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Differential effects of rapeseed, sunflower and linseed oils on rumen microbial functions in dual effluent fermenters on maize silage-based diet☆

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Abstract – Quantitative information on the concurrent changes in major rumen microbial functions induced by dietary lipids in relation with the degree of unsaturation of fatty acids is scarce. During a three-period essay (9 days per period), rapeseed (*Brassica napus* L., RO), sunflower (*Helianthus annuus* L., SO) and linseed (*Linum usitatissimum* L., LO) oils at the high level of 80 g/kg dry matter input (DMI) and a control without oil were compared in four 1-L dual outflow fermenters inoculated with bovine rumen microbiota and maintained on a maize silage-based diet. Neither the fermentation medium pH and redox potential nor starch and protein degradabilities were significantly altered. Oil supply significantly decreased butyrate and methane specific productions of to the benefit of propionate and hydrogen specific productions and decreased the microbial protein outflow and synthesis efficiency while increasing the outflows of ammonia and isovalerate, these effects being amplified by the fatty acids' unsaturation degree. Besides, with no impact of the degree of unsaturation, oil supply increased the amount of fermented hexose, the productions of all SCFAs except butyrate and the specific production of acetate. The rumen variables apparently sensitive to oil unsaturation degree were thus related either to metabolic hydrogen removal pathways or to protein metabolism.

Keywords: rapeseed oil / sunflower oil / linseed oil / rumen / microbial metabolism

Résumé – Effets différentiels des huiles de colza, de tournesol et de lin sur les fonctions microbiennes ruminales dans des fermenteurs à double effluent sur un régime à base d'ensilage de maïs. Les informations quantitatives sur les diverses actions simultanées des lipides alimentaires sur les principales fonctions microbiennes du rumen et leur lien avec le degré d'insaturation des acides gras sont rares. Au cours d'un essai de trois périodes (9 jours par période), des huiles de colza (*Brassica napus* L., RO), de tournesol (*Helianthus annuus* L., SO) et de lin (*Linum usitatissimum* L., LO) au taux élevé de 80 g/kg de matière sèche apportée (DMI) et un contrôle sans huile ont été comparés dans quatre fermenteurs à double flux inoculés avec un microbiote de rumen bovin et maintenus sur un régime à base d'ensilage de maïs. Ni le pH et le potentiel redox du milieu de fermentation, ni la dégradation de l'amidon et des protéines n'ont été significativement modifiés. L'apport d'huile a significativement diminué les productions spécifiques de butyrate et de méthane au profit des productions spécifiques de propionate et d'hydrogène et a diminué le flux de protéines microbiennes et l'efficacité de synthèse tout en augmentant les flux d'ammoniac et d'isovalérate, ces effets étant amplifiés par le degré d'insaturation des acides gras. Par ailleurs, sans impact du degré d'insaturation, l'apport d'huile a augmenté la quantité d'hexose fermenté, les productions de tous les AGCS sauf le butyrate et la production spécifique d'acétate. Les variables apparemment sensibles au degré d'insaturation de l'huile étaient donc liées soit aux voies d'élimination de l'hydrogène métabolique, soit au métabolisme des protéines.

Mots clés : huile de colza / huile de tournesol / huile de lin / rumen / métabolisme microbien

☆ Contribution to the Topical Issue "How oil- and protein- crops can help fight against climate change? / Contribution des oléoprotéagineux à la lutte contre le changement climatique".

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1 Introduction

In December 2019, the European Commission launched the Green Deal whose ultimate goal is to make the EU climate neutral by 2050, with a first step of compulsory 55% reduction of emissions in 2030. This ecological transition strategy is particularly relevant to agriculture insofar as the European Council adopted on March 17, 2022 a set of conclusions for a low-carbon agriculture aiming at climate neutrality by 2035 and increased environmental sustainability. One adaptation way among others lays in the promotion of local oil crops, in particular rapeseed (*Brassica napus* L.) and sunflower (*Helianthus annuus* L.), covering respectively 6.9 and 5.1% of the EU's cultivated area between 2016 and 2020 (FAO, 2022), as well as linseed (*Linum usitatissimum* L.). These plants require little water, fertilizer or treatments and are beneficial in crop rotations by breaking the cycle of cereal diseases not to mention their melliferous nature (Zegada-Lizarazu and Monti, 2011). In addition to these benefits in the fields, the use of their crops is also a factor that argues for their development. In this context, their seeds or the oils extracted from them have been incorporated into ruminant feed for various reasons: reducing the chance of ruminal acidosis in the diet of high-producing animals (Coppock and Wilks, 1991), sparing rumen bacterial proteins by partially depleting protozoa (Stern *et al.*, 1994), shifting the fermentative profile towards an increased specific production of propionate, a major glucogenic precursor (Wiltrout and Satter, 1972) or mitigating methanogenesis in the rumen (Van Nevel and Demeyer, 1996; Giger-Reverdin *et al.*, 2003). However, their broad spectrum of action on rumen microbial metabolism can include several adverse side effects, such as decreases in organic matter degradability and in microbial protein synthesis efficiency. Managing the trade-offs between these positive and negative consequences requires quantitative data on how the degree of dietary lipid unsaturation alters the main rumen functions. Yet, such information is scarce, mainly from meta-analyses on cattle (Patra, 2013) and sheep (Patra, 2014). Data from experiments simultaneously monitoring rumen fermentation, degradation and synthesis processes are still needed, as they are best able to document the different effects of dietary lipid on rumen functions and relate them to the unsaturation degree of fatty acids, which varies greatly between plant species, from oleic acid-rich rapeseed to linolenic acid-rich linseed.

The present essay was aimed at characterizing rapeseed (RO), sunflower (SO) and linseed (LO) oils on their potential effects on the *in vitro* metabolism of bovine rumen microbiota maintained on a maize silage-based diet and at identifying the metabolic responses most affected by the unsaturation degree of oil fatty acids. The three oils have been therefore tested at an unusually high level of intake in the field of ruminant nutrition but applicable in a continuous culture trial.

2 Material and methods

2.1 Experimental design

The main factor was the nature of oil and comprised 4 treatments: no supplementary oil (control, CTL) and the supply of rapeseed (RO), sunflower (SO) or linseed (LO) oil at the incorporation level of 80 g/kg dry matter input (DMI). On three independent periods comprising a 6-day equilibration phase and a 3-day measurement and sampling phase, the treatments were randomly assigned to four 1-L dual outflow fermenters (Broudiscou *et al.*, 1997). As a result, the experimental design comprised 12 runs resulting from the combination of the factor oil (df = 3) and the period, taken as a block factor (df = 2).

2.2 Preparation of oils

The oils were extracted in the facilities of Terres Inovia (Pessac, France) by cold pressing seeds. The batches of seeds were checked for their low oxidation levels by determining oleic acidity (7.0%) and peroxide indexes (3.5, 4.2 and 1.6 meq/kg respectively for rapeseed, sunflower seed and linseed) and the oils were analysed for fatty acid composition (Tab. 1) in the ITERG Laboratory (Pessac, France).

2.3 Incubations

On the first day of each period, the rumen fluid used to inoculate the fermenters was collected at the experimental facility of the MIXscience Sourches Research and Development Farm (Saint Symphorien, France) from two out of three rumen cannulated Holstein dry cows. The animals were held in carpet stalls and fed an alfalfa hay-based diet. Care and handling procedures complied with French regulations for animal experimentation (Anonymous, 2013a, 2013b) and the farm has an ISO 9001 certification. The experiment used 3 animals already fistulated and permanently cannulated and the collection of rumen fluid did not require an approval (as a painless procedure). The pooled rumen contents were coarsely filtered and maintained until inoculation under CO_2 atmosphere at 35–40 °C. Following filtration through a 2 mm metal sieve, 300 mL rumen fluid and 300 mL buffer solution were poured into each fermenter which was then thoroughly flushed with CO_2.

The solid substrate used in our trial was mainly based on corn silage and soybean cake (Tab. 2) to meet the request of a partner company to use a diet for dairy cows. The corn silage was stored at −20 °C and every morning, a batch of frozen silage was hand-chopped down to a particle size under 2–3 mm. The other constituents had been milled in a Retsch ZM1000 knife mill with a 1 mm opening grid then pooled and mixed with urea. Each fermenter received 11 g DM of solid substrate excluding oil at 11:00 and 23:00. At the same times, in accordance with the experimental design, we pipetted into three fermenters 0.961 mL RO, 0.951 mL SO or 0.921 mL LO, amounting to an incorporation level of 80 g/kg DMI. In order to maintain the medium pH above 6.3 and strongly reducing conditions, a buffer solution was infused at 1.11 ± 0.03 mL/min (Broudiscou *et al.*, 1999a). The turnover rates of liquid and solid phases were 0.09/h and 0.045/h, respectively. The daily control and effluent collection procedures are in Broudiscou *et al.* (1997).

On days 7 and 8, the pH and Eh of fermentative media were determined 11 h after substrate supply. The maize silage was sampled and pressed, the liquid fraction was stored at −20 °C following addition of 25% (v/v) of acid phosphoric acid 250 mL/L. On days 7 to 9, the fermentation gases were analysed by gas chromatography (Broudiscou *et al.*, 2014) to

Table 1. Oils composition (g/kg).

Fatty acid	Rapeseed	Sunflower	Linseed
C14:0	1	1	1
C16:0	53	65	68
C16:1	4	1	1
C18:0	15	40	32
C18:1	592	337	164
C18:2	218	536	151
C18:3	85	2	575
C20:0	6	3	2
C20:1	11	2	1
C22:0	3	8	1
C22:1	1	< 1	< 1
C24:0	1	3	1
C24:1	2	< 1	< 1
Other	8	2	3
Saturated	80	121	106
Monounsaturated	611	341	166
Polyunsaturated	303	538	726

Table 2. Chemical analysis of the diet.

Composition	(g/kg DM)
DM (g/kg)	446
OM	959
Starch	215
Crude protein	153
Ether Extract	23
aNDFom	358
ADFom	240
Feeds	(g/kg DM)
Maize silage	732.3
Soybean cake	112.5
Wheat grain	60.1
Rapeseed cake	48.7
Wheat straw	41.8
Urea	4.6

estimate CH_4, H_2S and H_2 daily productions. On days 7 to 9 as well, total effluents were collected and subsampled to measure DM (2 × 15 mL), determine concentrations of SCFA and ammonia nitrogen NH_3–N (2 × 4 mL mixed with 1 mL acid phosphoric acid 250 mL/L and stored at −20 °C until analysis) and isolate the bacterial pellets used in microbial biomass estimation (800 mL). In order to isolate one bacterial pellet per fermenter, the effluents were homogenised (Trabalza-Marinucci *et al.*, 2006), then subjected to differential centrifugation (5 min for 1000 g, 15 min for 15 000 g) and lyophilisation (Broudiscou *et al.*, 1999b). The remaining fractions of effluent were lyophilised.

2.4 Chemical analyses

Lyophilised substrates and effluents were ground in a Culatti grinder (Zurich, Switzerland) through a 0.8 mm aperture screen, prior to determination of starch (Faisant *et al.*, 1995), aNDFom (assayed without sodium sulfite and with alpha amylase), ADFom and Lignin (Van Soest *et al.*, 1991). The aNDFom and ADFom were measured from their ash content (550 °C for 5 h). The DM contents of feeds, effluents and bacterial pools were measured by oven drying for 48 h at 105 °C, and their OM contents by ashing at 550 °C for 16 h. Total nitrogen was analysed using the Dumas technique (Sweeney and Rexroad, 1987) on a LECO model FP-428 Nitrogen Determinator (LECO, St. Joseph, MI). Crude protein was calculated as N × 6.25. Individual SCFA concentrations in effluents and silage juice were quantified by reverse phase HPLC (De Baere *et al.*, 2013). The NH_3–N concentration was quantified using a specific probe (Broudiscou and Papon, 1994). The nucleobases, used as microbial markers, were quantified in effluents and bacterial pellets by reversed phase HPLC (Lassalas *et al.*, 1993).

2.5 Calculations and statistical analyses

The daily amount of hexoses fermented (HF) was determined as follows (Demeyer and Van Nevel, 1975):

$$HF = (C2 + C3) / 2 + C4 + C5 \ (mmol/d). \quad (1)$$

C2, C3, C4 and C5 respectively standing for the differences between the daily outflows of acetate, propionate, butyrate and valerate and their inflows from the maize silage supply (mmol/d).

The daily amount of ATP produced from OM fermentation was calculated as follows:

$$ATP = 2 \times C2 + 2 \times C3 + 3 \times C4 + 2 \times C5 \ (mmol/d). \quad (2)$$

The daily amount of fermented organic matter (FOM) was determined from HF as follows:

$$FOM = 162 \times HF \ (g/d). \quad (3)$$

The microbial nitrogen outflow (MNf) and the efficiency of microbial protein synthesis (EMPS) were calculated from DM outflow (DMf), nucleobase –used as a microbial marker– and Dumas nitrogen contents in DM outflows and in bacterial pellets (Broderick and Merchen, 1992).

$$EMPS = g \ MNf/kg \ daily \ flow \ of \ FOM. \quad (4)$$

The trues degradabilities of OM and nitrogen, tdOM and tdN were calculated as follows:

$$tOMd = 100 * (MOM \ outflow + FOM) / OM \ input. \quad (5)$$

MOM standing for the microbial organic matter.

$$tNd = 100 * (1 - (ONf - MNf) / INANf), \quad (6)$$

where ONf is the outflow of organic N (non-ammoniacal N) and INANf the inflow of non-ammoniacal N.

Table 3. Outflows of fermentation metabolites (mmoles/day) in dual-effluent fermenters.

	Treatments				SEM	*P*
	CTL	RO	SO	LO	(*N* = 3)	Oil
Acetate	59.3[a]	74.5[b]	70.4[ab]	73.2[ab]	2.86	0.033
Propionate	31.9[a]	46.9[b]	50.1[b]	46.7[b]	2.26	0.0049
Butyrate	19.1[a]	8.6[b]	8.5[b]	5.2[b]	0.98	0.0003
Valerate	2.9	4.5	5.3	5.3	0.52	0.051
CH_4	28.5[a]	24.0[a]	20.6[ab]	15.5[b]	1.65	0.0074
H_2	1.6[a]	4.7[a]	4.4[a]	9.0[b]	0.77	0.0032
H_2S	9.6[a]	20.3[a]	21.7[a]	44.7[b]	4.38	0.0069
ATP	245.4[a]	277.5[b]	277.0[b]	266.0[b]	3.49	0.0019

CTL: control; RO: rapeseed oil; SO: sunflower oil; LO: linseed oil, each supplied at 80 g/kg dry mater input; SEM: standard error of means. Different letters mean statistically different values (Tukey test, $P < 0.05$).

Table 4. Amount of hexoses fermented (HF) and fermentation pattern in dual effluent fermenters.

	Treatments				SEM	*P*
	CTL	RO	SO	LO	(N = 3)	Oil
HF (mmol/d)	67.6[a]	73.8[b]	74.0[b]	70.4[ab]	1.00	0.011
Specific productions (mol/100 mol HF)						
Acetate	87.6[a]	100.9[ab]	95.3[ab]	104.1[b]	3.19	0.043
Propionate	47.3[a]	63.8[ab]	67.5[b]	66.2[b]	3.39	0.018
Butyrate	28.3[a]	11.7[b]	11.5[b]	7.4[b]	1.41	0.0002
Valerate	4.3[a]	6.0[ab]	7.1[ab]	7.4[b]	0.64	0.043
CH_4	42.0[a]	32.5[ab]	27.9[bc]	21.9[c]	1.91	0.0017
H_2	2.4[a]	6.4[a]	6.0[a]	12.7[b]	0.96	0.0017

CTL: control; RO: rapeseed oil; SO: sunflower oil; LO: linseed oil, each supplied at 80 g/kg dry mater input; SEM: standard error of means. Different letters mean statistically different values (Tukey test, $P < 0.05$).

The partitioning of dietary OM input between the fermented, microbial and undegraded dietary outputs was evaluated by calculating the daily amounts of OM in the form of HF and in the form of microbial biomass (using nucleobases as a microbial marker). The difference between the dietary OM input and the sum of fermented and microbial OM outputs was attributed to undegraded dietary matter.

The results were subjected to analysis of variance and the main effects of oil and period were determined using the Minitab19 GLM procedure. The differences between the three oils and control were tested by performing pairwise multiple comparisons using the Tukey test with the experimentwise type I error set at $P = 0.05$ and the tendency threshold at $P = 0.10$.

3 Results

In the fermentation medium 11 h after addition of substrate, the oil supplementation did not significantly modify the pH, which averaged 6.65 ± 0.05, and equalled 6.69, 6.60, 6.63 and 6.66 in controls, with RO, SO and LO respectively. The Eh, as well, was not modified by oil supply. It averaged −214 ± 5.9 mV and equalled −224, −207, −210 and −217 respectively in controls, with RO, SO and LO. Both parameters were kept within optimal ranges for rumen microbial activity. The NH_3–N concentration 11 h following substrate supply averaged 29.8 ± 3.6 g/L, and it was not significantly modified by oil supplementation either, equalling 30.7, 22.8, 28.6 and 26.2 g/L in controls, with RO, SO and LO respectively.

The daily productions of end metabolites in the fermentation of polysaccharides were extensively altered by oil supplementation (Tab. 3). The production of acetate increased significantly by 26% with RO and in tendency with LO (+23%, $P = 0.07$). The daily production of propionate increased by 46% with RO and LO and by 57% with SO. On the contrary, butyrate production decreased by 55% with RO and SO and by 73% with LO. Valerate production tended to increase with SO ($P = 0.085$) and LO ($P = 0.09$). In parallel to the increase in propionate, the supply of polyunsaturated oils induced a shift in reducing equivalent disposal from methanogenesis to alternative mechanisms. The daily emission of CH_4 decreased significantly with LO by 46% and in tendency by 28% with SO ($P = 0.075$) to the benefit of H_2 and H_2S emissions which showed respectively a 6-fold and a 5-fold increases when LO was supplied. The production of ATP providing the energy for cell maintenance and growth was significantly higher with the addition of oil (up to 13% with SO).

Table 5. Feed degradation, outflows of nitrogen fractions and organic matter partitioning in dual effluent fermenters.

	Treatments				SEM	*P*
	CTL	RO	SO	LO	(N = 3)	Oil
tOMd	0.705[a]	0.722[a]	0.699[ab]	0.665[b]	0.0069	0.0063
Starch-d	0.908	0.914	0.917	0.915	0.0062	0.73
NDFd	0.242	0.146	0.230	0.256	0.024	0.052
ADFd	0.338	0.188	0.276	0.278	0.031	0.068
tNd	0.791	0.865	0.807	0.737	0.0368	0.21
Isovalerate	6.6[a]	8.7[ab]	9.4[ab]	11.5[b]	0.65	0.011
NH_3–N	37[a]	51[b]	58[b]	66[c]	1.27	< 0.0001
Organic N	490	427	437	445	14.6	0.089
Microbial N	379[a]	356[ab]	335[ab]	306[b]	13.7	0.043
EMPS	34.6[a]	29.8[ab]	27.9[ab]	26.7[b]	1.45	0.035
Organic matter partitioning						
Undegraded	29.5[a]	27.8[a]	30.1[ab]	33.5[b]	0.69	0.0063
Microbial	18.7	19.9	17.4	16.6	0.74	0.077
Fermented[1]	51.8	52.3	52.4	49.9	0.73	0.15

CTL: control; RO: rapeseed oil; SO: sunflower oil; LO: linseed oil, each supplied at 80 g/kg dry mater input; SEM: standard error of means. Different letters mean statistically different values (Tukey test, $P < 0.05$).
[1] OM in the form of fermented hexoses.

The comparison between controls and oil-supplemented incubations outlined significant differences in the intensity and pattern of fermentation (Tab. 4). The amount of HF was 9% higher with RO and SO. The specific productions of SCFA were characterised by a general decrease in butyrate to the benefit of propionate with SO and LO (+40%) and in tendency with RO (+35%, $P = 0.065$). Furthermore, the addition of LO significantly increased the specific production of acetate and valerate by 19% and 72% respectively. The addition of oil strongly decreased the specific production of CH_4., by 34% and 48% with SO and LO respectively and in tendency with RO (−23%, $P = 0.065$) while the H_2 specific production was increased 5-fold with LO.

The starch, NDF and ADF degradabilities averaged 0.91, 0.24 and 0.27 respectively and they were not significantly modified by the addition of oil (Tab. 5). The OM true degradability was decreased by LO supplementations (Tab. 5). All the variables relating to the nitrogenous compound anabolism were modified by oil supplementation the more the oil was unsaturated. The ammonia and isovalerate net outflows increased while the microbial nitrogen outflow and the EMPS decreased with LO by 19% and 23% respectively, the SO tending to lower the EMPS ($P = 0.09$). In the partitioning of OM outflow, LO increased the undegraded dietary fraction by 14%.

4 Discussion

The impact of dietary fatty acids on rumen microbiota depends on their kinetics of release in the medium, which cannot be easily controlled experimentally when the lipids are in a complex matrix, such as a seed, a fresh forage or a cake. Thus, we chose to test pure vegetable oils owing to the rapid hydrolyse of the bonds between glycerol and fatty acids in the rumen. The fatty acid compositions of the three oils were consistent with published data (Broudiscou and Lassalas, 1991; Perretti *et al.*, 2004; Váradyová *et al.*, 2008; Szterk *et al.*, 2010; Ding *et al.*, 2017). As expected, they differed markedly one from another on C18 unsaturated acids contents with an average number of double bonds per C18 fatty acid of 1.41, 1.55 and 2.38 in RO, SO and LO respectively. Moreover, since dietary characteristics such as the nature and amount of carbohydrates are likely to modify the effects of a given dietary lipid on rumen function (Jalc *et al.*, 2006; Benchaar *et al.*, 2015) we focused on a diet considered typical of intensive dairy farming by our partner companies and characterized by high proportions of maize silage and protein cake. The use of dual effluent fermenters allowed to implement an unusually high oil incorporation level of 80 g/kg DMI overlapping its common range of variation in the ruminant's diet around 40 g/kg DMI that may reach 60 g/kg DMI (Bionaz *et al.*, 2020) in order to clearly highlight the nature of the effects of lipids on rumen metabolism. Indeed, when supplementing the diet of cows with incremental levels of sunflower oil, Shingfield *et al.* (2008) observed significant changes in ruminal digestion and fermentation pattern only at the highest input level of 750 g/d. In a previous study on the dose effects of LO and RO on in vitro rumen metabolism, the comparison of 40 and 80 g oil/kg DMI has shown that the oil input rate did not modify the nature of the effects observed but only their extent (Broudiscou *et al.*, 2022). Dual-effluent fermenters allowed to explore more components of rumen microbial metabolism than *in vivo* in the search for compromises specific to each oil supply, for example methane remediation vs. efficiency of microbial protein synthesis when LO was added. Despite the obvious interest of the fermenter as a tool to achieve this goal, these results must be crossed with *in vivo* data to integrate issues on animal productivity, health or product quality. The limitations of *in vitro* systems must also be kept in mind, such as the difficulty of keeping protozoa viable which is at best be reduced to a third of its in vivo equivalent (Broudiscou *et al.*, 1997). Furthermore, the particulate phases kinetics *in vitro*

remain a coarse simulation of *in vivo* phenomena (Bernard *et al.*, 2000). Nevertheless, the fermentation processes in controls were efficient since they were characterised by high daily productions of SCFA and methane along with low di-hydrogen and valerate productions, in good accordance with literature data (Jarrige *et al.*, 1995).

In our trial, the consequences of vegetable oil addition were threefold. A number of variables were not significantly altered. A second group of variables were altered the higher the degree of unsaturation of the oil. A final group of variables were altered without the magnitude of these changes being associated with the degree of unsaturation of the oil.

Within the first group of variables, oil supply did not significantly change the fermentation medium physicochemical conditions eleven hours after the substrate supply, in accordance with the observations by Vargas *et al.* (2020) when 6% sunflower or linseed oil on rumen microbial metabolism were added in rumen simulating systems (Rusitec) on a concentrate diet. In the same way, adding sunflower oil up to 5% DMI in the diet of lactating cows on grass silage-based diet did not significantly change neither the rumen pH nor the omasal flows of NDF and OM (Shingfield *et al.*, 2008). Starch and protein degradabilities were not significantly altered by the addition of oil. In controls they were in the higher range of the values commonly reported possibly due to the feed-processing mode requiring a grinding step, thus intensifying the microbial colonization of feed particles. Otherwise, the limited plant cell wall degradation was partly due to an average particles' residence time set at 22 h instead of the common value of 33 h in order to simulate the rumen solid phase kinetics observed in a dairy cow which are faster than in a standard castrated sheep. The lack of significant effect of dietary oils on dietary nitrogen true degradability was consistent with Potkanski and Nowak (2000) who observed that supplementing heifers with 30 and 60 g/kg RO did not change the *in sacco* effective protein degradability of three feeds differing in protein degradability.

The variables whose variations appeared to depend on the oil unsaturation degree can be divided into two groups linked either to H_2 removal pathways or to protein metabolism. the former group comprised the decreased specific productions of butyrate and methane and the increased specific productions of propionate and hydrogen. The higher effect of the most unsaturated oil on methanogenesis was consistent with the literature (Demeyer and Henderickx, 1967). Vargas *et al.* (2020) investigated the effects of 6% sunflower or linseed oil on rumen microbial metabolism in Rusitec on a concentrate diet and they also reported significant reductions of methane production by 21–28% along with an increase in propionate production when compared to the control treatment. The decrease of butyrate production induced by the oils agreed with published data (Broudiscou and Lassalas, 1991; Jalc *et al.*, 2006; Vargas *et al.*, 2020). The greater toxicity of polyunsaturated fatty acids to butyrate-producing bacteria is a plausible cause (Maia *et al.*, 2007). In addition, the increases in valerate and propionate productions following the addition of oil can be viewed as substitutes for methane as metabolic hydrogen sinks. Among the latter group of variables linked to nitrogen metabolism, the supply of unsaturated fatty acids significantly affected the outflows of ammonia and isovalerate, that are the end products of aminoacid degradation, as well as the microbial protein outflow and synthesis efficiency. In controls, the energetical efficiency of protein synthesis estimated through EMPS calculation was within the commonly reported range, *i.e.* 25–35 g of N/kg FOM (Stern *et al.*, 1994). Our observations were consistent with Czerkawski *et al.* (1975) who reported a decrease in ruminal microbial synthesis at a dietary input of 66 or 100 g/kg LO, yet the effects of dietary LO on microbial biomass flow and EMPS being inconsistent in the literature. In Rusitec maintained on a high-concentrate diet, the incorporation of 60 g/kg LO did not affect the microbial protein synthesis but with 2.2 double-bonds per C18 fatty acids the oil composition had an unsaturation degree lower than ours (Vargas *et al.*, 2020). Yet, Knight *et al.* (1978) and Sutton *et al.* (1983) reported a significant increase in EMPS following LO incorporation to the diet of sheep. According to Van Nevel and Demeyer (1981) such discrepancies may stem from the balance between two antagonist actions of oil on bacterial biomass synthesis: a direct inhibition of bacteria vs. a lowered bacterial predation and lysis due to protozoa depletion, the latter effect being underrepresented *in vitro* as protozoa tend to rarefy in fermenters.

Among the variables altered by oils without consideration of their unsaturation degree, the positive action on the productions of all SCFA but butyrate, on acetate specific production and on the amount of hexose fermented has not been reported yet. It may derive from the glycerol fraction liberated through the hydrolysis of triglycerides. Increasing the level of glycerol in semi-continuous fermenters on a forage diet increased the production of SCFA and propionate (Avila Stagno, 2013). However, the fermentation of glycerol to lactate (Henderson, 1973) would account for at most a third of these increased propionate production and amount of hexose fermented in our essay. A fraction of fermentation end products might also derive from the degradation of released fatty acids although it is commonly accepted that fatty acids cannot be extensively degraded to acetate in the rumen as they are in other anaerobic ecosystems, due to shorter retention times (Mackie *et al.*, 1991). In our essay, the deposition of fatty acids on fermenter surfaces may have increased their residence time sufficiently to allow degradation, provided the presence of the involved bacterial species likely epimural in the rumen. The forthcoming analysis of the balance between individual long chain fatty acid fluxes in and out of our fermenters will provide an opportunity to test this hypothesis.

5 Conclusions

In the fermenters maintained on a maize silage-based ration, exposure of the ruminal microbiota to the three oils at high incorporation levels led to a distinction between two classes of effects depending on the degree of unsaturation of the oils. The rumen variables, which appeared to be sensitive to oil unsaturation degree, were related either to metabolic hydrogen removal pathways or to protein metabolism.

Conflict of interest

The authors declare that they have no conflicts of interest in relation to this article.

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