Effect of extruded linseed supplementation, grain source and pH on dietary and microbial fatty acid outflows in continuous cultures of rumen microorganisms

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Abbreviation: aNDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; anteiso-FA, anteiso methyl branched-chain fatty acid; BCFA, branched-chain fatty acids; BH, biohydrogenation; C18:2-BH, C18:2 9c,12c apparent biohydrogenation; C18:3-BH, C18:3 9c,12c,15c apparent biohydrogenation; CLA, conjugated linoleic acids; EMPS, efficiency of microbial protein synthesis; ESFA, even numbered fatty acids; FA, fatty acid; FAME, fatty acid methyl ester; FOM, fermented organic matter, GC, gas chromatography; HF, hexose fermented; iso-FA, iso methyl branched-chain fatty acids; OBCFA, odd-numbered and methyl branched-chain fatty acids; odd-FA, odd-chain fatty acids; PUFA, polyunsaturated fatty acids; tdOM, true organic matter degradability; VFA, volatile fatty acids.

Abstract

Using 6 continuous cultures of rumen microorganisms, we studied the effects of pH (low vs. high) and extruded linseed supplementation (10% of DM) in association with rapidly or slowly degraded starch sources (wheat vs. corn grains) 1) on fatty acid (FA) outflows and PUFA biohydrogenation (BH) processes 2) on FA microbial composition and its contribution to FA outflows, in two replicated periods of 10d (7d adaptation and 3d sampling). The control diet contained wheat. The buffer solutions infused in low or high pH cultures differed by the addition of 10 mL of 5N H_2SO_4 or NaOH so as to allow fermentation medium pH fluctuations with diets and time after feeding. The fermentation pattern, daily amounts of hexoses fermented (HF), efficiency of microbial protein synthesis (EMPS), and specific production of gases (CH_4 and H_2) were measured. FA compositions were determined in total effluents and bacterial pools isolated from effluents. Lowering pH (from 6.46 to 6.16 measured just before feeding) increased the VFA concentration in the control diet whereas it decreased it in all linseed supplemented diets. Lowering pH tended to decrease CH_4 specific production as well as acetate and propionate proportions and to increase butyrate and valerate proportions but did not alter HF and EMPS. Linseed but not grain source increased pH by 0.08 and did not modify fermentative parameters. Apparent BH of C18:3 9c,12c,15c and C18:2 9c,12c decreased at low pH but increased with linseed supplementation. Corn associated to linseed increased 18:3 9c,12c,15c BH compared to wheat. Consequently, C18:3 9c,12c,15c outflows increased at low pH and with linseed, and were higher with linseed-wheat diet than linseed-corn diet. For all treatments, the proportions of C18:0 (% of C18-FA outflows) remained low associated with high levels of BH intermediates (C18:2 11t, 15c and C18:1 11t) suggesting that BH did not proceed to completion. Lowering pH decreased C16:1 9c and C18:1 11c bacteria contents and tended to increase anteiso-FA. Linseed supplementation increased C18:0, C18:1 9c, C18:1 9t, C18:2 9c,12c bacteria contents without modifying the C18:3

9c,12c,15c and decreased their odd-FA content and anteiso-FA in tendency. Compared to wheat, corn decreased branched-FA bacteria content, as well as even-saturated FA. Lowering pH decreased the bacterial FA outflow whereas linseed increased it. Both decreased the bacterial FA contribution to total FA outflow. Results indicate that pH and diets modified PUFA BH while differing in their effect on odd- and branched-chain FA bacteria content and outflows.

Key Words: Continuous culture; Fatty acid; Biohydrogenation**;** Rumen Bacteria; Linseed; pH

Introduction

Public health policies in most developed countries recommend their population to decrease their intake in saturated fatty acids (SFA) and trans fatty acids (FA) as these FA in the diet are associated with higher cardiovascular disease (CVD) risks (Lichtenstein et al., 2006; EFSA, 2010; Gebauer et al., 2015). In human and animal models, CVD have been associated with C18:1 10t (Roy et al., 2007) and C18:1 11t (Gebauer et al., 2015), whereas C18:1 11t, metabolized to C18:2 9c,11t by Δ-9 desaturase enzyme in tissues may have numerous putative health benefits (McCrorie et al., 2011). An increase in consumption of polyunsaturated fatty acids (PUFA) and especially in n-3 PUFA (Gebauer et al., 2006; Harris et al., 2009) is also recommended. Ruminant derived foods (meat, milk and dairy products) are significant sources of these SFA and trans FA and are characterized by low PUFA content as dietary PUFA undergo lipolysis and extensive biohydrogenation (BH) in the rumen resulting in the formation of SFA, and of a variety of positional or geometric (cis, trans) isomers of unsaturated FA (Lourenço et al., 2010). The extent to which dietary unsaturated FA are incorporated into products is therefore partly dependent of lipolysis and BH process. These processes are affected by several factors such as forage:concentrate ratio driving fiber and starch proportions, dietary amount of unprotected lipids and type of lipid supplements (vegetable oil or oilseeds, marine oils…) among others (Lourenço et al., 2010; Buccioni et al., 2012; Gudla et al., 2012). However, these nutritional strategies to manipulate FA profile in ruminant products and BH process still need to have their optimal modes of implementation specified.

Changes in the ruminal environment initiated through the diet can lead to alteration of microbial activity or shift in the microbial population, and, to modifications of the BH process and amounts and type of BH intermediates accumulated in the rumen. Dietary lipids have an antimicrobial effect associated with the degree of unsaturation of the FA and their inclusion

92 rate. They usually decrease ruminal bacteria biomass outflow with diets rich in FA
93 (Schmidely et al., 2008; Lourenço et al., 2010). They also alter the rumen bacteria
94 communities which play the main role in FA BH in the rumen (Harfoot and Hazlewood,
95 1997; Lourenço et al., 2010; Gudla et al., 2012). Lipid supplementation modulates BH and
96 usually increases BH intermediates, especially trans-C18:1, in line with the increased dietary
97 inputs of C18 unsaturated FA and the potential inhibition of the reduction step leading to
98 trans-monoenoic acids (Lourenço et al., 2010; Gudla et al., 2012). The effectiveness of the
99 lipid supplement to control BH also depends on the basal diet and on the physical form of the
100 supplement. Most *in vitro* experiments supplied FA in the form of plant oils while long-term
101 fermentation experiments on extruded oilseeds, especially linseed, in association with low
102 fiber diets are lacking. Low pH can also affect the microbial population, especially
103 cellulolytic bacteria which also includes hydrogenating bacteria, resulting in reduced ruminal
104 lipolysis and BH, and modification of BH intermediates production *in vitro*, in batch cultures
105 as well as in continuous culture (Harfoot and Hazlewood, 1997; Qiu et al., 2004; Troegeler-
106 Meynadier et al., 2006; Fuentes et al., 2011). Low pH often results in a decreased production
107 of trans-11 isomers and in an increased production of trans-10 isomers, possibly due to
108 modulation of enzymatic or bacterial activity (Fuentes et al., 2009; Fuentes et al., 2011;
109 Troegeler-Meynadier et al., 2014). In most continuous culture experiments though, pH was
110 set at fixed levels around 6.4-6.5 and 5.8-5.4 for high and low pH values respectively (Qiu et
111 al., 2004; Fuentes et al., 2009; Fuentes et al., 2011; Gudla et al., 2012) rather than being
112 allowed to fluctuate between feeding times, which is observed *in vivo*. This study looks at
113 different levels of pH while, at the same, allowing pH fluctuation which has rarely been done
114 before in *in vitro* studies.
115 High starch diets decrease ruminal pH and efficiencies of some BH reduction steps leading to
116 increased BH intermediates (AbuGazaleh and Jacobson, 2007; Zened et al., 2011). Even

117 though the effects of starch and pH could not be completely separated, the level of starch has
118 been identified as the main factor for the shift from the trans-11 to trans-10 pathways *in vitro*,
119 in batch cultures as well as in continuous culture (AbuGazaleh and Jacobson, 2007; Fuentes et
120 al., 2009; Fuentes et al., 2011). However, the source of starch may also be a factor affecting
121 the intensity of BH and production of intermediates. Lascano et al. (2016) reported that
122 increasing corn starch degradability both in low or high fat diets led to rumen BH
123 intermediates accumulation, mainly trans-10 isomers. Yet Li et al. (2014) did not observe any
124 modification of these intermediates in the rumen FA content of dairy goats when increasing
125 rumen degradable starch. Jenkins et al. (2003) observed a greater accumulation of BH
126 intermediates, mainly C18:1 11t, with corn compared to barley diet and a decrease in C18:2
127 9c,12c BH, prompting the need to study the effect of source of starch on BH.
128 All these effects of pH, fat supplementation and sources of starch on BH processes suggest
129 potential alteration in the microbial ecosystem. It is thus of interest to study their effects on
130 the FA microbial contribution to FA outflow and on the microbial FA composition
131 modification as they are poorly documented. A special attention should be paid to the odd
132 (odd-FA) and branched-chain fatty acids (BCFA) (OBCFA) of bacteria origin, as large
133 differences in these OBCFA profiles among rumen bacteria might help to assess
134 compositional changes in rumen microbial populations (Ifkovits and Ragheb, 1968; Fievez et
135 al., 2012). Moreover, there is a growing interest in using these FA in milk or meat as
136 potential diagnostic tools of rumen function and microbial activity (Buccioni et al., 2012;
137 Fievez et al., 2012).
138 Therefore, the objectives of the present study were to quantify the effects of pH and linseed
139 supplementation associated with two grain sources 1) on FA outflows and PUFA BH
140 processes 2) on FA microbial composition and its contribution to total FA outflow in
141 continuous cultures of rumen microorganisms.

Materials and methods

Experimental design

Two factors were combined: 1) the fermentation medium pH at two levels (low vs. high), 2) the experimental diet at three levels, Control Wheat (CW) vs Linseed Wheat (LW) vs Linseed Corn (LC). The three diets differed either in the grain source (wheat vs. corn) or in the addition of extruded linseed. Their compositions are detailed in Table 1 and were designed to have similar content of net energy and CP. The six combinations of factors were randomly assigned to six continuous culture systems for two consecutive periods of 10 days in a randomized complete block design, the blocking factor being the incubation period.

Incubations

Dual-flow continuous culture systems (working volume of 1.1 L) were run under a standard procedure detailed in Broudiscou et al. (1997). At the beginning of each period, 1.5 L of rumen contents were collected by accredited personnel from 2 dry Holstein cows fitted with rumen cannula and fed 8.4 kg DM per day of a diet based on 69 % grass hay, 17 % sugar beet pulp and 14 % barley. Animal care and use procedures were approved by the French Ministry of Agriculture in agreement with French regulations for animal experimentation (Décret n°2013-118). Rumen contents were pooled, coarsely filtered, and kept at 39°C ± 2°C under CO_2 atmosphere until introduction into continuous cultures systems. Each continuous culture was permanently infused with a mineral buffer solution (Broudiscou et al., 1999b) so as to set particle and liquid phase dilution rates at 0.024 and 0.06/h respectively. The fermentation medium pHs were adjusted at low or high levels in accordance with the experimental design by adding 5 ml/d of 5*N* H_2SO_4 or 5*N* NaOH to infused solutions respectively. Each fermenter received twice daily 13 g DM of one out of the three pelleted diets, at 11:30 and 23:30.

On d 8, d9 and d 10 at 11:20, prior to feeding, fermentation broths were sampled for pH measurement, volatile fatty acids (VFA) analysis (2 x 4mL mixed with 0.25 vol of 410 g/L

167 H_3PO_4 and stored at -20°C until analysis), and protozoa counts (2 x 1mL mixed with 3mL of a
168 13.3 mL/L glutaraldehyde, 666 mL/L glycerol solution and stored at +4°C). On d 9 and d 10
169 the volumes of daily fermentation gas were measured. From d 8 to d 10, total effluents were
170 pooled by fermenters and sampled for DM measurement and VFA determination. Bacterial
171 pellets were isolated from 600 mL of total effluents following the procedure described by
172 Broudiscou et al. (1999a). Then the remaining effluents and bacterial pellets were stored at -
173 20°C until freeze-drying for OM, nucleobases and FA determinations.

174 *Fermentative parameters and microbial analysis*

175 Prior to analysis, feeds and freeze-dried effluents were ground using a Culatti grinder DFH48
176 (Zurich, Switzerland) with a screen of 0.8 mm aperture. DM was measured using an oven,
177 drying at 105°C for 48 h. OM was measured by ashing at 550°C for 16 h. Nucleobase
178 concentrations were assessed in freeze-dried effluents and microbial pools by high-
179 performance liquid chromatography on a Jasco instrument comprising a PU2089 pump and a
180 MD 2018 diode array detector (Tokyo, Japan) (Lassalas et al., 1993) so that they could be
181 used as microbial markers. Individual VFA concentrations in fermentation broths and
182 effluents were measured by gas chromatography (GC) on a Varian Chrompack CP 3800
183 system (Varian Inc., Walnut Creek, CA, USA) (Kristensen, 2000). Total protozoa were
184 enumerated in Jessen cells on a microscope Nikon Labophot (Tokyo, Japan) (Broudiscou et
185 al., 1997). Fermentation gas composition was measured by GC on a CP-2003 Micro-GC
186 (Varian Inc., Walnut Creek, CA, USA) (Broudiscou et al., 2014).

187 *FA analysis*

188 FA content and FA composition of feed, effluents and bacteria were determined using
189 procedures described by Bas et al. (2003) with adaptations. Briefly, on day one, 150 mg of
190 each sample were vortex-mixed during 4 min with 10 mL of chloroform:methanol (2/1,
191 vol/vol) containing nonadecanoic acid (0.25 mg/mL) as internal standard (IS) and BHT as

anti-oxidant (100 mg/L). After centrifugation (2000 g, 5 min, 4°C), the supernatant was filtered with a phase separator and stored. The remaining solid phase was dried under nitrogen and treated by vortex-mixing with 4 mL of an acidic solution (absolute ethanol: distilled water: hydrochloric acid 37 %, 5/4/1 by vol.). Then 5 mL of hexane were added and the hexane phase was filtered. This procedure was repeated three times and the whole hexane phase recovered was dried in a rotary vacuum evaporator. Lipid residues were recovered with 6 mL of chloroform and transferred into sealed tubes. The extract was twice mixed with distilled water that was then removed after centrifugation (2000 g, 5 min, 4°C). The chloroform phase was filtered with a phase separator and recombined with previously stored supernatant. It was dried in a rotary vacuum evaporator and purified by saponification over night with 3 ml of 2 *N* potassium hydroxide solution in 95% ethanol (vol/vol).

On day two, total extract was recovered with 3 ml of distilled water. Impurities were removed three times by mixing with hexane that was then removed. FAs of the KOH phase were released with 3 ml of 6 *N* HCl. They were then extracted twice with hexane, dried, recovered with chloroform, mixed with distilled water and centrifuged as describe on day one. Then they were filtered as describe above, dried on a rotary vacuum evaporator and recovered with 2 mL of hexane before being transferred into sealed tubes. After drying the extract under nitrogen, FAs were double-methylated with sodium methanolate and BF3 as described by Bas et al. (2007). GC was performed with a Varian Chrompack CP 3800 system (Varian Inc., Walnut Creek, CA, USA) equipped with a flame ionization detector, a split/splitless injector and a CP 8400 autosampler. Helium was used as carrier gas (flow of 1 mL/min) and nitrogen as make-up gas. Fatty acid methyl ester (FAME) were separated on a 100m x 0.25 mm ID fused silica capillary column (CP-Sil88, Varian S.A, Les Ulis, France). The split ratio was 20:1 and injector and detector temperatures were maintained at 250°C. The oven temperature program was as follows: temperature was held at 50°C for 1 min, increased to 170°C at

10°C/min, held at 170°C for 78 min, increased to 220°C at 5°C/min, before finally being held at 220°C for 10 min. FAME were identified with methyl ester standards and FAME mixture (purity at least 98%) purchased from Sigma Aldrich (St Louis, MO, USA).

Calculations and statistical analyses

The daily amounts of hexoses fermented (HF) were calculated from the relation given by Demeyer and Van Nevel (1975):

$$HF\ (mmol/d) = (C2 + C3)/2 + C4 + C5 + C6 \quad (1)$$

where C2, C3, C4, C5, and C6 are acetate, propionate, butyrate, valerate, and caproate productions (mmol/d) respectively.

The fermented OM (FOM) was calculated using equation (2)

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

The true OM degradability (tdOM) was calculated from dietary OM inflow and fermented and microbial OM outflows as detailed in Broudiscou et al. (1999a).

The efficiency of microbial synthesis (EMPS) was calculated using equation (3)

$$EMPS = g\ microbial\ N\ daily\ outflow\ /\ kg\ FOM\ daily\ outflow \quad (3)$$

C18:2 9c,12c and C18:3 9c,12c,15c BH (18:2-BH and C18:3-BH respectively, expressed in %) were calculated as follows:

$$C18:2\text{-}BH = 100 \times (C18:2\ 9c,12c\ inflow - C18:2\ 9c,12c\ outflow) / (C18:2\ 9c,12c\ inflow) \quad (4)$$

$$C18:3\text{-}BH = 100 \times (C18:3\ 9c,12c,15c\ inflow - C18:3\ 9c,12c,15c\ outflow) / (C18:3\ 9c,12c,15c\ inflow) \quad (5)$$

with FA inflow and outflow expressed in mg/d.

The FA bacterial contribution to total FA outflow (%) was calculated as follows:

$$FA\ bacterial\ contribution = 100 \times FA\ bacterial\ outflow / total\ FA\ outflow \quad (6)$$

242 with FA outflow expressed in mg/d.

243 Data were analysed with ANOVA using the GLM procedure (SAS Inst Inc., Cary, NC). The
244 effects of dietary treatments and pH on fermentative parameters and FA composition or
245 outflows were analysed according to

246 $Y_{ijk} = \mu + D_i + pH_j + (D \times pH)_{ij} + B_k + \varepsilon_{ijk}$

247 where Y_{ijk} is the individual observation, μ is the overall mean, D_i is the effect of dietary
248 treatments ($i \in [1,3]$), pH_j is the effect of pH ($j \in [1,2]$), $(D \times pH)_{ij}$ is the interaction between
249 dietary treatments and pH, B_k is the blocking variable (incubation period, $k \in [1,2]$) and ε_{ijk} is
250 the residual error. Two contrast analyses were also performed, the first one examined the
251 effect of linseed supplementation and the second one isolated the effect of grain source in
252 linseed-supplemented diets. Effects were considered significant at $P < 0.05$ and tendency was
253 declared when $0.05 < P < 0.10$.

254 **Results**

255 *Effects on fermentative and microbial parameters*

256 Protozoa were absent from all fermentation broth. The pH 11h30 after substrate supply was
257 6.16 and 6.46 for the low and high pH treatments respectively (P=0.0004) and tended to
258 increase with linseed supplementation (P=0.09) (Table 2). Total VFA concentrations 11h30
259 after substrate supply ranged from 68 to 73 mM. Lowering pH tended to decrease the
260 proportion of acetate (P=0.09) and propionate (P=0.09), and to increase the proportion of
261 butyrate (P=0.08) and valerate (P=0.07). Acetate, propionate, butyrate, valerate and
262 isovalerate proportions were not affected by linseed supplementation or grain source. Diet x
263 pH interactions affected total VFA (P=0.01) and isobutyrate concentrations (P=0.05).
264 Lowering pH increased total VFA concentration in non-supplemented linseed diet whereas it
265 decreased it in linseed supplemented diets whatever the grain source. Isobutyrate was only
266 detected in wheat linseed supplemented diet at high pH.

HF, ranging from 66 to 74 mmol per day, tended to be decreased by linseed supplementation (Table 3, *P*=0.10) but was not modified by grain source or pH. tdOM which averaged 0.55 in the present experiment was not modified by diets but tended to be lower with lower pH (*P*=0.10). EMPS which averaged 16.3 g N per kg FOM was not modified by diet or pH. In all runs, H_2 accumulated, resulting in a high specific production yield of 1 to 12 mol/100 mol HF (data not shown) and was not modified by treatments. CH_4 specific production yield was very low in 11 runs (from traces to 2 mol/100 mol HF) and low in one run (8 mol/100 mol HF) (data not shown). It tended to be decreased at low pH by 93 % (*P*=0.07) while it was not affected by diets.

Effects on FA composition of rumen bacteria

Fatty acid content and composition of rumen bacteria are presented in Table 4. Interactions between diet and pH were only significant for the C12:0 proportions. It was not possible to estimate the C18:1 10t proportion in 4 samples because of total overlapping with the C18:1 11t peak due its high proportion in these samples (18-28 % of total FA).

Lowering pH did not modify the total bacterial FA content but modified some individual bacterial FA contents. It significantly decreased C16:1 9c (*P*=0.02) and C18:1 11c (*P*=0.02) contents and only in tendency C14:0 (*P*=0.07) and iso-C13 (*P*=0.08) contents. It tended to increase C12:0 (*P*=0.06), anteiso-C15 (*P*=0.08) and anteiso-FA (*P*=0.07) bacterial contents.

Linseed supplementation did not modify the total FA content of bacteria. For SFA bacterial contents, it significantly decreased C12:0 (*P*=0.001), C13:0 (*P*=0.002), C15:0 (*P*=0.04), anteiso-C17 (*P*=0.01), odd-FA (*P*=0.05), OBCFA (*P*=0.05) and only in tendency anteiso-C15 (*P*=0.09) and anteiso-FA (*P*=0.07). It also increased the C18:0 (*P*=0.04) bacterial content. It did not modify the iso-FA except for an increase in the bacterial iso-C15 content (*P*=0.02). The other SFA contents were not modified by linseed supplementation. For MUFA, linseed supplementation increased the C18:1 9c (*P*=0.003) and C18:1 9t (*P*=0.0005) bacterial

292 contents without modifying the other isomers of C18:1. For PUFA, linseed supplementation
293 decreased the C18:2 9c,12c (P=0.0006) and C18:2 9c,12t (P=0.01) bacterial contents without
294 modifying the C18:3 9c,12c,15c content.
295 Compared to wheat, corn significantly decreased iso-C16 (P=0.05), iso-C18 (P=0.02) and iso-
296 FA (P=0.04) bacterial contents and only in tendency iso-C13 (P=0.08) and iso-C15 (P=0.06).
297 It also significantly decreased anteiso-FA (P=0.04) but only in tendency individual anteiso-
298 C15 (P=0.06) and anteiso-C17 (P=0.06) without modifying the odd-FA bacterial content. It
299 also significantly decreased the even-numbered saturated fatty acid (ESFA, P=0.04) bacterial
300 content, and, only in tendency C18:0 (P=0.07) and C18:1 9t (P=0.07). The other bacterial FA
301 were not modified by the grain source.
302 *Effects on the total fatty acid outflows and the FA bacterial contribution to these outflows*
303 As for bacteria FA profile, it was not possible to estimate the C18:1 10t proportion in effluent
304 for 4 samples because of total overlapping with the C18:1 11t peak due its very high
305 proportion in these samples (22 % of total FA).
306 Main FA outflows are presented in Table 5. Lowering pH tended to decrease the FA effluent
307 outflow (P=0.08) while increasing the FA bacterial outflow (P=0.01) and its contribution to
308 FA effluent outflows (P=0.01). It also decreased the outflow of C14:0 (P=0.01), C16:0
309 (P=0.01), iso-BCFA (P=0.05) and odd-FA (P=0.05) whereas C12:0, C18:0 and anteiso-FA
310 outflows remained unchanged. For MUFA, lowering pH decreased the outflows of C16:1 9c
311 (P=0.007) whereas it tended to increase the outflow of C18:1 9c (P=0.07). Lowering pH did
312 not alter cis-C18:1 and trans-C18:1 as well as all individual C18:1 trans outflows (data not
313 shown). For PUFA, lowering pH increased C18:2 9c,12c (P=0.0004) and C18:3 9c,12c,15c
314 (P=0.007) outflows.
315 Linseed supplementation significantly increased the FA effluent outflow (P<0.0001) and only
316 in tendency the FA bacterial outflow (P=0.08). It decreased its contribution to FA effluent

317 outflows (*P*=0.04) and also the C12:0 (*P*=0.003) outflow. It significantly increased C16:0
318 (*P*<0.0001) outflow and in tendency C18:0 (*P*=0.10) and iso-FA (*P*=0.10) outflows without
319 modifying C14:0, odd-FA and anteiso-FA outflows. For MUFA, linseed supplementation
320 increased the C16:1 9c (*P*=0.004), C18:1 9c (*P*=0.0004) and total cis-C18:1 (*P*=0.0005)
321 outflows whereas it did not modify the total trans-C18:1 outflows. For PUFA, linseed
322 supplementation increased the C18:2 9c,12c (*P*=0.05) and C18:3 9c,12c,15c (*P*=0.005)
323 outflows.
324 Compared to wheat, corn did not modify the FA effluent outflow, the FA bacterial outflow
325 and its contribution to FA effluent outflows. It did not modify most of the FA outflows except
326 significantly increased C16:0 (*P*=0.03) outflow. It also tended to increase C18:1 9c (*P*=0.06)
327 and total cis-C18:1 (*P*=0.09) and to decrease iso-BCFA (*P*=0.10) and C18:3 9c,12c,15c
328 (*P*=0.08) outflows. A significant interaction diet x pH was observed for the C18:3 9c,12c,15c
329 (*P*=0.05). When lowering pH, the C18:3 9c,12c,15c outflow increased from 6 to 17 mg/d for
330 the CW diet, from 54 to 184 mg/d for the LW diet and from 57 to 101 mg/d for the LC diet.
331 *Effects on the profile of C18-FA and on PUFA biohydrogenation*
332 For the apparent BH presented in table 5, lowering pH decreased C18:3-BH (-26.2 %,
333 *P*=0.001) and C18:2-BH (-30.4 %, *P*=0.0003). Linseed supplementation increased C18:3-BH
334 (+11.9 %, *P*=0.04) and C18:2-BH (+17.3 %, *P*=0.005). Compared to wheat, corn tended to
335 increase C18:3-BH (+ 10.9 %, *P*=0.06).
336 C18-FA biohydrogenation profiles are presented in Table 6. Total C18 outflow was not
337 modified by pH. Lowering pH significantly decreased the proportion of C18:0 (*P*=0.05) as
338 well as the proportions of C18:1 9t (*P*=0.05) and in tendency C18:1 15t (*P*=0.10) proportion
339 without modifying the other individual cis and trans C18:1 proportions. Moreover, it
340 increased the proportions of C18:2 9c,12c (*P*=0.02), C18:2 9c, 12t (*P*=0.02) and C18:3
341 9c,12c,15c (*P*=0.03) without modifying the others PUFA isomers.

342 Linseed supplementation increased the total C18 outflow (P=0.01) as well as the C18:3
343 9c,12c, 15c (P=0.04) and C18:3 9c,11t,15c (P=0.02) proportions. It significantly decreased
344 the C19:2 9c,12c (P=0.005), C18:2 9c,12t (P=0.002) as well as the C18:2 9t,12t (P<0.001)
345 proportions and tended to decrease the C18:1 11c (P=0.06) proportions. Trans C18:1 isomers
346 proportions were not modified by linseed supplementation.
347 Compared to wheat, corn did not modify the total C18-FA outflows. It significantly decreased
348 the C18:1 9t (P=0.05), C18:1 15t (P=0.01) and the C18:2 9t,12t (P=0.007) proportions and in
349 tendency the C18:0 (P=0.08) proportions. The others C18-FA proportions were not modified.
350 **Discussion**
351 *FA metabolism and rumen fermentation conditions*
352 In the present study, across all treatments, the apparent ruminal BH values ranged between
353 61-88 % and 46-76 % for C18:3 9c, 12c, 15c and C18:2 9c, 12c, respectively, well within the
354 range observed with high concentrate diets (Fuentes et al., 2011; Buccioni et al., 2012).
355 However, even though the apparent BH seemed in line with other experiments, the measured
356 C18:0 proportions were below 14% of the C18-FA outflows along with high levels of BH
357 intermediates (especially C18:2 11t,15c and C18:1 11t). It indicates that BH of unsaturated
358 FA did not proceed to completion. Martin and Jenkins (2002) also reported a reduced C18:0
359 concentration in continuous cultures of mixed rumen bacteria maintained on soybean oil
360 emulsion and a mixture of soluble carbohydrates at various extracellular pH. As a
361 consequence, our C18:0 content and proportion in ruminal mixed bacteria isolated from the
362 continuous culture were very low (between 3.6 to 10.3 % of total FA). Usually C18:0, along
363 with C16:0, are the two main FA in bacteria, with C18:0 proportions ranging from 20 to 65 %
364 depending on the type of bacteria (liquid-associated or solid-adherent bacteria; Bessa et al.,
365 2009; Vlaeminck et al., 2006) or to the diets used (high or low fiber diets with lipid
366 supplementation or not; Hussein et al. 1995; Bas et al., 2003; Varadyova et al., 2008). The

accumulation of these BH is related to lowered reductase activity, either by a direct inhibition or, more probably, by a rarefaction of the microorganisms active at this metabolic step (Buccioni et al., 2012).

FA metabolism is strongly linked to other metabolisms in the rumen through the microbial species involved and common reliance on H_2 metabolism (Lourenço et al., 2010). Utilization of [H] for fatty acid BH is considered to be small in regards to methane and propionate synthesis which are the two major sinks for ruminal [H] (Lourenço et al., 2010). In the present experiment, the gas production was unusual, with methane specific productions close to null and very high hydrogen specific productions, indicating the depression of methanogens-Archaea activity or presence and the disruption of interspecies H_2 transfer. This pattern was already observed by Broudiscou et al. (2014) in continuous cultures on low-NDF diets. The proportions of propionate were high (between 35 to 38 % of total VFA respectively) and the proportions of acetate were low (between 39 and 44 % of total VFA) with very low C2/C3 ratios ranging between 1.1 and 1.2. This suggests a partial redirection of [H] spared from CH_4 towards propionate production. However, Ungerfeld (2015), when conducting a meta-analysis on hydrogen metabolism in ruminal continuous cultures, observed a redirection of metabolic hydrogen towards atypical [H] sinks such as H_2 accumulation rather than toward propionate and butyrate in line with our results.

Even though the true degradability of organic matter was within the normal range, the efficiency of microbial protein synthesis varied from 13 to 20 g N /kg FOM which is low compared to most studies (Broudiscou et al., 2002; Fuentes et al., 2011). It suggests that microbial activity was hampered in our culture conditions across all treatments. The pH, which is known to inhibit microbial activity, was between 6.46 and 6.16 just before feeding which is not too low compared to others experiments in continuous cultures which maintained pH as low as 5.8 without depressing microbial activity (Qiu et al., 2004; Fuentes et al., 2009;

392 Fuentes et al., 2011). This poor microbial activity might be partly explained by the
393 accumulation of H_2 disrupting regeneration of cofactors playing a key role in bacteria
394 metabolism.
395 Implementing a 5-day adaptation period followed by a 2-day sampling period is common in
396 essays run in dual-outflow continuous cultures. Due to our severe experimental treatments,
397 high-starch high–fat diets at low pH, we increased both periods to 7 and 3 days respectively.
398 However, most of our results suggest that, although allowed to adapt for a longer time, rumen
399 microbiota had suffered disturbances of unusual magnitude which may be compared to
400 acidosis *in vivo*. With excessive grain feeding, the diversity of the rumen microbiota is
401 reduced with the potential loss of community function (Plaizier et al., 2018). In our study, this
402 reduction in microbiota richness was well illustrated by the disappearance of protozoa since
403 our continuous cultures are able to maintain protozoa population densities at the same order of
404 magnitude as *in vivo* when run in standard environmental and dietary conditions (Broudiscou
405 et al., 1997).
406 *Effects of pH*
407 In lipid supplemented diets, total VFA concentrations were decreased at low pH in agreement
408 with Qiu et al. (2004), Fuentes et al. (2009; 2011) and Sari et al. (2015). However, in contrast
409 to those studies, at low pH, both acetate and propionate proportions tended to decrease with
410 tendencies for increased butyrate and valerate proportions, leading to similar amounts of
411 hexoses fermented and C2/C3 ratios for all pH. The discrepancy may be a consequence of the
412 very high proportion of propionate (38 % of VFA) at our high pH level. In the literature, such
413 proportions are reported only at low pH values. These acetate and propionate decrease trends
414 might indicate a reduction of microbial fermentative activity at low pH in line with the
415 tendency for a decrease in the true OM degradability observed. Moreover, even though the
416 production of methane was already depressed in our trial, it tended to be further decreased by

lowering pH in agreement with Lana et al. (1998) who suggested that it has an inhibitory effect on methanogens Archaea.

In the present study, an increase of C18:2 9c,12c and C18:3 9c,12c,15c outflows was observed at low pH. It reflects C18:2-BH and C18:3-BH inhibition in agreement with the protective effect of low pH on C18:2 9c,12c and C18:3 9c,12c,15c losses (Troegeler-Meynadier et al., 2003; Qiu et al., 2004; Fuentes et al., 2011). It could be explained by an inhibition of lipolysis as the growth of the main lipolytic bacteria (*Anaerovibrio lipolytica*) has been shown to be decreased at low pH (Fuentes et al., 2011). However, in their study, pH reduced BH more strongly than lipolysis. The BH decreases observed following a drop of 0.3 pH unit were 30% and 26 % for C18:2 9c,12c and C18:3 9c,12c,15c respectively. This reduction is of the same magnitude as in the study of Fuentes et al. (2011) who reported a larger pH drop of 0.6 unit (6.4 vs 5.6) though.

Moreover, in line with the decrease in the C18:0 proportion of the C18-FA outflow, a large numeric decrease in the total trans-C18:1 outflow was observed with lower numeric C18:1 11t or significant C18:1 9t and C18:1 15t proportions, in accordance with Martins and Jenkins (2002). The decrease in BH intermediates at lower pH may be due to the decrease in the apparent BH of PUFA which reduced the precursor supply for the later steps in the BH process. However, in the present study, lowering pH did not shift BH from the major known pathway (via C18:2 9c,11t and C18:1 11t) to the second BH pathway (via C18:2 10t,12c and C18:1 10t) as the latter FA remained in very low proportions. This is contrary to other continuous culture studies which observed decreases in the C18:2 9c,11t and C18:1 11t proportions and increases in the C18:2 10t,12c and C18:1 10t proportions when decreasing pH from 6.4 to 5.6 (Fuentes et al., 2009; Fuentes et al., 2011). Fuentes et al. (2009) suggested that pH was the main factor affecting the BH processes resulting in the accumulation of C18:1 10t and C18:2 10t,12c. However, in our study, even at low pH, no shift or increase in those

BH intermediates was observed. This discrepancy could be explained by the absence of inclusion of oils rich in C18:2 9c,12c in the diets, as Zened et al. (2012) observed that the shift from trans-11 to trans-10 isomers was induced *in vitro* by high-starch diets with the addition of C18:2 9c,12c. But our unusually poor microbial activity and disrupted interspecies H_2 transfer might have affected the composition of the bacterial community differently than previously observed.

Even though it is difficult to describe the rumen bacterial populations at the species level from OBCFA, variations of OBCFA profile leaving the rumen are supposed to reflect changes in the relative abundance of rumen bacteria strains (Fievez et al., 2012; Zhang et al., 2017), since each organism possess a consistent and reproducible fatty acid profile (Ifkovits and Ragheb, 1968). In the present study, the similar bacterial FA compositions between low and high pH, especially on odd and iso branched-chain fatty acids, in line with the results of Troegeler-Meynadier et al. (2014), suggest the lack of significant modification of the bacterial species composition through pH modification. The tendencies for higher anteiso-C15 and anteiso-FA bacterial contents at low pH may be link to a modification in amylolytic bacteria as suggested by Vlaeminck et al. (2006) and Zhang et al. (2017). However, when looking at the daily outflow of OBCFA, a different pattern was observed with a significant decrease in the daily outflows of odd-FA and iso-FA and similar anteiso-FA outflow at low pH. OBCFA outflows actually derive from both OBCFA bacteria contents and the net synthesis of microbial biomass, the latter being possibly influenced by dietary treatments and pH. Moreover, lowering pH decreased FA bacterial contribution to total FA outflows in line with the decreased microbial activity and the numeric decreased in EMPS.

Effect of linseed supplementation

A tendency for an increased medium pH by 0.08 unit was observed with linseed supplementation along with tendencies for reduced total VFA concentration and HF amounts,

suggesting that linseed supplementation inhibited microbial fermentative activities. Extruded linseed supplementation increased apparent C18:2-BH and C18:3-BH, which may be partly explained by the pH increase between the control and the linseed supplemented diets. Other studies have also reported an increase in the apparent C18:3 9c,12c,15c BH when linolenic source was used, mainly as oil (Troegeler-Meynadier et al., 2003; AbuGhazaleh and Jacobson, 2007). An increase in PUFA-BH was also observed by Qiu et al (2004) following an addition of C18:2 9c,12c in a free FA form into continuous cultures. Even though linseed supplementation increased apparent PUFA BH, higher outflows of C18:2 9c,12c and C18:3 9c,12c,15c as well as higher C16:0, total-C18 and cis-C18:1 outflows were measured due to the higher supplies of dietary C16 and C18 FA with the linseed diet.

With linseed supplementation, we observed an increased outflow of C18:3 9c,11t,15c. This agrees with the proposed ruminal BH pathway of C18:3 9c,12c,15c which initiates with isomerization to C18:3 9c,11t,15c followed by reductions to C18:2 11t,15c then C18:1 11t and C18:1-15t or C18:1-15c to the final C18:0 (Lourenço et al., 2010). Although not significant, a numeric increase in the outflow of C18:2 11t,15c was observed associated with very high proportion of this FA in the effluent. The accumulation of these intermediates suggests a saturation of the BH process at the second reduction step, known as a metabolic bottleneck (Troegeler-Meynadier et al., 2006). Moreover, as for lowering pH, no shift in the BH pathway was observed, with further accumulation of C18:1 11t and not of C18:1 10t or C18:2 10t, 12c. Zened et al. (2011) also reported that C18:3 9c,12c,15c was mainly hydrogenated via the usual trans-11 pathway whatever the starch level in the diet.

Linseed supplementation decreased the bacterial FA contribution to effluent total FA flow from 53 % in the control diet to 35 %. This is in agreement with the meta-analysis of Schmidely et al. (2008) on *in vivo* experiments who observed that the proportion of bacterial FA fell below 50 % when diets rich in FA (> 40 g / kg DM) were used.

Both bacteria and protozoa can synthetize FA (Harfoot and Hazlewood, 1997). However, as no protozoa were detected in our fermentation broths, BH processes and net synthesis of FA were only of bacterial origin. In the present study, ruminal bacteria contained between 90 and 120 mg/g DM of FA which is in the normal range of what is usually observed with non-supplemented fat died (Hussein et al., 1995; Bas et al., 2003; Bessa et al., 2009). Linseed supplementation did not increase the bacteria FA content contrary to most studies which reported an increase in bacterial FA content in response to fat supplementation (Bauchart et al., 1990; Hussein et al., 1995). The discrepancy might be due to the type of fat source used in the present study (extruded oilseed rich in C18:3 9c,12c,15c vs oil supplementation in the other studies). Linseed supplementation did not increase the C18:3 9c,12c,15c bacterial content in agreement with Vlaeminck et al. (2006) and Bauchart et al. (1990) who failed to establish a relationship between dietary C18:3 9c,12c,15c intake and its concentration in bacteria, C18:3 9c,12c,15c being considered as toxic to rumen microbes. For the other FA and in agreement with Vlaeminck (2006), our bacterial fatty composition is closely related to dietary fatty acids i.e. for C18:0, C18:1 9c, and C18:2 9c,12c contents in line with the higher supply of FA, especially C18-PUFA, and higher BH with linseed diets. Moreover, contrary to pH, linseed supplementation greatly modified the OBCFA bacterial FA composition with increased iso-C15 content, decreased odd-FA (C15:0), anteiso-FA (especially anteiso-C17) and OBCFA bacterial content which might reflect greater changes in the relative abundance of specific bacterial populations (Vlaeminck et al., 2006). However, as for pH, the outflows of OBCFA were quite different from the pattern of OBCFA bacterial content as no effect of dietary linseed supplementation was observed on odd-FA and anteiso-FA outflows and a tendency for a higher iso-FA outflows with linseed supplementation due to integration of net synthesis microbial biomass.

Effect of grain source

To study the impact of starch degradability on BH process, we selected corn and wheat classified as slowly and rapidly degraded starch cereals, to modify ruminal pH and fermentations, as they have effective ruminal starch degradabilities of 0.60 and 0.95 kg/kg respectively (Offner et al., 2003). The grain source did not modify the fermentation broth pH measured 11h30 after the meal distribution, in line with Jenkins et al. (2003). The fermentative activities were similar with both grain sources. The only difference observed was the absence of isobutyrate in the LC broth compared to the LW broth. The LC diet increased apparent C18:3-BH compared to the LW diet and tended to increase C18:2-BH. It may be mediated by pH kinetics since Fulton et al. (1979) reported lower rumen pH values with wider fluctuations in steers on wheat based diet compared to corn based diet. This is in agreement with Lascano et al. (2016) who measured increased C18:3 9c,12c,15c and C18:2 9c,12c BH in low or high fat diets with decreased starch degradability. But the increased C18:3-BH and lower C18:3 9c,12c,15c outflows did not lead to higher C18:0 outflows since, as already discussed, BH of PUFA did not proceed to completion. However, contrary to Jenkins et al. (2003) and Lascano et al. (2016), we did not observe an increase in C18:1 11t and C18:2 9c,11t outflows with corn compared to wheat. The pattern of BH intermediates with the LC diet seems quite different from the LW diet with high numeric C18:2 11t,15c proportions, no 18:1 15t detected and a decrease in C18:1 9t which might indicate an inhibition of the C18:3-BH process at an earlier step with corn compared to wheat. It differ from the study of Li et al. (2014) who did not observe any modification of goat rumen content FA composition, except for the OBCFA, when decreasing rumen degradable starch.

The bacterial FA pattern modification with the LC diet compared to LW diet is in line with the FA effluent composition except for the C18:3 9c,12c,15c bacterial content as already stated. Bacteria from the LC diet have lower content of iso-FA and anteiso-FA which might indicate modification of the bacterial composition with different grain sources. However,

542 contrary to our study, Li et al. (2014) observed that decreasing starch degradability increased
543 the proportions of OBCFA, especially iso and anteiso-FA. The discrepancy might be due to
544 the inclusion of 10 % of corn oil in their diet when substituting corn with wheat which might
545 also change bacteria population (Enjalbert et al., 2017) and, in line with that, the OBCFA
546 proportion. However, the modification in OBCFA bacteria content in the present study is
547 different from the one observed with the decrease in pH : increase in the bacteria iso-FA
548 (tendencies for iso-C13 and iso-C15 and significant for iso-C16 and iso-C18) and anteiso-FA
549 (tendencies for both individual anteiso-C15 and anteiso-C17) content with the LW diet vs a
550 tendency for an increase in the anteiso-FA (anteiso-C15) proportion with the decrease in pH,
551 which might indicate that different bacterial composition with grain source was not only due
552 to mean pH modification.

553 **Conclusion**

554 The fermentative activities sustained by our experimental diets were atypical, of low intensity
555 causing a redirection of spared [H] from CH_4 towards H_2 production. PUFA BH processes did
556 not proceed to completion and led to unusually high levels of BH intermediates and low level
557 of C18:0. pH affected PUFA apparent BH and BH processes, though no shift in the BH
558 pathway was observed. Extruded linseed supplementation and grain source modified PUFA
559 apparent BH and BH processes resulting in modification of C18:3 9c,12c,15c outflows. pH
560 and diet composition altered the bacteria FA content. They affected the odd- and branched-
561 chain FA bacteria content and outflows differently. Therefore, OBCFA outflows as potential
562 markers of changes in the relative abundance of rumen bacteria strains should be used with
563 care.

564

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567

568 **References**

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715

716 Table 1, Ingredients and nutrient composition of diets

	Diets [1]		
	CW	**LW**	**LC**
Ingredient, g/kg of DM			
Dehydrated alfalfa	200	220	270
Wheat	590	410	-
Corn	-	-	360
Extruded linseed	-	100	100
Extruded wheat bran	-	60	60
Sunflower meal	-	40	40
Soyabean meal	100	-	50
Rapeseed meal	60	120	70
Molasses	30	30	30
Minerals and vitamins mix [2]	20	20	20
Chemical composition, g/kg DM			
DM	907	912	918
CP	188	181	184
aNDF	203	230	234
Starch	399	337	306
Fatty acid, g/kg DM			
C14:0	0.9	0.9	1.1
C16:0	2.6	4.3	5.0
C18:0	0.3	1.4	1.5
C18:1 9c	2.2	8.0	9.8
C18:2 9c,12c	6.6	11.6	15.3
C18:3 9c,12c,15c	1.3	16.3	16.6
Total fatty acids	15.9	48.6	56.7

717 [1] diet, CW = control wheat diet without fat supplement, LW = wheat diet with extruded
718 linseed, LC = corn diet with extruded linseed

719 [2] Composition: 330 mg kg^{-1} calcium, 90 mg kg^{-1} sulphur, 6 mg kg^{-1} magnesium, 8350 mg kg^{-1}
720 zinc, 6000 mg kg^{-1} manganese, 70 mg kg^{-1} iodine, 25 mg kg^{-1} cobalt, 20.5 mg kg^{-1} selenium,
721 1 000 000 UI kg^{-1} vitamin A, 200 000 UI kg^{-1} vitamin D3, 1000 mg kg^{-1} vitamin B1, 1500 mg
722 kg^{-1} vitamin E.
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Table 2, Fermentation parameters 11h30 after supply of a control wheat diet without fat supplement (CW), a wheat diet with extruded linseed (LW) or a corn diet with extruded linseed (LC) at high (H) or low (L) pH

	Diet			pH[1]			Probability of contrast			
	CW	LW	LC	L	H	SEM	CW vs (LW+LC)	LW vs LC	pH	Diet x pH
pH	6.26	6.33	6.34	6.16	6.46	0.05	0.09	0.81	0.0004	0.26
VFA (mM)	72.9	68.2	69.4	68.0	72.3	1.66	0.07	0.60	0.05	0.01
mol/100 mol										
Acetate (C2)	39.2	43.2	42.1	39.2	43.8	1.39	0.21	0.70	0.09	0.49
Propionate (C3)	36.7	35.9	37.0	34.9	38.2	1.30	0.91	0.61	0.09	0.15
Butyrate (C4)	12.1	10.2	9.9	12.7	8.7	1.46	0.33	0.89	0.08	0.89
Isobutyrate (iC4)	0.00	0.12	0.00	0.00	0.08	0.290	0.15	0.03	0.06	0.05
Valerate (C5)	6.6	4.8	5.3	7.4	3.7	0.93	0.42	0.83	0.07	0.86
Isovalerate (iC5)	0.57	0.51	0.68	0.53	0.64	0.06	0.81	0.23	0.34	0.31
C2/C3	1.09	1.20	1.12	1.09	1.19	0.05	0.51	0.51	0.32	0.35

[1] pH, L=low pH, H= high pH

Table 3, Effect of diets and pH on the true OM degradability (tdOM), amounts of hexoses theoretically fermented (HF, mmol per day), efficiency of microbial protein synthesis (EMPS, g N/kg FOM) and specific productions of gases (mol/100 mol HF).

	Diet[1]			pH[2]			Probability of contrast			
	CW	**LW**	**LC**	**L**	**H**	**SEM**	**CW vs (LW+LC)**	**LW vs LC**	**pH**	**Diet x pH**
tdOM	0.57	0.54	0.53	0.52	0.57	0.02	0.15	0.79	0.10	0.91
HF (mmol/d)	73.8	65.7	66.9	67.5	69.5	2.00	0.10	0.83	0.61	0.55
EMPS (g N/kg FOM)	15.7	18.4	14.9	14.0	19.0	2.22	0.85	0.53	0.30	0.38
CH_4 (mol/100 mol HF)	0.53	2.75	1.10	0.18	2.70	0.17	0.29	0.28	0.07	0.37
H_2 (mol/100 mol HF)	7.3	6.0	5.3	7.5	5.0	0.74	0.43	0.83	0.12	0.91

[1] diet, CW = control wheat diet without fat supplement, LW = wheat diet with extruded linseed, LC = corn diet with extruded linseed

[2] pH, L=low pH, H= high pH

740 Table 4, Effect of diets and pH on FA content of mixed rumen bacteria (mg/g DM)

	Diet[1]			pH[2]			Probability of contrast			
Fatty acid[2]	**CW**	**LW**	**LC**	**L**	**H**	**SEM**	**CW vs (LW+LC)**	**LW vs LC**	**pH**	**Diet x pH**
C12:0	1.85	0.97	0.60	1.30	0.98	0.221	0.001	0.26	0.06	0.05
C13:0	0.57	0.53	0.35	0.43	0.48	0.034	0.002	0.60	0.25	0.12
C14:0	2.92	2.81	2.30	2.24	3.12	0.302	0.42	0.33	0.07	0.12
C15:0	5.18	3.71	3.42	3.80	4.41	0.345	0.04	0.70	0.35	0.37
C16:0	30.8	30.44	28.5	30.3	29.5	1.58	0.53	0.45	0.67	0.18
C17:0	1.16	1.13	0.95	1.07	1.09	0.071	0.41	0.34	0.88	0.25
C18:0	3.16	12.13	6.25	5.62	8.74	1.57	0.04	0.07	0.21	0.24
iso-C13	0.07	0.11	0.06	0.06	0.10	0.014	0.51	0.08	0.08	0.30
iso-C14	0.52	0.53	0.35	0.45	0.49	0.064	0.52	0.24	0.74	0.18
iso-C15	0.44	0.61	0.51	0.50	0.54	0.027	0.02	0.06	0.27	0.15
iso-C16	0.34	0.40	0.19	0.29	0.32	0.047	0.54	0.05	0.71	0.14
iso-C17	0.27	0.25	0.19	0.26	0.22	0.016	0.19	0.15	0.20	0.48
iso-C18	0.02	0.03	0.01	0.02	0.02	0.004	0.96	0.02	0.46	0.64
anteiso-C15	6.1	5.7	4.0	5.8	4.6	0.45	0.09	0.06	0.08	0.33
anteiso-C17	0.49	0.44	0.25	0.41	0.37	0.037	0.01	0.06	0.33	0.14
C16:1 9c	0.61	0.81	0.66	0.45	0.94	0.098	0.44	0.41	0.02	0.86
C17:1 9c	0.27	0.21	0.20	0.21	0.25	0.021	0.12	0.79	0.26	0.54
C18:1 9c	9.8	17.4	18.2	15.8	14.5	1.41	0.003	0.63	0.41	0.67
C18:1 11c	3.44	4.02	3.66	2.73	4.69	0.41	0.53	0.61	0.02	0.84
C18:1 6-8t	0.03	0.37	0.01	0.01	0.26	0.102	0.46	0.18	0.24	0.38
C18:1 9t	0.25	0.89	0.71	0.67	0.56	0.089	0.0005	0.07	0.17	0.16
C18:1 10t	0.02	0.06	0.00	0.02	0.04	0.016	0.70	0.19	0.49	0.49
C18:1 11t	4.5	10.2	19.4	4.98	17.7	4.62	0.36	0.48	0.24	0.94
C18:1 12t	0.11	0.11	0.01	0.06	0.09	0.024	0.64	0.12	0.39	0.65
C18:2 9c,12c	4.58	1.65	1.47	2.44	2.70	0.451	0.0006	0.71	0.51	0.83
C18:2 9c,12t	0.06	0.01	0.01	0.02	0.03	0.007	0.01	0.78	0.60	0.48
C18:2 11t,15c	0.31	2.99	13.1	3.74	7.21	1.699	0.29	0.24	0.60	0.90
C18:2 9c,11t	0.03	0.09	0.22	0.06	0.17	0.054	0.40	0.43	0.46	0.91
C18:2 9t,11t	0.17	0.56	0.75	0.44	0.54	0.146	0.22	0.66	0.77	0.64
C18:3 9c,12c,15c	0.36	0.72	0.50	0.53	0.52	0.063	0.16	0.18	0.93	0.76
ESFA	39.3	47.0	38.5	40.3	43.0	2.24	0.26	0.04	0.34	0.11
odd-FA	7.19	5.42	4.96	5.5	6.21	0.435	0.05	0.62	0.36	0.31
iso-FA	1.67	1.94	1.31	1.58	1.69	0.131	0.84	0.04	0.58	0.19
anteiso-FA	6.5	6.1	4.2	6.3	5.0	0.475	0.07	0.04	0.07	0.29
OBCFA	15.4	13.5	10.5	13.3	12.9	0.855	0.05	0.12	0.74	0.25
MUFA	21.0	35.8	43.6	26.3	40.6	4.99	0.13	0.54	0.20	0.87
PUFA	5.9	7.4	18.6	8.4	12.9	3.257	0.41	0.27	0.57	0.94
Total FA (mg/g DM)	89	112	121	96	119	8.3	0.21	0.70	0.27	0.74

741 [1] diet, CW = control wheat diet without fat supplement, LW = wheat diet with extruded
742 linseed, LC = corn diet with extruded linseed

743 [2] ESFA = C10:0 + C12:0 + C14:0 + C16:0 + C18:0 + C20:0 + C22:0 + C24:0; Odd-FA =
744 C11:0 + C13:0 + C15:0 +C17:0, iso-FA = iso-C11 + iso-C13 + iso-C14 + iso-C15 + iso-C16
745 + iso-C17 + iso-C18, anteiso-FA= anteiso-C15 + anteiso-C17, OBCFA = odd-FA + iso-FA +

746 anteiso-FA, MUFA = ∑16:1 + ∑17:1 + ∑18:1 + C20:1 11c, PUFA = ∑C18:2 + ∑18:3 +

747 C20:2 + C20:3 + C20:5.

748 [2] pH, L=low pH, H= high pH

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752 Table 5, Effect of diets and pH on daily FA effluent and bacterial outflows (mg/d), on the FA
753 bacterial contribution to these total FA outflows (%) and extents of biohydrogenation

	Diet[1]			pH[2]			Probability of contrast			
	CW	LW	LC	L	H	SEM	CW vs (LW+LC)	LW vs LC	pH	Diet x pH
FA bacterial outflow, mg/d	213	305	286	202	334	26	0.08	0.68	0.01	0.79
FA bacterial contribution, %	53.5	32.0	28.0	28.6	41.1	5.49	0.04	0.70	0.01	0.28
FA outflow [2], mg/d										
Saturated										
C12:0	5.66	3.38	3.23	4.57	3.69	0.504	0.003	0.78	0.11	0.03
C14:0	6.14	7.42	7.17	5.71	8.10	0.551	0.12	0.74	0.01	0.05
C16:0	83	119	130	105	117	6.54	<0.0001	0.03	0.01	0.24
C18:0	13.8	82.7	39.6	29.1	61.5	14.51	0.10	0.17	0.20	0.26
odd-FA	14.7	14.7	16.5	13.7	16.9	0.98	0.66	0.44	0.05	0.64
iso-FA	3.99	6.99	4.79	4.12	6.39	0.74	0.10	0.10	0.05	0.31
anteiso-FA	13.8	15.7	14.1	15.2	13.9	0.77	0.61	0.54	0.57	0.64
Unsaturated										
C16:1 9c	2.21	3.89	3.77	2.61	3.97	0.328	0.004	0.76	0.007	0.79
C17:1 9c	0.86	0.97	1.14	0.99	0.10	0.067	0.24	0.39	0.93	0.48
C18:1 9c	54	145	182	141	113	17.38	0.0004	0.06	0.07	0.41
C18:2 9c,12c	88	112	118	149	62	14.72	0.05	0.67	0.0004	0.12
C18:3 9c,12c,15c	11.3	115	71.1	103	29.1	19.98	0.005	0.08	0.007	0.05
total cis-C18:1	68	168	204	159	135	18.4	0.0005	0.09	0.15	0.45
total trans-C18:1	26.0	80.5	88.5	23.2	108	27.2	0.37	0.91	0.17	0.77
Total FA	403	950	1011	739	836	85	<0.0001	0.31	0.08	0.29
Apparent biohydrogenation , outflow as % of input										
C18:2-BH	49.3	63.3	70.9	46.0	76.4	5.56	0.005	0.13	0.0003	0.24
C18:3-BH	66.5	73.0	83.9	61.4	87.6	4.90	0.04	0.06	0.001	0.14

754 [1] diet, CW = control wheat diet without fat supplement, LW = wheat diet with extruded
755 linseed, LC = corn diet with extruded linseed

756 [2] Odd-FA = C11:0 + C13:0 + C15:0 +C17:0, iso-FA = iso-C11 + iso-C13 + iso-C14 + iso-
757 C15 + iso-C16 + iso-C17 + iso-C18, anteiso-FA= anteiso-C15 + anteiso-C17, OBCFA = odd-
758 FA + iso-FA + anteiso-FA, total cis-18:1 =C18:1 9c + C18:1 11c + C18:1 12c + C18:1 13c,
759 total trans-C18:1 = C18:1 6-8 t + C18:1 9t + C18:110 t + C18:1 11t + C18:1 12t + C18:1 15t
760 + C18:1 16t

761 [2] pH, L=low pH, H= high pH

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764 Table 6, Effect of diets and pH on daily C18-FA outflows (mg/d) and on the proportion of
765 individual C18-FA (% C18-FA outflow)

	Diet[1]			pH[2]			Probability of contrast			
	CW	LW	LC	L	H	SEM	CW vs (LW+LC)	LW vs LC	pH	Diet x pH
Total C18 outflow, mg/d	216	603	633	510	458	68.3	0.01	0.79	0.58	0.82
Proportion of individual C18-FA, % of the C18-FA outflow										
C18:0	6.4	13.0	6.9	5.8	11.7	1.73	0.20	0.08	0.05	0.13
C18:1 9c	26.2	25.8	30.6	27.4	27.7	2.2	0.74	0.50	0.96	0.94
C18:1 11c	6.70	4.00	3.90	3.84	5.90	0.66	0.06	0.94	0.12	0.81
C18:1 6-8t	0.07	0.39	0.02	0.01	0.31	0.11	0.53	0.18	0.18	0.37
C18:1 9t	0.42	0.51	0.23	0.25	0.53	0.07	0.60	0.05	0.02	0.20
C18:1 10t	0.06	0.10	0.03	0.04	0.09	0.02	0.93	0.30	0.38	0.80
C18:1 11t	9.4	9.5	11.6	2.9	17.5	4.02	0.89	0.83	0.12	0.86
C18:1 12t	0.14	0.32	0.02	0.04	0.28	0.08	0.84	0.11	0.13	0.32
C18:1 15t	0.07	0.47	0.0	0.07	0.29	0.09	0.21	0.01	0.10	0.26
C18:1 16t	0.00	0.19	0.01	0.01	0.13	0.05	0.25	0.11	0.16	0.20
C18:2 9c,12c	42.4	19.4	19.4	34.8	19.4	4.53	0.005	0.99	0.02	0.54
C18:2 9c,12t	0.30	0.12	0.11	0.22	0.13	0.03	0.002	0.64	0.02	0.25
C18:2 9t,12t	0.18	0.08	0.03	0.11	0.10	0.02	<0.001	0.007	0.19	0.01
C18:2 11t,15c	0.54	2.9	9.16	3.13	5.26	1.80	0.23	0.24	0.60	0.97
C18:2 9c,11t	0.09	0.22	0.95	0.52	0.32	0.22	0.37	0.26	0.70	0.53
C18:2 9t,11t	0.44	0.69	1.06	0.70	0.76	0.19	0.40	0.53	0.91	0.50
C18:3 9c,12c,15c	5.40	19.7	12.1	17.9	6.9	3.01	0.04	0.13	0.03	0.15
C18:3 9c,11t,15c	0.00	0.24	0.30	0.20	0.24	0.06	0.02	0.61	0.17	0.54

766 [1] diet, CW = control wheat diet without fat supplement, LW = wheat diet with extruded
767 linseed, LC = corn diet with extruded linseed

768 [2] pH, L=low pH, H= high pH

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