RESEARCH ARTICLE

Evaluation of Complementary Effects of Exogenous Fibrolytic Enzymes and Fumaric Acid on Methane Emission and Ruminal Fermentation by *In Vitro* Method

Arumbaka Sudheer Babu*, Madhu Mohini, Sudarshan Singh Thakur, Goutam Mondal

Abstract

The objective of the present study was to investigate the hypothesis that exogenous fibrolytic enzymes (EFE, Cellulase with activity >4500-5000 µM glucose/g/min and Xylanase with activity >7500-8000 µM xylose/g/min, mixed in 50:50 w/w), in combination with fumaric acid (FA) and sodium sulfate (SS) may provide complementary effects to mitigate methane emission and enhance *in vitro* fermentation parameters of a sorghum stover based substrate (60% roughage and 40% concentrate). Three levels of EFE (0, 0.75 and 1.5 g/kg DM) (E_0, E_1 and E_2) with two levels of FA (F_0 and F_1) @ 0 and 8 mM (or 0 and 2 % of DM) and SS (S_0 and S_1) @ 0 and 1 g/kg DM, were tested in 3x2x2 factorial design using *in vitro* gas production technique. E_1 level reduced the methane production and improved TDDM (%) significantly while the combination of E_1 level with F_1 reduced methane % along with enhanced TDDM and TDOM (%). Fumarate alone was not efficient to decrease methane levels and increase digestibility but its combination with E_1 level showed the desired effects. Other levels (S_1 with E_1 and S_1F_1 with E_1) reduced methane but TDDM (%) and TDOM (%) were reduced. Overall, the *in vitro* results of fumaric acid in combination with EFE 0.75 g/kg DM can provide a possible strategy for mitigation of enteric methane emission to be proved by *in vivo* experiments.

Key words: Cellulase, Exogenous fibrolytic enzymes, Fumaric Acid, *In vitro,* Methane, Sodium sulfate, Rumen fermentation, Xylanase.

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Introduction

Ruminants are generating globally 80 million tones of methane annually by way of enteric fermentation (Beauchemin *et al.*, 2008), which is also responsible for a loss of 5-15% of dietary energy. Methane production can be mitigated by suppressing the methanogens, which play a key role in maintaining the low concentration of hydrogen in the rumen (Tavendale *et al.*, 2005). Accumulation of hydrogen in the rumen is disadvantageous because it has negative effect on fibre digestion (McAllister *et al.*, 1996). Sulphate is effective in reducing CH_4 emission by alternative H_2 sink as sulphate reduction, *i.e.,* $SO_4 + 4H_2 + 2H = H_2S + 4H_2O$. Sulphate reduction has been reported to occur in the rumen but it is considered a minor route of H_2 disposal compared with methanogensis (Takahashi *et al.*, 1989). Using hydrogen sinks such as fumaric acid (FMA) is an approach for reducing enteric methane production (Wood *et al.*, 2009). The objective of the present study was to investigate complementarity effects of exogenous fibrolytic enzymes, FMA (hydrogen sink) and sodium sulfate on ruminal fermentation using *in vitro* batch culture.

Materials and Methods

In Vitro Experimentation

The experiment was conducted following approval of the protocol by the Institutional Animal Ethics Committee of ICAR-National Dairy Research Institute, Karnal, India.

Animal Nutrition Division, ICAR-National Dairy Research Institute, Karnal-132001, Haryana, India

Corresponding Author: Dr Arumbaka Sudheer Babu, Associate Professor, Department of Animal Nutrition, College of Veterinary Science, Rajendranagar, Hyderabad-500030, Telangana State, India. e-mail: bakasudheer@gmail.com

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Rumen contents were collected from two rumen fistulated buffaloes (450 kg body weight) fed wheat straw *ad libitum* and concentrate 2.0 kg in equal proportions at 10:00 h and 16:00 h, mixed and strained through two layers of cheese cloth into pre-warmed thermo flask with an oxygen free headspace. Ruminal feed particles were allowed to settle to the bottom (5 min), and finally ruminal fluid was strained through two layers of nylon cloth (50 mm pore size). Particle-free ruminal fluid was mixed with the buffer solution (Menke and Steingass, 1988) in a proportion 1:2 (v/v) at 39°C, anaerobically.

The substrate comprised per 100 g dry matter (DM): 10 g of oats (*Avena sativa*) (fodder, hay), 50 g of (*Sorghum bicolor*)

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sorghum stover and 40 g of concentrate. The concentrate contained: maize grain, ground nut cake, deoiled mustard cake, wheat bran, rice bran, mineral mixture and common salt (33, 21, 12, 20, 11, 2 and 1 g/kg DM. respectively). The organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF) and acid detergent fibre (ADF) content of substrate determined were 89.29, 10.58, 1.56, 65.06 and 38.75 % DM, respectively. Samples of substrate ingredients were dried in oven (65°C for 72 h) and ground through a 1 mm screen.

Two hundred milligrams of substrate were accurately weighed into 100 mL calibrated glass syringes. Fumaric acid (FA) (Thomas Baker Chemicals, Ltd) was added to achieve final concentrations of 0 and 8 mM (0 and 2 % of DM) along with sodium sulfate (SS) 0 and 1 g/kg DM and exogenous fibrolytic enzymes (EFE) (0, 0.75 and 1.5 g/kg DM) of substrate on DM basis) in triplicates. Exogenous fibrolytic enzymes (EFE) included Cellulase with activity >4500-5000 µM glucose/g/min and Xylanase with activity >7500-8000 µM xylose/g/min, mixed in 50:50 w/w. The syringes were pre-warmed (39°C) prior to the dispensing 30 mL of buffered rumen contents into each one. The syringes were incubated in water bath at 39±0.5°C for 24 h. Three blanks containing only 30 mL of buffered rumen fluid were included to correct gas production values for gas released from endogenous substrates. Three additional blanks containing 30 mL of buffered rumen fluid, plus the corresponding additives, were also included for each treatment, in order to correct gas production values for gas released from endogenous substrates and the additive as recommended by Carro *et al.* (2005). The corrected gas values (net gas) were used for calculation of partitioning factor and microbial biomass production as explained below.

After 24 h incubation, fermentation was arrested by chilling the syringes to 4°C, and the quantities of fermentation products were determined in each syringe following the methods of analysis described later. The total and net gas values were recorded and a suitable aliquot of gas was withdrawn from the tip of the incubation syringes using gas tight syringe. Thereafter, the syringe contents were transferred to centrifuge tubes and the pH was measured immediately. The tubes then were centrifuged at 1600 x *g* for 10 min and an aliquot of supernatant was acidified with equal volume of 0.5 M HCl for measuring ammonia nitrogen (N) and kept at -20°C. Another aliquot of supernatant (4 mL) was added to 25% m-phosphoric acid (2 mL), kept overnight at 4°C and centrifuged at 1600 x *g* for 15 min and supernatant was stored at -20°C for volatile fatty acids (VFA) analysis. The pellets were transferred to spoutless beakers (500 mL) by dissolving with 70 mL of neutral detergent solution. Beakers were kept on heater and refluxed at 100°C for 1 h from when the boiling started. The contents in the beakers were filtered under vacuum through pre-weighed sintered (G1) crucibles and washed with hot water. The crucibles containing residue was oven dried (65°C for 48 h), weighed and the dried residue was ashed at 550°C.

Chemical Analysis

Chemical composition of formulated substrate estimated as follow: OM was determined by ashing at 550°C for 5 h; NDF and ADF were determined by the methods of Van Soest *et al.* (1991) and AOAC (2006), respectively; CP content (N*6.25) was determined using KEL PLUS-N analyser (Pelican, India), and EE was estimated using AOAC (2006). After 24 h incubation, suitable aliquot of gas was withdrawn from the tip of the incubation syringe using gas tight syringe and analyzed for its methane with the help of Gas chromatograph (Nucon 5700, India) fitted with stainless steel column packed with Porapak-N and Flame Ionization Detector (FID) as per Zhao *et al.* (2015).

The proportion of methane (%) and volume of methane (mL) were calculated as follows:

$$\text{Methane (\%)} = \frac{\text{Area covered by the sample}}{\text{Area covered by the standard of methane}} \times 50$$

Methane production (mL) = Total gas produced (mL) × % methane in the sample

For the estimation of ammonia nitrogen concentration, acidified supernatant (5 mL) was mixed with 10 mL of NaOH (1 N) and immediately steam distilled using KEL PLUS - N analyser (Pelican, India). The ammonia evolved collected in boric acid solution (20% w/v) having mixed indicator and titrated against N/100 sulfuric acid. Individual VFA (acetate, propionate and butyrate) in the samples were determined using gas chromatograph-GC (Nucon 5700, Nucon Engineers, New Delhi) equipped with flame ionization detector-FID and stainless steel column, packed with chromosorb 101 mesh 80-100 (length 1.5 m, o.d 3.175 mm and i.d. 2 mm). Analytical conditions for fractionation of VFA were as follow: injection port temperature, 210°C; column temperature, 180°C and detector temperature, 230°C. The flow rate of carrier gas (nitrogen) was 40 mL/min. *In vitro* dry matter digestibility (IVDMD) and true organic matter digestibility (TOMD) were calculated from the disappearances of DM and OM. Partitioning factor (PF) and microbial biomass production (MBP) were calculated based on truly degraded organic matter (TDOM) as described by Blummel *et al.* (1999) and Blummel *et al.* (2005).

$$\text{PF} = \frac{\text{mg TDOM}}{\text{Net gas production}}$$

Where TDOM was calculated by multiplying TOMD (%) by mg OM content of substrate

Microbial biomass production (MBP) was calculated from TDOM using equation: MBP (mg) = TDOM (mg) – (2.25 × net gas volume), Where, constant 2.25 is the stoichiometric factor.

Statistical analysis

The data were analysed by two-way ANOVA according to a 3x2x2 factorial design with EFE (three levels: 0, 0.75 and 1.5 g/kg DM), sodium sulfate (two levels, 0 and 1 g/kg DM) and FA (two levels, 0 and 8 ppm) as main factors of variation using SAS statistical software package version 8.2. Following

a significant F test ($p \leq 0.05$) the differences among means were examined by the Tukey's test.

Results and Discussion

Main Effects of Exogenous Fibrolytic Enzymes and Additives

The main affects of EFE (E_0, E_1 and E_2) and additives (sodium sulfate and fumaric acid) (S_0F_0, S_0F_1, S_1F_0, and S_1F_1) on fermentation variables of the substrate incubated with buffered rumen fluid are presented in Table 1. E_0 level has recorded higher in *in vitro* total gas production compared to E_1 and E_2 supplementation. E_1 level lowered methane percent significantly and the higher level of E_2 did not affect or mitigate methane production further. Enzyme supplementation (E_1 and E_2) significantly ($p<0.000$) reduced methane levels (when expressed as mL/24 h and g/kg IVOMD) when compared with E_0 level although the difference between E_1 and E_2 was not significant. The E_1 level improved *in vitro* DM digestibility significantly followed by E_2 and lowest in control. TOMD per cent was significantly higher and similar at E_1 and E_2 levels compared to control. The treatment of the substrate with enzymes non-significantly augmented partitioning factor and microbial biomass production compared to control. E_1 and control had lower acetate values compared with E_2 level. Addition of sodium sulfate and fumaric acid had no significant effect individually or combined on various *in vitro* fermentation parameters (Table 1).

In the present study, *in vitro* total gas production (GP) was significantly ($p<0.001$) higher in control, followed by E_1 and E_2 supplementation. In contrast, Almaraz *et al.* (2010), observed no significant increase of GP in control compared to the diet supplemented with various doses of EFE (3 and 6 g enzyme/kg DM) (diet contained 70% concentrate on % DM basis, having CP of 14 to 14.5 %). It was suggested that as the total fermentable material has not increased there was no effect on GP.

The positive increase of IVDMD and TOMD parameters compared to the control, suggest that the enzymes were able to degrade complex substrates to simpler ones, possibly on account of faster ruminal colonization and fermentation, as reported by Colombatto *et al.* (2003). While the lack of effects on final GP suggests that the substrates degraded by the enzymes, would have been degraded in the medium anyway, even though at a later time (Colombatto *et al.,* 2007). In contrast, Wallace *et al.* (2000) reported that cellulase and other commercial fibrolytic enzymes can increase the cumulative GP and rate of *in vitro* fermentation of grass. Increasing the levels of fibrolytic enzymes supplementation to rice straw increased GP (linear, $p<0.01$) (Tang *et al.,* 2008). Mao *et al.* (2013) observed that the addition of CEL and XYL increased the GP parameters. The observed variations on EFE addition are influenced by diet composition, type of enzyme used, enzyme addition level, enzyme stability and method of application (Yang *et al.,* 2000).

Methane expressed as per cent was significantly lower in E_1 followed by E_2 and was higher in control (E_0). In agreement

Table 1: Main effects of exogenous fibrolytic enzymes (E), and additives (A) fumaric acid (F) and sodium sulphate (S) on *in vitro* fermentation parameters of the incubated substrate

Parameters	Enzyme (E)			Additives (A)				P value			SEM
	E_0	E_1	E_2	S_0F_0	S_0F_1	S_1F_0	S_1F_1	E	A	E x A	
Total gas (mL/g DM)	215.83^b	197.80^a	196.03^a	201.41	206.68	201.16	203.64	<0.000	0.659	0.893	2.17
Methane (%)	26.32^c	23.87^a	24.54^b	24.65	24.74	25.08	25.19	<0.000	0.137	<0.000	0.22
Methane (mL/24 h)	11.36^b	9.44^a	9.62^a	9.95	10.23	10.11	10.28	<0.000	0.658	0.745	0.17
Methane (g/kg IVOMD)	50.76^b	41.70^a	42.87^a	44.08	45.67	44.73	45.96	<0.000	0.652	0.762	0.86
IVDMD (%)	65.41^a	67.37^c	66.57^b	66.62	66.16	66.53	66.49	<0.000	0.443	<0.000	0.20
TOMD (%)	65.56^a	67.67^b	67.13^b	66.75	66.53	67.06	66.81	<0.000	0.501	<0.000	0.23
PF (mg TDOM/mL net gas)	3.54^a	3.83^b	3.85^b	3.78	3.66	3.80	3.72	<0.008	0.641	0.998	0.04
MBP (mg)	44.23^a	51.62^b	51.92^b	50.18	47.55	50.64	48.66	<0.002	0.585	0.995	0.97
Ammonia - N (mg/dL)	13.67	13.19	13.67	13.06	14.22	13.38	13.38	0.486	0.172	0.975	0.18
Acetate (mM/L)	37.99^a	40.93^{ab}	41.64^b	38.44	39.92	41.63	40.77	<0.027	0.225	0.061	0.66
Propionate (mM/L)	13.21	12.92	12.77	13.05	12.96	12.79	13.08	0.905	0.994	0.951	0.35
Butyrate (mM/L)	7.33	7.64	7.63	7.37	7.57	7.73	7.47	0.064	0.199	0.105	0.07
A/P ratio	2.91	3.28	3.38	3.04	3.14	3.36	3.21	0.238	0.801	0.782	0.11

SEM: Standard error of the mean. IVOMD, *in vitro* organic matter digestibility; IVDMD, *in vitro* dry matter digestibility; TOMD, True organic matter digestibility; MBP, microbial biomass production; PF, Partitioning factor. Means within a row under each main factor lacking a common superscript letter differ ($p \leq 0.05$). SEM: standard error of treatments mean.

with the above results, Zhao *et al.* (2015) observed a decrease ($p<0.01$) in production of methane per DMD and NDFD, while the total absolute methane production did not change on addition of cellulase and xylanase to corn stover. In contrast, Wang and Xue (2016), observed no significant ($p<0.05$) differences among dietary treatments on total CH_4 emissions (g/d), CH_4 emission as a proportion of live weight or feed intake (DM, OM, digestible DM or digestible OM), or CH_4 energy output (CH_4-E) as a proportion of energy intake (GE, DE, or ME), on supplementing exogenous cellulase on Boer crossbred goats with basal diet (control, no cellulase), basal diet plus 2 g unitary cellulase/kg DM, and basal diet plus 2 g compound cellulase/kg DM.

Methane emissions significantly ($p<0.001$) reduced on enzyme supplementation and were lower than control for all the variables except for the methane expressed as per cent of total gas ($E_1 < E_2 < E_0$). Further the methane values were statistically similar for E_1 and E_2, suggesting that increase of enzyme level has no further reduction affect on methane production. In contrast, Chung *et al.* (2012) observed increased enteric methane production on increasing the dose of enzyme supplementation and compared to control diet, high enzyme level addition to the diet increased enteric CH_4 (g/head per day) by 16%. It was inferred that EFE caused shift of bacterial communities (or methanogenic species) with overall densities of protozoa, bacteria, or methanogens remaining same (Zhou *et al.,* 2011).

The IVDMD (%) and TOMD (%), increased significantly in E_1 and E_2 as compared to control. The results of the present study were in accordance with studies of Bowman *et al.* (2002), Shojaeian and Thakur (2007) and Alvarez *et al.* (2009), wherein increased digestibility of DM and OM were reported when EFE supplemented through the concentrate portion of the ration. Thakur *et al.* (2008) reported significant ($p<0.05$) improvement of DM degradability in an *in vitro* experiment while studying effects of various levels of EFE's added @ 1.5, 3.0 and 6.0 g/kg DM of substrates (prepared with various proportion of concentrate, wheat straw and green sorghum) and also observed that, EFE level beyond 3.0 g/kg DM, recorded no further enhancement of the observed positive response, probably due to the diminishing of the beneficial disruption of feed surface, as the excess EFE attached to the feed restricted microbial attachment and limited the digestion of feed.

In the present study, the substrate supplemented with E_1 and E_2 levels of enzymes have shown 8 and 9 % increase, respectively, for PF value, while MBP (mg) value increased by 17 % for both E_1 and E_2 compared to the control (E_0). The partitioning factor (PF: mg OM digested/ mL gas produced) is a valid indicator of the fermentation efficiency wherein, with the same amount of degradable substrate having a higher value of PF, will record less gas and higher microbial mass production (Blummel *et al.,* 1997). As implied in the previous statement, the present study also recorded higher PF and MBP values consequent to the higher IVDMD and TOMD for enzyme supplemented substrate.

There were no significant ($p>0.05$) changes in the production of propionate, butyrate and A/P ratio and ammonia nitrogen except for the significant ($p=0.027$) low acetate level in the control compared to the E_1 and E_2 level of enzyme supplementation. In contrast, Soltan *et al.* (2013) observed that two levels of fibrolytic enzyme products (having cellulase and xylanase) when administered at 7.5 or 0.46 enzymatic units/500 mg DM substrate had no effect on acetate, valerate, isobutyrate and isovalerate concentrations in the incubation fluid at either incubation time 24 or 48 h and the overall propionate concentration had increased ($p<0.05$). The acetate production is associated with the release of H_2, which can be used by methanogens to form methane (Stewart *et al.,* 1997), while in the present study methane production was significantly higher in control with a lower acetate production. Krueger and Adesogan (2008) reported decreased acetate and increased propionate on addition of cellulase and xylanase with ferulic acid esterase from Bahia grass hay.

Interactions of Exogenous Fibrolytic Enzymes (E) and Additives (A)

Significant interactions of E × A are presented in Table 1. The substrate had 60% roughage and 40% concentrate and methane (%) was lowest for E_2 supplementation with a similar effect achieved by supplementing E_1 level with F_1 additive along with significant ($p<0.001$) positive effect on IVDMD (%) and TOMD (%) values. Fumarate alone was not efficient to decrease methane levels and increase digestibility but its combination with E_1 level showed the desired effects. Other levels (S_1 with E_1 and S_1F_1 with E_1) reduced methane but also depressed IVDMD (%) and TOMD (%).

Conclusion

It can be inferred from the above study that substrate showed positive response in the aspect of digestibility and methane mitigation on enzyme supplementation compared to control and increasing the enzyme level showed no further response for the above variables. Additives sulfate and fumarate in combination or individually added to the substrate had no effect on the various *in vitro* digestibility, fermentation and methane emission parameters. Hence, it can be concluded that EFE @ 0.75 g/kg DM and fumeric acid @ 2% of DM is the optimum additive combination for substrate, having the best possible effect on methanogenesis and rumen fermentation than substrate without additives or each one alone.

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