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1 Dose response relationships between linseed or rapeseed oils supply and rumen microbial metabolism in
2 continuous culture on maize silage-based diet

3

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10 Abstract

11 Little quantitative information is available on how dietary lipids concurrently alter the main rumen
12 microbial functions in relation with their incorporation level. In a three-period experiment, linseed
13 (*Linum usitatissimum*, LO) and rapeseed (*Brassica napus* L., RO) oils were added at 0, 40 or 80 g/kg
14 dry matter input (DMI) to five 1-liter dual outflow fermenters that were on a maize silage-based diet for
15 nine days per period. RO supply decreased butyrate specific production. The amount of hexoses
16 fermented (HF) increased by 9% at 40 g/kg LO. The production of CH₄ was lower at 80 g/kg LO by
17 46% compared to controls. Conversely, the supply of LO significantly increased H₂ and H₂S
18 productions in an antagonistic mode. The specific productions of propionate, butyrate, CH₄ and H₂ were
19 altered by LO. The supply of RO increased the ammonia daily outflow (by 23% at 40 g/kg) and
20 decreased the organic N outflow (by 13% at 80g/kg). The degradabilities of dietary fractions were not
21 affected by RO, neither the OM partitioning between dietary, fermented and microbial outflows. The
22 OM true degradability decreased at 80 g/kg LO compared with controls and 40 g/kg LO. When LO was
23 supplied, isovalerate and ammonia-N outflows were higher, organic and microbial N outflows and
24 EMPS were lower along with changes in the the OM outflow partitioning. Overall microbial processes
25 appeared to differ in their responses to fatty acids saturation. Moreover, most effects were present at 40
26 g/kg DMI and diminished or even plateaued at 80 g/kg DMI.

27 Keywords: rumen, lipid, metabolism, bacteria, fermenter

28 Highlights

- 29 • 4% oil lowered butyrate specific production
- 30 • 4% Linseed oil lowered microbial protein synthesis efficiency
- 31 • Linseed oil lowered methanogenesis in proportion to input level

32

33 Introduction

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

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

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

67 At last, as with other dietary components such as protein sources (Brizga et al. 2021), the question of the
68 oil origin has recently emerged, with a growing preference for local sources and shortened supply chains
69 in an effort to reduce the environmental impact of ruminant husbandry (Herrero et al. 2009; Balmford et
70 al. 2018). In this regard, because linseed (*Linum usitatissimum* L.) and rapeseed (*Brassica napus* L.) are
71 common crops in France (Nag et al. 2015) and in Europe (Fridrihsone et al. 2018; Charbonnier et al.
72 2019) they appear to be good oil sources models.

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

77 Materials and Methods

78 *Experimental Design*

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

84 *Incubation Procedure*

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

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

91 On the first day of each period, the rumen contents of two cows were pooled, coarsely filtered and kept
92 at 35 -40 °C under CO₂ atmosphere until inoculated. The fermenters were filled with 300 mL of
93 artificial saliva then 300 mL of rumen fluid previously filtered through a 2 mm metal sieve. Each
94 fermenter received 22 g DM/d of solid substrate, excluding oil, in 2 equal supplies at 11:00 and 23:00.
95 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
96 DMI respectively, were introduced in accordance with the experimental design. Both oils were
97 produced in Terre Inovia facilities and their fatty acid compositions (table 1) were determined in Terre
98 Inovia and OLEAD laboratories (Pessac, France). The solid substrate (table 2) is typical of a dairy cow
99 standard diet, that is 16 kg DM maize silage, 2.8 kg soybean cake, 1.5 kg wheat grain, 1.2 kg rapeseed
100 cake, 1 kg wheat straw, and 100 g urea. Corn silage was stored at -20 °C. Every morning, a batch of
101 corn silage still frozen was hand-chopped down to a particle size under 2-3 mm (allowing particles to
102 flow through the 16-mm diameter overflow) and weighed. The other constituents had been milled into a
103 Retsch ZM1000 knife mill equipped with a 1 mm opening grid and then mixed with urea. A buffer
104 solution was continuously infused at 1.11 ± 0.03 mL/min to maintain the medium pH above 6.3 and
105 strongly reducing conditions (Broudiscou et al. 1999a). The liquid and solid phases turnover rates were
106 set at 0.09 /h and 0.045 /h respectively. The daily control and effluent collection procedures are in
107 Broudiscou et al. (1997).

108 On days 7 and 8, 11h after substrate supply, fermentative media were collected. Their pH and Eh were
109 measured. Then they were sampled (1.2 mL of medium mixed with 0.3 mL of acid phosphoric acid 250
110 mL/L) and stored at -20°C until short-chain fatty acids (SCFA) and ammonia nitrogen (NNH₃)
111 analyses. Batches of maize silage were pressed, the liquid fractions were sampled and stored at -20°C
112 until SCFA analysis. On days 7 to 9, total effluents were collected and subsampled to measure DM (2 x
113 15 mL), determine concentrations of SCFA and NNH₃ (2 x 4 mL) and isolate reference bacterial pellets

114 (800 mL). The remaining fractions of around 800 mL were stored at -20°C until lyophilisation. To
115 isolate reference bacterial pellets used in microbial biomass estimation, the effluents were milled
116 (Trabalza-Marinucci et al. 2006), then treated by differential centrifugation (5 min 1000g, 15 min
117 15000g) and freeze-dried (Broudiscou et al. 1999b). On days 7 to 9, the fermentation gases were
118 collected in sealed bags (10 L, Linde Gas) for methane, hydrogen sulphide and hydrogen daily
119 productions determination.

120 *Laboratory Analyses*

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

133 *Calculations and Statistical Analysis*

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

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

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

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

138 (2) $FOM = 162 \times HF$ (g/d)

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

142 (3) $ESPM = \text{g MNf} / \text{kg daily flow of FOM}$

143 The true degradabilities of OM and nitrogen, tOM and tN were calculated as follows:

144 (4) $tOMd = 100 * (\text{MOM outflow} + \text{FOM}) / \text{OM input}$

145 Where MOM is the microbial organic matter.

146 (5) $tNd = 100 * (1 - (\text{ONf} - \text{MNf}) / \text{INANf})$

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

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

152 Results

153 Whatever their incorporation levels, LO and RO did not tamper the physicochemical parameters of the
 154 fermenter contents 11h after substrate supply (Table 3). These remained within optimal ranges for
 155 rumen microbial activity. Fermentation broth pH varied from 6.55 to 6.78, averaged 6.69 in controls and
 156 was not significantly modified by oil supply. The redox potential, averaging -224 mV in controls, was
 157 significantly higher at 40 and 80 g/kg RO (by 17 mV) and at 40 g/kg LO (-20 mV). The concentrations
 158 of NH₃-N (30.7 g/L in controls) and SCFA were not significantly modified by oil supply. The nature of
 159 oil significantly affected the broth Eh, molar proportions of propionate, butyrate and valerate at 11h, the
 160 amount of hexoses fermented, the specific productions of propionate and butyrate and the individual gas
 161 productions. When RO was supplied the molar proportion of butyrate at 11h and its specific production
 162 were significantly decreased (by 52% and 53% respectively at 40 g/kg) along with an increase in the
 163 molar proportions of propionate and valerate. The supply of LO significantly decreased butyrate molar
 164 proportion (by 63% at 40 g/kg) and increased both the propionate and valerate proportions. The
 165 production of CH₄ averaged 28 mmol/d in controls and was lowered at 80 g/kg LO by 46%.

166 Conversely, the supply of LO significantly increased H₂ and H₂S productions in an antagonistic mode.
167 The specific productions of propionate, butyrate, CH₄ and H₂ were significantly altered by LO.
168 The degradabilities of dietary fractions were not significantly affected by oils, neither the OM
169 partitioning between dietary, fermented and microbial outflows (Table 4). Otherwise, the nature of oil
170 significantly affected the variables associated with nitrogen fractions outflows. The supply of RO
171 significantly increased the ammonia daily outflow (by 23% at 40 g/kg) and decreased the organic N
172 outflow (by 13% at 80g/kg). When LO was supplied, isovalerate and ammonia-N outflows were higher,
173 organic and microbial N outflows as well as EMPS were lower.

174 Discussion

175 The present study focused on the oils extracted from two plants, lin and rapeseed, commonly cultivated
176 in the temperate European regions so as to contribute to promoting local resources as ruminant feeds.
177 The fatty acid compositions of our oils were typical of both crops and consistent with literature data
178 (Broudiscou and Lassalas 1991; Varadyova et al. 2000; Szterk et al. 2010; Ding et al. 2017). As
179 expected, they differed markedly one from another on oleic and linolenic acids contents and the average
180 number of double bonds per C18 fatty acid was 1.41 and 2.38 in RO and LO respectively, allowing to
181 investigate the role of unsaturation degree, along with dietary incorporation rate, in the changes induced
182 in rumen metabolism. The effect of a given dietary lipid source on rumen also depends on its chemical
183 form and supply rate that both drive the fatty acid release rate. Opting for oils supplied along with solid
184 substrate helped to maximize this release rate since oil triglycerides were readily available to microbial
185 lipolysis and the bonds between glycerol and fatty acids quickly hydrolysed. The effect of a dietary fat
186 on rumen metabolism also depend on the characteristics of the other dietary components, in particular
187 the nature and amount of carbohydrates (Jalc et al. 2006a; Jalc et al. 2006b; Benchaar et al. 2015). Our
188 trial was thus intended to focus on a ration commonly used in intensive dairy farming, characterised by
189 high proportions of maize silage and protein cake.
190 Both oils altered in a similar way the fermentative profile and the medium redox potential eleven hours
191 after the substrate supply. The variations in butyrate molar proportions in oil-supplemented cultures
192 were similar to those previously reported with RO (Potkanski and Nowak 2000; Jalc, Potkanski, et al.

193 2006) as well as with LO supplementation (Broudiscou et al. 1994; Benchaar et al. 2015; Vargas et al.
194 2020). When LO was supplied, the changes in redox potential, along with the effects on propionate
195 proportion, suggest that the microbial activity impacted redox potential through the fermentation
196 pathways modulating metabolic hydrogen flows. In addition to fermentation profiles shared with *in vivo*
197 trials, continuous cultures give access to the daily productions of individual fermentation end-products.
198 The fermentative activities in controls were consistent with the literature (Jarrige et al. 1995). Major
199 SCFA and methane productions in controls were high along with low di-hydrogen and valerate
200 productions, which indicate an efficient fermentation process. The favorable effect in our study of the
201 LO on the productions of all SCFA but butyrate has not been reported yet. The fermentation of glycerol
202 to lactate (Henderson 1975) could account for at most a third of this increased propionate production
203 and amount of hexose fermented (in tendency). It may stem from the differential susceptibility of rumen
204 microbes to PUFA (Henderson 1973; Maia et al. 2007) giving a competitive advantage to some
205 microbial species in the access to limiting substrates. As for SCFA profile 11h following substrate
206 supply, the decrease of butyrate induced by both oils agreed with previous findings (Broudiscou and
207 Lassalas 1991; Jalc, Potkanski, et al. 2006; Vargas et al. 2020). One commonly accepted cause is the
208 greater toxicity of PUFA to butyrate-producing bacteria (Maia et al. 2007). In addition, the increase in
209 valerate and propionate proportions when LO was supplied can be interpreted as a substitute for
210 methane in the role of metabolic hydrogen sink.

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

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

222 Starch and protein degradabilities in controls were in the higher range of the normal values observed in
223 the rumen possibly due to the feed-processing mode requiring a grinding step, thus favoring feed
224 particle colonization by rumen microorganisms. Otherwise, the limited plant cell wall degradation was
225 partly due to an average particles' residence time set at 22 h instead of the common value of 33 h in
226 order to simulate the rumen solid phase kinetics observed in a dairy cow which are faster than in a
227 standard castrated sheep. With regards to nitrogen metabolism in our study, LO supply significantly
228 affected microbial protein outflow and synthesis efficiency. The lack of significant effect of both LO
229 and RO on dietary nitrogen true degradability was consistent with Potkanski and Nowak (2000) who
230 observed that supplementing heifers with 30 and 60 g/kg RO did not change the in sacco effective
231 protein degradability of three feeds differing in protein degradability. However, a possible outcome of
232 oil supply in the rumen could be a change in deamination yield altering differentially the amounts of
233 individual amino acids available for microbial protein synthesis. Supplying sheep with 60 g/kg DM LO
234 significantly lowered the *in vitro* degradation of glutamine by half without any significant effects on
235 aspartic acid, glutamic acid and threonine degradation (Broudiscou and Lassalas 1991). The intensity of
236 microbial synthesis in controls was consistent with the published data in continuous fermenters or in the
237 rumen using nucleobases as a microbial marker (Stern et al. 1994). Its energetical efficiency estimated
238 through EMPS calculation was within the commonly reported range, ie 25-35 g of N/kg FOM. The
239 outflow of microbial nitrogen and the EMPS were lowered even at low LO levels. Our findings agreed
240 with Czerkawski et al. (1975) who observed a fall in the microbial synthesis in the rumen following the
241 dietary input of 66 or 100 g/kg LO, yet the effects of dietary LO on microbial biomass flow and EMPS
242 have been inconsistent in the literature. In Rusitecs (*Rumen simulating technique*) maintained on a high-
243 concentrate diet close to ours, the incorporation of 60 g/kg LO did not affect the microbial protein
244 synthesis but with 2.2 double-bonds per C18 fatty acids the oil composition presented an unsaturation
245 degree lower than ours (Vargas et al. 2020). Opposite to our *in vitro* results, Knight et al. (1978) and
246 Sutton et al. (1983) reported a significant increase in EMPS when LO was added tho the diet of sheep.

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

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

265 Conclusions

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

272

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277

278 Data availability statement

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

281

282 Declaration of Interest: None.

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- 399

400

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 |

402

403

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

406

407 Table 3. fermentation parameters 11h after substrate supply, daily amount of hexoses fermented (HF)
 408 daily gases productions, and specific productions of short-chain fatty acids (SCFA) and gases in
 409 continuous cultures.
 410

| | Treatments | | | | | SEM (n=3) | P | |
|---|------------|----------|----------|----------|-----------|--------------|--------|-------|
| | CTL | RO 40 | RO 80 | LO 40 | LO 80 | | 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 ₂ S (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 |

411 SEM: standard error of the mean

412

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 (n=3) | P | |
|--|------------|---------|---------|---------|---------|--------------|--------|-------|
| | CTL | RO 40 | RO 80 | LO 40 | LO 80 | | 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 outflow;

418 EMPS: Efficiency of microbial protein synthesis