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EFFECTS OF SUPPLEMENTATION WITH MONENSIN AND VEGETABLE OILS ON *IN VITRO* ENTERIC METHANE PRODUCTION AND RUMEN FERMENTABILITY OF GOATS

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Seven combinations of monensin (33 mg/kg of the dietary DM) and vegetable oils (coconut oil, palm oil, soybean oil, sunflower oil, canola oil and linseed oil) (33 g/kg of the dietary DM) supplementations were tested to reduce *in vitro* enteric methane production in goats. Data showed that all treatments from supplementation of monensin and vegetable oils significantly reduced *in vitro* enteric methane production compared to control from 40.55-48.58%. In addition, total gas production also decreased from7.37-29.47% and number of protozoa decreased from 64.15-75.47% compared with the control. Supplementation of monensin and vegetable oils had no effect on rumen pH, microbial protein, *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD), but had effect on ammonia concentration. Supplementation of monensin and vegetable oils effectively lowered total gas production, enteric methane production and number of protozoa in the rumen. However, the best supplementation was monensin-sunflower oil (MSf) which can reduce total gas production up to 29.47%, enteric methane production up to 48.58%, number of protozoa up to 75.47% compared to control (CD), without affecting the process of fermentation in the rumen.

Keywords: Monensin, vegetable oils, methane, protozoa, rumen fermentability.

INTRODUCTION

Methane is a potent greenhouse gas (GHG) because it has 14.3% contribution on anthropogenic global anthropogenic GHG emissions, second behind carbon dioxide at 76.7% (IPCC, 2008). 17 to 37% of global anthropogenic methane (CH_4) emissions have been estimated from ruminant livestock (Hünerberg *et al*., 2013) and related with livestock production around 10.8% of the global GHG emissions (O'Mara, 2011). Besides cows, goats also have great potential in methane emissions. Level of methane gas emissions from goats is 5 kg/head/year from process of digestion and 0.22 kg/head/year from manure management (IPCC, 2006). Goats can produce CH_4 until 26.9 L/kg of DM intake (Abecia *et al*., 2013). Mean CH_4 yield from individual sheep was 18.4±0.38 g/kg of DM intake (Pinares-Patino *et al*., 2011).

Methane is a product of methanogenic bacteria in the rumen. In ruminant livestock (cattle, buffalo, sheep and goats), organic compounds of feedstuffs are fermented by rumen microbes produce which volatile fatty acids, carbon dioxide (CO_2), hydrogen (H_2) and microbial mass (Haryanto and Thalib, 2009). Through the process of methanogenesis by methanogenic bacteria, CO_2 is reduced with H_2 to form CH_4 (Moss *et al*., 2000). 6.1-7.9% of intake energy (GEI) consumed is lost as CH_4 energy by enteric fermentation of beef heifers (Jordan *et al*., 2006). Decreased production of methane gas (CH_4) from ruminant livestock is a strategy to reduce GHG emissions and to improve the efficiency of feed. Freetly and Brown-Brandl (2013) reported that CH_4 production decreases with increased feed efficiency and Patra (2012) stated that if CH_4 emissions per unit of product can be reduced so total animal production can be increased.

Monensin has been the most studied ionophore and extensively used to manipulate rumen fermentation (Iqbal *et al*., 2008). A meta-analysis showed that monensin (given at 32 mg/kg of DM) in ruminant livestock can reduce CH_4 emissions (Hristov *et al*., 2013). Diets monensin at 33 mg/kg (DM basis) can decrease enteric CH_4 emissions 30% and total ciliate protozoa populations 82.5% (Guan *et al*., 2006).

In previous study, supplementation of vegetable oil can decrease enteric CH_4 production. In cattle, addition of sunflower oil (5% of DM) reduced CH_4 emissions 16.92% (McGinn *et al*., 2004). Mao *et al*. (2010) reported that daily CH_4 production of growing lambs was decreased 13.9% after supplementing with soybean oil (3% of DM). Inclusion of linseed oil (4.5% of DM) decreased enteric CH_4 production 26.9% of feedlot Nellore steers (Fiorentini *et al*., 2014).

The application of ionophores and dietary lipids (including vegetable oils) recommended for mitigation enteric CH_4 emissions because effective, not give long-term effect, safe for the environment and animals (Hristov *et al*., 2013). Supplementations with a combination of monensin and

vegetable oils are expected to be better in reducing enteric CH_4 production from livestock, including goats.

The main purpose of this experiment was to examine effect of supplementation combination of ionophores monensin and vegetable oils on mitigation of enteric CH_4 production of goats.

MATERIALS AND METHODS

***Experimental design and treatments*:** Completely randomized design with 7 treatments and 4 replications was used in this experiment. The treatments were supplemented with ionophores monensin and vegetable oils in the feed. CD = without supplementation (controls substrate), MCo = monensin 33 mg/kg of the dietary DM + coconut oil 33 g/kg of the dietary DM, MP = monensin 33 mg/kg of the dietary DM + palm oil 33 g/kg of the dietary DM, MS = monensin 33 mg/kg of the dietary DM + soybean oil 33 g/kg of the dietary DM, MSf = monensin 33 mg/kg of the dietary DM + sunflower oil 33 g/kg of the dietary DM, MC = monensin 33 mg/kg of the dietary DM + canola oil 33 g/kg of the dietary DM, ML = monensin 33 mg/kg of the dietary DM + linseed oil 33 g/kg of the dietary DM. Concentrations of monensin of 33 mg/kg of the dietary DM equal with 6 mg/L of culture volume and concentrations of vegetable oils of 33 g/kg of the dietary DM equal with 6 g/L of culture volume.

***Goats and rumen fluid collection*:** Three Crossbreed Boer female goats (25.33±1.61 kg) were used as rumen fluid donors for this experiment. Basal diet given to goats at least 14 d before collection of rumen fluid and fed feed twice daily (08:00 and 17:00 h) total 1.2 kg. The basal diet was commercial pellet feed with nutrient content: dry matter 86.94%, crude protein 13.44%, ether extract 1.83%, crude fiber 17.09%, nitrogen free extract 41.17%, ash 13.41% and gross energy 15.13 MJ/kg. Rumen fluid was obtained before afternoon feeding and strained using 4 layers of cheesecloth before saved in a sealed thermos.

***In vitro batch fermentation*:** The substrates (china field grass and corn) for *in vitro* incubation were dried at 55°C and grounded through a 1-mm screen Wiley mill. Prior to the incubation, 500 mg dry substrates (50: 50, china field grass and corn) and supplementation treatments were added to a 165 mL serum bottle (4 replicates per treatment, 28 bottles for methane production test and 28 bottles for in vitro digestibility test). In addition, 8 serum bottles, four for each analysis (4 for methane production test and 4 for *in vitro* digestibility test), without the substrates and treatments were prepared as blanks. Before incubation, all bottles were pre-warmed using water bath at 39°C. In every serum bottle, 40 mL of McDougall's buffer (McDougall, 1948) and 10 mL rumen fluid were added then flushed with CO_2, crimp-sealed with butyl rubber stoppers, and oscillated (125 rpm) using Environ-Shaker incubator at 39°C for 24 h. Fermentation will be stopped after 24 h incubation with placing serum bottles in an ice-water bath for 15 min.

***Measurement of in vitro digestibility and total gas production*:** A water displacement method was used for determination of total gas production. A 50-mL inverted burette connected with an 18-gauge needle was used to puncture the butyl-rubber stopper of every serum bottle for *in vitro* digestibility test, and then determined by measuring the milliliters of water displacement. After release the pressure was centrifuged serum bottle at 3,000 ×*g* for 15 min. After centrifugation throw away the supernatant, and then prepared the pepsin solution (2 g of pepsin (1:10,500 pepsin from porcine stomach mucosa) + 100 mL of 1 *N* HCl, diluting to 1 L use distilled water). In every serum bottle, 50 ml of pepsin solution was added and oscillated (125 rpm) using Environ-Shaker incubator at 39°C for 48 h. Furthermore, samples were filtered with quantitative filter paper ashless (Whatman-Xinhua Co. Ltd., Hangzhou, China), rinsed with water, and dried in a forced-air oven (100°C for 24 h) to determine the residue dry weight. The percent loss in weight was calculated and presented as *in vitro* dry matter digestibility (IVDMD). The residue left was ashed in a muffle furnace (550°C for 6 h) to determine *in vitro* organic matter digestibility (IVOMD).

***Measurement of methane production, rumen pH, number of protozoa and in vitro fermentability*:** CH_4 analysis was measured using CH_4 Gas detector (Wost CH4-IR Model DR95C; Shenzhen Wosaite Technology Co. Ltd., China) by inserting the attached 18 gauge needle through the rubber stopper. The bottles were then uncapped. The pH of the incubation mixture was measured by a pH meter**.** A part from fermentation fluid (5 mL) was used for counting total protozoa numbers in rumen. Then, 0.5 mL of a 20% (v/v) H_2SO_4 solution was added into serum bottles. Fermentation fluids used for the determination of *in vitro* fermentability. If not analyzed immediately, all the samples were stored at -20°C until further processing. Ruminal protozoa were counted by a 0.2-mm depth counting chamber (hemocytometer) with the method of Methyl green Formalin Saline (Ogimoto and Imai, 1981). Before being counted, samples were fixed by the addition of 5 mL/mL of MFS solution. Then, the samples were shaken to ensure homogeneity and stored in dark place at least 30 min and transferred via a pipette to the edge of the cover slip, allowing the 0.2-mm deep chamber to fill by capillary action and thereby ensuring that there is no bubble formation under the cover glass. The primary square of the counting chamber was visualized under a microscope at 40 x magnification. Ammonia concentration was analyzed by the phenol hypochlorite method (Chaney and Marbach, 1962) wherein based on indophenols reaction resulting stable blue color. Sample preparation for NH_3 analysis was performed by taking 0.4 mL of rumen fluid-buffer after 24 h incubation, added 0.2 mL solution A (10% sodium tungstate) and 0.2 mL solution B (H_2SO_4 1 *N*), then centrifuged (15,000 x *g* for 10 min).

Amount of 20 μl of supernatant was taken, added 2.5 mL of solution C (phenol solution) and 2.5 mL of solution D (hypochloride solution). Mixture solution was heated at 40°C for 30 minutes and then read the sample absorbance with the spectrophotometer in the wavelength (l) 630 nm. The standard solution prepared from $(NH_4)_2SO_4$ to determine the standard curve. Fermentation fluid was centrifuged (3,000 rpm for 15 min), and then 1 mL of the supernatant was taken and centrifuged (10,000 rpm for 15 min). The precipitate was used for determination of microbial protein. The pellets were resuspended using a 1 mL 1 *N* NaOH solution, then boiled at a temperature of 90°C for 10 minutes and analyzed for microbial protein with Lowry method using bovine serum albumin (BSA) as the standard (Waterborg, 2002). 1 mL of the test solution was added with 5 mL of 'alkaline solution', and saved at room temperature for 10 min. Then it was added by 0.5 mL of diluted Folin-Ciocalteau reagent. After 30 min the result of absorbance was read with spectrophotometer at 750 nm. The values obtained were converted to milligrams of bacterial mass protein per milliliter of broth. Analysis of VFA was performed by gas chromatography method as followed by Yang and Choong (2001). Sample preparation was conducted by taking samples of rumen fluid buffer after 24 hours incubation and centrifuged (28,000 x *g* for 30 min at 4°C). Supernatant of 1 mL injected into the packed column type Chrompack CP-Wax 52 CB fused silica column (30 m x 0.53 mm i.d., 1.0 mm film thickness) on Gas Chromatograph (GC) (Model 2010, Shimazu, Japan) and equipped with Flame Ionization Detector (FID). Detector temperature was 280°C and injector port temperature was 240°C. The carrier gas used helium with flow rate 3 mL/min. The program for oven temperature was heated at 75°C for 1 min, increased to 180°C at 6°C /min, then raised to 230°C at 10°C /min, and the last held at 230°C for 5 min. Furthermore, VFA concentrations in fermentation fluid samples were calculated used response areas from standards.

***Statistical analysis*:** Data were analyzed with an analysis of variance (ANOVA) and Duncan's new multiple range test used to determine the differences of mean value. The GLM procedure of SAS was used for performing computations (SAS, 2008). Significant differences among the least squares means were declared at $P< 0.05$.

RESULTS

***Total gas and methane production*:** Supplementation with monensin and vegetable oils had effect on total gas and enteric methane (CH_4) production ($P<0.05$). MCo and MSf treatments had significant difference with CD on total gas production but other treatments did not differ with CD. Enteric methane production from all treatments was decreased after supplementation with monensin and vegetable oils, but that effect was not different among the supplementation treatments (Table 1). The results showed that total gas production and enteric methane production decreased from 40.55-48.58% compared to control ($P<0.05$) and MSf was lowest among the treatments. MSf can reduce enteric methane production up to 48.58% compared to CD (63.37 vs. 123.25 mL/L).

***Amount of protozoa*:** The results showed that monensin supplementation and vegetable oils can reduce amount of protozoa in the rumen from 64.15-75.47% compared to control ($P<0.05$). Supplementation treatments had different effect with control ($P<0.05$) but not different each other ($P\geq0.05$) (Table 1). Amount of protozoa from MSf was lowest among the treatments and can reduce up to 75.47% compared to CD (13 vs. 53 x 10^5 cell/mL). Decrease amount of protozoa in line with decrease enteric methane production in the rumen.

***Volatile fatty acid production (VFA)*:** In this study VFA production decreased after supplementation monensin and vegetable oils. All supplementation treatments had lower VFA production than that of control ($P<0.05$), but not

Table 1. Effects of supplementation monensin and vegetable oils on total gas, methane, protozoa and VFA of *in vitro* culture using rumen fluid from goats.

Observed Variables	CD	MCo	MP	MS	MSf	MC	ML	SEM
Total Gas (mL)	39.77^{a}	28.89^{b}	32.24^{ab}	32.24^{ab}	28.05^{b}	35.59^{ab}	36.84^{ab}	0.99
Methane (CH_4) (mL/L)	123.25^{a}	66.55^{b}	68.46^{b}	73.27^{b}	63.37^{b}	65.43^{b}	67.15^{b}	4.16
Protozoa (x 10^5 cells/mL)	53.00^{a}	19.00^{b}	17.00^{b}	14.00^{b}	13.00^{b}	16.00^{b}	16.00^{b}	2.62
Total VFA (mM)	97.24^{a}	80.91^{b}	78.22^{b}	77.78^{b}	76.83^{b}	83.98^{b}	82.69^{b}	1.35
Acetate	48.493^{a}	39.03^{bc}	37.68^{bc}	37.36^{bc}	36.46^{c}	37.62^{bc}	40.89^{b}	0.79
Propionate	26.04^{ab}	26.06^{ab}	25.95^{ab}	26.27^{ab}	26.11^{ab}	29.54^{a}	25.05^{b}	0.39
Butyrate	17.84^{a}	11.77^{b}	10.67^{b}	10.27^{b}	10.41^{b}	12.62^{b}	12.62^{b}	0.50
A:P Ratio	1.87^{a}	1.50^{bc}	1.45^{bc}	1.43^{bc}	1.40^{bc}	1.28^{c}	1.65^{ab}	0.04

^{a,b,c} Values with different superscript in the same row indicate significant differences ($P <0.05$). CD: control. MCo: monensin-coconut oil. MP: monensin-palm oil. MS: monensin-soybean oil. MSf: monensin-sunflower oil. MC: monensin-canola oil. ML: monensin-linseed oil. SEM: standard error of a mean. VFA: volatile fatty acid. A: acetate and P: propionate.

Table 2. Effects of supplementation monensin and vegetable oils on rumen pH, ammonia concentration, microbial protein, IVDMD and IVOMD of *in vitro* cultures using rumen fluid from goats.

Observed Variables	CD	MCo	MP	MS	MSf	MC	ML	SEM
Rumen pH	6.54	6.46	6.49	6.44	6.4	6.41	6.42	0.02
Ammonia N (mg/100 mL)	12.23[a]	9.84[b]	9.51[b]	9.85[b]	9.55[b]	9.90[b]	10.74[ab]	0.21
Microbial Protein (mg/100	31.96	28.48	31.59	28.7	28.02	27.08	29.28	0.51
mL) IVDMD (%)	48.28	46.51	47.6	46.05	45.68	45.68	46.18	0.47
IVOMD (%)	52.76	47.5	50.93	52.08	49.78	49.46	50.81	0.50

[a,b] Values with different superscript in the same row indicate significant differences (P <0.05). CD: control. MCo: monensin-coconut oil. MP: monensin-palm oil. MS: monensin-soybean oil. MSf: monensin-sunflower oil. MC: monensin-canola oil. ML: monensin-linseed oil. SEM: standard error of a mean. IVDMD: *in vitro* dry matter digestibility. IVOMD: *in vitro* organic matter digestibility.

different each other (P≥0.05). However, the result showed that supplementation with monensin and vegetable oils can decrease (P<0.05) A:P ratio where MC was lowest among the treatments, but no significant difference was found between MC and other supplementation treatments (Table 1). A:P ratio from MC had significant difference with CD were 1.28 and 1.87 respectively (*P*<0.05). The statistical analyses showed that no significant difference was found between MC and MSf.

***Parameter of fermentation*:** Supplementation with monensin and vegetable oils had no effect on rumen pH, microbial protein, IVDMD and IVOMD (P≥0.05), but had effect on ammonia concentration (P<0.05). Supplementation treatments on ammonia concentration had different effect with control, however, the multiple comparisons analyses showed that no significant difference was found between supplementation treatments (Table 2). Ammonia concentration from supplementation treatments can reduce from 12.18–22.24% compared to CD (9.51-10.74 vs. 12.23 mg/100 mL).

DISCUSSION

Monensin and vegetable oils supplementation effectively reduces total gas and enteric methane production by methanogenic bacteria in the rumen. This can happen because monensin and vegetable oil can suppress the growth of protozoa. These results indicate that a decrease in protozoa resulted in a decrease of enteric methane production. Supplementation with vegetable oils interferes with the metabolic activity of protozoa because protozoa do not have lipolytic activity as well as bacteria and protozoa eventually led to many dead (Sitoresmi *et al*., 2009). Machmüller *et al*. (2003) stated that methanogenesis influenced by the existence of a mechanism ecto- and endo-symbiosis between ciliate protozoa with methanogens, i.e. through a specific path way in the transfer of H_2 interspecies and affect up to 9-25% in the process of methanogenesis in the rumen. Decrease symbiosis between ciliate protozoa with methanogens cause a decrease in the availability of hydrogen to methane formation (Jordan *et al*., 2006). Decreased availability of hydrogen also caused by decreased production of acetate and increased propionate production, due to supplementation of monensin. Table 1 showed that A:P ratio decreased with supplementation of monensin and vegetable oils, from 1.28-1.65 compared to control 1.87. This effect was lower than the result from Aderinboye *et al*. (2012) by 1.6 due to added monensin 45 mg/kg of the dietary DM compared to control by 2.5 in West African dwarf (WAD) goats. Methane was a good negative correlation with VFA production (methane and propionate (r^2=0774); methane and A:P ratio (r^2=0772)) (Moss *et al*., 2000). Monensin changes the ratio of VFA in the rumen, increase feed efficiency, decrease dry matter intake and increase average daily gain (Duffield *et al*., 2012).

Supplementation of monensin (33 mg/kg of the dietary DM) and vegetable oils (33 g/kg of the dietary DM) in goats can reduce the production of *in vitro* enteric methane (CH_4) up to 48.58% compared to the control. These results are higher than Guan *et al*. (2006) which uses monensin (33 mg/kg of the dietary DM) in cattle by 30% and the use of soybean oil 3% of DM in growing lambs by 13.9% (Mao *et al*., 2010). This suggests that the combination of monensin and vegetable oils is effective to reduce methane production in the rumen. Combinations of monensin-sunflower oil (MSF) most effectively lowering total gas production, enteric methane production and amount of protozoa in the rumen. This occurs due monensin can increased production of propionate and play a role in inhibiting H_2-producing bacteria (Smith *et al*., 2010), other than that sunflower oil can reduce population of protozoa because have rich linoleic acid (C18:2) or linolenic acid (18:3) (Wanapat *et al*., 2011). Subsequently reported by Wanapat *et al*. (2011) that supplementation of sunflower oil 6% of concentrate can reduce amount of protozoa by 21.43%. Results of total gas production from MSF (62.10 mL/g of fermented DM) is much lower than that reported by Smith *et al*. (2010) of 264 mL/g of fermented DM due to supplementation of monensin by *in vitro*(6 mg/L of culture volume).

The process of fermentation in the rumen can run well even if supplemented with monensin and vegetable oils. Almost all

fermentation parameters (rumen pH, microbial protein, IVDMD, IODMD) due to supplementation of monensin and vegetable oils are not significantly different from the control, only the ammonia concentration which showed different results with control. But all still within normal limits the need for passage of fermentation in the rumen. Supplementation monensin and vegetable oils reduced ammonia concentration in the rumen. A reduced ammonia concentration in the rumen occurs due to inhibition of the growth of protozoa, may be because of depressed bacterial lysis (Hanim *et al*., 2009). Concentration of ammonia due to supplementation by monensin and vegetable oils were 9.51-10.74 mg/100mL. This result is lower than reports Otaru *et al*. (2011) by 25.98 mg/100mL (after feeding) due 8% palm oil supplementation on Red Sokoto goats, but relatively similar to the results Bhatt *et al*. (2011) 6 mg/100 mL due to supplementation with coconut oil 25 g/kg of DM in Malpura lambs, Wanapat *et al*. (2011) 13.5mg/100 mL due to supplementation with sunflower oil 6% of concentrate in swamp buffaloes.

Average of rumen pH from this study was 6.46 and little higher than found in reports of Safaei *et al*. (2014) by 6.38 due to supplementation of monensin 30 mg/kg of the dietary DM in Gezhel lambs, Aderinboye *et al*. (2012) by 6.1 due to supplementation of monensin 45 mg/kg of the dietary DM in West African dwarf (WAD) goats. Microbial protein had no difference with controls were 27.08-29.28 vs. 31.96 mg/100 mL. Sitoresmi *et al*. (2009) reported that added vegetable oils until 7.5% of DM substrate (20% king grass + 80% rice bran) had no effect with result of microbial protein 39 mg/100 ml by *in vitro* in crossbreed Ongole. IVDMD and IVOMD were not affected by supplementing monensin and vegetable oils. This result is in line with Smith *et al*. (2010) who reported that added monensin 6 mg/L of culture volume did not affect on IVDMD.

***Conclusion*:** All treatments from supplementation of monensin and vegetable oils significantly reduced enteric methane production compared to control. However, the results of this study have demonstrated that supplementation of monensin and sunflower oil (MSf) was the best treatment for decrease *in vitro* enteric methane production in the rumen from goats. MSf can decrease enteric methane (CH_4) production up to 48.58% compared to CD, but the process of fermentation in the rumen still can run very well.

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