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RESEARCH NOTE

Effect of Unconventional Oils on *in Vitro* Rumen Methane Production and Fermentation

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Abstract

M. Embaby, M. Günal, and A. AbuGhazaleh. 2019. Effect of unconventional oils on in vitro rumen methane production and fermentation. Cien. Inv. Agr. 46(3): 276-285. The effects of unconventional oils high in polyunsaturated fatty acids (PUFAs) (blackberry, blueberry, raspberry, pomegranate, black seed and hemp oils) on *in vitro* rumen fermentation and methane (CH_4) production were examined in a 24-h batch culture experiment. Treatments consisted of a control (no oil supplement), a control plus corn oil, or a control plus the unconventional oils. Oils were added to rumen cultures at 500 mg L^{-1} (equivalent to 3.3 g oil.kg^{-1} of dietary dry matter (DM)). After 24 h of incubation, CH_4 production was not different between the control and the corn oil treatment. Of the six unconventional oils tested, only the hemp and blueberry oils reduced CH_4 production by 10-16% relative to that of the control and corn oil treatments. Dry matter degradability and total volatile fatty acids (VFAs) were not affected by the addition of oils. Except for a reduction in the acetate concentration with the raspberry and hemp oils, all tested unconventional oils had no effects on fermentation and the VFA profile relative to those of the control. In conclusion, our results showed that hemp and blueberry oils were moderately effective in reducing rumen CH_4 formation without compromising rumen fermentation and digestibility.

Keywords: Blueberry oil, hemp oil, methane, raspberry oil, rumen culture.

Introduction

The microbial fermentation of feeds consumed by ruminant animals produces methane (CH_4) as a byproduct. Although methanogenesis prevents adverse effects of hydrogen accumulation in the rumen, CH_4 production has a detrimental effect on the atmosphere and represents a loss of feed energy from the diet. Different mitigation strategies to reduce CH_4 emissions from ruminants, either as a direct effect on the methanogenic bacteria or an indirect effect through inhibiting microorganism interaction with methanogenic bacteria, has been examined (Beauchemin *et al.,* 2008). Dietary lipid supplementation was reported to reduce CH_4 production in rumens by reducing rumen ciliated protozoa (Ivan *et*

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al., 2013), reducing the activity of methanogens, or through the use of hydrogen during the biohydrogenation process (Chilliard *et al.,* 2009). However, when lipid supplements were added at levels greater than 4% of the dietary dry matter (DM), the reductions in rumen CH_4 production were also associated with reductions in substrate fermentation and digestibility (Whitney *et al.,* 2000; Wang *et al.,* 2017).

As a result of their anti-microbial properties that could affect methanogenic bacteria or protozoa in the rumen, phenolic compounds, such as flavonoids, are one of the most popular plant extracts that have attracted the interest of researchers to reduce CH_4 emissions (Garcia-Gonzalez *et al.,* 2008; Oskoueian *et al.,* 2013). Pomegranate, black seed, hemp and berry oils are characterized by their high polyunsaturated fatty acid (PUFA) content. The seeds of these oils are also rich in polyphenolic compounds, such as proanthocyanidins, hydrolyzable tannins, and flavonols, that have potent antioxidant and antimicrobial properties (McPartland, 1984; Dahham *et al.,* 2010). During cold processing, these compounds are extracted into cold pressed oil in significant quantities (Parry and Yu, 2004). Although plant oils high in PUFAs, such as rapeseed, sunflower, soybean, corn and linseed oils, have been intensively studied for their potential to reduce CH_4 production (Machmüller *et al.,* 1998; Lillis *et al.,* 2011; Ivan *et al.,* 2013), unconventional oils high in PUFAs and rich in phenolic compounds have not yet been investigated for their efficiency in mitigating rumen CH_4 production. Therefore, the main objective of this study was to investigate the effects of blackberry, blueberry, raspberry, pomegranate, black seed and hemp oils on rumen CH_4 production and fermentation *in-vitro*.

Materials and Methods

This study was approved by the Ethical Committee of the Institutional Animal Care and Use Committee-Southern Illinois University Carbondale, with a tracking number of 18–025.

Oil sources

The six unconventional oils used in our experiment were blackberry (*Rubus fruticosus*) seed oil, blueberry (*Vaccinium corymbosum*) seed oil, raspberry (*Rubus idaeus*) seed oil, pomegranate (*Punica granatum*) seed oil, black seed (*Nigella sativ*a) oil, and hemp (*Cannabis sativa*) seed oil. Oils were acquired from NOW Foods Essential Oil Company (Bloomingdale, IL, USA), Nutiva (Richmond, CA, USA), and Puressant (Missouri city, TX, USA). All oils were unrefined and cold-pressed.

Experimental design, in vitro incubation procedure and laboratory analysis

Rumen fluid was collected 3 h after morning feeding from a lactating cannulated Holstein cow fed a total mixed ration consisting of alfalfa and grass hay mix (600 g kg^{-1}), ground corn (250 g kg^{-1}), soy hulls (100 g kg^{-1}), and soybean meal (50 g kg^{-1}; all on a DM basis). The rumen contents were strained through two layers of cheesecloth (1 mm pore size) and then used within approximately 15 min after collection. Twelve ANKOM gas jars (3 per treatment) containing finely ground (1 mm screen) diet (3 g), strained ruminal fluid (70 ml) and preheated buffer media (130 ml) were used as batch rumen cultures. The buffer was prepared according to Goering and Van Soest (1970). The diet consisted of (on a DM basis) alfalfa hay (250 g kg^{-1}), corn silage (250 g kg^{-1}), ground corn (300 g kg^{-1}), soybean meal (100 g kg^{-1}), soy hulls (80 g kg^{-1}), and a mineral-vitamin mix (20 g kg^{-1}). Treatments were a control (no lipid supplement), a control plus corn oil, and a control plus each of the six unconventional oils (pomegranate oil, raspberry oil, blackberry oil, blueberry oil, black seed oil or hemp oil). Oil supplements were added

to jars at a rate of 500 mg L^{-1} of rumen culture (amounts equivalent to 3.3 g g kg^{-1} of dietary DM), and treatments were run in triplicate. To estimate the effects of treatment diets on DM degradability, approximately 1.5 grams of ground Timothy hay (1 mm screen) weighed into a Dacron bag (5-cm × 10-cm bags with a 20-µm pore size; Ankom Inc., Fairport, NY, USA) and placed in each of the ANKOM jars. After 24 h of incubation, the bags were removed and rinsed in cold water for a total of six rinses. The bags were then dried in an oven at 55 ºC for 48 h, placed in a desiccator for 3 h, and weighed. The samplers and diets were then analyzed for DM (AOAC, 2000).

Each jar was gassed with carbon dioxide (CO_2) before sealing and then connected to a Tedlar gas collection bag (CEL Scientific Corp., Santa Fe Springs, CA, USA). Jars were placed in a water bath at 39 ºC for 24 h. Gasses from jars were programmed to be released into connected bags when the psi exceeded 1.0. Every two hours, the jars were shaken by hand for approximately 30 seconds. After 24 h, gas bags were disconnected from jars and analyzed immediately for gas composition. From each collected gas bag, three separate gas samples were collected using a 1-ml gas tight needle syringe (27G 1 1.4^{-1}; Fisher Scientific, Chicago, IL, USA) and analyzed for gas composition using a gas chromatograph (SRI 8610C, Torrance, CA, USA) equipped with a TCD detector (6" × 0.125" S.S. Shin Carbon) and an ST 80/800 column (2 m × 2 mm ID). The oven temperature was programmed at 38 °C for five min, then increased at 5 °C.min^{-1} to 270 °C and held for five min. The carrier gas was argon. Sample gas peaks (CO_2 and CH_4) were identified by comparing the retention times with those of the corresponding standard (Scotty Analyzed Gases 14, Sigma-Aldrich, St. Louis, MO, USA).

The relative proportion of each gas in the collected gas bags was calculated using the response factor (RF) equation:

$$RF = (CC_i \,.\, Area_i^{-1}) \times (Area_{ref} \cdot CC_{ref}^{-1})$$

where RF is the response factor, CC_i is the proportion of gas *i* in the sample of the gas being tested, $Area_i$ is the area of the gas *i* peak, CC_{ref} is the proportion of the reference gas (helium) in the internal standard, and $Area_{ref}$ is the area of the peak of the reference gas.

To calculate the relative proportion of each gas in the collected gas bags, Avogadro's Law was used:

$$N = P\,(V{\cdot}RT^{-1})$$

Where N is the amount of gas produced in moles, P is the pressure in kilopascals, V is the head–space volume in the gas jars in liters, T is the temperature in Kelvins, and R is the gas constant.

At the end of each experiment, two 5 mL samples were also collected from each culture jar for VFA and ammonia-N (NH_3-N) determination. Collected samples were placed immediately in an ice bath and then stored at -20 °C until analyses. The pH was measured immediately after samples were collected from each jar with a portable pH meter. Samples for VFA analysis were mixed with 1 mL of freshly prepared 25% meta-phosphoric acid and centrifuged (IEC Centra GP8R, Needham Heights, MA, USA) at 20,000 g and 4 °C for 20 min. The supernatant fluid was then collected and analyzed for VFAs using 2-ethylbutyric acid as an internal standard (Jenkins, 1987). A Shimadzu GC-2010 gas chromatograph (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) equipped with a flame-ionization detector and a 30-m SP-2560 fused silica capillary column (Restek Stabil WAX DA column, Bellefonte, PA, USA) was utilized for VFA analysis. The helium carrier gas was maintained at a linear velocity of 23 cm s^{-1}. The oven temperature was programmed to 65 °C for 3 min and increased at 12 °C min^{-1} to a final temperature of 225 °C, which was held for 9 min. The column temperature was maintained at 65 °C, and the flame ionization detector temperature was maintained at 225 °C. For ammonia-N, the 5 mL collected sample was centrifuged at 20,000 g and 4 °C for 10 min. The supernatant

was then acidified with 0.5 mL of 0.1 N HCl and analyzed for ammonia-N, as outlined by Cotta and Russell (1982).

Data were analyzed using the MIXED procedures of SAS (Version 9.1, Statistical Analysis System 2003) using treatment as the fixed effect and replicate as the random effect. Differences among treatment means were tested using PDIFF. All results were expressed as least-square means, and significance was declared at $P<0.05$.

Results

The fatty acid composition for the tested oils is presented in Table 1. Polyunsaturated fatty acids were the dominant fatty acids in all oils, ranging from 65% to 80% of total fatty acids. The highest PUFA levels were in hemp and raspberry oils, and the lowest PUFA levels were in corn oil. Except for pomegranate oil, linoleic acid (C18:2 *c*9*c*12) was the main PUFA in all oils, ranging from 44.7% to 64% of total fatty acids. Linolenic acid (C18:3 *c*9*c*12*c*13) was highest in blueberry oil at approximately 32%. In pomegranate oil, punicic acid (C18:3 *c*9*t*11*c*13) was the main fatty acid at 61% of total fatty acids.

The effects of oils on rumen fermentation and CH_4 production are presented in Table 2 and Figure 1. Relative to the control and corn oil treatments, only the addition of blueberry oil and hemp seed oil reduced ($P<0.05$) CH_4 production, while blackberry oil tended to decrease ($P>0.12$) CH_4 production. The DM degradability, total VFAs, propionate, butyrate and NH_3-N concentrations were all similar ($P>0.05$) across treatments. Relative to that of the control, total gas production decreased ($P<0.05$) only with the addition of corn oil and black seed oil. The acetate concentration was lower ($P<0.05$) in the raspberry oil and hemp oil treatments relative to that of the control.

Discussion

Previous studies reported linear reductions in rumen CH_4 production with the addition of lipid supplements. However, at dietary lipid supplements greater than 4% of dietary DM, the reduction in CH_4 was confounded with a decrease in DM degradability (Whitney *et al.,* 2000; Wang *et al.,* 2017). Therefore, oils in our experiments were added at levels that are reported to have minimum effects on substrate fermentation and digestibility. The addition of corn oil and the unconventional oils to rumen cultures in our experiments had no effects on DM degradability and total VFAs compared with those of the control, suggesting that these oils had no negative effects on rumen fermentation. These effects are consistent with the findings of others who reported little or no effects of oils on fermentation when added at lower levels (Patra, 2013; El-Sherbiny *et al.,* 2016; Roy *et al.,* 2017).

Table 1. Fatty acid composition for oil supplements (g 100 g fatty acid^{-1}).

	C16:0	C18:0	C18:1c9	C18:2 c9c12	C18:3 c9c12c15	C18:3 c9t11c13	PUFA
Corn oil	7.53	0.58	26.31	64.00	0.85	0.00	64.85
Blackberry oil	7.71	2.29	20.40	59.00	9.70	0.00	68.70
Blueberry oil	2.63	0.52	18.74	44.73	32.15	0.00	76.88
Black seed oil	7.71	1.55	22.62	63.71	0.24	0.00	63.95
Hemp oil	3.22	1.03	7.08	60.20	19.38	0.00	79.58
Raspberry oil	2.11	1.05	16.98	60.46	18.54	0.00	79.00
Pomegranate oil	5.79	1.95	15.60	3.83	7.09	61.28	72.20

PUFA= Polyunsaturated fatty acid

Table 2. Effect of oil supplements on dry mater degradability and fermentation.

	Control	Corn oil	Pomegranate oil	Raspberry oil	Blackberry oil	Blueberry oil	Black seed oil	Hemp oil	SEM
Dry matter degradability, %	29.95	28.79	32.74	31.59	31.03	30.69	29.46	28.83	0.767
pH	6.15	6.16	6.18	6.09	6.18	6.11	6.19	6.06	0.12
Gas production, ml	158.72^{a}	140.75^{b}	145.04^{ab}	147.95^{ab}	146.23^{ab}	148.26^{ab}	135.52^{b}	147.62^{ab}	4.604
NH_3-N, mg.dL^{-1}	5.81	6.92	6.43	6.19	6.44	7.93	6.14	7.96	0.84
VFA total, m*M*	42.15	41.86	42.54	38.27	43.14	39.26	38.83	37.36	2.698
Acetate (C2)	17.81^{a}	16.65^{ab}	17.36^{a}	15.26^{b}	18.05^{a}	16.12^{ab}	16.47^{ab}	14.97^{b}	1.037
Propionate(C3)	12.18	12.03	12.25	11.13	13.76	12.1	11.49	10.93	0.859
Butyrate (C4)	9.7	10.03	10.4	9.56	10.63	9.09	9.04	9.16	0.56
Iso-butyrate	0.37^{a}	0.31^{a}	0.35^{a}	0.33^{a}	0.29^{a}	0.29^{a}	0.21^{b}	0.21^{b}	0.031
Valerate	1.2	1.26	1.32	1.27	1.31	1.14	1.18	1.15	0.056
Iso-valerate	0.88	0.9	0.85	0.83	0.47	0.42	0.76	0.76	0.096
C2:C3	1.54^{a}	1.52^{a}	1.42^{ab}	1.40^{b}	1.35^{b}	1.33^{b}	1.43^{ab}	1.37^{b}	0.033

^{ab} Values within rows followed by the same letter are not significantly different at $P<0.05$
VFAs=volatile fatty acids; NH3-N=ammonia nitrogen; SEM= standard error of the mean

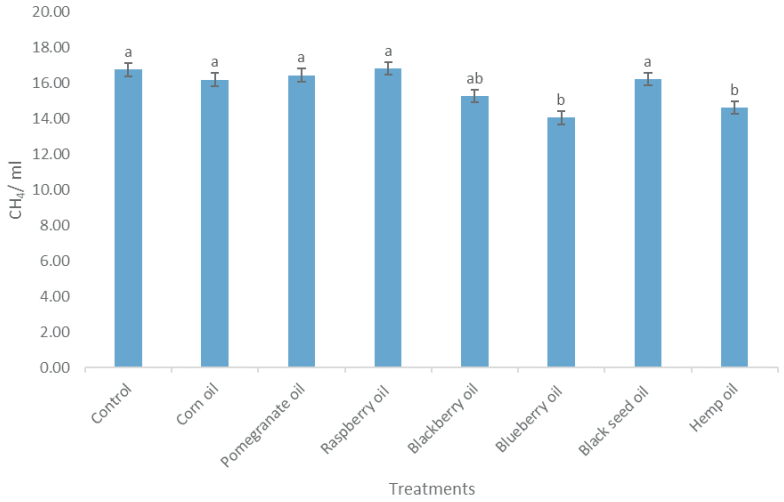

Figure 1. Effect of oil supplements on methane (CH_4) production.
^{ab}the same letters are not significantly different at P<0.05.

The rumen fermentation parameters, total VFA concentration and profile and NH_3-N in our experiments were also not significantly affected by the addition of oils, suggesting again that the tested oils had minimum effects on rumen microbial fermentation. A decline in acetate production is usually associated with reductions in fiber digestibility, and in the current experiment, the acetate concentration decreased only with the addition of raspberry oil and hemp oil. Although previous studies (Lillis *et al.,* 2011; Ivan *et al.,* 2013) showed that unsaturated fatty acids had adverse effects on fibrolytic bacteria, it is unlikely that fatty acids had an effect in this experiment, as all

oils had almost similar total unsaturated fatty acid contents. It is possible that raspberry oil and hemp oil had other components (e.g., flavonoids) that may have negatively affected fibrolytic bacteria. In the present study, the unchanged propionate concentration with the tested oils suggested that these oils had no or little effect on the degradation of other carbohydrates, such as starch. Salem *et al.* (1993) and Hervas *et al.* (2008) also observed that oil supplementation resulted in no variation in rumen amylolytic activity.

Supplementary oils can inhibit CH_4 emission by reducing the activity/number of rumen methanogens and protozoa (Lillis *et al.,* 2011), decreasing the amount of fermentable substrate, and/or through the biohydrogenation of unsaturated fatty acids (Chilliard *et al.,* 2009). However, the biohydrogenation of unsaturated fatty acids is not quantitatively relevant and has a low potential of CH_4 reduction compared to the decreased microbial activity and reduction of fermentable substrate (Ramin and Huhtanen, 2013). In the present study, the reduction in CH_4 production with the blueberry and hemp oil diets may not be attributed to the reductions in substrate fermentation, as the DM degradability and total VFA with these two oils were not different from the other diets. The blueberry and hemp oil effects on CH_4 production therefore most likely resulted from adverse effects on the rumen methanogens rather than decreasing substrate fermentation. The unsaturated fatty acids can directly inhibit rumen methanogenic archaea and may change their metabolic activity, as well as the composition of the rumen methanogenic population. Prins *et al.* (1972) observed that the growth of *Methanobacterium ruminantium* was severely inhibited in a pure culture by long-chain fatty acids. Popova *et al.* (2011) reported that the metabolic activity of methanogens in bulls decreased with the feeding of extruded linseed. Czerkawski *et al.* (1966) observed that the extent of in the suppression of CH_4 production with supplementation of fatty acids is far greater than that of the suppression of fiber digestibility in sheep. Zhang *et al.* (2008) observed that *Archaea* populations were decreased only by linoleic and linolenic acids among stearic, oleic, linoleic and linolenic acids. In the current study, the fatty acid profile for the tested oils may explain in part the differences between oils regarding their effects on CH_4 production. For example, the linolenic acid-to-linoleic acid ratio was greater in blueberry oil than in corn and blackberry oils. A meta-analysis study by Patra (2013) reported that linolenic acid had more inhibitory effects on methanogenesis than linoleic acid. However, the increased ratio of linolenic acid-to-linoleic acid cannot explain the observed reduction in CH_4 production with the hemp oil, as both hemp and raspberry oils had the same ratio. Wang *et al.* (2017) reported that the level of methanogenesis and the ratios of linoleic acid to linolenic acid in seed oils were not related; therefore, the reductions in CH_4 production with the blueberry and hemp oils may be largely due to their bioactive compounds. The polyphenolic and antioxidant compounds in blueberry and hemp oils may have negative effects on microbial fermentation (McPartland, 1984; Khalifa *et al.,* 2015). Hemp contains many classes of resinous compounds, such as terpenes, terpenols, cannabinoids and dihydrostilbenes. Cannabinoids have been reported to exhibit antimicrobial activity against bacteria and fungi (Klingeren and Ham, 1976; McPartland, 1984). Hemp oil also contains important terpenes, such as α-pinene, β-pinene, limonene, α-selinene, β-caryophyllene and β-humulene (Romano and Hazekamp, 2013). It was observed that the sesquiterpenes caryophyllene and humulene were only mildly inhibitory to methanogens. However, polar phenols, monoterpenes and terpenols have been reported to exert high methanogenic toxicity (Kortekaas *et al.,* 1995). Blueberries also contain flavonoids and hydrolyzable tannins, such as quercetin and kaempferol, with a small amount of gallic acid, gallotannic, pyrogallol, caffeic acid, and catechin (Yi *et al.,* 2006). Oskoueian *et al.* (2013) observed that flavonoids such as catechin and kaempferol decrease the rumen microbial population and that quercetin reduces the total populations of protozoa and methanogens *in vi-*

tro. The derivates of hydrolyzable tannins, such as gallic acid, gallotannic acid and pyrogallol, have been reported to exert high methanogenic toxicity (Field and Lettinga, 1987). Furthermore, tannins are known to reduce the protozoal population (Bhatta *et al.,* 2009). Flavonoids have been reported to serve as alternative H_2 sinks during the degradation of their metabolites (Williamson and Clifford, 2010). For example, Becker *et al.* (2014) indicated that 1.0 mol catechin prevented the emission of 1.2 mol CH_4.

In the present experiment, CH_4 production was not affected by the addition of corn, raspberry, pomegranate or black seed oils. A review by Beauchemin *et al.* (2008) estimated that CH_4 production in ruminants is reduced by 5.6% for every 1% increase in added dietary lipid. Girón *et al.* (2016) observed that the addition of 40 g kg^{-1} of corn oil to the diet of dairy cows decreased CH_4 production by 16%. Maleki *et al.* (2016) reported a decrease in CH_4 concentration when pomegranate oil was added to rumen cultures at 10 mg 75 ml^{-1}. Sallam *et al.* (2009) also reported significant reductions in CH_4 concentration when black seed extract was added to rumen cultures at 0.5, 0.75 and 1.0 ml 75 ml^{-1}. In contrast, in Rusitec fermenter**,** the addition of 50 and 500 mg L^{-1} black seed oil to rumen cultures (forage-to-concentrate*, 48:52)* did not affect the protozoal count (Klevenhusen *et al.,* 2015). The dietary lipid supplementation effects on rumen methanogenesis can vary by the composition of the basal diet. According to Patra (2013), the inhibitory effect of lipids on methanogenesis is more pronounced in ruminants fed concentrate-based diets than in ruminants fed forage-based diets. Dong *et al. (*1997) found that the depressive effect of lipid supplementation on CH_4 production was greater with a concentrate-based diet than with a hay diet. Hook *et a*l. (2011) reported an altered methanogen community and diversity in high-concentrate feeding dairy cattle. Machmuller *et al.* (1998) found that dietary supplementation with sunflower seed reduced CH_4 production up to 40% in a concentrate-based diet, whereas it decreased by 23% with a medium-concentrate diet. The forage-to-concentrate ratio in our basal diet (50:50) most likely weakened the oil's potential CH_4-suppressing effects. However, the present study confirms that hemp and blueberry oil supplementation to ruminant diets can decrease CH_4 production by 10-16% relative to that of the control. This result is similar to the results of a meta-analysis study by Patra (2013), who noted that lipid supplementation up to 60 g kg^{-1} of the diet (DM) can decrease CH_4 emissions (up to 15%) in cattle without a reduction of feed digestion and fermentation.

The results from the present study showed that, except for hemp oil and blueberry oil, the unconventional oils had no effects on rumen CH_4 production. The addition of hemp and blueberry oils reduced CH_4 production without negatively affecting rumen fermentation and total VFA concentration. The reductions in CH_4 production observed with hemp and blueberry oils most likely resulted from their effects on methanogens. Although these two oils slightly affected rumen methane production, questions remain to be answered about the long-term effects of supplementation with these unconventional oils on rumen fermentation and the ability of rumen microbes to adapt to these oils or their active compounds. Additionally, further studies are needed to elucidate the mechanisms by which these different unconventional oils and/or their bioactive compounds affect rumen fermentation and the rumen microbial community.

Resumen

M. Embaby, M. Günal, y A. AbuGhazaleh. 2019. Efecto de los aceites no convencionales en la producción y fermentación *in vitro* de metano del rumen. Cien. Inv. Agr. 46(3): 276-285. . Los efectos de los aceites no convencionales altos en ácidos grasos poliinsaturados (PUFA) (mora, arándano, frambuesa, Granada, semilla negra y cáñamo) en la producción de rumen in vitro de fermentación y metano (CH_4) fueron examinados en experimentos de cultivo por lotes de tres 24-h. Los tratamientos en cada experimento consistían en control (sin suplemento de aceite), control más aceite de maíz, o control más dos de los aceites no convencionales. Se añadieron aceites a las culturas rumen en 500 mg L^{-1} (equivalente a 3,3 g aceite kg^{-1} de materia seca dietética (DM)). Después de 24 horas de incubación, la producción de CH_4 no fue diferente entre el control y los tratamientos de aceite de maíz. De los seis aceites no convencionales probados, sólo los aceites de cáñamo y arándano redujeron la producción de CH_4 en un 9–16% en relación con los tratamientos de control y aceite de maíz. La degradabilidad de la materia seca y los ácidos grasos volátiles totales (VFA) no se vieron afectados por la adición de aceites de cáñamo y arándano. A excepción de una reducción en la concentración de acetato con el aceite de frambuesa, y un aumento en la concentración de valerato con el aceite de Granada, todos los aceites no convencionales probados no tuvieron efectos sobre la fermentación y el perfil de VFA en relación con el control. En conclusión, nuestros resultados mostraron que los aceites de cáñamo y arándano eran moderadamente efectivos para reducir la formación del rumen CH_4 sin comprometer la fermentación y digestibilidad del rumen.

Palabras clave: Aceite de arándano, aceite de cáñamo, aceite de frambuesa, cultura rumen, metano.

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