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EFFECT OF FEED ADDITIVES ON *In vitro* FERMENTATION KINETICS AND SUBSTRATE UTILIZATION WITH AND WITHOUT BUFFER

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ABSTRACT

The study was aimed to evaluate the effect of various categories of feed additives on rumen fermentation. Fermentation kinetics and substrate utilization by rumen microbes was studied by fortifying the substrate having roughage to concentrate of 60:40 with glucogenic *viz*., glycerol and propylene glycol (PG), rumen fermentation modifiers *viz*., para aminobenzoic acid (PABA), exogenous fibrolytic enzymes (EFE) and probiotic feed additives by applying first order kinetics model to *in vitro* gas production (IVGP) technique. Glycerol was more effective than PG for substrate fermentation kinetics and its utilization, particularly IVTVFA production. Buffer addition was more effective with PG but not with glycerol. PABA had non-significant impact on either substrate fermentation kinetics or utilization. EFE fortification significantly improved the extent of fermentation, IVNDFD and IVTVFA; however, probiotics showed only significant increase in IVDMD (P<0.01) and IVTVFA (P<0.05) by 11%. Study concluded that PG and glycerol being glucogenic compounds improved the extent of fermentation and TVFA production but glycerol was more effective than PG. Probiotics supplementation also had positive effect on IVTVFA production. EFE fortification improved fibre digestion. Buffer addition along with feed additives may enhance substrate kinetic efficiency of fermentation but it was selective.

Key words: Buffer, enzymes, glycerol, PABA, probiotics, propylene glycol, fermentation kinetics

INTRODUCTION

Rumen fermentation is central point in the performance of ruminant animals. Pattern of gases produced during rumen anaerobiosis and their kinetics are helpful in understanding the substrate fermentation, microbial characteristics, rate determination and biological value of feedstuffs (Singh and Srinivas, 2012). Manipulation of rumen fermentation by fortifying diets with feed additives can enhance the feed utilization and productivity of dairy animals (Tarek *et al*., 2015). For instance, exogenous fibrolytic enzymes (EFE) and probiotics supplementation increases rumen microbial activity (Malik and Srinivas, 2010), glycerol and propylene glycol (PG) provides energy for rumen microbes by increasing total volatile fatty acids (TVFA) and helps in utilization of ammonia nitrogen (Shingfield *et al*., 2002; Silva *et al*., 2015) and, PABA being a structural component of folic acid, acts as growth promoter for the cellulolytic bacteria (Scott and Dehority, 1965). Additive

effect of any feed additives is also dependent on optimum environment of rumen, particularly rumen pH. Modern feeding practices in dairy animals with high proportion of concentrate supplements lead to higher incidence of sub-acute ruminal acidosis (Doepel and Hayirli, 2011). Decreased activities of rumen microflora under acidic condition (Li *et al*., 2012) affects EFE, probiotics activity and also decrease the utilization of glycerol and PG. Sodium bicarbonate has been supplemented to dairy cows for many years as rumen buffer (Le Ruyet and Tucker, 1991) and many trials showed an extra increase in rumen pH and production benefits (Bach *et al*., 2007; Thrune *et al*., 2009). Bipin *et al*. (2016) reported improvement in rumen pH and reduction in serum acute phase protein during subacute rumen acidosis within 72 h of supplementation of sodium bicarbonate buffer. The present study was aimed to evaluate the effect of different feed additives with and without sodium bicarbonate buffer on fermentation kinetics and substrate utilization *in vitro*.

MATERIALS AND METHODS

Feed additives and substrate

Glucogenic feed additives (M/s Thomas Bakers, Mumbai); glycerol (@ 20 mg 200 mg^{-1}) and PG (@ 6 mg 200 mg^{-1}), rumen fermentation modifiers (M/s Varsha Group, Bangalore); EFE (mixture of cellulase, xylanase, hemicellulase and pectinase; supplemented @ 0.2 mg 200 mg^{-1}), probiotic (mixture of *Lactobacillus acidophilus*, *Saccharomyces cerevisiae* and *Saccharomyces boulardii*; supplemented @ 0.2 mg 200 mg^{-1}) and PABA (@ 8 μg 200 mg^{-1}) were selected and supplemented based on their reported optimum levels (Nocek *et al*., 2002; Chung *et al*., 2012; Pechova *et al*., 2014; Ariko *et al*., 2015) with and without sodium bicarbonate (1% of CS) buffer to the total mixed ration (TMR) as a substrate. TMR consisted of roughage to concentrate supplement (CS) in the ratio of 60:40. Roughage part of TMR consisted of $3/4^{th}$ ragi straw (RS; *Eleusine coracana*) and $1/4^{th}$ mixed green forages (3 parts para grass (*Brachiaria mutica*) and 1 part each of Guinea grass (*Megathyrsus maximus*), napier (*Pennisetum purpureum*) and maize (*Zea mays*) dried at 32°C. Dried samples were ground to pass through 1 mm screen and stored in polyethylene terephthalate (PET) container.

Fermentation kinetics in vitro (IVFK)

IVFK were studied by *in vitro* gas production technique (IVGP) using 100 mL glass syringes. Rumen liquor (RL) was collected from 3 cows fitted with flexible rumen cannula (large cannula #1C, M/s Bar Diamond, USA) fed with roughage to concentrate ratio of 60:40. RL was drawn before offering feed at 8 a.m. with a minimum of 6 h gap from last offering of drinking water and strained (SRL) through a double layered muslin cloth. SRL was used immediately as incubation media. SRL (10 mL) was taken along with 200 mg substrate and 20 mL buffer medium (Menke and Steingass, 1988). Piston was lubricated with petroleum jelly and fixed into the cylinder of syringe while removing any excess air. Syringes were kept in BOD incubator at 39°C. IVGP was measured at 0, ½, 1, 2, 4, 8, 12, 18, 24, 36, 48, 60 and 72 h intervals. IVGP values at different intervals were adjusted to blank which contained only buffer and inoculum. Blank corrected IVGP values at different time intervals were fitted to the first order generalized Mitscherlich kinetic model with 2 rate constants (France *et al*., 1993); $Y_{max} = A\ [1\text{-}e^{-k(t-\lambda) - d(\sqrt{t} - \sqrt{\lambda})}]$ where, Y_{max} was upper asymptote for total gas production, 'A' total gas produced during incubation, 'k' and 'd' are rate constants of gas production, 'λ' was lag time during the initiation of gas production and adjusted to time 't'.

Substrate utilization

After 72 h incubation, the glass syringes were filtered through muslin cloth. Filtrate was used for TVFA estimation by using Markham distillation apparatus (Barnett and Reid, 1956). Unfermented

substrate recovered was dried overnight in drought oven to constant weight. IVDMD of the substrate was determined as weight loss in substrate before and after incubation in 100 mL glass syringes. NDF in substrate before and after incubation was estimated according to AOAC (2005). IVNDFD was calculated as difference in the substrate before and after incubation.

Statistical analysis

IVGP at different intervals were analyzed using ANOVA model included the repeat measure. Dose effects were considered as between group variance and data for time intervals used for within group measures. IVFK were subjected to completely randomized design (CRD) based on the model: $Y_{ij} = \mu + T_i + B_j + e_{ij}$ where, 'Y_{ij}' was any observation for which 'μ' was general mean, 'T_i' was treatment effect, 'B_j' was block effect, and 'e_{ij}' was random error component (Das and Giri, 1991). Pair-wise comparison between group means was tested by Duncan multiple rank test and significance denoted by different alpha superscripts with probability ranging from 0.01 to 0.10.

RESULTS AND DISCUSSION

IVGP and IVFK

Total gas production (Table 1) and fermentation kinetics (Table 2) on substrate with PG or glycerol fortification was higher than control up to 36 and 24 h, respectively. Their impact at subsequent intervals was nullified thus indicating that PG and glycerol need supplementation at 24 h cycles for continuous impact on fermentation. Addition of buffer to PG resulted in significantly reduced ($P<0.01$) Y-max and improved ($P< 0.01$) kinetic parameters such as λ, $T_{1/2}$, k and d. This indicated

Table 1: Total gas production (ml 200 mg^{-1}) on different feed additives as % of total gas at different time intervals in crossbred cows

Feed additive	TGP (mL)	12 h	24 h	36 h	48 h	60 h
Glucogenic feed additives						
Control	37.76^{b}	30.5^{a}	61.0^{a}	77.8^{a}	89.6^{a}	98.0^{b}
Propylene glycol (6 mg 200 mg^{-1})	39.07^{b}	39.8^{b}	72.0^{b}	85.5^{b}	93.7^{b}	98.1^{b}
Propylene glycol + buffer$^{\#}$	35.31^{a}	47.9^{c}	73.9^{b}	83.4^{b}	90.4^{a}	96.2^{a}
SEM	0.66**	1.48**	1.48**	0.88**	0.67**	0.49*
Control	40.31^{a}	38.4^{a}	67.6^{a}	84.1	92.0	97.5
Glycerol (20 mg 200 mg^{-1})	44.31^{b}	45.1^{b}	73.9^{b}	85.6	93.8	98.1
Glycerol + buffer$^{\#}$	44.26^{b}	44.3^{b}	73.1^{b}	84.4	91.8	97.7
SEM	1.06*	1.60**	1.04**	0.75	0.76	0.28
Rumen fermentation modifiers						
Control	41.64	37.5^{b}	70. 8^{b}	81.5	90.5	97.6^{b}
PABA (8 µg 200 mg^{-1})	42.95	36.2^{ab}	66.8^{a}	81.9	90.4	96.3^{a}
PABA + buffer$^{\#}$	41.28	33.0^{a}	66.1^{a}	80.7	90.1	96.4^{a}
SEM	0.62	1.2*	0.79**	0.45	0.29	0.29**
Control	33.77^{a}	31.2	62.3	78.2^{a}	89.1^{b}	95.6^{b}
EFE (0.2 mg 200 mg^{-1})	36.09^{b}	31.6	62.3	78.2^{a}	87.0^{a}	94.5^{a}
EFE + buffer$^{\#}$	35.19^{b}	30.7	63.2	80.3^{b}	90.2^{b}	96.7^{b}
SEM	0.40**	0.95	0.50	0.26**	0.49**	0.28**
Control	33.8^{a}	31.2	62.3	78.2^{b}	89.1^{b}	95.6
Probiotic (0.2 mg 200 mg^{-1})	38.4^{c}	32.7	63.0	78.3^{b}	89.7^{b}	95.9
Probiotic + buffer$^{\#}$	36.6^{b}	32.5	63.4	63.4^{a}	80.4^{a}	96.2
SEM	0.55**	0.80	1.13	1.09**	0.79**	0.46

$^{\#}$Buffer was added @ 800 µg 200 mg^{-1} (1% of concentrate supplement in substrate); The values having different alpha superscripts in a column differ significantly from each other: *P<0.05 and **P<0.01

that effect of PG was accelerated in presence of buffer without altering the extent of fermentation but was not true with glycerol. PG produces organic acids which decreased pH up to 5.0. In contrast, 1 M glycerol had pH of 6.88 which is optimum rumen pH. Hence, buffer addition to PG showed impact on kinetic parameters rather with glycerol. Glycerol fortification had more beneficial effects on Y-max and kinetic parameters of the substrate.

PABA fortification had no influence on either substrate fermentation or kinetic parameters. PABA fortification to substrate showed adverse effect than control. Masoud *et al*. (2015) suggested that even though PABA acts as growth promoter for cellulolytic bacteria, its requirement is negligible when folic acid is available in diet. EFE and probiotic addition significantly increased TGP on substrate after 36 h, thus both the supplements require longer duration to exert their impact on substrate fermentation. Buffer addition to EFE or probiotics was not effective when roughage to concentrate ratio was 60:40. Although Y-max of substrate was significantly higher on EFE ($P<0.01$) and probiotics ($P<0.01$) fortification, kinetic parameters; λ, T1/2, k and d were unaltered. According to Malik and Srinivas (2010), increased TGP or Y-max of substrate with EFE fortification could be because of the hydrolysis and utilization of fiber while probiotics fortification could be helpful in improving the anaerobic environment resulting in increased microbial population in inocula. According to Srinivas and Krishanmoorthy (2005) Yα and λ represents microbial characteristics and Srinivas and Swain (2009) reported k and T½ represents feed characteristics.

Substrate utilization

Effect of different feed additives on substrate utilization revealed that PG in the presence of buffer significantly increased IVDMD compared to control and PG exclusively (Table 3). PG fortification showed no effect on IVNDFD. IVTVFA was significantly increased with PG fortification to substrate

Table 2: *In vitro* fermentation kinetics of different types of feed additives with mixed substrate in crossbred cows

Feed additives	Lag time (h)	$T_{1/2}$ time (h)	Rate constant (%)		Y max (mL)	Ferm. rate* (%)
			k_1	k_2		
Control	0.39^{c}	19.68^{c}	3.52^{a}	7.03^{a}	36.01^{a}	4.32^{a}
PG (6 mg 200 mg^{-1})	0.31^{b}	15.84^{b}	4.50^{b}	9.20^{b}	38.15^{b}	5.69^{b}
PG + buffer$^{\#}$	0.25^{a}	12.87^{a}	5.45^{c}	11.05^{c}	35.01^{a}	7.01^{c}
SEM	0.01**	0.56**	0.19**	0.35**	0.69**	0.25**
Control	0.32^{b}	16.76^{b}	4.14^{a}	8.86^{a}	39.28^{a}	5.22^{a}
Glycerol (20 mg 200 mg^{-1})	0.27^{a}	13.85^{a}	5.04^{b}	10.23^{b}	43.64^{b}	6.62^{b}
Glycerol + buffer$^{\#}$	0.28^{a}	14.09^{a}	5.18^{b}	10.41^{b}	43.58^{b}	6.43^{b}
SEM	0.01*	0.60**	0.21**	0.37*	0.96*	0.29*
Control	0.32^{a}	16.51	4.22	8.66^{b}	40.59	5.29^{b}
PABA (8 µg 200 mg^{-1})	0.33^{b}	16.89	4.04	8.36^{ab}	41.68	5.05^{ab}
PABA+ Buffer$^{\#}$	0.34^{b}	17.06	3.86	7.63^{a}	39.81	4.76^{a}
SEM	0.02•	0.34	0.08	0.01**	0.67	0.12*
Control	0.39	19.24	3.60	7.2	32.30^{a}	4.43
EFE (0.2 mg 200 mg^{-1})	0.38	19.17	3.62	7.3	34.56^{b}	4.46
EFE + buffer$^{\#}$	0.39	19.12	3.63	7.1	33.69^{b}	4.44
SEM	0.01	0.19	0.04	0.22	0.39**	0.06
Control	0.39	19.24	3.61^{a}	7.2	32.30^{a}	4.43
Probiotic (0.2 mg 200 mg^{-1})	0.37	18.98	3.69^{b}	7.5	36.77^{c}	4.56
Probiotic + buffer$^{\#}$	0.37	18.87	3.68^{b}	7.5	35.13^{b}	4.54
SEM	0.01	0.39	0.06*	0.18	0.56**	0.08

PG = Propylene glycol; PABA = Para aminobenzoic acid; EFE= Exogenous fibrolytic enzymes; $^{\#}$*Buffer was added @ 800 µg 200 mg^{-1}*(1% of concentrate supplement in substrate); * Ferm rate = fermentation rate

*Values bearing different alpha superscripts in a column differ significantly: •P<0.10, *P<0.05 and **P<0.01*

Table 3: *In vitro* substrate utilization on different types of feed additives with mixed substrate in crossbred cows

Feed additives	IVDMD (%)	IVNDFD (%)	IVTVFA (m mol L^{-1})
Control	73.61[b]	62.23	77.50[a]
PG (6 mg 200 mg^{-1})	73.12[a]	62.81	83.75[b]
PG + buffer[#]	74.92[c]	62.23	80.00[ab]
SEM	0.10**	0.49	0.12*
Control	73.61[a]	63.04[a]	78.75[a]
Glycerol (20 mg 200 mg^{-1})	75.68[b]	66.19[b]	90.00[b]
Glycerol + buffer[#]	75.25[ab]	64.18[a]	83.75[ab]
SEM	0.28•	0.33*	0.18**
Control	73.57	65.79[b]	75.00
PABA (8 µg 200 mg^{-1})	74.80	64.92[ab]	73.75
PABA+ Buffer[#]	74.57	63.79[a]	72.5
SEM	0.63	0.32*	0.34
Control	73.49[a]	62.75[a]	82.50
EFE (0.2 mg 200 mg^{-1})	75.01[c]	65.29[b]	83.75
EFE + buffer[#]	73.96[b]	64.72[b]	82.50
SEM	0.07**	0.15**	0.32
Control	73.49[a]	64.72	73.75[a]
Probiotic (0.2 mg 200 mg^{-1})	77.16[c]	65.90	82.5[b]
Probiotic + buffer[#]	74.17[b]	64.72	81.25[b]
SEM	0.06**	0.68	0.17*

substrate with or without buffer than control. Glycerol fortification increased significantly IVDMD, IVNDFD and IVTVFA. According to Fisher *et al.* (1973), improvement in dry matter intake in dairy cows was better with glycerol compared to PG when supplemented at 3% of CS. Increased TVFA with PG or glycerol fortification could be due to their glucogenic property (Chung *et al*., 2009). Shingfield *et al*. (2002) reported higher proportion of propionic acid in increased TVFA due to PG supplementation. Wang *et al*. (2009) reported significant increase in TVFA and NDFD in dairy animals with 200 g day^{-1} glycerol supplementation. Ivanor (2014) even suggested replacement of maize with glycerol by 16%.

PABA fortification had no significant effect on substrate utilization with or without buffer. Buffer addition significantly ($P< 0.05$) but negatively affected the IVNDFD of substrate. EFE and probiotic supplementation significantly increased IVDMD while the addition of buffer had no such effect. EFE with and without buffer significantly increased NDFD compared to control but addition of buffer had no additional advantage. Many workers have also reported positive impact of EFE on NDFD (Malik and Srinivas, 2010; Salem *et al*., 2011). Although probiotic fortification had no significant impact on NDFD, TVFA was significantly increased by 11% as compared to CG. This may be probably due to the impact of live microbial cells on anaerobiosis (Srinivas and Srinath, 2015). Present results are consistent with *in vitro* experiment of Wang *et al*. (2016) who reported significant increase in TVFA with probiotic addition.

Conclusion: Study revealed that PG and glycerol being glucogenic compounds improved the extent of fermentation and TVFA production in rumen, but glycerol was more effective than PG. Probiotics supplementation had positive effect on TVFA production. EFE fortification would improve fibre digestion. Buffer addition along with feed additives may enhance substrate kinetic efficiency of fermentation but it was selective.

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