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An *in vitro* and *in situ* evaluation of a diet for cattle added with organic oils

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ABSTRACT

Objective: To perform *in vitro* and *in situ* evaluation of a diet for dairy cattle at different rates of fish oil and soybean oil.

Designmethodology/approach: Four treatments with different rates of fish oil (FO) and soybean oil (SO) were evaluated (Control: no added oil; diet 1: 2% FO; diet 2: 2% FO and 1.5% SO; diet 3: 2% FO and 3% SO). *In vitro* digestibility and *in situ* degradability were evaluated. Ammonia nitrogen, lactic acid, volatile fatty acids (VFAs), and microbial protein were determined. For the *in situ* evaluation, a protein degradability kinetic was carried out. The means were compared using a Tukey test at a 5% confidence level.

Results: The proposed diets increased gas production in *in vitro* kinetics, as the addition of oils increased ($p<0.001$) and the kinetic latency time decreased ($p<0.001$). All diets decreased the production of short-chain fatty acids ($p<0.001$). The production of ammonia nitrogen and lactic acid did not differ compared to the control ($p<0.05$). Diet 3 had a higher production of propionic acid in comparison to diet 1 and 2. In the *in situ* kinetic, the "*kd*" rate increased as more oils were added.

Study limitations/implications: Although all treatments increased the production (milliliters) of CH_4 and CO_2, the gas production had a proportional increase, as a result of a better use of the diets.

Findings/conclusions: The addition of oils produced changes in the fermentation patterns and in the degradation of the protein at the ruminal level, increasing bypass protein. This offers an opportunity to improve performance in certain production situations.

Keywords: *in vitro* digestibility, *in situ* degradability, gas production.

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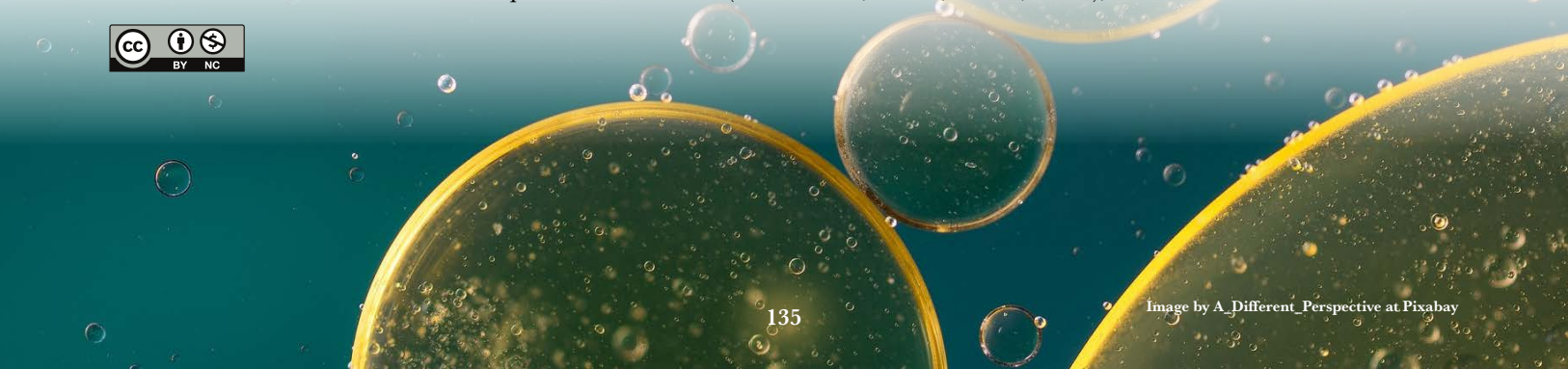
INTRODUCTION

Conjugated linoleic acid (CLA) —a fatty acid found naturally in the dairy and meat products of ruminants— has been identified as a potential anti-obesogenic, anti-cancerogenic, and enhancer of the immune and inflammatory response (Den Hartigh, 2018; Whigham, Watras, and Schoeller, 2018). CLA is an 18-carbon PUFA formed as an intermediate, during the biohydrogenation (BH) of linoleic acid (C18:2 *cis*-9, *cis*-12, LA) and alpha-linolenic acid (C18:3 *cis*-9, *cis*-12, *cis*-15, ALA), and their transformation into



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stearic acid (C18:0, SA), or by the endogenous conversion of *trans*-vaccenic acid (C18:1 t-11, TVA) by the action of the Δ9-desaturase enzyme in the mammary gland (Khanal and Olson, 2004). LA and ALA are found in high proportions in the lipids of fodder and in some supplements, such as oils. Castillo *et al.* (2013) point out that supplementing diets with LA- and ALA-rich soybean oil reduces short-chain fatty acids and increases long-chain unsaturated fatty acids (UFA) —especially TVA and CLA. For its part, fish oil contains eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA); adding these acids to ruminant diets increases CLA concentration in milk and meat (Wąsowska *et al.*, 2006). This has an impact on the BH process of the UFA, increasing the contribution of TVA —which serves as a substrate for the endogenous formation of CLA (Shingfield *et al.*, 2006).

Furthermore, ruminants make a significant contribution to the total greenhouse gas (GHG) emissions of the agricultural sector (Sejian and K. Naqvi, 2012). One way to decrease methanogenesis in ruminants is to include PUFAs in the diet, since UFAs serve as electron acceptors during the biohydrogenation process, causing a depression in methane production (Broucek, 2018). Methane represents a significant loss of dietary energy: a 2-12% loss of gross energy intake (Johnson and Johnson, 1995). Reducing enteric methane production could also increase feed efficiency.

Taking into account that both the production of GHG and the fatty acids accumulated in the milk and meat of the ruminant take place at the metabolic level, determining the effects of the addition of organic oils in its diet is an important step. Therefore, the objective of the present study was to evaluate the effects of adding FO and SO on the parameters of ruminal fermentation and the *in vitro* and *in situ* degradability kinetics. This evaluation allowed us to find out the metabolic differences between the different oil levels.

MATERIALS AND METHODS

The evaluated diets contained a balanced feed mixture (6% soybean husk, 16% distillers' dried grains, 6% sugarcane molasses, 20% rolled corn, 17.5% ground corn, 3.5% mineral mixture, 4% cottonseed, 5.8% soybean paste, 11.2% ground sorghum, 4% cottonseed meal, and 6% wheat bran), silage, and alfalfa (30, 29, and 41%, respectively) added with different ratios of SO and FO (Control: without added oil; diet 1: 2% FO; diet 2: 2% FO and 1.5% SO; diet 3: 2% FO and 3% SO). For the gas production technique proposed by Menke *et al.* (1979), the components of the diets were dried in an oven at 55 °C for 48 h, ground, and sieved (1mm mesh) separately. One gram of dry matter (DM) from each diet (with their respective oils) was added to 250-mL fermentation flasks with 120 mL of a 2:1 mixture of ruminal fluid and artificial saliva (Macromineral solution: 1 L distilled H_2O, 5.7 g Na_2HPO_4, 6.2 g KH_2HPO_4, 0.6 g $MgSO_47H_2O$; micromineral solution: 100 mL distilled H_2O, 13.2 g $CaCL_2 \cdot H_2O$, 10 g $MnCl_2 \cdot 4H_2O$, 1 g $CoCl_2 \cdot 6H_2O$, and 8 g $FeCl_3 \cdot 6H_2O$; buffer solution: 1 L distilled H_2O and 39 g $NaHCO_3$; reducing solution: 100 mL distilled H_2O, 4 mL 1N NaOH and 625 mg $Na_2S \cdot 9H_2O$; and resazurin solution: 100 mL distilled H_2O and 100 mg resazurin); CO_2 was used to establish an anaerobic environment; the fermentation flasks were incubated in an ANKOM Daisy^{II} equipment for 96 h. The accumulated gas pressures were measured using an automated measuring

equipment (ANKOM RF Gas production System) equipped with a pressure transducer connected to each fermentation flask. Measurements were taken at 0, 0.5, 1, 2, 3, 6, 12, 24, 36, 48, 72, and 96 h after inoculation. The gas production profiles were adjusted to the Gompertz equation (Tjørve and Tjørve, 2017) (Equation 1).

$$GP = Gmax\ e^{-Ae^{-kt}} \quad (1)$$

Where GP=gas production, $Gmax$=maximum gas production, A=adaptation phase, and k=gas production rate.

Gas samples were taken from the upper section of the fermentation flasks at 24 h, to measure the production of CO_2 and CH_4, using a Biogas 5000 equipment. At the end of the fermentations (96 h), the residues of fermentation were centrifuged at 2,500 rpm for 5 min, then they were filtered, and used to analyze VFAs, ammonia nitrogen, and lactic acid. The VFAs were analyzed in a 6890N gas chromatograph (Agilent Technologies, Wilmington, DE) equipped with a flame ionization detector and an HP-Innowax polyethylene glycol capillary column (30 m×0.32 mm×0.15 μm, J&W Scientifics). The oven was set up at 80 °C; after 1 min, the temperature was raised to 120 °C, with a 20 °C/min increase, until it reached 205 °C; afterwards, the temperature was increased 10 °C/min (for 2 minutes). Nitrogen was used as carrier gas at a constant flow rate of 40 ml/min, injecting 1 μL of sample.

The ruminal environment was evaluated through the analysis of ammonia nitrogen, lactic acid, *in vitro* digestibility of dry matter (*IVDDM*), and microbial protein. The ammonia nitrogen in the samples (Galyean, 2010) was subject to a spectrometry analysis at a wavelength of 630 nm in a Genesys 10S VIS Thermo Scientific spectrometer; the lactic acid in the samples (Borshchevskaya *et al.*, 2016) was analyzed using the same equipment at 390 nm with 0.2% $FeCl_3$. The *IVDDM* was evaluated using nylon bags F57 (ANKOM)s with 0.5 g of each diet in 1.6 L of a mixture of solution A:solution B (5:1) (Solution A: 10 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.5 g/L NaCl, 0.1 g/L $CaCl_2 \bullet 2H_2O$, and 0.5 g/L urea; Solution B: 15 g/L Na_2CO_3 and 1 g/L $Na_2S \bullet 9H_2O$) and 400 mL of ruminal fluid; 5-L glass incubation jars were used in an ANKOM Daisy^{II} incubator at 39 °C for 48 h; CO_2 was used to establish an anaerobic environment. At the end of the incubation period, the nylon bags F57 (ANKOM) were dried in an oven at 55 °C for 48 h and the *IVDDM* was determined by weight difference. The *IVDDM* value of each diet and its gas production at 48 h were used to calculate the microbial protein using Equation 2, according to Blümmel, Steingaβ, and Becker (1997).

$$MP\left(\frac{mg}{g\ \text{de}\ MS}\right) = IVDDM - \left(GP_{48} * 2.2\right) \quad (2)$$

Where: MP=microbial protein, $IVDDM$=*in vitro* digestibility of dry matter, and GP_{48}=gas production at 48 hours.

The *in situ* evaluation was carried out in three "Angus" cattle with ruminal fistula, using the nylon bag technique (Mehrez & Ørskov, 1977): 20×10 cm bags with an average pore of 50 microns were used. The bags were previously dried at 60 °C for 24 h; 10 g of DM of each diet (with their respective oils) were placed in each one; and two repetitions of each time were made (0, 3, 6, 12, 18, 24, 36, 48, 72, and 96 h). The bags were placed in the ventral part of the rumen and were introduced in the reverse order of their incubation time; all of them were removed together. The zero-hour bags were introduced and immediately removed, once they were moistened with ruminal fluid (Nocek & Russell, 1988). The bags were thoroughly washed with running water and then dried in an oven at 55 °C for 48 h. Crude protein (CP) was determined from the residues of each bag with the Kjeldahl method, using the N×6.25 factor (AOAC 991.20). *In situ* degradation parameters were estimated with the modifications made by McDonalds (1981) to the model proposed by Orskov and Mcdonald (1979) (Equation 3).

$$d = a + b\left(1 - e^{-kd*t}\right) \quad (3)$$

Where: d=degradability at t time, t=incubation time, a=soluble or rapidly degradable fraction, b=insoluble but potentially degradable fraction (%), and kd=degradation constant of "b".

The non-digestible fraction ("*C*") and potentially digestible fraction ("*PD*") were calculated using Equation 4, and the effective degradability ("*ED*") was calculated using Equation 5.

$$C = 100 - (a + b) \quad (4)$$

Where: C=non-digestible fraction (%), PD=potentially digestible fraction ($a+b$).

$$ED = a + \left[(b * kd) / (kd + kp)\right] \quad (5)$$

Where: ED=effective degradability, kp=5%/h constant ruminal passage rate, and kd=degradation constant of "b".

A completely randomized experimental design was used; a mean comparison Tukey test was performed. We considered that there was a significant difference when $p<0.05$. A statistical analysis was performed with the SigmaPlot [12.0] statistical software.

RESULTS AND DISCUSSION

In the parameters obtained for the Gompertz equation (Table 1), the maximum gas production ("*Gmax*") increased ($p<0.001$) as more oil was added and the "A" lag phase decreased ($p<0.001$). In diet 3, the "*k*" gas production rate increased ($p<0.05$) compared to control. The increase in "*Gmax*" indicates a greater fermentation of organic matter (Blümmel and Ørskov, 1993). This increase differs from the results of Toral *et al.* (2009)

in sheep, who used a mixture of FO with sunflower oil (1 and 2%), observing a slight decrease in the accumulated gas production, without changing the gas production rate. These differences may be the result of the type and quantity of oils, as well as of the different concentrate rates used in each study (Wachira *et al.*, 2000; Ueda *et al.*, 2003; Shingfield *et al.*, 2008). Beuvink and Spoelstra (1992) point out that the total amount of gas produced depends on the composition of the final fermentation products. Starch fermentation produces more gas than glucose or cellulose fermentation. Therefore, the increase in "*Gmax*" obtained in the present study can be attributed to an increase in the population of amylolytic bacteria, as a consequence of the toxic effect that oils have on cellulolytic bacteria and protozoa (Harfoot and Zealand, 1997; Yang *et al.*, 2009; Palmquist and Jenkins, 2017; Roy *et al.*, 2017; Lima *et al.*, 2019). The decrease in the number of cellulolytic bacteria could also explain the decrease in total volatile fatty acids (VFA) (Table 4), since cellulose fermentation produces the highest quantity of VFAs (Beuvink and Spoelstra, 1992).

Within the parameters of ruminal fermentation (Table 2), the *IVDDM* decreased in diet 2 (63.9%) compared to control and diet 1 (68.2 and 67%, respectively; $p<0.05$). The ammonia nitrogen and lactic acid concentration is not affected by the addition of oils compared to the control diet. Microbial protein synthesis decreased as more oils were added ($p<0.001$). The decrease in *IVDDM* observed in diet 2 differs from the results obtained by Roy *et al.* (2017), who did not observe differences in *IVDDM* when they used different oils (0, 3, and 4%). In contrast, El-Sherbiny *et al.* (2016) observed a decrease in *IVDDM* with FO and SO mixtures at 5 and 7% of total addition.

Table 3 shows an increase ($p<0.001$) in the production of CO_2 and CH_4, related to higher gas production (*Gmax*). On one hand, the ratio is not significantly affected, indicating that diets do not change the rate in which it is produced. No significant differences were

Table 1. Effect of the addition of organic oils on the parameters of the Gompertz equation.

	Control	DIET 1	DIET 2	DIET 3	EE*	p-value
Gmax (mL)	62.1 ± 3.38^{c}	96.6 ± 4.01^{b}	138.7 ± 12.09^{a}	140.4 ± 10.46^{a}	18.75	<0.001
A (h)	15.5 ± 2.21^{a}	7.2 ± 1.09^{b}	4.5 ± 0.59^{bc}	3.7 ± 0.21^{bc}	2.69	<0.001
k (h^{-1})	0.06 ± 0.004^{b}	0.14 ± 0.029^{ab}	0.24 ± 0.117^{ab}	0.29 ± 0.075^{a}	0.05	<0.05

[a, b, c, d]: the same letters indicate that there is no statistically significant difference, EE*: Standard error.

Table 2. Effect of the addition of organic oils on the parameters of ruminal fermentation.

	Control	DIET 1	DIET 2	DIET 3	EE*	p-value
DIVMS: (%)	68.2 ± 1.20^{a}	67.0 ± 2.21^{a}	63.9 ± 2.42^{b}	65.9 ± 1.10^{ab}	0.91	<0.05
Ammonia nitrogen (mg/dL)	13.0 ± 1.48^{ab}	10.8 ± 1.72^{b}	13.8 ± 0.39^{a}	12.8 ± 0.04^{ab}	0.63	<0.05
Lactic acid (g/L)	2.6 ± 0.38^{ab}	3.0 ± 0.12^{a}	2.1 ± 0.18^{b}	2.2 ± 0.28^{ab}	0.20	<0.05
Calculated microbian protein (mg/g DM)	564.6 ± 16.11^{a}	462.9 ± 17.1^{b}	364.6 ± 37.39^{c}	381.0 ± 14.57^{c}	45.80	<0.001

[a, b, c, d]: the same letters indicate that there is no statistically significant difference, EE*: Standard error. *In vitro* digestibility of dry matter (*IVDDM*).

Table 3. Effect of the addition of organic oils on the production of methane and carbon dioxide.

	Control	DIET 1	DIET 2	DIET 3	EE*	p-value
CO_2 (ml)	33.1±1.14^{c}	64.4±0.02^{b}	58.7±5.05^{b}	83.0±7.94^{a}	10.30	<0.001
CH_4 (ml)	5.1±0.08^{c}	10.3±0.14^{b}	9.5±0.31^{b}	12.7±0.52^{a}	1.58	<0.001
CO_2 (%)	71.7±0.25	76.9±0.3	75.6±3.7	73.1±3.32	1.17	nssd
CH_4 (%)	11.2±0.60^{b}	12.3±0.15^{a}	12.2±0.05^{a}	11.2±0.25^{b}	0.30	<0.05
CH_4/CO_2	0.15±0.007	0.16±0.002	0.16±0.008	0.15±0.008	0.002	nssd

$^{a, b, c, d}$: the same letters indicate that there is no statistically significant difference, nssd: no statistically significant difference, EE*: Standard error.

found in the percentage of that was produced. On the other hand, the increase of the propionic acid production (Table 4) in diet 3 and the control compared to diets 1 and 2 (23.8 and 23.1%: 16.7 and 18.3%, respectively; $p<0.05$), decreases the percentage of (11.2 and 11.2%: 12.3 and 12.2%, respectively; $p<0.05$), as well as the A/P ratio (1.8 and 1.9: 2.8 and 2.7, respectively; $p<0.05$). The production of TVFAs decreased with the addition of oils ($p<0.001$); consequently, there was a greater decrease in diet 3 (10.1 mM) than in control (17.5 mM). These results match the findings of several authors (Broudiscou and Lassalas, 1991; Zhang *et al.*, 2008; Martin *et al.*, 2009; Vargas *et al.*, 2020); as propionic fermentation increases, the hydrogen uptake increases and methane production decreases (Boadi *et al.*, 2004).

In the case of *in situ* protein degradability (Table 5), diet 3 presents highly significant effects ($p<0.001$) in the "*C*" non-digestible fraction —*i.e.*, a greater increase with respect to control (48.9 and 41.0%, respectively). Likewise, its potentially digestible fraction (*PD*) shows a greater decrease (51.0 and 58.9%). A similar effect is observed in diet 1 and 2. The *ED* in diets 2 and 3 is greater than in control (31.6 and 30.6: 25.5%, respectively; $p<0.05$); a similar effect is observed with "*kd*" (0.045 and 0.061: 0.025 h^{-1}, respectively; $p<0.001$). The "*a*" fraction of the diets did not present a significant difference ($p>0.05$) compared to control. The decrease in the *PD* fraction and increase in *C* is consistent with a study carried out by Ferreira *et al.* (2016) in lambs, in which the digestibility of CP in the total digestive tract decreased when a mixture of FO and SO was supplemented: the higher the SO concentration, the lower the apparent digestibility. This decrease in protein degradability is related to the decrease in microbial protein synthesis (Table 2), which could be the result of an inhibition in microbial growth caused by the PUFAs present in FO and SO (Maia *et*

Table 4. Effect of the addition of organic oils on the production of volatile fatty acids (VFAs).

	Control	DIET 1	DIET 2	DIET 3	EE*	p-value
Acetic acid (%)	44.2±6.92	46.5±2.63	48.6±2.73	44.7±1.78	0.99	nssd
Propionic acid (%)	23.1±0.94^{a}	16.7±1.41^{b}	17.6±1.15^{b}	23.8±1.78^{a}	1.83	<0.05
Butiric acid (%)	27.8±5.97	31.9±1.22	28.8±2.65	26.6±3.56	1.13	nssd
TVFA (mM)	17.5±1.11^{a}	11.8±1.12^{bc}	13.1±0.99^{b}	10.1±0.40^{c}	1.58	<0.001
A/P ratio	1.9±0.37^{b}	2.8±0.39^{a}	2.7±0.16^{a}	1.8±0.06^{b}	0.26	<0.05

$^{a, b, c, d}$: the same letters indicate that there is no statistically significant difference, nssd: no statistically significant difference, EE*: Standard error. TVFA: total volatile fatty acids.

Table 5. Effect of the addition of organic oils on the *in situ* ruminal degradation parameters and effective protein degradability of each diet.

	Control	DIET 1	DIET 2	DIET 3	EE*	p-value
a (%)	8.3±2.49^{ab}	13.4±0.33^{a}	7.6±1.75^{ab}	5.7±2.78^{b}	1.64	<0.05
b (%)	50.5±4.22	43.2±0.15	50.1±1.54	45.3±3.64	6.73	nssd
kd (h^{-1})	0.025±0.0001^{c}	0.027±0.005^{c}	0.045±0.000^{b}	0.061±0.0032^{a}	0.008	<0.001
Non-digestible fraction "*C*" (%)	41.0±1.72^{c}	43.2±0.48^{b}	42.1±0.21^{bc}	48.9±0.86^{a}	1.75	<0.001
Potentially digestible fraction "*DP*" (%)	58.9±1.72^{a}	56.7±0.48^{b}	57.8±0.21^{ab}	51.0±0.86^{c}	1.75	<0.001
Effective degradability "*DE*" (%); para *kp*=5%/ h	25.5±1.12^{b}	28.8±1.62^{ab}	31.6±1.17^{a}	30.6±1.36^{a}	1.34	<0.05

$^{a, b, c, d}$: the same letters indicate that there is no statistically significant difference, nssd: no statistically significant difference, EE*: Standard error, *C*=non-digestible fraction (*C*=100−(*a*+*b*)), *PD*= potentially digestible fraction (*PD*=*a*+*b*), *ED*: effective degradability for *kp*=5%/hour (*ED*=*a*+[(*b***kd*)/(*kd*+*kp*)]).

al., 2010; Ferreira *et al.*, 2016). These findings match the tests carried out by El-Sherbiny *et al.* (2016), who recorded a reduction in the total count of bacteria as a mixture of SO and FO increased to 3, 5, and 7% of total addition.

CONCLUSIONS

The addition of oils produced changes in the fermentation patterns that suggest a modification in the microbial populations. Protein degradation is also modified, increasing bypass protein. This offers an opportunity to improve performance in certain production situations.

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