In vitro methane production and quality of corn silage treated with maleic acid

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Abstract

This study aimed to determine the effects of maleic acid (MA) addition to corn at ensiling on silage quality and in vitro methane and total gas production, metabolisable energy (ME), and organic matter digestibility (OMD) parameters by using in vitro gas production techniques. Forage corn was ensiled either without (control group: MA 0) or with three different dosages of maleic acid, 0.5% (MA 0.5), 1.0% (MA 1.0), and 1.5% (MA 1.5) w/w of the fresh material for 60 days. As a result of this study, neutral detergent fibre level was decreased in the MA 1.5 group (P<0.05). The 0.5, 1.0, and 1.5% addition of maleic acid to forage corn at ensiling increased lactic acid concentration (P<0.05) in silage and reduced propionic acid (P<0.05). Iso-valeric acid concentration in the organic acids of the silage was decreased with maleic acid addition (P<0.05). The maleic acid addition decreased in vitro ruminal methane production (P<0.01). The silage pH value, and acetic, butyric and iso-butyric acid concentrations and in vitro total gas production, OMD, and ME values did not change by MA addition (P>0.05). It was concluded that MA addition could reduce methane emission without any negative effects on silage nutrient composition or in vitro ruminal fermentation parameters.

Introduction

The most important greenhouse gasses are carbon dioxide (CO2), methane (CH4) and nitrous oxides (N2O), all of which have increased in the last years (Merbold et al., 2015). Methane is an especially potent trace gas due to its global warming potential (Hook et al., 2010). The major factors influencing CH4 emissions from ruminants are: i) level of feed intake, ii) type of carbohydrate fed, and iii) the ruminal microflora (Johnson and Johnson, 1995; Lascano and Cardenas, 2010). Feed consumed by ruminants is fermented in the rumen and as a result polysaccharides in the feed are converted into volatile fatty acids (VFA) and microbial protein accompanied by the release of gaseous by-products (CO2 and hydrogen) (Kamra, 2005). As a result of this process, ruminants lose 2-12% of gross dietary energy in the form of CH4, depending on the type of diet (Johnson and Johnson, 1995). Approximately 87% of the enteric CH4 is produced in the rumen (Lascano and Cardenas, 2010). As indicated earlier, enteric CH4 arises from the conversion of hydrogen to CH4 by means of a specific group of microorganisms, collectively described as methanogens. Other microorganisms break down feed to produce VFA, carbon dioxide and hydrogen. Increasing the production of one of these fatty acids (propionate) reduces hydrogen production, resulting in less being available for conversion to CH4. Theoretically, a number of organic acids (malate, fumarate, and pyruvate) 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. The potential of dicarboxylic organic acids such as fumaric acid, maleic acid (MA), and cycloexedrin diallyl maleate as inhibitors of methanogenesis is well documented in vitro (Carro and Ranilla, 2003; Newbold et al., 2005; Li et al., 2012; Sirohi et al., 2012) and in vivo (Lila et al., 2004; Wallace et al., 2006; Foley et al., 2008) studies. Organic acids are propionate precursors in the succinate-propionate pathway, and by acting as alternative H2 sinks in the rumen, they have the potential to decrease ruminal methanogenesis (Newbold et al., 2005).

The corn silage is wet roughage the most widely used in the diet of dairy cows and beef cattle in the world. However, this roughage, so common used, should be determined the effect of its on methane emissions. This study was conducted to determine the effects of maleic acid addition to forage corn at ensiling on silage quality (pH, nutrient matter composition, silage acids) and in vitro methane and total gas production, metabolisable energy (ME), and organic matter digestibility (OMD) parameters in rumen fluid inoculum by using in vitro Hohenheim gas production techniques.

Materials and methods

Silage preparation

In this study, corn was grown at the Kayseri (38° 56 N, 34° 24 E, 1050 m), Turkey. It were planted on 10 April and harvested with a conventional forage harvester (chop-length of approximately 2.0 cm) at soft dough stage of seed maturity (after about 110 days of planting). The chopped forage was ensilled within 6 h of harvesting time in 1.5 L glass jars with clips and a rubber gasket (Sisecam, Istanbul, Turkey). At ensiling, it was treated with different doses of maleic acid (C4H2O4, M0375; Sigma Chemical Co., St Louis, MO, USA). Chopped corn was ensiled either without (control group: MA 0) or with three dosages of maleic acid, 0.5% (MA 0.5), 1.0% (MA 1.0), and 1.5% (MA 1.5) of the fresh material in five replicates. Silage jars were stored a compartment of laboratory at 20-25°C. The material was pressed tightly into the jars and sealed airtight. All jars in treatment were opened on days 60 post-ensiling and were analysed.

Chemical analysis

The dry matter (DM) levels of the silage samples were determined after waiting for 48 hours at 60°C. Dried samples were milled in an IKA A10 basic analytical grinder mill (IKA-Werke, Staufen im Breisgau, Germany) to a maximum particle size of 1 mm for chemical analysis and in vitro gas production. The ash levels were determined after waiting at 525°C for 8 h. Nitrogen content was measured by the kjeldahl method (AOAC, 1995). Crude protein (CP) was calculated as NX6.25. The diethyl ether extract (EE) and crude fibre (CF) levels
were determined according to the method reported by the AOAC (1995). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents, which form the cell wall components in the samples, were analysed according to the methods reported by Van Soest et al. (1991). The neutral detergent fibre was determined using sodium sulfate and heat stable amylase. Both NDF and ADF were not inclusive of residual ash. All chemical analysis was carried out in triplicate.

Determination of pH, lactic acid and volatile fatty acids in silage samples

Lactic acid, VFA, and pH were determined in silage extracts, prepared by adding 450 mL of distilled water to 50 g of silage, homogenising for 5 min in a laboratory blender (Waring, Odessa, FL, USA) and filtered through four layers of cheesecloth. The silage pH value was determined using a digital pH meter (Mettler Toledo S220; Mettler Toledo, Greifensee, Switzerland). These analyses were carried out in duplicate.

After extraction, the liquid was decanted into centrifugation tubes and centrifuged at 26,000 g for 30 min (Tjardes et al., 2000). The supernatant was filtered and analysed with a high-performance liquid chromatography device (Agilent 1100 HPLC; Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (HP 1047A). An Aminex Hpx 87H column (Bio-Rad, Hercules, CA, USA) (300x7.8 mm column) was used. The flow rate of the mobile phase (0.005 M H2SO4) was 0.6 mL/min at 41°C (Canale et al., 1984).

In vitro Hohenheim gas production technique

Rumen fluid, which required for in vitro gas production technique, was obtained from a Simmental steer beef (at 12 months of age and in 450 kg live weight). Animal care procedures for the experiment were conducted under a research protocol approved by the Local Ethics Committee for Animal Experiments of Erciyes University (Kayseri, Turkey). Steer beef has fed with 10.8 kg/day of total mix ration (DM basis) containing about 40% of forage (alfalfa hay and corn silage) and about 60% of concentrate feed since one month.

Rumen fluid was collected into a glass bottle (Isolab, Wertheim, Germany). The bottle was transported to the laboratory in a sealed thermos container at 39±2°C and filtered through four layers of cheesecloth under CO2 gas. In vitro gas production was analysed in four different silage samples of 0, 0.5, 1.0, and 1.5% maleic acid addition in five replicates. The samples were incubated in rumen fluid and buffer mixture in 100 mL glass syringes (Model Fortuna; Hayerle Labortechtechnik GmbH & Co. KG, Lonsee, Germany) following the procedures of Menke and Steingass (1988). About 200±10 mg dry samples were weighed in triplicate into glass syringes. The syringes were precultured at 39°C in a thermostatically controlled cabinet (Lovibond, Dortmund, Germany), before 10 mL of rumen fluid and 20 mL of prewarmed buffer mixture were dispensed anaerobically in each syringe using a automatic bottletop dispenser (Isolab). The syringes were closed using one position polypropylene clamps and incubated at 39±0.5°C for 24 h. In addition, three blank syringes (no sample; rumen fluid + buffer mixture) were used to calculate the total gas production.

Determination of total gas and methane production

After 24 h of incubation, the total gas volume was recorded from the calibrated scale on the syringe. After measuring the total gas volume, 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 percent of the total gas was displayed on a PC.

Determination of metabolisable energy and organic matter digestibility

The metabolisable energy and OMD levels of silage samples were calculated using the equations of Menke et al. (1979) and Blümmer et al. (1997) as follows:

\[ ME (MJ/kg DM) = 2.20 + 0.136 \times GP + 0.057 \times CP \]
\[ OMD (\%) = 14.88 + 0.889 \times GP + 0.45 \times CP + 0.0651 \times A \]
\[ GP = 24 \text{ h net gas production (mL/200 mg)} \]

where, A is ash content (g/kg DM).

Statistical analysis

The statistical analysis of data was performed, using SPSS 15.0 software. The difference among groups was determined by one-way ANOVA analysis. The Duncan’s Multiple Range Test, one of the multiple comparison tests, was used when the difference among groups was found to be significant. Orthogonal contrasts for linear and quadratic effects of maleic acid addition were used to evaluate different addition levels. Significance was defined at P<0.05.

Results

Table 1 gives the chemical analyses of the silages on day 60. Maleic acid did not affect the DM, ash, CP, EE, CF, ADF, or ADL values of silages. The neutral detergent fibre level was decreased in the MA 1.5 group is (P<0.05). The 0.5, 1.0, and 1.5% addition of maleic acid to forage corn at ensiling increased lactic acid concentration (P<0.05) and reduced propionic (P<0.05) in silage. Isovaleric acid concentration in the organic acids of the silage was decreased with maleic acid addition (P<0.05). The maleic acid addition decreased in vitro methane production (P<0.01), and increased in vitro ruminal pH (in MA 1.0 and 1.5 groups) at 24 h ruminal fermentation (P<0.05). The silage pH value, and acetic, butyric and isobutyric acid concentrations did not change with maleic acid addition to the silage (P>0.05). Besides, the in vitro total gas production, OMD, and ME values were not affected by the addition of 0.5, 1.0, and 1.5% maleic acid (Tables 2-4).

Discussion

In the present study, the decreased NDF in silage with increasing dose of MA is in agreement with the study of Sniffen et al. (2006). The decrease of NDF was linear significant in relation to the increase in dose of MA. Liu et al. (2009) stated that in situ ruminal NDF degradation of corn stove was improved but the CP degradability of concentrate mix was decreased linearly by MA. Montano et al. (1999) also reported that MA addition to steer fed an 81% steam-flaked barley-based diet did not affect ruminal digestion of OM. In the current experiment, CP, ADF and ADL levels, which are plant cell wall components, also did not change statistically with MA addition. According to the results of the study, MA in corn silage may be considered to increase the solubility of fibrous compounds in silage environment. The 11.56-12.43% of ash levels and 7.79-8.10% of CP levels of silage in present study are similar to the findings of Karakozak and Ayasan (2010). Levels of CP and EE in present study were lower than those found by Podkowka and Podkowka (2011), which were determined as 11.45 and 4.91, respectively.
the main product. Silage supplements have been added silage to stimulate lactic acid fermentation (by increase to lactic acid and decrease to butyric acid), decrease to pH and thus improving silage preservation (Filya et al., 2006). In study, lactic acid composition of silage was increased with MA addition. Generally, lactic and acetic acids composition of corn silages was lower than previous studies (Filya et al., 2006; Podkowka and Podkowka, 2011). Increasing of butyric acid composition and butyric acid bacteria count in corn silage was associated with an increasing of pH (Visser, 2011). Butyric acid and butyric acid bacteria can have large negative effects on the preservation quality, nutritive value, and palatability of silage and the quality of cheese milk and cheese (by transferring) (Driehuis, 2013). In present study, butyric acids composition of corn silage decreased with increasing dose of MA as linear, but not statistically significant.

Silage pH values in the study were determined between 4.04 and 4.11. Baytok et al. (2005) stated that formic acid addition (0.5%) to corn silage did not change pH value (3.77-3.96). Corn silage pH values of some studies were determined 3.63-4.70 (bacteria inoculants addition to corn silage) (Filya et al., Karakozak and Ayasan, 2010) and 4.31 (no addition to corn silage) (Podkowka and Podkowka, 2011). Dolezal et al. (2008) stated that mixture of organic acids, comprised of propionic acid, formic acid, benzoic acid and ammonium formiate addition to lupine silage decreased to 3.81 from 4.07 of silage pH value. The total gas production did not affect by the addition of 0.5, 1.0, and 1.5% maleic acid. This result was similar to previous in vitro studies that investigated the influence of malic acid on rumen fermentation (Li et al., 2012; Sirohi et al., 2012). In some of the studies conducted to investigate the effects of malic acid and malate on in vitro rumen fermentation, an increase in total gas production was reported (Martin, 1998; Tejido et al., 2005). Carro and Ranilla (2003) determine that disodium fumarate (4, 7, and 10 mM) did not change in vitro gas production of concentrated feeds (corn, sorghum, barley, wheat, and cassava meal). In vitro ME, and OMD values did not affect by the maleic acid addition to forage in the present study. On the other hand, Sirohi et al. (2012) stated that in vitro dry matter digestibility of total mix diet was higher with 5 and 10 mM maleic acid addition. In another a study, the organic matter apparent disappearance of corn meal was increased with 10 mM-fumarate, but did not change with 4 and 7 mM-fumarate (Carro and Ranilla, 2003). In vitro gas produ-

| Parameters                                      | Treatment                                      | SEM | P       |
|-------------------------------------------------|-----------------------------------------------|-----|---------|
| pH                                              | MA 0, MA 0.5, MA 1.0, MA 1.5                  |     |         |
| Butyric acid, g kg⁻¹ DM                         | 11.64b                                      |     |         |
| Acetic acid, g kg⁻¹ DM                          | 4.62                                        |     |         |
| Propionic acid, g kg⁻¹ DM                       | 1.16<0.01b                                  |     |         |
| Iso-butyric acid, g kg⁻¹ DM                     | 4.45                                        |     |         |
| Butyric acid, g kg⁻¹ DM                         | 1.79                                        |     |         |
| Iso-valeric acid, g kg⁻¹ DM                     | 1.63<0.01b                                  |     |         |
| pH                                              | 4.11                                        |     |         |
| Total gas production, mL/200 mg DM              | 28.33                                        |     |         |
| Methane, %/200 mg DM                            | 18.34<18.60                                 |     |         |
| Methane, %/200 mg DM                            | 100.00                                      |     |         |
| OMD, %                                          | 18.40                                       |     |         |
| OMD, %                                          | 16.60                                       |     |         |
| OMD, %                                          | 88.83                                       |     |         |
| pH                                              | 6.98                                       |     |         |
| pH                                              | 6.98                                       |     |         |
| pH                                              | 6.98                                       |     |         |

**Table 1. Effect of maleic acid addition to corn silage on crude nutrient matter composition.**

**Table 2. Effect of maleic acid addition to corn silage on in vitro total gas and methane production.**

**Table 3. Effect of maleic acid addition to corn silage on in vitro total gas and methane production.**

**Table 4. Effect of maleic acid addition to corn silage on in vitro ME, OMD, and pH at 24 h of incubation.**
tion is influenced by nutrient composition (cell wall components, starch, sugar) of tested feed, the presence of compound inhibiting gas production (such as condensed tannin, microflora and microfauna content of the rumen fluid (donor animal’s diet feeding direction) and provided fermentation quality (Johnson and Johnson, 1995; Goel et al., 2008; Hook et al., 2010).

The linear effect (increased dose-related) of reducing the CH4 emission of MA additions was seen very clearly in the present study. The reduction of CH4 emission in silage supplemented with maleic acid in this study is consistent with the findings of other in vivo (Martin, 1998) and in vitro (Tejido et al., 2005) studies. Lopez et al. (1999) observed that the addition of fumarate to an in vitro continuous culture system (rumen simulation technique) led to a reduction in methane emission of approximately 17%. In threepresent study, 0.5, 1.0, and 1.5% of maleic acid addition to forage corn at ensiling reduced methane concentration as negative linear by up to 16.07, 11.77, and 9.09%, respectively. Li et al. (2012) determined that unsaturated C18 fatty acids (oleic, linoleic, linolenic acids) either alone or in combination with maleic acid decreased in vitro methane production. In a previous study, the addition of disodium maleate (4 and 8 mmol/l, buffer) to low concentrate diet (80:20, % dry matter, forage: concentrate) reduced in vitro CH4 production but did not do so in high concentrate diet (Tejido et al., 2005). Organic acids (fumaric acid, lactic acid, malic acid) have the potential to decrease ruminal methanogenesis by reducing the ruminal protozoa population (Ok et al., 2012). They are also propionate precursors in the succinate-propionate pathway, and act as alternative H2 sinks in the rumen (Newbold et al., 2005).

According to findings of in vitro gas production, ruminal pH was increased with increasing maleic acid addition, which is in agreement with earlier studies using malic acid and its salts (Montano et al., 1999; Foley et al., 2008). Li et al. (2012) found that 5 and 10 mM malic acid added to 60 mL of in vitro fermenter fluid increased ruminal pH. In another a study, 5-15 mM malic acid added to 60 mL of in vitro fermentation fluid did not change ruminal pH (Sirohi et al., 2012). A decrease in ruminal pH may be related to the fact that malic acid compounds reduce lactic acid concentration in the rumen by stimulating lactate utilisation by Selenomonas ruminantium (Toprak and Yilmaz, 2013).

Conclusions

Collectively, the results suggest that maleic acid addition to corn forage at ensiling reduced in vitro methane production in the rumen, increased lactic acid concentration, reduced silage propionic acid and iso-valeric acid concentration, did not affect negatively silage nutrient composition or in vitro ruminal fermentation. Besides, the neutral detergent fibre level of corn silage was decreased with 1.5% maleic acid addition. However, there are needs to further in vivo studies for generalising the conclusions of this study.

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