Effect of protected and non-protected corn oil supplementation on \textit{in vitro} rumen fermentation

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\textbf{Abstract.} This study aimed to determine the effect of supplementation of corn oil (CO) and protected corn oil (PCO) mixture using formaldehyde on rumen fermentation. Protection of corn oil was carried out by mixing skim milk powder and CO (2:1) using formaldehyde 1.5\%. Rumen fluid for \textit{in vitro} dry matter digestibility (IVDMD), \textit{in vitro} organic matter digestibility (IVOMD) as well as for \textit{in vitro} gas production was collected from rumen fistulated Bali cattle which has been adapted with elephant grass and wheat bran (60:40). The combination of CO and PCO in several rations was added as the supplement to feed substrate (dry matter basis). Ratios of CO: PCO supplementation that given were T0 (0\%:0\%) as a control, T1 (5\%:0\%), T2 (3.75\%:1.25\%), T3 (2.5\%:2.5\%), T4 (1.25\%:3.75\%), and T5 (0\%:5\%). The T1 and T2 had lower (P<0.05) total gas production at 48 hours incubation and fewer protozoa compared to the other treatments. The T1 had lower (P<0.05) NH\textsubscript{3} and IVDMD.

It is concluded that supplementation with the ratio of 3.75\% CO and 1.25\% PCO reduced the production of total gas and the number of protozoa, but did not decrease the pH, NH\textsubscript{3}, IVDMD, and IVOMD.

1. \textbf{Introduction}

Corn oil contains a high level of polyunsaturated fatty acids (PUFA) \cite{1,2}. During the digestive process in the rumen, PUFA undergo hydrogenation by rumen bacteria to become saturated fatty acids, thus affect the post-ruminal PUFA availability that will be absorbed by the circulatory system \cite{3}. To escape from the ruminal hydrogenation process, PUFA protection is necessary. Protection can be conducted, among others, by encapsulation of fat using protein-bond that have been protected from formaldehyde using aldehyde \cite{4}. Formaldehyde forms a cross-link with the amino acid in the protein, called methylene bridge (\(-\text{CH}_2\)), which make protein resistance from microbial degradation \cite{5}.

Moreover, non-protected oil supplementation can reduce methane (CH\textsubscript{4}) emission from the rumen. Oil inhibits CH\textsubscript{4} formation due to toxic effect on protozoa and methanogens and some other bacteria that digest fiber \cite{6}. Oil contains high unsaturated fatty acid has the double bond which can act as a hydrogen sink in the hydrogenation process \cite{7}. This study was conducted to determine the effect of supplementation of corn oil (CO) and protected corn oil (PCO) mixture in several ratios on fermentation parameters and total gas production using the \textit{in vitro} gas production technique.
2. Materials and methods

2.1. Materials
The materials used in this study were corn oil (CO), skim milk powder, and formaldehyde 37% pro analysis. Feed substrate consisted of elephant grass (Pennisetum purpureum) and wheat bran (60:40), thoroughly homogenized and ground to pass 1 mm screen. Rumen fluid was taken from fistulated Bali cattle.

2.2. Capsulation of corn oil
The capsulation procedure of PUFA in corn oil was based on the method described by Menke and Steingass [9]. The corn oil was mixed evenly with skim milk powder with the ratio of 1:2. Formaldehyde 37% pro analysis was added to the mixture with the level of 1.5% by weight of the mixture, mixed evenly to form a protected corn oil (PCO). Then, CO and PCO combination was added as the supplement to feed substrate based on the dry matter (DM basis). There were six combination treatments of CO:PCO which were evaluated using in vitro gas production technique: T0 (0%:0%) as a control, T1 (5%:0%), T2 (3.75%:1.25%), T3 (2.5%:2.5%), T4 (1.25%:3.75%), and T5 (0%:5%).

2.3. Incubation
The in vitro gas production technique was measured following the procedure described by Menke and Steingass [8]. Rumen fluid was collected before morning feeding from fistulated Bali cattle. The culture medium contained 660 ml rumen fluid, 1095 ml H2O, 730 ml buffer (4 g NH4HCO3 + 35 g NaHCO3 diluted to 1000 ml on H2O), 365 ml macro-mineral (5.7 g Na2HPO4 + 6.2 g KH2PO4 + 0.6 g MgSO4.7 H2O, diluted to 1000 ml on H2O), 0.23 ml micro-mineral (13.2 g CaCl2.2H2O + 10 g MnCl2.4 H2O + 1 g CoCl2.6 H2O + 8 g FeCl3. 6 H2O diluted to 100 ml on H2O), 1 ml resazurin 0.1% (w/v), 60 ml reducing solution (3.7 ml NaOH 1N + 580 mg Na2S.9 H2O diluted to 60 ml on H2O). Before rumen fluid fluid, the medium had been extensively reduced by continuous bubbling CO2 and warmed at 39°C. Thirty milliliters of buffered rumen fluid were anaerobically dispensed into syringes containing 300 g of feed substrate that has been inserted the CO:PCO (weighed according to treatment). Triplicate syringes for each treatment were incubated at 39°C for 48 h.

2.4. Analytic methods
After 48 h incubation, gases were measured and syringe contents were transferred to the centrifuge tubes and centrifuged at 500 x g for 20 min at 4°C. The pH of the medium was determined using a digital pH meter. A milliliter supernatant was preserved by adding 0.8 ml of formaldehyde solution (37% formaldehyde (v/v):0.9% (w/v) NaCl, 1:9) as the sample for protozoa number calculation using hemocytometer according to Diaz et al. [10]. To one milliliter supernatant was added with 1 ml 20% NaCl for ammonia (NH3) analysis according to Weatherburn [11]. Feed substrate of the syringe was used to determine in vitro dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD). Chemical analysis including dry matter and organic matter were measured by AOAC [12].

3. Results and discussion
The effect of protected and non-protected CO supplementation in several ratios on total gas production, pH, NH3, number of protozoa, IVDMD and IVOMD can be seen in Table 1. The study found that T1 and T2 treatments reduced total gas production significantly at 48 hours of incubation (P<0.05). Compared to the control group, the total gas production of T1 and T2 declined by 13.70% (58.25 vs 67.50 ml/300mg) and 11.11% (60.00 vs 67.50 ml/300mg), respectively. Oil supplementation has the ability to hamper the metabolic activity of microbes as it can cover the feed surface, thus, preventing membrane cells of microbes to intact with the feed. Moreover, oil can also disrupt the enzyme production which digests the feed [13].

According to Table 1, the increased number of PCO on the diet was followed by the increment of total gas production. It indicated that formaldehyde has the ability to bind protein from skim milk that
eventually protects the CO on the feed ration. The protein-formaldehyde complex is stable at neutral condition, while labile on acidic condition. The neutrality of rumen liquid enables to maintain the stability of the protein-formaldehyde complex from the hydrolytic enzyme produced by rumen microorganism [14].

Table 1. Total gas production, pH, NH₃, number of protozoa, IVDMD, and IVOMD of feed with supplementation of corn oil (CO) and protected corn oil (PCO) in several ratios

| Parameter                  | T0            | T1            | T2            | T3            | T4            | T5            |
|---------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Total gas production at 48 h, ml/300mg | 67.50±2.53ab | 58.25±1.73a   | 60.00±2.45a   | 62.83±2.27ab  | 67.62±2.85b   | 68.58±2.77b   |
| pH                        | 6.70±0.02     | 6.70±0.02     | 6.72±0.02     | 6.73±0.03     | 6.71±0.03     | 6.72±0.02     |
| NH₃, mg/100ml             | 42.78±2.66b   | 33.81±3.52a   | 38.33±3.01ab  | 40.56±9.27ab  | 40.66±4.43ab  | 39.09±2.94ab  |
| Number of protozoa, 10³ cell/ml | 7.31±0.16c   | 6.12±0.13a   | 6.56±0.25ab  | 6.81±0.10bc  | 6.95±0.16bc  | 7.29±0.55c   |
| IVDMD, %                  | 51.50±0.14    | 45.32±2.65    | 47.01±3.32    | 48.09±2.46    | 48.97±0.22    | 53.68±4.21    |
| IVOMD, %                  | 50.86±0.91b   | 42.59±3.90a   | 45.75±1.82ab  | 46.35±1.51ab  | 47.56±2.57ab  | 51.67±3.45b   |

abc Superscripts within the same row indicate the significant difference (P<0.05)

IVDMD: in vitro dry matter digestibility, IVOMD: in vitro organic matter digestibility, T0: control, T1: CO 5% and PCO 0%, T2: CO 3.75% and PCO 1.25%, T3: CO 2.5% and PCO 2.5%, T4: CO 1.25% and PCO 3.75%, T5: CO 0% and PCO 5%.

The study found that there was no significant difference between CO and PCO supplementation on the acidic level of rumen liquid. It might be a result because of the similar non-structural carbohydrate contents on each treatment group. The acidic level of liquid rumen decreased once high-soluble carbohydrates contained in the feed are available. Feed with a high energy content reduces the rumen acidic level [15]. However, the CO supplementation could affect the fiber-digesting bacteria as indicated by the reduction of total gas production (Table 1) on the 5% CO-supplemented group with no altered acidic level of rumen liquid. Hence, the buffer provided in the study was able to keep the pH stability. Buffer plays a role in maintaining the pH levels during the instability condition [16].

The result showed that the T1 group reduced the NH₃ concentration significantly by 20.97% (P<0.05) compared to the control group. This result is similar to previous report in which the NH₃ production was reduced as an effect of high UFA-containing oil supplementation [17]. High UFA-containing oil affects to reduce of the ruminal NH₃ concentration [18]. The effect may be associated with the oil’s ability to reduce the protozoa population as protozoa can degrade protein in the rumen. Santra et al. [19] also reported that most studies using oil as supplementation reduced the NH₃ concentration. Oil supplementation can decrease protein degradation on rumen, thus reducing the NH₃ concentration. It is supported by Wanapat et al. [20] who obtained an insignificant difference in the NH₃ concentration of buffalo that fed with vegetable oil supplementation.

This study also demonstrated that T1 and T2 treatments reduced the protozoa number of post-fermentation rumen liquid significantly (P<0.05) by 16.28% (6.28 vs 7.31 10³ cells/ml) and 10.26% (6.56 vs 7.31 10³ cells/ml) compared to control group. It indicated that CO supplementation had a toxic effect on protozoa. Protozoa covered with oil will have limited lipolytic activity. As a result, it disturbs the metabolic activity and leads to a great number of dead protozoa [21]. Beauchemin et al. [6] added that oil supplementation will hinder the CH₄ formation due to its toxic effects on protozoa, methanogenic bacteria, and fiber-digesting bacteria. Hristov et al. [22] reported that supplementation monounsaturated fatty acids (MUFA) such as oleic acid and PUFA such as linoleic acid at the 5% level (w/vol) on the same substrate, reduced the number of protozoa significantly by 10.74% and 14.90%.

All treatments did not alter the IVDMD. However, T1 treatment tends to have less IVDMD, while T5 treatment had higher IVOMD. The same trend was also observed on the IVOMD in which T1 treatment reduced IVOMD significantly (P<0.05). As the CO supplementation was replaced with the increased PCO supplementation, the organic matter digestibility tends to increase. It indicated that 5%
supplementation of CO perturbed the fiber-digesting bacteria, demonstrated by the declined organic matter digestibility. The toxicity level is varied, depending on the number and type of the fatty acids [22]. Oil supplementation on feed can impair and prevent the metabolic activity of microbes because of its ability to cover the substrate surface (feed) with the cell membrane of bacteria [13].

4. Conclusion
Supplementation of corn oil and protected corn oil at a certain ratio on feed can affect the parameter of rumen fermentation. The ratio of 3.75% corn oil and 1.25% protected corn oil reduced the number of protozoa without had a negative effect on pH, ammonia concentration, IVDMD, and IVOMD.

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