British Journal of Nutrition (2007), **97**, 938–948
doi: 10.1017/S0007114507675187
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https://doi.org/10.1017/S0007114507675187 Published online by Cambridge University Press

The effect of absence of protozoa on rumen biohydrogenation and the fatty acid composition of lamb muscle

David R. Yáñez-Ruiz, Selwyn Williams and Charles J. Newbold*

Institute of Rural Sciences, University of Wales Aberystwyth, Llanbadarn Campus, Aberystwyth SY23 3AL, UK

(Received 5 June 2006 – Revised 8 December 2006 – Accepted 11 December 2006)

The effects of the absence of protozoa in the rumen of lambs on animal growth, rumen fermentation, microbial diversity and fatty acid profiles in abomasal fluid and intramuscular fat were measured in ten control and ten protozoa-free (PF) lambs. PF lambs were prepared by isolating them from ewes within the first 24 h after birth. The PF and control lambs were kept for 4 months in two different fields and received a daily supplement of 250 g concentrate. The bacterial populations visualised by denaturing gradient gel electrophoresis differed between the two groups of animals and showed a higher bacterial diversity in control lambs than in PF lambs. Abomasal contents from control lambs contained higher concentrations of 22 : 5*n*-3 and 22 : 6*n*-3 and lower concentrations of vaccenic acid (*trans*-11–18 : 1) and 20 : 3*n*-6 than PF lambs. The rest of the fatty acids, including the conjugated linoleic acid (CLA) isomers, were present at the same concentrations in abomasal contents from both experimental groups. Fatty acid composition in intramuscular fat showed differences between the groups. PF lambs had higher proportions of 18 : 0, 18 : 3, *trans*-10, *cis*-12-CLA and total SFA than control lambs. Control lambs had higher proportions of *cis*-9–18 : 1, *cis*-9, *cis*-12–18 : 2, 20 : 3*n*-6, 22 : 6*n*-3 (DHA) and MUFA. In conclusion, rumen defaunation led to higher tissue levels of the *trans*-10, *cis*-12-CLA isomer and SFA and lower PUFA:SFA ratio and *n*-3 PUFA in lamb muscle.

Conjugated linoleic acid: Protozoa: Rumen biohydrogenation: Fatty acids

Conjugated linoleic acid (CLA) is a collective term used to describe positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12-18 : 2). Food products derived from ruminant animals are the major source of CLA in human diets. The main form of CLA, *cis*-9, *trans*-11–18 : 2 (*c*9,*t*11-CLA), can be produced directly by microbial hydrogenation in the rumen or by the enzyme stearoyl-CoA desaturase in the animal's tissues using vaccenic acid (VA; *trans*-11-18 : 1), formed in the rumen, as its substrate (Bauman *et al.* 1999). Another important CLA isomer, *trans*-10, *cis*-12-18 : 2 (*t*10,*c*12-CLA) is also formed in the rumen. Some isomers of CLA have been associated with inhibition of some types of cancer (De la Torre *et al.* 2005), reductions in atherosclerosis (Toomey *et al.* 2005), enhancement of the immune response (Zhang *et al.* 2005) and body fat repartitioning (Blankson *et al.* 2000). However, some experiments on laboratory animals appear to suggest that the *t*10,*c*12 isomer may have detrimental effects on health (Wahle *et al.* 2004).

Recently, it has been shown that the rumen protozoa have a higher content of CLA and VA than the rumen bacteria, suggesting that protozoa may make a significant contribution to VA and CLA flow from the rumen (Devillard *et al.* 2006). By using real-time PCR to quantify protozoal 18s rDNA genes we have observed that protozoal cells contain a significant proportion (40 %) of the CLA and VA reaching the duodenum of animals (Yáñez-Ruiz *et al.* 2006). Studies on the effect of defaunation on lipid metabolism in the rumen are contradictory and not conclusive. In an early study Dawson & Kemp (1969) reported that biohydrogenation in ruminal digesta was only slightly decreased following the removal of protozoa from the rumen (defaunation) and the presence of protozoa was not necessary for biohydrogenation to occur. Klopfenstein *et al.* (1966) reported a decrease in 18 : 1 in blood in the absence of protozoa, which is not consistent with protozoa being a major source of 18 : 1 supply to the small intestine. More recently, Jouany & Lassalas (2003) observed that CLA formation actually increased when protozoa were removed. However, no attempts have been made to look at the effect of the absence of protozoa on the CLA content of the ruminant product (milk or meat).

The present experiment was carried out to determine the effect of the absence of protozoa in the rumen of lambs on ruminal biohydrogenation, rumen microbial ecology and the fatty acid composition of lamb muscle.

Material and methods

Experimental design and animal management

Twenty crossbred lambs were used. Ten were taken from the ewes within the first 24 h of life and kept isolated from adult

Abbreviations: *c*9,*t*11, *cis*-9, *trans*-11; CLA, conjugated linoleic acid; DGGE, denaturing gradient gel electrophoresis; PF, protozoa-free; *t*10,*c*12, *trans*-10, *cis*-12; VA, vaccenic acid.

* **Corresponding author:** Professor C. J. Newbold, fax + 44 1970 611264, email cjn@aber.ac.uk

https://doi.org/10.1017/S0007114507675187 Published online by Cambridge University Press

animals (protozoa-free (PF) group) and fed commercial milk replacer (Volac® Instant; Volac International Ltd, Royston, Herts, UK) while the other ten were left with the ewes (control group). Both groups were provided grass hay *ad libitum* and commercial concentrate supplemented with retinol (30 000 μg/kg), cholecalciferol (62·5 μg/kg), vitamin E (25 mg/kg) and Se (500 μg/kg) (Wynnstay LambMaster; Wynnstay plc, Llansantffraid, Powys, UK) and were weaned at 6 weeks. On week 7, animals in both groups were each given orally 7·5 ml of a mineral (14 g Ca/kg, 4·5 g P/kg, 150 mg Zn/kg, 108 mg Fe/kg, 50 mg Mn/kg, 2 mg Co/kg, 6·5 mg I/kg, 0·182 mg Se/kg) and vitamin (1800 retinol μg/kg, 30 μg/kg cholecalciferol/kg, 13·4 μg alphatocopherol/kg) supplement (Liquithrive; Agri Lloyd International Ltd, Leominster, Herefordshire, UK) and 1 mg diclazuril/kg body weight (Vecoxan®; Janssen Animal Health, Saunderton, Bucks, UK) as an anticoccidial treatment. This last treatment was then repeated at 4-week intervals three more times. Once weaned, at 6 weeks of age, the lambs grazed on two different pastures (predominantly perennial ryegrass) supplemented with a lamb commercial concentrate supplement (Wynnstay LambMaster; 250 g/head per d) for 4 months before slaughter. The main ingredients in the commercial concentrate were wheat, barley, sunflower, molasses, sugarbeet pulp, soya and mineral–vitamin mix. The fields were 7300 m^2 and 4800 m^2 for the control and PF groups, respectively. PF pasture had not been used by any ruminant in the 6 months before the experiment, ensuring no contamination by rumen protozoa. Animals were weighed every week to monitor weight gain. Electric fences were used to prevent grazing in an area of approximately 51 m^2 in each experimental field in order to estimate daily grass intake (Stockdale & King, 1983).

Sample collection

Samples of grass and concentrate were collected every week, pooled and frozen at −20°C for chemical (ten subsamples) and fatty acid (five subsamples) analysis.

On week 11 of the grazing period an area of wool (50 mm × 50 mm) was clipped from the left hindquarters of each lamb. After 7 weeks the wool grown within these patches was clipped and packaged into paper bags. The wool and the bags were oven-dried overnight at 60°C and the dry weight of the wool produced was then calculated.

The daily herbage intake was calculated using the difference between the grass growth in the isolated area compared with that in the grazed field over a 9-week period. Grass growth was measured in the grazed and fenced areas after the 9-week period using random sampling with a 1 m^2 quadrat, which was thrown randomly ten times in the restricted area and thirty times in the grazed area. Quadrats were cut to ground level, dried at 100°C for 24 h, for the conversion to DM mass.

On the day of slaughter animals were transported 30 miles to a commercial slaughter facility (Hamer International Ltd, Llanidloes, Powys, UK) then weighed and slaughtered within 2 h. Animals were fasted overnight before slaughter. At slaughter samples from the rumen, abomasum and tail muscle (*sacrocaudalis ventralis lateralis*) were collected, kept in ice, pH of rumen content measured and samples frozen at −20°C within 1 h. After slaughter, cold carcass weight was recorded. In order to have enough intramuscular fat to extract and accurately detect the different fatty acids by GC, muscle and abomasal samples (5 g) were pooled from pairs of animals. Thus five samples per group were analysed and not ten.

Samples of rumen fluid from defaunated lambs were examined by light microscopy (40 ×) to confirm the absence of protozoa.

Chemical and molecular analysis

Ash and, by mass difference, organic matter in samples of herbage and concentrate were analysed by combusting the ground samples at 550°C for 6 h in a muffle furnace. Total N was determined by a micro-Kjeldahl technique using 'kjeltec' equipment (Foss UK Ltd, Warrington, Cheshire, UK). Neutral-detergent fibre and acid-detergent fibre analysis was performed by the sequential procedure of van Soest & Mason (1991), using the Ankom$^{200/220}$ fibre analyser (Ankom, 2000). Neutral-detergent fibre was assayed with sodium sulfite and without α-amylase. Neutral-detergent fibre and acid-detergent fibre were expressed without residual ash.

The fatty acids in the grasses and concentrate were measured using the one-step extraction–transesterification procedure described by Sukhija & Palmquist (1988). Abomasal fatty acids were obtained by direct hydrolysis at 60°C, with added internal standard (100 μl methyl ester C21, 15 mg/ml $CHCl_3$), in 5 M-KOH in aqueous methanol. Potassium carboxylates were converted into fatty acids by the addition of 10 ml 0·5 M-H_2SO_4 and methylated using 5 % (w/w) HCl in methanol at 50°C (Kramer & Zhou, 2001). Total intramuscular muscle lipids were extracted as described by Folch *et al.* (1957) and saponified and methylated as described for abomasal fatty acids.

After saponification and methylation, methyl esters of fatty acids were recovered in 5 ml hexane and quantified by GC on a CP-Select chemically bonded for FAME column (100 m × 0·25 mm internal diameter; Varian Inc., Palo Alto, CA, USA) with ultra-high-purity He carrier gas at a flow rate of 7 ml/min. Injector and detector temperatures were 250 and 255°C, respectively. The splitting ratio to the flame ionisation detector was 1:80. The oven temperature schedule for abomasal samples was: 70°C for 1 min; increase to 100°C at 5°C/min; constant 100°C for 2 min; increase to 175°C at 10°C/min; constant 175°C for 34 min; increase to 225°C at 4°C/min; constant 225°C for 22 min. The total run time was 85 min. The oven temperature schedule for muscle samples was: 100°C for 0 min; increase to 175°C at 20°C/min; constant 175°C for 25 min; increase to 204°C at 2°C/min; increase to 230°C at 10°C/min and hold for 2 min; increase to 240°C at 20°C/min and hold for 8 min. The total run time was 60 min. Individual fatty acids were quantified by reference to the internal standard and using external standards: *c*9,*t*11-18 : 2; *t*10,*c*12–18 : 2; *trans*-11–18 : 1 (Matreya, Pleasant Gap, PA, USA) and standard mixture of 37 FAME (Supelco, Poole, Dorset, UK). Data from abomasum and muscle samples were calculated as normalised area percentages of fatty acids.

Volatile fatty acids in rumen liquor were determined by HPLC (Rooke *et al.* 1990). Ammonia-N concentration was determined by a colorimetric method (Weatherburn, 1967).

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Molecular analysis on rumen samples

DNA was extracted from frozen rumen samples using a QIAamp® DNA Stool Mini Kit (Qiagen Ltd, Crawley, West Sussex, UK) following the manufacturer's instructions.

Denaturing gradient gel electrophoresis (DGGE) was used to compare bacterial diversity between rumen samples for each experimental animal. Approximately 200 bp of the 16S rDNA gene were amplified using the primers: forward 5′-CCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCTACGGGAGGCAGCAG-3′; reverse 5′-ATTACCGCGGCTGCTGG-3′ (Nubel *et al.* 1996). Use of a GC-clamp has been shown to help obtain a better profile on the denaturing gel (Muyzer *et al.* 1993). PCR was performed using the following steps: one cycle (94°C for 4 min); twenty-five cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min); one cycle (94°C for 1 min, 55°C for 1 min, 72°C for 10 min). The size of amplicon was confirmed by electrophoresis on a 2·5 % agarose gel, visualised after staining with ethidium bromide.

DGGE was performed in a C.B.S. Scientific® system (C.B.S. Scientific, Inc., Del Mar, CA, USA). Gels contained 40–60 % denaturant gradient in 8 % acrylamide gels and were run at 130 V, 200 mA and 250 W for 16 h at 60°C. DNA was visualised by silver staining with DNA Silver Staining Kit (Amersham Biosciences, Uppsala, Sweden). Gels were scanned using a GS-800 Calibrated Imaging Densitometer (BioRad, Hercules, CA, USA). Scanned DGGE images were analysed with Quantity One® Software (BioRad) by scoring for the presence or absence of bands at different positions in each lane. DGGE banding profiles were compared by using similarity trees. Each band position present in the gel was binary coded for its presence or absence within a lane and each lane was compared by using a similarity matrix (Regensbogenova *et al.* 2004). Trees were constructed by using the NEIGHBOUR program (PHYLIP version 3·6; Felsenstein, 2002).

Protozoal rDNA concentration was determined using the real-time PCR assay developed by Sylvester *et al.* (2004, 2005), using the ciliate-specific primers: forward -5′-GCTTTCGWTGGTAGTGTATT-3′-; reverse -5′-CTTGCCCTCYAATCGTWCT-3′-. A no-template (sterile distilled water) negative control was loaded on each plate run to screen for possible contamination and dimer formation and to set the background fluorescence for plate normalisation. The extracted DNA from washed protozoal solutions from all ruminal samples (Williams & Coleman, 1992), after DNA quantification, was diluted to generate the necessary standard curves. Real-time PCR was performed using a DNA Engine Opticon® System, PTC-200 DNA Engine™ Cycler (MJ Research, Braintree, Essex, UK). DNA extract (1 μl) was added to amplification reactions (50 μl) containing 50 pmol of each primer, 25 μl of SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, Poole, Dorset, UK) containing 20 mM-tri(hydroxymethyl)-aminomethane-HCl (pH 8·3), 100 mM-KCl, 7 mM-$MgCl_2$, 0·4 mM each dNTP, stabilisers, Taq DNA polymerase (0·05 unit/μl), JumpStart Taq, antibody and SYBR Green I. Cycling conditions were 94°C for 4 min; forty-five cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min; and a final extension of 72°C for 6 min. Fluorescence readings were taken after each extension step, and a final melting analysis was obtained by slow heating with 0·1°C/s increment from 65 to 95°C, with fluorescence collection at 0·1°C intervals. The threshold cycle (i.e. the amplification cycle in which product formation exceeds background fluorescence) of each standard dilution was determined during the exponential phase of amplification and regressed against the logarithm (base 10) of known protozoal DNA standard. All post-run data analyses were performed using MJ Research Opticon Monitor Software (version 1·06; Waltham, MA, USA).

Total bacterial rDNA concentration in rumen samples was measured by real-time PCR using the primers designed by Maeda *et al.* (2003) to target the conserved region on the 16S rDNA: forward 5′-GTGSTGCAYGGYTGTCGTCA-3′, reverse 5′-ACGTCRTCCMCACCTTCCTC-3′. DNA extract (1 μl) was added to amplification reactions (50 μl), containing 20 pmol of each primer, 25 μl of SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) containing 20 mM-tri(hydroxymethyl)-aminomethane-HCl (pH 8·3), 100 mM-KCl, 7 mM-$MgCl_2$, 0·4 mM each dNTP, stabilisers, Taq DNA polymerase (0·05 unit/μl), JumpStart Taq, antibody and SYBR Green I. Cycling conditions were 95°C for 5 min, forty cycles of 95°C for 15 s, 61°C for 1 min and 72°C for 30 s. Fluorescence readings, melting analysis and standard analyses were performed as described earlier for the protozoal assay. A bacterial rDNA standard curve was generated from DNA extracted from a mix (equal volumes) of 24 h cultures of the following rumen bacterial strains all grown on Hobson's medium 2 (Stewart *et al.* 1997): *Prevotella ruminicola* 23, *Butyrivibrio fibrisolvens* SH13, *Ruminococcus albus* SY3, *Prevotella albensis* M384, *Clostridium sticklandii* 12 662, *Peptostreptococcus anaerobius* 27 337, *R. flavefaciens* Fd1, *Mitsuokella multiacidus* 46/5, *Selenomonas ruminantium* 2388, *Lachnospira multipara* D15d, *Veillonela parvula* L59, *Prevotella bryantii* B14, *Prevotella brevis* GA33, *Lactobacillus casei* LB17, *C. aminophilum* 49 906, *Streptococcus bovis* ES1 and *Megasphera elsdenii* J1, all obtained from the Rowett Research Institute's (Aberdeen, UK) culture collection. The DNA concentration of the bacterial standard was determined by fluorescence following the bisbenzimide–DNA assay described by Labarca & Paigen (1980).

Calculations and statistical analysis

Species richness on DGGE gels was calculated as the mean numbers of bands present. The Shannon's diversity index (proportional abundance of species in a community) was determined according to Marrugan (1988): H' = −SUM ($pi \times * \ln(pi)$), where the proportion of single bands (i) relative to the total number of bands (pi) is calculated, and then multiplied by the natural logarithm of this proportion ($\ln pi$). The resulting product is summed across species and multiplied by −1.

Data were subjected to ANOVA (Genstat 7; Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, Herts, UK) with absence of protozoa as the sole treatment effect.

Results

The rumen of PF lambs remained free of protozoa during the experiment as neither protozoa nor protozoal DNA was found in their rumen at slaughter.

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Diet composition

The chemical composition of the grasses and concentrate are presented in Table 1. The composition of the two grasses (field control and field PF) was very similar, although the control grass contained more DM and contained a relatively higher amount of organic matter and structural carbohydrates (acid-detergent fibre and neutral-detergent fibre). Fatty acid analysis revealed very similar profiles between both grasses with a high content of linolenic and palmitic acids whereas the experimental concentrate contained high levels of palmitic, stearic and oleic acids.

Grass intake and animal growth

PF lambs had numerically a slightly higher grass intake over the 9 weeks of measurement (Table 2). As the values represented a whole group measurement and not individual animal intakes we were not able to perform any statistical analysis. We assume that concentrate intake was the same in both groups as there were no refusals and it was served in long feeding troughs (total length 3 m) with enough space for each lamb to have access. Assuming equal concentrate intakes, total daily DM intake would be 837 and 893 g DM for the control and PF groups, respectively. Live-weight gain and final live weight at slaughter (Table 2) values did not differ between the experimental groups. Wool growth was greater ($P<0{\cdot}05$) in PF lambs than in control lambs.

Ruminal fermentation and microbial ecosystem

Rumen pH did not differ between samples taken from control and PF lambs (Table 3). However, ammonia concentrations were significantly affected by the absence of protozoa, with 2·5-fold decrease concentrations in PF animals. Ruminal volatile fatty acid concentration and composition differed between control and PF lambs; butyrate molar proportions decreased in PF lambs compared with control lambs, whose rumen content had higher total volatile fatty acid concentrations than PF lambs. As we expected, no protozoal DNA was detected in PF lambs whereas rumen samples from control animals had 2·21 μg protozoal DNA/ml. Total bacteria DNA quantified by real-time PCR targeting 16S bacterial rDNA did not differ between the experimental groups.

Fig. 1 shows the DGGE image and the dendrogram derived from the cluster analysis. Rumen content from control and PF animals had a different bacterial species distribution, as the banding profiles were clearly clustered into two different groups, representing control and PF lambs. The two groups showed no more than 50 % similarity in banding patterns, whilst within groups similarity was between 75 and 80 %. Additionally, PF lambs had a lower average total number of bands (40·7 (SD 4·8) *v.* 54·5 (SD 5·0) for PF and control lambs, respectively) and lower diversity (Shannon index: 3·99 *v.* 4·22 for PF and control lambs, respectively). The present results suggest that, even though total bacterial numbers were similar, the distribution of the different bacterial groups was changed due to the lack of protozoa.

Abomasal and intramuscular fatty acid composition

The fatty acid compositions of both abomasal and intramuscular fat samples are shown in Table 4. Abomasal contents from control lambs had lower concentrations of *trans*-11–18 : 1 and higher of 20 : 3*n*-6, 22 : 5*n*-3, 22 : 6*n*-3 and total PUFA than PF lambs. The rest of the fatty acids, including the CLA isomers, were present at the same concentrations in abomasal contents from both experimental groups.

The total lipid in tail muscle samples was 24·2 and 27·3 mg lipid/g muscle (wet weight) for control and PF groups, respectively ($P=0{\cdot}403$; SED 4·93). Fatty acid concentrations in intramuscular fat showed differences between the groups. PF lambs had higher proportion of 18 : 0, 18 : 3*n*-3, *t*10,*c*12-CLA and total SFA than control lambs. The other CLA isomer analysed in the present study (*c*9,*t*11) was present in higher concentrations than the *t*10,*c*12 and did not differ between groups. On the other hand, control lambs had higher proportion of *cis*-9-18 : 1, *cis*-9, *cis*-12–18 : 2, 20 : 3*n*-6, 22 : 6*n*-3 and MUFA.

Overall the PUFA:SFA ratios in fatty acids detected in intramuscular fat in the present study were 0·39 and 0·28 for control and PF groups, respectively.

Table 1. Chemical composition and fatty acid profile of grasses and concentrate (g/kg dry matter)
(Mean values with their standard errors)

	Grass (control)	Grass (protozoa-free)	SEM†	*P**	Concentrate
DM (g/kg fresh matter)	337	224	32·5	0·025	854
Organic matter	878	846	28·9	0·435	925
Crude protein	197	202	4·69	0·452	162
Acid-detergent fibre	351	297	23·1	0·113	78
Neutral-detergent fibre	540	436	35·5	0·053	163
Diethyl ether extract	29	30	0·60	0·361	35
Fatty acid					
14 : 0	0·50	0·47	0·077	0·859	2·60
16 : 0	2·78	3·20	0·375	0·419	27·1
18 : 0	0·37	0·38	0·114	0·962	26·33
18 : 1*n*-9	0·18	0·16	0·049	0·888	22·73
18 : 2*n*-6	1·92	1·83	0·345	0·821	17·4
18 : 3*n*-3	5·62	5·44	0·810	0·882	2·71
20 : 0	0·12	0·11	0·037	0·794	1·13

* Control grass *v.* protozoa-free grass.
† SEM values for *n* 10 for chemical composition and *n* 5 for fatty acid profile.

Table 2. Effect of the absence of protozoa on production parameters and carcass characteristics

(Mean values with their standard errors)

Item	Control	Protozoa-free	SEM*	*P*
Grass intake (g DM/d)	587	643		
Live-weight gain (kg/d)	0·197	0·206	0·0127	0·637
Final live weight (kg)	32·4	33·6	0·834	0·323
Carcass weight (kg)	12·3	13·5	0·442	0·071
Carcass yield (%)	38·0	40·2	0·838	0·081
Wool growth (g)	0·97	1·11	0·045	0·043

* SEM values for *n* 10.

Discussion

Rumen defaunation can be achieved by different methods, one of which consists of separating the young animals from their dam after birth and preventing contact with adult animals, being the one used in our work (Abou Akkada & El Shazly, 1964). The literature and the present results confirm that this method allows the rumen to be naturally contaminated with strictly anaerobic bacteria and is also reliable at stopping protozoa developing in the rumen (Williams & Coleman, 1992).

Grass intake and animal growth

In the present experiment PF lambs had a higher daily live animal weight and carcass yield than control lambs. Published results on the effects of eliminating rumen protozoa on animal growth and wool production are contradictory (Veira, 1986; Coleman, 1988). In agreement with the present results, several authors (Demeyer *et al.* 1982; Bird & Leng, 1984; Van Nevel *et al.* 1985; Santra & Karim, 2002) have shown that exclusion of protozoa from the rumen has a beneficial effect on the growth rate, wool growth and feed conversion efficiency in animals under certain feeding conditions. However, some reports have concluded that protozoa are an essential component of the microbial ecosystem in the rumen, and their exclusion has a harmful effect on the productivity of the host (Eadi & Gill, 1971; Ramprasad & Raghacan, 1981). It is, however, clear that selective retention of protozoa in the rumen and the engulfment and digestion of bacteria by protozoa in the rumen increases the recycling of microbial N therein (Koenig *et al.* 2000). Thus, removing protozoa from the rumen causes an increase in bacterial and total N flows to the duodenum (Ushida *et al.* 1986; Ivan *et al.* 2000, 2006). This is manifested in the present experiment by the numerically higher wool growth; wool growth might be an indication of total flow of microbial protein to the duodenum because wool growth is often limited by the sulfur-containing amino acids which are high in microbial protein formed in the rumen (McNabb *et al.* 1993). The only published paper (Van Nevel *et al.* 1985) on the effect of the absence of protozoa on carcass composition found a trend towards more meat and less fat in the carcass of defaunated lambs, but this was not significant.

Ruminal fermentation and microbial ecosystem

Previous studies have found that defaunation had no effect on rumen pH when diets were rich in cell-wall carbohydrates (Collombier, 1981), which is in agreement with the present results. However, ammonia concentrations were significantly affected by the absence of protozoa. With the exception of the results published by Demeyer *et al.* (1982), all authors agree that by defaunating the rumen the NH_3-N concentration is greatly decreased (Jouany *et al.* 1988), mainly due to a reduction in breakdown of bacterial proteins and lower N recycling in the rumen (Koenig *et al.* 2000).

The lower butyrate molar proportion observed in PF lambs compared with control is commonly associated with a decreased ruminal fauna (Sutton *et al.* 1983; Williams & Coleman, 1992). Many authors have reported that defaunation frequently results in an increase in the molar proportion of propionic acid (Jouany *et al.* 1988); however, we found no effect, with similar acetate:propionate ratios in the experimental

Table 3. Effect of the absence of protozoa on ruminal fermentation and microbial numbers

(Mean values with their standard errors)

Item	Control	Protozoa-free	SEM*	*P*
PH	7·02	6·98	0·054	0·663
NH_3-N (mg/100 ml)	8·60	3·12	1·293	0·008
Total protozoa (cells × 10^5/ml)	1·08	ND		
Lactate (mm)	0·88	1·74	0·473	0·213
Total volatile fatty acids (mM)	41·0	30·9	1·97	0·002
Molar proportion (mol/100 mol)				
Acetate	61·6	62·8	1·32	0·464
Propionate	25·8	26·9	1·70	0·643
iso-Butyrate	0·92	0·48	0·081	<0·001
Butyrate	9·09	5·56	0·591	<0·005
iso-Valerate	1·08	1·02	0·079	0·568
Valerate	1·37	3·25	0·274	<0·001
Acetate : propionate (mol/mol)	2·4	2·3	0·19	0·712
Total bacteria DNA (μg/ml)	1·98	1·93	0·179	0·848
Total protozoal DNA (μg/ml)	2·21	ND		

ND, not detected.
* SEM values for *n* 10.

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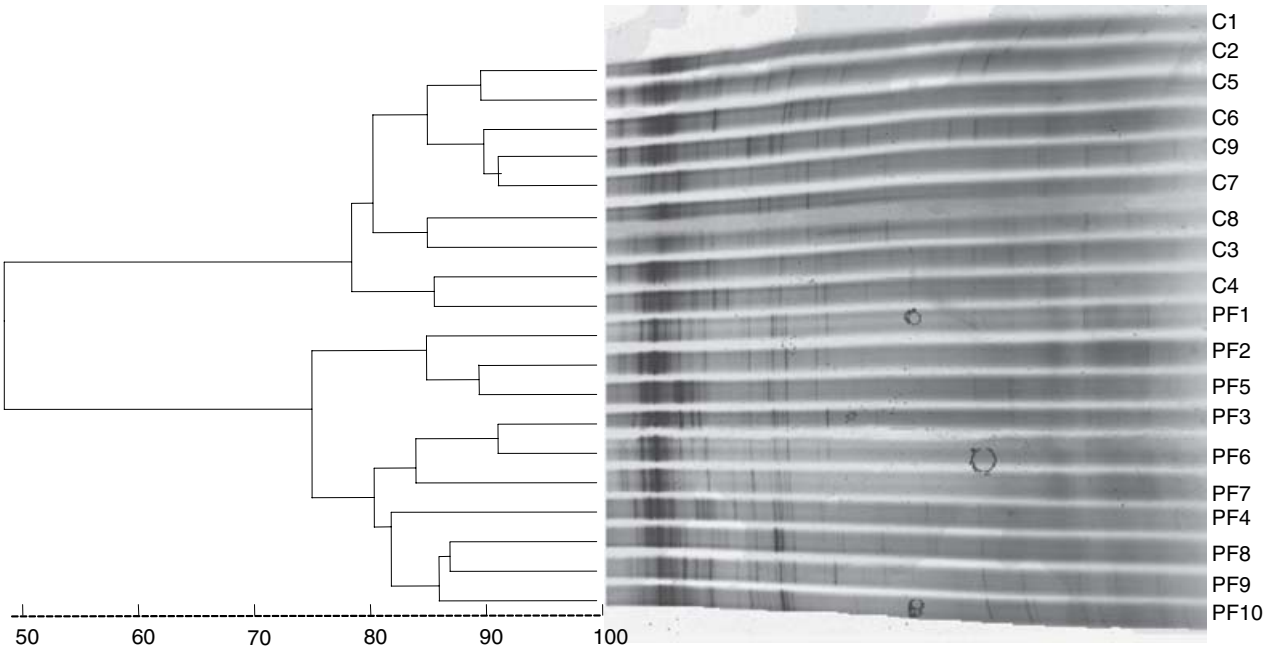

Fig. 1. Denaturing gradient gel electrophoresis of rumen bacterial 16S rDNA from control (C) and protozoa-free (PF) lambs and dendrogram derived after cluster analysis (unweighted pair group method with arithmetic mean; UPGMA) of banding profiles. The scaled bar indicates percentage similarity coefficients.

Table 4. Effect of the absence of protozoa on fatty acid composition (proportion (× 100) of total fatty acids) of abomasal content and *sacrocaudalis ventralis lateralis* intramuscular fat

(Mean values with their standard errors)

	Abomasum				Muscle			
Fatty acid	Control	PF	SEM*	*P*	Control	PF	SEM*	*P*
14 : 0	3·46	4·26	0·452	0·245	3·30	3·50	0·367	0·718
16 : 0	15·1	17·0	0·69	0·088	18·3	19·4	1·45	0·601
16 : 1	1·49	1·59	0·107	0·536	1·47	1·38	0·141	0·684
18 : 0	39·8	42·9	1·65	0·226	23·6	30·0	0·89	<0·001
trans-11-18 : 1	5·25	6·80	0·429	0·034	5·10	3·92	0·379	0·059
cis-9-18 : 1	10·36	9·25	0·644	0·258	30·5	26·8	0·86	0·015
18 : 2*n*-6	9·68	7·06	1·270	0·182	8·79	6·17	0·446	0·003
18 : 3*n*-3	6·28	4·72	0·533	0·072	1·27	1·82	0·074	<0·001
cis-9, *trans*-11-18 : 2	0·05	0·04	0·006	0·135	1·31	1·75	0·195	0·150
trans-10, *cis*-12-18 : 2	0·02	0·01	0·005	0·596	0·10	0·39	0·027	<0·001
20 : 3*n*-6	1·40	0·80	0·152	0·023	1·16	0·65	0·097	0·006
20 : 4*n*-6	3·66	3·41	0·328	0·603	3·07	2·78	0·265	0·463
20 : 5*n*-3	1·93	1·37	0·261	0·168	1·20	0·80	0·158	0·113
22 : 5*n*-3	1·04	0·59	0·078	0·004	0·51	0·48	0·054	0·727
22 : 6*n*-3	0·44	0·19	0·051	0·008	0·27	0·15	0·037	0·048
SFA†	58·4	64·2	1·84	0·057	45·2	52·9	1·34	0·004
MUFA‡	17·1	17·6	0·80	0·645	37·1	32·1	0·92	0·005
PUFA§	24·5	18·2	1·51	0·018	17·7	15·0	0·95	0·080

PF, protozoa-free.
* SEM values for *n* 5.
† Sum of 14 : 0, 16 : 0 and 18 : 0.
‡ Sum of 16 : 1, *trans*-11-18 : 1 and *cis*-9-18 : 1.
§ Sum of 18 : 2*n*-6, 18 : 3*n*-3, *cis*-9, *trans*-11-18 : 2, *trans*-10, *cis*-12–18 : 2, 20 : 3*n*-6, 20 : 4*n*-6, 20 : 5*n*-3, 22 : 5*n*-3 and 22 : 6*n*-3.

groups. The decreased concentrations of *iso*-butyrate may be indicative of decreased proteolysis (Wolin *et al.* 1997). Our observations suggest that defaunation had no effect on total bacterial numbers, which differs from the majority of the experiments carried out on the effect of defaunation (Hsu *et al.* 1991). However, most of these measurements were made over a short time period. Williams & Withers (1990) reported that in the medium to long term, bacterial numbers in the rumen of defaunated sheep tended to decrease to the original values observed before defaunation.

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https://doi.org/10.1017/S0007114507675187 Published online by Cambridge University Press

Despite there being no effect on total bacterial numbers, DGGE 16S rDNA banding profiles showed different bacterial populations in PF and control lambs. Previous studies have reported that defaunation causes an increase in Gram-negative rod-type bacteria (Kurihara *et al.* 1968), of amylolytic bacteria (Kurihara *et al.* 1978) and modifications in the balance between cellulolytic bacteria (Whitelaw *et al.* 1972). More recently, Ozutsumi *et al.* (2005) observed different bacterial communities in faunated and defaunated cattle by using 16S rDNA clone libraries. In the former study 151 sequences from faunated animals were classified into 98 operational taxonomic units, while the 161 sequences from defaunated animals were classified into 72 operational taxonomic units. However, for complex microbiomes such as those in the rumen or soil, it is estimated that thousands to ten of thousands clones are required for more robust comparison (Dunbar *et al.* 2002). In addition, only about twenty to twenty-two clones per animal were sequenced, which limits inter-animal variation. The DNA fingerprint technique used in the present study (PCR-DGGE), although less detailed in terms of bacterial phylogeny, ensures a broader analysis of the bacterial community in comparison with 16S rDNA libraries with limited number of sequences and confirmed the higher bacterial diversity reported by Ozutsumi *et al.* (2005) in faunated animals compared with defaunated animals and showed that the differences are consistent amongst animals. The effect of the absence of protozoa in different bacterial numbers may be explained by the preferential uptake of specific groups of bacteria by protozoa. Coleman (1964, 1986) showed that *Selenomonas ruminantium* and *B. fibrisolvens* were taken up and digested at faster rates than other bacteria by *Entodinium caudatum*. Kurihara *et al.* (1978) found a significant increase in *Megasphera* numbers in the rumen after defaunation. However, we found that eliminating protozoa from the rumen leads to lower bacterial diversity and the disappearance of some bacterial groups compared with faunated animals. That might be explained by the close metabolic relationship between protozoa and some groups of bacteria, in addition to methanogens, found to be attached to protozoa (Vogels *et al.* 1980; Bonhomme, 1990).

Abomasal and intramuscular fatty acid composition

Tail muscle samples were taken from the carcass in the present study to study muscle fatty composition to enable carcasses to be sold. Although most of the published work studying the fatty acid composition of intramuscular fat used *longissimus dorsi* muscle as a reference, there is evidence that differences in the fatty acid composition (in percentage terms) of the intramuscular fat is very small between different esqueletic muscles (Enser *et al.* 1998). In the former work the authors did not find major differences in the fatty composition of three esqueletic muscles from different parts of the carcass (*triceps branchi*, *longissumus dorsi* and *gluteobiceps*) from lambs fed grass. Intramuscular fat is metabolically very active in all esqueletic muscles compared with perineal and subcutaneous adipocytes (Gardan *et al.* 2006), and that could explain the similarity between different muscles in the body.

Ruminant fats are among the richest natural dietary source of CLA, in particular the *c*9,*t*11 and *t*10,*c*12 isomers (Chin *et al.* 1992). Both isomers are produced as a result of the partial biohydrogenation of linoleic and linolenic acid in the rumen by ruminal micro-organisms (Harfoot & Hazlewood, 1997). Although, biohydrogenation in the rumen has been considered only in terms of bacterial activity, recent observations have suggested that rumen protozoa may play an important role given the high concentration in VA and CLA in protozoa compared with bacteria (Devillard *et al.* 2006). Indeed, by using real-time PCR to quantify rumen protozoa (Sylvester *et al.* 2005), we have estimated that protozoa accounted for between 30 and 43 % of CLA and 40 % of the VA reaching the duodenum (Yáñez-Ruiz *et al.* 2006).

Various studies have demonstrated that little CLA flows out of the rumen (Duckett *et al.* 2002; Sackmann *et al.* 2003), although this is the first intermediate in the biohydrogenation of linoleic acid (Harfoot & Hazlewood, 1997). The flow of VA to the duodenum was more than twenty times greater than CLA in steers fed typical finishing diets (Duckett *et al.* 2002; Sackmann *et al.* 2003). Thus, the key to increasing the CLA content of animal tissues may be to produce CLA using the Δ^9 desaturase enzyme in the animals tissue which has been shown to be important for accumulation of *c*9,*t*11-CLA in milk (Griinari *et al.* 2000). Thus, the higher VA content found in the abomasum of PF lambs could explain the numerically higher *c*9,*t*11-CLA synthesis and deposition in the muscle observed in the present study. This higher VA production is in agreement with lower 18 : 2 concentration as a result of an increase in the biohydrogenation of 18 : 2 to 18 : 1 and 18 : 0, suggesting that a shift in the biohydrogenation pathway has occurred in PF lambs compared with control lambs. However, meat fat from control lambs had higher VA concentrations, which could represent an advantage over PF lambs, considering that about 20 % of the dietary VA is desaturated to *c*9,*t*11-CLA in the human liver (Turpeinen *et al.* 2002). Early work (Klopfenstein *et al.* 1966; Lough, 1968) studied the effect of defaunation on lipid metabolism and indicated that 16 : 0, 16 : 1 and 18 : 0 levels in plasma were unchanged after defaunation; however, the plasma concentrations of 18 : 3 and 18 : 2 were increased in defaunated animals, whereas 18 : 1 concentrations fell. These results might indicate that dietary fatty acids are hydrogenated more effectively when ciliate protozoa are present in the rumen, which does not agree with our observations. Different ruminal bacteria groups involved in biohydrogenation have been described. Two complementary groups are believed to be involved: group A biohydrogenate long-chain unsaturated fatty acids to VA whereas group B bacteria biohydrogenate VA to stearic acid (Kemp & Lander, 1984; Harfoot & Hazlewood, 1997). *B. fibrisolvens* from group A hydrogenates linoleic acid to *c*9,*t*11-CLA and VA and *Fusocillus* from group B completes the hydrogenation of VA to stearic acid. More recently, Van de Vossenberg & Joblin (2003) isolated from a grazing cow a bacterium phylogenetically close to *B. hungatei* which could form stearate from linoleic acid. As noted earlier, it has been previously shown that *B. fibrisolvens* are taken up and digested at faster rates than other bacteria by *E. caudatum* (Coleman, 1986). Thus it seems possible that in the absence of protozoa *B. fibrisolvens* numbers increased, hence the higher VA production, but that the complete hydrogenation to stearic acid was not enhanced. In any case, because of the complexity of the number of different species

https://doi.org/10.1017/S0007114507675187 Published online by Cambridge University Press

involved in biohydrogenation and CLA synthesis (Ogawa *et al.* 2005), changes in biohydrogenation pathways might not be explained by changes in the numbers of only two or three bacterial species.

The fatty acid composition of *sacrocaudalis ventralis lateralis* intramuscular fat agrees with values obtained in abomasal contents, even though we did not measured absolute fatty acid flows from the rumen. The fatty acids 16 : 0, 18 : 0 and *cis*-9–18 : 1 make up the greatest proportion of the fatty acids in the muscle, which is in agreement with other work carried out with grazing lambs (Enser *et al.* 1998; Rowe *et al.* 1999). As might be expected from grass-fed animals, significant proportions of *n*-3 PUFA were also observed, although not at the levels reported by others (Díaz *et al.* 2005), probably due to the concentrate supplement given to the experimental animals which contributed to increase *n*-6 PUFA intake. With regards to intramuscular fat content, the fact that there was no difference between groups rules out the possibility of explaining some differences in fatty acids due to changes in phospholipid and neutral lipids (Raes *et al.* 2004). From the present results, it seems that keeping protozoa in the rumen is beneficial for human health in terms of obtaining a higher PUFA:SFA ratio in ruminant meat. In addition, control lambs had higher levels of 22 : 6*n*-3, a fatty acid that has been associated with reduction in the thrombotic tendency of blood and a lower risk of heart disease (Department of Health, 1994).

The proportions observed for the main CLA isomer (*c*9,*t*11) are in the same range of those found by others in lamb meat (Wachira *et al.* 2002; Cooper *et al.* 2004) and are higher than values reported for lambs fed on concentrate diets (Aurousseau *et al.* 2004). Although only a few papers report the *t*10,*c*12-CLA content in lamb muscles, our values observed for the PF lambs appear to be slightly higher than those published (Bolte *et al.* 2002). The three-fold rise observed in *t*10,*c*12-CLA in PF lambs compared with control lambs is of the same order to that achieved by Boles *et al.* (2005) in lambs after supplementing the basal diet with 6 % of safflower oil. This increase is in agreement with Jouany & Lassalas (2003) who observed, *in vitro*, that three times more *t*10,*c*12-CLA was produced in vessels inoculated with rumen fluid from defaunated animals compared with those inoculated with rumen fluid from faunated animals. They suggested that defaunating activates certain isomerisation reactions and pushes biohydrogenation pathways towards *trans*, *trans* and *t*10,*c*12 isomer production. Interestingly, Kim *et al.* (2002) identified a strain of *Megasphaera elsdenii* (YJ-4) capable of producing *t*10,*c*12-CLA, whilst Kurihara *et al.* (1978) showed a ten-fold increase in *Megasphera* after defaunation, which could explain the higher production of this isomer observed in PF lambs. A number of recent observations using individual isomers in animals and human subjects seem to suggest that some CLA isomers, particularly the *t*10,*c*12 isomer, may elicit detrimental effects on human health (Medina *et al.* 2000; Riserus *et al.* 2001, Roche *et al.* 2002) although no detrimental effects on health have been reported for the more commonly employed 50:50 mixtures of the two main isomers of CLA or the most abundant *c*9,*t*11 isomer (Wahle *et al.* 2004). The present results show a higher concentration of the *t*10,*c*12 isomer in muscle from PF animals which, if the deleterious effects of this isomer are confirmed, suggests that defaunating the rumen might not be desirable in terms of producing a diet for optimal human health.

As noted before, PUFA:SFA ratios of the fatty acids detected in intramuscular fat in the present study were 0·39 and 0·28 for control and PF groups, respectively. Chalupa & Kutches (1968) believed that the increased levels of oleic and linoleic acids in the milk of animals with depressed milk fat tests were attributable to the virtual absence of protozoa in the rumen of these cattle. Investigations with pure isomers have shown that *t*10,*c*12-CLA is a potent inhibitor of milk fat synthesis (Bauman & Griinari, 2003), which could explain the relationship between absence of protozoa and milk fat depression reported by Chalupa & Kutches (1968). In contrast, Abaza *et al.* (1975) found a decrease in SFA levels in the plasma of sheep lacking protozoa compared with faunated animals. Protozoa rapidly engulf chloroplasts released from plant cells (Hall *et al.* 1974) and possibly as a result the fatty acids of rumen protozoa are less saturated (between 22 and 35 % of the fatty acids present as 18 : 1, 18 : 2 or 18 : 3) compared with bacteria (between 7 and 25 % of the fatty acids present as 18 : 1, 18 : 2 or 18 : 3) (Harfoot & Hazlewood, 1997). Girard & Hawke (1978) estimated that 20–25 % of the linoleic acid in the rumen of a cow receiving a hay diet was present in the lipids of the relatively small *Isotricha* population (10^3–10^4 cells/ml). Different diets and means of providing forages (grazing, silage, hay) will lead to differing levels of chloroplast entering the rumen and hence a possible different role of protozoa. Based on the present results, it might be speculated that unsaturated dietary fatty acids may be partially protected from biohydrogenation by incorporation into structural phospholipids of protozoa. Given that protozoa make up approximately half of the rumen biomass (Jouany, 1995), this suggests that ciliate protozoa represent a major pool of unsaturated fatty acids in the rumen as a result of their storage capacity. This is supported by our recent results (Yáñez-Ruiz *et al.* 2006) which show that protozoa make a greater contribution to the flow of unsaturated fatty acids from the rumen (30–50 % of the fatty acids entering the duodenum of protozoal origin) than to the flow of SFA (10–20 %).

In conclusion, the elimination of protozoa from the rumen leads not only to greater animal lamb growth, but also to changes in bacterial populations and in the biohydrogenation pathways, which results in more VA being produced in the rumen and less PUFA, more SFA and *t*10,*c*12-CLA in intramuscular fat, which is not desirable from a human health perspective. More research needs to be undertaken on the effect of rumen protozoa in animals fed different types of diets with different lipid sources to confirm such a hypothesis.

Acknowledgements

The authors acknowledge expert analytical advice from Dr Susan Girwood, and Evita Gonzalez and Dr Stephane Duval for technical assistance. D. R. Y.-R. gratefully acknowledges the receipt of a research contract from the European Commission (Marie Curie Programme RUMENOMICS 010972).

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