Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content

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The present experiment was designed to estimate the quantitative contribution of rumen protozoa to the total N, conjugated linoleic acid (CLA) and vaccenic acid (VA; trans-11-18:1) flow to the duodenum of steers fed two silage diets: control silage (CS) and silage high in water-soluble carbohydrates (HS). Protozoal duodenal flows were estimated using a real-time PCR assay to quantify the genes encoding protozoal 18S ribosomal RNA. Denaturing gradient gel electrophoresis was used to confirm that the rumen protozoa populations were similar to the protozoal population flowing to the duodenum. Estimated duodenal flow of protozoal N was 14·2 and 18·2 g/d (P<0·05) for animals fed the CS and HS diets respectively. Protozoal flow thus represented between 12 and 15 % of the total N duodenal flow. In terms of fatty acid flow, protozoa accounted for between 30 and 43 % of the CLA and 40 % of the VA reaching the duodenum. The contribution of protozoa to 16:0 and 18:0 flows to the duodenum was less than 20 and 10 %, respectively. These results show that the fatty acids within protozoa make up a significant proportion of the CLA and VA reaching the duodenum of ruminants.

Conjugated linoleic acid: Protozoa: Rumen: Vaccenic acid: Water-soluble carbohydrates

Microbial cell matter that flows to the duodenum from the rumen is an important source of nutrients for the host animal. To date the contribution of microbial cell matter has been mainly considered in terms of N supply; however, microbial cells also account for a significant proportion of the fatty acids leaving the rumen. There is currently an increasing interest in the fatty acid composition of ruminant products, particularly in regards to conjugated linoleic acid (CLA). 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 (c9,t11-CLA), can be produced directly by microbial hydrogenation in the rumen or by a Δ9-desaturase in the animals’ tissues, using vaccenic acid (VA; trans-11-18:1) formed in the rumen as its substrate (Bauman et al. 1999). The second most abundant isomer of CLA, trans-10, cis-12-18:2 (t10,c12-CLA) is also formed in the rumen. Some isomers of CLA have been associated with the inhibition of some types of cancer (De la Torre et al. 2005), reduction of atherosclerosis (Toomey et al. 2005), enhancement of the immune response (Zhang et al. 2005) and body fat repartitioning (Blankson et al. 2000). However, recently, some experiments on laboratory animals appear to suggest that the t10,c12-CLA isomer may have detrimental effects on health (Wahle et al. 2004).

To date the production of CLA and VA in the rumen has been considered solely from the perspective of bacterial metabolism and it has been suggested that protozoa are of minor importance (Harfoot & Hazlewood, 1997). The role of ciliate protozoa in fatty acid biohydrogenation in the rumen remains unclear; Wright (1959) suggested that protozoa rapidly reduced trienes to dienes and monenes to saturated acids in vitro but the conversion of dienes to monoenes was slow. Abaza et al. (1975) found that mixed entiodinomorphs reduced oleic acid to stearic acid even in the presence of antibiotics. However, this was disputed by Singh & Hawke (1979) who, using fractionated rumen contents incubated with 14C-labelled monogalactosyl diacylglycerol, suggested that the contribution made by protozoa to biohydrogenation was due to the activity of ingested bacteria. Recently, Devillard et al. (2004) have extended this observation to suggest that the CLA and VA content of rumen protozoal cells was 4–5-fold higher than found in bacteria, suggesting that protozoa may also represent a major pool of CLA and VA in the rumen.

It is, however, difficult to calculate what the contribution of protozoa to fatty acid supply at the duodenum would be, as it is clear from many studies that protozoa tend to be retained in the rumen and that outflow to the duodenum does not always reflect ruminal concentrations (Dehority, 2003). However, research into protozoal passage to the duodenum has been hindered by the lack of a suitable marker of protozoal flow (Firkins et al. 1998).

Recently, Sylvester et al. (2005) have re-examined this problem using an approach based on the use of real-time PCR,
utilising primers specific to protozoal 18S ribosomal DNA (rDNA), to quantify protozoa in duodenal digesta. In the present study we have taken advantage of a trial in which duodenal flows were measured in steers receiving diets based on silages containing different levels of water-soluble carbohydrate to apply the technique described by Sylvester et al. (2005) to access the protozoal contribution to the flow of N, CLA and VA reaching the duodenum.

Materials and methods

Animals and experimental design

Six Hereford × Friesian steers, initial live weight 173 ± 7·1 kg, each prepared with a rumen cannula and simple ‘T’-piece cannulae in the proximal duodenum were used. They were housed in individual pens, and transferred to metabolism crates for the measurement period. The building was well ventilated and animals had free access to fresh water. A vitamin–mineral pre-mix (Rumins Cattle Regular; Rumenco Ltd, Burton-on-Trent, Staffs, UK) was offered at 100 g/head per d, sprinkled on the top of the morning meal. This pre-mix included: macro-minerals Ca, P, Mg and Na at 200, 40, 50 and 80 g/kg, respectively; micro-minerals Se, Co, I, Mn, Zn, Fe and Cu at 0·02, 0·15, 0·25, 3·5, 3·5, 3·0 and 1·8 g/kg, respectively; vitamins (retinol, cholecalciferol and alphatocopherol at 90, 1·5 and 667 mg/kg, respectively).

The experimental design was a two-period changeover, with two dietary treatments: control grass silage (CS) and high-water-soluble carbohydrates grass silage (HS), commercially named ‘FEN’ and ‘AberDOVE’, respectively. Each period consisted of 20 d, consisting of 17 d adaptation to the diet, and 3 d for rumen and duodenal sampling. Silage was made from a grass silage offered for periods 1 and 2, respectively. The flow was measured in steers receiving diets based on silages with 18·5 % (v/v) formaldehyde for protozoa cell counting (Dehority, 1984). The rest of the rumen sample was filtered through two layers of cheesecloth; the dry particulate fraction was washed by hand with 39°C Coleman buffer (Williams & Coleman, 1992) and added to the first filtrate to maximise the recovery of particulate-associated protozoa. Approximately 10 ml was kept at −80°C for DNA extraction and subsequent denaturing gradient gel electrophoresis (DGGE) analysis in order to compare rumen protozoal populations with the population recovered at the duodenum. The rest (300 ml) was immediately used for isolation of washed protozoa as follows. The sample was diluted with 300 ml Coleman buffer and placed in a separation funnel at 39°C and allowed to flocculate for 45 min. The flocculent layer was aspirated and the bottom layer was drawn off and filtered through a nylon filter cloth with a 400 µm pore size to remove the remaining plant material. The last filtrate was filtered through a new nylon filter cloth with a 10 µm pore size to remove contaminating bacteria that were externally associated with protozoa. The retained protozoal cells were washed four more times (125 ml each time) with Coleman buffer in each washing to ensure minimal bacterial contamination. A sample of the protozoal solution obtained after each filtration step was taken and kept at −80°C for DNA extraction to monitor bacterial contamination by real-time PCR. The final protozoal solution obtained from each animal was split into two subsamples; one kept at −80°C for DNA extraction and the other kept at −20°C. Samples of protozoal solutions and duodenal contents kept at −20°C were freeze-dried and ground to pass a 0-5 mm screen before fatty acid analysis.

Molecular analyses

DNA was extracted from frozen protozoal solutions and duodenal samples using the QIAamp® DNA Stool Mini Kit (Qiagen Ltd, Crawley, West Sussex, UK) following the manufacturer’s instructions. DNA concentrations in isolated protozoal solutions were determined using BisBenzimide (Labarca & Paigen, 1980), in order to use them as standards for the real-time PCR assay.

Denaturing gradient gel electrophoresis analysis

DGGE was used to compare protozoal diversity between rumen and duodenal samples for each experimental animal. In addition, rumen protozoal sample profiles were also compared with the protozoal preparation obtained by filtering to check whether the protozoal population in the standards represents those in the rumen.

Approximately 400 bp of the 18S rDNA gene was amplified using rumen ciliate-specific primers (Regensbogenova

These were subsequently freeze-dried, ground and retained frozen for analysis. On day 20, samples of rumen content (2 litres) were collected 2 h after the first feeding time. A subsample (250 ml) was used to isolate microbial cells by centrifuging at 3000g for 25 min, then the supernatant fraction was centrifuged at 30000g for 25 min followed by a distilled water wash and a further spin (30000g) repeated three times, to minimise contamination from whole rumen contents (Lee et al. 2002). The pellet was then freeze-dried before analysis.

Another subsample (5 ml) was treated with an equal volume of 18·5 % (v/v) formaldehyde for protozoa cell counting (Dehority, 1984). The rest of the rumen sample was filtered through two layers of cheesecloth; the dry particulate fraction was washed by hand with 39°C Coleman buffer (Williams & Coleman, 1992) and added to the first filtrate to maximise the recovery of particulate-associated protozoa. Approximately 10 ml was kept at −80°C for DNA extraction and subsequent denaturing gradient gel electrophoresis (DGGE) analysis in order to compare rumen protozoal populations with the population recovered at the duodenum. The rest (300 ml) was immediately used for isolation of washed protozoa as follows. The sample was diluted with 300 ml Coleman buffer and placed in a separation funnel at 39°C and allowed to flocculate for 45 min. The flocculent layer was aspirated and the bottom layer was drawn off and filtered through a nylon filter cloth with a 400 µm pore size to remove the remaining plant material. The last filtrate was filtered through a new nylon filter cloth with a 10 µm pore size to remove contaminating bacteria that were externally associated with protozoa. The retained protozoal cells were washed four more times (125 ml each time) with Coleman buffer in each washing to ensure minimal bacterial contamination. A sample of the protozoal solution obtained after each filtration step was taken and kept at −80°C for DNA extraction to monitor bacterial contamination by real-time PCR. The final protozoal solution obtained from each animal was split into two subsamples; one kept at −80°C for DNA extraction and the other kept at −20°C. Samples of protozoal solutions and duodenal contents kept at −20°C were freeze-dried and ground to pass a 0-5 mm screen before fatty acid analysis.

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et al. 2004): forward 5'-GGTTGATCGCATGCGC-3'; reverse 5'-ATGCTTACTATATCTC-3'. PCR was performed using the following steps: one cycle (94°C for 4 min); thirty cycles (94°C for 1 min, 57°C for 30 s, 72°C for 1 min); one cycle (94°C for 1 min, 57°C for 30 s, 72°C for 10 min). The size of amplicons was confirmed by electrophoresis on a 2.5% agarose gel, visualised after staining with ethidium bromide. 

DGGE was performed on a C.B.S. Scientific system (C. B. S. Scientific Company, Inc., Del Mar, CA, USA). Gels contained 30 to 50% denaturant gradient in 8% acrylamide gels and were run at 130 V, 200 mA, 250 W for 16 h at 60°C. DNA was visualised by Ag staining with a commercial DNA Silver Staining Kit (Amersham Biosciences, Uppsala, Sweden). Gels were scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad, Laboratories Ltd, Hemel Hempstead, Herts, UK). Scanned DGGE images were analysed with Quantity One Software (Bio-Rad) by scoring for the presence or absence of bands at different positions in each lane. Two different DGGE gels were run; one including samples from animals 90, 91 and 92 (periods 1 and 2) and another from animals 93, 94 and 95 (periods 1 and 2). DGGE profiles of rumen and duodenal samples within the same gels 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 (Regenbo genova et al. 2004). Trees were constructed by using the NEIGHBOUR program (PHYLLIP (Phylogeny Inference Package) version 3.6a; F. Felsentein, University of Washington, Seattle, WA, USA).

Real-time polymerase chain reaction assay

Protozoal rDNA concentration was determined using a real-time PCR assay developed by Sylvester et al. (2004, 2005) in duodenal samples, using ciliate-specific primers: forward 5’-GCTTTGCWGTTGAGTGATT-3’; reverse 5’-CTTCGCCCTCY.AATCGTWCT-3’. Three replicate extractions of DNA were performed on each sample and three replicates of the pooled DNA extracts were used. A no-template (sterile distilled water) negative control was loaded on each plate run to screen for possible contamination and dimmer for-
in duodenal fractions and microbial pellets obtained from the rumen using the HPLC as described by Cozzi et al. (1993).

Fatty acids in freeze-dried duodenal digesta and ruminal protozoa samples were extracted and methylated following the procedure of Kramer & Zhou (2001). Briefly, 300 mg dried digesta was placed in 15 ml tubes with Teflon-lined screw caps, followed by the addition of 100 µl methyl heneicosanoate (15 mg/ml) (21:0, internal standard) and 5 ml of a 2:1 mixture of chloroform and methanol. The sample was vortexed for 5 min and centrifuged at 900 g for 5 min. The top layer was aspirated and placed in a new tube. The original sample was extracted twice more with 5 ml chloroform–methanol. The lipid extract in chloroform–methanol was dried under N2 in a water-bath at 50°C and re-dissolved in 1 ml hexane. Then, 3 ml of 0.5 N NaOH in methanol was added, vortexed and heated for 15 min at 50°C. Another 3 ml of 5 % HCl in methanol was added, vortexed and heated for 60 min at 50°C. 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 a fatty acid methyl ester (FAME) column (100 m × 0.25 mm internal diameter; Varian Inc., Palo Alto, CA, USA) and a standard Supelco 37 component FAME mixture (Supelco, Poole, Dorset, UK).

Calculation and statistical analysis

Digesta flows were calculated after mathematical reconstitution of true digesta as described by Faichney (1975). Microbial N flow to the duodenum was computed by taking into account the purine:N ratio calculated in the microbial pellets from the rumen and in the duodenal samples.

The N:total DNA ratios and the N:individual fatty acid ratios were recorded for the concentrated protozoal standard for every experimental animal, and the duodenal flows of protozoal DNA, N and individual fatty acids were calculated.

Data were subjected to ANOVA fitted with orthogonal polynomials (Genstat 7; Lawes Agricultural Trust, Rothamsted, Herts, UK) with diet as the treatment effect and blocking for period and animal.

Results

The chemical composition of the silages is given in Table 1. The composition of the silages was typical of well-preserved grass silage and very similar between the HS and CS, although the HS contained slightly higher N and diethyl ether extract contents and lower neutral-detergent fibre content than the CS. Water-soluble carbohydrate content of the HS (739 g/kg DM) was 27 % higher than in the CS (581 g/kg DM). Fatty acid composition was similar between the HS and CS.

Table 1. Chemical composition (g/kg DM) and fatty acid profile of the experimental diets: control silage (CS) or high-water-soluble carbohydrates silage (HS)

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>416</td>
<td>380</td>
</tr>
<tr>
<td>Crude protein</td>
<td>153</td>
<td>169</td>
</tr>
<tr>
<td>Acid-detergent fibre</td>
<td>379</td>
<td>363</td>
</tr>
<tr>
<td>Neutral-detergent fibre</td>
<td>622</td>
<td>585</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>21</td>
<td>33</td>
</tr>
<tr>
<td>Water-soluble carbohydrates</td>
<td>58</td>
<td>74</td>
</tr>
<tr>
<td>Fatty acid composition (g/kg DM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0 (Myristic)</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>14:0 (Lauric)</td>
<td>0.58</td>
<td>0.64</td>
</tr>
<tr>
<td>16:0 (Palmitic)</td>
<td>4.25</td>
<td>3.89</td>
</tr>
<tr>
<td>16:1 (Palmitoleic)</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>18:0 (Stearic)</td>
<td>0.56</td>
<td>0.44</td>
</tr>
<tr>
<td>18:1n-9 (Oleic)</td>
<td>0.62</td>
<td>0.57</td>
</tr>
<tr>
<td>18:2n-6 (Linoleic)</td>
<td>4.21</td>
<td>3.89</td>
</tr>
<tr>
<td>18:3n-3 (Linolenic)</td>
<td>9.21</td>
<td>10.2</td>
</tr>
<tr>
<td>20:0 (Arachidic)</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>Total fatty acids</td>
<td>20.4</td>
<td>20.8</td>
</tr>
</tbody>
</table>

Comparative denaturing gradient gel electrophoresis banding profiles and bacterial contamination of washed protozoal standards

The DGGE banding profiles were generally more similar between the ruminal and duodenal samples within each animal than when compared between sampling sites across animals as shown by the phylogenetic tree obtained after unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis (Fig. 1). The set of primers used produced twenty-one major different bands. Every band obtained from a ruminal sample was also found in the corresponding duodenal sample from the same animal, showing the same protozoal species distribution in rumen and duodenal samples.

Bacterial DNA contribution to total DNA decreased from 53.7 % in strained rumen fluid filtered through 400 µm pore-size nylon cloth to 7.4 % after the third filtration-washing on a 10 µm pore-size nylon cloth and remained constant after further washing. The washed protozoal standard obtained from animal 90 in period 1 showed a high bacterial DNA content, which accounted for 39 % of the total DNA even after repeated washing on a 10 µm pore-size nylon cloth. Data from this animal in period 1 were thus excluded from all analysis. For the rest of the animals every band found in the rumen was also detected in the protozoal standards, as shown in Fig. 2 for animal 91 fed the HS diet.

Protozoal counts and fatty acid content of rumen protozoa

Concentrations and distribution of ciliates based on visual counts are shown in Table 2. Animals offered the HS (13.2 × 105 cell/ml) diet had higher (P<0.05) total protozoa counts compared with those fed the CS (8.12 × 105 cell/ml) diet. Entodinium sp. were the predominant protozoa found in the rumen of all animals and accounted for 60 and 65 % of the total protozoa, respectively, for the CS and HS diets. Holotrich protozoa (Isotrichidae) only appeared in the rumen fluid of animals offered the HS diet, accounting for 1.5 % of the total protozoa.
The fatty acid composition of washed protozoal suspensions is also shown in Table 2. Stearic acid (18:0) was the most abundant fatty acid, followed by palmitic acid (16:0), giving 16:0:18:0 ratios of 0.72 and 0.85, respectively for the CS and HS diets. VA content was slightly lower than \( c_{9}, c_{12} \)-18:2 content at 8.73 to 12.2 % of the total fatty acids. The content of the \( c_{9}, t_{11} \)-CLA isomer was 5.66 and 4.95 mg/mg N, respectively, for the CS and HS diets, three times higher than the other measured CLA isomer (\( t_{10}, c_{12} \)).

The content of all fatty acids did not differ (\( P > 0.05 \)) between diets, with the exception of 15:0, which was higher (\( P < 0.05 \)) in the HS diet. Linolenic acid (18:3) was found in protozoal standards at lower concentrations than \( c_{9}, c_{12} \)-18:2 and 18:0.

Duodenal flows

Table 3 shows DM, organic matter and N intakes and duodenal flows. There were no diet effects on intakes or flows. However, microbial N flow tended to be higher (\( P = 0.154 \)) in animals fed the HS v. CS diet (73.8 v. 67.8 g/d, respectively). Estimated duodenal flow of protozoal N was 14.2 and 18.2 g/d for animals fed the CS and HS, respectively (\( P > 0.05 \)). They represented, respectively, 12.3 and 15.4 % of total N and 21 and 24.7 % of microbial N flowing to the duodenum.

Duodenal flows (g/d) of long-chain fatty acids and biohydrogenation intermediates are shown in Table 4. Fatty acid profiles in duodenal digesta reflected the long-chain fatty acid profiles found in the silage and the biohydrogenation process that takes place in the rumen; higher contents of C\(_{18}\) than C\(_{16}\) and the majority of C\(_{18}\) fatty acids in the form of stearic acid. In agreement with dietary concentrations, long-chain fatty acid flow did not differ (\( P > 0.05 \)) between experimental diets.

The protozoal contribution to total fatty acids flowing to the duodenum varied depending on the fatty acid considered. As a general pattern, protozoal contribution appears to be higher for

### Table 2. Protozoa counts and type distribution (%) and composition of protozoal standard solutions in the rumen of steers offered control silage (CS) or high-water-soluble carbohydrates silage (HS) (Mean values and standard errors of the difference)

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>HS</th>
<th>SED</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protozoa (x10^5/ml)</td>
<td>8.12</td>
<td>13.2</td>
<td>1.26</td>
<td>0.025</td>
</tr>
<tr>
<td>Entodiniinae</td>
<td>60.1</td>
<td>65.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diplodiniinae</td>
<td>37.8</td>
<td>31.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diplodinium</td>
<td>40.9</td>
<td>54.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eudiplodinium</td>
<td>55.8</td>
<td>45.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ophrysolecinae</td>
<td>2.1</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotrichidae</td>
<td>0</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protozoal standards</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (g/ml)</td>
<td>0.17</td>
<td>0.21</td>
<td>0.012</td>
<td>0.586</td>
</tr>
<tr>
<td>DNA:N ((\mu)g/mg)</td>
<td>34.3</td>
<td>27.7</td>
<td>1.238</td>
<td>0.725</td>
</tr>
<tr>
<td>Fatty acids ((\mu)g/mg N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>8.62</td>
<td>9.93</td>
<td>2.3</td>
<td>0.931</td>
</tr>
<tr>
<td>15:0</td>
<td>1.76</td>
<td>3.71</td>
<td>0.4</td>
<td>0.014</td>
</tr>
<tr>
<td>16:0</td>
<td>243</td>
<td>235</td>
<td>56.4</td>
<td>0.826</td>
</tr>
<tr>
<td>17:0</td>
<td>1.54</td>
<td>2.54</td>
<td>0.7</td>
<td>0.246</td>
</tr>
<tr>
<td>18:0</td>
<td>338</td>
<td>278</td>
<td>142.7</td>
<td>0.503</td>
</tr>
<tr>
<td>Trans-11-18:1</td>
<td>81.0</td>
<td>72.0</td>
<td>15.0</td>
<td>0.999</td>
</tr>
<tr>
<td>Cis-9, cis-12-18:2</td>
<td>106</td>
<td>107</td>
<td>1.42</td>
<td>0.943</td>
</tr>
<tr>
<td>18:3</td>
<td>53.6</td>
<td>52.2</td>
<td>5.49</td>
<td>0.157</td>
</tr>
<tr>
<td>Cis-9, trans-11-CLA</td>
<td>5.66</td>
<td>4.95</td>
<td>2.59</td>
<td>0.644</td>
</tr>
<tr>
<td>Trans-10, cis-12-CLA</td>
<td>1.96</td>
<td>1.95</td>
<td>6.09</td>
<td>0.989</td>
</tr>
</tbody>
</table>

CLA, conjugated linoleic acid.

![Fig. 1. Dendrograms derived after unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis of denaturing gradient gel electrophoresis banding profiles of rumen (R) and duodenal (D) samples in animals 90, 91 and 92 (a) and 93, 94 and 95 (b) fed either control (C) or high-water-soluble carbohydrates (H) silages.](image)

![Fig. 2. Comparative protozoal banding profile between duodenal (D) and ruminal (R) samples and a protozoal standard solution (P) in animal 91 fed the high-water-soluble carbohydrates silage diet.](image)
unsaturated compared with saturated fatty acids. Protozoa were responsible for between 36 to 39% of $c_9,t_{11}$-CLA flow, 48 to 64% of the $t_{10},c_{12}$-CLA flow and 39 to 40% of the VA flow but no more than 10% of 18:0 and 20% of 16:0 flows.

**Discussion**

*Measuring duodenal flow of protozoa*

By comparing the protozoal DGGE banding profile obtained with specific protozoa 18s RNA primers between rumen fluid, duodenal digesta and washed protozoal standards we found no evidence of selective protozoal loss during protozoa isolation nor through digesta passage to the duodenum (Fig. 2), which confirms the suitability of the protozoa standards to represent the whole protozoal population in the rumen. Thus, accepting that some errors may remain because of differences in the relative numbers of different protozoal groups between the fractions as indicated by differences in the intensities of bands on the DGGE gels, in the present study we have used washed protozoal suspensions obtained by filtration as standards for real-time PCR quantification of protozoal flow to the duodenum and for fatty acid profiling within cells. Quantification as to what if any errors in our calculations will result from a change in the relative distribution of different protozoal groups in different fractions will require a more detailed knowledge of the fatty acid composition of individual protozoal species plus the development of specific primer set to quantify individual protozoal species in digest samples. However, based on a semi-quantitative examination of band intensities in our DGGE profiles we estimate that there was no more than a 9.2% difference in the relative distribution of total ciliate biomass between bands in profiles of rumen compared with duodenal samples from the same animal, suggesting the resultant error might also be small.

Given the significant contribution of protozoa to the total microbial protein in the rumen, their important role in bacteria predation and their high recycling rates, several methods have been used to estimate the protozoal flow leaving the rumen (Steinhour et al. 1982; Michalowski et al. 1986; Punia et al. 1992; Dijkstra, 1994; Shabi et al. 2000). As noted previously, research in this area is hindered by the lack of an accurate...

| Table 3. Daily intakes and duodenal flows of dry matter, organic matter (OM) and nitrogen and rumen protozoa in steers fed control silage (CS) or high-water-soluble carbohydrates silage (HS) (Mean values and standard errors of the difference) |
|---|---|---|---|---|---|
| Intakes | CS | HS | SED | P |
| DM (kg/d) | 3.54 | 3.53 | 0.021 | 0.944 |
| OM (kg/d) | 3.29 | 3.28 | 0.027 | 0.952 |
| N (g/d) | 111 | 114 | 0.001 | 0.303 |
| Flows | | | | |
| DM (kg/d) | 2.68 | 2.75 | 0.562 | 0.245 |
| OM (kg/d) | 1.94 | 1.97 | 0.028 | 0.622 |
| Total N (g/d) | 115 | 118 | 0.857 | 0.611 |
| Microbial N (g/d) | 67.5 | 73.8 | 0.432 | 0.154 |
| Protozoal N (g/d) | 14.2 | 18.2 | 0.502 | 0.058 |

| Table 4. Daily intake, duodenal flow and protozoal flow of long-chain fatty acids and biohydrogenation intermediates and protozoal fatty acid duodenal flows of steers fed control silage (CS) or high-water-soluble carbohydrates silage (HS) (Mean values and standard errors of the difference) |
|---|---|---|---|---|---|
| Intake (g/d) | Duodenal flow (g/d) | Protozoal flow (g/d) | Contribution $^*$ |
| Fatty acids | CS | HS | SED | P | CS | HS | SED | P | CS | HS | SED | P |
| 12:0 | 0.21 | 0.35 | 0.025 | 0.514 | 0.3 | 0.355 | 0.97 | 0.74 | 0.35 | 0.003 | 0.133 | 0.03 |
| 14:0 | 2.05 | 2.27 | 0.181 | 0.614 | 0.2 | 0.172 | 0.731 | 0.84 | 0.17 | 0.11 | 0.18 |
| 16:0 | 11.6 | 13.8 | 0.126 | 0.629 | 1.7 | 0.424 | 3.45 | 4.49 | 1.6 | 0.625 |
| 18:0 | 14.9 | 13.8 | 1.01 | 0.649 | 22.0 | 5.78 | 3.04 | 3.04 | 1.65 | 0.626 |
| 18:1 (trans11) | ND | ND | – | – | 2.58 | 0.421 | 1.52 | 0.541 | 1.52 | 0.421 |
| 18:1 (cis9) | 2.19 | 2.01 | 0.037 | 0.481 | 3.08 | 0.402 | 1.27 | 1.27 | 0.18 |
| 18:2 (cis9, cis12) | 16.9 | 13.8 | 0.301 | 0.481 | 2.01 | 1.01 | 0.301 | 0.481 |
| 18:3 | 32.5 | 32.0 | 0.396 | 0.857 | 5.28 | 5.19 | 3.4 | 0.626 |
| 20:0 | 0.78 | 0.60 | 0.025 | 0.207 | 1.25 | 1.21 | 0.061 | 0.361 |
| 20:1 (trans11, cis12) | ND | ND | – | – | 2.88 | 0.421 | 1.52 | 0.541 |
| 20:1 (cis9, trans11) | ND | ND | – | – | 0.26 | 0.003 | 0.003 | 0.003 |
| 20:2 | 8.27 | 8.27 | 0.409 | 0.268 | 8.27 | 8.27 | 0.409 | 0.268 |
| 20:3 | 0.384 | 0.384 | 0.112 | 0.03 | 0.384 | 0.384 | 0.112 | 0.03 |
| Total fatty acids | 80.2 | 82.7 | 4.09 | 0.384 | 112.9 | 117.0 | 7.98 | 0.268 |

**ND**, not detected; **CLA**, conjugated linoleic acid.

$^*$ (Protozoal flow/duodenal flow) £ 100.

† Values may be overestimated as a consequence of other isomers which co-elute on the GC column.
Protozoa contribution to duodenal flow

Fatty acid composition of rumen protozoa

Determining the fatty acid composition of rumen protozoa is complicated by the technical difficulties involved in obtaining protozoal preparations uncontaminated by bacteria and feed particles and because of the variation in protozoal populations between individual animals and experimental diets.

In the present study we used 16S rDNA quantified by real-time PCR as a marker of bacterial contamination of our protozoal samples during the successive washes and filtrations.

The levels of contamination achieved (7-4 %) during protozoal isolation compares favourably with values obtained by others (23 %; Volden et al. 1999). Thus we conclude that our washed protozoal standards contained only bacterial material within or strongly bound to the surface of the protozoal cells which would in effect leave the rumen as part of the protozoal pool. Additionally, it is well established that the diet fed to animals determines the profile of the major long-chain fatty acids in ruminal micro-organisms, so that the C_{16}C_{18} ratio in the diet will be reflected in the ruminal digesta (Cook et al. 1972). Thus, in terms of the C_{16}C_{18} ratio and the extent to which unsaturated C_{18} fatty acids have been hydrogenated to 18:0, our measurements found a higher C_{16}C_{16} ratio than previously reported (Devillard et al. 2004), possibly reflecting the dietary fatty acid profile found in the experimental silage. However, not only the fatty acid content of the diet drives the fatty acid profile in the protozoal fraction. Our DGGE results show different protozoal diversity present in the rumen of different animals fed the same diet. Chalupa & Kutches (1968) found no hydrogenation of [1-14C]linoleic and [1-14C]oleic acids by Holotrich protozoa cultivated in vitro, while in the same experiment they observed that Entodiniomorphid protozoa were capable of hydrogenating the same fatty acids. Therefore, difference in the protozoal distribution might also explain different fatty acid profiles. In the present study, Holotrich protozoa were only found in those animals fed the HS diet, possibly reflecting the preference of these protozoa for the use of soluble carbohydrates (Williams & Coleman, 1992). It is possible that the different proportions of protozoal types observed between the two experimental diets via the presence or absence of Holotrichs could help explain the different fatty acid composition of the protozoal population isolated from animals fed the two experimental diets used in the present study compared with the study of Devillard et al. (2004).

In agreement with the present study, Devillard et al. (2004) observed higher concentrations of VA than CLA (c9,t11 isomer) in the ciliates. They also reported a higher VA content in protozoa than in bacteria.

Devillard et al. (2004) did not speculate on the reasons for the high VA content of rumen protozoa. One theoretical mechanism could be the de novo synthesis of C_{18} long-chain fatty acids and subsequent desaturation to 18:1. Emmanuel (1974) observed net incorporation of radioactive SCFA into octadecanoic acid with the double bounds in the Δ^{11} position compared with oleic acid (Δ^{9}), suggesting the above mechanism may operate. However, the possibility of rumen protozoa producing 18:2 iso- mers via desaturation has not yet been examined.

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). Given that protozoa make up approximately half the rumen biomass (Jouany, 1995), this suggests that ciliate protozoa represent a major pool of unsaturated fatty acids in the rumen.

The capacity of protozoa to store fatty acids is unknown and it is believed to differ between different types of protozoa. The presence of particulate matter and its capacity to bind NEFA should affect the maximal fatty acid concentration achieved in the in vitro media (Harfoot et al. 1973; Girard & Hawke, 1978). It might be speculated that the capacity of protozoa to store fatty acids depends on the combination of factors: rate of incorporation of NEFA to cellular lipids; presence of particulate matter; symbiotic relationship with rumen bacteria; rate of hydrogenation of unsaturated fatty acids. These factors are of crucial importance, as unsaturated fatty acids appear to be partially protected from biohydrogenation by their incorporation into microbial phospholipids.

Protozoal contribution to vaccenic acid and conjugated linoleic acid supply from the rumen

The present study is the first to attempt to quantify the contribution of protozoa to the fatty acid flow reaching the duodenum. We conclude that protozoa contribute to almost 40 % of the c9,t11-CLA and 30 to 36 % of the t10,c9-CLA isomer flow and, more importantly, that 40 % of the VA leaving the rumen does so in ciliate protozoa. Given that the two silage diets used were very similar in chemical composition, it would be interesting to confirm the protozoa contributions on diets high in concentrate that increased the proportion of Holotrichs in the rumen. It is interesting to speculate on the effect that the presence or absence of protozoa in the rumen would have on the CLA content of ruminant products. On initial observation, the present results suggest that defaunation would reduce CLA concentrations; however, other studies on the effect of defaunation on lipid metabolism in the rumen are
contradictory. While rumen protozoa are known to hydrogenate lipids, the biohydrogenation activity of rumen contents was only slightly reduced by defaunation (Dawson & Kempt, 1969). 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 irreplaceable source of 18:1 supply to the small intestine. More recently, Jouany & Lassalas (2004) failed to find any significant difference in fatty acid metabolism and CLA synthesis between rumen fluid collected from faunated and non-faunated animals. However, they did find significant differences between short- (2 months) and long-term (12 months) defaunated animals suggesting that different bacterial populations developed in the two different defaunated animals. Ozutsumi et al. (2005) observed significantly different bacterial communities in faunated and defaunated cattle by using 16S rDNA clone libraries, which suggests that the comparison between ciliate-free and faunated animals should be interpreted carefully, as bacterial metabolic activities would also change.

In conclusion, a significant proportion of the CLA and VA leaving the rumen do so in protozoal cells. Several reasons could explain the high CLA and VA content of rumen protozoa, although none have been proven yet. Given that protozoa populations vary considerably even between animals receiving the same diet and that they can be affected by certain additives it would seem necessary to re-investigate the role of protozoa in fatty acid biohydrogenation in the rumen.

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