Range Mgmt. & Agroforestry 41 (2) : 343-350, 2020
ISSN 0971-2070

Effect of mineral supplementation on rumen fermentation, microbial population, nutrient intake and utilization in sheep fed sorghum stover based diets

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Received: 12th October, 2019 Accepted: 30th August, 2020

Abstract
Study was conducted to record the effect of area specific mineral mixture (ASMM) supplementation on rumen fermentation, microbial population, nutrient intake and utilization in sheep fed sorghum stover based diets. A concentrate mixture was prepared with barley, mustard cake and wheat bran, which was then fortified with 0% (T_1), 1% (T_2), 2% (T_3) of ASMM and 2% (T_4) of standard mineral mixture available in the market. Female Jalauni sheep (16) were selected and distributed randomly into 4 dietary treatment groups of 4 animals in each group. The animals were offered required amount of sorghum stover and concentrate mixture fortified with different levels of mineral mixture for a maintenance dietary regime. Study showed that pH of strained rumen liquior was highest in non-supplemented diet of T_1 group (6.54) and lowest in T_3 group (6.29) and the differences were significant among the groups. On the contrary, higher TVFA concentration (meq/dl) was recorded in T_3 (13.53) when compared to other groups like T_1 (10.98). The carboxymethyl cellulose and xylanase activities (μg sugar/h/day) also followed the same trend, being highest in T_3 and lower in T_1 group. Population of total viable bacteria (x10^9/ml) was highest ($P<0.01$) in T_3 (15.63) and lowest in T_1 group (8.27). Similar was the trend with respect of amylolytic and cellulolytic bacterial population. But dietary treatments had no influence on proteolytic bacterial population. Fungal population was significantly higher in T_3 group (5.68) as compared to T_2 (3.97) and T_1 (2.63) groups. Digestible crude protein intake (DCPI) was higher ($P<0.05$) in mineral supplemented groups, being maximum in T_3 group (36.8 g/d). Average daily total digestible nutrient intake (TDNI; g) was maximum ($P<0.05$) in sheep of T_3 group (424) followed by T_2 (352), T_4 (329) and T_1 (302) groups. Thus ASMM supplementation had positive effect on rumen fermentation, microbial population and nutrient utilization in sheep fed sorghum stover based diets.

Keywords: ASMM, Nutrient utilization, Rumen enzymes, Rumen metabolites, Rumen microbes, Sheep

Abbreviations: **ADF**: Acid detergent fiber; **ASMM**: Area specific mineral mixture; **CP**: Crude protein; **CPI**: Crude protein intake; **DCP**: Digestible crude protein; **DM**: Dry matter; **DMI**: Dry matter intake; **EE**: Ether extract; **NDF**: Neutral detergent fiber; **OM**: Organic matter; **SRF**: Strained rumen fluid; **TDN**: Total digestible nutrient; **TDNI**: Total digestible nutrient intake; **TVB**: Total viable bacteria; **TVFA**: Total volatile fatty acids

Introduction

The rumen of a ruminant animal is a complex ecosystem where nutrients consumed by the microorganisms such as bacteria, protozoa, and fungi are fermented anaerobically (Castillo-Gonzáleza *et al*., 2014). The main end products of fermentation are volatile fatty acids and microbial biomass, which are used by the host animal. The interaction between microorganisms and host animal results in a symbiotic relationship, which permits animal to digest even low quality forages (Peripolli *et al*., 2017). In rumen, the environment favours microorganisms to provide the enzymes necessary to digest nutrients. Animal thus converts low quality forages into high quality products such as meat and milk (Suttle, 2000). The ability of ruminal microorganisms to produce the enzymes necessary for fermentation processes allows animal to efficiently obtain the energy contained in forages (Burns, 2008). However, the ruminal fermentation process is not optimum or efficient in many feeding regimens (Kingston-Smith *et al.,* 2012). Indeed, the anatomical adaptation of digestive system allows animal to use cellulose as an energy source without requiring external sources of vitamin B complex (Russell and Mantovani, 2002) or essential amino acids because ruminal microorganisms are able to produce such products (Cole *et al.,* 1982). But ruminant animals require

minerals in the diets for efficient rumen fermentation.

Minerals are required for an array of important functions in the animal body (Underwood and Suttle, 1999; Das *et al*., 2018; Singh *et al*., 2020). The concentration of minerals within feed resources vary greatly, in addition to difference among feed type and plant species mineral content can be affected by production and management practices (Gowda and Pal, 2020). Although required in small quantities, minerals are essential for optimal functions of the rumen as well as the animal itself (Hilal *et al*., 2016). Bioavailability of minerals contained in feed resources is an important consideration in evaluating dietary adequacy (Ammerman *et al.,* 1995). Minerals contribute to the regulation of the physiochemical characteristics of rumen such as osmotic pressure, buffering capacity, redox potential and dilution rate. Animal feeds and fodders often do not include all the requisite minerals and therefore, need to be supplemented. Supplementation of area specific mineral mixture (ASMM) to producing animals showed noticeable improvement in growth, milk production and performances (Tiwari *et al*., 2012; Mushtaq *et al*., 2017). However, there was paucity of information on its effect on rumen metabolites and enzymes in animals. Hence, in the present investigation effects of ASMM (containing Ca, P, Na, Cu, and Zn) supplementation on rumen metabolites and enzymes, microbial population, nutrient intake and utilization were recorded in sheep fed sorghum stover based diets.

Materials and Methods

Study site and experimental design: The study was carried out in Sheep-Goat Unit under Plant Animal Relationship Division, ICAR-Indian Grassland and Fodder Research Institute, Jhansi, which is situated at 25^0 27' N latitude and 78^0 37' E longitudes. It has an altitude of 275 m above mean sea level. Sixteen female, non-lactating, non-pregnant, Jalauni sheep were used in this study under maintenance rations. All the animals were dewormed with broad spectrum anthelmintic and they were kept in well ventilated shed having the facility of individual feeding.

A concentrate mixture was prepared with barley (40 parts), mustard cake (35 parts) and wheat bran (23 parts). An area specific mineral mixture (ASMM) comprising of dicalcium phosphate, zinc sulphate, copper sulphate, potassium iodide and common salt was also prepared. Concentrate mixture was then fortified with 0% (T_1), 1% (T_2), 2% (T_3) of prepared ASMM and 2% (T_4) of standard mineral mixture available in the market. A maintenance dietary regime of roughage to concentrate ratio of 80:20 was then prepared using sorghum stover as a roughage source. The animals were allocated in four different groups of 4 in each group, and offered required amount of sorghum stover and concentrate mixture fortified with different levels (T_1: 0%, T_2: 1%, T_3: 2% of ASMM and T_4: 2% of standard) of mineral mixtures. Concentrate mixture was offered to the animals first followed by sorghum stover daily between 9:30 am to 10:30 am. However, the animals were provided with ad libitum to drinking water. Thus the animals were exposed to the adaption period of 30 days before the beginning of the metabolism trial.

***Sampling and analysis*:** After 30 days of experimental feeding, metabolism trial of 7-days duration was conducted. The animals were put in metabolic cages and representative samples of feed offered, residue left and faeces of each animal were taken daily during digestibility measurements for determining DM intake. The feeds and dried residues were bulked, sampled, grinded and stored for chemical analysis. The daily collected faeces samples for dry matter digestibility were also stored for chemical analysis.

After the metabolism trial rumen fluid samples were collected from individual animal and filter through double layer muslin cloth. Rumen fluid samples were collected in thermos-flask having temperature around 39 ^oC and CO_2 was purged within the flask. The strained rumen fluid was then immediately analyzed for pH (digital pH meter 5652), and processed for enzymatic activities (Somogyii, 1952). The rumen fluid/ inoculums were also used for bacterial and fungal counts following serial dilution technique with anaerobic dilution fluid and appropriate culture media (Brayant and Burkey, 1953). The most probable number of bacteria/ fungi was then computed using Mc Crady's (1918) table on the basis of positive tubes. The total number as well as differential number of protozoa in rumen fluid samples was calculated following Kamra *et al.* (1991). The rumen fluid samples were then preserved with saturated solution of mercuric chloride for further estimation of TVFA (Barnett and Reid, 1957) and other rumen metabolites. The feed and faeces samples were also analyzed for proximate constituents (AOAC, 2012) and cell wall fractions (Goering and Van Soest, 1970). The data were subjected to statistical analysis (Snedecor and Cochran, 1989; SAS, 2012) following completely randomized design.

Results and Discussion

***Chemical composition of feeds*:** Chemical composition of experimental feeds viz. concentrate mixture containing different level of mineral mixture (0%, 1%, 2% of ASMM and 2% Standard) and sorghum stover was recorded (Table 1). The crude protein content (%) of concentrate mixture was maximum in T_4 group (21.69) followed by T_2 group (21.60) and almost similar in T_1 and T_3 groups. However, the values of NDF, ADF and cellulose in T_1, T_2, T_3 and T_4 were ranged from 36.61 to 39.34, 17.23 to 18.46, and 10.49 to 11.81%, respectively. Average crude protein content as well as cell wall constituents of concentrate mixture fed T_1 group (concentrate mixture containing no mineral supplement) were within the range of observed values of Dev *et al*. (2009). But the values were not in agreement with the reported values of Dutta and Singh (2009). This variation was probably due to change in ingredients and their composition of concentrate mixture. However, the proximate compositions as well as cell wall constituents of the sorghum stover used in this study were within the range as reported earlier by Pailan *et al*. (2008). Although there were some variations in values of sorghum stover constituents because of differences in crop maturity, fertilizer, temperature, variety etc (Dhore *et al.,* 2005).

***Rumen metabolites and enzymes*:** Data on concentration of rumen metabolites in strained rumen fluid (SRF) of sheep fed sorghum stover and concentrate mixture with varying level of mineral mixture indicated the lowest ($P<0.01$) pH on T_3 group followed by T_2, T_4 and T_1 group (Table 2). On the contrary, concentration of total volatile fatty acids (TVFA) in SRF was highest in T_3 group (13.53 meq/dl) followed by other groups which did not differ significantly from each other, showing inverse relationship between the values on pH and TVFA. However, concentration of ammonia nitrogen, total nitrogen and TCA perceptible nitrogen were comparable among the groups and remained unaffected by dietary supplementation of mineral mixtures. Ammonia nitrogen and total nitrogen in the present study were within the range of values reported by Pandey *et al*. (2009). Higher TVFA and nitrogen metabolite production in mineral mixture supplemented diets might be due to relatively higher organic matter digestibility including cell wall fractions recorded in the present study. These results were indicative of better rumen fermentation in the sheep fed ASMM than non-supplemented and supplemented with standard mineral mixture containing all the elements. Carboxy methyl cellulase and xylanase enzyme activities in terms of mg sugar/mg protein were maximum (919.7 and 1370.5) in SRF of sheep fed sorghum stover and concentrate mixture supplemented with 2% area specific mineral mixture (T_3). This higher enzyme activity in ASMM supplemented groups was partly attributed to enhanced cellulotytic bacteria resulting in more cellulase and xylanase production compared to non-supplemented dietary regimens (Krishnamurti and Kitts, 1969; Upadhye and Kashavamurthy, 1982).

Table 1. Chemical composition (% DM basis) of experimental feeds

Attributes	OM	CP	EE	NDF	ADF	Cellulose
Concentrate mixture-I	92.17	20.76	3.89	39.34	17.85	11.79
Concentrate mixture-II	90.92	21.60	3.77	36.53	18.46	11.81
Concentrate mixture-III	90.80	20.75	3.83	37.98	18.01	10.87
Concentrate mixture-IV	90.77	21.69	3.68	36.61	17.23	10.49
Sorghum stover	92.42	5.21	1.21	73.34	49.27	40.10

Table 2. Changes in rumen metabolites and enzymes activities in SRF of sheep

Attributes	Treatment groups			
	T_1	T_2	T_3	T_4
pH**	6.54^{a}±0.04	6.44^{a}±0.03	6.29^{b}±0.04	6.48^{a}±0.04
TVFA (meq/dl)**	10.98^{b}±0.35	11.98^{b}±0.43	13.53^{a}±0.29	11.33^{b}±0.28
Ammonia N (mg/dl)	12.84±0.93	13.48±1.31	15.36±1.86	13.90±1.77
Total N (mg/dl)	87.25±3.63	91.50±2.07	96.85±1.96	88.63±2.45
TCA ppt. N (mg/dl)	67.33±3.05	70.50±2.82	75.95±3.76	65.95±2.90
CMCase (µg sugar/h/day)*	782^{b}±15.11	813^{b}±34.92	919^{a}±31.62	790^{b}±20.94
Xylanase (µg sugar/h/day)*	1209^{b}±26.20	1259^{b}±27.91	1370^{a}±25.41	1182^{b}±27.42

Values bearing different superscripts in a row differed significantly (*$P<0.05$; **$P<0.01$)

***Microbial population*:** Microbial population *i.e.* bacteria, protozoa and fungi in SRF of sheep fed sorghum stover and concentrate mixture diets supplemented with varying level of mineral mixture was also recorded (Table 3). Population of total viable bacteria (x10^9/ml) was highest in T_3 (15.63) and lowest in T_1 group (8.27) and T_4 (9.51). Similar was the trend with respect of amylolytic and cellulolytic bacterial population, being maximum in T3 group. On the contrary, dietary treatments had no influence on proteolytic bacterial population, which ranged from 1.79 to 2.78 x 10^9 in SRF. The fungal population (x10^4/ml) was higher (P<0.01) in T_3 group (5.68) as compared to all treatment groups. However, there was no significant difference in T_1 and T_4 groups. Similarly, the protozoal number (x10^5/ml) was also highest in T_3 (2.11) and lowest in T_1 (1.51) group. Indeed, animals under T_4 group were also supplemented with standard mineral mixture but its effects on rumen microbes were not significant and probably associated with excess quantities of mineral elements having negative effects on rumen function similar to mineral deficient diets (Tripathi and Karim, 2008). The reported values of TVB and cellulolytes in the present study were within the range of values reported earlier (Singh, 2005; Malik and Singhal, 2009). Overall results indicated that there was significant effect of mineral supplementation as ASMM on the bacteria and fungi population. Although the popu-lation of rumen microbes is affected by several factors like concentration of soluble carbohydrates, degradable protein source, minerals and other critical nutrients (Singh *et al.,* 1980). Galindo *et al.* (1990) observed that mineral mixture and mineral salts increased the anaerobic viable bacteria and cellulolytic bacteria population, which corroborated with the present findings.

Data on generic distribution of protozoa in SRF of sheep fed sorghum stover based diets fortified with different levels of mineral mixture indicated that dietary variation did not have any influence on generic distribution of protozoa (Table 4). Average population (x10^4/ml) of holotrichs and spirotrichs were comparable among the groups and ranged from 2.50 to 3.47 and 12.57 to 17.67. However, population of spirotrichs was higher than holotrichs. Santra and Karim (2001) also observed that holotrichs contributed to a smaller fraction (15.5%) of the total rumen protozoa than spirotrichs (84.5%) in sheep. They recorded linear increase in rumen ciliate protozoa (both holotrichs and spirotrichs) in sheep up to 6 hours of post feeding and this increase was associated with migration of protozoa from the rumino-reticular fold to the rumen. This established that rumen protozoa sequester to the rumen medium in response to chemical stimuli originating from the diets (Kamra *et al.*, 1991).

Table 3. Ruminal microbiota in SRF of experimental sheep

Attributes	Treatment groups			
	T_1	T_2	T_3	T_4
TVB (x10^9)**	8.27^{c}±0.74	12.33^{b}±0.46	15.63^{a}±0.44	9.51^{c}±0.38
Proteolytic (x10^9)	1.79±0.07	2.19±0.12	2.78±0.38	1.99±0.27
Amylolytic (x10^9)**	3.45^{b}±0.40	4.39^{b}±0.29	5.86^{a}±0.29	3.72^{b}±0.37
Cellulolytic (x10^9)**	1.81^{c}±0.18	2.93^{b}±0.35	4.90^{a}±0.14	2.52^{bc}±0.47
Fungi (x10^4)**	2.63^{c}±0.55	3.97^{b}±0.13	5.68^{a}±0.50	2.96^{c}±0.29
Protozoa (x10^5)	1.51±0.33	1.84±0.09	2.11±0.03	1.64±0.17

TVB: Total viable bacteria; Values bearing different superscripts in a row differed significantly (**P<0.01)

Table 4. Generic distribution of ciliate protozoa in experimental sheep

Attributes	Treatment groups			
	T_1	T_2	T_3	T_4
Isotricha (x10^4)	1.20±0.31	1.50±0.06	1.67±0.09	1.37±0.07
Dasytrica (x10^4)	1.30±0.31	1.57±0.09	1.80±0.03	1.40±0.15
Holotrich (x10^4)	2.50±0.61	3.07±0.13	3.47±0.12	2.77±0.22
Large spirotrich (x10^4)	3.20±0.70	3.87±0.15	4.47±0.03	3.43±0.34
Small spirotrich (x10^4)	9.37±2.02	11.43±0.61	13.20±0.13	10.20±1.15
Total spirotrich (x10^4)	12.57±2.72	15.30±0.75	17.67±0.16	13.63±1.49
Total protozoa (x10^4)	15.07±3.33	18.37±0.87	21.13±0.27	16.40±1.70

***Nutrient intake*:** The values on dry matter intake (g/d) were comparatively higher in mineral mixture fortified diets of sheep than control diets (Table 5). Similarly, the values of dry matter intake as % body weight and (g/ $kgw^{0.75}$) were also comparatively higher in mineral supplemented diets than control diet, although they were statistically similar. Indeed, the increase in DMI in sheep supplemented with mineral mixture was expected, due to better ruminal fermentation of feed materials as supplemental elements like P, Zn, Na and Cu stimulate the microbial synthesis/ activity in the rumen (Tripathi and Karim, 2008). Tiwari *et al*. (2000) observed that supplementation of trace minerals significantly (P<0.05) improve the dry matter intake in Sahiwal cows. Average CP intake (CPI) was also statistically similar among the groups. On the contrary, digestible crude protein intake (DCPI) was higher (P<0.05) in mineral supplemented groups, being maximum in T_3 group (36.8 g/d). Similar was the trend with respect DCPI in terms of g per $kgw^{0.75}$. Average daily total digestible nutrient intake (TDNI) was maximum in sheep under T_3 group (424 g) followed by T_2 (352 g), T_4 (329 g) and T_1 (302 g) groups. The differences were significant (P<0.05) among the groups. This higher DCP and TDN intakes were probably associated with relatively higher DM intakes as well as their better utilization in the rumen through increased microbial activity.

***Nutrient digestibility*:** Study revealed that there was increase in digestibility of nutrients in mineral mixture fortified diets as compared to control (Table 6). Maximum digestibility of nutrients was recorded in T_3 group followed by other dietary treatment groups. The differences in digestibility of nutrients were also significant among the dietary treatment groups except CP digestibility. Average CP digestibility (%) was 50.63, 54.10, 55.67 and 53.86 in T_1, T_2, T_3 and T_4 groups, respectively. Average DM and OM digestibilities were higher (P<0.01) in T_3 group when compared to other treatment groups. However, the digestibility values were statistically comparable between T_1 and T_4 groups. Similar trend was observed on ADF, NDF and cellulose digestibilities. The effect of different forms of mineral on nutrients utilization in different animal species was reported earlier (Toppo *et al.,* 1997; Tiwari *et al.,* 2000), which confirmed the result of present study. The higher nutrient utilization in mineral mixture supplemented animals might be due to proliferation of more cellulolytic bacteria resulting in better cell wall digestion as observed in present study.

Table 5. Intake of nutrients in experimental sheep

Attributes	Treatment groups			
	T_1	T_2	T_3	T_4
DMI (g/day)	589±19.45	632±18.90	703±16.14	634±55.83
DMI (kg/100 kg bwt)	2.09±0.05	2.24±0.15	2.46±0.15	2.22±0.11
DMI (g/kg $w^{0.75}$)	48.17±0.43	51.61±2.61	56.91±2.86	51.28±2.40
CPI (g/day)	58.25±1.08	62.11±1.04	64.42±0.75	62.22±2.90
CPI (g/kg $w^{0.75}$)	4.77±0.11	5.07±0.23	5.21±0.21	5.06±0.20
DCPI (g/day)**	29.48^{c}±0.58	34.13^{ab}±1.84	36.87^{a}±0.86	33.23^{b}±0.74
DCPI (g/kg $w^{0.75}$)*	2.42^{b}±0.08	2.77^{ab}±0.09	2.97^{a}±0.05	2.73^{ab}±0.21
TDNI (g/day)**	302.5^{c}±13.01	352.7^{b}±18.53	424.7^{a}±6.81	329.0^{bc}±19.69
TDNI (g/kg $w^{0.75}$)**	24.71^{c}±0.30	28.68^{b}±1.21	34.37^{a}±1.35	26.71^{bc}±0.82

bwt: Body weight; Values bearing different superscripts in a row differed significantly (*P<0.05; **P<0.01)

Table 6. Digestibility of nutrients in experimental sheep

Attributes	Treatment groups			
	T_1	T_2	T_3	T_4
DM (%)**	51.27^{c}±0.86	56.40^{b}±1.73	61.84^{a}±0.65	53.15^{bc}±1.50
OM (%)**	54.38^{c}±0.83	59.22^{b}±1.65	64.18^{a}±0.63	55.67b^{c}±1.39
CP (%)	50.63±0.84	54.10±1.67	55.67±1.16	53.86±3.34
EE (%)*	56.12^{b}±0.69	60.93^{ab}±1.54	64.63^{a}±0.98	58.19^{b}±2.59
NDF (%)**	41.63^{c}±1.39	46.64^{b}±2.40	54.93^{a}±0.78	42.55^{bc}±1.06
ADF (%)**	39.45^{c}±1.69	45.02^{b}±2.61	53.36^{a}±0.82	42.25^{bc}±0.83
Cellulose (%)**	52.10^{c}±1.25	60.26^{b}±1.79	66.97^{a}±0.58	54.11^{c}±0.59

Values bearing different superscripts in a row differed significantly (*P<0.05; **P<0.01)

Table 7. Nutritive values in experimental maintenance rations of sheep

Attributes	Treatment groups			
	T_1	T_2	T_3	T_4
CP (%)	9.90±0.15	9.83±0.13	9.16±0.10	9.91±0.36
DCP (%)	5.01±0.13	5.39±0.22	5.26±0.22	5.37±0.50
TDN (%)**	51.31^{c}±0.79	55.69^{b}±1.61	60.42^{a}±0.66	52.24^{bc}±1.33

Values bearing different superscripts in a row differed significantly (**P<0.01)

***Nutritive value*:** Nutritive value of maintenance rations of experimental sheep in terms of CP and DCP contents were comparable among the dietary treatment groups and the values were ranged from 9.16 to 9.91% and 5.01 to 5.39%, respectively (Table 7). On the contrary, TDN content (%) was highest in T_3 group (60.42) when compared to all dietary treatment groups. This higher TDN contents in ASMM supplemented groups was probably associated with relatively higher, intake as well as higher (P<0.05) digestibility of organic matter including cell wall constituents. However, all the values were within the range of values observed earlier in ruminant animals by Santra and Pathak (2009).

Conclusion

It was concluded that sorghum stover based diets supplemented with minerals (as ASMM @ 2% of concentrate mixture) in sheep resulted significant improvement in rumen fermentation, microbial population, carboxy methyl cellulase and xylanase activities. Mineral supplementation had also positive effect on nutrient utilization of diets in sheep.

Acknowledgement

The senior author is thankful to the Director, ICAR-IGFRI, Jhansi and the Head, Plant Animal Relationship Division for providing the facilities to carry out this piece of work.

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