Dietary Supplementation of Phytoncide and Soybean Oil Increases Milk Conjugated Linoleic Acid and Depresses Methane Emissions in Lactating Holstein Cows

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Abstract

The objective of this study was to determine whether adding phytoncide oil (PO) and soybean oil (SBO) to the dairy cow diet could increase milk conjugated linoleic acid (CLA) and depress methane (CH_4) emissions in lactating Holstein cows. Rumen fermentation was conducted at four levels of SBO (0, 1, 2, and 4%, on DM basis) and two levels of PO (0 and 0.1%, on DM basis) with *in vitro* experiment. To evaluate blood parameters, fecal microbe population, milk yield fatty acid compositions, and methane production, *in vivo* experiment was conducted using 38 Holstein dairy cows divided into two groups of control (fed TMR) and treatment (fed TMR with 0.1% PO and 2% SBO as DM basis). In the *in vitro* study (Experiment 1), PO or SBO did not affect ruminal pH. However, SBO tended to decrease ruminal ammonia-N ($p = 0.099$). Additionally, PO or SBO significantly decreased total gas production ($p = 0.041$ and $p = 0.034$, respectively). Both PO and SBO significantly decreased methane production ($p < 0.05$). In addition, PO significantly increased both CLA (c9, t11 and t10, c12 CLA) ($p < 0.001$). Collectively, 0.1% PO and 2% SBO was selected that improved CLA and methane production most effectively and did not induce abnormal ruminal fermentation. In the *in vivo* study (Experiment 2), 0.1% PO with 2% SBO (PSO) did not affect CBC. However, it decreased BUN and MG levels in blood ($p = 0.021$ and $p = 0.01$, respectively). PSO treatment decreased pathogenic microbes ($p < 0.05$). It increased milk yield ($p = 0.017$) but decreased percentage of milk fat ($p = 0.013$) and decreased MUN level ($p < 0.01$). In addition, PSO treatment increased both CLA and PUFA in milk ($p < 0.01$). Finally, it decreased methane emissions from dairy cows. These results provide compelling evidence that PSO supplemented with a TMR-based diet can simultaneously increase CLA synthesis and decrease methane production with no influence on the percentage of milk fat in lactating Holstein cows.

Introduction

Recently, research on various health and eco-friendly substances is being conducted due to the ever-increasing interest in consumer health. Several studies have shown that plant or plant extracts can increase production quality in cows, including conjugated linoleic acid (CLA) ¹. CLA is known as a functional natural product that is produced in the rumen. It has been proven to have many beneficial effects on the health of humans. In addition, CLA has been shown to be an effective anticancer agent ². It is an antioxidant ³. It can improve atherosclerosis ⁴ and immune response ⁵. CLA can also lower cholesterol ⁶ and prevent diabetes ⁷. According to previous research, although CLA form may vary greatly, cis-9, trans-11 CLA is the main form and its role is the greatest among all forms. Interestingly, cis-9, trans-11 CLA levels in ruminant products are higher than those in other animal products. This is because the cis-9, trans-11 CLA can be produced by microbes in the rumen of ruminant. To increase cis-9, trans-11 CLA levels in ruminant products, lots of soybean and sunflower compounds have been used because they contain high levels of linoleic acid ^{8,9}. Their extract in oil form is known to be the direct source that can increase CLA ¹⁰ and inhibit methane production ^{11,12}. Although such sources of plant oil contain considerable linoleic acid, they also have a negative effect of increasing trans-10, cis-12 CLA which inhibits the synthesis of milk fatty acids ^{13–15}. Phytoncide oil (PO) has attracted attention as an additional source to deal with such problem. POs are secondary metabolites from many plants ¹⁶. PO has been reported to show antimicrobial properties similar to tannin and saponin ^{17,18}. This is because PO contains a large amount of β -carotene which is effective in regulating Gram-negative and Gram-positive bacteria ¹⁹ known to participate in the metabolism of fatty acid in rumen, termed bio-hydrogenation.

From what we discussed above and according to Oh et al. ²⁰, plant extracts can increase propionate and decrease acetate. Propionate can regulate methane production in rumen ^{20,21}. Biohydrogenation is involved in the metabolism to produce CLA from linoleic acid. It is related to the formulation of methane and volatile fatty acid (VFA) because of their competition with hydrogen ion ^{12,22}. Based on our previous study, we hypothesized that combining soybean oil (SBO) with phytoncide oil from pine nut cone (PO) could increase CLA yield and decrease methane production, while maintaining or increasing milk fat, by regulating the formulation of methane and VFA in rumen. There is a lack of information as to whether phytocides can affect final ruminant production and reduce methane emissions. Therefore, the objective of this study was to investigate *in vitro* and *in vivo* effects of phytocide oil plus soybean oil on increasing milk CLA and depressing methane emissions in lactating Holstein cows.

Materials And Methods

Experimental materials and procedures for *in vitro* experiment

The procedures involving animals in this study followed the ARRIVE guidelines. All cannulation procedures involving animals were performed according to the Animal Experimental Guidelines in Konkuk University and the ethical approval was obtained by the Animal Care and Use Committee (IACUC) of Konkuk University, Republic of Korea (Approval No.: KU18058).

The ruminal fluid was collected after filtering with two layers of cheese cloth through rumen cannula at 2 hours before feeding. The collected ruminal fluid was kept in a 2 L thermos bottle that was filled with preheated CO_2 gas and with oxygen (O_2) completely removed, maintained at 39 °C, and then moved to the research lab. After removing feed particles by vacuum pump, the supernatant was used for the test.

The basal diet was dried in a drying oven (JS-CO2-AT100, Johnsam Co., Ltd. Bucheon, Korea) at 60°C for 24 hours. The forage and concentrate were then crushed with a 2 mm screen and used as a TMR at a ratio 6:4. Then 0.2 g of TMR and 20 mL of anaerobic McDougall buffer ²³ were added to a 60 mL serum bottle containing 10 mL of ruminal fluid. After adding 30 mL of culture solution to the culture bottle, two levels of PO (0 and 0.1% on DM basis arranged by Phylus Co., Ltd. Seoul, Korea) were added according to each treatment and four levels of SBO (0, 1, 2, and 4% on DM basis arranged by Haepyo Co., Ltd) as a precursor of CLA in the rumen were added, respectively. Each ruminal sample was kept at the maximum anaerobic conditions by flushing with CO_2 gas and capping with a rubber cap and an aluminum cap to prevent gas leakage as much as possible. The experiment was conducted with 8 treatments (three replicates per treatment) at time intervals of 0, 6, 12, 24, 48 h in a shaking incubator (JSP Corp, SI-900R; 100 rpm) at 39°C.

Analysis

After each incubation, total gas and methane were detected and the portion of ruminal sample was distributed for analysis of pH, VFA, ammonia-N, and fatty acid. Measurement of total gas was conducted using a 50 mL gas cylinder (Habdong Co., Anyang, Korea). Methane was detected using a Gas Chromatography/TCD (HP 6890, Hewlett Packard Co., CA, USA) equipped with an HP-PLOT-A column (dimension: 30 m × 0.32 mm × 20.00 µm) according to Owen et al.²⁴. Culture solution of 2 mL was collected for ammonia (1 mL) and VFA analyses (1 mL). All samples collected were kept frozen at -20 °C until analyzed. Ammonia concentration was determined with the method of Fawcett and Scott²⁵ using a spectrophotometer (Model 680, BIO-RAD, CA, USA). Culture solution of 1 mL was mixed with 0.1 mL 25% phosphoric acid. Then 0.2 mL pivalic acid solution (2%, w/v) was added as an internal standard. The mixed solution was centrifuged at 15,000 × *g* for 15 min and the supernatant was used to determine the concentration and composition of VFA using a gas chromatograph (HP 6890, Agilent Technologies, Santa Clara, CA, USA). Incubation solution of 20 mL for long chain fatty acids was also collected at each incubation time and freeze dried. Lipids were extracted using Folch's solution and analyzed with the gas chromatograph/FID (HP 7890 series GC System, Agilent technologies, California, USA) equipped with an Sp-2560 capillary column (dimension: 100 m × 0.25 mm × 0.2 µm film thickness).

Animals and design for in vivo experiment

A total of 38 Holstein dairy cows (milk fat: 5.0 ± 0.30%; milk yield: 37.1 ± 2.85 kg/d/head; days in milk: 99.4 ± 24.32; parity: 3.1 ± 0.68) were randomly assigned to two groups to analyze milk fatty acids, methane production, fecal microbe population, and blood parameters and studied for 35 days. The cows were housed in a roofed shelter equipped with sunlight-roof and bedded with dry manure for comfort (4.5 m² per cow). Sunlight-roof covered the barn and winch curtain helped ventilation. The cows were fed in mangers and each pen was equipped with separate water throughs. The procedures involving animals in this study followed the ARRIVE guidelines. The ethical procedure was according to the Animal Experimental Guidelines in Konkuk University and approved by the Animal Care and Use Committee (IACUC) of Konkuk University, Republic of Korea (Approval No.: KU18058).

Diets and treatments

Dairy cows were fed total mixed ration (76.8%), roughage (8.4%), and concentrated feed (14.8%). These experimental diets were formulated to meet or exceed the NRC recommendations²⁶. They were provided for *ad libitum* intake. Supplemental Table S1 shows ingredients and chemical compositions of the experimental diet. According to results of *in vitro* experiment, PSO (0.1% PO and 2% SBO on DM basis) were mixed with the vehicle and added to the TMR by top dressing. The control group (Control) was fed TMR added with the same amount of vehicle (not including PO or SBO).

Feed samples were analyzed for dry matter (DM), crude protein, ether extract, crude fiber, crude ash, neutral detergent fiber (NDF), and acid detergent fiber (ADF) according to AOAC (1990) procedures. DM content was determined by drying samples in a vacuum oven at 100°C overnight. Ash content was determined by incineration at 550°C overnight in a muffle furnace. Crude protein (N×6.25) was determined using the Kjeltac System (Kjeltac 2400, FOSS, Hillerød, Denmark). Crude fiber, NDF, and ADF contents of feed samples were analyzed using a Fibertec System (Fibertec 2010, FOSS, Hillerød, Denmark). Ether extract contents were determined using an ether extraction system (ANKOM XT15 Extractor, ANKOM Technology, NY, USA). Mineral contents of the feed were determined with inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo, Waltham, MA, USA).

Blood collection and analysis

For blood cell counting and metabolite analysis, blood samples were collected from each Holstein dairy cow via a jugular vein at 3 h before feeding and at 0, 21, and 35 days after feeding. Using EDTA tube (BD 367844 Vacutainer, Becton Dickinson, NJ, USA), whole blood was subjected to complete blood count (CBC) test using a VetScan HM2 Hematology System (Abaxis, Union City, CA, USA). In addition, the plasma portion of blood was obtained by using heparin tube (BD 367874 Vacutainer, Becton Dickinson, NJ, USA) and centrifuging at 3,000 rpm for 15 min in order to determine blood metabolites using a Chemistry analyzer (Furuno CA-270, Nishinomiya, Japan).

Fecal collection and analysis of microbes

Feces were collected on the same day at the same time as blood sampling. They were obtained from each lactating Holstein cow from the rectum, kept in liquid nitrogen, transferred into ice box at -4°C, transported to laboratory, and maintained in a deep freezer (-80°C) until analysis. One gram of fecal sample was diluted with 10 mL of 0.85% NaCl solution. Then 1 mL of the mixture was subjected to ten-fold serial dilution (10¹ to 10¹¹). The dilution was vigorously shaken with a vortex mixer. Then 0.1 mL of the dilution was spread onto a agar plate. Enumeration of bacteria was performed on MRS agar plates for *Lactobacillus spp.* and LB agar (Luria-Bertani broth diluted with bacto agar) plates for *bacillus spp.* Plates were incubated in anaerobic condition at 37°C for 24 h. Under the same condition, enumeration was done for pathogenic microbes *Salmonella* and *E. coli* on MacConkey agar plates. The number of colony-

$$\log_{10} \frac{CFU}{g} \text{ feces.}$$

forming unit (CFU) was expressed as

Methane emission analysis

At 0, 7, 21, and 35 d, measurement of methane emission was conducted using Laser Methane mini-Green (LMm-G; Tokyo gas engineering solution, Tokyo, Japan). Between 10:00 and 11:00 hours when gas emission was the most active during feed intake, methane emission levels from all dairy cows in each group were measured in triplicates. To increase the reliability of measurement, a fixed distance was used for each measurement and external environment (temperature, humidity, wind speed) was also measured. Finally, the total ppm value was calculated for each treatment by dividing the value of ppm-m (parts per million × meter) to the distance (meters) measured after derivation.

Milk collection and analysis

During the experimental period, milk was collected twice a day (at 3 am and 3 pm). Total milk yield was then calculated and the mean milk yield for 7 days was measured. At the end of the experiment, average milk yield was calculated. After mixing the collected milk in the morning and afternoon at each sampling day (0, 21 and 35 days), samples were stored at 4°C for milk composition analysis. Subsamples were stored at -20°C for fatty acids analysis.

Milk composition was measured with a MilkoScan (CombiFoss FT+500 S/H, Hillerød, Denmark). Milk lipids were extracted and detected using the same methods as described above for the *in vitro* experiment. Analysis conditions were: Sp-2560 capillary column (dimension: 100 m × 0.25 mm × 0.2 µm film thickness); injection: split, 30:1; heater temperature, 255°C; pressure, 32.64 psi; total flow, 39.5 mL/min; split flow, 36.0 mL/min; injection volume, 1.0 µL; carrier gas, helium 1.2 mL/min; oven program, 70 to 100°C at 5 °C/min and hold for 2 min, then 100 to 175°C at 10 °C/min and hold for 40 min, then 175 to 225°C at 5°C/min and hold for 40 min; detector, FID System; and heater temperature, 260 °C (H₂ flow: 40 mL/min, air flow: 400 mL/min).

Statistical analyses

Results obtained from *in vitro* and *in vivo* experiments were subjected to least squares analysis of variance according to ANOVA and MIXED procedure of SAS (SAS Inst., Inc., Cary, NC, USA), respectively. Duncan multiple range test was used for ranking treatment means within a significant F test. Animals in *in vivo* were randomly assigned to control and treatment groups based on completely randomized design. Statistical differences were considered significant at $P < 0.05$. Differences among means with $0.05 < P < 0.10$ were accepted as representing tendencies of differences.

Results

In vitro experiment

The Changes in ruminal pH, ammonia-N (NH₃-N) and gas production is shown in Table 1. Oil supplementation materials did not affect the pH of the culture solution over the incubation time. No significant differences were observed in ammonia-N concentration by phytoncide oil. However, numerically lower values were observed in phytoncide oil treatments. In contrast, ammonia concentration was increased by SO treatment ($p = 0.099$).

The production of total gas and methane was detected by using GC at 0, 12, 24 and 48 h incubation time. There were significant decreases in the production of total gas and methane among treatment groups ($p < 0.05$). Total gas production was lower ($p = 0.041$ and 0.034 , respectively) when PO or SBO was added, especially from 48 h incubation. Additionally, from 6 h incubation, methane production was lower ($p < 0.05$) in treatment groups containing PO or soybean oil. Except for 12 h incubation, methane was much lower ($p < 0.05$) when both PO and SBO were added to the diet, compared with separate supplementation and the most decreases in methane was observed when 0.1% PO and 2% SBO supplementation.

The properties of volatile fatty acids at 48 h incubation time was analyzed by using GC (Table 2). There was no significant difference in individual VFAs.

In the variation of long chain fatty acids. From 6 to 48 h, the trans vaccenic acid (TVA) was higher ($p < 0.05$) in treatment groups containing PO or SBO, except for 4% dosage (Table 3). Additionally, linoleic acid was higher ($p < 0.001$) at all time in the treatment groups containing PO or SBO. From 24 h incubation, both conjugated linoleic acids (c9, t11 CLA, t10, c12 CLA) were higher ($p < 0.001$, $p < 0.05$), and total CLA was significantly higher ($p < 0.001$) in the PO treatment groups than the non-PO treatment groups. Additionally, soybean oil significantly increased CLA at 24 and 48 h after incubation ($p < 0.05$ at 24 h, $p < 0.001$ at 48 h). Moreover, more higher CLA level from 24 to 48 h was observed in the treatment group containing more SBO with PO ($p < 0.05$).

Collectively, 0.1% PO and 2% SBO supplementation on TMR was selected for *in vivo* study because its dosage indicated most activation of inducing hydrogen ion from methane production to biohydrogenation of fatty acids when considering the result of most decrease in methane and increase in CLA simultaneously with no influence on ruminal fermentation.

In vivo experiment

Blood analysis

The result of CBC analysis was conducted by using whole blood (Supplemental Table S2). There were no significant differences in white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin or mean corpuscular hemoglobin concentration between the PSO treatment and control groups. In the result of the blood metabolic parameters (Supplemental Table S3), There was no significant difference ($p > 0.05$) in most of the parameters, except for blood urea nitrogen (BUN) and magnesium (MG). The two metabolites, BUN and MG, were lower ($p = 0.021$, $p = 0.01$) in the PSO treatment group.

Fecal microbes

The beneficial microbes in the fecal were not affected by the treatment. However, the pathogenic microbes were significantly lower ($p < 0.05$) from 21 to 35d in the fecal of the PSO treatment group (Figure 1).

Milk yield, composition and fatty acids

In the result of milk yield. There was no difference from the initiation of the study until 2 weeks. However, from 15 d, an increase ($p < 0.01$) in milk yield was observed in the PSO group (Table 4). Thus, the total average of milk yield was higher ($p = 0.017$) in the PSO group than in the control group. Although milk fat content (%) was lower in PSO group, 4% FCM was not difference between two groups. In addition, lower value of MUN was observed ($p < 0.01$) in the treatment group (Table 5). Although the metabolites of CLA, stearic acid, or trans vaccenic acid were not changed consistently, cis-9, trans-11 CLA in milk was higher at nearly double ($p < 0.01$), and trans-10, cis-12 CLA was higher ($p < 0.05$) in the treatment group. Additionally, the total CLA was higher ($p < 0.01$) in the PSO group (Table 6).

Methane emission analysis

The analysis of methane emission from the cows was conducted by using laser detector in each group (Figure. 2). There were 4 detection times during the experiment at (0, 7, 21, 35) d. Although the data could not be analyzed statistically because we measured in total for each group with 3 replicates, we could find that lower methane gas emissions was observed in the treatment group than in the control group from 7 to 35 d.

Discussion

In vitro experiment

This *in vitro* experiment was conducted to select optimal dosage of PO and SBO which could be used in *in vivo* experiment by considering the effect of the oils on production of methane, CLA and influence on ruminal fermentation.

In this experiment, ruminal pH was not affected by PO or SBO (Table 1). Some studies have reported that ruminal pH could be variously changed by high-concentrate diets in feed and the addition of fat source. High-concentrate diets supplemented with sunflower oil can promote the fermentation of rumen microbes and consequently decrease ruminal pH²⁷. Doreau and Ferlay²⁸ have reported that ruminal pH does not change even in high-energy diets such as SBO. As mentioned before, the variety of ruminal pH could be affected by changes of ruminal fermentation patterns. Considering that neither SBO nor PO affected ruminal pH value, these sources might not directly affect the environment of ruminal fermentation.

Results of our experiment showed that there was no significant difference in the amount of ammonia concentrate in the group treated with 0% PO at 48 h (Table 1). However, ammonia was affected by SBO (increase of ammonia in SBO supplementation group with PO at 0.1%). The synthesis of ruminal ammonia nitrogen is also known to be influenced by the composition of fat source. Beauchemin et al.²⁹ reported that the supplemented fat sources can be used as an energy source for rumen microbes, affecting ruminal fermentation patterns and the synthesis of microbial protein. However, it is also known that fat additives, which can be used as an energy source, have various effects on ruminal pH and microbial activity, depending on the fat composition²⁸. In this study, when considering that the ammonia-N concentrate was numerically higher in 0% than in 0.1% PO treatments and the ammonia was increased by the SBO at 0.1% PO treatments, it is speculated that PO and SBO affected the synthesis of microbial protein and ammonia-N differently.

Generally, total gas production and methane levels in rumen are negatively correlated in *in vitro* experiments with fat supplement. This is because the fat source can facilitate ruminal lipolysis, resulting in decreased methane and increased total gas production by affecting ruminal microbes^{12,22}. Wettstein et al.³⁰ have reported that fat supplement in diet can reduce total gas production, which means exhausted energy. Thus, they suggested that fat supplement could improve the energy availability of microorganisms by reducing the exhausted energy in rumen. On the other hand, it has been reported that the addition of unsaturated fatty acids in feeds can act as a hydrogen sink in the rumen besides lipolysis, inducing hydrogen ions that could be used for methane production, to the process of converting unsaturated fatty acid into saturated fatty acid²².

In addition, as with PO, the essential oil has antimicrobial effects in the rumen, and results in lowering gas production during ruminal fermentation³¹. Considering all these phenomena, the results of this study indicate that the reduced total gas and methane production were affected by the hydrogen sink effect and especially accelerated the lipolysis effect of the oil; the effect could be confirmed more severely at 0.1% PO and 2% SBO addition (Table 1).

Factors affecting VFA compositions in association with the rumen fermentation environment are various, including the ratio of forage to concentrate, high protein, carbohydrate and fat content in feed, supplemented fat composition, and so on^{30,32}. Depending on the fermentation rate of feed in rumen, ruminal pH and VFA composition changes are associated with each other. For example, the rate of fermentation in the rumen in the case of overfeeding concentrate could be faster than that in the case of feeding low concentrate, resulting in lower ruminal pH, lower levels of acetate and butyrate, and increased propionate ratio in the VFA composition. In particular, it has been reported that oil supplement in feed can be fermented by ruminal microbes and used as an energy source, thereby modifying the VFA composition by reducing the pH in the rumen³³. As previously mentioned, the addition of unsaturated fatty acids in the rumen can increase the production of propionate, act as a hydrogen sink, and reduce methane production. Castillejos et al.³⁴ have reported that antimicrobial materials such as essential oil can increase the ratio of acetate to propionate in calves fed a 6: 4 alfalfa hay: concentrate diet. In contrast, Cardozo et al.³⁵ have observed that the ratio of acetate to propionate is decreased in calves fed a 1: 9 straw: concentrate (based on corn, barley, and soybean meal) diet

supplemented with essential oil. It has been reported that effects of essential oil on ruminal pH and feed composition are very diverse³¹. Considering these results, further research is needed to determine the effect of plant-derived essential oil on ruminal VFAs.

In results of long chain fatty acids in rumen, consecutive changes of linoleic acid were observed (Table 3). In the early stage of fermentation, TVA from linoleic acid was increased by the SBO supplement. CLA was increased by SBO and PO. Linoleic acid, which is found in SBO, is known to produce CLA and TVA through isomerization and biohydrogenation by microorganisms in the rumen³⁶. According to Kairenius et al.³⁷, fatty acid metabolism is affected by the composition of the added fat. Higher degree of unsaturation of the added fat causes more extensive metabolism of fatty acids. In addition, Kim et al.¹⁶ have reported that the addition of not only fat, but also antimicrobial essential oil such as PO, can induce an increase of CLA in the milk of ruminants. Taking all these studies into account, the increase of CLA in the present study might be due to effects of SBO containing a large amount of unsaturated fatty acids and PO. Furthermore, it was speculated that the most activation of inducing hydrogen sink might be occurred by 0.1% PO and 2% SBO addition when considering most decreases in methane and VFAs and increase in CLA production simultaneously.

In vivo experiment

This *in vivo* experiment was carried to confirm the effect of selected supplementation (0.1% PO and 2% SBO) in *in vitro* experiment on increasing milk CLA and depressing methane emission by using dairy cows.

As expected from results of *in vitro* experiment (Table 6), CLA percentage and CLA yield were higher in the PSO group. According to Wang et al.³⁶, CLA in milk could be increased when linoleic acids, the same as SBO, are added to the diet. The problem is that increased t10, c12 CLA often decreases milk fat as a result of increased total CLA. In the current study, milk fat percentage was decreased. However, milk yield was higher in PSO group than control thus, amount of milk fat (kg/d) was not difference between two groups. In addition, total CLA yield was not decreased, although t10, c12 CLA was higher in the treatment group than that in the control group. Considering all these phenomena, PO might have induced more c9, t11 CLA isomerization than t10, c12 CLA isomerization by affecting related microbes (e.g., *Butyrivibrio fibrisolvens*). In addition, increased UFA and PUFA levels were observed at the same time in the treatment group. All these differences between the two groups could be explained by the amount of linoleic acid in added SBO and PO inducing hydrogen ion to isomerization of linoleic acid from what might be methanogenesis results of *in vitro* experiment. Thus, induced isomerization in the treatment group might have increase UFA, PUFA, and CLA compared to the control group. In addition, stearic acid was higher in the PSO group at 35 d. This higher stearic acid could be explained by greater microbe biohydrogenation of linoleic acids in the PSO group fed more linoleic acid than the control group.

In the methane production results, one lack was the measuring methane per pen which was not individual animal, even though we tried to redress this limitation by measuring the methane 3 times during the experiment. However, interestingly, Figure 2 shows the adaptability of cows to the PSO treatment, as it shows considerable increase in 7 d, and gradual increase after 7 d to the end of the experiment. Generally, the production of methane in ruminant is decreased when dietary fat is added, and it is reported that the effect is varies greatly, depending on the constituents or unsaturation of added fat^{12,22}. As mentioned earlier, the addition of unsaturated fatty acids is known to induce fatty acid metabolism and inhibit methane production in rumen microbes. In the result of gas production in *in vitro* experiment, it is observed that the addition of SBO, which contains a large amount of unsaturated fatty acid, decreased methane production, and PO addition resulted in more decrease in methane. Considering the results of fatty acid in rumen and milk, the CLA enhancement effect of the two oil sources confirmed in this study may ultimately lead to a decrease in methane production, by competition of hydrogen ion between biohydrogenation and methane production.

Complete blood cell counting (CBC) is a representative physiological index of animals. It was used in this study for the purpose of verifying the safety of experimental additives through animals. Some researchers have shown that oil supplements can affect animal disease because of the negative effect of unsaturated fatty acid in rumen³⁸. However, in this study, there were no significant differences in results of CBC (white blood cell, lymphocyte, monocyte, or other indices) between control and treatment groups containing PO and SBO. Thus, the addition of PO and SBO supplement to the diet did not lead to disease-related chemical changes in dairy cows (Supplemental Table S2).

Some researchers have found a relationship between fat supplement and blood metabolites^{39,40}. According to Henderson et al.³⁹, fat addition in ruminant diet affects the synthesis of microbial protein in rumen. The added fat is used as an energy source for protein synthesis, resulting in increasing amino acid-N and decreasing ammonia-N in the rumen, eventually reducing nitrogen content in the blood. It is known that fat degraded by lipolysis in rumen is fermented to increase propionate content in VFA compositions, thereby increasing glucose content in the blood by gluconeogenesis⁴¹. Similar to results of previous studies, the present study also showed that the PSO group containing oil supplement in feed had lower blood BUN but higher glucose concentration than the control group (Supplemental Table S3). Blood magnesium was also higher in the oil supplement group compared to the control. In general, most of the digestive and absorption lipid is transferred itself or binding to protein. However, some proportions of the lipid binding to magnesium are presented in the blood. Thus, the higher blood magnesium in our result might be affected by the oil supplement in the diet⁴². The reason why blood Ca showed a tendency to be significant was unknown. This parameter was inconsistent with other plasma outcomes.

Fecal microbes are microorganisms involved in the digestive physiology of livestock. Their digestibilities have been evaluated according to their populations⁴³. Currently, antimicrobial effects of essential oils have been extensively studied in rumen. However, their effects on post-ruminal digestive have not been studied in detail yet. Furthermore, although the essential oil PO used in the experiment is also known to have antimicrobial effects, it is not yet known whether such effect could affect the whole ruminant digestive system⁴⁴. However, as shown in the present study, numbers of pathogens in fecal microbes decreased due to the addition of PO and SBO. This demonstrates that the antimicrobial effect of PO also affects post-ruminal digestive system. Thus, the result of decrease in pathogenic microbes by PSO could support that PO might also be able to improve the energy availability of feed in ruminant (Figure 1).

Generally, some studies have reported that milk production is positively correlated with the energy content in feed ⁴⁵. As expected, this study also showed that the addition of 2% fat source, a high energy source, resulted in an increase in milk yield. Bauman ⁴⁵ has reported that the increase in glucose in the blood is positively correlated with milk yield. In particular, the fat supplement used in this study increased glucose in the blood by increasing propionate in the VFA composition due to the effect of SBO and PO on rumen fermentation in *in vitro* experiment. Furthermore, the antimicrobial effect of PO might have affected fecal microbes. Thus, PO might be able to increase milk yield by improving the energy availability of SBO and basal diet (Table 4).

From milk composition data (Table 5), milk fat content (kg/d) and 4% FCM were similar between control and treatment groups. This is different from the result of Rico and Harvatiné ¹⁵. This might be because SBO has an effect on milk fat depression by increasing trans-10, cis-12 CLA known to inhibit the synthesis of milk fat. In our result, the unchanged milk fat in the treatment group may be explained by the effect of PO on isomerization to cis-9, trans-11 CLA rather than to trans-10, cis-12 CLA. Milk nitrogen is generally influenced by blood nitrogen and ammonia-N in rumen. Thus, decreased MUN in the PSO group could be explained by the flow of decreased BUN in blood (Supplemental Table S3).

In summary, a mixture of 0.1% phytoncide oil (PO) and 2% soybean oil (SBO) in diet can lead to an increase in CLA and a decrease in methane production in rumen by inducing hydrogen ion from methane production to biohydrogenation, especially isomerization of linoleic acid with no change in milk fat. Therefore, a combination of PO and SBO could result in increased milk CLA and depressed methane production with no influence on milk fat.

Declarations

Acknowledgments

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Authors' contributions

TBK and MHB conducted this study and wrote the first draft of the manuscript. JGN and JSL assisted in the farm works, reviewing and editing the manuscript. HGL designed the study and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

Data availability

Datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Tables

Table 1												
Effects of phytoncide essential oil and soybean oil on ruminal pH, NH ₃ -N and gas production												
Item	Incubation time (h)	Treatment ¹								SEM ²	P-value	
		PO, 0 %				PO, 0.1 %					PO	SO
		SO				SO						
		0%	1%	2%	4%	0%	1%	2%	4%			
pH	0	6.29	6.30	6.31	6.29	6.28	6.29	6.30	6.30	0.007	0.225	0.435
	6	6.27	6.29	6.28	6.28	6.25	6.26	6.25	6.27	0.015	0.315	0.288
	12	6.21	6.20	6.21	6.20	6.19	6.19	6.20	6.19	0.008	0.202	0.273
	24	6.18	6.17	6.17	6.17	6.18	6.18	6.16	6.17	0.004	0.236	0.199
	48	6.11	6.12	6.10	6.13	6.12	6.11	6.09	6.10	0.002	0.261	0.325
NH ₃ -N (mg/100mL)	0	12.17	12.14	12.15	12.14	12.25	12.25	12.25	12.25	0.092	0.522	0.211
	6	12.75	12.61	12.68	12.645	15.74	14.31	15.02	14.66	1.037	0.422	0.182
	12	16.43	16.07	16.25	16.16	18.39	18.38	18.38	18.38	1.161	0.164	0.144
	24	20.12	19.53	19.82	19.67	21.04	22.45	21.74	22.09	1.319	0.215	0.101
	48	29.94	29.78	29.86	29.82	23.78	28.53	26.15	27.34	0.753	0.156	0.099*
Total gas (mL)	6	14.31	12.96	10.62	9.45	14.56	11.88	10.31	8.24	0.225	0.136	0.045**
	12	20.03	16.07	15.71	14.58	19.44	15.47	14.77	11.82	0.642	0.115	0.033**
	24	24.85	19.89	20.88	21.51	25.32	18.05	19.24	15.39	0.851	0.215	0.053**
	48	30.50	24.12	25.02	24.75	29.76	23.27	22.62	18.09	0.461	0.041**	0.034**
Methane gas (mL)	6	1.41	1.19	1.00	0.89	0.98	0.89	0.74	0.60	0.034	0.014**	0.01**
	12	2.43	1.55	1.65	1.64	1.65	1.53	1.03	0.95	0.488	0.002**	0.005**
	24	3.19	2.12	2.45	2.51	2.65	1.95	1.63	1.45	0.515	<0.001**	0.003**
	48	4.61	2.57	2.87	3.01	3.42	2.49	1.76	1.95	0.563	<0.001**	0.005**

*P < 0.1, **P < 0.05.

¹Treatments: PO, phytoncide oil; SO, soybean oil, mg.

²SEM: Standard error of the mean.

Effects of phytoncide essential oil and soybean oil on volatile fatty acid (VFA) in rumen fluid

Item	Treatment								SEM	P-value		
	PO, 0 %				PO, 0.1 %					PO	SO	PO × SO
	SO				SO							
	0%	1%	2%	4%	0%	1%	2%	4%				
Individual VFAs (mM) at 48h												
Acetic acid (C2)	90.40	83.82	85.23	86.75	78.25	78.42	77.82	78.43	3.611	0.716	0.265	0.451
Propionic acid (C3)	41.31	38.92	40.12	40.39	39.72	36.85	35.22	37.53	2.643	0.096*	0.243	0.398
Iso-butyric acid (C4)	0.45	0.43	0.43	0.55	0.32	0.36	0.37	0.46	0.551	0.671	0.36	0.256
Butyric acid (C4)	19.59	18.91	20.65	19.99	17.77	18.72	20.37	19.22	1.422	0.491	0.082*	0.682
Iso-valeric acid (C5)	3.73	3.58	3.70	3.94	3.43	3.65	3.67	3.85	0.242	0.888	0.382	0.399
Valeric acid (C5)	2.68	2.54	2.67	2.90	2.66	3.00	2.84	3.10	0.201	0.168	0.486	0.174
A:P (C2/C3)	2.19	2.15	2.12	2.15	1.97	2.13	2.21	2.09	1.37	0.132	0.344	0.281
¹ Treatments: PO, phytoncide oil; SO, soybean oil, mg.												
² SEM: Standard error of the mean.												

Table 3

Effects of phytoncide essential oil and soybean oil on long chain fatty acids (LCFAs) in rumen fluid

LCFA (mg/mL)	Treatment								SEM	P-value		
	PO, 0 %				PO, 0.1 %					PO	SO	PO × SO
	SO				SO							
	0%	1%	2%	4%	0%	1%	2%	4%				
0h												
SA	0.701	0.691	0.731	0.798	0.687	0.697	0.672	0.779	0.021	0.783	0.462	0.911
TVA	0.041	0.044	0.048	0.047	0.036	0.032	0.037	0.042	0.003	0.719	0.910	0.417
LA	0.035	2.061	4.121	7.552	0.036	2.091	4.182	7.572	0.760	<0.001**	<0.001**	0.891
LnA	0.009	0.011	0.013	0.016	0.007	0.008	0.010	0.012	0.002	0.781	0.522	0.150
6h												
SA	0.771	0.760	0.804	0.878	0.756	0.767	0.739	0.857	0.610	0.031	0.512	0.124
TVA	0.039	0.041	0.061	0.098	0.051	0.056	0.091	.081	0.009	<0.001**	<0.001**	0.212
LA	0.021	1.041	2.081	4.111	0.024	1.076	2.151	4.231	0.381	<0.001**	0.032**	0.412
LnA	0.006	0.007	0.009	0.010	0.007	0.008	0.010	0.012	0.001	0.790	0.736	0.124
12h												
SA	0.848	0.836	0.885	0.966	0.831	0.843	0.813	0.943	0.033	0.230	0.731	0.699
TVA	0.042	0.045	0.057	0.075	0.024	0.046	0.066	0.064	0.008	0.080	0.373	0.518
LA	0.012	0.581	1.162	2.116	0.011	0.665	1.329	2.226	0.038	<0.001**	<0.001**	0.525
LnA	0.009	0.011	0.013	0.016	0.007	0.008	0.010	0.012	0.001	0.210	0.125	0.325
24h												
SA	1.601	1.621	1.712	1.801	1.555	1.626	1.623	1.799	0.126	0.337	0.162	0.893
TVA	0.050	0.053	0.068	0.089	0.029	0.055	0.079	0.076	0.053	0.091*	0.119	0.912
LA	0.010	0.465	0.930	1.693	0.009	0.532	1.063	1.781	0.174	<0.001	<0.001	0.411
LnA	0.011	0.013	0.016	0.019	0.008	0.010	0.012	0.015	0.022	0.426	0.621	0.776
Sum of CLAs	ND	0.109	0.161	0.294	ND	0.123	0.269	0.452	0.189	<0.001	0.047**	0.031**
c9,t11 CLA	ND	0.088	0.131	0.242	ND	0.114	0.224	0.381	0.141	<0.001	0.051*	0.128
t10,c12 CLA	ND	0.021	0.030	0.052	ND	0.009	0.045	0.071	0.078	0.031**	<0.001**	0.624
48h												
SA	1.913	1.837	1.996	2.152	1.894	1.921	1.874	2.152	0.127	0.141	0.102	0.451
TVA	0.055	0.059	0.062	0.072	0.055	0.046	0.051	0.072	0.011	0.112	0.049**	0.902
LA	0.009	0.301	0.672	1.092	0.007	0.281	0.728	1.210	0.428	<0.001**	<0.001**	0.268
LnA	0.008	0.009	0.011	0.013	0.006	0.007	0.008	0.010	0.002	0.290	0.217	0.933
Sum of CLAs	ND	0.149	0.298	0.522	ND	0.232	0.472	0.710	0.115	<0.001**	<0.001**	0.045**
c9,t11 CLA	ND	0.121	0.241	0.444	ND	0.201	0.401	0.622	0.108	<0.001**	<0.001**	0.032**
t10,c12 CLA	ND	0.029	0.057	0.078	ND	0.031	0.071	0.088	0.048	0.021**	<0.001**	0.082*
¹Treatments: PO, phytoncide oil; SO, soybean oil, mg.												
²SEM: Standard error of the mean.												
*LA, Linoleic acid (C18:2); LnA, Linolenic acid (C18:3); TVA, trans-11 vaccenic acid (C18:1); SA, Stearic acid (C18:0); LCFAs, Long chain fatty acids; ND, not detectable.												

Table 4

Effect of the mixture material on milk yield in dairy cow

Period (d)	Control	PSO ¹	SEM	P-value
Milk yield, kg				
0	33.6	34.3	1.32	0.807
1–7 (AVG)	34.0	35.0	1.31	0.334
8–14 (AVG)	33.9	35.1	1.25	0.202
15–21 (AVG)	32.7	35.8	1.32	< 0.01
22–28 (AVG)	32.2	35.9	1.37	< 0.01
29–35 (AVG)	32.8	35.4	1.34	0.012
1–35 (AVG)	33.1	35.4	1.30	0.017

¹PSO: group fed TMR with 0.1% PO and 2% SBO as DM basis

Table 5

Changes in milk compositions of dairy cows given diet containing a mixture of 0.1% PO and 2%SO for 35 days

Items ² (%)	Control			SEM	PSO ¹			SEM	P-value		
	0 d	21 d	35 d		0 d	21 d	35 d		PSO (P)	Days (D)	P × D
Milk fat	5.2	5.0	5.3	0.13	5.6	4.2	4.2	0.20	0.021	<0.01	0.013
4 % FCM (kg/d)	38.0	35.4	37.5	1.89	44.4	38.5	38.0	2.32	0.291	0.487	0.734
Milk fat (kg/d)	1.7	1.5	1.7	0.09	2.0	1.6	1.5	0.11	0.576	0.205	0.450
Milk protein	3.4	3.2	3.3	0.04	3.4	3.2	3.4	0.04	0.572	0.007	0.719
Lactose	4.7	4.4	4.7	0.03	4.7	4.4	4.8	0.04	0.973	0.022	0.732
SNF	8.6	8.3	8.7	0.05	8.7	8.2	8.8	0.07	0.694	0.015	0.626
SCs	106.7	193.3	208.5	41.94	128.4	628.2	233.5	141.12	0.277	0.259	0.421
MUN	18.6	17.0	20.7	0.36	18.1	12.4	16.8	0.47	<0.01	<0.01	<0.01
Acetone	0.003	0	0.007	0.00	0	0	0.026	0.01	0.146	<0.01	0.062
BHB	0.001	0	0	0.00	0	0	0.003	0.00	0.531	0.502	0.283
B-Casein	2.6	2.5	2.6	0.03	2.67	2.5	2.62	0.04	0.764	0.011	0.618

¹PSO: group fed TMR with 0.1% PO and 2% SBO as DM basis

²Items: SNF, solid-not-fat; MUN, milk urea nitrogen; BHB, B-Hydroxybutyrate.

Table 6

Changes in milk fatty acids of dairy cows given diet containing a mixture of 0.1% PO and 2% SO for 35 days

items	Control			SEM	PSO ¹			SEM	P-value		
	0 d	21 d	35 d		0 d	21 d	35 d		PSO (P)	Days (D)	P × D
% of total FAs											
STA (stearic acid)	0.12	0.19	0.12	0.010	0.14	0.15	0.17	0.010	0.049	0.017	0.295
TVA (Trans vaccenic acid)	3.03	1.39	0.83	0.417	2.24	1.20	1.10	0.589	<0.01	<0.01	<0.01
C18:2n6t (Linolelaidic)	0.45	0.81	0.21	0.051	0.42	0.80	0.37	0.072	<0.01	<0.01	0.003
C18:2n6c (Linoleic)	0.18	0.15	0.03	0.181	0.42	0.09	0.04	0.162	0.146	0.004	0.378
cis-9, trans-11-CLA ²	0.29	0.38	0.37	0.031	0.39	0.55	0.65	0.044	0.437	<0.01	<0.01
trans-10, cis-12-CLA	0.02	0.02	0.01	0.011	0.06	0.02	0.05	0.015	0.048	0.311	0.041
SFA (Saturated fatty acids)	36.26	37.94	31.54	1.124	33.66	32.67	25.42	1.215	<0.01	<0.01	0.547
UFA (Unsaturated fatty acids)	63.57	61.41	68.28	1.140	68.11	67.90	74.34	1.634	<0.01	0.014	0.911
PUFA	1.64	2.08	1.42	0.103	2.29	2.39	2.10	0.076	<0.01	<0.01	0.387
CLA	0.31	0.39	0.38	0.035	0.45	0.57	0.70	0.049	0.922	<0.01	<0.01
¹ PSO = group fed TMR with 0.1% PO and 2% SBO as DM basis											
² CLA = conjugated linoleic acid.											

Figures

Figure 1

Effects of the mixture on fecal coliform and beneficial microbe population (35 days). Values are expressed as means. Contrasts of the same microbes between Control and PSO in each period are shown (e.g. Beneficial-Control vs Beneficial-PSO). *Differences in superscript indicate significance at $P < 0.05$. Beneficial fecal microbes: *Bacillus* and *L. bacillus* spp; Pathogenic fecal microbes: *E. coli* and *Salmonella* spp.

Figure 2

Changes in methane gas (CH_4) of dairy cows fed with a mixture of 0.1% PO and 2% SO for 35 days. Values are expressed as the mean of methane production from all cows in each treatment.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalTables.docx](#)