Effect of dietary formic acid on the in vitro ruminal fermentation parameters of barley-based concentrated mix feed of beef cattle

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ABSTRACT
This study aimed to determine the effect of 0 (FA0) and 1, 2, 4 or 8 mL/kg (FA1, FA2, FA4 and FA8) formic acid addition to barley-based concentrated mix feed on in vitro gas kinetics [gas production from quickly soluble fraction (a gas), gas production constant rate (c gas), gas production of insoluble fraction (b gas)], potential gas production (a + b) gas, methane production, organic matter digestibility (OMD), metabolic energy (ME), net energy lactation (NE L), pH, ammonia-N, volatile fatty acids (VFAs), total bacteria count and number of ciliate protozoa. The in vitro cumulative gas production, c gas, b gas, (a + b) gas, ME, NE L and OMD values and ammonia-N concentration were decreased by formic acid (P < .05). Formic acid supplementation increased in vitro methane production up to about 12% (P < .05). Total bacteria count and number of protozoa and molar concentrations of total VFAs, acetic, propionic and butyric acids decreased with formic acid supplementation (P < .001). The numbers of Isotrichia spp. and Dasytricha spp. increased with high formic acid (P < .001). The numbers of Diplodiniinae and Entodiniinae decreased in FA4 and FA8 groups (P < .05). The results indicated that formic acid addition to feed decreased ruminal microbiota count, digestibility, proteolysis and molar VFA values also increased ruminal methane emission. Besides, formic acid supplementation could increase energy loss during ruminal fermentation of feed.

1. Introduction
Following the European ban of antibiotic growth promoters in 2006 (EC Regulation No. 1831/2003), the use of organic acids (also called acidifiers) in animal feed has gained importance. Their positive effects on feed quality and animal performance have been known for decades. Most organic acids with specific antimicrobial activity are short-chain acids (C1–C7) and the pKa value is from 3 to 5 (Papatisros et al. 2013). Almost all of the organic acids used in animal nutrition, such as formic, propionic, lactic, acetic, malic or citric acids, have an aliphatic structure and represent a source of energy for the cells (Dibner & Buttin 2002; Kara et al. 2014). Formic acid which is used as a safe feed additive with E236 code, have preservative, antibacterial, antifungal, strong odour-flavour properties and inhibitory effects on biogenic amines in digestive tract. In the Europe Union Feed Additives List, it has been advised that formic acid can add up to 10 g/kg in livestock animal rations (Papatisros & Billinis 2012; EFSA 2014). This volatile organic acid is used as a silage additive for stimulating the lactic acid fermentation and inhibiting the butyric acid production (Baytok et al. 2005) and the conversion of feed protein to non-protein-nitrogen (Jaakkola 2006). However, higher doses of formic acid in diet may cause increasing dietary acidity, disturbing the acid-base status, lowering feed intake, damaging the stomach and duodenal mucosa, and also corrosive structure materials of cage and feeder (Eckel et al. 1992; Papatisros & Billinis 2012) and increase the in vitro methane emission of ruminant feed (Kara et al. 2015a). Normally, formic acid has been found a maximum 5% of volatile fatty acids (VFAs) in rumen contents and metabolized more rapidly than it is formed in the rumen. Formic acid, which is formed in the production of acetate in rumen, can be used by archaea methanogen as a substrate for methanogenesis (Hook et al. 2010). Janssen and Kirs (2008) stated that most species of methanogen can grow using hydrogen and often formic acid as their energy sources and use the electrons derived from hydrogen (or formic acid) to reduce carbon dioxide to methane.

This study was conducted to determine the effects of formic acid addition to the concentrated mix feed on the in vitro methane production, gas kinetics, organic matter digestibility, energy, pH, ammonia-N, short-chain fatty acids, total bacteria count and number of protozoa.

2. Materials and methods

2.1. Formic acid
In the study, formic acid was used as a commercially feed additive (Amasil85 liquid, BASF The Chemical Company). This feed
additive is a product easily soluble in water, colourless liquid and contains a minimum 85% of formic acid (Product code: 10001972, PRD number 30041102, molecule formula HCOOH, molecule weight 46.03 g). It has 1.190 g/cm³ density (at 20°C) and pH 2.2 (10 g/L H₂O).

2.2. The chemical analysis of feed

The concentrated mix feed of beef cattle was milled through a 1-mm sieve (IKA MF 10.1, Germany) for use in chemical analysis and in vitro gas production. Dry matter (DM) (AOAC 1984, method 14.081), crude ash (AOAC 1990, method 942.05), crude protein (CP) (AOAC 1990, method 954.01), diethyl ether extract (EE) (AOAC 1990, method 920.39) and crude fibre (CF) (AOAC 1980, methods 7.066–7.070) compositions of the feed were analysed using the AOAC methods. The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin contents were analysed using a fibre analyser (Velp FIWE3, Italy) according to the methods reported by Van Soest et al. (1991). The NDF was determined using sodium sulphite and thermo-stable α-amylase (Mega-Zyme, Ireland) (aNDF). Neither NDF nor ADF was inclusive of residual ash (aNDFom and ADFom). Non-fibrous carbohydrate (NFC) levels were calculated using the following formula (NRC 2001):

\[
\text{NFC} \% = 100 - (\text{NDF} \% + \text{CP} \% + \text{EE} \% + \text{Ash} \%)
\]

The ingredient and nutrient matter compositions of the concentrated mix feed are presented in Table 1.

2.3. In vitro gas production technique

The technique was performed with different doses of formic acid supplementation to concentrated mix feed. Formic acid was not added to the control group (FA0 group) and treatment groups were added 1, 2, 4 or 8 mL/kg formic acid supplementation (treatment groups; FA1, FA2, FA4 and FA8).

Rumen fluid which is necessary for in vitro fermentation was obtained from two beef cattle feed with a diet containing rough feed (approximately 30% of total mix feed on a DM basis, maize silage + alfalfa hay + wheat straw) and concentrate feed (approximately 70% of total mix feed on a DM basis). Rumen fluid of approximately 1 L (totally) was collected in a thermos including water at 39°C using CO₂ gas, and filtered with 4 layers of cheesecloth in the laboratory. The technique was carried out according to the procedures of Menke et al. (1979). The feed samples, which were milled through a 1-mm sieve, were incubated in rumen fluid and buffer mixture in 100-mL glass syringes (Model Fortuna, Germany). One litre of buffer mixture included 474 mL of bi-distilled water, 237.33 mL of macro-mineral solution (5.7 g of Na₂HPO₄, 6.2 g of KH₂PO₄ and 0.6 g of MgSO₄ in 1 L of bi-distilled water), 237.33 mL of buffer solution (35 g of NaHCO₃ and 4 g of NH₄HCO₃ in 1 L of bi-distilled water), 0.12 mL of trace-mineral solution (13.2 g of CaCl₂*2H₂O, 10 g of MnCl₂*4H₂O, 1 g of CoCl₂*6H₂O and 0.8 g of FeCl₃*6H₂O in 100 mL of bi-distilled water), 1.22 mL of resazurin solution (0.1 g of resazurin in 100 mL of bi-distilled water) and 50 mL of reducing solution (285 mg of Na₂S*7H₂O and 4 mL of 1 N NaOH in 96 mL of bi-distilled water). Dried samples (200 ± 10 mg) and 30 mL of the rumen fluid + buffer mixture at a 1:2 (v/v) ratio were incubated to syringes in triplicate. In addition, three blank syringes (no template; rumen fluid + buffer mixture) were used to calculate the total gas production. The clips of syringes were closed, the initial volume recorded and the syringes were incubated in a water bath at 39°C for 96 h.

2.4. Determination of total gas and methane production

In incubation, the total gas volume was recorded from the calibrated scale in the syringe for 3, 6, 12, 24, 48, 72 and 96 h. After measuring the total gas volume at 24 h, the tubing of the plastic syringe outlet was inserted into the inlet of the methane analyser (Sensor, Europe GmbH, Erkrath, Germany) and the piston was pushed to insert the accumulated gas into the analyser. The methane as a percent (%) of the total gas was displayed on a computer (Goel et al. 2008; Kara 2015).

Cumulative gas production data were fitted to the exponential equation of Ørskov and McDonald (1979):

\[
Y = a + b(1 - 2ex^{-ct}),
\]

where \(a\) is the gas production from the immediately soluble fraction (mL); \(b\) is the gas production from the insoluble fraction (mL); \(c\) is the gas production constant rate; \(a + b\) is the potential gas production (mL); \(t\) is the incubation time (h); \(y\) is the gas produced at time \(t\).

The fermentation kinetics was estimated using a computer package programme called Fig P (Biosoft, Cambridge, UK).

| Ingredient | Percent, as-feed basis |
|------------|------------------------|
| Barley     | 44.15                  |
| Wheat      | 17.00                  |
| Corn       | 15.00                  |
| Wheat bran | 10.00                  |
| Cotton seed meal, with 31% CP | 5.25 |
| Sugar beet molasses | 5.00 |
| Limestone, with 38% Ca | 2.50 |
| Di-calcium phosphate | 0.50 |
| Salt       | 0.60                   |
| Total      | 100.00                 |

**Analysis values**

| Percent, in DM basis |
|----------------------|
| CP                   | 12.70                |
| NFC                  | 47.48                |
| aNDFom               | 28.87                |
| ADFom                | 12.47                |
| ADL                  | 6.60                 |
| CP                   | 8.90                 |
| EE                   | 3.13                 |
| Ash                  | 7.82                 |

*Note: ADFom, acid detergent fibre expressed exclusive of residual ash; ADL, acid detergent lignin determined by solubilization of cellulose with sulphuric acid; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; CF, crude fibre; CP, crude protein; DM, dry matter; EE, diethyl ether extract; NFC, non-fibre carbohydrates.*
Table 2. In vitro cumulative gas production of formic acid addition to the feed (mean ± standard error).*

| Item          | FA0       | FA1       | FA2       | FA4       | FA8       | SD       | P-value |
|---------------|-----------|-----------|-----------|-----------|-----------|----------|---------|
| Gas 3 h       | 19.33 ± 0.76   | 14.33 ± 0.42 | 12.00 ± 1.27 | 11.66 ± 0.42 | 11.83 ± 0.47 | 3.39     | <.001   |
| Gas 6 h       | 42.00 ± 1.36   | 26.83 ± 0.87 | 23.50 ± 2.50 | 24.66 ± 0.76 | 24.83 ± 0.87 | 8.13     | <.001   |
| Gas 12 h      | 55.33 ± 2.40   | 34.00 ± 1.09 | 33.50 ± 1.28 | 32.00 ± 1.00 | 31.33 ± 4.75 | 11.19    | <.001   |
| Gas 24 h      | 69.33 ± 0.88   | 47.70 ± 0.93 | 47.25 ± 0.89 | 47.25 ± 1.89 | 45.75 ± 1.54 | 9.58     | <.001   |
| Gas 48 h      | 76.00 ± 1.15   | 60.91 ± 2.02 | 58.58 ± 0.88 | 56.25 ± 1.00 | 52.58 ± 4.66 | 9.04     | 0.04    |
| Gas 72 h      | 78.66 ± 1.33   | 63.92 ± 2.60 | 57.58 ± 1.45 | 58.25 ± 1.15 | 54.25 ± 5.03 | 9.80     | <.001   |
| Gas 96 h      | 81.00 ± 1.52   | 64.25 ± 2.64 | 59.58 ± 1.85 | 58.58 ± 1.20 | 54.25 ± 5.00 | 10.45    | <.001   |

Note: FA0: without formic acid supplementation to feed (control group). FA1, FA2, FA4 and FA8: 1, 2, 4 and 8 mL/kg formic acid supplementation, respectively (treatment groups).

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Table 3. Effect of formic acid addition on ruminal fermentation parameters and gas kinetics to the feed (mean ± standard error).

| Item          | FA0        | FA1        | FA2        | FA4        | FA8        | SD        | P-value |
|---------------|------------|------------|------------|------------|------------|-----------|---------|
| c<sub>gas</sub> | 0.122 ± 0.010 | 0.089 ± 0.003 | 0.077 ± 0.006 | 0.063 ± 0.004 | 0.089 ± 0.004 | 0.02      | <.05    |
| a<sub>gas</sub> | -0.66 ± 0.072 | 0.64 ± 0.356 | 1.49 ± 0.600 | 1.77 ± 0.261 | 0.72 ± 0.268 | 1.12      | <.05    |
| b<sub>gas</sub> | 78.66 ± 0.38  | 58.80 ± 0.39  | 57.51 ± 0.30  | 57.13 ± 0.52  | 56.86 ± 0.75  | 8.78      | <.001   |
| (α + β)<sub>gas</sub> | 78.00 ± 1.08  | 60.58 ± 0.60  | 59.01 ± 0.78  | 57.78 ± 0.86  | 57.59 ± 1.07  | 8.15      | <.001   |
| Methane (%)   | 20.30 ± 0.10  | 21.13 ± 0.20  | 21.90 ± 0.52  | 21.96 ± 0.48  | 22.73 ± 0.77  | 1.11      | <.05    |
| ME (MJ/kg DM) | 12.06 ± 0.13  | 8.66 ± 0.14  | 8.59 ± 0.14  | 8.83 ± 0.29  | 8.35 ± 0.24  | 1.50      | <.001   |
| NE<sub>i</sub> (MJ/kg DM) | 7.68 ± 0.10  | 5.19 ± 0.10  | 5.14 ± 0.10  | 5.74 ± 0.21  | 4.97 ± 0.17  | 1.10      | <.001   |
| OMD (% DM)    | 79.18 ± 0.88  | 57.58 ± 0.89  | 57.12 ± 0.93  | 57.92 ± 1.80  | 55.62 ± 1.54  | 9.57      | <.001   |
| NH<sub>3</sub>-N (mg/dL) | 180 NH<sub>3</sub>-N | 180 NH<sub>3</sub>-N | 180 NH<sub>3</sub>-N | 180 NH<sub>3</sub>-N | 180 NH<sub>3</sub>-N | 180 NH<sub>3</sub>-N | 180 NH<sub>3</sub>-N |
| pH            | 6.77 ± 0.03  | 6.76 ± 0.01  | 6.80 ± 0.02  | 6.82 ± 0.02  | 6.82 ± 0.01  | 0.03      | >.05    |

Note: FA0: without formic acid supplementation to feed (control group). FA1, FA2, FA4 and FA8: 1, 2, 4 and 8 mL/kg formic acid supplementation, respectively (treatment groups). c<sub>gas</sub> = gas production constant rate (mL/h 0.2 g DM), a<sub>gas</sub> = gas production (mL/0.2 g DM) from quickly soluble fraction, b<sub>gas</sub> = gas production (mL/0.2 g DM) from insoluble fraction. (α + β)<sub>gas</sub> = potential gas production (mL/0.2 g DM), SD: standard deviation of means.

2.5. Estimating of metabolic energy (ME), net energy lactation (NE<sub>L</sub>) and organic matter digestibility (OMD) levels

The ME, NE<sub>L</sub> and OMD values in feeds were calculated using equations for concentrate feed of Menke and Steingass (1987).

ME (MJ/kg DM) = 0.157 × GP + 0.0084 × CP + 0.022 × EE - 0.0081 × CA + 1.06

NE<sub>L</sub> (MJ/kg DM) = 0.115 × GP + 0.0054 × CP + 0.014 × EE - 0.0054 × CA - 0.36

OMD (% DM) = 0.9991 × GP + 0.0595 × CP + 0.0181 × CA + 9.00,

where GP is 24-h net gas production (mL/200 mg DM), and CP, EE, CA, OMD are CP, EE, crude ash (mg/kg DM) and organic matter digestibility, respectively.

2.6. Determination of ammonia-N and pH in rumen fluid

The pH of the rumen medium was determined using a digital pH meter (Mettler Toledo, USA). The ammonia-N (NH<sub>3</sub>-N, mg/dL) concentration of the rumen medium was estimated by a distillation system, without acid digestion and after distillation with potassium hydroxide (2.0 N) in boric acid and titration with diluted hydrochloric acid (0.1 N), after previous centrifugation of the sample at 1000 × g for 15 min (Souza et al. 2010).

2.7. Determination of the number of protozoa and bacteria count in rumen fluid

Total numbers and generic composition of ciliate protozoa were determined according to the procedures described by Dehority (1984). Fermentation fluid (0.1 mL) samples were collected and fixed by 0.9 mL methyl green-formal-saline solution (100 mL formaldehyde (35%), 900 mL distilled water, 0.6 g methyl green and 8.0 g NaCl). The diluted sample was pipetted into a Sedgewick Rafter counting chamber by a wide-orifice pipette. The total numbers and generic composition of ciliate protozoa were determined using a microscope (Nikon Eclipse E-100, the Netherlands). Determination of total bacteria count was carried out using a spectrophotometer (T80+ UV/VIS Spectrophotometer, PG Instruments Ltd, UK).

2.8. Determination of volatile fatty acids in rumen fluid

The ruminal fluid was squeezed through four layers of cheese-cloth, mixed with 25% (w/v) meta-phosphoric acid and kept frozen (−20°C) for the analysis of VFA. The frozen samples were thawed and centrifuged. The supernatant (0.5 mL) was mixed with the same volume of 20 mmol 4-methyl N-valeric acid as an internal standard. Ruminal VFA concentration (acetic, propionic and butyric acids, mmol/L) was assessed by a gas chromatograph (Perkin Elmer Autosystem XL, USA) as described by Erwin et al. (1961).

2.9. Statistical analysis

The statistical analysis of data was performed using SPSS 17.0 software. The statistical significance among groups was determined by one-way ANOVA analysis. Levene’s test was performed to check the homogeneity of variances. ‘Tukey’s multiple range test’, one of the multiple comparison tests, was used when the difference among groups was found to be significant.
be statistically significant. The data were presented on the basis of mean and ± standard error of mean.

The correlations, r, among the addition level of formic acid and gas production kinetics, and some estimated parameters, were determined using the SPSS procedure.

3. Results

The *in vitro* cumulative total gas production (mL) up to 96 h of formic acid addition to the feed is shown in Table 2. The *in vitro* cumulative total gas production, $c_{gas}, b_{gas}$ (a + b)$_{gas}$, ME, NEL, and OMD levels and ammonia-N concentration were decreased by formic acid addition to the concentrated mix feed ($P < .05$; Tables 2–4). The *in vitro* methane production of the formic acid groups increased up to 12% compared to FA0 group ($P < .05$; Table 3). Considering *in vitro* methane production (14.07–10.40 mL/0.2 g DM) and OMD (79.18–55.62%) in the present study, in vitro methane produced by 0.2 g DM digested was 17.81, 21.44, 22.02, 22.09 and 22.86 mL for FA0, FA1, FA2, FA4 and FA8 groups, respectively. Ruminal pH did not change in FA1, FA2, FA4 and FA8 groups compared to FA0 ($P > .05$; Table 3).

Total bacteria count and number of total protozoa were low in all formic acid groups compared to the control group ($P < .001$). The numbers of *Isotricha spp.* (in both FA4 and FA8 groups) and *Dasytricha spp.* (only FA8 groups) protozoa increased with high formic acid doses ($P < .001$). The numbers of families *Diplodiniinae* and *Entodiniinae* protozoa decreased with 4 and 8 mL/kg formic acid supplementation to feed ($P < .001$) (Table 4).

| Items                              | FA0       | FA1       | FA2       | FA4       | FA8       | SD         | P-value |
|------------------------------------|-----------|-----------|-----------|-----------|-----------|------------|---------|
| Total bacteria                     | 12.86 ± 0.52 $^a$ | 10.56 ± 0.14 $^b$ | 10.48 ± 0.04 $^b$ | 10.20 ± 0.05 $^b$ | 8.11 ± 0.24 $^b$ | 1.61       | <.001   |
| Total ciliate protozoa             | 8.13 ± 0.84 $^a$ | 7.58 ± 0.90 $^b$ | 6.21 ± 0.23 $^b$ | 6.01 ± 0.14 $^b$ | 5.99 ± 0.29 $^b$ | 1.48       | <.001   |
| *Isotricha spp.*                    | 0.31 ± 0.01 $^a$ | 0.46 ± 0.06 $^b$ | 0.48 ± 0.01 $^b$ | 0.54 ± 0.02 $^b$ | 0.77 ± 0.06 $^b$ | 0.17       | <.001   |
| *Dasytricha spp.*                  | 0.47 ± 0.01 $^a$ | 0.47 ± 0.01 $^b$ | 0.48 ± 0.01 $^b$ | 0.53 ± 0.04 $^b$ | 0.59 ± 0.04 $^b$ | 0.14       | <.001   |
| *Diplodiniinae*                    | 2.59 ± 0.41 $^a$ | 2.00 ± 0.02 $^a$ | 1.60 ± 0.05 $^b$ | 1.47 ± 0.16 $^b$ | 1.13 ± 0.34 $^b$ | 0.63       | <.05    |
| *Entodiniinae*                     | 4.76 ± 0.14 $^a$ | 4.65 ± 0.35 $^a$ | 3.65 ± 0.05 $^b$ | 3.47 ± 0.01 $b$ | 2.98 ± 0.23 $b$ | 0.79       | <.05    |

Note: FA0: without formic acid supplementation to feed (control group). FA1, FA2, FA4 and FA8: 1, 2, 4 and 8 mL/kg formic acid supplementation, respectively (treatment groups).

The molar total VFAs and molar acetic acid values decreased with 2, 4 and 8 mL/kg formic acid supplementation ($P < .001$). In this study, 4 and 8 mL/kg formic acid supplementation decreased molar propionic ($P < .001$), iso-butyric ($P < .001$) and valeric acids ($P < .01$) and A:P ratio ($P < .01$). However, molar butyric acid decreased with all supplementation doses of formic acid ($P < .001$). Molar iso-valeric acid in rumen fluid also did not change by formic acid supplementations ($P > .05$) (Table 5).

The addition level of formic acid had a negative correlation with the $c_{gas}$ ($r = -0.615; P < .05$), $b_{gas}$ ($r = -0.699; P < .01$), ($a + b_{gas}$) ($r = -0.683; P < .01$), ME, NEL, OMD ($r = -0.695; P < .01$), and NH$_3$-N ($r = -0.803; P < .001$) and a positive correlation with methane production ($r = 0.742; P < .01$) (Table 6).

4. Discussion

Total gas produced by the *in vitro* fermentation technique is related to the digestion level of substrate (feed). Digestion level of the substrate changes according to some factors such as diet supplements (condensed tannin, organic acids), easy soluble nutrient composition and microbiota count of rumen fluid (Kara 2015; Kara et al. 2015a, 2015b). In the present study, *in vitro* gas production of FA0 reached 81 mL/0.2 g DM for 96 h. This high gas production and digestion value can be relation with high NFC value of the barley-based concentrated mix feed (47.48%). Potential gas production was negatively correlated with up to 9.52 g/kg (8 mL/kg = 8*1.19 g/kg) formic acid addition and decreased up to about 26%. The gas production constant rate with 0.122 (ml/h) of barley grain-based

Table 5. Effects of formic acid addition to feed on VFAs (mmol/L) in rumen fluid (mean ± standard error).

| Items                              | FA0       | FA1       | FA2       | FA4       | FA8       | SD         | P-value |
|------------------------------------|-----------|-----------|-----------|-----------|-----------|------------|---------|
| Total VFAs                         | 113.58 ± 1.52 $^a$ | 110.49 ± 2.12 $^a$ | 106.17 ± 1.57 $^b$ | 34.72 ± 0.24 $^c$ | 31.85 ± 0.81 $^c$ | 39.08       | <.001   |
| Acetic acid (A)                    | 67.44 ± 1.65 $^a$ | 67.75 ± 1.73 $^a$ | 62.69 ± 0.15 $^b$ | 17.85 ± 0.52 $^b$ | 16.59 ± 0.50 $^b$ | 24.82       | <.001   |
| Propionic acid (P)                 | 20.00 ± 0.84 $^a$ | 20.78 ± 0.15 $^a$ | 21.40 ± 0.75 $^b$ | 8.21 ± 0.43 $^b$ | 7.34 ± 0.82 $^b$ | 6.66       | <.001   |
| Butyric acid                      | 18.16 ± 0.12 $^a$ | 14.59 ± 0.20 $^b$ | 14.91 ± 0.18 $^b$ | 6.15 ± 0.15 $^b$ | 5.41 ± 0.28 $^b$ | 5.29       | <.001   |
| Iso-butyric acid                   | 6.96 ± 0.91 $^a$ | 6.56 ± 0.73 $^a$ | 6.47 ± 0.63 $^a$ | 2.04 ± 0.11 $^a$ | 2.14 ± 0.07 $^a$ | 2.48       | <.001   |
| Valeric acid                       | 0.09 ± 0.00 $^a$ | 0.09 ± 0.01 $^a$ | 0.09 ± 0.01 $^a$ | 0.07 ± 0.01 $^b$ | 0.03 ± 0.01 $^b$ | 0.03       | <.01    |
| Iso-valeric acid                   | 0.02 ± 0.02 $^a$ | 0.02 ± 0.02 $^a$ | 0.02 ± 0.02 $^a$ | 0.03 ± 0.02 $^a$ | 0.03 ± 0.02 $^a$ | 0.03       | <.01    |
| A:P                               | 3.37 ± 0.08 $^a$ | 3.26 ± 0.09 $^a$ | 2.92 ± 0.16 $^a$ | 2.20 ± 0.17 $^a$ | 2.32 ± 0.28 $^a$ | 0.55       | <.001   |

Note: FA0: without formic acid supplementation to feed (control group). FA1, FA2, FA4 and FA8: 1, 2, 4 and 8 mL/kg formic acid supplementation, respectively (treatment groups).

Table 6. Correlation coefficient (r) relationship of the addition level of formic acid with gas production kinetics and some estimated parameters.

| Pearson correlation | Methane | $c_{gas}$ | $b_{gas}$ | ($a + b_{gas}$) | ME | NEL | OMD | pH | NH$_3$-N |
|---------------------|---------|-----------|-----------|-----------------|----|-----|-----|----|----------|
| Addition level of Formic acid | 0.742 | -0.615 | -0.699 | -0.683 | -0.695 | -0.695 | -0.695 | 0.509 | -0.803 |
| P-value             | <.01    | <.05     | <.01     | <.01            | <.01 | <.01 | <.01 | .05 | <.001    |
concentrated mix feed decreased up to a range from 0.089 to 0.063 by formic acid supplementation. In the current study, decrease in total gas production and estimated digestibility parameters may be connected with antimicrobial effect on ruminal total bacteria and Entodiniinae and Diplodiniinae ciliate protozoa. As a result of the reduction in feed digestion, molar VFAs in rumen fluid decreased by formic acid. Castiloo-Gonzalez et al. (2014) reported that CO₂ which comprises 60–65% of total gas produced in rumen is produced by cellulose-degrading bacteria, lactate-degrading bacteria and pectin-degrading bacteria. Partanen and Jalava (2005) determined that formic acid had the greatest effect on the maximum rate of gas production, thereby exhibiting a greater inhibiting effect on microbial fermentation than the other organic acids (propionic, lactic, fumaric, citric and its organic acid salts). Even if, supplementation doses (1.19–9.52 g/kg) of formic acid to barley-based concentrated mix feed in the present study were lower than advice dose (up to 10 g/kg) in the Europe Union Feed Additives List (EFSA 2014) had antimicrobial effect on rumen bacteria and protozoa.

Organic acids such as malate, fumarate, lactate and pyruvate (in the succinate-propionate pathway) are needed as precursors to propionate and if the rumen concentrations of these acids could be increased, propionate production would increase and methane production would decrease (Kara 2015). Formic acid is a the methane precursor in the succinate-methane pathway, and by acting as an alternative H₂ sink in the rumen, it has the potential to increase ruminal methanogenesis (Hook et al. 2010; Castiloo-Gonzalez et al. 2014). In the present study, formic acid addition increased ruminal methane production, especially 8 mL/kg formic acid addition. In the study, in vitro methane produced by 0.2 g of OM digested of diet was 17.81 ml for FA0 group, and 22.86 ml for FA8 group. Addition level of formic acid was positively correlated with methane and was connected with increase in numbers of Holotrich (Isotricha and Dasytricha) ciliates, especially 8 mL/kg formic acid supplementation.

In rumen fluid, two important ciliate protozoa as ‘entodiniomorphs (in Entodiniomorphida order)’ and ‘holotrichs (in Tri-cho stomatida order)’ were identified. Entodiniinae and Diplodiniinae subfamilies from rumen ciliate protozoa were belong to Ophryoscolecidae family in Entodiniomorphida order (Kreier & Baker 1993). The numbers of total protozoa and genera in rumen fluid are associated with diet type and changes with ruminal organic matter digestion, fibre and proteolytic activities (Veira 1986). In the current study, the total number of Ophryoscolecidae family, have ciliates significantly decreased. In agreement with our result, it has been reported that formic acid decreased the number of total protozoa in a previous study (Donmez et al. 2003). The antimicrobial effect mechanism of formic acid on protozoa and bacteria may be connected that changing cell membrane permeability and lysis of cell (Francis et al. 2002). In the present study, number of Diplodiniinae and Entodiniinae which are abundant ciliate protozoa of rumen fluid decreased by formic acid supplementation was similar with the results of Donmez et al. (2003). Increasing of methane production may be connected with increasing Isotricha spp. and Dasytricha spp. numbers by formic acid supplementation dose (Kara et al. 2016). In agreement with our result, previous researchers have observed that Holotrich protozoa seem to be the key players in rumen methanogenesis (Belache et al. 2012, 2015).

The estimated OMD that was decreased by formic acid addition may be connected to the antimicrobial effect on ruminal total bacteria count and number of ciliate protozoa of formic acid. The ME and NEₜ levels in formic acid groups decreased up to about 31% and 34%, respectively. In the present experiment, low digestibility of feed has decreased molar VFAs in rumen fluid. It can be said that formic acid, which causes high methane production and low digestion, increased energy loss via ruminal fermentation of feed.

Volatile fatty acids are one of the fermentation end-products of feedstuffs in rumen. Even though the concentration of VFAs highly differ among diets, it generally ranges from 60 to 120 mmol/L in rumen fluid (Donmez et al. 2003; Jakkola et al. 2006; Belanche et al. 2015). In the present study, reducing of total VFAs, acetic acid and propionic acid concentration by formic acid addition was similar with findings of Partanen and Jalava (2005). The decreasing molar total VFAs and individual VFAs in rumin fluid may be in relation with the antimicrobial effect of formic acid on rumen bacteria and ciliate protozoa. In the study of Belanche et al. (2015), ruminal total VFA concentration increased progressively with the increasing number and type of ciliate protozoa in rumen.

The positive effects of some dietary organic acids on animal performance have been explained in part by decreased/prevented proteolysis (supply that flow essential amino acids to the duodenum from the rumen) and the decreased release of ammonia-N and amines (Dibner & Buttin 2002; Baytok et al. 2005; Jaakkola 2006). In the present study, the increasing level of formic acid supplementation decreased ruminal ammonia-N concentration to 58%, being negatively correlated. Decreasing ammonia-N concentration in rumen fluid may be relation with negative effect of formic acid on number of Entodiniinae ciliate which is proteolytic protozoa produced ruminal ammonia-N and ruminal bacteria count (Ivan et al. 2000; Castillo-Gonzalez et al. 2014). In contrast, Jaakkola et al. (2006) stated that ruminal ammonia-N concentration did not change in cattle fed with grass silages preserved with formic acid (2–6 L/tonnes grass herbage). According to the results in the present study, 1–8 mL/kg formic acid supplementation in concentrated mix feed has a reducing effect on ruminal proteolysis and ammonia-N concentration.

5. Conclusion

The results suggest that 1–8 mL/kg formic acid addition to the barley-based concentrated mix feed decreased ruminal microbiota count, digestibility, proteolysis and molar VFAs, but increased ruminal methane emission. The results of the current study it was found that formic acid increased energy loss during ruminal fermentation of feed.

Disclosure statement

No potential conflict of interest was reported by the authors.
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