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EFFECT OF TARTARIC ACID ADDITION ON RUMEN FERMENTATION, METHANE PRODUCTION AND DIGESTIBILITY IN DIFFERENT DIETS CONTAINING WHEAT STRAW *IN VITRO*

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ABSTRACT: *The aim of the current study was to evaluate the effect of tartaric acid addition in diets on in vitro methanogenesis and rumen fermentation. Different levels of tartaric acid (5, 10, and 15 ppm) were tested for their effect on methanogenesis, rumen fermentation and digestibility in three wheat straw containing diets i.e. Low fiber diet (LFD, 40R:60C), medium fiber diet (MFD, 50R:50C) and high fiber diet (HFD, 60R:40C). Evaluation of tartaric acid was carried out using in vitro gas production technique. Methane production and individual fatty acids were estimated by Gas Chromatography. Results of different levels of tartaric acid on in vitro methanogenesis indicated that the maximum methane reduction (22.60% in term of mM/gDM) was observed in LFD at the supplementation dosage of 15 mM and a similar trend was seen, when methane was expressed in ml/gDM. Non-significant (P≤0.05) effect of tartaric acid addition on in vitro dry matter digestibility (IVDMD) was observed in almost cases. Protozoal population decreased with increasing concentration of tartaric acid and maximum reduction (54.64%) was in the MFD. Acetate to propionate ratio was decreased in tartaric acid supplemented diets which reflects increase in propionic acid production in comparison to control diet. Microbial biomass yield also increased due to the addition of tartaric acid in most of the diets.*

Key words: *Tartaric acid; Rumen fermentation; IVDMD, Microbial biomass; Methane production*

ORIGINAL ARTICLE

INTRODUCTION

Methane is one of the major end products of anaerobic fermentation of feeds in the rumen. Nutritionally, ruminal methanogenesis is a wasteful process which represents 2 to 12% gross energy loss from a mature animal (Moss, 1993). Methane production by animals, mainly from ruminants, is estimated to constitute 15 to 20% of the global production of methane (Crutzen et al. 1986). Its emissions to the atmosphere may result in a detrimental impact on the environment because of its greenhouse effect. Therefore, extensive research interests of animal nutritionists and ruminant microbiologists have been focused on developing methods of reducing methane production and manipulate the ruminal microbial ecosystem to improve the feed conversion efficiency. Many strategies such as processing of forages (Takahashi, 2001; Santoso et al., 2003), increasing the proportion of concentrates in the diet (Lee et al. 2003), and supplementation of some methane inhibitors such as halogenated compounds (Martin and Macy, 1985), ionophores (Van Nevel and Demeyer, 1988), organic acids (Martin, 1998), sarsaponin (Lila et al. 2003), and unsaturated fatty acids (Czerkawski et al., 1966) have been proposed as a means of reducing methane production in the rumen. Another method to reduce the methane formation in the rumen is diverting H_2 from CH_4 production to increase alternative electron sink metabolic pathways to dispose of the reducing power (Lopez et al. 1999; Ungerfeld et al. 2003 and Newbold et al. 2005). Dicarboxylic acid can act as alternative hydrogen sinks in the formation of methane in enteric fermentation; consequently, they are precursors to propionate production in the rumen. Fumarate and malate are the key propionate precursors in the dicarboxylic acid pathway (Castillo et al., 2004) and may act as a hydrogen acceptor (Martin and Park, 1996) hence both malate and fumarate have been increased pH, total volatile

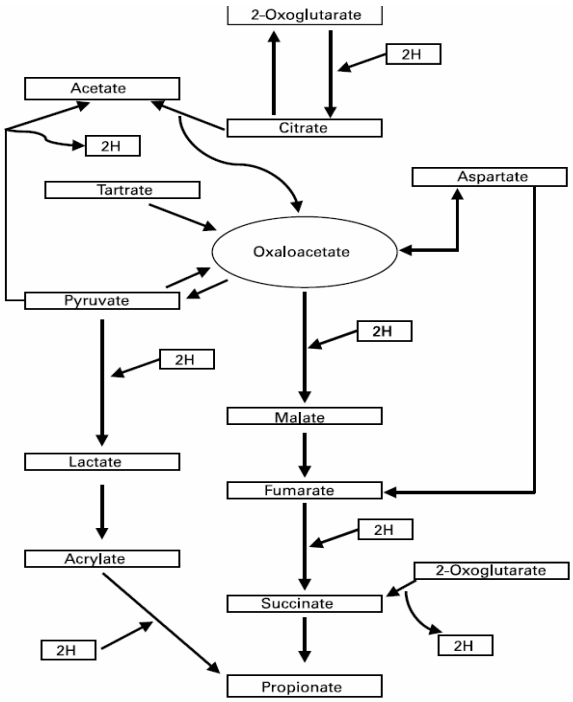

Fig 1- Possible fermentation pathway for tartaric acid (Newbold et al. 2005)

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fatty acid production and concentration of propionate in the rumen (Martin and Streeter, 1995; Carro and Ranilla, 2003). Tartaric acid is also converted into oxaloacetate and enters into TCA cycle and converted to propionic acid (Figure 1).The objective of the present study was to examine the effect of the addition level of tartaric acid in rumen fermentation characteristics and methane production by rumen microbes *in vitro*.

MATERIALS AND METHODS

Feeds and experimental design

To evaluate the response of Tartaric acid three diets were prepared by taking different roughage and concentrate ratio i.e. High fiber diet (HFD, 60R:40C), medium fiber diet (MFD, 50R:50C) and low fiber diet (LFD, 40R:60C) and milled to pass through a 1 mm sieve and used as substrate. The roughage part composed of wheat straw and the concentrate part composed of maize (33%), GNC (21%), mustard cake (12%), wheat bran (20%), de-oiled rice bran (11%), mineral mixture (2%) and salt (1%) respectively. Tartaric acid (Sigma-Aldrich, EC203-743-0) was added in incubation medium to achieve a final concentration of 0, 5, 10 and 15 mm. All the treatment combinations were arranged in 4 x 3 factorial design with three replicates. A set was incubated devoid of substrate with and with out tartaric acid which served as blanks for a particular treatment and values were corrected for different parameters with these blanks.

Preparation of inoculums and *in vitro* gas production

Rumen liquor was collected after manual mixing of rumen contents from a fistulated mature male buffalo (*Bubalus bubalis*) maintained on a standard diet (60 parts roughage: 40 parts concentrate) before the morning feeding into a pre-warmed insulated flask and brought into the laboratory. Permission has been taken from Animal ethics committee of institute for taking rumen liquor from male fistulated buffalo. The rumen liquor filtered through four layers of muslin cloth and then the required amount of filtered rumen liquor used as a source of inoculum. The incubation medium was prepared as per previously described method (Menke and Steingass, 1988).

The tartaric acid solution was injected as per the dose by small syringe into 100 ml glass syringe containing 200±10 mg of milled (1mm) three type wheat straw based diets. The 30 ml incubation medium was dispensed anaerobically in each syringe. The plungers of syringes applied with petroleum jelly for smooth movement and stop any leakage. Syringes were closed using clamps and were incubated at 39±0.5^{0}C for 24 h.

Estimation of methane production by Gas Chromatography

Methane content in fermentation gas was determined by gas chromatography (GC) using Nucon-5765 gas chromatograph described by (Sirohi et al., 2012). For methane estimation, each gas sample (250 µl) was manually injected using Hamilton airtight syringe. Methane content in the sample was calculated by external calibration, using a certified gas mixture with 50% CH_4 and 50% CO_2 (Spantech calibration gas, Surrey, England). The peak of methane gas was identified on the basis of retention time of standard methane gas and the response factor obtained was used to calculate methane percentage in the gas sample. The methane produced from substrate during 24 h incubation was compared to the blank values. The volume of methane produced was calculated as follows: Methane production (ml) = Total gas produced (ml) × % methane in the sample.

Rumen fermentation parameters

TVFA concentration (mM/100 ml) in the supernatant was estimated according to prescribed method (Barnet and Reid, 1957). For the estimation of IVFA, 1 ml of the supernatant was treated with 25% meta-phosphoric (4 ml) and kept for 3-4 h at ambient temperature (Erwin et al. 1961). Thereafter, IVFA was estimated using gas chromatograph according to the prescribed method (Sirohi et al. 2012). Sample (2 µL) was injected through the injection port using Hamilton syringe (10 µL). Individual VFAs of the samples were identified on the basis of their retention time and their concentration (mmol) and calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values. For the estimation of ammonia nitrogen, the supernatant of each syringe including that of blank was used for NH_3-N estimation. Supernatant (5 ml) was mixed with 1 N NaOH (12 ml) and steam passed on this using KEL PLUS-N analyzer (Pelican, India) and the NH_3 evolved was collected in a boric acid solution having a mixed indicator and titrated against N/100 H_2SO_4.

Partitioning factor (PF) and microbial biomass yield (MBM)

The PF is calculated as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (ml) produced by it. Substrate provides important information about partitioning of fermentation products. The MBM yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor (Blummel et al., 1997).

Microbial biomass (mg) = Substrate truly degraded - (gas volume × stoichiometrical factor) Where the stoichiometrical factor used was 2.25.

Protozoa counting

For protozoa count one milliliter of the fermentation fluid was diluted with 1 ml of formalin (18.5% formaldehyde) and 3-4 drops of brilliant green and then incubated for 24 h at room temperature. The stained

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protozoa were diluted (if needed) and counted by haemocytometer as per the method described by Dehority (1984).

In vitro true DM degradability

To estimate true DM degradability of feed sample of each syringe containing residues after incubation was estimated as per the prescribed method (Van Soest et al., 1991).

Proximate analyses and Cell wall constituents

The proximate analysis of substrate was carried out as per the methods (AOAC, 1995). The cell wall constituents of substrates were determined according to the described method (Van Soest et al., 1991).

Statistical analysis

Experimental data was fitted into 3x4 factorial arrangement for different parameters and analyzed in complete randomized block design with three replicates for analysis of variance (Snedecor and Cochran, 1968).

RESULTS AND DISCUSSION

The physical and chemical composition of all the three wheat straw based diet was shown in Table 1. The effects of tartaric acid addition on *in vitro* rumen fermentation pattern and methane production of different diets were shown in table 2 and 3, respectively.

Table 1- Physical and chemical composition diets used as substrates in *in vitro* incubations

Ingredient of diets		
Diets	G/kg on DM basis	
	Wheat straw	Concentrate
HFD	600	400
MFD	500	500
LFD	400	600

Ingredient of concentrate	
Particulars	G/kg on DM basis
Maize	330
Ground nut cake	210
Mustard cake	120
Wheat bran	200
Deoiled rice bran	110
Mineral mixture	20
Salt	10

Chemical constituents of diets (g/kg on DM basis)							
Diets	OM	CP	EE	NDF	ADF	HC	TA
HFD (60R:40C)	867.6	108.6	23.4	623.1	372.0	251.1	132.4
MFD (50R:50C)	878.4	125.3	30.4	604.5	329.5	275.0	121.6
LFD (40R:60C)	875.6	142.7	34.8	538.7	298.7	240.0	124.4

HFD: High fiber diet, MFD: Medium fiber diet, LFD: Low fiber diet, OM: Organic matter, CP: Crude protein, EE: Ether extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, HC: Hemicelluloses, TA: Total Ash

Table 2 - Effect of tartaric acid on rumen fermentation pattern in different diets *in vitro*

Diets	Dose (mM)	IVDMD (%)	PF	MBM (mg)	CH_4 (ml/gDM)	CH_4 (mM/gDM)	Protozoa (x10^4/ml)
HFD	0	55.67	2.24	63.92	38.53	3.83	1.67
	5	57.83	2.71	75.08	32.83	3.26	1.00
	10	51.83	2.74	68.08	41.41	4.12	1.00
	15	53.67	3.77	80.92	32.06	3.19	0.83
MFD	0	57.83	2.23	66.08	42.70	4.25	1.83
	5	58.67	2.69	75.92	38.08	3.79	1.17
	10	56.00	2.67	72.25	37.06	3.69	1.17
	15	55.67	3.63	82.92	33.40	3.32	0.83
LFD	0	64.67	2.21	72.92	40.94	4.07	1.50
	5	63.00	2.63	80.25	37.69	3.75	1.50
	10	66.33	2.54	82.58	40.65	4.04	1.00
	15	65.83	3.25	93.08	31.67	3.15	1.00
SEM	D	1.04	0.03	1.04	NS	NS	NS
	T	NS	0.03	1.20	0.91	0.09	NS
	D*T	NS	0.06	NS	NS	NS	NS

HFD: High fiber diet, MFD: Medium fiber diet, LFD: Low fiber diet, PF: Partition factor, MBM: Microbial biomass yield, CH_4: Methane, SEM: Standard of Means, D: Diets, T: Dose

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Table 3 - Effect of tartaric acid on rumen fermentation pattern in different diets *in vitro*

Diets	Dose (mM)	TVFA (mM/100ml)	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	A/P ratio	NH_3-N (mg/100ml)
HFD	0	8.23	5.31	2.70	0.22	1.97	14.75
	5	7.10	3.62	3.17	0.31	1.14	14.56
	10	4.68	2.31	2.18	0.19	1.05	15.40
	15	5.83	2.52	3.04	0.27	0.83	15.59
MFD	0	5.40	1.47	3.67	0.26	0.40	15.77
	5	5.58	2.67	2.65	0.26	1.01	15.87
	10	5.28	2.40	4.26	0.40	0.56	15.96
	15	7.03	2.80	3.78	0.44	0.74	13.53
LFD	0	9.05	4.97	3.99	0.19	1.25	19.23
	5	9.15	5.09	3.97	0.19	1.28	21.65
	10	6.38	4.42	4.67	0.32	0.95	21.93
	15	5.83	2.52	3.65	0.21	0.69	16.05
SEM	D	NS	0.26	NS	NS	0.22	0.34
	T	NS	0.31	0.27	0.03	0.25	0.40
	D*T	0.50	0.53	0.46	0.04	0.43	0.69

HFD: High fiber diet, MFD: Medium fiber diet, LFD: Low fiber diet, TVFA: Total Volatile Fatty Acid, A/P: Acetate to propionate Ratio, NH_3-N: Ammonia nitrogen, SEM: Standard of Means, D: Diets, T: Dose

In the current experiment, results of IVDMD was non-significantly ($P \leq 0.05$) affected due to the addition of tartaric acid. IVDMD values almost remained similar as control at all levels of tartaric acid supplementation and different types of diets. These results were more or less in accordance with previous studies. Increases in DM degradation were not observed when free acids were used (Newbold et al., 2005), while, small increase, approximately 4% in the apparent *in vitro* digestibility of maize supplemented with fumarate (Carro and Ranilla, 2003). The partition factor (PF) and microbial biomass production (MBM in mg) values were increased ($P<0.05$) with supplementation of tartaric acid at different concentration in all types wheat straw based diets. The highest increase 68.30, 62.78 and 47.06% in PF and the highest increase in MBM (mg) was 26.68, 25.48 and 27.65% found at 15 mM concentration, as compared to control in HFD, MFD and LFD, respectively. A reduction in methane production (ml/gDM, mM/gDM) was seen except 10 mM level in all cases, which however does not, reduced the methane production significantly. The maximum methane reduction was observed at highest level i.e. 15 mM in all diets. Results indicate that the highest methane reduction (16.71, 21.88 and 22.60%) was noticed at 15 mm level in HFD, MFD and LFD, when expressed in mM/gDM respectively. The similar trend was noticed when methane reduction was expressed in ml/gDM (table -2). Previous studies indicate that methane production was decreased with increase the concentration of free acid or propionate precursor (Asanuma et al., 1999; Lopez et al., 1999a; Iwamoto et al., 1999). In the present experiment, a reduction in protozoa number was also observed with the increasing concentration of tartaric acid. At the 15 mM dosage/concentration of tartaric acid, the maximum reduction in protozoa number was found i.e. 50.30, 54.64 and 33.33 percent in HFD, MFD and LFD, respectively. Tartaric acid supplementation showed non-significant ($P<0.05$) effect on TVFA concentration in comparison to control. Apparently slight changes in TVFA concentration were observed in all cases except in HFD at 10 mM level, whereas, maximum 43.13% reduction was observed, while, in case of MFD, the maximum increase (30.19%) was noticed at 15 mM level. Acetate concentration decreased in almost all cases except MFD, although the decrease was non-significant and maximum reduction (52.54%) was observed in HFD at 10 mM level. In case of MFD, it increased and maximum increased (47.50%) was noticed at 15 mM level. A slight effect of tartaric acid inclusion on propionic acid concentration (mM/100ml) was seen in all the three types of diets. Results indicated that the maximum increase (17.41%) was noticed in HFD at 5 mM level, and at the same level in MFD show the maximum reduction (27.79%) in propionic acid concentration. Slight change in butyrate concentration was also observed in the present study (table -3). In the present study, reduction in A/P ratio was observed in HFD and LFD in all concentration of tartaric acid, while in case of MFD, it was increased and highest increase (60.60%) was seen at 5 mM concentration. In the present experiment, slight change in NH_3-N concentration was observed due to tartaric acid supplementation. The concentration of ammonia nitrogen decreased with high level i.e. 15 mM in MFD and LFD, while increasing in HFD at the same level. The maximum reduction was (16.54%) found in LFD at 15 mM level. The results of the study was in accordance of results of Newbold et al. (2005) as they reported that with addition of organic acid in general able to decrease methane production due rechanneling the available hydrogen towards propionic acid production without affecting dry matter digestibility even with fumaric acid and malic acid supplementation digestibility was rather increased in some cases (Carro and Ranilla, 2003; Sirohi et al., 2012)

CONCLUSIONS

In the present study it was concluded that tartaric acid addition in wheat straw containing diets is able to significantly decrease the methane production by diverting the available hydrogen towards propionate production without affecting the digestibility, but more studies are required to validate the results under *in vivo* conditions.

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