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Effect of supplemental malic acid on methane mitigation in paddy straw based complete diet for sustainable animal production in indigenous dairy cattle

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ABSTRACT

A study was conducted to evaluate the effect of supplemental malic acid on mitigation of methane emission for dairy cattle by *in vitro* and *in vivo* methods. The *in vitro* finding was validated by *in vivo* feeding trial in indigenous dairy cattle. Ten dairy cattle with uniform milk production were selected and divided into two groups with five animals each and they were fed with and without supplementation of malic acid at 0.39% in 60% paddy straw and 40% concentrate mixture based complete diet. The malic acid at 0.39% was the minimum level which resulted in highly significant reduction of methane by 15.95% and methane (ml) per 100 mg of truly digested substrate by 15.69%, respectively than control in *in vitro* study. The methane emission per animal per day and per kg dry matter intake (DMI) was significantly decreased by 3.26% and 3.11%, respectively in malic acid supplemented group than control. The methane emission per kg milk production was significantly reduced by 5.43% in malic acid supplemented group than control. The total volatile fatty acid (TVFA) and propionic acid were significantly increased by 2.69% and 11.71%, respectively in malic acid supplemented group than control. It was concluded that the supplementation of malic acid at 0.39% of paddy straw based complete diet significantly reduced the methane emission per animal per day and per kg milk production than control in indigenous dairy cattle.

Keywords: Dairy cattle, *In vitro*, *In vivo* methane reduction, Malic acid

Methane is one of the major end products of anaerobic fermentation of feeds in the rumen and represents a loss of 8–12% of the feed energy (Moss 2000). It is also considered as one of the most important green house gases that causes global warming (IPCC 2001). Methane production is an unavoidable and inefficient product during ruminal fermentation and is usually associated with decreased propionate production and increased acetate to propionate ratio (Russell 1998). Methane is produced by strict anaerobes belonging to the sub-group of the archaea like *Methanobacterium formicicum*, *M. ruminantium*, *M. bryanti,* etc. Recently, various feeding strategies/dietary modifications have been emphasized for the reduction of methane emission (Vargas *et al*. 2022). For instance, antibiotics like ionophores, many chemical feed additives and defaunating agents were tried and found to decrease the methane emission from the rumen (Patra *et al*, 2010). These feed additives are either toxic to host animals or have only a transient effect on methanogenesis or possibility of development of resistance to antibiotics. This has led to the utilization of other sources for methane mitigation. Supplemental organic acids such as malate and fumarate are acted as precursors to propionate and if the rumen concentrations of these acids could be increased, propionate production would increase and methane production would fall. Recent research has shown that organic acids can stimulate the growth of the prominent ruminal bacterium *Selenomonas ruminantium* which favourably alters the mixed ruminal micro-organisms fermentation and improves the performance of animals (Martin 1998). The supplementation of malic acid decreased the methane emission by *in vitro* (Bharathidhasan 2020) and *in vivo* (Foley *et al*. 2009a). Furthermore, the supplementation of malic acid is one of the potential feeding strategies to reduce the methane emission without affecting the rumen fermentation characteristics and it also improves the performance of the dairy cattle (Bharathidhasan 2016). Hence the present research was carried out to study the effect of supplemental malic acid on reduction of methane emission by *in vitro* and *in vivo* methods in indigenous dairy cattle for sustainable animal production in paddy straw based complete diet.

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MATERIALS AND METHODS

In vitro *gas production technique (IVGPT*: The *in vitro* gas production technique (*IVGPT)* (Menke and Steingass 1988) was carried out by incubating the malic acid (MA) at 0, 0.13%, 0.26%, 0.39% and 0.52% of 60% paddy straw and 40% concentrate mixture based complete ration with rumen liquor in shaking water bath for a period of 24 h. The rumen

fluid was collected from three cattle maintained on grazing and it was squeezed through four layers of muslin cloth into an Erlenmeyer flask under continuous flushing with CO_2 and it was maintained at the temperature of 39°C. Then rumen fluid was mixed with media as described by Menke and Steingass (1988). The paddy straw based complete diet was used as substrate and it was taken as 200 mg in 100 ml calibrated syringes and weighed quantity of malic acid was added to the syringes in triplicate. Then, 30 ml of rumen inoculum was anaerobically transferred to glass syringe and it was incubated in a shaking water bath at 39°C for 24 h. At the end of the incubation period the methane was estimated in Gas Chromatography and *in vitro* true dry matter digestibility (*IVTDMD*) was determined (Van Soest and Robertson 1988).

Estimation of methane emission by in vitro*:* Methane concentration was estimated using Gas Chromatography (Perkin Elmer, Claurus 500 model) fitted with Flame Ionization Detector (FID) and capillary column (30 m length and 250 micrometer diameter). Helium was used as carrier gas with oven temperature at 60°C, injector temperature at 100°C and detector temperature at 110°C (Sitaula *et al.* 1992). Methane concentration in samples (%) was calculated using the following formula:

$$\text{Methane concentration (\%)} = \frac{\text{Peak area of sample gas}}{\text{Peak area of standard gas}} \times \text{Methane concentration in standard}$$

In vivo *feeding trial:* The *in vitro* finding of the effect of supplemental malic acid on methane reduction was validated by *in vivo* in paddy straw based complete ration for indigenous dairy cattle. The methane emission was estimated by using sulfur hexa fluoride (SF_6) tracer gas technique (Johnson and Johnson 1995). Ten indigenous dairy cattle (Gir) with uniform body weight and milk production in mid phase were selected and divided into two groups with five animals each. The animals were fed with and without supplementation of malic acid at 0.39% in 60% paddy straw and 40% Concentrate mixture based complete diet (Table 1). The feeding trial was conducted for a period of 30 days and the collection period was seven days. The dairy cattle were fed with measured quantity of paddy straw and concentrate mixture separately and left over were recorded to study the dry matter intake (DMI) per day per animal. They were allowed to drink free access of water and reared under standard manage mental practices.

Table 1. Quantity of paddy straw and concentrate mixture offered to the indigenous dairy cattle per animal per day

Feed ingredient	Without malic acid (Control)	With malic acid (Treatment)
Paddy straw (60 %)	4.2 kg	4.2 kg
Concentrate mixture (40 %)	2.8 kg	2.8 kg
Total	7.0 kg	7.0 kg
Malic acid	0	27.30 g (0.39 % of complete feed)

The dung samples were collected during the last week of trial for digestibility study. The rumen liquid was also collected during last week for estimation of rumen fermentation characteristics like volatile fatty acids (Chase 1990) bacterial count (Gall *et al.* 1949) and protozoal count (Moir 1951).

Estimation of methane emission by sulfur hexafluoride (SF_6) tracer gas technique: Methane (CH_4) emission in indigenous dairy cattle fed with and without supplementation of malic acid was estimated using sulfur hexafluoride (SF_6) tracer gas technique (Johnson and Johnson 1995). In this technique, the brass permeation tubes (12.5 mm × 40 mm) containing SF_6 tracer gas were pre-determined for the release rates and they were placed in the rumen by one per animal for fourteen days prior to the start of the experiment to allow the tracer gas to reach steady state in the rumen. The release rate of SF_6 from each permeation tube was determined before their placement in animal by measuring weight loss from the tubes during six weeks of incubation at 39°C. Stainless steel frit and Teflon frit were fixed to facilitate the release of SF_6 from brass permeation tube and kept at 39°C. The predetermined SF_6 release rate of the permeation tubes was ranged from 3 to 4 mg per day. Gases expired by animals were sampled using a gas collection PVC canister fitted around the neck and attached to a halter (Chaves *et al*. 2006). The canister consisted of a two liters volume PVC yoke evacuated between –0.700 bar and –0.900 bar pressure. The PVC canister was placed around the neck of each dairy cattle just behind the ears and attached to a halter fitted with an air-tight connection to a 90 cm length of restriction tubing (1.5875 mm internal diameter) with an in-line 15 mm filter and flexible nose piece. The animals were adopted for fixing the PVC canister with halter for 3 days before the commencement of gas collection. Samples of expired air accumulated in evacuated collection canisters for 24 h period in consecutive five days were collected and evaluated for methane and SF_6 gas emission.

Also, a canister was hung in animal shed to record the background concentration of CH_4 and SF_6 gases and they were subtracted from the CH_4 and SF_6 gases collected from the indigenous dairy cattle. The concentration of CH_4 and SF_6 gases in collected gas samples were determined using the gas chromatography fitted with Flame Ionization Detector (FID) and Electron Capture Detector (ECD) for methane and SF_6 respectively. The methane emission was determined from CH_4 to SF_6 ratio using the release rate of SF_6 as given in the following formula (Williams *et al*. 2011).

$$RCH_4 \text{ (g/animal/ day)} = RSF_6 \times \frac{\{(CH_4 \text{ canister}) - (CH_4 \text{ background})\}}{\{(SF_6 \text{ canister}) - (SF6 \text{ background})\}} \times (MWCH_4/ MWSF_6) \times 1000$$

Where, the values in square brackets are concentrations and R represents rates of CH_4 emission and SF_6 release. Methane emissions have been expressed as absolute values for individual cows (CH_4 g/animal/ day). MW represents molecular weight.

Statistical analysis: All data collected were statistically analyzed as per the Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

Effect of supplemental malic acid (MA) on reduction of methane emission by IVGPT : The effect of supplemental malic on methane (ml), *IVTDMD* and methane (ml) per 100 mg of truly digested substrate were presented in Table 2. The methane emission was significantly ($p<0.01$) decreased in all the malic acid added groups than control. The lowest concentration of malic acid that resulted highly significant ($p<0.01$) reduction of methane by 15.95%, which was observed in 0.39% malic acid added group than control. Similarly, there was a highly significant ($p<0.01$) reduction of methane (ml) per 100 mg of truly digested substrate in treatment 4 (15.69%) when compared to control. The lowest concentration of malic acid that induced highly significant ($p<0.01$) reduction in methane (ml) per 100 mg of truly digested substrate was 0.39% and it was identified level for *in vivo* study.

Table 2. Effect of supplemental malic acid on methane (ml), *IVTDMD (*%), methane (ml) per 100 mg of truly digested substrate by *IVGPT* (Mean# ± S.E)

Treatment*	Methane (ml)	*IVTDMD*% NS	Methane (ml) per 100 mg of truly digested substrate
T_1	3.01 ± 0.06 c	46.28 ± 0.89	3.25 ± 0.08 c
T_2	2.83 ± 0.07 b	46.11 ± 1.78	3.08 ± 0.16 b
T_3	2.71 ± 0.08 b	46.23 ± 0.84	2.93 ± 0.04 b
T_4	2.53 ± 0.18 a	46.22 ± 2.36	2.74 ± 0.06 a
T_5	2.51 ± 0.05 a	46.20 ± 1.84	2.73 ± 0.07 a

#Mean of six observations, NS, Not significant; Means bearing different superscripts in the same column differ significantly ($p<0.01$); *T_1-0 % MA, T_2-0.13 % MA, T_3-0.26 % MA, T_4-0.39 % MA and T_5-0.52 % MA.

Similar finding was also reported earlier by Asanuma *et al*. (1999) who reported that the malic acid supplementation decreased the methane production by *in vitro*. Jalc *et al.* (2002) also reported that the addition of malic acid decreased the methane emission by 1.9% than control by *in vitro*. Further Li *et al*. (2009) observed that the supplementation of 24 mM malate and 24 mM fumarate with linolenic acid was decreased ($p<0.0020$) the methane (µmol) emission by 71.53% and 84.54% than control.

In accordance to the present study the methane: dry matter disappearance decreased ($p=0.001$) by the supplementation of disodium malate at 15.1 g/kg DM in grass hay and concentrate (60:40) and 4.87 g/kg DM in barley straw and concentrate (10:90) based diet, respectively (Gomez *et al*. 2005) by *in vitro*.

As methanogenesis is principally a sink for metabolic hydrogen in the rumen and diverting hydrogen away from methane formation would decrease the methane production. Malic acid is a potential precursor for propionate and may act as electron sink competing with methanogen with available hydrogen ultimately reduce in the methane production. (Lopez *et al*. 1999). The decrease in methane emission was due to the reduction of organic acids by the ruminal bacterial species that utilize hydrogen to form succinate then propionate (Asanuma *et al*. 1999) could possibly decrease the availability of hydrogen for methane production. This was the reason for decreased methane production in the present study.

The *IVTDMD* was not differed among all the treatment groups. Similar to the present study the supplementation of disodium malate at 0, 4, 8 mmol/litre malate in three substrate differing forage: concentrate ratio with low, medium and high concentrate based diet did not influence the organic matter effective degradability (OMED) by *in vitro* (Tejido *et al*., 2005). Similarly, Sniffen *et al*. (2006) observed that the effects malic acid at 0, 50, 100 g per animal per day supplementation in continuous culture *in vitro* rumen fermentation study did not alter the dry matter digestibility. The addition of malic acid at 0.39 % of substrate did not influence the rumen fermentation and hence the unaltered *IVTDMD* was observed in the present study.

Effect of supplemental malic acid on reduction of methane emission in paddy straw based complete diet for indigenous dairy cattle: The effect of supplemental malic acid with and without supplementation on body weight (BW), DMI, DDMI, methane emission (g) per animal per day, g per kg DMI and g per kg DDMI are presented in Table 3.

Table 3. Effect of malic acid with and without supplementation on BW, DMI, DDMI, methane emission gram per animal per day, per kg DMI, per kg DDMI in paddy straw based complete diet for indigenous dairy cattle (Mean# ± SE)

Parameter NS	Without malic acid (Control)	With Malic acid (Treatment)
BW (kg) NS	263.43 ± 4.83	260.81 ± 7.02
DMI (kg/day) NS	6.69 ± 0.01	6.68 ± 0.01
DMI (g/kg BW) NS	25.41 ± 0.47	25.66 ± 0.68
DDMI (g/day) NS	3771.87 ± 27.97	3739.12 ± 16.36
CH_4 g/animal/day	94.77 ± 0.77 a	91.68 ± 0.37 b
CH_4 g/kg DMI	14.17 ± 0.12 a	13.73 ± 0.08 b
CH_4 g/kg DDMI NS	25.13 ± 0.39	24.52 ± 0.15

#, Mean of five observations; NS, Not significant; Means bearing different superscripts in the same row differ significantly ($p<0.05$).

There was no significant difference in BW, DMI and DDMI with and without supplementation of malic acid in paddy straw based complete diet for indigenous dairy cattle. Similar to the present study, Carro *et al*. (2005) also observed that the supplementation of malate at 0, 4, 8 g/kg DM in growing lambs for a period of 35 days did not influence the apparent digestibility of organic matter. Further an earlier studies also reported that the supplementation of malic acid did not alter the DMI in

dairy cattle (Sniffen *et al*. 2006, Foley *et al*. 2009a)

The methane emission per animal per day was 94.77 g and 91.68 g in without and with supplementation of malic acid respectively in paddy straw based complete diet. There was a significant ($p<0.05$) decrease of methane emission by 3.26% in malic acid supplemented group than control group. Foley *et al*. (2009b) also reported that the supplementation of DL malic acid at 3.75% and 7.5% decreased ($p<0.001$) the methane emission by 6.30% and 15.77% respectively than control in beef cattle.

The methane emission per kg DMI or methane emission per 100 g DMI was also significantly decreased by 3.11% in supplemental malic acid added group than without added group in indigenous dairy cattle. Similar to the present study, Foley *et al*. (2009b) also observed a decrease ($p<0.001$) in methane (g) per kg total dry matter intake than control in beef cattle.

The methane per kg or per 100 g DDMI was numerically decreased by 2.43% in malic acid supplemented group than un-supplemented group, but it was not significant. The present study was also simulated by Foley *et al*. (2009b) who observed a non-significant influence on methane emission on dry matter digestibility while supplementation of DL malic acid from 3.75% to 7.5% in beef cattle. Also, in earlier findings supplementation of encapsulated fumaric acid to wethers had deceased the CH_4 emissions by up to 75% per kg of DMI (Wallace *et al*. 2006, Molano *et al*. 2008).

Further it was observed that the malic acid supplementation on methane reduction was more evident by *in vitro* than *in vivo*. This highlights the lack of consistency between *in vitro* and *in vivo* studies. As the dietary inclusion of malic acid reduce the acetate: propionate ratio (Moss *et al*. 2000), and this in turn has been associated with lower CH_4 emissions, it is possible that malic acid inclusion in high-concentrate rations may further augment the reduction in ruminal acetate: propionate ratio. This may provide some explanation toward the apparent improved inhibiting effects of dietary organic acid on methanogenesis under high concentrate regimens. Carro *et al.* (2006) suggested that inconsistent responses between *in vivo* and *in vitro* studies could be related to the different experimental conditions found between the two systems. Differences in dose or supplementation rate are possibly other factors leading to variation in the ruminal fermentation response to organic acids. Carro *et al*. (2006) suggested that it is possible that greater dietary inclusion rates of malate would be necessary to detect significant effects on *in vivo* volatile fatty acid production.

The effect of supplemental malic acid with and without supplementation on rumen fermentation characteristics (volatile fatty acids and microbial population), milk production and methane emission per kg milk production are presented in Table 4.

The total volatile fatty acid was significantly ($p<0.05$) increased by 2.69% in malic acid supplemented group than without supplemented group. Li *et al*. (2009) also reported that the supplementation of 24 mM malate with linolenic acid increased the volatile fatty acids in fermented solution. The highly significant ($p<0.05$) increase in propionic acid by 11.71% in malic acid added group when compared to control. The acetic acid was significantly ($p<0.05$) decreased by 4.04% in malic acid added group than without added group. The A/P ratio was also significantly ($p<0.05$) decreased by 14.86% in malic acid added group when compared to control.

Table 4. Effect of malic acid with and without supplementation on rumen fermentation characteristics (Mean ± SE)

Parameter	Without malic acid (Control)	With malic acid (Treatment)
TVFA (mg/dl)*	60.75±0.28 [a]	62.43±0.48 [b]
Acetic acid (%)*	68.58±0.37 [a]	65.81±0.97 [b]
Propionic acid (%) **	23.15±0.24 [a]	26.22±0.63 [b]
Butyric acid (%)[NS]	8.27±0.41	7.97±0.41
A/P ratio**	2.96±0.04 [a]	2.52±0.10 [b]
Bacterial population ($\times 10^8$) [NS]	4.59±0.03	4.54±0.10
Protozoal Population ($\times 10^5$) [NS]	3.58±0.05	3.69±0.02
Milk production per day [NS]	4.68±0.03	4.79±0.06
CH_4 g/kg milk production*	20.24±0.24 [a]	19.14±0.29 [b]

Note: [#]Mean of five observations; [NS], Not significant; Means bearing different superscripts in the same row differ significantly *($p<0.05$), **($p<0.01$).

Foley *et al*. (2009b) also reported that supplementation of DL malic acid at 0, 2.5, 5.0 and 7.5 % increased ($p<0.001$) the propionic acid and decreased ($P<0.001$) the acetic acid and acetate to propionate ratio than control in beef cattle. The supplementation of sodium salts of malate and fumarate at 0, 8, 16 and 24 mM in 70% concentrate: 30% ground alfalfa roughage based diet increased the propionic acid and decreased the acetic acid & acetate to propionate ratio significantly ($p<0.0001$) in both malate and fumarate added groups than control (Li *et al*. 2009).

The addition of malic acid reduced the concentration of acetic acid, with increased concentration of propionic acid, led to an overall decrease in A:P ratio. Beauchemin and McGinn (2006) found that the ruminal fermentation characteristics being consistent with a reduction in CH_4 production with increase in molar proportions of propionate and decrease in A:P ratio. Also, Lila *et al.* (2004) reported a reduction in acetate and an increase in propionate, which led to a substantial decrease in CH_4 emission when β-cyclodextrin diallyl maleate was supplemented to cattle. The change in A:P ratio here because of increasing malic acid supplementation was not as extensive as reported previously (Lila *et al*. 2004, Beauchemin and McGinn 2006), but it may have assisted in the reduction of CH_4, with less H_2 available for methanogenesis.

There was no significant difference in bacterial and

protozoal population among the treatment groups. On contrary to the present findings the earlier report was dealt with increased bacterial count (Khampa *et al*. 2009) and decreased protozoal count (Foley *et al*. 2009b) while supplementation of malic acid. The unaltered microbial population in the present study could be due to the minimal inclusion of malic acid when compared to the earlier study.

The total milk production was numerically increased by 2.29% in malic acid supplemented group than control group, but it was not significant. However the methane emission per kg milk production was significantly ($p<0.05$) decreased by 5.43% in malic acid supplemented group than control. Similarly, Kung *et al*. (1982) who observed that the malic acid supplementation in dairy cows fed with corn and corn silage based diet had recorded a higher feed conversion efficiency to milk in animals. Similarly, Sniffen *et al*. (2006) reported that the dairy cattle fed 50 g of supplemental MA per day increased the milk yield with minimal effect on milk composition.

It was concluded that the supplementation of malic acid at 0.39% of paddy straw based complete diet significantly ($p<0.05$) reduced the methane emission per animal per day by 3.26% and methane per kg milk production by 5.43% than control in indigenous dairy cattle. The decrease in methane emission in the present study might be due to the supplementation of malic acid which was more effective alternate hydrogen sink competing with methanogenesis (Newbold *et al*. 2002). Also, the malic acid was the direct metabolic precursor of propionic acid and it has the potential to decrease the methane emission by directing hydrogen into succinate rather than in to methane production (Lopez *et al*. 1999). Further, the energy saved through decrease in methane emission was used for increasing the milk production and it may also decrease the negative effect of climate change.

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