Spanish Journal of Agricultural Research
16 (3), e0608, 10 pages (2018)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2018163-13010
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, O.A, M.P. (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Differences in nutritional characteristics of three varieties of sorghum grain determine their *in vitro* rumen fermentation

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Abstract

The effect of phenolic compounds and protein matrix on microbial fermentation of three sorghum grains (S1, S2 and S3) were studied *in vitro*. Total phenolics and total tannins concentration (g/kg dry matter, DM) were 1.5 and 0.2 for S1, 19.3 and 8.1 for S2 and 2.6 and 1.3 for S3. Protein enzymatic digestibility was 0.614, 0.226 and 0.454, respectively. Trial 1 was conducted for 24 h, without or with polyethylene glycol (PEG) to determine the effect of phenolic compounds on fermentation. Without PEG, gas production for S1 was the highest after 24 h (257, 237 and 238 mL/g organic matter, for S1, S2 and S3; $p<0.05$), and higher proportion of propionate at the expense of acetate was recorded with S3 after 6 h. Gas produced with S1 and S3 remained unaffected ($p>0.10$), but increased in S2 by 0.21 to 0.30 with *vs.* without PEG. No differences in gas production between S1 and S2 were observed with PEG, S3 recording the lowest ($p<0.05$) values from 8 h onwards. Addition of PEG reduced proportion of butyrate ($p<0.05$) and increased three-fold lactate (1.62 *vs.* 4.98 mM; $p<0.001$). In Trial 2 (12 h) gas production followed a similar pattern. Without PEG, starch disappearance was the highest in S1 (0.356, 0.231 and 0.216, respectively), but no differences were recorded with PEG. Considering differences in protein digestibility and the effect of phenolic compounds, the effect of starch nature and structure on fermentation is apparently minor. Colour of grain is not necessarily related to phenolic compounds proportion or rumen utilization.

Additional keywords: starch; tannins; protein; polyethylene glycol; gas production.

Abbreviations used: ADF (acid detergent fibre); ADL (acid detergent lignin); aNDFom (neutral detergent fibre treated with amylase and excluding residual ashes); BCFA (branched chain fatty acids); BE (biological effect); CP (crude protein); DM (dry matter); DMd (dry matter disappearance); EE (ether extract); OM (organic matter); PEG (polyethylene glycol); SEM (standard error of means); TP (total phenolics); TT (total tannins); VFA (volatile fatty acids).

Authors' contributions: Conception and design, analysis and interpretation of data: ZA and MF. Performing of experiments: ZA and SY. Drafting of the manuscript: ZA, AV and MF. All authors read and approved the final manuscript.

Citation: Amanzougarene, Z.; Yuste, S.; de Vega, A.; Fondevila, M. (2018). Differences in nutritional characteristics of three varieties of sorghum grain determine their *in vitro* rumen fermentation. Spanish Journal of Agricultural Research, Volume 16, Issue 3, e0608. https://doi.org/10.5424/sjar/2018163-13010

Received: 10 Feb 2018. **Accepted:** 31 Jul 2018.

Funding: Spanish Government, MINECO (project AGL2013-46820); Department of Industry and Innovation of the Government of Aragón; European Social Fund; Government of Aragón–DGA (grant to SY).

Competing interests: The authors declare that no competing interests exist.

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Introduction

Sorghum (*Sorghum bicolor*) grain is a common feed for ruminant and monogastric animals in Africa, Asia and America because of its high starch and energy content, similar to that of maize (Streeter *et al.*, 1990; Rowe & Pethick, 1994). However, it is seldom used in Europe because of its highly variable feeding quality in terms of protein and starch availability, and digestibility (Bramel-Cox *et al.*, 1990; Opatpatanakit *et al.*, 1994). Besides, the presence of condensed tannins and other phenolic compounds in some varieties is assumed to limit nutrient availability (Dykes & Rooney, 2006; Barros *et al.*, 2012). Variability among genotypes in nutritive value has been related to the content in amylose and proportion of vitreous starch, the nature of the endosperm protein, and the concentration of phenolic compounds (Rooney & Pflugfelder, 1986). However, improvements in nutritive value are often associated with reductions in agronomic traits such as grain yield, maturity and bird resistance (O´Brien, 1999).

In general, tannins (mostly proanthocyanidins) are assumed to reduce rumen microbial fermentation of substrates by binding to nutrients, which will be available to intestinal digestion after low pH dissociation when passing through the abomasum

(Mueller-Harvey, 2006). In contrast, tannins may also have a positive effect, preventing bloat or enhancing efficiency of energy utilisation by reducing methanogenesis (Mueller-Harvey, 2006; Jayanegara *et al.*, 2009). Besides, the low digestible proteins from the sorghum endosperm (kafirins) that are embedded in the starch matrix may reduce access to microbial enzymes (Rooney & Pflugfelder, 1986; McAllister *et al.*, 1993). Because of this restricted rumen fermentation, sorghum probably delivers an important proportion of starch to the lower gut (Rowe & Pethick, 1994), and the non-degraded starch that passes to the intestine provides 42% more energy than that digested in the rumen (Owens *et al.*, 1986). In this regard, Hibberd *et al.* (1985) found that up to 25% of variation in the nutritive value of sorghum varieties largely depends on the digestion site. Complexes between tannins and nutrients that are stable at common rumen pH can be dissociated at pH under 3.5 (Jones & Mangan, 1977), thus making nutrients available for digestion at the lower gut.

In a previous study (Amanzougarene *et al.*, 2017), major differences in *in vitro* fermentation were observed between three varieties of sorghum grain, but the relative importance of the nutritive factors determining the magnitude of those differences was not defined. Therefore, this *in vitro* study aimed to evaluate the potential differences in ruminal fermentation of grains from three sorghum varieties (a white sorghum, S1, and two brown sorghums, S2 and S3), and to determine to what extent either the content of tannins and other phenolic compounds or the endosperm protein matrix may affect their microbial fermentation characteristics and starch degradability. In a first incubation trial (Trial 1), the effect of phenolic compounds on sorghum fermentation pattern was monitored for 24 h of incubation; after, a subsequent incubation (Trial 2) for 12 h was carried out to explore the effect of the three sorghum varieties on substrate degradation. Gas production and fermentation parameters were compared between Trials 1 and 2.

Material and methods

Substrates and inocula

Grains of three different varieties of sorghum (variety names not available), a white sorghum (S1) and two brown sorghums (S2 and S3) were chosen for their diversity in nutritional characteristics, based on their chemical composition (Table 1). The three substrates, harvested in the previous year session, were screened and ground through a sieve of 1 mm using a hammer mill (Retsch GmbH/SK1/417449, Haan, Germany). Observation of ground sorghum under a dissecting microscope at ×20 magnification showed a high proportion of vitreous starch in both S1 and S2, and a high proportion of floury starch in S3.

Table 1. Chemical composition (g/kg DM) of the three varieties of sorghum (S1, S2 and S3).

	S1	S2	S3
Organic matter	984	985	979
Crude protein	109	106	113
Ether extract	40	41	11
Starch	689	638	647
Neutral detergent fibre	98	93	97
Acid detergent fibre	47	60	60
Lignin in sulphuric acid	14	17	5
Total phenolic compounds[1]	1.5	19.3	2.6
Total tannins[1]	0.2	8.1	1.3

[1] g tannic acid equivalents/kg DM.

Four adult Rasa Aragonesa ewes (average weight 70 ± 2.7 kg) fitted with a cannula in the dorsal sac of the rumen, were used as donors of inoculum. Animals were housed in the facilities of the *Servicio de Apoyo a la Experimentación Animal* of the *Universidad de Zaragoza* and were daily fed with 600 g of alfalfa hay plus 300 g of barley grain. Sheep also received a commercial vitamin-mineral mixture throughout all the experiment. Management and extraction procedures of rumen inoculum from donor animals were approved by the Ethics Committee for Animal Experimentation, Care and Management of Animals agreed with the Spanish Policy for Animal Protection RD 53/2013, which complies with EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Rumen contents (approx. 300 mL) were extracted from each sheep before the morning feeding, filtered through cheesecloth and transferred to the laboratory in thermos bottles preheated to 39° C for their use as incubation inoculum.

Experimental design

In Trial 1, four *in vitro* incubation series were carried out, using rumen contents from a different donor ewe on each series, thus considering animal (*i.e.*, incubation series) as a block. On every series, triplicate glass bottles (116 mL total volume) containing 500 mg of each substrate were incubated for each treatment, either in the presence or in absence of polyethylene glycol 6000 (PEG, Panreac, Barcelona; 1 mg/mg substrate) as tannin binder. The bottles were filled under anaerobic conditions with 8 mL of rumen inoculum and 72 mL of an incubation solution made up with (mL/L) 238 buffer solution (14 g $NaHCO_3$ and 1.5 g $(NH_4)HCO_3$ per L), 238 macrominerals solution (5.7 g Na_2HPO_4, 6.2 g

KH_2PO_4 and 0.6 g $MgSO_4 \cdot 7\ H_2O$ per L), 474 distilled water and 50 reduction solution (47.5 mL distilled water, 2 mL of 1N NaOH and 313 mg HCl-cysteine). Concentration of bicarbonate ions in the buffer solution was adjusted to 0.044 M in order to adjust incubation pH to 6.4 (Kohn & Dunlap, 1998). Microminerals and resazurin were not added (Mould *et al.*, 2005). Bottles were filled with the incubation solution under a CO_2 stream, sealed and incubated for 24 h in a water bath at 39ºC. After 6 h of incubation, one of the bottles from each treatment was transferred to an ice water bath, opened and samples of the incubation medium were taken for volatile fatty acids (VFA) analysis (2 mL, collected over 0.5 mL of a deproteinizing mixture of 0.5M PO_4H_3 with 2 mg/mL 4-methyl valeric acid) and lactate (2 mL) and immediately frozen and stored at -20ºC until analysis. During the experiment, pressure into the remaining two bottles was recorded every 2 hours up to 12 h, and then at 24 h, by means of an HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy). Readings were converted into volume by a linear regression equation pre-established under the same environmental conditions, considering the pressure recorded after injecting known air volumes in the same type of bottles (n= 103; R^2= 0.996), expressed per unit of incubated organic matter (OM).

A similar procedure was conducted in Trial 2, but with only three incubation runs, each one with rumen inoculum from a different donor sheep. In this case, at the end of the incubation period that lasted for 12 h, samples for analysis of VFA and lactate were collected from the liquid medium, and the solid residue of the bottles was filtered through 45 µm pore size nylon cloth and dried at 60ºC for 48 h to determine dry matter disappearance (DMd) and residual starch analysis.

The biological effect of tannins (BE) on gas production from both incubation trials was estimated as the ratio between gas production results with and without adding PEG (Makkar *et al.*, 1995; Rodríguez *et al.*, 2014).

Chemical analyses

Substrates were analysed following the procedures of AOAC (2005) for their content of dry matter (DM; 934.01), organic matter (OM; 942.05), crude protein (CP; 976.05) and ether extract (EE; 2003.05). Concentration of neutral detergent fibre (aNDF) was analysed using an Ankom 200 Fiber Analyzer (Ankom Technology, NY) as described by Mertens (2002), and acid detergent fibre (ADF) and lignin in sulphuric acid as described by Robertson & Van Soest (1981). The aNDF is expressed exclusive of residual ashes, and α–amylase was used in the analysis. Sodium sulphite was not used. Total starch content was determined enzymatically from samples ground to 0.5 mm using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). The total phenolic (TP) contents was analysed following the colorimetric method of Makkar *et al.* (1993) using the Folin-Ciocalteau reagent and with tannic acid (MERCK Chemicals, Madrid, Spain) as the reference standard, and total tannins (TT) were estimated as the difference between TP before and after treatment with polyvinyl polypyrrolidone. The solubility and digestibility of the protein fraction of sorghum grains, in the presence or absence of PEG, as an index of protein susceptibility to microbial degradation was assayed by incubating in 0.1M phosphate buffer (pH 7.5) without or with protease (Pronase 81748, Fluka Analytical), respectively, for 4 h in a shaking water bath at 37ºC (McAllister *et al.*, 1993). The frozen samples of incubation media were thawed and centrifuged at 20,000 g for 15 minutes before their analysis of VFA and lactate. The determination of VFA was carried out by gas chromatography on an Agilent 6890 apparatus (Agilent Technologies España S.L., Madrid), equipped with a capillary column (HP-FFAP polyethylene glycol TPA, 30 m × 530 µm Id). The total lactate concentration was determined by the colorimetric method proposed by Barker & Summerson (1941), using blanks with PEG for correction in this case.

Statistical analysis

The results of each incubation trial were analysed using the Statistix 10 software package (Analytical Software, 2010). The effect of the substrate, the addition of PEG and the interaction of both factors on the fermentation characteristics were studied for each time of incubation as a two-way ANOVA, considering the average of the two bottles of the same substrate on each incubation time as the experimental unit and the incubation series as a block. Differences between substrates were considered significant when $p<0.05$, and as a trend to significance when $0.05<p\leq0.10$. The Tukey *t* test ($p<0.05$) was used for the multiple comparison between means.

Results

Protein of the three substrates was characterised in terms of buffer solubility and enzymatic digestibility, either in presence or absence of PEG, to estimate the possible effect of tannins on those variables (Fig. 1). Inactivation of tannins by the addition of PEG to the medium scarcely affected the proportion of soluble

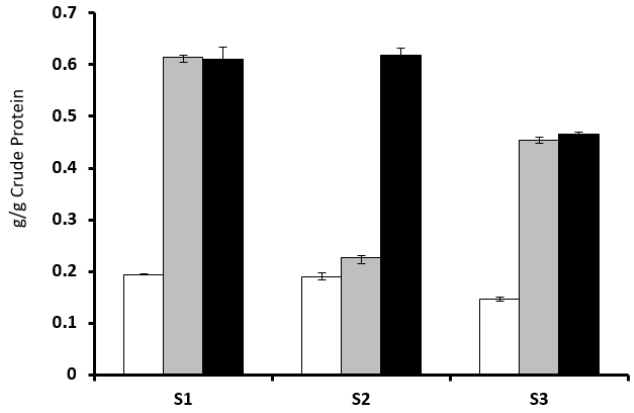

Figure 1. Solubility in buffer (white columns) and protease digestibility of the protein fraction of the sorghum substrates, without (grey columns) or with (black columns) PEG. Upper bars show standard errors of the means (n=3).

protein of the varieties tested (average differences between means below 0.0024 g/g), and therefore the effect of PEG on protein solubility was not considered; thus, average protein solubility was 0.194 ± 0.0009, 0.190 ± 0.0073 and 0.146 ± 0.0036 g/g for S1, S2 and S3, respectively. Protein enzymatic digestibility was the highest in S1 (0.614 ± 0.0100 g/g), intermediate in S3 (0.454 ± 0.0053 g/g) and lowest in S2 (0.226 ± 0.0104 g/g). The inclusion of PEG only showed an effect on protein digestibility in the case of S2 (0.618 ± 0.0139 g/g when PEG was added).

Trial 1

An interaction between the effects of the addition of PEG to inactivate tannins, and the type of sorghum incubated as substrate was observed ($p<0.05$) throughout all the experimental period. Consequently, for a better understanding of differences, gas production from the three substrates in the absence or presence of PEG is presented in Figs. 2a and 2b, respectively. In the absence of PEG (Fig. 2a), gas production from S1 was higher ($p<0.05$) than that of S2, whereas S3 showed intermediate values ($p>0.05$). The addition of PEG to the incubation solution did not result in differences ($p>0.05$) in the volume of gas produced from S1 or S3 at any time of incubation; however, the gas production from S2 increased ($p<0.05$) in a proportion ranging from 0.21 to 0.30 when the effect of tannins was inactivated by addition of PEG, reaching similar values to those recorded in S1, and higher ($p<0.05$) than those recorded for S3 with PEG from 8 h onwards (Fig. 2b).

There were no effects of the factors studied on the total VFA concentration at 6 h of incubation (Table 2). In absence of PEG, acetate proportion was lower, and that of propionate higher ($p<0.05$) with S3 compared with S2, and acetate was also lower with S3 compared with S1; however, differences between sorghum varieties were not detected with PEG (interaction substrate × PEG, $p=0.012$). Proportion of branched chain fatty acids was higher ($p<0.05$) with S3 than S1 (Table 2). The addition of PEG resulted in a lower proportion of butyrate for all sorghum varieties (average proportions of 0.157 *vs.* 0.147, without and with PEG; $p<0.05$). Lactate concentration after 6 h incubation was not affected by the sorghum variety but increased three-fold with the addition of PEG (on average, from 1.62 to 4.98 mM, SEM=0.5028; $p<0.001$).

The addition of PEG to the incubation medium highlighted a clear BE of tannins on *in vitro* gas production for S2, ranging from 1.23 to 1.30-fold from 4 to 12 h incubation, and being higher ($p<0.05$) than that on S1 and S3 at all incubation times (Fig. 3). The maximum BE values were recorded for S2 and S3 at 8 h incubation (average values of 1.05, 1.31 and 1.10), whereas those on S1 were almost constant (from 1.01 to 1.05) throughout all the incubation period.

Trial 2

Although of a lower overall magnitude, the gas production pattern (Fig. 4) agreed with observations in the first incubation trial. Thus, in absence of PEG, higher values were recorded from S1 than from S2 at 8 h ($p<0.05$). Gas production was also higher ($p<0.05$) for S1 than for both S2 and S3 at 10 and 12 h incubation. When PEG was added, gas production from S2 increased at 6 h ($p=0.08$) and from 8 h onwards ($p<0.05$), in a proportion of 0.26 to 0.40, whereas there was no effect of PEG addition on gas production from either S1 or S3 ($p>0.05$).

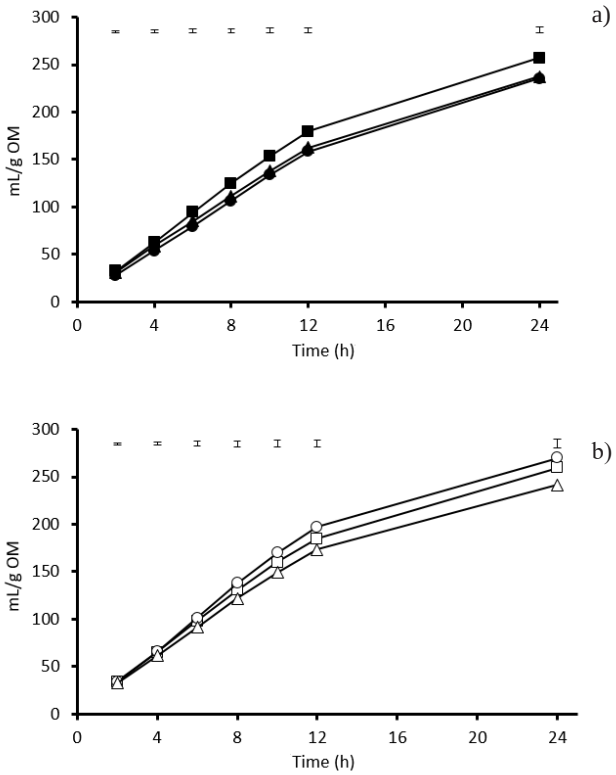

Figure 2. Pattern of *in vitro* gas production (mL/g organic matter, OM) from the three varieties of sorghum (S1 ■, □; S2●, ○; S3 ▲, △), without (a) or with (b) added PEG, in Trial 1. For each time of incubation, upper bars show standard errors of the means.

Table 2. Total volatile fatty acids (VFA, mM) concentration and molar VFA proportions, together with lactate concentration (mM), recorded at 6 h of incubation of the three sorghum grains (S1, S2 and S3) without or with polyethylene glycol (PEG), in Trial 1.

Substrate	PEG[1]	VFA	Acetate	Propionate	Butyrate	BCFA[2]	Valerate	Lactate
S1	N	21.3	0.637a	0.172ab	0.151	0.0029c	0.0011b	2.56ab
S2	N	20.7	0.638a	0.161b	0.160	0.0030abc	0.0011ab	1.18b
S3	N	22.2	0.613b	0.182a	0.162	0.0032a	0.0012ab	1.13b
S1	Y	22.2	0.636a	0.177a	0.146	0.0030bc	0.0011ab	5.28a
S2	Y	23.2	0.640a	0.176ab	0.142	0.0030abc	0.0011ab	4.52ab
S3	Y	21.7	0.631ab	0.174ab	0.152	0.0032ab	0.0012a	5.13ab
SEM[3]		0.81	0.0050	0.0032	0.0049	0.0005	0.0002	0.871

[1] N, without PEG; Y, with PEG. [2] BCFA: branched-chain fatty acids (sum of isobutyrate and isovalerate). [3] SEM: standard error of the means. Means within a column with different superscripts differ (p<0.05).

Compared with results in Trial 1, total VFA concentration increased when recorded after 12 h of incubation (Table 3), and the average concentration with S1 tended to be higher (p=0.052) than with S3. Average molar proportions of acetate were lowest for S1 (p<0.05), and propionate was higher with S1 than S3 (p<0.05), mainly when PEG was added, although no effect on the VFA molar pattern was observed with the addition of PEG. Despite an increase in lactate concentration due to PEG addition was again observed in this trial (0.11 *vs.* 1.82 mM, SEM = 0.1391; p<0.001) and no differences were detected between sorghum varieties, concentration in this trial reached lower values than in the former.

In vitro DMd after 12 h incubation increased (0.247 *vs.* 0.286 g/g; p<0.001) when PEG was added to the medium (Table 4). Although in the absence of PEG, the highest DMd was recorded in S1, no differences between varieties were observed when PEG was added (interaction substrate × PEG, p=0.001). The proportion

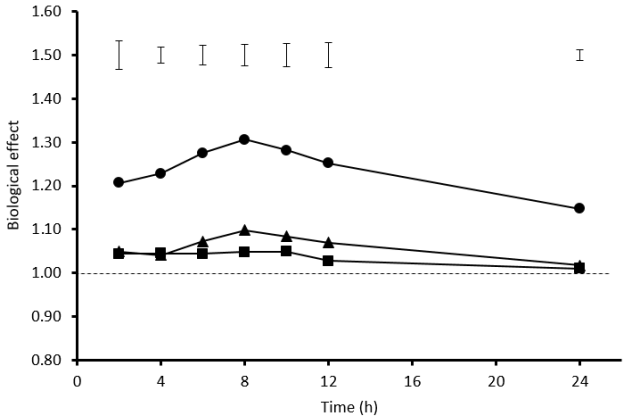

Figure 3. Biological effect of tannins on *in vitro* gas production from the three varieties of sorghum grain (S1 ■; S2●; S3 ▲) in Trial 1. For each time of incubation, upper bars show standard errors of the means. Dotted line shows the absence of a tannin effect (biological effect = 1).

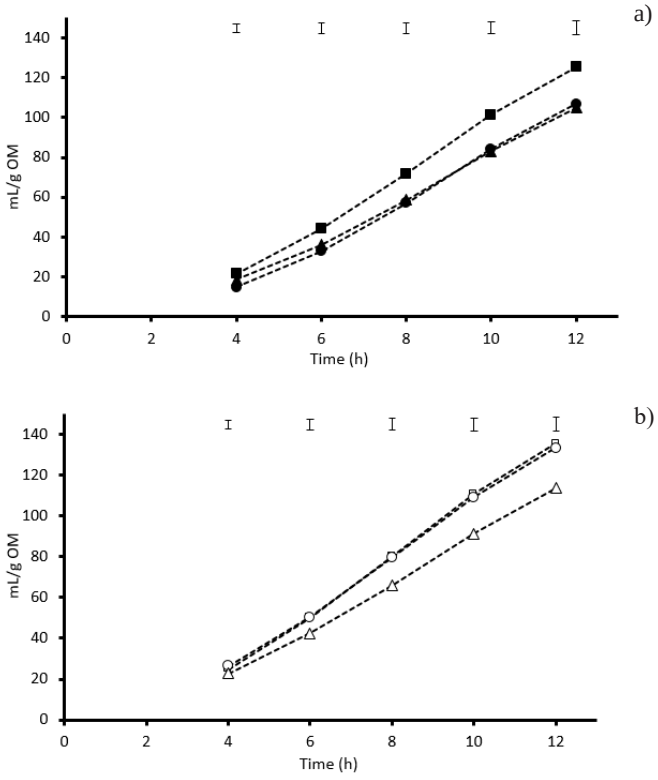

Figure 4. Pattern of *in vitro* gas production (mL/g organic matter, OM) from the three varieties of sorghum (S1 ■, □; S2●, ○; S3 ▲, △), without (a) or with (b) added PEG, in Trial 2. For each time of incubation, upper bars show standard errors of the means.

of starch disappearance followed a similar pattern, differences between S1 and both S2 and S3 disappearing when substrates were incubated with PEG (interaction substrate × PEG, $p = 0.014$). Consequently, the amount of starch per unit of incubated DM remaining at 12 h incubation when PEG was not added was lowest in S1 ($p<0.05$).

No differences in the BE of tannins were detected on gas production in Trial 2 ($p>0.05$) because of the high magnitude of the error term (coefficients of

Table 3. Total volatile fatty acids (VFA, mM) concentration and molar VFA proportions, together with lactate acid concentration (mM), recorded at 12 h of incubation of the three sorghum grains (S1, S2 and S3) without or with polyethylene glycol (PEG), in Trial 2.

Substrate	PEG[1]	VFA	Acetate	Propionate	Butyrate	BCFA[2]	Valerate	Lactate
S1	N	32.0	0.597ab	0.276a	0.104	0.0085	0.0066	0.11a
S2	N	29.5	0.612a	0.253ab	0.109	0.0096	0.0066	0.10a
S3	N	29.7	0.613a	0.254ab	0.108	0.0092	0.0064	0.11a
S1	Y	31.9	0.593b	0.277a	0.106	0.0087	0.0067	6.84b
S2	Y	32.1	0.612a	0.254ab	0.109	0.0091	0.0066	6.83b
S3	Y	29.0	0.612a	0.247b	0.113	0.0104	0.0069	7.44b
SEM[3]		0.92	0.0059	0.0054	0.0034	0.0005	0.0002	0.241

[1] N, without PEG; Y, with PEG. [2] BCFA: branched-chain fatty acids (sum of isobutyrate and isovalerate). [3] SEM: standard error of the means. Means within a column with different superscripts differ (p<0.05).

variation ranging from 0.09 to 0.28 from 4 to 12 h of incubation); however, differences between sorghum varieties were similar to those observed in the first trial, with estimated BE values of 1.07, 1.27 and 1.08 (SEM= 0.061) at 12 h of incubation for S1, S2 and S3, respectively (values of 1.03, 1.25 and 1.07 in the first trial).

Discussion

In a previous study (Amanzougarene *et al.*, 2017), we compared the fermentation pattern of three varieties of each corn, barley and sorghum grain, and observed the major range of differences in the response occurring in the latter. The aim of this work was to define the influence of nutritional characteristics on the extent of microbial fermentation, rather than to characterise the nutritive value of three varieties of sorghum grain for ruminants.

Table 4. Proportions of dry matter (DMd) and starch disappearance (g/g), and of resistant starch (mg/g incubated DM), after 12 h of incubation, for the three varieties of sorghum (S1, S2 and S3), in the presence or absence of polyethylene glycol (PEG), in Trial 2.

Substrate	PEG[1]	DMd	Starch disap.	Resistant starch
S1	N	0.289a	0.356a	444c
S2	N	0.214b	0.231bc	490ab
S3	N	0.239b	0.216c	507a
S1	Y	0.289a	0.360a	441c
S2	Y	0.284a	0.338a	423c
S3	Y	0.287a	0.296ab	456bc
SEM[2]		0.0057	0.0145	9.5

[1] N, without PEG; Y, with PEG. [2] SEM: standard error of the means. Means within a column with different superscripts differ (p<0.05).

In general, chemical composition of the experimental sorghum substrates agreed with values recorded from 23 samples by Lanzas *et al.* (2007), but starch proportion was lower than those reported by Rowe & Pethick (1994) and Offner *et al.* (2003). There were no major differences in CP or NDF proportions between the three sorghum varieties (Table 1), despite O'Brien (1999) reported that CP content of sorghum grains greatly varies among genotypes. Besides, only a slightly higher starch proportion was detected in S1 (0.06 and 0.08 higher than in S2 and S3, respectively). The phenolic and tannin contents of the three varieties studied here were within the range cited by Dykes & Rooney (2006) for white and brown sorghums.

The pattern of *in vitro* gas production of raw sorghum varieties (*i.e.*, when PEG was not added) showed rates and extent of fermentation in the range of those observed by Streeter *et al.* (1990), Opatpatanakit *et al.* (1994) and Lanzas *et al.* (2007). The white sorghum S1 was more extensively fermented than the brown varieties S2 and S3 in both incubation trials (Figs. 2a and 4a, respectively). Throughout both incubation trials, the volume of gas produced from S1 was over 0.10 higher than that from S2 and S3, in a proportion of 0.10 to 0.18 after 10-12 h. These results agree with the greater DM and starch disappearance recorded with S1 (Table 4), although the magnitude of treatment differences in gas production was two-fold higher to that observed among treatments in DMd. It has to be mentioned that incubation conditions in the second trial were adjusted to 12 h for the *in vitro* study of DM and starch degradation considering that, from the calculations of Offner *et al.* (2003), up to 0.73 sorghum degradation occurs at that time and, consequently, differences between substrates in *in situ* starch degradation are maximised.

Differences in the rate and/or extent of *in vitro* fermentation of sorghum varieties by rumen microbes have been mainly associated to three factors: the

content and nature of starch, the interaction with the protein matrix embedded in the endosperm, and the presence of tannins and phenolic compounds. The nature of starch in terms of either its amylose to amylopectin ratio or the floury or vitreous endosperm was not studied here. Some authors have associated a higher DM degradation and gas production of sorghum or maize grains to the proportion of waxy starch, because its branched nature allows for a better microbial access (Streeter *et al.*, 1990), or to a lower vitreousness (Philippeau & Michalet-Doreau, 1997). Rooney & Pflugfelder (1986) attributed some effect of chemical composition and physical structure of sorghum starch on grain digestibility; however, differences were minimised when they studied isolate starches. Therefore, as hypothesized by van Barneveld (1999), minor differences in fermentation between the three studied sorghum varieties can be assumed related to their type or amount of starch. In any case, the higher starch content in S1 might partly explain the higher volume of gas produced, associated to the higher starch disappearance observed (Table 4).

In the endosperm of cereal grains such as maize or sorghum, the nature and structure of the protein matrix in which the starch granules are embedded may restrict access of amylases to starch (Rooney & Pflugfelder, 1986). In maize, McAllister *et al.* (1993) observed an increase in starch degradation after 4 h of preincubation with protease. Such interaction between endosperm proteins and starch in reducing rate and extent of degradation may partly explain differences between vitreous or floury endosperm (Philippeau *et al.*, 2000). Sorghum protein fraction includes kafirins, accounting for around 0.80 of total endosperm protein, and non-prolamin proteins (Belton *et al.*, 2006). The sorghum varieties studied here had similar total CP content (106 to 113 g/kg, Table 1) and proportion of soluble proteins (0.15 to 0.19 of total CP, Fig. 1); however, 4 h protease digestion was the highest in S1, intermediate in S3 and lowest in S2. This should imply a wider access of microbial enzymes to endosperm starch in S1, partly explaining its highest starch disappearance (Table 4) and gas production (Figs. 2a and 4a). However, range of differences in protein digestibility did not apparently affect comparison in microbial fermentation between S2 and S3, since both recorded similar results in gas production and starch disappearance, suggesting that other factors should affect comparison between these two varieties.

The third mentioned factor on sorghum starch utilisation was the presence of phenolic compounds and tannins. Tannins may inhibit amylases but, more importantly, bind to carbohydrates and restrict enzyme access to nutrients, limiting their utilisation in the rumen (Makkar *et al.*, 1995; Mueller-Harvey, 2006). Streeter *et al.* (1990) observed a 1.4-fold higher gas production from tannin-free than from bird-resistant sorghum varieties. In practical situations, the inclusion of sorghum grain in ruminant diets is commonly linked to its content of condensed tannins, flavonoids, and simple phenolic compounds, with ferulic and p-coumaric acids being dominants (Dykes & Rooney, 2006), that may hamper nutrient utilisation in the rumen. Although flavonoids and simple phenolic acids may form complexes with proteins, there is no conclusive evidence that such interaction would cause a reduction in protein and starch digestibility like condensed tannins (Duodu *et al.*, 2003, Belton *et al.*, 2006), and thus the effect of these compounds on fermentative activity may differ according to their chemical nature. Barros *et al.* (2012) observed a two-fold increase of *in vitro* resistant starch with condensed tannins than with phenolic monomers.

The sorghum varieties used in this experiment had variable proportions of phenolic compounds, with negligible contents in S1, minor concentration in S3 and moderate proportions of both phenolic acids and tannins in S2 (Table 1). It is worth mentioning that even being a brown variety, tannins content in S3 were quite low, showing that pericarp colour is not a reliable indicator of tannins in sorghums (Dykes & Rooney, 2006). The ability of PEG to bind to tannins allows for estimating the effect of tannins on fermentative activity of rumen microbial population (Makkar *et al.*, 1995; Silanikove *et al.*, 2001) and, although some minor changes in microbial community have been reported (Belenguer *et al.*, 2011), it is consider to be inert to rumen function (Makkar *et al.*, 1995). To our knowledge there is no information about the binding capacity of PEG to simple phenolic compounds; however, Khazaal *et al.* (1996) and Getachew *et al.* (2002) observed almost the same correlation coefficients for the relationship between either TT or TP and the increase in gas production when fibrous feeds were incubated in the presence of PEG. Since in those works tannins were, on average, 0.53 and 0.69, respectively, of TP of the substrates, it could be assumed that other phenolic compounds would react similarly.

The increase in lactate concentration when adding PEG to the media might be attributed to an increase in starch availability when the effect of tannins was inactivated; however, the lack of response differences between the tannin-free S1 and S2 and S3 might partly suggest an artefact in the colorimetric method of analysis. As it can be seen in Figs. 2 and 4, as well as in Table 4, the inclusion of PEG in the incubation solution showed no effect on gas production or starch disappearance when S1 was incubated, indicating the

absence of any measurable effect of phenolic compounds on the activity of microbes. A minor response was found with S3, because of its low proportion of TP and TT. In this case, the lower gas production from S3 should thus be mostly a response to the lower digestibility of the protein fraction, interfering in starch utilisation at a higher extent than in S1, as explained above. In contrast, the negative effect of tannins on fermentation of S2 was clearly manifested, since their inactivation increased gas production values (BE) over 1.20 to 1.30 times that of the substrate without PEG in the first incubation trial (Fig. 3). The magnitude of such response is relatively high, considering the amount of TP and TT in S2, since our group previously observed BE of tannins in browse forage legumes ranging from 1.1 to 1.3 in *Acacia cornigera*, with almost two-fold higher TP and TT concentration, and from 1.3 to 1.6 in *Leucaena leucocephala* with five and eight-fold higher TP and TT contents (Rodríguez *et al.*, 2014). Fig. 1 shows that inactivation of tannins when PEG was added could also allow for a high digestibility of the protein fraction of S2, and thus protein was not the limiting factor in fermentation of starch with this variety.

From the results obtained it can be concluded that differences in nutritive value between sorghum varieties can be mainly attributed to the digestibility of its protein fraction, which may restrict the access of microbial enzymes to starch, and the biological effect of the phenolic compounds (tannins and simple phenolic compounds), whereas the chemical and physical structure of starch may be less important. Colour of grain does not necessarily imply a high proportion of phenolic compounds, and consequently a lower extent of rumen utilization.

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