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Phenolic Composition, Fermentation Profile, Protozoa Population and Methane Production from Sheanut (*Butryospermum Parkii*) Byproducts *In vitro*

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ABSTRACT: Sheanut cake (SNC), expeller (SNE) and solvent extractions (SNSE) samples were evaluated to determine their suitability in animal feeding. The CP content was highest in SNSE (16.2%) followed by SNE (14.7%) and SNC (11.6%). However, metabolizable energy (ME, MJ/kg) was maximum in SNC (8.2) followed by SNE (7.9) and SNSE (7.0). The tannin phenol content was about 7.0 per cent and mostly in the form of hydrolyzable tannin (HT), whereas condensed tannin (CT) was less than one per cent. The *in vitro* gas production profiles indicated similar y max (maximum potential of gas production) among the 3 by-products. However, the rate of degradation (k) was maximum in SNC followed by SNE and SNSE. The $t^{1/2}$ (time taken for reaching half asymptote) was lowest in SNC (14.4 h) followed by SNE (18.7 h) and SNSE (21.9 h). The increment in the *in vitro* gas volume (ml/200 mg DM) with PEG (polyethylene glycol)-6000 (as a tannin binder) addition was 12.0 in SNC, 9.6 in SNE and 11.0 in SNSE, respectively. The highest ratio of CH_4 (ml) reduction per ml of the total gas, an indicator of the potential of tannin, was recorded in SNE (0.482) followed by SNC (0.301) and SNSE (0.261). There was significant ($p<0.05$) reduction in entodinia population and total protozoa population. Differential protozoa counts revealed that *Entodinia* populations increased to a greater extent than *Holotricha* when PEG was added. This is the first report on the antimethanogenic property of sheanut byproducts. It could be concluded that all the three forms of SN byproducts are medium source of protein and energy for ruminants. There is a great potential for SN by-products to be incorporated in ruminant feeding not only as a source of energy and protein, but also to protect the protein from rumen degradation and suppress enteric methanogenesis. (**Key Words:** Sheanut Meal, *In vitro*, Tannin, Phenolics, Methane, Protozoa)

INTRODUCTION

At the global level, the reasons to push for recycling of organic by-products and wastes in animal feeding fall into two different and complementary trends: in highly-developed industrialized regions of the world use of such products as unusual feedstuffs ensures more economical and ecological disposal, while in the developing countries like India this is a way to augment both quantity and quality of feed supply. There is acute shortage of conventional feeding ingredients with a consequent dwindling in productivity and profitability. As the production and processing of food becomes more and more industrialized, the sources of supply are increasingly located near large urban centres, i.e. near the demand. This in turn logistically enhances the conditions for industrializing the processing of by-products as livestock feed. Since the beginning of the 20th century the utilization of by-products as feed was accompanied by efforts in feed legislation to avoid negative effects on animals' health and the quality of food. On the one hand the utilization is intended due to ecological and economical reasons, on the other hand the use of by-products will be impaired more and more due to reduced social acceptance, i.e. the willingness of people to accept food from animals that were fed some groups of by-products (for example meat and bone meal). Therefore, incorporation of byproducts in animal feeding can help food processors earn revenue while preventing pollution.

The green fruit of Shea (*Butryospermum parkii*) tree has a pulp that covers the seed or nut. The harvest follows 3 to 5 years cycle and yields 80 kg of nuts and from these nuts, oil will be extracted and leaving the residue (Abidemi et al., 2009; Kumar et al., 2010). Sheanut meal is now receiving increased attention as a potential feed ingredient due to the increased amounts that are available as a result of high demand for shea fat for cosmetics and as a cocoa butter

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substitute in chocolate (Lipp and Anklam, 1998). The phenolic components and the antimethanogenic potential of sheanut products have not yet been fully explored. The present *in vitro* study was therefore undertaken to evaluate the sheanut cake, expeller and solvent extractions for their suitability as animal feed.

MATERIALS AND METHODS

Experimental samples

The sheanut cake, expeller (obtained after oil extraction by mechanical pressing) and solvent extractions (obtained after oil extraction using an organic solvent such as hexane) were obtained from the local market. The samples were ground to pass through 1 mm sieve and used for further analysis. The dried and milled samples were analyzed for their chemical composition. The protein contents were analyzed using standard method (AOAC, 1995). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analyzed according to Van Soest et al. (1991). The samples were analyzed without α-amylase and sodium sulfite. Detergent fibre values were expressed including residual ash.

Tannin assays

For tannin assays, samples were ground to fine powder in a Cycoltec mill (Foss, Denmark) and 0.2 g of samples were extracted in 10 ml aqueous acetone (acetone: water, 7:3) twice for 20 min in an ultrasonic water bath. The extracted samples were centrifuged (6,000×*g*, 10 min, 4°C), and the supernatants were combined and used for tannin analysis on the same day.

Tannin assays were according to Makkar (2003). Total phenols and total tannins in the extract were determined by a modification of the Folin-Ciocalteu method (Makkar, 2003) using polyvinylpolypyrrolidone (PVPP) to separate tannin phenols from non-tannin phenols, and condensed tannins (CT) were determined by the butanol-HCl-iron method. Both total phenols and total tannins were expressed as tannic acid equivalent and condensed tannins as leucocyanidin equivalent. The hydrolysable tannin was calculated as the difference between total tannin and CT.

In vitro gas production test

For the *in vitro* gas production test, rumen liquor was collected 1.5 h after morning feeding from two cannulated Holstein Friesian crossbred bulls fed with a total mixed ration based on finger millet (*Elusine coracana*) straw (*ad libitum*) and commercial concentrate mixture (about 3.0 kg). The rumen liquor, strained through 3 layers of muslin cloth, was pooled and used as the source of inoculum. A total of 200 mg air equilibrated samples were incubated with 30 ml buffered rumen inoculum (10 ml rumen fluid + 20 ml buffer) (Menke et al., 1979) in 100 ml calibrated syringes and placed in a water bath maintained at 39°C. The incubations were conducted in triplicate for each sample on 2 successive days and these incubations were repeated three times, with an interval of 1 wk. Incubations without sample served as the blanks with every set. The difference in the composition and activity of the rumen inoculum among incubations was controlled by parallel incubation of reference standard feedstuffs as suggested by Menke et al. (1979). Incubations were run for 24 h with recording of gas production after 6 and 24 h. The interference of tannins on *in vitro* fermentation was assessed using PEG-6000 as a tannin binder. The magnitude of the increase in gas volume on PEG addition to the substrate at a ratio of 2:1 was taken as an index of tannin's interference on rumen fermentation. To determine the rate of degradation ($t^{1/2}$) of the substrate, the samples were incubated for 96 h, with recording of the gas volumes after 2, 4, 6, 8, 10, 12, 24, 36, 48, 72 and 96 h. The data was subjected to graph-pad prism programme to determine Y min (minimum amount of gas production), Y max (maximum potential of gas production), $t^{1/2}$ and k (rate of gas production).

The metabolizable energy (ME) was calculated using the equation suggested by Krishnamoorthy et al. (1995).

Gas and methane estimation

After 24 h of incubation, total gas was recorded from visual assessment of the calibrated scale on the syringe. The gas produced due to fermentation of the substrate was calculated by subtracting gas produced in blank syringe (containing no substrate, but only the inoculum and buffer) from total gas produced in the syringe containing substrate and inoculums. The gas produced in standard syringe (containing concentrate and hay standard from Hohenheim University) was used to check variation in the quality of inoculum. For methane estimation 1 ml gas was sampled from the head space of syringe in an airtight syringe (Hamilton) and injected into Thermo-Fisher gas chromatograph equipped with thermal conductivity detector and stainless steel column packed with Porapak-Q. Temperature of injector oven, column oven and detector were 60, 100 and 110°C, respectively. Based on the methane percentage estimated in the gas sample, methane production was calculated (methane volume (ml) = methane %×total gas produced (ml) in 24 h).

Rumen fluid analysis

After the termination of the incubation (24 h) the supernatant was kept under -20°C until further analysis. The rumen fluid samples were analyzed for ammonia N (Conway, 1957) and total volatile fatty acid (Barnett and Reid, 1957).

Enumeration of protozoa

Rumen ciliates were identified according to Hungate (1966). Spirotrichs not identified to generic level were classified into small Spirotrichs (mainly Entodinia with an average size 42 μm×23 μm) and large Spirotrichs (mainly Diplodinia with an average size of 132 μm×66 μm). The protozoa numbers were calculated according to Kamra et al. (1991).

Statistical analysis

Data on all the parameters such as methane per cent, per cent increase in methane, protozoa count were analyzed using ANOVA model using SAS 9.0/SPSS and standard error of mean and LSD were computed. The model $Yij = \mu+\alpha i+\beta j+ \varepsilon ij$, where Yij is the dependent variable, μ is the least squares mean, αi is the effect of PEG and βj is the effect of sheanut byproducts was used for evaluating the effect of PEG.

RESULTS AND DISCUSSION

Phenolic components and antimethanogenic potential of sheanut byproducts have not yet been explored. The nutrient composition and phenolic fractions of sheanut cake (SNC), expeller (SNE) and solvent extractions (SNSE) are presented in Table 1. The CP (%) was highest in SNSE (16.2) followed by SNE (14.7) and SNC (11.6). Metabolizable energy (ME, MJ/kg) was maximum in SNC (8.2) followed by SNE (7.9) and SNSE (7.0). However, Okai et al. (1995) recorded 19.0 MJ/kg, ME in SNC, probably due to the difference in the nutrient composition. The protein and energy contents of SN byproducts indicated that they are medium source of protein and energy for ruminants. The nutrient composition is almost similar to palm kernel meal (INRA, 2004). The tannin phenol content was about 7.0 per cent and most of them were in the form of hydrolyzable tannin (HT). The condensed tannin (CT) was less than 1.0 per cent in SN byproducts. The concentrations of tannins in sheanut products were high relative to other feedstuffs such as sorghum and peas (Dei et al., 2007).

The gas production profiles presented in Table 2 indicated similar y max (maximum potential of gas production) among the samples. However, the maximum rate of degradation (k) was highest in SNC followed by SNE and SNSE. The $t^{1/2}$ (time taken for reaching half asymptote) was lowest in SNC (14.4 h) followed by SNE (18.7 h) and SNSE (21.9 h), therefore SNC is a better source of energy for ruminants as compared to SNE or SNSE.

Table 1. Nutrient composition and phenolic fractions (% DM) of sheanut cake, expeller and solvent extraction

	SN-cake	SN- expeller	SN-solvent extracted
Organic matter	84.9	84.9	85.0
Crude protein	11.6	14.7	16.2
NDF	52.9	55.2	61.9
ADF	35.5	35.9	41.9
ADL	6.70	7.10	4.80
Ether extract	4.90	2.80	1.60
Total ash	5.10	5.10	5.00
ME (MJ/kg DM)	8.2	7.9	7.0
Total phenols	7.73	6.98	7.06
Total tannin phenols	7.37	6.77	6.81
Condensed tannin	0.66	0.49	0.40
Hydrolysable tannin	6.71	6.28	6.41

SN-cake = Sheanut cake; SN-expeller = Sheanut expeller; SN-solvent extracted = Sheanut solvent extracted.
NDF = Neutral detergent fibre, ADF = Acid detergent fibre; ADL = Acid detergent lignin.

The gas production with PEG is presented in Table 3. The increment in *in vitro* gas volume (ml/200 mg DM) with PEG over and above that of without PEG was 12.0 in SNC, 9.6 in SNE and 11.0 in SNSE, respectively. Tannin bioassay (% increase in gas volume with PEG-6000), a measure of the biological activity of tannin was highest in SNC. In an earlier study Bhatta et al. (2001) recorded similar findings with tamarind (*Tamarindus indica*) seed husk (containing 140 g CT kg^{-1} DM) when incubated with PEG. The highest ratio of CH_4 (ml) reduction per ml of the total gas due to the presence of active tannin was recorded in SNE (0.482) followed by SNC (0.301) and SNSE (0.261). This ratio could be used as a effective tool to screen and categorize large number of samples to determine their capacity to suppress methanogenesis *in vitro* because it takes into account the total gas production (rumen fermentation) also. Higher ratios of CH_4 reduction per ml of the total gas indicate that a particular candidate would be better as a rumen modifier for methane reduction than compounds yielding lower ratios. Reduced rumen methanogenesis could be linked to the role of sheanut tannins in reducing

Table 2. Fermentation kinetics of sheanut cake, expeller and solvent extractions

	SN-cake	SN- expeller	SN-solvent extracted	SEM	p
Y min (ml)	0.008[a]	0.122[b]	2.268[c]	0.002	0.0236
Y max (ml)	15.13[b]	15.41[c]	14.82[a]	0.569	0.0451
K (Rate of degradation)	0.04802[c]	0.03702[b]	0.03154[a]	0.002	0.0365
($t^{1/2}$) Half-time (h)	14.43[a]	18.72[b]	21.98[c]	1.030	0.0249

Means in a row with different superscript differ significantly ($p<0.05$).

Table 3. Fermentation kinetics of sheanut cake, expeller and solvent extractions with PEG-6000 *in vitro*

Gas production (ml/200 mg DM)	SN-cake	SN- expeller	SN-solvent extracted	SEM	p
8 h	42.0 [a]	44.3 [b]	45.6 [c]	0.56	0.0356
With PEG	52.0 [a]	49.0 [b]	53.0 [c]	1.06	0.0426
24 h	53.0 [a]	57.0 [b]	59.0 [c]	1.25	0.0365
With PEG	65.0 [a]	67.6 [b]	69.6 [c]	1.05	0.0435
Total gas	23.0 [a]	27.0 [b]	28.3 [c]	1.26	0.0365
With PEG	35.0 [a]	37.6 [b]	39.3 [c]	0.98	0.0345
Net gas	6.00 [a]	10.0 [b]	11.3 [c]	1.96	0.0375
With PEG	18.0 [a]	19.6 [b]	22.3 [c]	0.96	0.0356
Tannin bioassay (% increase in gas production)	200.0 [b]	96.0 [a]	97.4 [a]	3.26	0.0423
Methane (ml/200 mg DM)	1.89 [a]	2.86 [b]	3.04 [c]	0.256	0.0365
With PEG	5.59 [a]	7.42 [b]	5.37 [a]	0.958	0.0240
Methane reduction/ml of total gas reduction	0.301 [b]	0.482 [c]	0.261 [a]	0.001	0.0461

SN-cake = Sheanut cake; SN-expeller = Sheanut expeller; SN-solvent extracted = Sheanut solvent extracted; PEG = Polyethylene glycol.
Means in a row with different superscript differ significantly ($p<0.05$).

protozoa and/or methanogenic archea population (Bhatta et al., 2009). Phenolic acids such as *p*-coumaric acids, ferulic acids, cinnamic acids and phloretic acids and some monomeric phenolics were reported to decrease rumen CH_4 (Field and Lettinga, 1987; Asiegbu et al., 1995).

The total and differential protozoa counts with and without PEG are presented in Table 4. There was significant ($p<0.05$) reduction in the entodinia population and in turn total protozoa population in samples without PEG as compared to that of samples with added PEG. Differential protozoa counts revealed that *Entodinia* populations increased to a greater extent than *Holotricha* with added PEG thus confirming higher susceptibility of *Entodinia* to phenolics present in sheanut byproducts. Wang et al. (2009) reported that protozoa help methanogens reduce CO_2 to CH_4. Hence, anti-protozoal effect of tannins could decrease CH_4 production ridding methanogen populations attached to protozoa. Therefore, CH_4 production could be higher when protozoa are present in greater numbers in the rumen than when absent or present in low number (Jouany and Lassalas, 1997). There are conflicting reports on the effects of HT and CT on ruminal ciliated protozoa counts such as HT were reported to have no effect on protozoa counts (Sliwinski et al., 2002), whereas, Leinmuller (1991) reported that CT was more inhibitory than HT on protozoa. Terrill et al. (1992) did not observe any adverse effect of CT-containing Sulla (*Hadysarum coronarium*) on protozoa numbers. Hess et al. (2001) suggested that only a small portion of total CH_4 production was due to the presence of methanogens attached with the ciliate protozoa. Dohme et al. (1999) also reported inhibition of *in vitro* CH_4 emission both in defaunated and faunated rumen liquor with coconut oil. Machmüller (2003) reported an increased number of methanogens in defaunated sheep and suggested that

Table 4. Total and differential protozoa population (per ml) with and without PEG from sheanut cake, expeller and solvent extractions with PEG-6000 *in vitro*

	SN-cake	SN- expeller	SN-solvent extracted	SEM	p
Entodinia (10^5)					
Small	0.172 [b]	0.162 [a]	0.158 [a]	0.028	0.0502
With PEG	0.183 [a]	0.185 [b]	0.170 [a]	0.021	0.0464
Large	0.060	0.054	0.057	0.001	0.0658
With PEG	0.074	0.071	0.061	0.001	0.0560
Holotricha (10^5)					
Isotricha	0.009	0.007	0.009	0.001	0.0654
With PEG	0.005	0.006	0.012	0.001	0.1256
Dasytricha	0.003	0.002	0.005	0.001	0.1547
With PEG	0.003	0.004	0.006	0.001	0.1635
Total (10^5)	0.243	0.226	0.229	0.025	0.0496
With PEG	0.264 [b]	0.265 [b]	0.248 [a]	0.035	0.0469

SN-cake = Sheanut cake; SN-expeller = Sheanut expeller; SN-solvent extracted = Sheanut solvent extracted; PEG = polyethylene glycol.
Means in a row with different superscript differ significantly ($p<0.05$).

Table 5. Ammonia-N and TVFA from sheanut cake, expeller and solvent extractions with PEG-6000 *in vitro*

	SN-cake	SN- expeller	SN-solvent extracted	SEM	p
NH_3-N (m mol/dl)	13.6 [c]	12.8 [b]	11.5 [a]	0.965	0.0325
With PEG	16.4 [c]	14.7 [b]	13.1 [a]	0.026	0.0436
TVFA (mg/dl)	15.6 [b]	11.3 [a]	11.6 [a]	1.236	0.0365
With PEG	15.1 [b]	12.1 [a]	15.2[b]	0.965	0.0523

SN-cake = Sheanut cake; SN-expeller = Sheanut expeller; SN-solvent extracted = Sheanut solvent extracted; PEG = Polyethylene glycol; NH_3-N = Ammonia-N; TVFA = Total volatile fatty acids.
Means in a row with different superscript differ significantly ($p<0.05$).

association between protozoa and methanogens does not play an important role in rumen methanogenesis.

The ammonia N and TVFA concentrations with and without PEG are presented in Table 5. Reduced TVFA concentration ($p<0.05$) in SNSE indicated adverse effect on the *in vitro* fermentation. Effect of tannin on volatile fatty acid production varied. Tannic acids have been reported to reduce VFA production (Vogels et al., 1980; Hristov et al., 2003). Inhibition of fibre degradation also shift fatty acid composition away from acetate and hence less production of hydrogen and less CH_4 fermentation (Jayanegara et al., 2012). Methane reduction could be due to direct effect (reduction in archea) or indirect effect (reduction in protozoa) (Bhatta et al., 2009) or due to reduced digestibility of nutrients. Since there was reduction in the protozoa and but no significant reduction in the TVFA (except SNSE), we assume that methane suppression recorded in SNC and SNE was due to direct/indirect effect of tannin.

There was significant reduction ($p<0.05$) in the NH_3-N concentration in the absence of PEG confirming the protein binding properties of tannin present in sheanut byproducts (Bhatta et al., 2002; 2005). Since tannins are recognized as protein binders, there was reduced *in vitro* NH_3-N concentration ($p<0.05$) in all the samples. According to Leinmuller and Menke (1990) HT and protein usually form complexes at an optimal pH range of 3 to 4, but complexes also occur under typical rumen pH of 6 to 7. Some ruminal bacteria dissociate the protein-HT complexes. However, dissociation of protein-CT complexes is difficult (McSweeny et al., 2001). The observed difference in the NH_3-N concentration among samples was attributable to the reversible nature of protein-HT complex. In the absence of tannin, degradability of protein was higher, resulting in greater NH_3-N concentration, possibly because of inhibition of microbial deaminase by tannins (Leinmuller and Menke, 1990). Lower ($p<0.05$) NH_3-N concentration may be attributed to the inhibition of the bacteria-degrading activity of tannins (Jouany, 1994). Lower NH_3-N in our samples was a result of protein binding property of tannins. This is another reason for incorporating SN byproducts in ruminant diets to protect highly degradable protein. However, long term animal feeding studies need to be carried out to determine the palatability of these byproducts and performance of animals.

CONCLUSIONS

The protein and energy contents of SN extractions established that all the three forms are medium source of protein and energy for ruminants. There is a great potential for SN byproducts to be incorporated in ruminant feeding not only as a source of energy and protein, but also to suppress enteric methanogenesis.

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