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Abstract

Little quantitative information is available on how dietary lipids concurrently alter the main rumen microbial functions in relation with their incorporation level. In a three-period experiment, linseed (*Linum usitatissimum*, LO) and rapeseed (*Brassica napus* L., RO) oils were added at 0, 40 or 80 g/kg dry matter input (DMI) to five 1-liter dual outflow fermenters that were on a maize silage-based diet for nine days per period. RO supply decreased butyrate specific production. The amount of hexoses fermented (HF) increased by 9% at 40 g/kg LO. The production of CH_4 was lower at 80 g/kg LO by 46% compared to controls. Conversely, the supply of LO significantly increased H_2 and H_2S productions in an antagonistic mode. The specific productions of propionate, butyrate, CH_4 ans H_2 were altered by LO. The supply of RO increased the ammonia daily outflow (by 23% at 40 g/kg) and decreased the organic N outflow (by 13% at 80g/kg). The degradabilities of dietary fractions were not affected by RO, neither the OM partionning between dietary, fermented and microbial outflows. The OM true degradability decreased at 80 g/kg LO compared with controls and 40 g/kg LO. When LO was supplied, isovalerate and ammonia-N outflows were higher, organic and microbial N outflows and EMPS were lower along with changes in the the OM outflow partionning. Overall microbial processes appeared to differ in their responses to fatty acids saturation. Moreover, most effects were present at 40 g/kg DMI and diminished or even plateaued at 80 g/kg DMI.

Keywords: rumen, lipid, metabolism, bacteria, fermenter

Highlights

- 4% oil lowered butyrate specific production
- 4% Linseed oil lowered microbial protein synthesis efficiency
- Linseed oil lowered methanogenesis in proportion to input level

Introduction

For several decades, a great deal of research has been carried on the nutritional consequences of the addition of lipid-rich materials to the ruminant's diet, with fat content well above the values of 20-50 g/kg DM usually measured in the diets high in forages on which the ruminal microbiota and its host have coevolved. The aims under consideration have been diverse, at first designed to partially replace starch in the diet of high-producing animals (Coppock and Wilks 1991) and thus reduce the risk of ruminal acidosis. The introduction of lipids in the ruminant diet has then been aimed at reducing the protozoan population in the rumen (Stern et al. 1994) and thus sparing the proteins synthesized by rumen bacteria , at shifting the fermentative profile towards an increased specific production of propionate, a major glucogenic precursor (Wiltrout and Satter 1972). Finally, it has been promoted as a strategy to mitigate methanogenesis (Van Nevel and Demeyer 1996; Giger-Reverdin et al. 2003; Benchaar et al. 2015), especially with rations rich in plant cell-walls, or eventually as a strategy to improve the nutritional quality of animal products, meat and milk, by increasing the unsaturation degree of their fatty acids (Lock and Bauman 2004). However, several possible side effects have been deemed undesirable in the rumen, such as decreases in organic matter degradability and in microbial protein synthesis efficiency. Assessing how valuable is a lipid source in the ruminant diet thus requires to quantitatively determine its influence simultaneously on the degradation of the other dietary components, in particular structural carbohydrates, on the individual productions of fermentative metabolites –short-chain fatty acids and gases - and on the synthesis of microbial biomass in order to better manage trade-offs between positive and negative consequences of its dietary supply.

Yet, quantitative data on how the incorporation rate and unsaturation degree of dietary lipids concurrently alter the main rumen functions are scarce. They mainly arise from compiling experimental studies through meta-analysis implementation on cattle (Patra 2013) and sheep (Patra 2014). Data from experiments monitoring rumen fermentation, degradation and synthesis processes at the same time are still needed, because best able to document the positive and negative effects of lipid intake on rumen functions and thus facilitate the choice of their intake level based on the resulting trade-offs. The unsaturation degree of fatty acids varies greatly depending on the plant species, from rapeseed rich in

oleic acid to linseed rich in linolenic acid. As the action of dietary fatty acids on the microbiota depends also on their kinetics of release in the rumen, the implementation of vegetable oil hydrolysates can avoid this additional experimental factor, but a more practical and equally acceptable solution is to test pure vegetable oils owing to their rapide and complete lipolysis in the rumen. Besides, when the lipid amount in the diet is to be considered *in vitro*, the experimental levels chosen for this factor can widely overlap its common range of variation in the ruminant's diet, that may reach 6% DM (Bionaz et al. 2020) in order to highlight the nature of the various effects of fat on rumen metabolism.

At last, as with other dietary components such as protein sources (Brizga et al. 2021), the question of the oil origin has recently emerged, with a growing preference for local sources and shortened supply chains in an effort to reduce the environmental impact of ruminant husbandry (Herrero et al. 2009; Balmford et al. 2018). In this regard, because linseed (*Linum usitatissimum* L.) and rapeseed (*Brassica napus* L.) are common crops in France (Nag et al. 2015) and in Europe (Fridrihsone et al. 2018; Charbonnier et al. 2019) they appear to be good oil sources models.

In the present trial in dual outflow fermenters inoculated with bovine rumen microbiota, we aimed at quantifying individually the dose effects of linseed oil (LO) and rapeseed oil (RO) on feed degradation, fermentation yields and microbial biomass synthesis, in order to estimate the possible trade-offs and help rationalise the choice of an incorporation level into a maize silage-based diet.

Materials and Methods

Experimental Design

For each oil, two incorporation levels (40 and 80 g/kg DMI) were tested against a control. On three independent periods each comprising a 6-day equilibration phase and a 3-day measurement and sampling phase, the treatments were randomly assigned to 5 1-liter dual outflow fermenters (Broudiscou et al. 1997). Hence, the experimental design comprised 15 runs, the periods being treated as block effects.

Incubation Procedure

Three Holstein dry cows, fitted with rumen cannulas, served as rumen fluid donors to inoculate fermenters at the beginning of each period. They were kept in carpet stalls at the experimental facility of

the MIXscience Sourches Research and Development Farm (Saint-Symphorien, France) and received a diet mainly composed of alfalfa hay. Care and handling of the cows followed the procedures approved by the French Ministry of Agriculture in agreement with French regulations for animal experimentation (Anonymous 2013a; Anonymous 2013b) and the farm has a ISO 9001 certification.

On the first day of each period, the rumen contents of two cows were pooled, coarsely filtered and kept at 35 -40 °C under CO_2 atmosphere until inoculated. The fermenters were filled with 300 mL of artificial saliva then 300 mL of rumen fluid previously filtered through a 2 mm metal sieve. Each fermenter received 22 g DM/d of solid substrate, excluding oil, in 2 equal supplies at 11:00 and 23:00. At the same times, 0.921 and 0.461 mL of LO, 0.961 and 0.480 mL of RO, equaling 80 and 40 g/kg DMI respectively, were introduced in accordance with the experimental design. Both oils were produced in Terre Inovia facilities and their fatty acid compositions (table 1) were determined in Terre Inovia and OLEAD laboratories (Pessac, France). The solid substrate (table 2) is typical of a dairy cow standard diet, that is 16 kg DM maize silage, 2.8 kg soybean cake, 1.5 kg wheat grain, 1.2 kg rapeseed cake, 1 kg wheat straw, and 100 g urea. Corn silage was stored at -20 °C. Every morning, a batch of corn silage still frozen was hand-chopped down to a particle size under 2-3 mm (allowing particles to flow through the 16-mm diameter overflow) and weighed. The other constituents had been milled into a Retsch ZM1000 knife mill equipped with a 1 mm opening grid and then mixed with urea. A buffer solution was continuously infused at 1.11 ± 0.03 mL/min to maintain the medium pH above 6.3 and strongly reducing conditions (Broudiscou et al. 1999a). The liquid and solid phases turnover rates were set at 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, 11h after substrate supply, fermentative media were collected. Their pH and Eh were measured. Then they were sampled (1.2 mL of medium mixed with 0.3 mL of acid phosphoric acid 250 mL/L) and stored at -20°C until short-chain fatty acids (SCFA) and ammonia nitrogen (NNH3) analyses. Batches of maize silage were pressed, the liquid fractions were sampled and stored at -20°C until SCFA analysis. On days 7 to 9, total effluents were collected and subsampled to measure DM (2 x 15 mL), determine concentrations of SCFA and NNH3 (2 x 4 mL) and isolate reference bacterial pellets

(800 mL). The remaining fractions of around 800 mL were stored at -20°C until lyophilisation. To isolate reference bacterial pellets used in microbial biomass estimation, the effluents were milled (Trabalza-Marinucci et al. 2006), then treated by differential centrifugation (5 min 1000g, 15 min 15000g) and freeze-dried (Broudiscou et al. 1999b). On days 7 to 9, the fermentation gases were collected in sealed bags (10 L, Linde Gas) for methane, hydrogen sulphide and hydrogen daily productions determination.

Laboratory Analyses

Feeds and effluents were ground prior to analysis in a Culatti grinder (Zurich, Switzerland) with a screen of 0.8 mm aperture. They were analysed for aNDFom (assayed without sodium sulfite and with alpha amylase), ADFom and Lignin (Van Soest et al. 1991), starch (Faisant et al. 1995). The aNDFom and ADFom were calculated from the determination of their ash content (550°C, five hours). Feeds, effluents and bacterial pools DM contents were determined by oven drying at 105°C for 48h. OM contents were determined by ashing at 550°C for 16h. Total nitrogen was determined using the Dumas technique (Sweeney and Rexroad 1987) on a LECO model FP-428 Nitrogen Determinator (LECO, St Josef, MI). Crude protein was calculated as N x 6.25. Nucleobases, used as microbial markers, were determined by reversed phase HPLC (Lassalas et al. 1993). Individual VFA concentrations in culture medium were determined by reverse phase HPLC. NNH3 concentration was determined using a specific probe (Broudiscou and Papon 1994). The composition of fermentation gases was determined by gas chromatography (Broudiscou et al. 2014).

Calculations and Statistical Analysis

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

(1) HF = (C2 + C3) / 2 + C4 + C5 (mmol/d)

Where C2, C3, C4 and C5 are the daily outflows of acetate, propionate, butyrate and valerate (mmol/d).

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

(2) FOM = 162 x HF (g/d)

The microbial nitrogen outflow (MNf) and the efficiency of microbial protein synthesis (EMPS) were determined from DM outflow (DMf), nucleobase and Dumas nitrogen contents in DM outflows and in bacterial pellets.

(3) ESPM = g MNf / kg daily flow of FOM

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

(4) tOMd = 100 * (MOM outflow + FOM) / OM input

Where MOM is the microbial organic matter.

(5) tNd = 100 * (1 - (ONf - MNf) / INANf)

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

The results were subjected to analysis of variance and the effects of oil, incorporation level and period (blocking factor) were determined using the Minitab19 GLM procedure and a nested model. The level factor was nested within oil. The differences between control and oil levels were tested by peforming pairwise multiple comparisons using the Tukey'method with an experimentwise type I error set at 0.05.

Results

Whatever their incorporation levels, LO and RO did not tamper the physicochemical parameters of the fermenter contents 11h after substrate supply (Table 3). These remained within optimal ranges for rumen microbial activity. Fermentation broth pH varied from 6.55 to 6.78, averaged 6.69 in controls and was not significantly modified by oil supply. The redox potential, averaging -224 mV in controls, was significantly higher at 40 and 80 g/kg RO (by 17 mV) and at 40 g/kg LO (-20 mV). The concentrations of NH_3-N (30.7 g/L in controls) and SCFA were not significantly modified by oil supply. The nature of oil significantly affected the broth Eh, molar proportions of propionate, butyrate and valerate at 11h, the amount of hexoses fermented, the specific productions of propionate and butyrate and the individual gas productions. When RO was supplied the molar proportion of butyrate at 11h and its specific production were significantly decreased (by 52% and 53% respectively at 40 g/kg) along with an increase in the molar proportions of propionate and valerate. The supply of LO significantly decreased butyrate molar proportion (by 63% at 40 g/kg) and increased both the propionate and valerate proportions. The production of CH_4 averaged 28 mmoles/d in controls and was lowered at 80 g/kg LO by 46%.

Conversely, the supply of LO significantly increased H_2 and H_2S productions in an antagonistic mode. The specific productions of propionate, butyrate, CH_4 ans H_2 were significantly altered by LO. The degradabilities of dietary fractions were not significantly affected by oils, neither the OM partionning between dietary, fermented and microbial outflows (Table 4). Otherwise, the nature of oil significantly affected the variables associated with nitrogen fractions outflows. The supply of RO significantly increased the ammonia daily outflow (by 23% at 40 g/kg) and decreased the organic N outflow (by 13% at 80g/kg). When LO was supplied, isovalerate and ammonia-N outflows were higher, organic and microbial N outflows as well as EMPS were lower.

Discussion

The present study focused on the oils extracted from two plants, lin and rapeseed, commonly cultivated in the temperate European regions so as to contribute to promoting local resources as ruminant feeds. The fatty acid compositions of our oils were typical of both crops and consistent with literature data (Broudiscou and Lassalas 1991; Varadyova et al. 2000; Szterk et al. 2010; Ding et al. 2017). As expected, they differed markedly one from another on oleic and linolenic acids contents and the average number of double bonds per C18 fatty acid was 1.41 and 2.38 in RO and LO respectively, allowing to investigate the role of unsaturation degree, along with dietary incorporation rate, in the changes induced in rumen metabolism. The effect of a given dietary lipid source on rumen also depends on its chemical form and supply rate that both drive the fatty acid release rate. Opting for oils supplied along with solid substrate helped to maximize this release rate since oil triglycerides were readily available to microbial lipolysis and the bonds between glycerol and fatty acids quickly hydrolysed. The effect of a dietary fat on rumen métabolism also depend on the characteristics of the other dietary components, in particular the nature and amount of carbohydrates (Jalc et al. 2006a; Jalc et al. 2006b; Benchaar et al. 2015). Our trial was thus intended to focus on a ration commonly used in intensive dairy farming, characterised by high proportions of maize silage and protein cake.

Both oils altered in a similar way the fermentative profile and the medium redox potential eleven hours after the substrate supply. The variations in butyrate molar proportions in oil-supplemented cultures were similar to those previously reported with RO (Potkanski and Nowak 2000; Jalc, Potkanski, et al.

2006) as well as with LO supplementation (Broudiscou et al. 1994; Benchaar et al. 2015; Vargas et al. 2020). When LO was supplied, the changes in redox potential, along with the effects on propionate proportion, suggest that the microbial activity impacted redox potential through the fermentation pathways modulating metabolic hydrogen flows. In addition to fermentation profiles shared with *in vivo* trials, continous cultures give access to the daily productions of individual fermentation end-products. The fermentative activities in controls were consistent with the literature (Jarrige et al. 1995). Major SCFA and methane productions in controls were high along with low di-hydrogen and valerate productions, which indicate an efficient fermentation process. The favorable effect in our study of the LO on the productions of all SCFA but butyrate has not been reported yet. The fermentation of glycerol to lactate (Henderson 1975) could account for at most a third of this increased propionate production and amount of hexose fermented (in tendency). It may stem from the differential susceptibility of rumen microbes to PUFA (Henderson 1973; Maia et al. 2007) giving a competitive advantage to some microbial species in the access to limiting substrates. As for SCFA profile 11h following substrate supply, the decrease of butyrate induced by both oils agreed with previous findings (Broudiscou and Lassalas 1991; Jalc, Potkanski, et al. 2006; Vargas et al. 2020). One commonly accepted cause is the greater toxicity of PUFA to butyrate-producing bacteria (Maia et al. 2007). In addition, the increase in valerate and propionate proportions when LO was supplied can be interpreted as a substitute for methane in the role of metabolic hydrogen sink.

Individual gases productions were significantly altered only by LO which inhibited methanogenesis and the magnitude of this effect was clearly related to the LO input level. The higher effect of the most unsaturated oil is consistent with published observations (Demeyer and Henderickx 1967). At the same time, dihydrogen and hydrogen sulphide accumuled but tended to co-evolve according to LO level in an antagonistic mode. Thus, hydrogen sulphide microbial production might require di-hydrogen partial pressure to build up to a threshold to occur. Lending support to this hypothesis, hydrogenotrophic bacteria other than methanogens have a reduced affinity for di-hydrogen. Furthermore, these data suggest that in ruminants fed on a diet rich in readily available sulfur such as inorganic forms, the intake of PUFA in large amounts could lead to an increase in ruminal production of hydrogen sulfide

potentially toxic to the animal (Sarturi et al. 2013), this possible metabolic interaction being undocumented so far.

Starch and protein degradabilities in controls were in the higher range of the normal values observed in the rumen possibly due to the feed-processing mode requiring a grinding step, thus favoring feed particle colonization by rumen microorganisms. 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. With regards to nitrogen metabolism in our study, LO supply significantly affected microbial protein outflow and synthesis efficiency. The lack of significant effect of both LO and RO 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. However, a possible outcome of oil supply in the rumen could be a change in deamination yield altering differentially the amounts of individual amino acids available for microbial protein synthesis. Supplying sheep with 60 g/kg DM LO significantly lowered the *in vitro* degradation of glutamine by half without any significant effects on aspartic acid, glutamic acid and threonine degradation (Broudiscou and Lassalas 1991). The intensity of microbial synthesis in controls was consistent with the published data in continuous fermenters or in the rumen using nucleobases as a microbial marker (Stern et al. 1994). Its energetical efficiency estimated through EMPS calculation was within the commonly reported range, ie 25-35 g of N/kg FOM. The outflow of microbial nitrogen and the EMPS were lowered even at low LO levels. Our findings agreed with Czerkawski et al. (1975) who observed a fall in the microbial synthesis in the rumen following the dietary input of 66 or 100 g/kg LO, yet the effects of dietary LO on microbial biomass flow and EMPS have been inconsistent in the literature. In Rusitecs (*Rumen simulating technique*) maintained on a high-concentrate diet close to ours, 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 presented an unsaturation degree lower than ours (Vargas et al. 2020). Opposite to our *in vitro* results, Knight et al. (1978) and Sutton et al. (1983) reported a significant increase in EMPS when LO was added tho the diet of sheep.

As an explanation of these discrepancies, Van Nevel and Demeyer (1981) suggested that oil supplementation had two antagonist effects on bacterial biomass synthesis: a direct inhibition of bacterial metabolism opposing a protozoa depletion sparing bacterial predation and lysis, the latter being underrepresented in *in vitro* systems.

Aggregating observations on the various aspects of rumen microbial metabolism makes it possible to search for compromises and associate it with the definition of an optimal range of oil incorporation level. Despite the obvious interest of the fermenter as a tool to achieve these objectives, it must be kept in mind that these observations must in practice be crossed with *in vivo* experimentation to integrate questions regarding animal productivity, product quality and animal health. The limitations of the *in vitro* model must also be kept in mind, such as the difficulty of keeping protozoa viable. Depending on the design of a continuous fermenter, the ciliate population will at best be reduced to a third of its *in vivo* equivalent, at worst washed out (Broudiscou et al. 1997). The kinetics of particulate phases will be a coarse simulation of *in vivo* phenomena (Bernard et al. 2000). Both issues are of interest when questionning the incorporation of fat in the ruminant diet. Nevertheless, if aiming at reducing methanogenesis and orienting fermentation towards a specific production of propionate is counterbalanced by the need not to deteriorate the OM degradability and fermentation extent and to reduce the negative impact on microbial proteosynthesis, setting the rate of dietary incorporation of LO, and to a lesser extent RO, within the range of 30-50 g/kg DMI seemed to be satisfactory in our study.

Conclusions

In our experimental conditions, LO and RO were ranked according to the magnitude of their impact on fermentation extent and profile, as well as on the microbial protein outflow and its synthesis efficiency, in good accordance with the fatty acids unsaturation degree. The quantification of dose effects for both oils suggest that most effects were present at the incorporation level of 40 g/kg DMI and attenuated or even plateaued at 80 g/kg DMI, with the notable exception of methane and microbial protein outflows when LO was supplied.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, Broudiscou L.P., upon reasonable request.

Declaration of Interest: None.

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401 Table 1. Fatty acid composition of the rapeseed and linseed oils (g/kg)

Fatty acids	Rapeseed	Linseed
C14:0	1	1
C16:0	53	68
C16:1	4	1
C18:0	15	32
C18:1	592	164
C18:2	218	151
C18:3	85	575
C20:0	6	2
C20:1	11	1
C22:0	3	1
C22:1	1	<1
C24:0	1	1
C24:1	2	<1
Other	8	3
Saturated	80	106
Monoinsaturated	611	166
polyinsaturated	303	726

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404 Table 2: composition of the solid substrate

Feeds	g/kg DM	DM (g/kg)
Maize silage	732.3	377
Soybean cake	112.5	878
Wheat grain	60.1	876
Rapeseed cake	48.7	887
Wheat straw	41.8	912
Urea	4.6	1000
Composition (g/kg DM)		
DM (g/kg)	446	
OM	959	
aNDFom	358	
ADFom	240	
Starch	215	
Crude protein	153	
Ether Extract	23	
NFC concentration	425	

405

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Table 3. fermentation parameters 11h after substrate supply, daily amount of hexoses fermented (HF) daily gases productions, and specific productions of short-chain fatty acids (SCFA) and gases in continuous cultures.

	Treatments					SEM	P	
	CTL	RO 40	RO 80	LO 40	LO 80	(n=3)	Oil	Level
11h pH	6.69	6.60	6.60	6.62	6.66	0.026	0.054	0.67
11h Eh (mV)	-223.7 a	-206.2 b	-206.7 b	-202.5 b	-217.3 ab	3.31	0.008	0.039
11h SCFA (mM)	64.4	74.9	80.1	73.7	76.3	4.27	0.094	0.65
11h SCFA molar proportion (mol/100mol)								
acetate	56.9	53.7	55.8	52.7	56.5	1.65	0.50	0.24
propionate	27.4 a	34.8 b	35.7 b	36.4 b	35.3 b	1.35	0.002	0.78
butyrate	15.34 a	7.28 b	5.35 bc	5.65 bc	3.04 c	0.813	<0.001	0.064
valerate	0.35 a	4.22 b	3.14 ab	5.30 b	5.12 b	0.686	0.001	0.55
11h NH_3-N mg/L	30.7	37.5	33.8	29.2	26.2	3.10	0.090	0.58
HF (mmol/d)	67.6	74.3	73.8	73.6	70,4	1.59	0.031	0.40
CH_4 (mmol/d)	28.5 a	23.8 ab	24.0 a	21.5 ab	15.5 b	1.71	0.004	0.098
H_2 (mmol/d)	1.64 a	3.37 ab	4.68 abc	8.0 bc	9.0 c	1.13	0.002	0.62
H_2S (mmol/d)	9.6 a	16.7 a	20.3 a	19.8 a	44.7 b	3.87	0.003	0.006
Specific productions (moles /100 moles hexoses fermented)								
acetate	87.6	96.2	100.9	96.7	104.1	3.78	0.060	0.32
propionate	47.3 a	63.6 ab	63.8 ab	68.6 b	66.2 b	3.39	0.004	0.89
butyrate	28.3 a	13.4 b	11.7 b	11.5 b	7.4 b	1.24	<0.001	0.093
valerate	4.3	6.7	6.0	5.84	7.45	0.972	0.18	0.48
CH_4	42.0 a	32.2 ab	32.5 ab	29.4 bc	21.9 c	2.09	0.001	0.095
H_2	2.44 a	4.47 ab	6.41abc	11.0 bc	12.7 c	1.48	0.001	0.50

SEM: standard error of the mean

413 Table 4. OM true degradability, NDF, ADF and starch degradabilities, nitrogen true degradability,
414 isovalerate and nitrogen-bound daily outflows, biomass synthesis and OM outflow partitioning in
415 continuous cultures
416

	Treatments					SEM	P	
	CTL	RO 40	RO 80	LO 40	LO 80	(n=3)	Oil	Level
tOMd	0.705	0.742	0.722	0.708	0.665	0.018	0.089	0.236
NDFd	0.252	0.187	0.146	0.240	0.256	0.030	0.050	0.61
ADFd	0.338	0.221	0.189	0.281	0.278	0.036	0.040	0.81
STARCHd	0.908	0.912	0.914	0.932	0.915	0.007	0.22	0.32
tNd	0.791	0.843	0.865	0.805	0.737	0.032	0.076	0.33
				Outflows				
isovalerate (mmol/d)	6.58 a	8.01 ab	8.73 ab	10.24bc	11.46 c	0.556	0.001	0.26
NH_3-N (mg/d)	36.6 a	45.0 b	51.4 bc	54.2 c	66.2 d	1.53	<0.001	0.001
organic N (mg/d)	489.5 a	443.9 b	427.2 b	427.8 b	445.4 b	8.50	0.002	0.19
microbial N (mg/d)	379. a	360. a	356. a	324. b	306. b	17.4	0.032	0.75
EMPS (gN/kg FOM)	34.6 a	30.0 a	29.8 ab	27.1 b	26.7 b	1.61	0.014	0.98
OM outflow partitioning (g /100 g outflow)								
undegraded	29.5	25.8	27.8	29.2	33.5	1.78	0.089	0.23
microbial	18.7	19.5	19.9	16.5	16.6	1.18	0.077	0.97
fermented	51.9	54.8	52.3	54.2	49.9	1.16	0.40	0.045

417 SEM: standard error of the mean; , organic N outflow: total N outflow minus ammonia-N ouflow;
418 EMPS: Efficiency of microbial protein synthesis