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In vitro fermentation response to alkaline treated sorghum grain

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ABSTRACT

Effects of three alkaline treatments: NaOH, $NaHCO_3$ and wood ash on the crude protein (CP), condensed tannin (CT), neutral detergent fiber (NDF), *in vitro* gas production kinetics and dry matter (DM) digestibility of sorghum grain were determined. The NaOH (2% w/v), $NaHCO_3$ (2% w/v) and wood ash (5% w/v) treatments were completed by soaking of sorghum grain with treatment solutions in the proportion of 1 L of solution to 1 kg of grain for 12 h. Gas production was measured at 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h post incubation. Alkaline treatment decreased significantly the CT ($P<0.001$) and NDF content ($P<0.05$) of sorghum grain, where it had no effect on the CP content. Treated grain with wood ash extract showed the highest ($P<0.05$) maximum gas production (*A*), and NaOH treatment trended ($P<0.06$) to the fastest fractional fermentation rate. Fractional rate of gas production and cumulative gas production overall incubation times except of 48 h ($P<0.05$) were not changed by $NaHCO_3$ and wood ash treatment. Maximum ($P<0.01$) *in vitro* DM digestibility of alkaline treatment of sorghum grain was observed by NaOH. Cumulative volatile fatty acids concentration was increased ($P<0.01$) at 4h for NaOH treated compared to untreated sorghum and then decreased ($P<0.001$) at 48 h post incubation. Alkaline treatment of sorghum grain may become attractive due to raise in nutritive values of sorghum and hide the negative effects of its tannin in the future if the costs of other processing continue to rise.

Key words: *in vitro* dry matter digestibility, *in vitro* gas production, sorghum grain, sodium hydroxide, sodium bicarbonate, wood ash

Introduction

Sorghum (*Sorghum bicolor*), a tropical grain, requires less water and can be grown successfully under a wider variety of dry lands or irrigated conditions than corn, but it is often discriminated against because of variable quality, inconsistent cattle growth rates and efficiencies, and a lower feeding value than that of corn (Streeter et al., 1990). Sorghum contains 71% starch, which makes it reliable source of energy for ruminants (Herrera-Saldana et al., 1990).

With increasing dependence upon sorghum grain as advantageous replacement for cereal grains (especially maize) to provide the energy and protein requirements of ruminant in developing countries and regarding the present competence of cereal sources for human and animal nutrition in such countries, the need for raising the overall nutritional status of sorghum grain as potential substitute for industrial cereal grain has become increasingly important. Sorghum grain has a resistant protein matrix and corneous endosperm that reduced its starch degradability by ruminal bacteria and is slower than the starch degradability of corn, oats, or barley (McAllister et al., 1993; Herrera-Saldana et al., 1990) that requires to be processed.

Another important factor affecting the nutritional value of sorghum grain is the presence of free and condensed phenols (tannins). Tannins are complex phenolic compounds with molecular weight range 3000–20000, classified either as hydrolysable or condensed. Van Buren & Robinson (1969)

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reported that tannins affect the growth of animals in three main ways: they have an astringent taste, which effects palatability and decreases feed consumption; they form complexes of reduced digestibility with proteins; and they act as enzyme inhibitors. All these factors result from the interaction of tannins and proteins to form soluble and insoluble complexes, an interaction that depends primarily on the relative proportions of phenol and protein. Though tannins mainly exert their effects on proteins, they also have effects on carbohydrates, particularly hemicellulose, cellulose, starch and pectin (McSweeney et al., 2001; Leinmüller et al., 1991; Schofield et al., 2001).

It has been observed that chemical treatment of whole grain with alkaline solution, such as NaOH, disrupts the seed coat by partial hydrolysis of hemicellulose and lignin and causes swelling of the outer starch granules so that ruminal bacteria and digestive enzymes gradually gain access to the starchy endosperm (Ørskov, 1979; Ørskov et al., 1980).

The aim of present study was to determine whether alkali treatment could affect chemical composition, fermentation characteristics, digestibility sorghum grain and cumulative volatile fatty acid production in the *in vitro* batch culture.

Materials and Methods

Sample preparation and alkaline treatment

Samples of annual autumn's sorghum grain (2009) were supplied from a local commercial supplier at Mianeh city, Eastern Azerbaijan province at northwest of Iran. In this study, different types of chemical processing, sodium hydroxide (NaOH, Merk), sodium bicarbonate ($NaHCO_3$, Merk) and wood ash were used to monitor effects of alkaline treatment on sorghum grain. Wood from an *Elaeagnusan gastifolial* tree was made into a fire to become ash completely and 2 kg of cooled and ground (2 mm sieve) ash was collected. A 20 % (w/v) wood ash solution was prepared by dissolving 500 g of prepared wood ash in 10 L (5% w/v) of distilled water in plastic buckets. The mixture was stirred for five minutes and was allowed to settle for 15 h. The resulting supernatant was carefully removed and filtered through cotton cloth. The pH of the ash extract was 11.3. One kilogram of whole sorghum grain was soaked in 1 L of wood ash extract and shacked 10 rpm in a rotary shaker for 12 h. For NaOH and $NaHCO_3$ processing, sorghum grain was mixed with 20 g/L of each of their solution (2% w/v) in the proportion of 1 L of solution to 1 kg of grain and settled for 12 h. All treated grains then washed and dried in exposure of the sun. Untreated and alkaline treated samples (300 mg) were weighed into 50 ml serum vial.

In vitro batch culture incubation

Rumen liquor samples were obtained from the three adult (16 months old) Ghezel×Arkhar-merino crossbred male sheep (weight 41±1.7 kg) that were fed on a diet containing 60% alfalfa hay and 40% commercial concentrate at maintenance level (NRC, 1985). Diet offered to the animals twice daily at 9.00 AM and 4.00 PM in equal sized meals. The animals had access to fresh water and mineral lick *ad libitum*. Rumen fluid was collected 2 h after the morning feeding. Rumen fluid was pumped with a manually operated vacuum pump and transferred into pre-warmed thermos flask, combined, filtered through four layers of cheesecloth and kept at 39°C under a continuous CO_2 stream according to Parnian et al. (2013). McDougall (1948) buffer solution was prepared and placed in a water bath at 39°C. In procedure one, untreated and alkaline treated sorghum grains were incubated in triplicate 50 mL serum vials with 20 mL of rumen liquor and McDougall buffer (1948) solution (1:2) for each time of incubation. Three vials containing only the rumen fluid/buffer solution and no feed sample was allocated for each time of incubation as blank. The vials were sealed immediately after loading and were affixed to a rotary shaker platform (lab-line instruments Inc Melors dark, USA) and housed in an incubator at 39°C. For each incubation time (2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h), the vials were removed from the incubator and gas production (mL/g dry matter) was measured using a water displacement technique (Fedorak & Hrudey 1983).

In vitro dry matter (DM) digestibility

Simultaneously with procedure one, *in vitro* digestibility for dry matter was determined. In procedure two, each treatment was adjusted to triplicate vials for four incubation times (2, 12, 24 and 48 h). Three vials were also allocated to blank for each incubation time. All vials were loaded with treated and untreated sorghum grain and buffered rumen liquor then incubated similar to procedure one but the produced gas was emptied during incubation via needles that installed on rubber caps of each vial. Upon removal at each incubation time, vials were at -20 °C for further analysis. A 10 ml aliquot from the supernatant were transferred into eppendorf tubes of 2 mL and stored at -20°C until analyzed for VFA concentration. Then, vials were thawed at room temperature and content of each vial was transferred into pre-weighed 50 mL falcon tubes (Cole-Palmer, Montreal, QC)

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and centrifuged at 1000×*g* for 10 minutes at 4°C and contents washed thoroughly one time by distilled water and more time by saline buffer, to ensure removal any microbial contamination of the contents by rumen microorganisms. After this, falcon tubes containing the incubated diets residua were transfers to oven and the contents have been dried at 55°C for 48 h and weighed to determine dry matter concentrations for the estimation of *in vitro* DM digestibility.

Chemical analysis

Sorghum grain dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (method ID 920.30) and crude protein (CP, method ID 984.13) were determined by procedures of AOAC (1999). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined with an ANKOM^{200} (Ankom Technology, USA) Fiber Analyzer using the manufacturer recommended reagents and filter bags (#F57). Analysis of NDF was conducted without a heat stable amylase and expressed exclusive of residual ash. ADF also expressed exclusive of residual ash. Total Tannins were measured at 725 nm using the Folin Ciocalteau spectophotometric method (Makkar, 2000). Tannic acid was used as the standard to evaluate the amount of tannins. Total volatile fatty acids were determined by the Markham's method (1942).

Curve peeling and statistical analysis

Gas production kinetic parameters were estimated using DUD method with NLIN option of GLM procedure of SAS 9.1 (2003) according to the non-linear exponential model:

$$GP = A\,(1 - e^{(-ct)})$$

where GP (mL) is cumulative gas production at incubation time *t* (h), *A* is the maximum gas production (mL/g DM) after the asymptote is reached, and *c* (/h) is the fractional fermentation rate. The analysis of variance was performed using the GLM procedures of SAS (2003) using the model:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

where Y_{ij} is the value of each individual observation for the dependent variables, μ the overall mean, α_i the effect of treatment, and ε_{ij} the random residual error. Differences among treatments within incubation were determined using LSMEANS with PDIFF statement (SAS Institute Inc., 2003).

Results and Discussion

Sorghum grain chemical composition before and after chemical treatment are presented in Table 1 and Table 2, respectively. Unlike the CP contents that were similar for untreated and alkaline treated grain, each of chemical treatments markedly decreased (P<0.001) the tannin and fiber content of the sorghum grain. Decreased tannin was observed with NaOH following wood ash and $NaHCO_3$, respectively. The chemical composition of the sorghum grain (Table 1) shows that the values are different to some extent from those in NRC (2001) tables of feed composition. These differences in nutrient composition could be attributed to grain species, soil type, fertilization level, season, geographical location and weather conditions, as reported by Van Straalen & Tamminga (1990).

Results indicate that alkaline treatment changed the tannin content of sorghum significantly (P<0.001) when grain soaked in alkalis for 12 h. This result was in agreement with previous reports (Banda-Nyirenda & Vohra, 1990; Ali et al., 2009). The active group on tannin, phenolic hydroxyl group, facilitates phenols to dissolve readily in alkaline solutions. showed that when 0.05 M NaOH was used it removed 84% of tannins from sorghum after 24 h at 30°C.

Table 1. *Chemical composition of Sorghum grain (g/kg DM)*

	Sorghum grain
Dry matter	897
Organic matter	962
Crude protein	108
Neutral detergent fiber	224
Acid detergent fiber	139
Ether Extract	28
Non fiber carbohydrate	499
Hemicellulose	85
Tannin	23.4

NFC: DM - (CP+EE+NDF+Ash)
Hemicellulose = Neutral detergent fiber - Acid detergent fiber

Cumulative gas production overall different incubation times upon *in vitro* fermentation of the sorghum grain are shown in Figure 1 and Table 3. In general, GP was greatest for alkaline treated sorghum after 4 h of incubation compared to untreated grain. As for the fermentation parameters estimated with the exponential model, treated grain with wood ash extract for 12 h showed the highest (P<0.05) maximum gas production (*A*), and NaOH treatment trended (P<0.06) to the fastest fractional fermentation rate. $NaHCO_3$ and wood ash treatment had no effect on fractional rate of GP and cumulative gas production overall incubation times except of 48 h (P<0.05).

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Maximum effect of NaOH treatment of sorghum grain on *in vitro* DM digestibility (Table 3) was occurred at initial time of incubation and then continued to fall down but this potential in wood ash treatment had a considerable raise from 4 h to 12 h of incubation (12.5 to 25%, respectively). VFA concentration was increased (P<0.01) at 4h for NaOH treated compared to untreated sorghum and then decreased (P<0.001) at 48 h of incubation time. This trend was the same for $NaHCO_3$ and wood ash treatment.

Marked increases in cumulative gas production (12 to 25% increases for 48 h of incubation) with chemical treatment can be attributed to enhanced *in vitro* dry mater digestibility. Berger et al. (1981) evaluated the effect of 3% and 6% NaOH treated sorghum on *in vitro* and *in situ* dry matter digestibility and observed that improvements on dry matter digestibility were due the effects of NaOH on the grains and not related to other effects such as increased pH. Although the exact mechanism by which alkali improves sorghum grain digestibility is not known yet, part of the improvement may resulted by solubilization of hemicellulose in the seed coat, which renders the starch portion of the kernel more available for microbial attack.

Table 2. *Effects of alkaline treatment on crude protein and tannin contents (g/kg) of sorghum grain.*

		Alkaline treated				Comparisons					
	Untreated	NaOH	$NaHCO_3$	Wood ash	SEM	Raw *vs.* NaOH	Raw *vs.* $NaHCO_3$	Raw *vs.* Wood ash	NaOH *vs.* $NaHCO_3$	NaOH *vs.* Wood ash	$NaHCO_3$ *vs.* Wood ash
Sorghum grain											
CT	23.4	3.6	14.2	10.2	1.34	<.0001	0.0013	0.0001	0.0005	0.0085	0.0685
CP	108	104	106	105	2.1	0.1817	0.5187	0.2932	0.4537	0.7444	0.6646
NDF	224	195	208	205	3.6	0.0004	0.0135	0.0062	0.0335	0.0760	0.6133

CT: Condense tannin; CP: Crude protein; NDF: Neutral detergent fiber

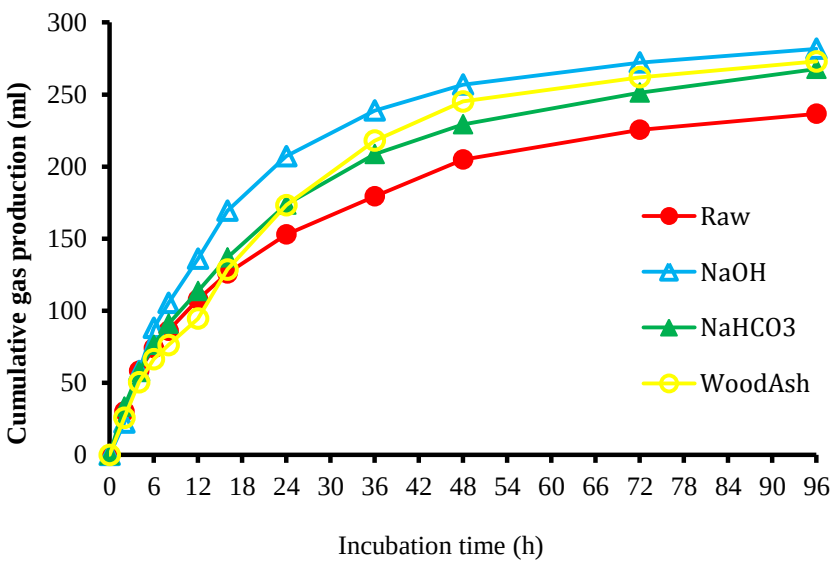

Figure 1. *Cumulative gas production curves of alkaline treated and untreated sorghum grain overall incubation times (Raw, Untreated sorghum grain; NaOH, sorghum grain soaked in 2% NaOH solution; $NaHCO_3$, sorghum grain soaked in 2% $NaHCO_3$ solution; Wood Ash, sorghum grain soaked in 5% wood ash extract solution).*

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Table 3. *Effects of alkaline treatment of sorghum grain on in vitro dry matter digestibility, cumulative volatile fatty acids, and rumen fermentation characteristics.*

Incubation time (h)	Untreated	Alkaline treated			SEM	Comparisons					
		NaOH	$NaHCO_3$	Wood ash		Raw *vs.* NaOH	Raw *vs.* $NaHCO_3$	Raw *vs.* Wood ash	NaOH *vs.* $NaHCO_3$	NaOH *vs.* Wood ash	$NaHCO_3$ *vs.* Wood ash
Cumulative gas production (mL/g dry matter)											
4	58.4	58.17	57.8	50.4	4.43	0.9726	0.9178	0.2372	0.9452	0.2494	0.2752
12	107.8	136.0	113.6	94.5	10.54	0.0949	0.7084	0.3972	0.1706	0.0236	0.2357
24	152.9	207.2	173.7	173.2	7.67	0.0010	0.0918	0.0977	0.0148	0.0139	0.9683
48	204.8	256.8	229.3	245.2	6.87	0.0007	0.0360	0.0032	0.0219	0.2640	0.1405
*In vitro gas production parameters**											
A	238.8	277.3	269.6	286.9	12.68	0.0641	0.1237	0.0278	0.6809	0.6062	0.3637
c	0.0401	0.0585	0.0410	0.0404	0.00575	0.0531	0.9114	0.9651	0.0635	0.0569	0.9461
In vitro dry matter digestibility (mg/g DM)											
4	80	153	122	90	5.9	<.0001	0.0010	0.2635	0.0057	<.0001	0.0047
12	160	261	211	200	6.1	<.0001	0.0004	0.0020	0.0004	0.0001	0.2328
24	318	350	329	327	6.2	0.0063	0.2415	0.3412	0.0063	0.0290	0.8067
48	474	521	473	447	10.4	0.0119	0.9418	0.1085	0.0119	0.0010	0.0966
Cumulative volatile fatty acids (mmol/L liquor)											
4	5.3	14.7	6.0	8.0	1.34	0.0012	0.7348	0.1981	0.0018	0.0080	0.3233
12	23.3	25.6	19.0	22.0	3.06	0.6049	0.3466	0.7662	0.1625	0.4221	0.5083
24	58.0	48.0	40.0	49.3	5.49	0.2335	0.0490	0.2965	0.3327	0.8678	0.2635
48	82.0	57.7	73.3	61.7	2.16	<.0001	0.0219	0.0002	0.0009	0.2268	0.0051

**A*: the maximum gas production (mL/g DM); *c*: the fractional fermentation rate (/h).

Hemicellulose is solubilized effective via NaOH in comparison with wood ash and $NaHCO_3$. Miron et al. (1997) reported an increased NDF digestibility (54.2 *vs.* 45.7) in cows fed a diet including NaOH treated sorghum grain, which indicates possible improved susceptibility of grain's cell wall to rumen microorganism by alkaline treatment. Additional beneficial effect of treatment of sorghum grain with alkaline solutions is that tannin content was decreased considerably (Table 2). Tavendale et al. (2005) proposed 2 mechanisms whereby condensed tannins reduce methane emissions from ruminants: 1) indirectly through a reduction in fiber digestion, which decreases H_2 production, and 2) directly through an inhibition of the growth of methanogens. It is known that bacteria involved in fiber digestion are more sensitive to condensed tannins than proteolytic bacteria (Bae et al., 1993; Jones et al., 1994). In our study, it seems that the lack of inhibitory effects of tannins on methane production in treated sorghum grain as well as possible deactivation of these components through chemical treatment were associated with increased *in vitro* gas production and dry matter digestibility.

Decreased VFAs concentration at 24 h and subsequent times of incubation was in agreement with Miron et al. (1997) who reported 7% decrease in ruminal VFAs concentration with cows fed diet containing NaOH treated sorghum than untreated grain, although the molar percentages of VFA and the ratio of acetate to propionate were similar. However, higher molar propionate in the *in vitro*

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fermentation system and lower protozoan count (Wang et al., 1994) produced by tannins has been reported (Makkar et al., 1995).

Conclusion

Alkaline treatment reduced condensed tannin levels in sorghum grain confirming other studies showed that alkalis can be used to reduce the tannin level of tannin rich feeds. Chemical treatment of sorghum grain may become attractive in the future if the costs of mechanical processing continue to rise. Processed sorghum grain can be considered as potential substitute for conventional similar cereal grains in developing countries, which availability and economic justification of common feed resources are limiting factors in sustainable ruminant nutrition.

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