**Effects of linseed oil and sunflower oil alone or both with fish oil on *in vitro* rumen fermentation and gas production**

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**ABSTRACT**

This study was conducted to test the effects of supplementing different oils on *in vitro* gas and CH4 production, ruminal fermentation, and digestibility. The study was carried out as a completely randomized design using rumen fluid obtained from three non-lactating Holstein Friesian dairy cows. The dietary treatments included: 1) high-concentrate diet without oil addition (Control), 2) linseed oil (LO), 3) sunflower oil (SO), 4) linseed oil and fish oil (LOFO), 5) sunflower oil and fish oil (SOFO), and 6) mixture of linseed oil with sunflower oil as well as fish oil (MIXO). The amount of added oils were at 3% DM. Cumulative gas production was recorded at 2, 4, 6, 8, 10, 12, 18, 24, 36, and 48 h incubation. *In vitro* digestibilities were determined after 48 h incubation. Ruminal pH, NH3-N, VFA, and CH4 values were measured at 0, 2, 4, 6, and 24 h post incubation. Cumulative gas production at 48 h incubation and protozoa population were lower (P<0.01) in the oils added in combination, such as LOFO, SOFO, and MIXO. Methane production was remarkably reduced (P<0.001) after 24 h incubation by oil inclusion (except SO). Inclusion of SOFO had lower total VFA concentration, lower acetate proportion, and higher propionate proportion than the control. Supplementation of LOFO and SOFO declined (P<0.05) microbial protein (MCP) synthesis and *in vitro* digestibilities of true DM, OM, and NDF. Based on this study, it suggests that inclusion of MIXO could maintain ruminal fermentation and digestibility, but could decrease gas and CH4 production.

**Keywords**: linseed oil, sunflower oil, fish oil, rumen fermentation, gas production

**Please note:**

* Abstract: An abstract with a single paragraph of no more than 300 words that should states important objectives, materials, results and principal conclusions of the study. The results were summarized in an understandable form using statistical evidence (P-values). Abbreviations are defined at first use. Exclude references to other works. In the case authors want to add a Figure/Table into the abstract (not more than one Figure/Table), the abstract should not more than 200 words.
* Key words: List up to 5 words in alphabetical order and separated by a comma. Capitalize only proper nouns. Do NOT use abbreviations. Place at the end of the abstract.

## INTRODUCTION

Conjugated linoleic acid (CLA), a group of geometric and positional isomers of linoleic acid (LA) with conjugated double bonds, is a fatty acid (FA) which can detect easily in ruminants-derived foods. Among CLA isomers in dairy products, *cis*-9,*trans*-11 has been known to exert various potent physiological functions such as anti-carcinogenic, anti-diabetic, anti-hypertensive, and anti-obese properties (Koba and Yanagita, 2014). Omega-3 polyunsaturated fatty acids (PUFA) including alpha-linolenic acid (ALA), EPA, and DHA have important roles in anti-atherogenic, anti-inflammatory, immunemodulatory, and anti-arrhythmic properties (Sekikawa et al., 2015). Supplementation of vegetable oils such as linseed oil (LO) and sunflower oil (SO) improved the contents of CLA and ALA in milk (Benchaar et al., 2012; Rego et al., 2009), whereas fish oil (FO) addition in the dairy cattle diet increased milk n-3 PUFA (Vahmani et al., 2013). Moreover, lipid supplementation has been researching extensively as an enteric CH4 mitigation strategy in ruminant feeding system (Hristov et al., 2013; Knapp et al., 2014). Climate Change Central (2010) in Alberta, Canada already recognizes oil addition as a strategy to abate CH4 emission from dairy cattle under their protocols. However, supplemental oil may cause to reduce digestibilities of dry matter (DM) and neutral detergent fiber (NDF) (Patra, 2014), reflecting in decreased animal performance, which may limit the use of oils to mitigate CH4 emissions in ruminants. The effect of added oils on CH4 production depends on the source, FA profile, and inclusion level (Knapp et al., 2014). Therefore, an ideal oil addition which doesn’t have negative effects on digestibility and ruminal fermentation, but still has greater lowering influence on CH4 production should be studied. This study aimed to investigate the effects of supplementing different oils on *in vitro* gas and CH4 production, ruminal fermentation, and digestibility.

## MATERIALS AND METHODS

**Experimental design and treatments:**This experiment was carried out using a syringe gas production technique. The experiment was conducted as a completely randomized design with the treatments included: 1) high-concentrate diet without oil addition (Control), 2) linseed oil (LO), 3) sunflower oil (SO), 4) oil mixture (1:1, w/w) from linseed oil and fish oil (LOFO), 5) oil mixture (1:1, w/w) from sunflower oil and fish oil (SOFO), and 6) oil mixture (1:1:1, w/w) from linseed oil, sunflower oil, and fish oil. Addition of oil alone or mixtures in the current experiment was at 3% DM (15 mg/syringe).

**Substrates, added oils, and rumen inoculum:** Corn silage and concentrate were ground in a Retsch mill to pass a 1-mm mesh prior to analyze for chemical compositions and *in vitro* gas production measurements. The incubation substrate consisted of corn silage and concentrate were mixed at a ratio of 40:60 (w/w, on DM basis). Oils were prepared and added into incubation syringes as an oil-ethanol solution (185:15, v/w).

**Table 1. Chemical composition (%DM) of feeds and oils used in the experiment**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Item | Concentrate1 | Corn silage | LO | SO | FO |
| DM | 90.2 | 23.8 | - | - | - |
| OM | 90.9 | 91.1 | - | - | - |
| CP | 21.1 | 9.61 | - | - | - |
| EE | 3.77 | 1.81 | 100 | 100 | 100 |
| Ash | 9.14 | 8.90 | - | - | - |
| NFC2 | 24.6 | 14.2 | - | - | - |
| NDF | 41.4 | 65.5 | - | - | - |
| ADF | 28.5 | 42.1 | - | - | - |
| Lignin | 3.61 | 4.44 | - | - | - |

1 Ingredients of concentrate included (% DM): 32% cassava distillers dried meal, 20% soybean meal, 17.5% CDDGS, 10% rice bran, 10% wheat bran, 8% molasses and 2.5% Mineral and vitamin mix. 2 Calculated as 100 − (CP + NDF + EE + ash).

Rumen contents were obtained before the morning feeding from three fistulated non-lactating Holstein Friesian dairy cows (approximately 500 kg) fed a maintenance diet based on corn silage and 21% CP concentrate (R:C 70:30, w/w on DM basis). The animals were fed twice daily at 08:30 and 17:00 for 1-week period before taking the rumen contents. Approximately 1,000 ml of rumen liquor obtaining from donor cows were transported in three thermos flasks to the *in vitro* laboratory. The rumen fluid was filtered through 2 layers of cheesecloth into pre-warmed thermos flasks to retain small particles.

**In vitro fermentation:** Medium solution was prepared according to Menke and Steingass (1988) with some minor modifications. Substrates were weighed to 500 mg of DM into 100-ml glass syringes then supplemented with 200 µl of oil-ethanol solutions which providing 15 mg of added oil/syringe. Three blank syringes for gas production were added 200 µl of absolute ethanol (99.99%) without oil supplementation and substrate. Under continuous CO2 flushing, the filtrated rumen fluid was mixed (1:4, v/v) with pre-warmed medium and then introduced (50 ml of rumen fluid and medium mixture) into gastight glass syringes. The lower end of syringes was closed afterward, and the syringes were incubated in a water bath at 39°C for 48 h. Gas volume produced was recorded at 2, 4, 6, 8, 10, 12, 18, 24, 36, and 48 h incubation.

**Sampling, measurements, and chemical analysis:** Rumen fluid was collected at 0, 2, 4, 6, and 24 h post incubation. pH of syringe contents was immediately measured. One milliliter of rumen content was sampled and mixed with 9 ml of 10% formalin solution. Total protozoa were directly counted using hemocytometer following the methods of Galyen (2010). The samples for NH3–N and volatile fatty acids (VFA) analyses were acidified with 1 M sulfuric acid (10/1, v/v), centrifuged at 14,000×g for 15 min, and the supernatant was then stored at – 20°C. At 48 h post inoculation, some syringe samples were stopped to determine *in vitro* true digestibility (IVTD) following the method described by Van Soest & Robertson (1985). Chemical composition including DM, organic matter (OM), crude protein (CP), ether extract (EE) and Ash were determined following the standard methods of AOAC (1998). Acid detergent fiber (ADF) and NDF were determined using the methods described by Van Soest et al. (1991). The rumen NH3–N was determined by Kjeldahl method (AOAC, 1998). The (VFA) were analyzed using gas chromatography.

**Calculations:** Methane concentration was calculated from individual net molar of VFA with the equation proposed by Fievez et al. (2005) as follow: CH4 (mmol) = 0.5 × acetate – 0.25 × propionate + 0.5 × butyrate. IVTD (%) = 100 × (DM of feed used for incubation – NDF residue)/DM of feed used for incubation. IVDNFD (%) = 100 × (NDF of feed used for incubation – NDF residue)/NDF of feed used for incubation. The *in vitro* digestible organic matter (IVDOM) was calculated from the gas produced and chemical composition according to equation of Menke et al. (1979): IVDOM (g/kg DM) = (14.88 + 0.889 × Gp + 0.45 × CP + 0.0651 × XA) × 10, where CP is the crude protein (% of DM), XA is the ash (% of DM), and Gp is the net gas production (ml) from 200 mg (DM of sample) after 24 h incubation. Microbial protein production (MP) was calculated as 19.3 g microbial N per kg IVDOM according to Czerkawski (1986).

**Statistical analysis:** Data on mean values of CH4 production, protozoa population, pH, NH3-N, and VFA were analyzed according to a completely randomized design with the repeated measures (hours) using PROC MIXED procedure of SAS (2002). Data on gas production, MCP, and digestibility were analyzed by ANOVA procedure of SAS (2002) for a completely randomized design. Significant differences among treatment means were assessed by Tukey's multiple comparison tests after a significant F-test at α<0.05.

## RESULTS

**Gas production, methane production, and protozoa:** Table 2 shows that cumulative gas production at 48 h reduced (*P*<0.01) from 12.61 mmol/g DM in the control to 11.8-11.9 mmol/g DM in the combinative oil groups. It was found from this study that oil inclusion as combinative form was more effective to mitigate CH4 emission rather than single form. The SOFO had lower (*P*<0.05) CH4 production than the control. Population of ruminal protozoa were numerously decreased (*P*<0.01) in the combinative oil groups (6.93-8.20×105 cfu/ml) compared to 1.08×106 cfu/ml in the control.

**Table 2. Gas production, CH4 production, and protozoa**

| Item | Treatment | SEM | *P*-value |
| --- | --- | --- | --- |
| Control | LO | SO | LOFO | SOFO | MIXO |
| Gas (48 h) |  |  |  |  |  |  |  |  |
| ml/g DM | 282a | 271ab | 270ab | 264b | 264b | 267b | 8.27 | 0.005 |
| mmol | 6.31a | 6.04ab | 6.03ab | 5.89b | 5.89b | 5.95b | 0.19 | 0.004 |
| mmol/g DM | 12.6a | 12.1ab | 12.1ab | 11.8b | 11.8b | 11.9b | 0.37 | 0.005 |
| Methane |  |  |  |  |  |  |  |  |
| mmol | 0.74a | 0.68ab | 0.67ab | 0.60ab | 0.54b | 0.63ab | 0.10 | 0.009 |
| mmol/g DM | 1.48a | 1.35ab | 1.33ab | 1.21ab | 1.09b | 1.26ab | 0.20 | 0.009 |
| Protozoa (×105 cfu/ml) | 10.8a | 8.33b | 8.93ab | 8.20b | 6.93b | 7.50b | 1.92 | 0.003 |

a-b Means within a row with different superscripts are significantly different at *P*<0.05 (*n*=6 for gas production and *n*=3 for methane production and protozoa).

**Table 3. Volatile fatty acid production, nitrogen metabolism, and digestibility**

|  |  |  |  |
| --- | --- | --- | --- |
| Item | Treatment | SEM | *P*-value |
| Control | LO | SO | LOFO | SOFO | MIXO |
| pH | 6.63a | 6.61ab | 6.61ab | 6.61ab | 6.60b | 6.60ab | 0.02 | 0.029 |
| Volatile fatty acid |  |  |  |  |  |  |  |  |
| Total (mmol) | 2.00a | 1.85ab | 1.84a | 1.66ab | 1.51b | 1.74ab | 0.27 | 0.014 |
| Total (mmol/g DM) | 3.99a | 3.71ab | 3.68a | 3.33ab | 3.02b | 3.49ab | 0.54 | 0.014 |
| Acetate, C2 (%) | 71.5a | 71.2a | 70.9ab | 71.0ab | 70.5b | 70.9ab | 0.66 | 0.008 |
| Propionate, C3 (%) | 18.0c | 18.6b | 18.9ab | 18.8ab | 19.1a | 19.1a | 0.33 | <0.001 |
| Butyrate (%) | 10.5 | 10.2 | 10.2 | 10.2 | 10.4 | 10.1 | 0.43 | 0.078 |
| C2/C3 ratio | 4.03a | 3.87b | 3.82bc | 3.83bc | 3.74c | 3.78bc | 0.11 | <0.001 |
| Nitrogen metabolism |  |  |  |  |  |  |  |  |
| NH3-N (mg N/dl) | 27.0 | 26.8 | 26.7 | 26.7 | 26.6 | 26.7 | 0.64 | 0.550 |
| MCP (g/kg OM) | 12.5a | 12.3ab | 12.2ab | 12.0b | 12.0b | 12.2ab | 0.26 | 0.018 |
| Digestibility (%) |  |  |  |  |  |  |  |  |
| IVTD | 63.9a | 63.7ab | 61.8ab | 59.8b | 59.7b | 62.6ab | 1.66 | 0.032 |
| IVOMD | 64.8a | 63.9ab | 62.3ab | 62.3b | 62.3b | 63.2ab | 1.33 | 0.018 |
| IVNDFD | 29.2a | 28.8ab | 25.1ab | 21.2b | 21.1b | 26.7ab | 3.26 | 0.032 |

a-b Means within a row with different superscripts are significantly different at *P*<0.05 (*n*=6 for MCP and IVOMD and *n*=3 for other parameters).

**Volatile fatty acids, nitrogen metabolism, and digestibility:** Oil supplementation influenced on VFA production, microbial protein synthesis as well as nutrient digestibility (Table 3). The inclusion of SOFO had lower total VFA concentration (3.02 mmol/g DM) than the control (3.99 mmol/g DM). The molar proportions of acetate and propionate were strongly modified (*P*<0.01) by SOFO supplementation, whereas butyrate proportion seemed to be less effect by additional oils (*P*=0.078). Addition of SOFO resulted in decreased acetate proportion (70.46%) but increased propionate proportion (19.1%) compared to those in the control (71.5 and 18.01%, respectively). These accompanied by decreasing of C2 to C3 ratio in the SOFO (3.74) compared to the control (4.03). Combinative oil inclusion had strongly modified MCP, but individual oil inclusion did not. Supplementation of LOFO and SOFO showed 0.48-0.49 g MCP/kg OM decrease relative to the control (*P*<0.05). Compared to the control moreover, supplementing LOFO and SOFO declined (*P*<0.05) IVTD, IVOMD, and IVNDFD. Inclusion of MIXO showed intermediate results of ruminal fermentation, microbial protein synthesis, and digestibility.

## DISCUSSION

**Gas and methane production:** Castagnino et al. (2015) reported that inclusion of soybean oil or linseed oil at 80 g/kg DM strongly decreased CH4 production through direct effect on rumen methanogens. However, addition of LO or SO alone at 3% DM in the current study didn’t show different effect on gas and CH4 production. This might be due to the lower amounts of linseed and sunflower oils were used in this experiment. As expectation, treatments contained either LO or SO or both combined with FO reduced total gas production after 48 h incubation and protozoa population, whereas only mixture of both SO and FO showed greater reduction of CH4 production. Similar result was also found in the *in vitro* research of Toral et al. (2009). The greater effects of oil mixtures on gas production and protozoa than those in the control and individual oils might be a result of FO inclusion. Supporting this finding, Fievez et al. (2003) concluded that FO had high potent to mitigate CH4 production through reduced ruminal methanogenesis. However, Pirondini et al. (2015) didn’t see any significant effect on CH4 production as FO was added at low amount (0.8% DM) into the high or low starch contained-diets. A meta-analysis of Patra (2013) showed that CH4 depression could be only detected when dietary lipid content more than 5%. In this study therefore the treatments containing 5.05% EE (2.05% from substrates and 3.00% from added oils) were high enough to observe the depression of gas and CH4 production. Rumen protozoa are responsible for symbiotic transfers of H2 with methanogens, which is used to reduce CO2–CH4 (Newbold et al., 1995). The decreased CH4 production in the SOFO probably happened due to the UFA profile of these oil sources since supplementation of unsaturated oils can increase H2 consumption by BH (Czerkawski, 1972). In addition, UFA may reduce protozoa counts, hence protozoa-associated methanogens, and may be also direct inhibitory effect on the membrane transport of methanogens (Beauchemin et al., 2008). The greater mitigation of ruminal protozoa population, reflecting on reduced methanogens in the treatments of LO or/and SO with FO than those added oils alone seemed the results of synergistic effect of oil combination. This was also supported by Soliva et al. (2004). The observed decrease of ruminal protozoa in this study were a result of oil supplementation rich in UFA. In fact, dietary lipids are almost hydrolyzed in the rumen by microbial lipases, releasing free LCFA that may inhibit activity of ruminal microorganisms. Among of LCFA, UFA are more antimicrobial than SFA (Harfoot & Hazlewood, 1997). Microbial toxicity of EPA and DHA has been reported to be greater than those from ALA and LA (Maia et al., 2007). In other words, FO has higher toxicity to rumen microbes than LO and SO.

**Ruminal fermentation and digestibility:** Mean pH was significantly lower (6.60) in the SOFO, and reduction was probably large enough to cause a disturbance in bacterial populations due to numerous decrease occurred on VFA production. The effect of oil supplementation on ruminal VFA content is inconsistent in previous studies. That reduced total VFA concentration in the SOFO was in line with evidence showing low VFA content with the inclusion of SO and FO mixture into rumen incubations (Toral et al., 2009), whereas VFA content showed a higher result with SO supplementation (Razzaghi et al., 2015). AbuGhazaleh & Ishlak (2014) and Pirondini et al. (2015) didn’t find any change of total VFA concentration by FO addition. Concerning particular VFA proportions, supplementation of SOFO caused shift of rumen fermentation towards increase of propionate at the expense of acetate with no change in butyrate. The increase in molar proportion of ruminal propionate in the SOFO treatment is a consequence of a decrease in acetate molar proportion rather than an increase in propionate concentration. The unsaturated oil addition has been reported increase in propionate proportion and decrease in acetate proportion (Razzaghi et al., 2015; Shingfield et al., 2011). The reduced acetate proportion by SOFO in this study suggests that the growth of predominant cellulolytic bacteria, such as *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens,* which are considered as acetate-producing bacteria, have been more inhibited by PUFA from these oils (Huws et al., 2010; Maia et al., 2007). The lower production of microbial protein in the LOFO and SOFO suggested that these oil compounds not only affected protozoa counts but also ruminal bacteria which involve microbial protein synthesis. The lower ruminal VFA production could be accompanied by lower digestibility. It was found in this study that the LOFO and SOFO decreased *in vitro* digestibility, especially NDFD, reflecting on the negative influence of double bonds in the UFA present in these treatments. Supplementation of oils rich in UFA such as EPA, DHA, ALA, and LA can be harmful to microbial membrane in the rumen and cause metabolic disorders, mainly in fibrolytic bacterial populations (Huws et al., 2010; Patra & Yu, 2013; Yang et al., 2009).

## CONCLUSIONS

The supplementation of LOFO, SOFO, and MIXO at 3% DM showed a good strategy to reduce gas and CH4 production. Both LOFO and SOFO showed disturbances in microbial protein synthesis and nutrient digestibility in the rumen, but only SOFO impaired total VFA concentration. However, rumen function was maintained with MIXO inclusion, To reduce gas and CH4 production without affecting ruminal fermentation and digestibility, an ideal oil addition would be MIXO at 3% DM. Moreover, to understand deeply the effects of this oil compound on shift of ruminal FA and microbial diversity, further aspects involving ruminal biohydrogenation and molecular-based studies would be advisable.

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