6.1 and 6.8 (de Fombelle et al., 2003; Julliand et al., 2001). The fermentation of structural carbohydrates in horses occurs in the caecum and colon by microbial enzymes. End-products of the fermentation are volatile fatty acids (VFA), mainly acetic, butyric and propionic acids, which are absorbed and used as energy (Al Jassim and Andrews, 2009; Vermorel and Martin-Rosset, 1997). For horses, approximately 1.0 g VFA is produced per kg of body weight (Dicks et al., 2014).

Sainfoin can be provided as fresh material, hay and silage (Hayot Carbonero et al., 2011). There is little information on the preservation technology of drying sainfoin to a forage hay. The main reason for drying hay is usually to preserve it for storage and the moisture content needs to be reduced to 80–150 g/kg. A moisture content of more than 200 g/kg can have negative effects on chemical and hygienic quality. It may result in moulding, in Maillard reactions and facilitates fungi growth, which produce toxins (Siles et al., 2015). Industrial drying under high temperature is preferred over natural climatic drying on the field due to more rapid and controlled conditions for drying. For alfalfa it is known that drying temperatures can vary from 40 °C to 800 °C (Adapa et al., 2004) depending on the drying method and residence time. High temperatures in the product itself, however, need to be avoided to prevent loss of nutrients such as lysine, certain vitamins and bio-active compounds (Pickford, 1992; Larrauri et al., 1997). Considering the colour and protein solubility, 175 °C was shown to be an optimum air temperature for alfalfa drying using a rotary drum (Sokhansanj and Patil, 1996).

Another benefit of preserving forage by industrial drying and pelleting is that it allows for easier storage and transport conditions. For pelleting, material needs to be ground prior to the pelleting process, which results in a reduced mean particle size (Lyu et al., 2020). A reduction in particle size decreases the physically effective fibre fraction (Cozzi et al., 2002), a term originating from ruminant nutrition to describe the physicochemical properties of a feed in terms of particle size and fibre content, and its effect on feed intake behaviour, saliva production and rumen functioning (Zebeli et al., 2012). This concept was introduced in equine nutrition by Brøkner et al. (2008) and Vervuert et al. (2013), who studied chewing activity in horses as affected by different types of feed. A reduced feed particle size has been shown to reduce the chewing activity in horses (Ellis et al., 2010; Petz et al., 2023), which may be associated with a decrease in saliva production.

The impact of feed process technological treatments on digestive processes occurring in the equine hindgut can be studied using an *in vitro* gas production technique (IVGPT), where the measured gas production can be used as a proxy for the organic matter (OM) fermentation; Kujawa et al. (2020); Lowman et al. (1999); Murray et al. (2014)).

The objective of the experiment reported here was to determine the effect of drying temperature during the process of hay making and subsequent pelleting of sainfoin on the physicochemical properties and the extent and kinetics of fermentation using an automated *in vitro* gas production system with equine faecal inocula, and to determine whether the biological effect of the tannins remains after the technological treatments. It was hypothesized that the combination of increasing drying temperature and pelleting will have a negative effect on the overall fermentation activity (i.e. gas production) and will compromise the biological effect of tannins.

2. Materials and methods

2.1. Sainfoin, technological treatments, substrates

The sainfoin cultivar 'Esparcette' used for this study was grown on clay soil near the township Kortgene, Zeeland, The Netherlands (51° 34' N, 3° 48' E). The crop was harvested in the autumn in late September and dried for four days on the land with max day time temperatures ranging between 16.2 and 17.3 °C and no precipitation recorded (Weather station Vlissingen, Royal Netherlands Meteorological Institute (KNMI)). A representative sample of ~ 50 kg pre-wilted product was send to Wageningen University. To determine the effect of technological treatments on the fermentation characteristics, six different technological treatments were conducted.

The sainfoin hay batch was randomly divided into batches by spreading it on the floor and dividing the whole sample into 24 portions. Each portion was then randomly assigned to one of the three sub-batches, which were randomly assigned to three drying temperatures of 30, 70 and 130 °C. All three sub-batches sainfoin hay were dried at the assigned temperature on trays in a forced

draught air drying oven for 2.5 h. After the drying each sub-batch contained approximately 8 kg of sainfoin hay. The hay was then carefully divided into two groups to obtain a 1-kg sample (hay as a reference) and a 7-kg hay sample to be pelleted. For pelleting, hay samples were ground using a hammer mill (LHM20/16, 1.5 kW; Condux International, Mankato, United States of America) with a sieve size of 4.5 mm. These dried samples were conditioned with some water and thereafter pelleted with a (pre-heated) Monorol Labour pellet press using a die size of Ø 5 × 25 mm. As a result, six different sainfoin substrates for the *in vitro* fermentation studies were created, referred to as sainfoin hay (dried at 30, 70 and 130 °C) and subsequent hay pellets.

2.2. Animals and inocula preparation

Equine faecal samples, used as inocula, were collected from eight warmblood horses, randomly selected from the herd present at riding school Quadenoord, Renkum, The Netherlands. The horses were individually housed part of the day with day and night access to pasture. In addition, animals received haylage and concentrates, and had free access to water. As faecal samples were obtained opportunistically from animals at a riding school no exact details on the animals feed intake nor on the nutritional composition of the diet could be obtained. Fresh faecal samples were collected upon voiding of each horse in the morning of the gas production runs, preserved in insulated containers that were pre-warmed and pre-flushed with CO_2 to maintain an anaerobic environment at 39 °C, transported to the laboratory and processed within 2 h after collection. Per run, faecal samples of four horses were collected and equal portions (w/w) of two faecal samples (randomly selected) were pooled to create two different inocula mixtures. The faecal mixtures were mixed with a pre-warmed (39 °C) protein free bicarbonate-phosphate buffer (1:3 w/v) as described by Desrousseaux et al. (2012). Faeces and the bicarbonate-phosphate buffer were mixed with a commercially available immersion blender (Type 4191, 600 W, Braun GmbH, Germany), followed by filtering through two layers of cheese cloth, thus creating the inoculants. All handlings were done under a continuous flow of CO_2 to ensure anaerobic conditions.

2.3. In vitro fermentation studies

To prepare the substrates for the gas production technique, the sainfoin hay samples were ground with a cross beater mill (Peppink 200AN) and the hay pellet samples with a centrifugal mill (Retsch ZM200), both with a sieve size of 1 mm.

The fermentation characteristics of the substrates were determined by *in vitro* gas production technique based on the modified procedure of Cone et al. (1996) as described by Desrousseaux et al. (2012). Approximately 0.5 g of the substrates was weighed into 250-mL fermentation bottles (Schott, Mainz, Germany) in triplicate. In addition, triplicate bottles were prepared containing substrates (0.5 g) in combination with 1.0 g polyethylene glycol (PEG; MW 6000) to inactivate the tannins (Makkar et al., 1995). Polyethylene glycol has a high affinity for tannins, which makes it bind to tannins; in this way it inhibits the binding of tannins to proteins or other feed constituents (Hayot Carbonero et al., 2011; Azuhnwi et al., 2011). All bottles were placed in a shaking water bath and were pre-warmed at 39 °C and pre-flushed with CO_2 prior to inoculation with 60 mL of one of the inoculants. After inoculation, bottles were directly returned to the shaking water bath and incubated for 72 h at a temperature of 39 °C. Per inocula, two blanks were incubated. After 72 h, the incubation was stopped and the bottles were placed on ice to arrest microbial activity. The fermentation fluids of bottles were sampled for VFA analyses and the pH was recorded.

For VFA analyses, aliquots of 600 μL fermentation fluid were mixed with 600 μL of an internal standard solution composed of 19.68 mM of 4-methylpentanoic acid in a 4.25% ortho-phosphoric acid solution in 1.5-mL Eppendorf safe lock tubes. Subsequently, samples were thoroughly shaken and centrifuged at 14,000g for 5 min at 20 °C, where after the cleared supernatants were transferred to crimp neck vials and stored at −20 °C pending analyses.

In total, two gas production runs were conducted, with in each run the six sainfoin samples, each using two different inocula mixtures. Per inocula mixture, the samples were incubated in triplicate, with or without PEG and per inocula blanks were included in duplicate.

2.4. Laboratory chemical analysis

Samples of ground hay and ground hay pellets were analysed for dry matter (DM) by drying to a constant weight at 103 °C (ISO 6496) and for ash by incinerating at 550 °C (ISO 5984), to determine organic matter (OM) content.

Proanthocyanidin (PA) contents were analysed following the modified procedure of Grabber et al. (2013). In brief, 30 mg of finely ground (ball mill MM 2000; Retsch Technology GmbH, Haan, Germany) samples were incubated in a water bath at 70 °C for 2.5 h with 15 mL of a modified acetone-butanol-HCl-iron reagent (40 mg ammonium iron(III) sulphate dodecahydrate, 3.3 mL of water, 5.0 mL 12-M HCl, 42 mL n-butanol, 50 mL acetone) in 25-mL thick-walled glass tubes and sealed with screw caps. After 45 min cooling, reactants were decanted into 2-mL Eppendorf safe lock tubes and centrifuged for 2 min at 10,000g (Z383 universal highspeed centrifuge, Hermle, Germany). The supernatants were transferred into quartz cuvettes and absorbance measured at 550 nm spectrophotometrically (Evolution 201, Thermo Scientific, Breda, The Netherlands). Modified acetone-butanol-HCl-iron reagent was used as a blank and as a diluent to keep absorbance levels below 0.6. As no external PA standard for sainfoin was available, tannin content is expressed as absorbance readings at 550 nm per g DM.

Total lysine (TL) was analysed by acid hydrolysis at 110 °C for 23 h and ion-exchange chromatography with post-column derivatization with ninhydrin (ISO 13903; ISO 2005).

Reactive lysine (RL) was analysed using O-methylisourea (OMIU) according to the method described by Moughan and Rutherfurd (1996).

The pH of the fermentation medium with the inoculum was measured using a digital pH metre (Hanna Instruments Model HI 9024, IJsselstein, the Netherlands).

Volatile fatty acid concentrations were determined by injecting 0.1 μL sample volumes on a gas chromatograph (GC; Trace Ultra, Thermo Scientific, Breda, the Netherlands) with a split/splitless injector operated in split mode (split ratio 1:9) and fitted to a flame ionisation detector (FID), using a capillary column (Agilent HP-FFAP, 30 m, i.d. 0.32 mm, film thickness 0.25 μm) with H_2 as the carrier gas (25 kPa pressure). The starting temperature of the column was set at 80 °C for 1 min followed by an 20 °C/min increase to 120, 6.1 °C/min increase to 160 and 3.0 °C/min increase to 205 °C at which point the temperature was maintained for 2 min. The temperature of the injection inlet and of the FID were both set at 260 °C. 4-Methylpentanoic acid was included as the internal standard and used to identify the individual straight-chain (acetic acid, propionic acid, butyric acid and valeric acid) and branched chain (isobutyric acid and isovaleric acid) VFA.

The total VFA concentrations were calculated as the sum of straight and branched chain VFA and expressed as mmol/L. The percentage of branched chain VFA (BCP) was calculated as the sum isobutyric acid and isovaleric acid concentrations scaled to the total VFA concentrations.

2.5. Particle size and pellet physical quality analysis

The particle size distribution of the sainfoin material was determined in duplicate for the hay by dry sieving and for hay pellet samples by wet sieving procedures. A Retsch AS 200 Control apparatus was used. For wet sieving of hay pellets, tap water was used to soak the material, fill the sieving apparatus and to flush the sieves. The dry matter weight of the samples and filters were measured in advance. Representative samples of 25–30 g were soaked for 90 min, to allow the particles in the pellets to fall apart, and sieved for about 46 min with three times filling the apparatus with water under amplitude and three times emptying the apparatus without amplitude. Six different sieves sizes were used: 2.5, 1.25, 0.63, 0.315, 0.160 and 0.071 mm. The sieved fractions were transported to filters, oven dried at 70 °C overnight followed by 3-h drying at 103 °C and weighed to determine the weight per fraction. The average particle size was expressed as the geometric mean diameter in μm (GMD) with a geometric standard deviation (GSD) (ASAE, 2008).

Hay pellet samples were analysed for their bulk density (apparent density of pellets/L; duplicate samples), hardness (kg force using an automated Kahl device; ten samples) and durability (% intact pellets after mechanical stress in a Holmen pellet tester; duplicate samples) as described in detail by Van Rooijen et al. (2014).

2.6. Curve fitting and statistical analyses

The gas production curves were fitted with a modified bi-phasic Michaelis-Menten equation as described by Groot et al. (1996), with a lag time included in the first phase, by non-linear least squares regression analyses using the NLIN procedure of SAS (2017), Eq. 1:

$$GP = (A1/(1 + (C1/(t - TT1))^{B1})) + (A2/(1 + (C2/t)^{B2})) \quad (1)$$

where GP is the cumulative gas produced (mL/g OM) at time t (h), A1, A2 is the asymptotic gas production for phase one and two (mL/g OM), B1, B2 is the switching characteristic for phase one and two that determines the shape of the curve, C1, C2 is the time (h) at which halve of A1 or A2 is reached, and TT1 is the lag time (h) associated with the first phase. Based on the gas production results the time of the maximal gas production in hours (Tmax) and the maximum rate of gas production for phase one and two (Rmax1, Rmax2; in mL/h), and the time at which Rmax was reached (Tmax1, Tmax2) were calculated as described by Yang et al. (2005).

Per inoculum treatment combination, replicate bottles were checked for outliers and excluded based on the coefficient of variation (CV > 10%) in combination with visual evaluation of curves. The remaining replicates were used to calculate average values per individual inoculum-substrate-PEG treatment combination that were considered as statistical units.

The effect of drying temperature (T), sainfoin form (F) and the addition of PEG (P) were analysed as a 3 × 2 × 2 factorial design, with inocula nested within run considered as random factor, using the MIXED procedure of SAS (2017), Eq. 2. The statistical model was expressed as:

$$Y_{ijk} = \mu + T_i + F_j + P_k + (T \times F)_{ij} + (T \times P)_{ik} + (T \times F \times P)_{ijk} + \varepsilon_{ijk} \quad (2)$$

where Y_{ijk} is the dependent variable, μ the overall mean, T_i is the drying temperature (i = 30, 70, 130 °C), F_j is the form of the product after technological processing (j = Sainfoin hay, hay pellets), P_k is the effect of PEG (k = NoPEG, PEG), $(T \times F)_{ij}$, $(T \times P)_{ik}$ and $(T \times F \times P)_{ijk}$ are the interaction terms and ε_{ijk} is the error term. Differences among treatment means were analysed by Tukey-Kramer's multiple pairwise comparison procedure with results reported as least square means and declared significant at $P \leq 0.05$, and a trend in case $0.05 < P \leq 0.10$.

3. Results

3.1. Processing (drying and pelleting)

The DM content of samples after the 2.5 h drying procedure was ≥ 916.1 g/kg and numerically increased at increasing drying temperatures for both the dried sainfoin hay and hay pellet samples (Table 1). Expressed per kg DM, OM has similar values for the all

treatment samples. Drying at higher temperatures decreases the samples coarse fractions numerically, where the middle and fine fractions are increased. This shift in particle size distribution is reflected by a the numerical decrease of the GMD. The GMD of particles from pellets show a further decrease in size due to the pelleting process. For the pellet samples, higher drying temperatures numerically increase bulk density with no effect on pellet durability. Drying at 130 °C and subsequent pelleting numerically decreases the pellet hardness. For total and reactive lysine (TL and RL, respectively), differences in dried sainfoin samples are small. Hay pellets, dried at 70 and 130 °C show a numerical decrease in both TL and RL, paralleled by a numerical decrease in the TL/RL ratio. The tannin concentration in the sainfoin samples is expressed as the absorbance at 550 nm. The sainfoin hay samples show a small numerical decrease in absorbance when the drying temperature is increased; for the pelleted samples a numerically higher absorbance is shown at all drying temperatures.

3.2. *In vitro gas production and fermentation characteristics*

Drying temperature had a significant ($P \leq 0.041$) effect on GP, A1, A2 and B1 and the maximum rates of gas production for the first and second phase (Rmax1 and Rmax2, respectively) (Table 2). Gas production caused by fermentation of sainfoin samples dried at 130 °C (217.4 mL/g OM) was lower ($P < 0.0001$) than the GP of samples dried at 30 and 70 °C (230.1 and 229.0 mL/g OM, respectively). Sainfoin samples dried at 30 °C gave an asymptotic GP of the first phase (A1) of 196.9 mL/g OM, which tended ($0.056 \leq P \leq 0.068$) to be higher than A1-values of samples dried at 70 and 130 °C (185.1 and 184.6 mL/g OM, respectively). The asymptotic GP of the second phase (A2) for samples dried at 70 °C (67.9 mL/g OM) was higher ($P = 0.027$) than those dried at 130 °C, but did not differ from the A2 of samples that were dried at 30 °C (55.5 mL/g OM). The switching characteristic (B1) for samples dried at 130 °C (1.31) was higher ($P = 0.024$) compared to samples dried at 30 °C (1.14), but did not differ from those that were dried at 70 °C (1.26). The Rmax1 for samples dried at 130 °C (12.95 mL/h) was lower ($P < 0.0001$) than the Rmax1-values of samples when dried at 30 and 70 °C (15.76 and 14.97 mL/h, respectively). The Rmax2 showed a similar effect of drying temperature (30 °C, 6.60 mL/h; 70 °C, 6.38 mL/h; 130 °C, 5.75 mL/h), but difference was only significant ($P = 0.040$) between drying temperature treatments 30 and 130 °C.

The sainfoin form (hay or hay pellets) had a significant ($P = 0.045$) effect on B1 and tended ($0.054 \leq P \leq 0.073$) to affect A1, A2 and the halftime of the second phase (C2). Pelleted sainfoin hay tended ($P = 0.054$) to give more gas in the first phase (A1) compared to sainfoin hay (193.0 vs. 184.7 mL/ g OM), whilst the A2 for hay pellets was lower ($P = 0.073$) compared to that of hay (51.6 vs. 62.7 mL/g OM).

Addition of PEG increased ($P < 0.001$) the GP (232.5 vs. 218.5 mL/g OM, PEG vs. NoPEG), the A1 (196.8 vs. 180.9 mL/g OM) and Rmax1 (15.48 vs. 13.63 mL/h), and decreased ($P < 0.001$) the C1 (9.3 vs. 10.7 h).

A temperature × form interaction tended ($P = 0.071$) to be present only for GP (Table 2). Sainfoin hay dried at 130 °C showed a

Table 1
Chemical analysis, particle size distribution and physical quality of pellets of dried sainfoin hay and pellet samples.

	Sainfoin hay			Sainfoin hay pellets		
Drying temperature (°C)	30	70	130	30	70	130
Chemical analyses						
Dry matter (g/kg)	916	942	962	942	946	950
Organic matter (g/kg DM)	907	908	906	903	904	904
Total lysine (TL; g/kg OM)	8.0	8.3	7.9	8.2	6.7	6.4
Reactive lysine (RL; g/kg OM)[a]	7.2	7.5	7.2	7.3	5.6	5.1
RL/TL (%)	89	91	90	89	84	79
Condensed tannins (Abs 0.550 nm)[b]	10.5	10.0	9.3	12.5	10.5	15.2
Size fractions (% DM)[c,d]						
Coarse Fr > 2500 μm	5.2	7.5	4.5	0.1	0.3	0.0
1250 < Fr < 2500 μm	18.2	17.4	10.1	6.8	5.1	3.8
630 < Fr < 1250 μm	19.5	20.4	21.8	21.2	19.0	17.8
Middle 315 < Fr < 630 μm	16.2	16.7	23.2	20.0	22.0	25.3
160 < Fr < 315 μm	5.4	4.6	7.2	9.7	11.6	11.6
Fine 71 < Fr < 160 μm	1.8	1.1	2.1	4.4	4.3	6.1
Fr < 71 μm	33.7	32.3	31.1	37.8	37.7	35.4
GMD ± GSD (μm)	837 ± 2.2	894 ± 2.2	682 ± 2.1	532 ± 2.1	494 ± 2.1	451 ± 2.1
Pellet physical quality[e]						
Bulk density (g/l)				557 ± 6.6	588 ± 2.2	626 ± 4.9
Pellet durability (%)				80.4 ± 0.4	78.0 ± 0.9	79.9 ± 2.2
Pellet hardness (kg)				8.6 ± 3.4	8.5 ± 1.7	7.2 ± 1.6

[a] O-methylisourea (OMIU)-reactive lysine.
[b] Absorbance values at 550 nm in the dry matter.
[c] Fr: fraction retained on sieve; GMD: geometric mean diameter; GSD: geometric standard deviation. GMD and GSD calculated as described in ASAE (1997).
[d] Hay samples, results of dry sieving; hay pellet samples,' results of wet sieving.
[e] Mean ± SD values for bulk density and pellet durability determined from n = 3 analytical replicates. Pellet hardness was determined on n = 10 analytical replicates.

Table 2
The effect of drying temperature, form (sainfoin hay or hay pellets) and PEG addition on the cumulative gas production and gas production characteristics.[a].

		GP		A1		B1		C1		Rmax1		A2		B2		C2		Rmax2	
		-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG
Sainfoin hay	30	224.5	242.3	180.1	207.2	1.20	1.20	11.2	8.6	14.0	17.4	68.8	53.0	5.74	7.97	19.1	17.4	7.20	6.61
	70	229.6	231.9	174.8	180.9	1.37	1.33	9.8	8.5	14.5	15.3	89.0	71.0	4.47	5.32	24.8	18.8	6.72	5.79
	130	203.8	227.2	169.0	196.2	1.27	1.35	10.7	10.1	11.6	13.1	50.1	44.1	7.20	6.73	17.7	19.0	6.24	5.19
Sainfoin hay pellets	30	215.5	238.2	183.5	216.9	1.13	1.04	9.9	10.1	14.3	17.3	54.2	46.0	6.67	7.76	17.1	14.4	6.24	6.34
	70	223.5	231.1	195.6	189.2	1.19	1.15	11.1	9.5	14.5	15.5	48.3	63.4	7.29	6.44	17.6	17.9	6.22	6.81
	130	214.2	224.4	182.4	190.8	1.21	1.40	11.7	8.9†	12.8	14.3	51.0	46.9	6.51	7.62	17.4	19.2	6.15	5.42
SEM		13.11		17.16		0.191		1.05		1.51		12.93		1.305		6.76		1.687	
P-value[b]																			
Temperature (T)		< 0.0001		0.035		0.026		0.343		< 0.0001		0.034		0.180		0.157		0.041	
Form (F)		0.260		0.054		0.045		0.246		0.142		0.073		0.164		0.062		0.733	
PEG (P)		< 0.0001		0.001		0.703		< 0.001		< 0.0001		0.307		0.254		0.326		0.123	
T × F		0.071		0.566		0.315		0.291		0.282		0.222		0.358		0.366		0.396	
T × P		0.004		0.020		0.248		0.865		0.021		0.766		0.459		0.261		0.502	
T × F × P		0.146		0.465		0.880		0.047		0.967		0.453		0.635		0.468		0.395	

a GP: cumulative gas production (mL/g OM); A1 + 2: asymptotic gas production for phase one and two (mL); B1 + 2: switching characteristic of the gas production curve; C1 + 2: time in hours when half of the gas is produced (h); Tmax1 + 2: time of the maximal gas production in hours for phase one and two (h); Rmax1 + 2: maximal gas production rate per hour for phase one and two (mL/h); -PEG: without the addition of polyethylene glycol; +PEG: with the addition of polyethylene glycol; SEM: standard error of the mean; T: drying temperature (°C); F: form (hay or pellet); P: with or without PEG. The average of the inoculum-substrate-PEG treatment combination is considered as experimental unit.
b interaction term Form × PEG was in all cases non-significant, and therefore excluded from the model.
Symbols indicate a significant difference within rows between -PEG and +PEG (†: $P \leq 0.10$; *: $P < 0.05$).

lower ($P \leq 0.001$) GP than drying at 30 and 70 °C. Hay pellets showed a similar but non-significant ($P \geq 0.130$) response.

A temperature × PEG addition interaction ($P \leq 0.021$) was observed for GP, A1 and Rmax1. In case of PEG addition, drying at 130 °C showed a lower GP than drying at 30 °C ($P < 0.001$), but did not differ from drying at 70 °C ($P = 0.454$). Without PEG addition, drying at 130 °C gave a lower GP than drying at 30 °C ($P = 0.015$) and 70 °C ($P < 0.0001$). For substrates without PEG addition no

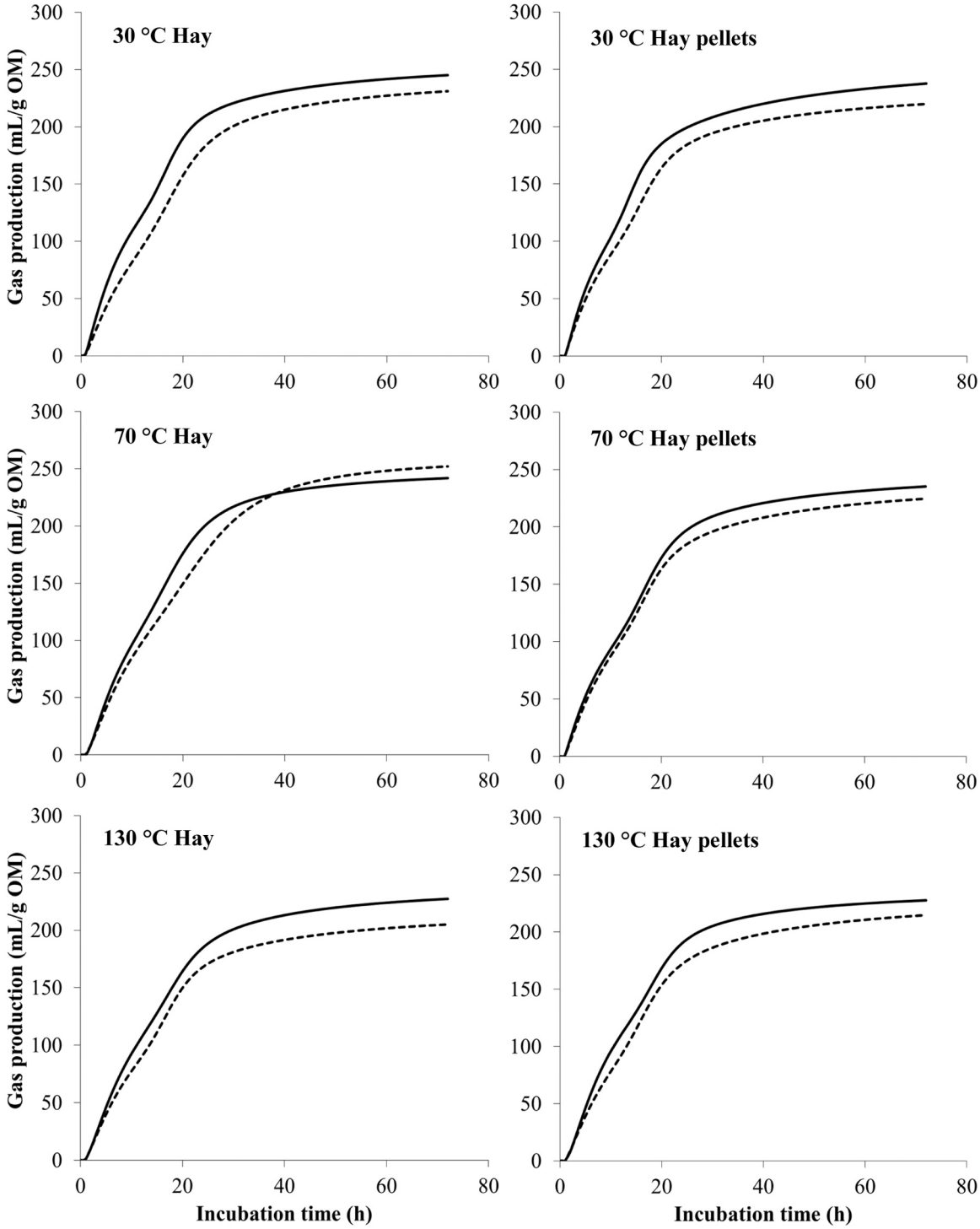


Fig. 1. The effect of polyethylene glycol (PEG) addition (with PEG (——) or without PEG (– – –)) on the cumulative gas production values for sainfoin hay dried at 30, 70 and 130 °C and sainfoin hay, dried at 30, 70 and 130 °C with pelleting afterwards. The average of the inoculum-substrate-PEG treatment combination is considered as experimental unit.

effects of drying temperature were observed for A1 ($P \geq 0.779$), whereas PEG addition showed a higher A1 for 70 °C ($P = 0.009$), but did not differ from 130 °C ($P = 0.137$). In the case of PEG addition, drying at 30 °C showed the highest Rmax1 and differed from Rmax1 observed after drying at 70 °C ($P = 0.020$) and 130 °C ($P \leq 0.0001$). Without PEG addition, drying at 130 °C gave a lower Rmax1 than drying at 30 °C ($P = 0.028$) and 70 °C ($P = 0.005$).

With respect to the three-way interaction term (temperature × form × PEG) only C1 was affected significantly ($P = 0.047$; Table 2).

The effect of drying temperature on the gas production curve is visualised in Fig. 1. Drying at 130 °C gives a lower cumulative gas production with slight changes in the shape of curves, suggestion an effect on the extent and kinetics of fermentation. In general, a small but clear lag time of 0.93 h ± 0.78 h (mean ± SD) was observed with a maximum of 2.30 h.

Neither temperature nor PEG treatment affected the lag time, but form did show a small but significant ($P = 0.019$) difference in lag time between sainfoin hay pellets (0.83 h) versus sainfoin hay (1.03 h).

3.3. Volatile fatty acids and pH

Drying temperature had a significant ($P \leq 0.026$) effect on the pH and all fermentation end-products except for butyric acid (Table 3). The pH at 130 °C was higher ($P = 0.021$) compared to 30 °C, but did not differ from 70 °C ($P = 0.215$). The total VFA at 130 °C (61.16 mM) was lower ($P \leq 0.009$) than the total VFA measured at 30 and 70 °C (62.52 and 63.14 mM, respectively). The proportion of acetic acid at 130 °C (0.640) was higher ($P < 0.001$) than the proportions measured at 30 and 70 °C (0.631 and 0.632, respectively). The proportion of propionic acid at 130 °C (0.255) was lower ($P < 0.001$) than those measured at 30 and 70 °C (0.262 and 0.262, respectively). The branched chain VFA percentage at 130 °C (2.95%) was lower ($P \leq 0.028$) than those at 30 and 70 °C (3.00% and 3.01%, respectively.

The form (sainfoin hay or hay pellets) had a significant ($P \leq 0.013$) effect on the total VFA and the percentage of branched chain VFA, but did not affect the relative proportions of acetic-, propionic-, and butyric acid, nor did it affect pH. Pelleted sainfoin hay had a lower ($P = 0.013$) total VFA compared to sainfoin hay (61.81 vs. 62.74 mM), and a lower ($P = 0.012$) branched chain VFA percentage compared to hay (2.97 vs. 3.01%).

Addition of PEG had a minor but significant ($P \leq 0.010$) effect on all fermentation end-product parameters (Table 3). Addition of PEG increased the pH (6.7 vs. 6.68, PEG vs. NoPEG; $P = 0.010$), the total VFA (63.26 vs. 61.29 mM; $P = 0.010$), the proportion of butyrate (0.065 vs. 0.063; $P < 0.0001$) and the branched chain VFA percentage (3.15 vs. 2.82%; $P < 0.0001$). In contrast, PEG addition decreased the proportions of acetic acid ($P = 0.007$) and propionic acid ($P < 0.0001$).

A temperature × form interaction ($P = 0.010$) was observed for the total VFA and a trend was observed for pH ($P = 0.076$) and branched chain VFA ($P = 0.068$). Within sainfoin hay treatment no effect ($P > 0.151$) of drying temperature was observed, whereas pelleted sainfoin hay dried at 130 °C resulted in a lower ($P \leq 0.004$) total VFA compared to drying temperatures of 30 and 70 °C. Sainfoin hay pellets dried at 130 °C had a lower ($P = 0.014$) total VFA compared to hay dried at 130 °C. For the other temperature treatments (i.e., 30 and 70 °C) no such effects of form were observed.

With respect to the temperature × PEG addition interaction only a trend ($P = 0.086$) was observed for the percentage of branched chain VFA.

For the three-way interactions term (temperature × form × PEG), no effects on fermentation end-products were observed.

Table 3
he effect of drying temperature, form (sainfoin hay or hay pellets) and PEG addition on the pH and volatile fatty acid production.[a].

		pH		Total VFA		HAc		HPr		HBu		BCP	
		-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG
Sainfoin hay	30	6.68	6.70	61.4	63.1	0.631	0.630	0.266	0.260	0.062	0.064	2.81	3.21
	70	6.68	6.69	62.3	65.2	0.631	0.630	0.265	0.260	0.063	0.065	2.89	3.20
	130	6.69	6.70	61.0	63.5	0.640	0.639	0.257	0.253	0.063	0.066	2.82	3.12
Sainfoin hay pellets	30	6.67	6.68	61.9	63.8	0.633	0.630	0.264	0.260	0.063	0.065	2.81	3.16
	70	6.69	6.70	61.9	63.2	0.635	0.631	0.263	0.260	0.063	0.064	2.80	3.16
	130	6.70	6.71	59.3	60.9	0.641	0.640	0.257	0.253	0.063	0.065	2.79	3.06
SEM		0.008		2.03		0.0066		0.0074		0.0052		0.267	
P-value[b]													
Temperature (T)		0.026		< 0.001		< 0.0001		< 0.0001		0.184		0.004	
Form (F)		0.569		0.013		0.110		0.228		0.760		0.012	
PEG (P)		0.010		< 0.0001		0.007		< 0.0001		< 0.0001		< 0.0001	
T × F		0.076		0.010		0.593		0.770		0.068		0.668	
T × P		0.683		0.925		0.687		0.605		0.717		0.086	
T × F × P		0.791		0.476		0.753		0.669		0.962		0.662	

Symbols indicate a significant difference within rows between -PEG and +PEG (†: $P \leq 0.10$; *: $P < 0.05$).

[a] VFA: volatile fatty acid production (mmol/L); HAc: proportion of acetic acid of total VFA; HBu: proportion of butyric acid of total VFA; HPr: proportion of propionic acid of total VFA; BCP: percentage of branch chain VFA (isobutyric and isovaleric acid/ total VFA × 100) (%); SEM: standard error of the mean; T: drying temperature (°C); F: form (hay or pellet); -PEG: without the addition of polyethylene glycol; +PEG: with the addition of polyethylene glycol. The average of the inoculum-substrate-PEG treatment combination is considered as experimental unit.

[b] interaction term Form × PEG was in all cases non-significant, and therefore excluded from the model.

4. Discussion

4.1. Physicochemical effects of processing conditions

Pelleting as a process has been shown to have a particle size reducing effect during the downstream processing of animal diets (Engberg et al., 2002), and when applied to forages it may affect the physicochemical properties. Forages are the vegetative parts of plants consumed by animals that are characterized by a high fibre content in the form of structural carbohydrates, that provide fibrous bulk (Lee, 1988; NRC, 2007) and increase the time spent on eating and chewing (Ellis, 2010).

In the current study, both sainfoin hay and hay pellets dried at 130 °C showed a reduced mean particle size, caused by an increased number of middle (hay) or fine (hay pellet) particle size fractions. Reduction of particle size due to a pelleting process decreased the physical effectiveness of the fibre fraction of lucerne, when lucerne was dehydrated and pelleted (Cozzi et al., 2002). The change in physical characteristics as observed in the current trial also suggests that the physical effectiveness of sainfoin is negatively affected by both the drying and the pelleting process. This may lead to a reduction in eating time and chewing activity, and hence, it may be that the sainfoin cannot be considered anymore as a forage source, which is in line with the observations of Cozzi et al. (2002). This could be an important limitation if sainfoin is intended to be used as a fibrous forage. Fibrous feeds are required to stimulate the chewing activity, which, especially for horses, is important for the excretion of saliva (Elia et al., 2010). In addition, the fibrous fractions in the feed may affect the degradation pattern in the hindgut of a horse differently as a function of particle size. In theory, when the particle size is reduced, the surface area of a substrate increases, which would make the substrate more accessible for microbial degradation, and hence, in theory should increase the extent of degradation relative to a given mean retention time. In contrast, Drogoul et al. (2000b) observed a reduced extent and fractional rate of *in situ* degradation of the DM and neutral detergent fibre (NDF) fraction in the caecum and colon for finely ground and pelleted hay compared to chopped hay. This, however, did not affect the overall apparent *in vivo* digestibility, which they contributed to the considerable increase in the mean retention time of small particles in the hindgut of ponies receiving a finely ground and pelleted hay (Drogoul et al., 2000a). When a forage is processed to become a pelleted feedstuff, losses of forage feeding value due to pelleting are evident (Cozzi et al., 2002). So, when sainfoin is a preferred feed for its tannin composition, it can be pelleted to become a tannin-containing concentrate, but then it can no longer be deployed for its fibrous characteristics.

The hardness and durability of the pellets are influenced by the material itself. Sainfoin is a forage legume, which makes it a fibre rich feed (Hayot Carbonero et al., 2011). Fibre rich material expands after the pelleting process, which leads to an increased porosity due to a decreased contact between particles. Porosity in the pellets influences the pellet hardness (Thomas et al., 1997). Fibres can cause problems in the pelleting process due to their elasticity and stiffness, which prevent binding between particles. Large fibre particles also cause an inhomogeneity, which result in weak spots within the pellets. Because of this pellets will break more easily and pellet quality becomes compromised. On the other hand, water soluble fibres reduce the porosity in pellets and cause a high viscosity, which results in a higher pellet durability and hardness (Thomas et al., 1998). Bochnia et al. (2019) observed that the pellet physical characteristics (pellet size and hardness) in combination with meal size affected the feed intake rate and the chewing process, where pellet hardness seemed to be a predictor of saliva production. With regard to the manufacturing of pellets from forages like sainfoin, pellet size and hardness would be important aspects to consider in order to conserve the fibrous characteristics typical to forages (i.e. physically effective fibre, prolong eating time, stimulate chewing activity and saliva production).

4.2. Effects of processing conditions on in vitro fermentation characteristics

The gas production results show a significant effect of drying temperature on the *in vitro* gas production characteristics. The gas production for hay dried at 130 °C is significantly lower compared to hay dried at 30 or 70 °C. A lower gas production indicates a lower fermentability of the feed (Theodoridou et al., 2011). This indicates that when the drying temperature of sainfoin is increased to 130 °C, the fermentability decreases. During the drying process it is important to avoid high temperatures to prevent nutrient and quality losses (Siles et al., 2015).

The drying of feeds at higher temperatures is likely to cause a Maillard reaction. This reaction has several drawbacks, being the degradation of carbohydrates, e.g. hemicellulose and sucrose, to phenolic compounds, which catalytically break down amino acids and polymerize with the remains of the amino acids (Van Soest and Mason, 1991) and the decrease of levels and reactivity of amino acids, especially lysine (Van Rooijen et al., 2013; Hulshof et al., 2016). Heated forages have cell walls with a higher lignin content and a lower hemicellulose content compared to unheated forages (Van Soest, 1965). The Maillard polymer is similar to lignin and indigestible. Moreover, processing of feedstuffs may also have negative effects on the content and reactivity of amino acids and in our study: both total lysine and OMIU-reactive lysine were decreased at higher drying temperatures. Therefore, the Maillard reaction is related to a lower protein (Van Soest and Mason, 1991) and amino acid (Hulshof et al., 2016) digestibility.

Although tannins can also be incorporated in the Maillard reaction, still a significant effect of PEG was observed with hay, dried at 130 °C. This means that although the digestibility decreases when the hay is dried at 130 °C, the effect of the tannins on the gas production remains and is not affected by the drying temperature. This may relate to the findings of the tannin analyses, which has shown no numerical difference of absorbance (550 nm) between the technological treatments. The protein fermentation results in the production of branched chain VFA as percentage of the total VFA production. The results of this study showed that a high temperature during pelleting gives a lower total VFA production as well as a lower percentage of branched chain VFA.

4.3. Effects of processing conditions on bioactivity of sainfoin tannins

Polyethylene glycol was used to test the effect of the tannins on the gas and VFA production. The PEG binds to the tannins in sainfoin and inhibits the effect of the binding of the tannin to the dietary protein (Hayot Carbonero et al., 2011; Azuhnwi et al., 2011). The effect of PEG thus reflects the effect of the tannins. The gas production results show there is an effect of the tannins; the cumulative gas production, the A1 and the Rmax1 are increased and the C1 is decreased when PEG is added. This means that when the biological effect of the tannins are not inhibited by PEG, the tannins are responsible for a lower gas production and a more gradual rate of fermentation. These results are supported by a study of Pellikaan et al. (2011), who reported that PEG increases the gas production showing that tannins were able to inhibit the gas production. In contrast, Theodoridou et al. (2011) observed no effect of PEG addition on the *in vitro* gas production of sainfoin upon incubation with ruminal fluid from sheep. The ability of tannins to bind to PEG (or proteins) depends amongst others on the type of tannin, i.e. plant source (Pellikaan et al., 2011), but the efficacy of tannins also depends on factors like the maturity at harvest (Koupai-Abyazani et al., 1993; Theodoridou et al., 2011) and variety (Hatew et al., 2014; Wang et al., 2015). Most of the studies do not specify these characteristics (Hayot Carbonero et al., 2011), which makes it difficult to compare results between studies.

The total VFA and branched chain VFA were significantly increased by the addition of PEG for all substrates. Without the addition of PEG tannins will bind to protein, which resulted in a decrease in the branched chain VFA indicating a lowered proteolytic activity. McMahon et al. (1999) confirms these findings and found that an increased amount of sainfoin in the diet of steers decreased the total VFA production and the production of branched chain VFA. Branched chain VFA originate from protein fermentation and can be used as biomarkers for bacterial proteolytic activity. Microbial proteases formed in the gut lumen can modulate immunogenicity of dietary antigens and reduce mucosal barrier function, and may contribute to gastro-intestinal disorders (Caminero et al., 2023).

Gas production results of sainfoin hay of an *in vitro* study with ruminal fluid range from 241 to 290 mL/g OM after an incubation of 120 h (Guglielmelli et al., 2011). These results are not comparable to the cumulative gas production of this study, because in this study the sainfoin was incubated for 72 h. However, when the asymptotic gas production results of phase one and two together (273.6 for hay dried at 30 °C, 272.7 for hay dried at 70 °C and 238.3 mL/g OM for hay dried at 130 °C) are compared to the results of Guglielmelli et al. (2011), there is little to no difference observed. *In vitro* fermentation of freeze dried grass and high temperature dried grass showed asymptotic gas production results of 326 and 220 mL/g OM, respectively (Murray et al., 2014), indicating a negative effect of high temperature drying.

The results that the tannins in sainfoin affects gas production kinetics, the VFA and branched chain VFA production show the potential that a horse can benefit from eating sainfoin. However, horses are monogastric hindgut fermenters and this *in vitro* study only simulated the fermentation in the hindgut of horses. One aim of the current study was to assess the effect of drying temperature and pelleting on the biological properties of tannins present in sainfoin using an *in vitro* fermentation assay. Sainfoin samples were used in the *in vitro* fermentation assay as such and not subjected to an *in vitro* pre-caecal digestion procedure. In recent years a limited number of *in vitro* pre-caecal digestion studies have been conducted to mimic digestive processes in the equine foregut (stomach and small intestine; Bachmann et al., 2020; Moore-Colyer et al., 2014; Strauch et al., 2023). The procedure often includes a one or two step procedure by incubating samples with HCl-pepsin and pancreatin, successively. Forages subjected to a two step *in vitro* pre-caecal digestion procedure showed that protein and simple sugars were degraded considerably (Strauch et al., 2017), but structural carbohydrate fractions seemed to remain unaffected. In addition, *in vitro* pre-caecal digestion procedures indicate a loss of fructans and fructooligosaccharides (Bachmann et al., 2020; Ince et al., 2014; Strauch et al., 2017). In general, these observations suggest that part of the (macro)nutrients in sainfoin might have been digested, but no information is available if pre-caecal digestive processes in the equine digestive tract would affect the efficacy of tannins. Future research in this area would be warranted.

To simulate fermentative processes in the equine hindgut, faecal inocula are commonly used in *in vitro* fermentation studies (Desrousseaux et al., 2012; Murray et al., 2014), although the approach to use faecal inocula as a representative for the entire hindgut (i.e., caecum, colon) is under debate (Costa et al., 2015; Julliand and Grimm, 2016; Kujawa et al., 2020). Lowland (1998) showed similar cumulative gas production results for faecal and caecal inocula in contrast to observations of Kujawa et al. (2020) and Murray et al. (2014). Although faecal inoculum does not appear to be fully representative for the microbial processes occurring in the different segments of the equine hindgut, it can be representative when investigating the extent and kinetics of fermentation along the hindgut.

5. Conclusions

Drying sainfoin hay at high temperature and subsequent grinding and pelleting affected the physicochemical properties. The current study showed that drying temperature of sainfoin hay and pellets clearly affected the *in vitro* measured cumulative gas production and fermentation end-products when using equine faecal inocula. An increase in temperature from 30 or 70–130 °C decreased the *in vitro* organic matter degradability, as reflected by a decrease in gas production. The sainfoin tannins affected the extent and kinetics of fermentation and decrease the proportion of branched chain VFA, suggesting a decrease in the proteolytic activity. The level and reactivity of lysine decreased at increasing drying temperatures in the case sainfoin hay was ground and pelleted. The efficacy of the tannins present in sainfoin seems to remain intact irrespective the height of the drying temperature or the pelleting process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Groenvoeder drogerij Timmerman, Kortgene, The Netherlands, for providing the sainfoin roughage and Research Diet Services BV for their services concerning the pelleting process. The study was financially supported by Wageningen University. Mr Tamme Zandstra of Wageningen UR is acknowledged for assistance with the application of the technological treatments and Mrs Erika Beukers-van Laar of Wageningen UR for assistance with the wet sieving technique and the VFA analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115740.

References

Adapa, P., Schoenau, G., Tabil Jr., L., Sokhansanj, S., 2004. Fractional drying of alfalfa leaves and stems: Review and discussion. In: Mujumdar, A.S. (Ed.), Dehydration of products of biological origin. Science Publishers, Oxford & IBH, p. 553.

Al Jassim, R.A.M., Andrews, F.M., 2009. The bacterial community of the horse gastrointestinal tract and its relation to fermentative acidosis, laminitis colic, and stomach ulcers. Vet. Clin. Equine 25, 199–215.

ASAE, 2008. American society of agricultural and biological engineers. method of determining and expressing fineness of feed materials by sieving. Standard 4 (No. S319).

Azuhnwi, B.N., Boller, B., Martens, M., Dohme-Meier, F., Ampuero, S., Günter, S., Kreuzer, M., Hess, H.D., 2011. Morphology, tannin concentration and forage value of 15 Swiss accessions of sainfoin (*Onobrychis viciifolia* Scop.) as influenced by harvest time and cultivation. Grass Forage Sci. 66, 474–487.

Bachmann, M., Glatter, M., Bochnia, M., Wensch-Dorendorf, M., Greef, J.M., Breves, G., Zeyner, A., 2020. *In vitro* gas production from batch cultures of stomach and hindgut digesta of horses adapted to a prebiotic dose of fructooligosaccharides and inulin. J. Equine Vet. Sci. 90, 103020.

Barrau, E., Fabre, N., Fouraste, I., Hoste, H., 2005. Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the in vitro larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. Parasitol 131, 531–538.

Bochnia, M., Goetz, F., Wensch-Dorendorf, M., Koelln, M., Zeyner, A., 2019. Chewing patterns in horses during the intake of variable quantities of two pelleted compound feeds differing in their physical characteristics only. Res. Vet. Sci. 125, 189–194.

Brøkner, C., Nørgaard, P., Hansen, H.H., 2008. Effect of feed type and essential oil product on equine chewing activity. J. Anim. Physiol. Anim. Nutr. 92, 621–630.

Caminero, A., Guzman, M., Libertucci, J., Lomax, A.E., 2023. The emerging roles of bacterial proteases in intestinal diseases. Gut Microbes 15, 2181922.

Collas, C., Sallé, G., Dumont, B., Cabaret, J., Cortet, J., Martin-Rosset, W., Wimel, L., Fleurance, G., 2018. Are sainfoin or protein supplements alternatives to control small strongyle infection in horses. Animal 12, 359–365.

Cone, J.W., Van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L., 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Technol. 61, 113–128.

Costa, M.C., Silva, G., Ramos, R.V., Staempfli, H.R., Arroyo, L.G., Kim, P., Weese, J.S., 2015. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet. J. 205, 74–80.

Cozzi, G., Burato, G.M., Berzaghi, P., Andrighetto, I., 2002. Evaluation of pellets from different industrial processing of dehydrated lucerne in dairy cattle feeding. Anim. Feed Sci. Technol. 99, 13–24.

de Fombelle, A., Varloud, M., Goachet, A.G., Jacotot, E., Philippeau, C., Drogoul, C., Julliand, V., 2003. Characterization of the microbial and biochemical profile of the different segments of the digestive tract in horses given two distinct diets. Anim. Sci. 77, 293–304.

Desrousseaux, G., Santos, A.S., Pellikaan, W.F., van der Poel, A.F.B., Cone, J.W., Guedes, C.M.V., Ferreira, L.M.M., Rodrigues, M.A.M., 2012. Effect of collection time on the fermentative activity of microbes in equine faeces. Anim. Feed Sci. Technol. 178, 183–189.

Dicks, L.M.T., Botha, M., Dicks, E., Botes, M., 2014. The equine gastro-intestinal tract: an overview of the microbiota, disease and treatment. Livest. Sci. 160, 69–81.

Drogoul, C., Poncet, C., Tisserand, J.L., 2000a. Feeding ground and pelleted hay rather than chopped hay to ponies 1. Consequences for in vivo digestibility and rate of passage of digesta. Anim. Feed Sci. Technol. 87, 117–130.

Drogoul, C., Tisserand, J.L., Poncet, C., 2000b. Feeding ground and pelleted hay rather than chopped hay to ponies 2. consequences on fibre degradation in the cecum and the colon Anim. Feed Sci. Technol. 87, 131–145.

Edwards, J.E., Shetty, S.A., van den Berg, P., Burden, F., van Doorn, D.A., Pellikaan, W.F., Dijkstra, J., Smidt, H., 2020. Multi-kingdom characterization of the core equine fecal microbiota based on multiple equine (sub)species. Anim. Micro 2, 6.

Elia, J.B., Erb, H.N., Houpt, K.A., 2010. Motivation for hay: effects of a pelleted diet on behaviour and physiology of horses. Physiol. Behav. 101, 623–627.

Ellis, A., Longland, A., Coenen, M., Miraglia, N., 2010. Biological basis of behaviour in relation to nutrition and feed intake in horses. EAAP Publ. 128, 53–74.

Engberg, R.M., Hedemann, M.S., Jensen, B.B., 2002. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. Br. Poult. Sci. 43, 569–579.

Grabber, J.H., Zeller, W.E., Meuller-Harvey, I., 2013. Acetone enhances the direct analysis of procyanidin- and prodelphinidin-based condensed tannins in lotus species by the butanol-HCl-iron assay. J. Agric. Food Chem. 61, 2669–2678.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for in vitro fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 77–89.

Guglielmelli, A., Calabrò, S., Primi, R., Carone, F., Cutrignelli, M.I., Tudisco, R., Piccolo, G., Ronchi, B., Danieli, P.P., 2011. *In vitro* fermentation patterns an methane production of sainfoin (*Onobrychis viciifolia* Scop.) hay with different condensed tannin contents. Grass Forage Sci. 66, 488–500.

Hansen, T.L., Bobel, J.M., Rankins, E.M., Sanchez, L.C., Warren, L.K., 2020. A pilot study exploring the relationship between digesta retention time in the equine gastrointestinal tract and compartment models. J. Equine Vet. Sci. 88, 102941.

Hayot Carbonero, C., Mueller-Harvey, I., Brown, T.A., Smith, L., 2011. Sainfoin (*Onobrychis viciifolia*): a beneficial forage legume. Plant Genet. Resour. 9, 70–85.

Hulshof, T.G., Bikker, P., van der Poel, A.F.B., Hendriks, W.H., 2016. Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products. J. Anim. Sci. 94 (3), 1020–1030.

Ince, J.C., Longland, A.C., Moore-Colyer, M.J.S., Harris, P.A., 2014. *In vitro* degradation of grass fructan by equid gastrointestinal digesta. Grass Forage Sci. 69, 514–523.

Julliand, V., Grimm, P., 2016. Horse species symposium: the microbiome of the horse hindgut: history and current knowledge. J. Anim. Sci. 94, 2262–2274.

Julliand, V., de Fombelle, A., Drogoul, C., Jacotot, E., 2001. Feeding and microbial disorders in horses: part 3-Effects of three hay:grain ratios on microbial profile and activities. J. Equine Vet. Sci. 21, 543–546.

Koupai-Abyazani, M.R., Muir, A.D., Bohm, B.A., Neil Towers, G.H., Gruber, M.Y., 1993. The proanthocyanidin polymers in some species of *Onobrychis*. Phytochem 34, 113–117.

Kujawa, T.J., van Doorn, D.A., Wambacq, W.A., Hesta, M., Pellikaan, W.F., 2020. Evaluation of equine rectal inoculum as representative of the microbial activities within the horse hindgut using a fully automated in vitro gas production technique system. J. Anim. Sci. 50..

Larrauri, J.A., Rupérez, P., Saura-Calixto, F., 1997. Effect of drying temperature on the stability of polyphenols and antioxidant activity of red grape pomace peels. J. Agric. Food Chem. 45 (4), 1390–1393.
Lee, J., 1988. II. 2. Forages. Livest. Prod. Sci. 19 (1), 13–46. https://doi.org/10.1016/0301-6226(88)90085-1.
Lowman, R.S., Theodorou, M.K., Hyslop, J.J., Dhanoa, M.S., Cuddeford, D., 1999. Evaluation of an in vitro batch culture technique for estimating the in vitro digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. Anim. Feed. Sci. Technol. 80, 11–27.
Lyu, F., Thomas, M., Hendriks, W.H., van der Poel, A.F.B., 2020. Size reduction in feed technology and methods for determining, expressing and predicting particle size: a review. Anim. Feed Sci. Technol. 261, 114347.
Makkar, H.P.S., Blummel, M., Becker, K., 1995. Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in in vitro techniques. Br. J. Nutr. 73, 897–913.
McMahon, L.R., Majak, W., McAllister, T.A., Hall, J.W., Jones, G.A., Popp, J.D., Cheng, K.J., 1999. Effect of sainfoin on in vitro of fresh alfalfa and bloat in steers. Can. J. Anim. Sci. 203–212.
Miyaji, M., Ueda, K., Nakatsuji, H., Tomioka, T., Kobayashi, Y., Hata, H., Kondo, S., 2008. Mean retention time of digesta in the different segments of the equine hindgut. Anim. Sci. J. 79, 89–96.
Moore-Colyer, M., O'Gorman, D.M., Wakefield, K., 2014. An in vitro Investigation into the effects of a marine-derived, multimineral supplement in simulated equine stomach and hindgut environments. J. Equine Vet. Sci. 34, 391–397.
Moughan, P.J., Rutherfurd, S.M., 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202–2209.
Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010–2037.
Mueller-Harvey, I., Bee, G., Dohme-Meier, F., Hoste, H., Karonen, M., Kölliker, R., Lüscher, A., Niderkorn, V., Pellikaan, W.F., Salminen, J.-P., Skøt, L., Smith, L.M.J., Thamsborg, S.M., Totterdell, P., Wilkinson, I., Williams, A.R., Azuhnwi, B.N., Baert, N., Brinkhaus, A.G., Copani, G., Desrues, O., Drake, C., Engström, M., Fryganas, C., Girard, M., Huyen, N.T., Kempf, K., Malisch, C., Mora-Ortiz, M., Quijada, J., Ramsay, A., Ropiak, H.M., Waghorn, G.C., 2019. Benefits of condensed tannins in forage legumes fed to ruminants: importance of structure, concentration, and diet composition. Crop Sci. 59, 861–885.
Murray, J.M.D., McMullin, P., Handel, I., Hastie, P.M., 2014. Comparison of intestinal contents from different regions of the equine gastrointestinal tract as inocula for use in an vitro gas production technique. Anim. Feed Sci. Technol. 187, 98–103.
NRC, 2007. Nutrient requirements of horses. 6th edition. Natl. Acad. Press Washington, DC.
Pellikaan, W.F., Stringano, E., Leenaars, J., Bongers, D.J.G.M., van Laar-van Schuppen, S., Plant, J., Mueller-Harvey, I., 2011. Evaluating effects of tannins on extent and rate of in vitro gas and CH_4 production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166–167, 377–390.
Petz, V., Khiaosa-Ard, R., Iben, C., Zebeli, Q., 2023. Changes in eating time, chewing activity and dust concentration in horses fed either alfalfa cubes or long-stem hay. Vet. Med. Sci. 9, 1154–1162.
Pickford, J.R., 1992. Effects of processing on the stability of heat labile nutrients in animal feeds. In: Garnsworthy, P.C., Haresign, W., Cole, D.J.A. (Eds.), Recent Advances in Animal Nutrition. Butterworth-Heinemann, pp. 177–192.
Rufino-Moya, P.J., Blanco, M., Bertolín, J.R., Joy, M., 2019. Effect of the method of preservation on the chemical composition and in vitro fermentation characteristics in two legumes rich in condensed tannins. Anim. Feed Sci. Technol. 251, 12–20.
SAS Institute Inc. 2017. SAS/STAT Software SAS® 9.4: Cary, NC, USA.
Siles, J.A., González-Tello, P., Martín, M.A., Martín, A., 2015. Kinetics of alfalfa drying: simultaneous modelling of moisture content and temperature. Biosyst. Eng. 129, 185–196.
Sokhansanj, S., Patil, R.T., 1996. Kinetics of dehydration of green alfalfa. Dry. Technol. 5, 1197–1234.
Strauch, S., Wichert, B., Greef, J.M., Hillegeist, D., Zeyner, A., Liesegang, A., 2017. Evaluation of an in vitro system to simulate equine foregut digestion and the influence of acidity on protein and fructan degradation in the horse's stomach. J. Anim. Physiol. Anim. Nutr. 101, 51–58.
Theodoridou, K., Aufrère, J., Niderkorn, V., Andueza, D., Le Morvan, A., Picard, F., Baumont, R., 2011. *In vitro* study of the effects of condensed tannins in sainfoin on the digestive process in the rumen at two vegetation cycles. Anim. Feed Sci. Technol. 170, 147–159.
Thomas, M., Van Zuilichem, D.J., Van der Poel, A.F.B., 1997. Physical quality of pelleted animal feed. 2. contribution of processes and its conditions. Anim. Feed Sci. Technol. 64, 173–192.
Thomas, M., Van Vliet, T., Van der Poel, A.F.B., 1998. Physical quality of pelleted animal feed. 3. contribution of feedstuff components. Anim. Feed Sci. Technol. 70, 59–78.
Van Rooijen, C., Bosch, G., Wierenga, P.A., Hendriks, W.H., van der Poel, A.F.B., 2014. The effect of steam pelleting of a dry dog food on the Maillard reaction. Anim. Feed Sci. Technol. 198, 238–247.
Van Rooijen, C., Bosch, G., van der Poel, A.F.B., Wierenga, P.A., Alexander, L., Hendriks, W.H., 2013. The Maillard reaction and pet food processing: effects on nutritive value and pet health. Nutr. Res. Rev. 26, 130–148.
Van Soest, P.J., 1965. Use of detergents in analysis of fibrous feeds. III. study of effects of heating and drying on yield of fibre and lignin in forages. J. Assoc. Agric. Chem. 48, 785–790.
Van Soest, P.J., Mason, V.C., 1991. The influence of the Maillard reaction upon the nutritive value of fibrous feed. Anim. Feed Sci. Technol. 32, 45–53.
Vermorel, M., Martin-Rosset, W., 1997. Concepts, scientific bases, structure and validation of the French horse net energy system (UFC). Livest. Prod. Sci. 47, 261–275.
Vervuert, I., Brüssow, N., Bochnia, M., Cuddeford, D., Coenen, M., 2013. Electromyographic evaluation of masseter muscle activity in horses fed (i) different types of roughage and (ii) maize after different hay allocations. J. Anim. Physiol. Anim. Nutr. 97, 515–521.
Wang, Y., McAllister, T.A., Acharya, S.N., 2015. Condensed tannins in sainfoin: composition, concentration and effects on nutritive and feeding value of sainfoin forage. Crop Sci. 55, 13–22.
Yang, H.J., Tamminga, S., Williams, B.A., Dijkstra, J., Boer, H., 2005. *In vitro* gas and volatile fatty acids production profiles of barley and maize and their soluble and washout fractions after feed processing. Anim. Feed Sci. Technol. 120, 125–140.
Zebeli, Q., Aschenbach, J.R., Tafaj, M., Boguhn, J., Ametaj, B.N., Drochner, W., 2012. Invited review: role of physically effective fiber and estimation of dietary fiber adequacy in high-producing dairy cattle. J. Dairy Sci. 95, 1041–1056.