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Description of development of rumen ecosystem by PCR assay in milk-fed, weaned and finished lambs in an intensive fattening system

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Introduction

At birth, the reticulo-rumen only constitutes approximately 25–35% of the ruminant stomach, but this portion grows rapidly to constitute 70% of the total digestive system in the adult ruminant. Rumen development occurs in three phases: a non-ruminant phase, from birth to 3 weeks; a transitional phase, at 3–8 weeks; and an adult stage, beyond 8 weeks (Lane et al., 2000). Rumen development is modulated by the access to a solid diet, microbial colonization and their interactions.

Lambs in an intensive fattening system are exposed to sudden dietary changes and rumen microbes need

Keywords
rumen development, bacteria, protozoa, DGGE, quantitative PCR

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Summary

This study examined the reticulo-rumen characteristics of the microbial community and its fermentative characteristics in milk-fed, at weaning and finished lambs in a conventional fattening system. Five lambs were assigned to each of three groups: milk-fed lambs slaughtered at 30 days (T30), weaned lambs slaughtered at 45 days (T45) and ‘finished lambs’ slaughtered at 90 days (T90). At slaughter, rumen size, fermentation parameters (pH, volatile fatty acids and microbial enzyme activity) and protozoal counts were recorded. Quantitative PCR was used to quantify the genes encoding 16S and 18S ribosomal DNA of the rumen bacterial and protozoal populations, respectively, and the sequential colonization of the rumen by cellulolytic (Ruminococcus albus, Ruminococcus flavefaciens) and amylolytic (Prevotella ruminicola, Streptococcus bovis) bacteria, and protozoa (Entodinium sp.). Denaturing gradient gel electrophoresis was used to study the development of rumen microbiota biodiversity. Intake of solid food before weaning caused a significant increase in rumen weight (p < 0.0001) and bacterial DNA (p < 0.05) and volatile fatty acid analysis concentration (p < 0.01), whereas pH declined. In milk-fed lambs, cellulolytic bacteria were evident after 30 days. Thereafter, in the 45-day and 90-day groups, the proportions of R. flavefaciens decreased and R. albus increased. Amylolytic bacteria were present in milk-fed lambs; the proportion of P. ruminicola increased in fattening lambs and S. bovis was the least abundant species. Protozoal concentrations were irregular; milk-fed lambs had a significant number of protozoa species from Entodinium and subfamily Isotrichiidae, but they disappeared at weaning. Lamb rumen were refaunated in some individuals at 90 days (Entodinium and subfamily Diplodiniinae spp.), although individual concentrations were variable.

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to adapt to changes in their environment particularly when a high-concentrate diet replaces milk as the main component of the diet (Zitnan et al., 1993; Beharke et al., 1998). In most studies in fattening lambs, in vitro cultures are used to identify target species, and populations isolated from adults and young lambs are compared (Fonty et al., 1983); however, in vitro cultures fail to identify organisms that cannot be grown in vitro, and comparisons between age classes fail to describe the changes of the microbial ecosystem at critical phases in development such as at weaning.

Molecular techniques are useful for detecting and describing microbial communities, e.g. DNA fingerprint profiles from denaturing gradient gel electrophoresis (DGGE) to study complex microbial communities (Ferrera et al., 2004) or quantitative PCR to measure specific microbial groups (Tajima et al., 2001).

This study examined the rumen microbiota and fermentative activity in lambs fed under an intensive system at the three critical stages of development: milk feeding (T30), at weaning (T45) and the fattening period (T90). Bacterial and protozoal profiling was assessed by DGGE and the population of selected bacterial species was monitored by quantitative PCR.

Materials and methods

Animals and experimental design

The study used 15 Rasa Aragonesa singleton lambs (3.78 ± 0.24 kg live weight) from the same weekly cohort. Animals were housed in individual pens with straw bedding and access to fresh water. At the beginning of the experiment, the lambs were injected intramuscularly with a Selenium and vitamin complex (A, D and E; VITASEL®, Laboratorios Ovejero, S.A., León, Spain). At 24 h after birth, lambs were separated from their mothers but they were allowed to suckle three times daily for approximately 10 min (at 9:00, 14:00 and 20:00 hours). Mothers presented a daily intake of fresh matter (FM) of 700 g of chopped alfalfa hay and 700 g of ground concentrate (60% barley grain, 20% soya, 15% maize, 3% wheat bran, 2% minerals and vitamin supplement) divided into two meals (9:10 and 20:10 hours), along with barley straw ad libitum. To quantify milk yield, lambs were weighed daily before and after suckling. Lambs were weighed and digesta content measured. Rumen digesta was filtered through two layers of cheesecloth, pH was recorded and three samples were collected for measurements of enzyme activity, volatile fatty acid analysis (VFA) and protozoal counts. The 4-ml samples collected for the analysis of enzyme activity were frozen in liquid N and stored at −80 °C. Samples for VFA analysis (4 ml) were acidified with 1 ml of 0.5 m H3PO4 including 50 mm 4-methylvalerate as internal standard and stored at −20 °C. Samples for protozoal counts (5 ml) were diluted 1:1 in 18.5% formaldehyde and stored at room temperature until processed.

Table 1 Chemical composition of milk, compound feed and straw

<table>
<thead>
<tr>
<th>Composition (g/kg)</th>
<th>Milk</th>
<th>Concentrate</th>
<th>Straw</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>190</td>
<td>932</td>
<td>940</td>
</tr>
<tr>
<td>OM</td>
<td>942</td>
<td>924</td>
<td>948</td>
</tr>
<tr>
<td>CP</td>
<td>305</td>
<td>201</td>
<td>29</td>
</tr>
<tr>
<td>NDF</td>
<td>95</td>
<td>695</td>
<td></td>
</tr>
<tr>
<td>ADF</td>
<td>81</td>
<td>509</td>
<td></td>
</tr>
<tr>
<td>ADL</td>
<td>17</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>384</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>Lactose</td>
<td>253</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Jes Cantos, Spain; composition in Table 1) and barley straw, until they were slaughtered at 45 days of age (live weight, 15.23 ± 0.57 kg). The T90 group was managed in the same manner as the T45 group, weaned at 45 days and permitted ad libitum access to compound food and straw until 90 days, when they were slaughtered (live weight, 23.55 ± 1.15 kg).

Experimental procedures, collection and preparation of samples

The experimental protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza. On the day of slaughter, lambs were anaesthetized 2 h after feeding by an intramuscular injection of xilacine (Xilagesic 2%, 0.3 mg Xylazine per kg live weight) and an intravenous dose of Thiopental (Tiobarbital Braun®; B. Braun Medical, S.A. Barcelona, Spain; 10 mg/kg live weigh). Lambs were slaughtered by exsanguination before the reticulum-rumen was excised, isolated, weighed and digesta content measured.

Rumen contents were collected and homogenized, and duplicate 0.2 g samples were placed in liquid nitrogen and stored at −80 °C until DNA extraction. Rumen digesta was filtered through two layers of cheesecloth, pH was recorded and three samples were collected for measurements of enzyme activity, volatile fatty acid analysis (VFA) and protozoal counts. The 4-ml samples collected for the analysis of enzyme activity were frozen in liquid N and stored at −80 °C until analysis. Samples for VFA analysis (4 ml) were acidified with 1 ml of 0.5 m H3PO4 including 50 mm 4-methylvalerate as internal standard and stored at −20 °C. Samples for protozoal counts (5 ml) were diluted 1:1 in 18.5% formaldehyde and stored at room temperature until processed.
To maximize the recovery of loose particulate-associated micro-organisms, the solid fraction was washed with approximately 500 ml of Coleman Buffer (Williams and Coleman, 1992) at 39 °C and added to the liquid. To extract bacteria, a fraction of the filtrate was used in a differential centrifugation procedure (Cecava et al., 1990) and two 0.2 g samples of the bacterial pellet were collected and frozen in liquid N until it was used in the quantitative PCR analyses. A second fraction of the filtrate was used in the isolation of protozoa (Coleman and Sandford, 1979; Sylvester et al., 2004) and two 0.2 g samples of the protozoal extract were collected to obtain protozoal DNA and used as a standard in the quantitative PCR.

**DNA extraction and quantification, DGGE and real-time PCR analyses**

DNA was extracted from frozen samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Carwley, West Sussex, UK). The only modification to the manufacturer’s protocol was to incubate at 95 °C for 5 min to ensure the lysis of micro-organisms by the stool lysis buffer. The QIAamp® procedure provides efficient homogeneous cell lysis and quality DNA (Li et al., 2003). The concentration and purity of the extracted DNA were assessed spectrophotometrically by calculating $A_{260}/A_{280}$ ratios.

In the analysis of microbial biodiversity, the set of ciliate protozoa-specific PCR primers that were designed to be specific to the rumen ciliates (Regensbogenova et al., 2004) included forward (1055F) 5'-GGTGGTGTGATGGCCG-3', reverse (1400R) 5'-AATTGCAAAATCTATCC-3' with a 45-nucleotide GC-clamp attached to the 5' end of the reverse primer, and approximately 300 bp of the 18S rDNA gene was amplified. The amplification conditions were 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 57 °C for 30 s and 72 °C for 1 min, and a cycle of 72 °C for 10 min. The set of rumen bacteria-specific 16S rDNA primers (Nubel et al., 1996) included forward (342F) 5'-ACGGGGGCTACGGGAGGCAGC-3' and reverse (1400R) 5'-ATTACCAGCCGTCGCTGG-3'. Approximately, 200 pb of the 16S rRNA gene was amplified. PCR was performed as follows: 94 °C for 4 min, 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final step of 72 °C for 5 min. In a volume of 50 µl with 1.5 mM of MgCl2, PCR was performed in a thermocycler (My Cycler® Bio-Rad Laboratories, Hemel Hempstead, Herts, UK), and amplicon sizes were identified and confirmed by electrophoresis on a 2.5% agarose gel. PCR products were concentrated using a Speed-Vac® (RVC 2-25, CHRIST Freeze Deuyers, Osterode am Harz, Germany) at 30 °C for 30 min.

Denaturing gradient gel electrophoresis was performed on a C.B.S. Scientific® system (Del Mar, CA, USA). Gels contained a 25–45% and 40–60% denaturing gradient of urea formamide in 8% polyacrylamide for protozoal and bacterial gels respectively. Gels were run at 80 V at 60 °C for 16 h. DNA was visualized by silver staining (Amersham Biosciences, Uppsala, Sweden). Gels were scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad Laboratories). Scanned DGGE images were analysed using Quantity One® Software (Bio-Rad Laboratories) by scoring for the presence or absence of bands at various positions in each lane. The DGGE profiles of samples from the same gel were compared using the similarity trees derived by the NEIGHBOUR program (PHYLIP Phylogeny Inference Package version 3.6a; F. Felsenstein, University of Washington, Seattle, WA, USA). The Shannon Index, that is used to measure the diversity in categorical data, was calculated following the equation given by Magurran (2003): $H' = -\Sigma (p_i \ln p_i)$, where the proportion of single bands ($p_i$) relative to the total number of bands ($p_i$) is calculated and multiplied by the natural logarithm of this proportion ($\ln p_i$). The product is summed across species and multiplied by $-1$. Quantitative PCR primers were used to detect the targeted species and reaction was performed using an ABI PRISM® 7000 Sequence Detection System using specific primers for total bacteria (Maeda et al., 2003), total protozoa (Sylvester et al., 2004), Entodinium sp. (Skillman et al., 2006), Ruminococcus albus (Koike and Kobayashi, 2001) and R. flavefaciens, Prevotella ruminicola and Streptococcus bovis (Tajima et al., 2001). Extracted DNA was added to an amplification reaction (25 µl total volume) that contained 25 pmol of each primer, 12.5 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Barcelona, Spain) and 0.5 µl of ROX Reference Dye (Invitrogen™). The cycling conditions for the quantification of protozoa were 95 °C for 10 min and 40 cycles of 94 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s, and those for bacteria were 95 °C for 10 min and 30 cycles of 95 °C for 15 s, 61 °C for 30 s and 72 °C for 30 s. The quantification rumen micro-organisms used cycling conditions of 95 °C for 10 min and 45 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s.

Two replicates of each DNA sample and a non-template negative control (sterile, distilled water) were loaded onto each plate and screened for...
possible contamination and dimer formation. To generate the calibration curves for the quantification of total rumen protozoa and rumen bacteria, after the spectrophotometric quantification of the DNA, the DNA extracted from the washed protozoal solution and the centrifuged bacterial pellet were diluted (from $10^{-1}$ to $10^{-6}$) to determine the linear relationships between the logarithms of the known DNA quantities of each standard (ng/µl) and their respective threshold cycles ($C_T$). Those standards allowed for the quantification of total rumen bacteria and protozoa; however, a $\Delta C_T$ relative quantification method (Livak and Schmittgen, 2001) was used to quantify species with respect to total bacteria ($\Delta C_T = C_T$ species $- C_T$ total bacteria). All analyses were performed using ABI Prism 7000 Sequence Detection System software (Applied Biosystems, Carlsbad, CA, USA). In every reaction, the efficiency of the quantitative PCR amplification was checked and only the reactions that had an amplification efficiency >85% were accepted and included in the analysis. Cross-contamination of bacterial and protozoa DNA in microbial extracts was quantified by quantitative PCR and subtracted from the corresponding spectrophotometrically quantified DNA.

**Chemical analysis**

Milk, concentrate and straw were sampled and their dry matter (DM) were determined by drying at 60 °C to a constant weight and to quantify organic matter ashed at 550 °C for 8 h in a muffle furnace. To calculate total N, we used a Kjeltec Analyser Unit 2300 (Foss Tecator AB, Höganäs, Sweden), Foss Tecator with Selenium as a catalyst, following the instructions of the AOAC (2004). The amounts of neutral detergent fibre, acid detergent fibre and acid detergent lignin were quantified using an ANKOM 220 Fibre Analyser equipment (Ankom Technology, Macedon, NY, USA), following Van Soest et al. (1991) and the fibre concentration was given including ash fraction.

To quantify the VFA concentrations, we injected 0.3 µl of filtered sample and 0.3 µl of 4-methylvalerate (1%) as an internal standard (Jouany, 1982) in a Hewett Packard gas liquid chromatography (Hewett Packard 5890, Ramsey, Minnesota, USA) at 185 °C in a Teknokroma column (Teknokroma, S. Coop. C. Ltda, Sant Cugat del Valles, Spain) with a constant flow (25 ml/min) of N₂. The enzyme fractions were extracted from 1 g FM samples following the procedure of Nossal and Heppel (1966) based on a 3-h incubation at 37 °C with lysozyme (5 mg/ml of buffer). Then, the samples were centrifuged at 26 000 g for 15 min at 4 °C to recover the enzymatic fraction, the protein content of which was quantified using the Bradford Protein Assay Procedure. Polysaccharidase activity of the enzyme extract against carboxymethyl cellulose [Sigma, Sigma-Aldrich, Saint Louis, MO, USA, carboxymethylcellulase (CMCase activity)] or xylan from oat spelts [Sigma-Aldrich, Xylanase activity] as substrates was determined by incubating in duplicate 0.45 ml of a 1 mg/ml substrate solution (pH 6.7) with 50 µl enzyme extract at 39 °C for 30 min. Released reducing sugars were measured according to the Nelson–Somogyi method (Ashwell, 1957) and expressed per weight unit of extract. Finally, absorbance at 600 nm was determined in a 96-well spectrophotometer (Green et al., 1989). The differentiation of genera and counts of protozoa were performed using an optical microscope at 100x magnification after staining with brilliant green (Dehority, 1993) and using Lee et al. (1985) classification protocol.

**Calculations and statistical analysis**

The efficiency of DNA extraction was assumed to be 100%, and to correct for differences in dilutions, a dilution factor (1.4 + sample volume/sample weight) was applied. Statistical inferences were derived from one-way ANOVA using the SAS statistical package, version 8 (SAS Institute Inc., Cary, NC, USA), with diet-age as the main factor and lambs within the same group as replicates.

**Results**

**Feed intake (DM)**

In milk-fed lambs (T30), after 10 days, ingestion rates were constant (0.811 ± 0.037 l/day). From day 15, compound feed and straw were available to the T45 group but, until day 25, intake of solid food was not significant. Subsequently, lambs gradually increased their consumption of compound feed and straw (up to 350 ± 33 g DM/day and 28 ± 9 g DM/day, respectively) and decreased their consumption of milk (0.753 ± 0.050 l/day). After 5–6 weeks of age, lambs had no difficulty in adapting to solid food, and rumination was evident. In the T90 group, the intake of compound feed increased linearly just after weaning and levelled off at 60–70 day, when total DM intake (DMI) was 1.022 ± 0.064 g DM/day. From weaning onwards, average consumption of straw was 0.10 ± 0.01 of total DMI. During their last 7 days, average daily weight gain was 129, 311 and 230 g/day for lambs in the T30, T45 and T90 groups respectively.
Table 2 Rumen physical (weight), chemical (pH and VFA) characteristics and enzyme activity of lambs slaughtered at 30, 45 and 90 days

<table>
<thead>
<tr>
<th></th>
<th>T30</th>
<th>T45</th>
<th>T90</th>
<th>SE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live weight (kg)</td>
<td>8.39c</td>
<td>15.23b</td>
<td>23.55a</td>
<td>0.76</td>
<td>***</td>
</tr>
<tr>
<td>Rumen complete (kg)</td>
<td>0.28c</td>
<td>1.08b</td>
<td>2.25a</td>
<td>0.12</td>
<td>***</td>
</tr>
<tr>
<td>Content (kg)</td>
<td>0.21c</td>
<td>0.80b</td>
<td>1.72a</td>
<td>0.11</td>
<td>***</td>
</tr>
<tr>
<td>Viscera (kg)</td>
<td>0.06c</td>
<td>0.28b</td>
<td>0.53a</td>
<td>0.02</td>
<td>***</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.48b</td>
<td>6.41b</td>
<td>6.12b</td>
<td>0.11</td>
<td>ns</td>
</tr>
<tr>
<td>Total VFA (mM)</td>
<td>22.6b</td>
<td>130.9a</td>
<td>160.7a</td>
<td>11.7</td>
<td>***</td>
</tr>
<tr>
<td>Relative abundance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>84.3a</td>
<td>71.8b</td>
<td>69.1b</td>
<td>1.8</td>
<td>***</td>
</tr>
<tr>
<td>Propionate</td>
<td>12.4b</td>
<td>21.2a</td>
<td>25.1a</td>
<td>1.7</td>
<td>***</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.7b</td>
<td>4.8 a</td>
<td>4.0a</td>
<td>0.7</td>
<td>**</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>1.3a</td>
<td>0.4b</td>
<td>0.4b</td>
<td>0.1</td>
<td>***</td>
</tr>
<tr>
<td>Valerate</td>
<td>0.6b</td>
<td>1.5a</td>
<td>1.0a</td>
<td>0.2</td>
<td>***</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>1.4a</td>
<td>0.3b</td>
<td>0.4b</td>
<td>0.1</td>
<td>***</td>
</tr>
<tr>
<td>Enzyme total activity (µmol sugar released/g FM per min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rumen liquor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxymethylcellulase</td>
<td>0.12b</td>
<td>3.72a</td>
<td>1.13b</td>
<td>0.52</td>
<td>**</td>
</tr>
<tr>
<td>Xylanase</td>
<td>0.45</td>
<td>10.02</td>
<td>8.11</td>
<td>3.19</td>
<td>ns</td>
</tr>
<tr>
<td>Amylase</td>
<td>0.43</td>
<td>2.86</td>
<td>1.87</td>
<td>0.88</td>
<td>ns</td>
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<tr>
<td>Particulate matter</td>
<td></td>
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<tr>
<td>Carboxymethylcellulase</td>
<td>0.00b</td>
<td>1.44a</td>
<td>0.32ab</td>
<td>0.37</td>
<td>*</td>
</tr>
<tr>
<td>Xylanase</td>
<td>3.19</td>
<td>3.59</td>
<td>0.88</td>
<td>1.20</td>
<td>ns</td>
</tr>
<tr>
<td>Amylase</td>
<td>0.48</td>
<td>1.54</td>
<td>0.00</td>
<td>0.41</td>
<td>ns</td>
</tr>
</tbody>
</table>

FM, fresh matter; VFA, volatile fatty acids; ns, not significant.
*p < 0.05; **p < 0.01; ***p < 0.001.

Rumen development: size, chemical characteristics and enzyme activity

Average rumen weight was the lowest in the T30 group (3.3% of body weight) and the highest in the T90 group (9.5% of body weight) (Table 2). Viscera growth rate was the highest before weaning (14 g/day) and declined steadily (5.5 g/day).

Total VFA concentrations increased widely from 22.6 mM in milk-fed lambs to 130.9 and 160.7 mM in weaning-age and fattening lambs respectively (p < 0.001). The proportion of acetate decreased from 84.2% to 69.1% (p < 0.001). Relative abundance of isobutyrate and isovalerate also decreased. Contrarily, propionate (from 12.4% to 25.1%; p < 0.001), butyrate (from 0.7% to 4.0%; p < 0.01) and valerate (from 0.0% to 1.0%; p < 0.001) increased in their concentration in weaning age and fattening lambs compared with milk-fed animals. Changes in rumen pH paralleled changes in VFA concentrations and tended (p = 0.073) to decrease over time (from 6.48 to 6.12 in the T30 and T90 groups respectively).

Bradford analysis reported a mean value of 2.58, 4.30 and 4.20 for liquid and 2.50, 3.58 and 3.08 mg of protein/ml for particulate rumen samples from T30, T40 and T90 respectively. However, in both the liquid and particulate rumen digesta, enzyme activities (µmol sugar released/g FM min) had high residual variability (Table 2). In the rumen liquor, the activity of CMCase, xylanase and amylase was the lowest in milk-fed lambs, although only CMCase activity differed significantly between groups (0.12 vs. 3.72 and 1.13 respectively; p < 0.01). In the particulate matter, no trends were evident in enzyme activity.

DGGE banding patterns of rumen microbiota

In the bacteria dendrogram, the lambs clustered into one of three groups (Fig. 1a), T30 lambs clustered together, but the samples from the T45 and T90 lambs were clustered separately. Three of the T90 lambs were clustered with the T45 lambs, which indicated similar bacterial biodiversity. The other two T90 lambs formed a distinct cluster. Among the bacteria, the Shannon Index increased gradually as the rumen developed (2.92, 3.11 and 3.33 units in the T30, T45 and T90 respectively), but the differences were not statistically significant (Table 3).

In the protozoal diversity, the distribution of hamming distances was similar to that observed among bacteria, with the lambs in the T30 group distinct from the others (Fig. 1b). Lambs in the T45 group and three of five lambs in the T90 group were clustered together. The samples of the other two lambs in the T90 groups were clustered separately and exhibited the highest biodiversity. The Shannon Index of diversity did not differ significantly among the T30 (2.56), T45 (2.15) and T90 (2.79) groups.

Quantification of protozoa based on optical counts

Milk-fed lambs showed a significant concentration of protozoa in the rumen (2.5 x 10⁵ cells/ml) but during weaning, protozoa were virtually absent. After weaning, the number of protozoa increased to the highest concentrations (15.1 x 10⁵ cells/ml in the T90 group), but only in two of the five lambs (Table 3). In all of the groups, Entodinium sp. predominated (up to 90% of the count). Holotrich protozoa (Isotrichidae) were detected in milk-fed lambs (only 6% of the protozoa and 1.6 x 10⁴ cells/ml), and Diplodiniinae spp. were a significant proportion in the T90 group (11% of the total protozoa and 1.7 x 10⁵ cells/ml).

Quantification of microbes by quantitative PCR

Rumen bacterial and protozoal DNA concentration were measured by quantitative PCR and are...
presented in Table 3. For total protozoa, total bacteria, *R. albus*, *R. flavefaciens*, *P. ruminicola* and *Entodinium sp.*, the amplification efficiencies $(E_f = 10^{-1/\text{slope}})$ were 1.92, 1.93, 1.92, 1.97, 2.01 and 1.95 respectively. Crossover contamination in the protozoal and bacterial DNA standards was quantified by crossing primers against samples of protozoal and bacterial extracts. The contamination of bacterial DNA by protozoal DNA was negligible (<0.02 ± 0.01), but protozoal extracts contained a significant concentration of bacterial DNA (0.39 ± 0.07). The concentration (µg DNA/g FM) of bacterial DNA increased from 245 to 317, but decreased to 272 for T30, T45 and T90 lambs respectively.

Samples of rumen contents were taken individually and analysed separately in duplicate; however, a high variability in the molecular procedures was observed in each group. For the protozoa, the PCR signal was lower than it was in the bacteria, and the changes in the concentration of protozoal DNA (µg DNA/g FM) that were identified by the quantitative PCR were similar to those that were detected by optical counts (cells/ml) (Fig. 2). Milk-fed lambs (T30) had a high DNA concentration (27 µg/g FM), but the signal almost disappeared during weaning before a significant concentration was detected in the samples from the T90 lambs (90 µg DNA/g FM), although individual concentrations were variable. Two had a high protozoal DNA concentration (225 µg DNA/g FM), but the others in the group contained a negligible amount of fauna (0.41 µg DNA/g FM).

Lambs in all of the groups harboured cellulolytic bacteria (*R. albus* and *R. flavefaciens*), but they were distributed heterogeneously. The concentrations of *R. albus* increased (lower $\Delta C_T$ value) with an increase in the consumption of solid food, but the concentrations of *R. flavefaciens* were the highest in the lambs in the T30 group ($p < 0.05$) (Table 3). *Prevotella ruminicola* was the most abundant species and its relative abundance was higher in T90 group than in the other groups ($\Delta C_T = 8.74$ vs. 12.56). *Streptococcus bovis* was the least abundant bacteria, and its highest concentrations were in the milk-fed lambs. The

![Fig. 1 Rumen bacterial (a) and protozoal (b) diversity obtained by denaturing gradient gel electrophoresis fingerprinting.](image-url)
distribution of gene copies in Entodinium sp. was similar to that of protozoa species overall, and levels were the highest in the T90 group ($\Delta C_T = 1.28$), lower in the T30 group ($\Delta C_T = 2.72$) and no PCR signal was detected in weaning lambs (T45).

**Discussion**

The aim of this study was to monitor rumen microbial populations at three stages in the growth of lambs: during milk feeding, at weaning and at the end of the fattening period, when they are eating high-concentrate diets. Rumen development is widely influenced by the management, intensity, weaning age and weaning methods. For those reasons, lambs in a conventional intensive fattening system were chosen as a model for an analysis of the three specific phases of development, assuming that the effect of age would be always confused with dietary changes. In fact, it is impractical to keep milk feeding throughout the entire fattening period or to feed newborn lamb with solid food.

Variations in the abundance of several selected microbial species, four bacteria, R. albus, R. flavefaciens, P. ruminicola and S. bovis, and one protozoal genera, Entodinium were studied through the rumen evolution and target species were chosen attending to their relevance in the mature rumen ecosystem. Its evolution in lactating and weaned lambs towards the mature ecosystem was a relevant purpose in the present assay. Moreover, changes in biodiversity of the rumen microbiota were analysed using a DGGE approach, assuming the limitations imposed for such experimental approach.

In reference of rumen protozoal quantification, optical counts substantiated the results from the quantitative PCR, with either the general or the specific primers, the relationship between the results of the quantitative PCR and the optical counts was very close ($R^2 = 0.9$) (Fig. 2).

**Pre-weaning period**

In our study, the development of the reticulo-rumen in lambs was similar to that observed elsewhere (Swan and Groenewald, 2000), and T30 lambs were in a typically non-ruminant phase. During suckling, the oesophageal groove closes, which shunts liquid past the rumen and into the abomasum; however, the oesophageal groove does not close completely and some liquid leaks into the reticulo-rumen. Residual milk allows for the establishment of an incipient microbial population (Lane et al., 2000).

The reticulo-rumen is colonized in a characteristic succession (Savage, 1977) from transient to indigenous morph types that are common to communities found in adults; however, our results showed that
T30 lambs had significant concentrations of the reticulo-rumen species that are common in adult sheep such as *R. albus*, *R. flavefaciens*, *P. ruminantium* and *S. bovis*. Both *R. albus* and *R. flavefaciens* are cellulolytic species and it is difficult to know which substrate might have been available in the milk-fed lambs. In other studies, cellulolytic bacteria appeared in lambs (Fonty et al., 1987) and calves at 1–3 weeks of age (Minato et al., 1992). In our study, however, the DGGE analysis showed that reticulo-rumen biodiversity was the lowest at 30 days. Moreover, the microbial populations in milk-fed lambs were clustered together and then differentiated from those in older lambs. Moreover, it is assumed that the acidic conditions in the rumen under milk feeding prevent the establishment of a protozoal population but, in our study, protozoa were present in milk-fed lambs, although in lower concentrations than those found in two of the T90 lambs. In pre-weaning and 90-day lambs, the DNA ratio between protozoa and total microbes (µg DNA/g FM, Table 3) varied from 0.10 to 0.24. Although we unknown other similar studies with milk-fed lambs, Minato et al. (1992) in calves reared with milk, protozoa colonized the rumen at approximately 8 weeks, when the animals were at a stage of maturity that was similar to the lambs in our study.

The sequence in which protozoal species appeared was similar to observed elsewhere. When the diet was rich in soluble carbohydrates (e.g. milk), *Entodinium sp.* were the most common ciliates in association with *Holotrichs* and they are the initial species in reticulo-rumen faunation (15–20 days) (Fonty et al., 1983).

**Transitional phase**

The relative growth coefficients of the viscera (reticulo-rumen/total body weight ratio) of the T30 (2.31) and T45 lambs (2.24) were higher than the coefficients calculated after weaning (1.36 and 1.71, respectively) for reticulo-rumen and contents, respectively, and it would confirm the impact of the solid food contact with reticulo-rumen development (Zitnan et al., 1993). Rumen bacterial concentrations derived from the quantitative PCR analysis (µg DNA/g FM) increased significantly with lamb age, and the bacterial dendrograms generated from the DGGE fingerprint analysis indicated that the samples from the T45 lambs were clustered with those from the T90 lambs, and both differed from those from the T30. However, differences in biodiversity indices did not reflect the changes that occurred in the populations in the T30 and the T45 lambs.

The proportions (ΔCt) of *R. albus*, *P. ruminicola* and *S. bovis* did not vary significantly over time with lamb age, and the proportion of *R. flavefaciens* decreased significantly in at-weaning lambs. The cellulolytic *R. flavefaciens* can survive and grow in a non-cellulolytic habitat because it can establish symbiotic relationships with other bacterial species (Bau-chop and Mountfort, 1981). In another study, *R. albus* and *R. flavefaciens* were 3–5% of the total bacterial 16S rDNA in lambs that were fed alfalfa hay and milk (Krause et al., 1999) but, in our study, in the weaning age group, these micro-organisms were approximately three magnitudes less abundant, possibly, because of the low proportion of forage in the diet (<5%).

Lambs slaughtered at 45 days were almost free of protozoa. Defaunation might be associated with the weaning process (Petkov and Enev, 1979; Fonty et al., 1988) and was evident when lambs were given a mixed diet, although defaunation was mitigated when alfalfa hay was provided as the sole diet (Fonty et al., 1983; Franzolin and Dehority, 1996).

**Post-weaning period**

In our study, the consumption of solid food increased ruminal activity and VFA concentrations (Table 2) and pH declined. The increase in concentrate intake reduced the acetate/propionate ratio, which has also been described in 5–11 week lambs (Zitnan et al., 1993) and calves (Sahoo et al., 2005). Changes in rumen fermentation allowed the re-faunation of the reticulo-rumen, which was highly variable among individual lambs. Two lambs in the 90-day group had protozoal communities and concentration that were similar to those found in adult animals (10⁶ cells/ml), but the other three lambs remained almost free of protozoa. It remains unclear why lambs that were kept under identical experimental conditions, but housed individually, exhibited such variability. Franzolin and Dehority (1996) observed the sporadic appearance of rumen protozoa in steers fed high concentrate diets at 1- or 2-week intervals. Among faunated lambs, *Entodinium sp.* were the dominant species followed by *Diplodinium sp.* and the distribution of species after weaning was similar to reported elsewhere (Fonty et al., 1983).

The quantitative PCR did not detect significant changes in the distribution of bacterial DNA, either...
in absolute terms (µg bacterial DNA/g FM) or relative to the bacterial species (16S rDNA copies) evaluated in this study. Apparently, the amylolytic and proteolytic Prevotella rumicola was the most abundant micro-organism (ΔC_T = 8.74) (Reilly et al., 2002), while the abundance of the cellulolytic bacteria R. albus or R. flavefaciens was within the range (0.16–3.0%) reported elsewhere in adult ruminants (Koike and Kobayashi, 2001; Michalet-Doreau et al., 2002). Although R. flavefaciens is usually more abundant than R. albus in animals receiving forage diets (Michalet-Doreau et al., 2002), differences may decrease with concentrate diets (Koike and Kobayashi, 2001) and greater ability of the former to ferment other substrates than cellulose (Dehority, 1973). In this sense, the capability of R. albus to produce substances able to inhibit growth of R. flavefaciens has been also described in the existing literature (Odenyo et al., 1994).

Streptococcus bovis was a minor component (less than 0.001%) and surprising was even lower than in the other experimental treatments. Underestimation of S. bovis concentrations attributed to interfering proteins in the PCR amplification could be not discarded (Tajima et al., 2001), and this point needs to be further developed.

Those faunated lambs from 90-day group clustered separately and exhibited the highest biodiversity not only for protozoa (13.5 bands) but also for bacterial species (17.5 bands), which suggests that the presence of protozoa affects the bacterial population in increasing bacterial biodiversity rather than concentration. This point is open to discussion, whereas the absence of protozoa can increase bacterial concentrations because of the predator–prey relationship between the communities (Fejes and Várady, 1996). Ozutsumi et al. (2005) in contrast, reported more operational taxonomic units in faunated calves than in defaunated (by isolation) animals.

The present study demonstrates the interest of using quantitative PCR for the quantification of microbial species in the complex microbial ecosystem through its development. However, the high variability observed within treatments suggests the utilization of a higher number of replicates in further studies.

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