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Rumen responses, microbial profile and antioxidant indices of crossbred cattle fed *Moringa oleifera* foliage

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ABSTRACT

This study scrutinized the implication of *Moringa oleifera* supplementation on rumen responses, microbial profile and antioxidant indices in crossbred cattle. The present research was carried out on three adult fistulated male cattle in 3×3 latin square designs The CON (control) group cattle were provided a diet comprising of a feed supplement, wheat straw and 10% berseem hay, whereas the experimental cattle in M-10 and M-20 group were given diets containing 10 and 20% moringa foliage, respectively, substituting the berseem hay and concentrate mixture of CON group to make the diets isonitrogenous. The fistulated cattle in all the three groups were provided iso-nitrogenous diets. The ruminal fermentation attributes and rumen microbes were measured individually at the end of each experimental period. The levels of rumen metabolites viz. ammonical-N (NH_3-N), TCA-precipitable nitrogen (TCA-ppt-N) volatile fatty acids (VFAs) and rumen enzymes protease and xylanase were increased (P<0.05) in *M. oleifera* foliage supplemented groups. However, CMCase, avicelase, amylase and urease were comparable among the treatments. The number of fungi, methanogens, total bacteria and total live protozoa remained comparable (P>0.05) across treatments. The serum glutathione-S-reductase (GSR), glutathione-S-transferase (GST) and superoxide dismutase (SOD) were equivalent (P>0.05) across the treatments. However, serum glutathione peroxidase (GPx) and catalase (CAT) activity were significantly (P<0.05) greater in the group supplemented with moringa relative to CON. Total antioxidant capacity (TOAC) in serum was substantially (P<0.05) higher in M-20 group followed by M-10 and CON. It may be revealed that supplementary moringa foliage up to 10 per cent of diet has beneficial influence on rumen environment, enzymes and antioxidant status in crossbred cattle.

Keywords: Crossbred cattle, Microbial outline, *Moringa oleifera*, Rumen environment

Dairy farmers in resource limited area utilize fodder trees and bushes for increasing the quality and quantity of feeds for their dairy animals in dry seasons. The trees provide a good and inexpensive alternative source of protein and vitamins. Therefore, it is essential to look for newer feed sources that are affordable protein sources with easily cultivable and balanced amino acid. Recently, the focus of nutritionists and plant scientists is more on un- utilized crops and trees. *Moringa oleifera* customarily well-known as 'Moringa' is a Himalayan tree species. It is a multi-purpose tree and provides low-cost nutrients with improved dry matter intake, digestibility coefficient of various nutrients and productive performance of animals. Moringa is a well-established tree due to its nutritional composition and presence of bioactive compounds in the world and is known as a 'Miracle tree' (Yisehak 2011, Ashfaq 2012). The chemical composition of moringa leaves powder roughly 24.6% DM, 20.9% crude protein, and 47% bypass protein, besides a sufficient supply of amino acids (Nouman *et al*. 2014). The moringa leaves are enriched with amino acids, vitamins, minerals and protein (Makkar and Becker 1997, Gidamis 2003). Its leaves also contain soluble carbohydrates, fat and some bioactive components viz. vitamins, saponins, carotenoids, phytates, polyphenol, flavonoids, glucosinolates, tannins and isothiocyanates (Leone *et al*. 2015). Considering its nutritional profile, moringa can be deemed as a suitable feed for ruminants, particularly those consuming low-quality fodders. Hence, this research work was performed to ascertain the role of moringa as rumen modifier, and its influence on rumen responses and rumen microbial outline in crossbred cattle.

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MATERIALS AND METHODS

Animals and dietary treatments: This research work was performed at Animal Nutrition sheds of ICAR-IVRI, Izatnagar, Bareilly (UP). M. oleifera leaves were collected from ICAR-IVRI campus and air-dried before feeding. Three adult crossbred male fistulated cattle with mean live weight of 474.97±1.8 kg were randomly assigned to a changeover 3×3 Latin square design. The cattle in CON group were provided a diet comprising of wheat straw; concentrate mixture and berseem (Trifolium alexandrinum)

hay (10%), while the M-10 and M-20 treatment groups were given diets with 10 and 20% moringa foliage, respectively, by replacing berseem and feed supplement of the CON group. All experimental animals received iso-nitrogenous diets. The study was performed for 3 periods, and each period lasted for 21 d. After every 21 d of feeding period, each animal was assigned to a new treatment, so that every-one gets the opportunity to all dietary treatments. The body weights of the cattle were measured initially and at the end of each feeding period.

Feeding management of the animals: The adult crossbred male cattle were confined to well-ventilated individual stalls with feeders. Deworming and vaccinated for prevailing diseases were done for both external and internal parasites for all animals before starting the experiment following standard schedule. The experimental cattle were offered maintenance feed at 9:15 AM daily (ICAR 2013). Potable water was provided *ad libitum* twice a day.

Rumen liquor sampling: The samples of rumen fluid were collected towards the end of each experimental period for 2 consecutive days, at 0 and 2 h post feeding. Ruminal content was collected manually by through rumen fistula from four different sites at the corners and centre. All the rumen contents were mixed properly in CO_2 environment. A representative sample from the whole rumen content was processed for enzyme estimation. Briefly, 5g of rumen content was mixed with PBS (pH 6.8) and carbon tetra chloride and 0.4% lysozyme (freshly prepared) in a beaker. The beaker was then kept at 39°C for 3 h with continuous shaker in a shaker incubator. After 3 h, the contents of the beaker were centrifuged to collect the central aqueous phase as enzyme extract, which was used for enzyme activity estimation.

Rumen metabolites: As soon as the rumen fluid was collected, its pH was measured using a calibrated electronic pH metre (pH Testr 30, Eutech). The VFAs estimation was performed by mixing of the rumen fluid (1.0 ml) with 200 µl of 25% metaphosphoric acid and samples were allowed to stand for 1 h room temperature flowed by centrifugation (10 min/ 10,000 rpm). VFA fractions in supernatants were determined using gas chromatography as described by Agarwal *et al*. (2008) using flow of various gases asr air (30), N_2 (30) and H_2 (320) ml/min, respectively. For assessing various nitrogen fractions, samples of rumen liquor were strained through muslin cloth and instantly one drop of 20% H_2SO_4 was added per 10 ml of sample and stored at -20^{0}C. The N fractions viz. non-protein N (NPN), total N (TN) and TCA-ppt-N were estimated using the method of digestion, distillation and titration (AOAC 2000). The NH_3-N level in ruminal fluid was assessed as per Weatherburn (1967).

Rumen enzymes: The enzymes present in rumen content were extracted in phosphate buffer solution (PBS) as per Kala *et al*. (2017a). Briefly, lysozyme (0.4%) and CCl_4 were added to PBS containing rumen sample and incubated at 39^{0}C with constant shaking for 3.0 hours followed by final freezing to finish the reaction. Activities of amylase, xylanase, avicelase as well as carboxymethyl cellulose were determined as the protocol described by Agarwal *et al*. (2000). Azocasein was utilized as a substrate to measure the activity of protease enzyme (Iversen and Jorgensen 1995) and presented as µg of protein hydrolysed $min^{-1}ml^{-1}$. The urease concentration was ascertained following the procedure given by Weatherburn (1967).

Microscopic count of ciliate protozoa: The microscopic count of protozoa is most reliable and practical methods for enumeration of ciliate protozoa. For counting of protozoa, strained rumen fluid sample was fixed immediately after reaching in laboratory by mixing one volume of methyl green formal saline solution with one volume of rumen liquor and allowed to stay overnight at ambient temperature for overnight. The protozoal number was counted in a predetermined volume of stained rumen liquor under microscope and the total number was calculated (Kamra *et al*. 1991).

Microbial profile by RT-PCR: The quantification of methanogens, fungi and total bacteria was performed by real-time PCR as per Kala *et al*. (2017b). The Qiagen Stool kit was utilized to extract genomic DNA and specific primers were used for its amplification. The purified product of PCR was cloned using pGEMT easy vector and transformation was done in *E. coli*. Plasmid extraction was done followed by calculation of copy number after sequential dilution of plasmid (Ritalahti *et al.* 2006). The PCR master mixture was prepared by adding template DNA (2 ng), Takyon™ SYBR master mix (10 µl), forward and reverse primer (10 µM).

Table 1. Primers for q-PCR

Name of Primer	Sequence of primer	Reference
Bacteria F-5'CGGCAACGAGCGCAACCC-3'		Denman *et al*. (2006)
R-5'CCATTGTAGCACGTGTGTAGCC-3'		
Fungi F-GAGGAAGTAAAAGTCGTAACAAGGTTTC		
R-CAAATTCACAAAGGGTAGGATGATT		
Methanogen F-5'-TTCGGTGGATCDCARAGRGC-3'R		
R-5'-GBARGTCGWAWCCGTAGAATC C-3		

Table 2. Nutritional composition of feed ingredients (% DM basis)

Constituents	Concentrate	Wheat straw	Berseem hay	Moringa foliage
DM	90.91	96.55	91.79	90.72
OM	86.53	88.35	78.29	76.85
CP	18.40	3.23	19.70	22.52
EE	3.35	1.18	3.12	6.86
TA	4.38	8.20	13.50	13.87
NDF	43.93	83.83	48.98	28.11
ADF	29.05	49.51	30.85	31.13

Composition of feed supplement (kg): Wheat bran-44, Maize-35, De-oiled soybean cake-18, Mineral mixture-2 and Salt-1 kg.

Blood collection and separation of serum: The samples of blood were collected by jugular vein from all the cattle at the beginning (0 day) and end of each experimental period before feeding and watering. About 5 ml blood was drawn and transferred to clean dry vacutainer tube and was kept in the slanting position for serum separation. The collected serum samples were relocated into Eppendorf tube and preserved at -20^{0}C for biochemical, antioxidant and hormonal examination.

Chemical analysis: The offered feeds were ground using a 1.0 mm sieve for chemical analysis. The DM (dry matter), OM (organic matter), EE (ether extract), CP (crude protein) and TA (total ash) contents of feed were analysed as per the AOAC methods (AOAC, 2000). The fibre fractions viz. NDF and ADF contents were assessed following the procedure of Van Soest *et al*. (1991). The serum CAT, GPx and SOD activity were assessed by biochemical assay kit manufactured by Cayman chemical, MI, USA. The GST and GSR activity were measured by biochemical assay kit manufactured by Elabscience Biotechnology, Houston, Texas, USA. Serum TOAC was measured by immunotag assay kit manufactured by Geno technology, USA.

Statistical analysis: The data obtained were analysed following factorial univariate ANOVA with contrast analysis by means of model intercept, period, treatment and period x treatment to assess and comparison of their effects and interaction were analysed using DMRT following SPSS 20.0 computer package.

RESULTS AND DISCUSSION

Rumen metabolites and enzymes profile: The pH of rumen, total-N and NPN (mg/dl) levels in rumen liquor were similar (P>0.05) across the treatments, though, NH_3-N (mg/dl) and TVFA (mmol/dl) were greater (P<0.01) in M-10 followed by M-20 and CON (Table 3). TCA-ppt-N (mg/dl) was significantly (P<0.001) higher in M-10 than M-20 and CON groups. The acetate, propionate and butyrate content and A:P ratio was similar (P>0.05) among the treatments. The findings are in alignment with Jadhav *et al.* (2018), who reported comparable rumen pH, levels of VFAs and other metabolites in goats supplemented *M. oleifera* leaves at 10 and 20% DMI. Similar results were also reported by Jelali and Salem (2014) who observed no change in the ruminal pH by addition of soybean meal or moringa leaves in oat hay-based diet to lambs.

The fermentation metabolites viz. TVFA, NH_3-N and TCA-ppt-N were significantly (P<0.05) greater in M-10 than CON and M-20 groups. TVFA was in accordance with the earlier report of Kholif *et al*. (2015), who found an increased (P<0.05) TVFA level in goats, who fed dried

Table 3. Rumen metabolites, microbial enzymes and protozoal count in crossbred cattle

Attributes	Dietary treatments			SEM	P-value		
	CON	M-10	M-20		G	H	G*H
pH	7.16	7.12	7.14	0.02	0.76	0.16	0.73
Ammonia-N (mg/dl)	13.49a	16.62c	14.65b	0.04	<0.001	<0.001	<0.001
Total-N (mg/dl)	48.83	51.10	49.70	1.32	0.78	0.03	0.94
TCA-N (mg/dl)	26.33a	28.98b	26.72a	0.32	0.01	<0.001	0.66
NPN (mg/dl)	23.87	21.42	22.98	1.34	0.75	<0.01	0.83
TVFA (mmol/dl)	9.08a	9.59c	9.47b	0.02	<0.001	<0.001	<0.001
Acetate, %	63.17	64.25	63.01	1.13	0.89	0.58	0.34
Propionate, %	20.77	21.43	19.16	0.44	0.14	0.51	0.92
Butyrate, %	12.86	12.46	12.35	0.59	0.93	0.99	0.32
A:P ratio	3.04	3.02	3.29	0.09	0.46	0.98	0.66
Rumen enzymes activity (nmol product/ml/min)							
Carboxymethyl cellulase	227.28	267.11	241.16	11.57	0.39	0.22	0.42
Avicelase	156.11	162.07	162.99	10.05	0.95	0.56	0.98
Amylase	120.26	139.26	137.01	17.14	0.88	0.08	0.82
Xylanase	538.12a	588.44c	562.81b	0.44	<0.001	<0.001	<0.001
Protease	3462.45a	3844.39c	3632.64b	1.91	<0.001	<0.001	<0.001
Urease	29.84a	45.69b	37.17ab	3.54	0.039	0.15	0.29
Protozoal count (Log 10 value)							
Holotrichs	1.19a	3.93b	2.49ab	0.71	0.05	0.95	0.99
Oligotrichs	5.63	5.82	5.67	0.05	0.06	0.63	0.69
Total protozoa	5.64	5.83	5.68	0.05	0.06	0.63	0.69

M-10: *M. oleifera* foliage @10% of DMI, M-20: *M. oleifera* foliage @20% of DMI. [abc]Mean values with different superscript in a row differ significantly

Table 4. Effect of sundried *M. oleifera* on serum antioxidant profile of crossbred cattle

Attributes	Dietary treatments			SEM	P-value
	CON	M-10	M-20		
GPx, nmol min^{-1} ml^{-1}	831.99[a]	1122.77[c]	935.99[b]	41.33	0.04
GSR, UmL^{-1}	16.07	16.14	16.13	0.14	0.98
GST, UL^{-1}	5.73	4.96	5.57	0.57	0.88
SOD, UmL^{-1}	3.84	4.24	4.14	0.13	0.44
Catalase, nmol min^{-1} ml^{-1}	37.66[a]	42.09[b]	48.23[c]	1.28	<0.001

M-10: *M. oleifera* @10% of DMI, M-20: *M. oleifera* foliage @20% of DMI [abc]Mean values with different superscript in a row differ significantly

moringa leaves at 15% level. In line with our results, Mohamed *et al*. (2018) reported a significant increase in total VFAs in growing does at 25 to 50% levels of *M. oleifera* as compared to berseem .

The rumen ammonia-N, propionic acid concentration and acetate to propionate proportion was in contrast to the previous study (Kholif *et al*. 2015), however, the butyric acid and acetic acid were comparable to the earlier finding (Kholif *et al*. 2015). Mahmoud *et al*. (2013) found an unaffected rumen pH after 6 h in growing lamb feed with ground leaves of *M. oleifera* at 20-25% level in diet, while NH_3-N level was (P<0.05) greater in moringa group than other treatments. They further reported significantly (P<0.05) elevated level of butyrate and propionate in moringa treated group relative to other treatments, though acetate: propionate proportion was noticeably decreased in treatment group compared to control. These findings were contrary to our results. Ahmed *et al*. (2019) also reported a significant (P<0.05) increase in ruminal pH and NH_3-N concentrations with 20% moringa fed group than CON.

The molar concentration of amylase, avicelase and carboxymethyl cellulase was similar (P>0.05) among the treatments. Though, xylanase, protease and urease activity were significantly (P<0.01) greater in M-10 group followed by M-20 and CON, respectively. The present results were analogous with Jadhav *et al.* (2018), who supplemented moringa in goats @ 10 as well as 20% of DMI. *Rumen microbial profile*

Total bacteria, archea and fungal population (log_{10} value) were comparable (P>0.05) irrespective of treatments (Fig.1). The microscopic count of total live protozoa and oligotrichs were similar (P>0.05) amongst treatments, however, the holotrichs counts were (P<0.05) greater in M-10 relative to CON (Table 3). The microscopic count of protozoa was unchanged in rumen which indicated that rumen conditions and microbial outline was not altered by *M. oleifera* foliage addition. Nevertheless, equivalent rumen protozoal population attributed to lower level of moringa foliage. The findings of total bacteria, fungi and methanogens are in align with the earlier report (Jadhav *et al*. 2018), who observed no change (P>0.05) in inhabitants of rumen (Log_{10} number) of total rumen bacteria, fungi, protozoa and methanogens of animals provided a ration containing 10 as well as 20% moringa foliage.

Serum antioxidant profile: The serum GSR (UmL^{-1}), GST (UL^{-1}) and SOD (UmL^{-1}) activities were analogous (P>0.05) amongst the treatments. The serum GPx and catalase activities ($nmolmin^{-1}mL^{-1}$) were significantly (P<0.05) enhanced in moringa groups as compared CON (Table 4). The results of the current study suggested that addition of moringa in the diet of crossbred cattle may have increased their natural antioxidant defense. This is in conformity with previous reports (Iqbal and Bhanger 2006, Pourmorad *et al*. 2006, Satish *et al*. 2013) as the flavonoids kaempferol, rhamnetin, isoquercetin and kaempferitrin found in sundried moringa leaves have the potential to considerably aid in the scavenging of free radicals. The findings of GPx activity were in line with Zhang *et al.* (2018), who reported increased GPx activity in cattle fed moringa based ration. Wafa *et al*. (2017) had earlier reported greater concentration of catalase in serum by feeding of 4 and 8% moringa leaves on buffalo diets as observed in present. The serum TOAC (UL^{-1}) activity was also considerably (P<0.05) increased in M-20 followed by M-10 and CON group. The increased TOAC activity of in M-10 and M-20 group may be accredited to the existence of antioxidant substances like different vitamins (A, C, E) and total phenolic contents in moringa foliage. These observations are compatible with prior findings (Babiker *et al*. 2017) who found the elevated levels of TOAC in sundried moringa leaves fed goats at dose 25% relative to alfalfa hay-based diet.

On the basis of above results, it may be concluded that dietary supplementation of 10 per cent *Moringa oleifera* in crossbred adult cattle resulted in significant improvement in rumen metabolites, rumen enzymes (xylanase, protease and urease) and antioxidant indices and does not alter the ruminal microbial profiles and normal fermentation. Hence, it may be used in the form of supplement in livestock feeding.

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