Effects of supplemental plant oils on rumen bacterial community profile and digesta fatty acid composition in a continuous culture system (RUSITEC)

Highlights

- Supplementing ruminant diets with plant oils changes the rumen microbial community composition
- Adding vegetable oils at 6% to a high concentrate diet has minor effects on rumen digestion
- Hydrogenation of unsaturated fatty acids is decreased by adding vegetable oils to ruminant diets
- Supplemental sunflower oil increases the formation of vaccenic and conjugated linoleic acids in the rumen

Effects of supplemental plant oils on rumen bacterial community profile and digesta fatty acid composition in a continuous culture system (RUSITEC)

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Abstract

Lipid supplementation of ruminant diets may trigger changes in the ruminal microbiota and in anaerobic digestion. Changes in the bacterial community composition and in the fatty acid hydrogenation caused by the addition of different supplemental plant oils to a high concentrate diet were investigated in vitro using RUSITEC (rumen simulation technique) fermenters. The control (CTR) diet was a high-concentrate total mixed ration for dairy sheep, with no supplementary oil. The other experimental diets were supplemented with olive (OLV), sunflower (SFL) or linseed (LNS) oils at 6 % (dry matter basis). RUSITEC fermenters were inoculated with rumen digesta of sheep and four fermentation vessels used per treatment. Extent of dry matter and fat degradation, composition of the bacterial community and long-chain fatty acids in digesta were determined. The addition of plant oils increased ($P < 0.001$) apparent degradation of fat in the fermenters, whereas fermentation kinetics (gas production and average fermentation rate) were lower ($P < 0.05$) with the LNS than with the CTR diet. Hydrogenation of C18 unsaturated fatty acids ($P < 0.05$), in particular that of oleic acid ($P < 0.001$), and stearic acid proportion ($P < 0.001$) were reduced, and oleic acid proportion was increased ($P < 0.001$) with all oil supplements. Addition of OLV decreased linoleic and LNS increased α -linolenic ($P < 0.001$), whereas conjugated linoleic was increased with SFL oil ($P = 0.025$) and vaccenic increased with both SFL and LNS oils ($P = 0.008$). Addition of 6 % OLV and LNS reduced ($P < 0.05$) microbial community diversity and quantity of total bacteria relative to the control. Some specific microbial groups were affected ($P < 0.001$) by oil addition, with less relative abundance of Clostridiales and Actinobacteria and increased Bacteroidales, Aeromonadales and Lactobacillales species. In conclusion, the supplementation of high-concentrate ruminant diets with plant oils, in particular from sunflower or linseed,

causes shifts in the rumen microbiota and fatty acid hydrogenation in the rumen increasing the formation of vaccenic and conjugated linoleic acids.

Keywords: rumen; RUSITEC fermenter; dietary fat; microbial community composition; anaerobic digestion

Abbreviations: CLA = conjugated linoleic acid; CTR = control diet (high-concentrate total mixed ration); LNS = control diet supplemented with 6% linseed oil; OLV = control diet supplemented with 6% olive oil; PCA = principal component analysis; PCR = polymerase chain reaction; PERMANOVA = permutational multivariate analysis of variance; PUFA = polyunsaturated fatty acids; RUSITEC = rumen simulation technique; SFL = control diet supplemented with 6% sunflower oil; SIMPER = similarity percentage; T-RFLP = terminal restriction fragment length polymorphism

1 Introduction

In ruminants, dietary compounds are degraded by anaerobic digestion in the rumen [1]. One of the benefits of this process is that structural carbohydrates from fibrous plant-based feedstuffs can be broken down by microbial carbohydrases [2,3], allowing these animals to digest low-quality feedstuffs. Nevertheless, high-yielding ruminants need to be fed high-concentrate total mixed rations to meet their nutritional requirements. Thus, rations for high-yielding ruminants, in particular dairy cows, are usually supplemented with fats to increase the dietary energy concentration.

Anaerobic ruminal digestion is characterised by a high release of metabolic hydrogen that is disposed of by different metabolic processes, such as the hydrogenation of polyunsaturated fatty acids (PUFA). There are multiple and reciprocal interactions between rumen microbes

and diet [1], so that dietary oil supplements may cause changes in the microbial community and in the ruminal fermentation, and the altered microbial population may shift the metabolic transformations of dietary fatty acids in the rumen. Oil supplements can affect the microbial community in the rumen either by a direct toxic effect on microbes (as for some gram positive bacterial species) [4,5] or by limiting the microbial colonisation of the digesta and microbial enzyme activity [6,7], thus affecting the digestion of feed [8–10]. Concomitantly, the changes in the microbial communities induced by dietary fats may also alter lipid metabolism in the rumen, in particular the unsaturated fatty acid biohydrogenation pathways [11–13]. Dietary oil supplements rich in PUFA may be harmful to the bacterial groups involved in the biohydrogenation processes [12,14], with differences among fatty acids in their potential toxicity [15]. As a result, fatty acid hydrogenation may be incomplete with less formation of saturated stearic acid [16] and the appearance of hydrogenation intermediates, such as *cis*-9, *trans*-11 18:2 conjugated linoleic acid (CLA) or *trans*-11 18:1 vaccenic acid. Consequently, the type of fatty acids leaving the rumen [17,18] and absorbed in the small intestine and transferred to the animal products can be changed if the rumen biohydrogenation processes are altered. Sunflower oil (rich in *cis*-9, *cis*-12 18:2 linoleic acid) or linseed oil (rich in *cis*-9, *cis*-12, *cis*-15 18:3 α -linolenic acid), can be added to ruminant diets to lessen the hydrogenation of fatty acids in the rumen [12,13,19], and to change the lipid composition of ruminant livestock products [20–24]. Supplementing ruminant diets with vegetable oils may affect the composition of milk and adipose fat by decreasing their content in saturated fatty acids, increasing that of PUFA [25], and enriching their concentrations in CLA [26,27] that may be beneficial to human health [28,29].

Most studies on plant oil supplementation have focused on the effects on milk and fat composition, with a paucity of data regarding effects on the processes occurring in the rumen [1,30], owing to the difficulty of tracing these processes in the animal *in vivo*. Therefore, the

objective of this study was to examine the effects of vegetable oils differing in their fatty acid composition on hydrogenation of the fatty acids and bacterial community composition in the rumen. An *in vitro* rumen simulation technique (RUSITEC) [31] was used for a precise control of the incubation conditions and the measurement of substrate fermentation, lipid metabolism, and microbial community composition.

2 Materials and Methods

2.1 Incubation and sampling procedures

Two RUSITEC apparatuses, each with eight fermentation vessels, were used to simulate rumen fermentation [31]. Each vessel had an effective volume of 650 mL and was inoculated with rumen digesta (solid and liquid) obtained from four Merino sheep fed a diet with 70% grass hay and 30% concentrate. The preparation of solid and liquid inocula from the rumen contents (mixed from the four sheep) was detailed by Vargas et al. [32]. Procedures for sampling rumen contents from sheep were authorised by the Animal Ethics Committees of CSIC and University of Leon (Spain), and the EU Directive 2003/65 was applied for protection of animals used for research.

After inoculation, each fermenter was operated daily following the procedures described in detail by García-González et al. [33] and Vargas et al. [32]. Incubation was maintained at 37 °C and a continuous infusion of McDougall artificial saliva (pH 8.4) at a rate of 556 ml/day (dilution rate of 3.6%/h) per vessel. Fermenters were fed diets to which different vegetable oils were added, resulting in four contrasting treatments according to the experimental design of the study. The control diet (CTR) was based on a standard high-concentrate total mixed ration for ewes during lactation (Table 1). Three other three experimental diets were obtained by adding one vegetable oil to the control diet at a concentration of 60 g/kg. The oils used for the oil-supplemented diets were olive oil (OLV), sunflower oil (SFL) or linseed oil (LNS) (Table

1). Each supplemented diet was prepared every three days by adding 12 g of each oil to 200 g dry matter of the control diet, and then mixing the blend thoroughly to integrate the oil into the diet complete and homogeneously. Each experimental diet was applied to four fermenters (two in each RUSITEC apparatus). In the case of oil treated diets, the amount prepared in each batch was enough for the day it was mixed and for the following two days. During this time, mixed diets were refrigerated at 4 °C in the dark to prevent fatty acid oxidation. Feed (each experimental diet) was provided inside nylon bags that remained in the vessel for 48 h. Bags with feed were provided daily so that every day there was one bag with 48-h undigested residue (which was replaced with a bag containing fresh diet) and another bag with a 24-h incubation residue (which was replaced the day after).

The RUSITEC experiment lasted 17 days, with an initial period of 6 days until steady state conditions were achieved, and a measurement period lasting from day 7 to day 17. Each diet was weighed (15 g dry matter) into a nylon bag (100 µm pore size), which was closed at the top edge with a nylon string, enclosed within a perforated feed container and placed into each fermentation vessel. Dry matter and fat digestibility were estimated from disappearance from the bags after 48 h of incubation. Bags withdrawn from the fermenters on days 7, 8, 9, 14, 15 and 16 of the experiment were used to determine digestibility. For each vessel, digestibility values were averaged across the six measurement days.

On day 17, samples of the liquid content and of the undigested residue remaining in the nylon bags were collected from each vessel as described by Vargas et al. [32]. The samples were mixed thoroughly, frozen (-80 °C) and then freeze-dried. These samples were used for lipid extraction and fatty acid analysis and for DNA extraction.

Just after taking the aforementioned sample, batch cultures were inoculated with the remaining liquid contents of each RUSITEC vessel to assess the effects of the experimental treatments on

ruminal fermentation kinetics, using the CTR diet as the incubation substrate. The only difference among cultures was the initial inoculum reflecting variations in the microbial composition and fermentative activity of the RUSITEC fermenter caused by the oil supplementation during the RUSITEC study. Serum bottles (120 mL volume) were used, to which 500 mg of dry matter of the CTR diet (ground to 1 mm) were added and 50 mL of supernatant (liquid content) of each RUSITEC vessel (three bottles per vessel) were anaerobically dispensed. No other culture medium or buffer solution was added to the RUSITEC inoculum. Then, bottles were sealed and incubated at 39 °C. Gas released by fermentation to the headspace was measured using a pressure transducer at different time intervals post-inoculation up to 92 h. The cumulative gas production profiles were used to estimate fermentation kinetics parameters as described by France et al. [34].

2.2 Chemical, PCR and T_RFLP analyses

Conventional analytical methods (as described by García-González et al. [33]) were used to determine the chemical composition of diets and incubation residues. Long-chain fatty acid profile in samples of digesta was determined according to Morán et al. [35].

DNA from digesta was isolated and purified using the QIAamp DNA Stool Mini Kit (Qiagen Inc., USA) [32]. Bacterial DNA was amplified by PCR using universal bacterial fluorescein-labelled oligonucleotide probes, the labelled PCR products were purified and each DNA sample was digested with the restriction enzymes *HhaI*, *HaeIII* and *MspI* as detailed by Vargas et al. [32]. The terminal restriction fragments were separated and quantified by capillary electrophoresis using an automatic sequence analyser (MegaBACE 500, GE Healthcare) with internal Et-ROX labelled DNA size standards (Amersham Biosciences, GE Healthcare). The spectrum obtained for each sample was analysed by the Gene Marker

program to derive the matrix of relative height of peaks. These data were used to calculate bacterial richness and diversity [36,37].

A real time PCR equipment (Applied Biosystems StepOne Plus™) was used for quantitative PCR analyses. Primers were selected to target the 16S rRNA, and SYBR Green Supermix (Takara Bio Inc.) was used. All reactions were performed in triplicate as detailed by Vargas et al. [32]. The molecular beacon approach with the primers and probe designed by Paillard et al. [38] was used to quantitate *Butyrivibrio* bacteria assumed to be involved in the complete hydrogenation of unsaturated 18 C fatty acids to 18:0 stearic acid (*B. proteoclasticus*). Copy numbers of each bacterial group were calculated using standards prepared as described by Andrés et al. [39].

2.3 Statistical analysis and calculations

The calculations proposed by Wu and Palmquist [40] were used to estimate the ruminal hydrogenation of unsaturated C18 fatty acids.

Data were subjected to ANOVA using the SAS software package [41]. The experiment followed a randomized complete block design, with each fermenter (fermentation vessel) as the experimental unit (four replicates per treatment), the RUSITEC apparatus as the blocking factor and type of oil added to the diet (CTR, OLV, SFL or LNS) as the treatment factor (fixed effect). Digestibility data on the same vessel were averaged across the six measurement days. The Tukey test was used for the multiple comparisons of means.

Multivariate cluster analysis and principal components analysis (PCA) were used to assess similarity and grouping of treatments based on T-RFLP spectra, respectively. Permutational (non-parametric) multivariate analysis of variance (PERMANOVA) was applied to compare the diets (CTR vs. oil supplemented diets) considering all variables in the T-RFLP spectra.

The variables that could likely explain the observed differences between diets were identified by the similarity percentage (SIMPER) method [42]. PERMANOVA and SIMPER analyses were run with the Bray-Curtis similarity measure using the Paleontological Statistics (PAST) software package, version 3.16 [43].

3 Results

Fat disappearance from the bags was increased ($P < 0.001$) when the CTR diet was supplemented with any oil, and was higher with LNS than with OLV and SFL supplemented diets (Table 2). Gas production (asymptotic or after 24 h of incubation) and average fermentation rate) in batch cultures inoculated with the liquid content of the RUSITEC vessels was significantly reduced in the diet containing LNS compared to CTR (Table 2).

The addition of oil to the diet did not change ($P > 0.05$) the proportions of 16:0 and total C18 fatty acids in the digesta (Table 3). However, supplementing the diet with vegetable oils changed the C18 fatty acid profile, in particular the proportions of 18:0 (greater with CTR than with any oil diet, $P < 0.001$) and *cis*-9 18:1 (highest with OLV and lowest with the CTR diet, $P < 0.001$). Compared with the other diets, the addition of LNS increased ($P < 0.001$) the percentage of *cis*-9, *cis*-12, *cis*-15 18:3 in rumen digesta, whereas the relative percentage of *cis*-9, *cis*-12 18:2 was reduced ($P < 0.001$) when the diet was supplemented with OLV oil. The percentage of *trans*-11 18:1 was increased ($P = 0.008$) when the control diet was supplemented with either SFL or LNS oil, whilst *cis*-9, *trans*-11 18:2 was increased ($P = 0.025$) only when SFL was added to CTR diet (Table 3). Compared to the CTR diet, the addition of any vegetable oil reduced the overall hydrogenation (using the calculations of Wu and Palmquist [40]) of C18 unsaturated fatty acids ($P < 0.05$), in particular that of *cis*-9 18:1 ($P < 0.001$). Biohydrogenation of *cis*-9, *cis*-12, *cis*-15 18:3 was greater ($P = 0.011$) with OLV than with LNS diet, and that of *cis*-9, *cis*-12 18:2 was decreased ($P = 0.022$) with LNS

190 compared with OLV or SFL diets. There were no differences ($P > 0.05$) among supplemented
 191 diets (OLV, SFL or LNS diets) in total C18 fatty acid biohydrogenation.

192 The PERMANOVA revealed a significant ($P < 0.001$) effect of oil addition on T-RFLP
 193 microbial profiles. The pairwise comparisons showed that there were not significant
 194 differences among oils ($P > 0.05$), whereas the microbial profile in the digesta from the control
 195 diet was different ($P = 0.035$) to any of the oil supplemented treatments. Both cluster analysis
 196 (Figure 1) and PCA (Figure 2) also revealed a clear discrimination between bacterial profiles
 197 in the vessels fed CTR diets and those receiving the oil supplemented diets (OLV, SFL or
 198 LNS). Replicates of each of the different oil treatments formed paired groups within the
 199 dendrogram that seemed to be related to differences between the two RUSITEC apparatuses.

200 In the PCA plot, treatments were clearly separated along component 1 (explaining up to 28 %
 201 of the variance), indicating that this principal component would reflect the effects of dietary
 202 oil addition on the dominant bacterial communities. The more eccentric terminal restriction
 203 fragments with the greatest contributions to this principal component were identified. The
 204 relative contribution of these fragments in the T-RFLP spectra were further examined by the
 205 SIMPER analysis, confirming that the same fragments were the most influential on the
 206 differences between treatments observed by PERMANOVA. Then, the fragments were
 207 theoretically assigned to compatible bacterial species using the tap-tRFLP tool of the
 208 Ribosomal Data Project [44]. Based on this analysis it would seem that supplementation with a
 209 vegetable oil would reduce the relative abundance of Clostridiales (*Ruminococcus*,
 210 *Butyrivibrio*, *Eubacterium*, *Lachnospira*, *Clostridium*) and Actinobacteria, and would increase
 211 bacteria belonging to taxa Bacteroidales (*Bacteroides* and *Prevotella*), Aeromonadales
 212 (*Ruminobacter*, *Aeromonas*) and Lactobacillales (*Enterococcus* and *Lactobacillus*).

The supplementation with a vegetable oil (at 6 %) led to a significant decrease ($P < 0.05$) in the apparent richness and diversity of species (Table 4). Evenness was not significantly affected indicating a proportional reduction in numbers with rarer species reduced to below detection levels. The addition of 6 % OLV and LNS led to a reduction ($P < 0.05$) in fold copy number of total bacteria relative to the control (Figure 3), whereas the same concentration of SFL led to a non-significant reduction. The PCR copy numbers of *Butyrivibrio* groups were numerically decreased by dietary oil supplementation, but differences with CTR diet did not reach statistical significance (Figure 3).

4 Discussion

Each vessel of the RUSITEC is a fermenter (chemostat) for the continuous culture of mixed rumen microorganisms. This *in vitro* technology has been tested and used extensively to reduce the use of experimental animals, and has been considered as a valuable tool to investigate effects of dietary lipid supplementation on rumen fermentation [45]. Our study used this system to simulate the effects of supplementing a high-concentrate diet with vegetable oils differing in their fatty acid composition on microbial community composition and processes of fatty acid hydrogenation in the rumen.

Supplementary fat may affect microbial fermentation and feed degradation in the rumen [10]. Although gas production kinetics measured from the *in vitro* batch cultures were depressed to some extent by oil supplementation (in particular with LNS supplementation), dry matter degradation was not apparently affected by oil supplementation in the RUSITEC fermenters. The average dry matter disappearance coefficient was higher for the LNS than for the SFL diet. The amount and type of lipid added [6] and the diet composition [46] determine the magnitude of the effects of dietary lipid supplementation on nutrient degradation. Fat digestibility was increased when oils were added to the control diet, although this can be an

237 artefact occurring as the result of digestibility being measured as the disappearance of
238 substrate from the nylon bag. Oils are liquids and, although forming globules in an aqueous
239 phase or being adsorbed on to feed particles, they can pass freely through the pores of the bag
240 fabric, even with no previous hydrolysis or digestion. This will predictably overestimate fat
241 digestibility. Conversely, when lipid digestibility is measured *in vivo* from the duodenal
242 outflow of fatty acids, the estimates obtained clearly underestimate the actual rates of
243 triglyceride hydrolysis [6,47], because long-chain fatty acids are not absorbed in the rumen but
244 only liberated by lipolysis, reaching the small intestine as free fatty acids.

245 Oils may also cause changes in the ruminal microbiota. In our study, oil supplementation was
246 associated with a reduction in total viable bacteria, but had no effect on *Fibrobacter*
247 *succinogenes*, one of the main fibrolytic species. Oils may depress rumen bacteria as reported
248 in some studies *in vivo* [39,48] and *in vitro* pure cultures [14], with results supporting a dose-
249 related effect. Nur Atikah et al. [49] did not observe changes in *Fibrobacter succinogenes* or
250 *Ruminococcus* spp. abundance following addition (6 %) of either olive or sunflower oil to
251 high-concentrate diets fed to goats. Similar results (lack of effects on rumen microbiota) were
252 reported *in vitro* with either 3 % soybean [19] or 2 % sunflower [32] oil supplementation in
253 RUSITEC. Richness and diversity of bacterial species assessed from T-RFLP spectra were not
254 affected by the supplementation with OLV or SFL, and were decreased only with LNS oil
255 compared with the control, in agreement with Cremonesi et al. [50] with a diet for dairy goats
256 (>60% forage) . In both the dendrogram and principal component analyses, the control diet
257 vessels clustered separately from those receiving an oil-supplemented diet, confirming the
258 differences detected by PERMANOVA, with no distinctive discrimination among the three oil
259 types. High concentrate diets supplemented with a lower dose (2 % SFL) clustered according
260 to the ruminal microbial community composition compared to controls [32] similar to our
261 study. An interpretation of the T-RFLP spectra seemed to indicate that whereas some bacteria

taxa would be affected adversely, others are apparently benefitted from the addition of the plant oils. Cremonesi et al. [50] reported that linseed (9 %) was associated with increased relative abundance of *Fibrobacter* spp. and *Succinivibrio* spp. and reduced *Prevotella* spp. in the rumen of goats fed a diet with more than 60 % forage, whereas hemp seed (with a fatty acid composition similar to sunflower) had no effect on the most abundant bacterial phyla. Adding marine oils to a high-concentrate diet, Vargas et al. [32] observed that more nutritionally versatile species of bacteria (e.g., *Prevotella*) might be favoured when other species are depressed. Based on the T-RFLP results, species of Clostridiales would be reduced by oil supplementation, which include known butyrate-producing bacteria (such as some *Butyrivibrio* species).

Addition of fatty acids may cause saturation of microbial enzymes involved in lipolysis and hydrogenation [51], and may also cause changes in ruminal bacteria playing the most relevant role in the biohydrogenation of fatty acids in RUSITEC fermenters. The biohydrogenation of unsaturated fatty acids has been associated with bacterial species assigned to the *Butyrivibrio* group [16], although other bacteria may be involved in this process [15,52]. Compared to the CTR diet, the relative abundance of *Butyrivibrio* spp. seemed to be reduced with all the oil supplemented diets, although the difference did not reach statistical significance. These species seem to be involved in the hydrogenation of long chain fatty acids and also in the fermentation of sugars to butyrate [53], but at the same time the unsaturated fatty acids may be detrimental to *Butyrivibrio* spp.. This inhibitory effect has been observed *in vitro* [14] and *in vivo* [54]. The relative abundance of Lactobacillales seemed to be increased in supplemented vessels, and some species of this group may be involved in the production of conjugated PUFA [51].

285 The differences among experimental treatments in the digesta fatty acid profile can be
286 attributed to two factors: the fatty acid composition of the oil supplemented in each case and
287 the effect of the oil added on the biohydrogenation processes in the rumen. Thus, the relative
288 abundance of *cis*-9 18:1 was highest in the OLV digesta and that of *cis*-9, *cis*-12, *cis*-15 18:3
289 when LNS oil was added, related to the composition of each oil added. On the other hand, in
290 all cases oil supplementation caused a decline in the overall fatty acid hydrogenation rates, in
291 particular that of *cis*-9 18:1. Although hydrogenation of each acid (C18:1, C18:2 or C18:3)
292 was reduced to a greater extent with LNS (rich in more unsaturated fatty acids) than with
293 OLV, no significant differences in overall hydrogenation were observed among the three oils
294 studied. As a result, the relative percentage of saturated 18:0 was reduced with all the
295 supplemental oils in comparison with the control unsupplemented diet. Among unsaturated
296 fatty acids, hydrogenation was greatest for *cis*-9, *cis*-12, *cis*-15 18:3 and lowest for *cis*-9 18:1
297 [8]. The effect of the addition of unsaturated fatty acids inhibiting hydrogenation can be dose
298 related [55], as in the present study the reduction was significant with SFL oil added at 6 %,
299 whereas in a previous study with SFL added at 2 % the difference with the control diet was not
300 significant [32]. The effects of oil supplementation on fatty acid profile and biohydrogenation
301 in ruminal digesta depend not only on the type and amount of oil added but also on the diet to
302 which the oil is incorporated [56], with fatty acids being hydrogenated to a lesser extent when
303 oils are added to concentrate diets [57]. Fatty acid hydrogenation can be also affected by
304 ruminal pH [58,59]. The culture medium of RUSITEC fermenters is highly buffered through
305 the continuous infusion of artificial saliva, and pH is maintained with minor fluctuations and
306 no differences among dietary treatments (average pH ranging from 6.66 to 6.73). Therefore, it
307 could be expected that pH would not be an influential factor on the comparison among
308 supplemental oils in their effect on digesta fatty acid hydrogenation.

The fatty acid profiles observed in response to the addition of vegetable oils are in line with the hydrogenation routes proposed for each unsaturated fatty acid [60,61]. Thus, in the rumen *cis*-9, *cis*-12 18:2 is isomerised to *cis*-9, *trans*-11 18:2 and this latter is hydrogenated to *cis*-11 18:1 and ultimately to 18:0 under highly reducing conditions. However, with extra *cis*-9, *cis*-12 18:2 (provided by SFL oil) the isomerization becomes saturated, and the hydrogenation is inhibited, resulting in the accumulation of intermediates *cis*-9, *trans*-11 18:2 and *cis*-11 18:1 [62]. These intermediate fatty acids were significantly increased in rumen digesta with the SFL oil supplemented diet, similarly to other results obtained either *in vitro* [19,32,63,64] or *in vivo* [65,66]. The addition of SFL oils to the diet could, in theory, contribute to the increase of conjugated linoleic acid concentration in meat and/or milk [67]. α -Linolenic acid is converted to *cis*-9, *trans*-11, *cis*-15 18:3 and *trans*-11, *cis*-15 18:2 as intermediates (but not *cis*-9, *trans*-11 18:2) and finally hydrogenated to vaccenic acid. Dietary supplementation with LNS oil (rich in *cis*-9, *cis*-12, *cis*-15 18:3) will also limit the isomerization of 18:2 fatty acids and the complete hydrogenation to 18:0, resulting in the accumulation of *trans* 18:1 isomers, in particular *trans*-11 18:1 [62,68], as shown in our results. Oleic acid is hydrogenated to 18:0 though the formation of *trans* 18:1 isomers other than *trans*-11 18:1 [69]; therefore, supplementation with OLV oil is not expected to increase vaccenic acid in ruminal digesta [61].

5 Conclusions

Supplementation of high-concentrate ruminant diets with plant oils, in particular from sunflower or linseed, causes shifts in the rumen microbiota with some potentially favourable effects. Thus, there are changes in the C18 fatty acid profile so that the relative abundance of saturated stearic acid in the rumen digesta is reduced as result of exceeded fatty acid hydrogenation capacity, appearing intermediates such as vaccenic (with both sunflower and

linseed oil) or conjugated linoleic acid (only with sunflower). As these unsaturated fatty acids can be absorbed in the small intestine, the addition of vegetable oils lipids to high-concentrate ruminant diets could affect milk or meat fatty acid composition, although these effects need to be confirmed under field *in vivo* conditions.

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563

Table 1. Ingredients and chemical composition of control and oil supplemented high-concentrate diets

Treatment	Control	Olive Oil	Sunflower Oil	Linseed Oil
Ingredients (g/kg dry matter)				
Cracked corn grain	250	238	238	238
Barley grain	150	143	143	143
Soybean meal	200	190	190	190
Lucerne hay	200	190	190	190
Beet pulp	90	86	86	86
Molasses	60	57	57	57
Sodium bicarbonate	15	14	14	14
Mineral vitamin premix	35	33	33	33
Olive oil	-	60	-	-
Sunflower oil	-	-	60	-
Linseed oil	-	-	-	60
Composition (g/kg dry matter)				
Organic matter	925	927	930	929
Crude protein	153	145	145	147
Neutral detergent fibre	231	220	222	219
Acid detergent fibre	115	109	110	108
Ether extract	22	70	73	71
Dietary fatty acid (FA) profile (as % of total FA)				
14:0	0.10	-	0.02	-
16:0	22.1	15.3	12.3	11.1
Total C18	76.9	82.1	85.2	87.4
Dietary C18 fatty acids (as % of total C18 FA)				
18:0	5.1	5.1	5.7	5.1
<i>cis</i> -9 18:1	35.1	71.0	38.7	25.3
<i>cis</i> -9, <i>cis</i> -12 18:2	52.8	20.7	53.2	30.1
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	7.0	3.3	2.2	39.5

Fatty acid profile of oils (as % of total fatty acids)

Olive oil: 12.7 % 16:0. 4.2 % 18:0. 71.2 % *cis*-9 18:1. 7.0 % *cis*-9, *cis*-12 18:2. 1.6 % *cis*-9, *cis*-12, *cis*-15 18:3.

Sunflower oil: 8.5 % 16:0. 4.9 % 18:0. 35.4 % *cis*-9 18:1. 47.8 % *cis*-9, *cis*-12 18:2. 0.3 % *cis*-9, *cis*-12, *cis*-15 18:3.

Linseed oil: 6.9 % 16:0. 4.6 % 18:0. 19.7 % *cis*-9 18:1. 19.6 % *cis*-9, *cis*-12 18:2. 47.5 % *cis*-9, *cis*-12, *cis*-15 18:3

Table 2 Effects of oils added to a high-concentrate diet on substrate degradation in RUSITEC fermenters and gas production kinetics in batch cultures

Item	Control (CTR)	Olive oil (OLV)	Sunflower oil (SFL)	Linseed oil (LNS)	SEM (n=4)	<i>P</i> value	<i>P</i> value contrast CTR vs OIL
Disappearance coefficients, g digested/100 g incubated							
Dry matter	68.2 ^{ab}	71.4 ^{ab}	66.9 ^b	72.6 ^a	1.21	0.023	0.175
Ether extract	51.8 ^c	70.5 ^b	73.8 ^b	80.9 ^a	1.43	<0.001	<0.001
Fermentation kinetics*							
Asymptotic gas production, mL/g	311 ^a	291 ^{ab}	287 ^{ab}	277 ^b	11.0	0.053	0.014
Fractional fermentation rate, h ⁻¹	0.044	0.046	0.045	0.042	0.0002	0.425	0.759
Lag time, h	2.7	2.5	2.6	2.5	0.15	0.287	0.129
Gas production at 24 h, mL/g	189 ^a	184 ^{ab}	176 ^{ab}	165 ^b	6.3	0.017	0.023
Average fermentation rate, mL/h	8.40 ^a	8.36 ^{ab}	7.90 ^{ab}	7.33 ^b	0.350	0.037	0.091

CTR = Control diet; OLV = control diet supplemented with 6% olive oil; SFL = control diet supplemented with 6% sunflower oil; LNS = control diet supplemented with 6% linseed oil

SEM = standard error of the mean

Contrast CTR vs OIL: comparison between CTR and all treatments supplemented with oil

^{a,b,c} Within a row, mean values without common superscript letters differ ($P < 0.05$)

* Gas production kinetics when the diet was incubated in batch cultures inoculated with the liquid content of the RUSITEC vessels

Table 3. Effects of oils added to a high-concentrate diet on ruminal digesta fatty acid (FA) profile and C18 fatty acid biohydrogenation in RUSITEC fermenters

Item	Control (CTR)	Olive oil (OLV)	Sunflower oil (SFL)	Linseed oil (LNS)	SEM (n=4)	P value	P value contrast CTR vs OIL
Digesta fatty acids (as % of total FA)							
14:0	2.12 ^a	0.54 ^b	1.25 ^{ab}	0.87 ^{ab}	0.324	0.029	0.007
16:0	19.8	17.7	19.7	19.8	1.43	0.666	0.678
Total C18 FA	78.1	81.8	79.0	79.3	1.49	0.384	0.284
C18 FA profile (as % of total C18 FA)							
18:0	13.2 ^a	5.1 ^b	5.6 ^b	5.7 ^b	1.11	<0.001	<0.001
<i>cis</i> -9 18:1	18.9 ^c	56.3 ^a	37.6 ^b	30.1 ^b	2.22	<0.001	<0.001
<i>cis</i> -9, <i>cis</i> -12 18:2	27.4 ^a	9.3 ^b	24.4 ^a	20.5 ^a	2.15	<0.001	0.003
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	1.83 ^b	0.60 ^b	0.70 ^b	15.47 ^a	1.005	<0.001	0.008
<i>trans</i> -11 18:1	5.54 ^b	7.63 ^{ab}	10.46 ^a	10.26 ^a	0.907	0.008	0.003
<i>cis</i> -9, <i>trans</i> -11 18:2	0.40 ^b	0.49 ^{ab}	1.39 ^a	0.55 ^{ab}	0.213	0.025	0.126
Biohydrogenation %*							
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	73.9 ^{ab}	81.9 ^a	68.0 ^{ab}	60.8 ^b	3.66	0.011	0.415
<i>cis</i> -9, <i>cis</i> -12 18:2	48.2 ^{ab}	55.3 ^a	54.1 ^a	31.9 ^b	4.91	0.022	0.850
<i>cis</i> -9 18:1	46.0 ^a	20.7 ^b	2.7 ^{bc}	-18.8 ^c	5.58	<0.001	<0.001
Total C18 biohydrogenation	46.8 ^a	28.8 ^b	31.3 ^b	28.9 ^b	4.46	0.045	0.007

CTR = Control diet; OLV = control diet supplemented with 6% olive oil; SFL = control diet supplemented with 6% sunflower oil; LNS = control diet supplemented with 6% linseed oil

SEM = standard error of the mean

Contrast CTR vs OIL: comparison between CTR and all treatments supplemented with oil

^{a,b,c} Within a row, mean values without common superscript letters differ ($P < 0.05$)

* Calculated as proposed by Wu and Palmquist [40]

Table 4. Diversity indices of the RUSITEC bacteria community provided with control diets or diets containing 6 % vegetable oil supplementation

Index	Control (CTR)	Olive oil	Sunflower oil	Linseed oil	SEM (n=4)	<i>P</i> value	<i>P</i> value contrast CTR vs OIL
Species richness	58.3 ^a	45.3 ^{ab}	53.3 ^{ab}	40.8 ^b	4.03	0.043	0.027
Shannon's diversity index	3.07 ^a	2.83 ^{ab}	2.98 ^{ab}	2.59 ^b	0.101	0.029	0.040
Shannon's evenness index	0.756	0.746	0.751	0.700	0.019	0.196	0.318
Simpson's index of diversity	0.909 ^a	0.879 ^{ab}	0.900 ^a	0.842 ^b	0.012	0.011	0.027
Number of species accounting for 50% total relative peak height	3.25	2.75	2.75	2.00	0.431	0.287	0.160
Number of species accounting for 70% total relative peak height	9.25	7.50	9.00	7.25	1.613	0.756	0.489

SEM = standard error of the mean

^{a-b} Within a row, mean values without common superscript letters differ ($P < 0.05$)

Contrast CTR vs OIL: comparison between CTR and all treatments supplemented with oil

Figure Legends

Figure 1. Dendrogram derived from the analysis of terminal restriction fragment length polymorphism data, showing the grouping of RUSITEC fermenters receiving either a control diet (CTR, black) or diets supplemented with 6% olive (OLV, red) oil, 6% sunflower (SFL, green) oil or 6% linseed (LNS, blue) oil. The number after the treatment code indicates the RUSITEC apparatus and replicate number (there were two RUSITEC apparatuses and two replicates of each treatment in each apparatus).

Figure 2. Principal components plot derived from the analysis of terminal restriction fragment length polymorphism data, showing the discrimination along principal components 1 and 2 of RUSITEC fermenters receiving either a control diet (CTR, black) or diets supplemented with 6% olive (OLV, red) oil, 6% sunflower (SFL, green) oil or 6% linseed (LNS, blue) oil. The number after the treatment code indicates the RUSITEC apparatus and replicate number (there were two RUSITEC apparatuses and two replicates of each treatment in each apparatus).

Figure 3. Relative quantitation compared to control diet of copy numbers of total bacteria and rumen microbial groups involved in biohydrogenation after supplementation with olive (OLV), sunflower (SFL) or linseed (LNS) oil. Fold-changes for specific amplicon groups were calculated as the (log 2) ratio of normalized abundances.