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# Effects of fumaric or malic acid and 9, 10 anthraquinone on digestibility, microbial protein synthesis, methane emission and performance of growing calves

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ABSTRACT

This study was aimed to test whether combination of methanogens inhibitor and a hydrogen sink may result in any complementary effects *in vivo*. Growing calves (15; average 7- month-old, and weighing 130 kg) were arranged into 3 groups in a randomized complete block design. Treatments were: Control (no additives), FA and AQ (control + 6.5 mM of FA and AQ at the level of 4 ppm) and MA and AQ (control + 6.5 mM of MA and 4 ppm of AQ). AQ in combination with FA or MA had no effect on final BW, ADG and daily feed intake. There was no effect of supplementation of additives on intestinal flow of microbial-N, apparent digestibility of DM, OM, CF, EE, NDF, and ADF but CP digestibility increased as a result of feeding AQ and organic acids. Nitrogen intake was similar in three groups but supplementation of diet with AQ and FA or MA decreased nitrogen losses through feces and urinary losses of nitrogen also slightly declined resulting in a nonsignificant improvement of nitrogen retention in treated groups than control. Feeding AQ and OAs resulted in a significant reduction of 9.5% methane per unit DMI in animals fed AQ and MA. It can be concluded that reduction of methane production caused by feed additives in the present study was not notable and could not improve animal performance and nutrients utilization which challenges previous *in vitro* findings observed in the use of organic acids and methane inhibitors in combination.

**Key words**: Anthraquinone, Cattle, Digestibility, Methane, Organic acid

Since reducing equivalents generated during ruminal fermentation are disposed of mainly by the formation of the $CH_4$, inhibition of methanogens led to accumulation of hydrogen in the ruminal environment (Kung *et al.* 2003). One method of utilizing the excess hydrogen would be to couple feed additives that inhibit methanogens directly and hydrogen sinks that use hydrogen to produce purposeful fermentation products (Ebrahimi *et al.* 2011, Mohammed *et al.* 2004, Lin *et al*. 2013). The objective of the present study was to investigate effects of feeding anthraquinone (AQ) in combination with fumaric acid (FA) and malic acid (MA) on growth performance, methane emission and nutrient digestibility and utilization in Sahiwal calves.

## MATERIALS AND METHODS

*Animals, treatments and experimental procedures:* The experimental protocol was approved by Institute Ethics committee of Animal Experiments, National Dairy Research Institute, Karnal, India. Sahiwal male calves (15; 6–8 month-old; average body weight, 102 kg; range 69–153 kg), were selected from the Cattle Yard of National Dairy Research Institute, Karnal, and allotted to 3 treatments in a randomized complete block design (RCBD). The treatments were: control (no additives), FA and AQ (control + 6.5 mM of FA and AQ at the level of 4 ppm) and MA and AQ (control + 6.5 mM of MA and 4 ppm of AQ). The calves were housed in a well ventilated stall having facilities for individual feeding and watering. Animals were dewormed using Albendazole @ 10 mg/ kg body weight before starting the experimental feeding. During the experiment (120 d), calves were fed with a basal diet (Table 1) containing wheat straw, mature sorghum (fresh-cut) and concentrate mixture (40: 10: 50) *ad lib.* (15% in excess of the previous day's intake) once daily at 09: 00, and had access to freshwater all time. Additives were pre-mixed with concentrate at the time of feeding. The concentrations of organic acids and AQ were selected based on the results of an *in vitro* experiment (Ebrahimi *et al*. 2011). Before starting the growth trial, the actual feed intake for each group of animals was measured and total rumen fluid volume was calculated (Owens and Goetsch 1986). Organic acids and AQ doses were adjusted at the end of every fortnight according to new average BW and DMI for each treatment group.

*Body weight and dry matter intake*: The animals were weighed before feeding and watering in the morning on 2

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Table 1. Chemical analysis of diet ingredients (% of DM)

| Item | Wheat straw | Sorghum fodder | Concentrate mix.[1] |
|---|---|---|---|
| OM | 93.35 | 91.17 | 92.33 |
| CP | 3.33 | 7.88 | 22.93 |
| EE | 0.84 | 2.65 | 2.57 |
| CF | 44.60 | 34.51 | 9.50 |
| NDF | 84.68 | 74.42 | 51.68 |
| ADF | 57.18 | 46.14 | 14.52 |

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; CF, crude fibre; NDF, neutral detergent fibre; ADF, acid detergent fibre. [1]Concentrate contained: rice bran de-oiled, mustard cake, maize, wheat bran, ground nut cake, common salt and mineral mixture (11, 12, 33, 20, 21, 1 and 2% of DM, respectively).

consecutive days at the start of experimental feeding and thereafter at fortnightly intervals during the experimental period of 120 days. Wheat straw, sorghum fodder and concentrates were sampled weekly to determine their DM content, and diets were adjusted weekly to account for changes in DM content. DM intake was recorded twice a week by subtracting the residual DM from the quantity of DM offered.

*Growth performance*: Growth rate was calculated on the basis of increase in body weight at fortnightly intervals and feed conversion efficiency was expressed as the ratio of feed intake on gain (kg/kg).

*Metabolism trial:* On day 60, a metabolism trial was conducted with 7 days collection period to determine nutrient digestibility and nitrogen balance. Animals were housed in metabolism shed (equipped for quantitative collection of faeces and urine separately) 1 week prior to the metabolism trial for their adaptation to the surroundings. Animals were weighed before and after trial consecutively for 2 days. Allotted rations were fed to each animal as explained earlier. Feeds and their respective residues were collected in separate polythene bags daily for DM estimation. These samples were pooled at the end of the collection period and ground to pass through 1 mm screen and preserved in air tight polythene bags until analyzed for proximate principles.

Faeces voided during 24 h, was collected and weighed at 9.30 AM daily. After thorough mixing, an aliquot (1%) in duplicate dried at 80°C in oven for DM estimation and pooled for 7 days. Another aliquot (1/500) was mixed thoroughly with 5 ml of 25% $H_2SO_4$ and stored in pre-weighed air tight plastic container. At the end of collection period, the plastic container was weighed and contents were emptied in a tray and mixed thoroughly and an aliquot (10 g) was analyzed for total N. Dried dung samples were ground to pass through 1 mm sieve size and analyzed for proximate principles and cell wall constituents (NDF and ADF) as per standard procedure described below.

Total urine output was collected from each calf. Calves were fitted with urine collection bag, attached to the big plastic container through a plastic tube. Each container was added 100 ml of 10% $H_2SO_4$ daily to prevent bacterial destruction of purine derivatives in the collected urine. Total urine output for 24 h was measured daily at 9.30 AM and an aliquot (1/500 of total output) was taken for the nitrogen estimation. This aliquot was stored in plastic container containing 2 ml of 25% $H_2SO_4$. For urinary purine derivatives estimation, 1% of urine excreted/ day by each animal was taken and diluted five times with water and 20 ml of diluted urine was stored in the plastic container at –20°C daily throughout the collection period.

*Enteric methane emission*: Enteric $CH_4$ emissions were measured for 5 days starting on day 80 of the trial, using the $SF_6$ tracer gas technique described by Johnson *et al.* (1994). A permeation tube containing $SF_6$, an inert gas tracer, was placed into the rumen of each animal approximately 2 week before $CH_4$ measurements commenced. The permeation tubes were manufactured at Environmental lab, National Dairy Research Institute, India and were filled with excess of 1 g of $SF_6$ per bolus. The average release rate was 2.2±0.09 mg/d, which was predetermined over the preceding 8 week period by weighing each permeation tube at the same time point once weekly. After collection of a sample, the canisters were pressurized with nitrogen, and the concentration of $SF_6$ in the canisters was analyzed by gas chromatography, fitted with an electron capture detector (250°C) and 3.3 m molecular sieve column with an i.d. of 0.32 mm to determine $SF_6$ concentraions. Another gas chromatograph was fitted with a flame-ionization detector (100°C) and stainless steel column packed with Porapak-Q (length 1.5m; o.d. 3.2 mm; i.d. 2 mm; mesh range 80–100) to determine $CH_4$ concentration. The column and injector temperatures were 50 and 40°C in both gas chromatographs. All samples were analyzed in duplicate except standards, which were analyzed in triplicate. Nitrogen was used as the carrier gas at a pressure of 1kg/cm$^2$. The standards were run at the beginning and end of each day with the medium standard run every 10 samples throughout the day. Gas concentrations ($SF_6$ and $CH_4$) were determined from peak areas and identified from their different retention times relative to the known standards. The methane output calculated using following formula:

$$CH_4(g/d) = \left(\frac{S_{CH4} - B_{CH4}}{S_{SF6} - B_{SF6}}\right) \times \left(\frac{M_{CH4}}{M_{SF6}}\right) \times Q_{SF6} \times 1000$$

where, $S_{CH4}$ and $B_{CH4}$ are methane concentrations in sample and background's canisters (ppm), $S_{SF6}$ and $B_{SF6}$ represent the concentrations of $SF_6$ in sample and background's canisters (ppt), $M_{CH4}$ and $M_{SF6}$ are molecular weight of methane and $SF_6$ (g), respectively and $Q_{SF6}$ represents release rate of $SF_6$ (mg/d).

*Laboratory analysis:* The analytical DM content of samples was determined by oven drying at 105ºC for 24 h. Representative samples dietary ingredients were dried by oven at 65ºC for 72 h ground to pass a 1 mm screen and stored for subsequent analyses. Organic matter (OM) was

determined by ashing at 550ºC for 5 h, Neutral detergent fibre (NDF) and acid detergent fibre (ADF), both expressed inclusive of residual ash, were determined by the method of Van Soest *et al* (1991) and AOAC method (973.18, A-D, 2006), respectively. Crude protein (CP) content (N×6.25) was determined using KEL PLUS - N analyzer, which is based on the method described by AOAC (method 984.13 A-D, 2006). Ether extract (EE) was estimated using AOAC (method 920.39 A, 2006). Allantoin and uric acid in urine were determined by an ultravioletvisible spectrophotometer UV-2100 and intestinal flow of microbial-N was calculated using the procedure of IAEA (1997).

*Statistical analysis:* Data were analyzed as a randomized complete block design (RCBD). When a significant F value ($P < 0.05$) was detected, means were compared by the Duncan test. Statistical analyses were performed using the GLM procedures of SAS V8.

## RESULTS AND DISCUSSION

Chemical composition of diet ingredients are presented in Table 1. The feeding of additives on weekly DM intakes during 120 days growth trial which indicated no effect of treatments on DM intakes throughout the trial (Table 2). Growth rate and performance of calves are given in Tables 3 and 4. Animals fed AQ and FA or MA had significantly similar BW all over growth trial (Table 3), final BW, ADG and feed conversion efficiency (Table 4).

Table 2. Effect of feeding organic acids and anthraquinone on DMI (kg/day) of Sahiwal males during 120 days growth trial

| Days | Control | FA and AQ[1] | MA and AQ | SEM[2] | *P*-Value[3] |
|---|---|---|---|---|---|
| 1—7 | 2.37 | 2.46 | 2.43 | 0.15 | 0.92 |
| 8—14 | 2.36 | 2.55 | 2.59 | 0.15 | 0.54 |
| 15—19 | 2.69 | 2.91 | 2.94 | 0.13 | 0.34 |
| 20—27 | 3.09 | 3.17 | 3.13 | 0.13 | 0.90 |
| 28—34 | 3.03 | 3.11 | 2.99 | 0.17 | 0.87 |
| 35—41 | 3.14 | 3.17 | 3.04 | 0.13 | 0.76 |
| 42—48 | 2.88 | 2.95 | 3.06 | 0.15 | 0.70 |
| 49—54 | 3.48 | 3.49 | 3.59 | 0.12 | 0.78 |
| 55—60 | 3.43 | 3.62 | 3.36 | 0.17 | 0.54 |
| 61—70 | 3.70 | 3.72 | 3.48 | 0.15 | 0.49 |
| 71—77 | 3.60 | 3.85 | 3.37 | 0.16 | 0.17 |
| 78—85 | 3.84 | 3.91 | 3.46 | 0.22 | 0.34 |
| 86—94 | 4.49 | 4.52 | 3.98 | 0.21 | 0.19 |
| 95—101 | 4.46 | 4.61 | 4.12 | 0.15 | 0.13 |
| 102–108 | 4.70 | 4.59 | 4.59 | 0.19 | 0.88 |
| 109—120 | 4.67 | 4.71 | 4.24 | 0.27 | 0.45 |
| Avgerage (121days) | 3.58 | 3.66 | 3.45 | 0.14 | 0.58 |

Control (no additives), FA and AQ (control + 6.5 mM of fumaric acid and anthraquinone at the level of 4 ppm) and MA and AQ (control + 6.5 mM of malic acid and 4 ppm of anthraquinone). DMI, dry matter intake;[1]Doses are expressed in mM and ppm of total rumen fluid volume (Owens and Goetsch, 1986); [2]SEM: Standard error of means;[3] Probability of significance effects because of treatment.

Table 3. Effect of feeding organic acids and anthraquinone on change of BW (kg) during 121 days growth trial

| Days | Control | FA and AQ[1] | MA and AQ | SEM[2] | *P*-Value[3] |
|---|---|---|---|---|---|
| 1 | 102.24 | 102.23 | 102.79 | 4.72 | 0.99 |
| 15 | 110.63 | 109.67 | 109.77 | 5.38 | 0.99 |
| 30 | 116.24 | 117.66 | 115.8 | 5.27 | 0.97 |
| 44 | 124.65 | 124.82 | 124.27 | 5.65 | 0.99 |
| 63 | 130.97 | 128.75 | 130.17 | 5.96 | 0.97 |
| 72 | 141.08 | 139.61 | 137.67 | 5.88 | 0.92 |
| 86 | 147.72 | 146.51 | 145.46 | 6.31 | 0.97 |
| 95 | 156.9 | 154.45 | 151.96 | 6.54 | 0.87 |
| 109 | 163.11 | 164.34 | 161.33 | 6.24 | 0.94 |
| 121 | 171.85 | 172.76 | 165.93 | 7.00 | 0.76 |

Control (no additives), FA and AQ (control + 6.5 mM of fumaric acid and anthraquinone at the level of 4 ppm) and MA and AQ (control + 6.5 mM of malic acid and 4 ppm of anthraquinone).[1]Doses are expressed in mM and ppm of total rumen fluid volume (Owens and Goetsch 1986). [2]SEM, standard error of means; [3]probability of significance effects because of treatment.

Table 4. Effect of supplementation of organic acids and anthraquinone on growth performance of growing Sahiwal calves

| | Control | FA and AQ[1] | MA and AQ | SEM[2] | *P*-Value[3] |
|---|---|---|---|---|---|
| Initial BW (kg) | 102.24 | 102.23 | 102.79 | 4.72 | 0.99 |
| Final BW (kg) | 171.85 | 172.76 | 165.93 | 7.00 | 0.76 |
| ADG (kg) | 0.58 | 0.58 | 0.52 | 0.03 | 0.30 |
| Daily feed intake (kg) | 3.58 | 3.66 | 3.45 | 0.14 | 0.58 |
| Feed: gain (kg/kg) | 6.17 | 6.31 | 6.63 | 0.49 | 0.38 |

Control (no additives), FA and AQ (control + 6.5 mM of fumaric acid and anthraquinone at the level of 4 ppm) and MA and AQ (control + 6.5 mM of malic acid and 4 ppm of anthraquinone). BW, body weight; ADG, average daily gain.[1]Doses are expressed in mM and ppm of total rumen fluid volume (Owens and Goetsch, 1986). [2]SEM, Standard error of means;[3] Probability of significance effects because of treatment.

Results of metabolism trial are presented in Tables 5 and 6. There was no effect of supplementation of additives on apparent digestibility coefficient of DM, OM, CF, EE, NDF, and ADF but CP digestibility increased (P=0.04) because of feeding AQ and organic acids. Nitrogen intake was same in 3 groups but supplementation of diet with AQ and FA or MA decreased nitrogen loss through faeces (P=0.03) and urinary losses of nitrogen also slightly declined resulting a nonsignificant improvement of nitrogen retention in treated groups than control. Urinary excretions of allantoin and uric acid were not affected by treatments and there was no significant difference between control and groups fed AQ and organic acid on their intestinal flow of microbial-N (Table 5).

During methane estimation trial, DMI was also measured simultaneously and results have been summarized in

Table 5. Final BW (kg), Intake (kg/day) and apparent digestibility of nutrients (%), digestible nutrient intake (kg/day), nutritive value (%), urinary excretion of purine derivatives (mM/d) and intestinal flow of microbial-N (g/d) of Sahiwal males fed with organic acids and anthraquinone

| Items | Control | FA and AQ[1] | MA and AQ | SEM[2] | *P*-Value[3] |
|---|---|---|---|---|---|
| BW | 128.67 | 126.42 | 128.08 | 5.89 | 0.96 |
| DMI | 3.58 | 3.60 | 3.5 | 0.16 | 0.87 |
| Digestibility [4] | | | | | |
| DM | 57.21 | 59.75 | 60.55 | 1.61 | 0.36 |
| OM | 59.92 | 62.36 | 63.07 | 1.44 | 0.32 |
| CP | 65.25[a] | 66.92[b] | 66.09[b] | 1.14 | 0.04 |
| CF | 49.57 | 51.81 | 53.97 | 1.97 | 0.34 |
| EE | 72.72 | 68.69 | 69.11 | 1.83 | 0.29 |
| NFE | 63.80 | 65.88 | 66.24 | 1.54 | 0.51 |
| NDF | 52.97 | 55.16 | 55.70 | 1.8 | 0.55 |
| ADF | 41.47 | 43.55 | 46.19 | 2.28 | 0.39 |
| Digestible nutrient intake | | | | | |
| DOMI | 1.98 | 2.10 | 2.04 | 0.14 | 0.85 |
| DCPI | 0.33 | 0.36 | 0.35 | 0.02 | 0.45 |
| TDNI | 2.04 | 2.15 | 2.09 | 0.14 | 0.88 |
| Nutritive value | | | | | |
| DCP | 8.71[a] | 9.37[b] | 9.25[b] | 0.16 | 0.04 |
| TDN[5] | 56.88 | 58.97 | 59.63 | 1.41 | 0.40 |
| Urinary excretion | | | | | |
| Allantoin | 38.15 | 38.89 | 39.02 | 0.98 | 0.24 |
| Uric acid | 9.78 | 9.35 | 9.49 | 0.28 | 0.11 |
| Total PD | 47.93 | 48.24 | 48.51 | 1.01 | 0.23 |
| Intestinal flow | | | | | |
| Microbial N | 28.41 | 28.84 | 28.95 | 0.23 | 0.76 |

Control (no additives), FA and AQ (control + 6.5 mM of fumaric acid and anthraquinone at the level of 4 ppm) and MA and AQ (control + 6.5 mM of malic acid and 4 ppm of anthraquinone).

BW, body weight; DMI, dry matter intake; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NFE, nitrogen free extract; CF, crude fibre; NDF, neutral detergent fibre; ADF, acid detergent fibre; DOMI, digestible organic matter intake; DCPI, digestible crude protein intake; TDNI, total digestible nutrients intake; PD = purine derivatives; Microbial N, microbial nitrogen.[1]Doses are expressed in mM and ppm of total rumen fluid volume (Owens and Goetsch, 1986);[2]SEM: Standard error of means; [3]Probability of significance effects because of treatment; [4]Apparent digestibility: [(kg nutrient eaten - kg nutrient excreted)/ kg nutrient eaten)] × 100; [5]Total digestible nutrients = DCP + DNFE + DCF + 2.25 (DEE) and NFE = 100 - (%Moisture +%Protein +%Fiber +%Ash +%Fat).

Table 7. Similar to overall period of growth trial, DMI in group fed AQ and FA was higher and in group fed AQ and MA was lesser than control but the differences were not significant. The absolute methane production (g/d) in animals fed AQ and MA was significantly (P<0.001) lower than control and group treated with AQ and FA. Feeding AQ and FA or MA caused a reduction of 5.43 and 9.52% in methane production per unit DMI respectively, however, the difference between control and animals fed with AQ and FA was not significant.

Table 6. Effect of feeding organic acids and anthraquinone on nitrogen intake, losses and retention (g/day) of growing Sahiwal males

| Items | Control | FA and AQ[1] | MA and AQ | SEM[2] | *P*-Value[3] |
|---|---|---|---|---|---|
| Intake | 85.58 | 86.24 | 84.79 | 2.76 | 0.93 |
| Losses through: | | | | | |
| *Faces* | 32.30[a] | 27.94[b] | 28.81[b] | 0.94 | 0.03 |
| *Urine* | 28.14 | 27.99 | 26.18 | 1.85 | 0.68 |
| Retention | 24.88 | 30.27 | 29.81 | 1.86 | 0.14 |

Control (no additives), FA and AQ (control + 6.5 mM of fumaric acid and anthraquinone at the level of 4 ppm) and MA and AQ (control + 6.5 mM of malic acid and 4 ppm of anthraquinone).[1]Doses are expressed in mM and ppm of total rumen fluid volume (Owens and Goetsch 1986); [2]SEM: Standard error of means; [3]Probability of significance effects because of treatment.

Complementary effects of methanogens inhibitors and propionate precursors on methanogenesis reduction and utilization of the accumulated hydrogen (Mohammed *et al*. 2004, Ebrahimi *et al*. 2011) showed that under *in vitro* condition: (i) the doses of additives were adequate to affect mixed ruminal bacteria and (ii) a kind of synchronization occurred between two process of methane inhibition and use of the excess of hydrogen. Our results showed a reduction of 5.43% by AQ and FA versus 9.52% fall in methane (g/kg DMI) caused by AQ and MA. These reductions were not reflected in growth performance of calves during 4-month trial (Table 4). It might be possible that dose of AQ as primary essential substance was low under *in vivo* which could not inhibit methane production very much.

On the other hand, MA is highly soluble and there was a chance of quick escaping from the rumen as Martin *et al.* (1999) reported that malate disappeared within 30 min of reaching to the rumen *in situ*. Decreasing the release rate of FA by encapsulating with partially hydrogenated vegetable oil also resulted in a huge reduction in methane emission (Wood *et al.* 2009) compared with unprotected form of FA which confirms propionate precursors (malate and fumarate) provided by MA or FA were not probably utilized according to our assumption for efficient hydrogen utilization. The result of *in vivo* experiments with organic acids either in free acid or salt forms are inconclusive, for instance, Molano *et al.* (2008) reported FA supplementation at 10% of DMI had no effect on methane emissions per unit of feed intake in lambs. In some other studies also no significant differences were found in daily methane output of animals fed with organic acids (McGinn *et al.* 2004, Beauchemin and McGinn 2006, Foley *et al.* 2009a,b). However, feeding fumaric acid (at 2% of DMI) caused a significant decrease in daily methane production (Bayaru *et al.* 2001, Mohini *et al.* 2009).

Sawyer *et al*. (1974) found that feeding bromochloromethane resulted in about 85% inhibition of methane production but the energy saved did not transfer for

Table 7. Effect of supplementation of organic acids and anthraquinone on DMI (kg/day) and methane production of Sahiwal males during gas collection period

| Items | Control | FA and AQ[1] | MA and AQ | SEM[2] | *P*-Value[3] |
|---|---|---|---|---|---|
| BW (Kg) | 144.05 | 145.94 | 143.54 | 7.81 | 0.97 |
| DMI | 3.91 | 4.23 | 3.52 | 0.3 | 0.33 |
| Methane (g/d) | 68.48[a] | 72.83[a] | 56.64[b] | 2.61 (Control and FA and AQ)<br>2.55 (Control and MA and AQ)<br>2.64 (MA and AQ and FA and AQ) | <0.0001 |
| Methane (g/KgDMI) | 17.85[a] | 16.88[ab] | 16.15[b] | 0.45 (Control and FA and AQ)<br>0.44 (Control and MA and AQ)<br>0.45 (MA and AQ and FA and AQ) | 0.005 |

Control (no additives), FA and AQ (control + 6.5 mM of fumaric acid and anthraquinone at the level of 4 ppm) and MA and AQ (control + 6.5 mM of malic acid and 4 ppm of anthraquinone).[1]Doses are expressed in mM and ppm of total rumen fluid volume (Owens and Goetsch 1986).

[2]SEM: Standard error of means = $\sqrt{\frac{MSE}{n}}$ , where MSE represents Mean Square Error and n is the number of observations. Despite the number of animals were similar in each treatment groups, during methane collection period we missed some values of the methane and therefore different SEMs for methane values indicate non-equal number of observations in each treatment and calculated as: SEM $= \sqrt{(\frac{1}{n_1} + \frac{1}{n_2}) \times \frac{MSE}{2}}$ where $n_1$ and $n_2$ are the number of observations in two groups which are different in number.

[3] Probability of significance effects because of treatment.

productive purposes. Although hydrogen output was not estimated in their report but, energy losses through hydrogen might be a possible reason why animal performance was not affected. By combination of AQ and FA or MA in the present study it was tried to enhance rumen's capacity for hydrogen utilization in the process rather than methane and finally saving the energy for animals, however, results (Table 4) were not successful and probably energy saved by lower methane emission in the treatment groups was not high enough to be appeared in the performance parameters. Another fact which must be mentioned here is that when energy saved through inhibition of methane production in the ruminants alone or along with organic acids, there must be potential of energy utilization by animal. In another word, probably in our study there was no energy deficiency and extra gain potential for the animals in both groups fed with AQ and FA or MA.

An increase in CP digestibility in groups fed with additives than that of control was accompanied with significantly lower nitrogen losses through faeces, which resulted in nonsignificant improvement in nitrogen retention in animal supplemented with AQ and FA or MA (Tables 5, 6). This effect is probably because of presence of organic acid since Bayaru *et al*. (2001) also observed greater digestibility of CP and nitrogen retention in the steers fed with FA.

When malate salt was fed to animals, there was no effect of treatment on urinary excretion of purine derivatives and microbial N flow at duodenum (Carro *et al*. 2006). In contrast, feeding malic acid increased allantoin in urine and uric acid also was slightly greater than control (Liu *et al.* 2009). It should be noted that in the present study, both FA and MA were used in combination with AQ and their effects on excretion of purine derivatives and microbial N flow at duodenum might be different of them when they used alone.

It can be concluded from the results of the current research that reduction of methane production caused by feed additives in the present study was not notable and could not improve productivity and nutrients utilization for one, two or all of the following reasons: (i) low doses of additives especially methane inhibitor, (ii) disharmony between two process of methane inhibition and hydrogen utilization, (iii) lack of energy deficiency in animals fed with additives and not having extra gain potential. Positive *in vivo* responses of the using present strategy (combination of hydrogen sinks and methane inhibitors) may be achieved by considering above findings and further investigation.

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## REFERENCES

AOAC. 2006. Official methods of analysis. *Association of Official Analytical Chemists.* 17th edn. Arlington, Virginia, 22201, USA.

Bayaru E, Kanda S, Kamada T, Itabashi H, Andoh S, Nishida T, Ishida M, Itoh T, Nagara K and Isobe Y. 2001. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. *Animal Science Journal* **72** (2): 139–46.

Beauchemin K A and McGinn S M. 2006. Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. *Journal of Animal Science* **84**: 1489–96.

Carro M D, Ranilla M J, Giraldez F J and Mantecon A R. 2006. Effects of malate on diet digestibility, microbial protein synthesis, plasma metabolites, and performance of growing lambs fed a high-concentrate diet. *Journal of Animal Science* **84**: 405–10.

Ebrahimi S H, Mohini M, Singhal K K, Heidarian Miri V and Tyagi A K. 2011. Evaluation of complementary effects of 9,10–anthraquinone and fumaric acid on methanogenesis and ruminal fermentation in vitro. *Archives of Animal Nutrition* **65**(4): 267–77.

Foley P A, Kenny D A, Lovett D K, Callan J J, Boland T M and O'Mara F P. 2009a. Effect of DL-Malic acid on feed intake, methane emission, and rumen fermentation in beef cattle. *Journal of Animal Science* **87**: 1047–57.

Foley P A, Kenny D A, Lovett D K, Callan J J, Boland T M and O'Mara F P. 2009b. Effect of Dl-malic acid supplementation on feed intake, methane emissions, and performance of lactating dairy cows at pasture. *Journal of Dairy Science* **92**: 3258–64.

International Atomic Energy Agency (IAEA) 1997. *Estimation of rumen microbial protein production from purine derivatives in urine*. IAEA-TECDOC-945, pp. 22–24. IAEA, Vienna. Austria.

Johnson K A, Huyler M, Westberg H, Lamb B and Zimmerman P. 1994. Measurement of methane emissions from ruminant livestock using a sulphur hexafluoride tracer technique. *Environmental Science and Technology* **28**: 359–62.

Kung L, Smith K A, Smagala A M, Endres K M, Bessett C A, Ranjit N K and Yaissle J. 2003. Effects of 9,10 anthraquinone on ruminal fermentation, total-tract digestion, and blood metabolite concentrations in sheep. *Journal of Animal Science* **81**(1): 323–28.

Lin B, Wang J H, Lu Y, Liang Q and Liu J X. 2013. In vitro rumen fermentation and methane production are influenced by active components of essential oils combined with fumarate. *Journal of Animal Physiology and Animal Nutrition* **97**(1): 1–9.

Liu Q, Wang C, Yang W Z, Dong Q, Dong K H, Huang Y X, M. Y X and He D C. 2009. Effects of malic acid on rumen fermentation, urinary excretion of purine derivatives and feed digestibility in steers. *Animal* **3**(1): 32–39.

Martin S A, Streeter M N, Nisbet D J, Hill G M and Williams S E. 1999. Effects of Dl-malate on ruminal metabolism and performance of cattle fed high-concentrate diet. *Journal of Animal Science* **77**: 1008–15.

McGinn S M, Beauchemin K A, Coates T and Colombatto D. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *Journal of Animal Science* **82**: 3346–56.

Mohammed N, Lila Z A, Ajisaka N, Hara K, Mikuni K, Kanda S and Itabashi H. 2004. Inhibition of ruminal microbial methane production by beta-cyclodextrin iodopropane, malate and their combination in vitro. *Journal of Animal Physiology and Animal Nutrition* **88**(5–6): 188–95.

Mohini M, Singhal K K, Sirohi S S and Mohanta R K. 2009. Methane emission from Sahiwal cows on dieatary supplementation of fumaric acid as a feed additives. *Indian Journal of Animal Nutrition* **26**(1): 56–60.

Molano G, Knight T W and Clark H. 2008. Fumaric acid supplements have no effect on methane emissions per unit of feed intake in wether lambs. *Australian Journal of Experimental Agriculture* **48**(2): 165–68.

Owens F N and Goetsch A L. 1988. Ruminal fermentation. *The Ruminant Animal Digestive Physiology And Nutrition*. pp 145–47. (Ed.) Church D C. Waveland Press Inc, Illinois, USA.

Sawyer M S, Hoover W H and Sniffen C J. 1974. Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. *Journal of Animal Science* **38**(4): 908–14.

Van Soest P J, Robertson J B and Lewis B A. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science***74**: 3583–97.

Wood T A, Wallace R J, Rowe A, Price J, Yánez-Ruiz D R, Murray P and Newbold C J. 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. *Animal Feed Science and Technol*ogy **152**: 62–71.