Preliminary study of the changes in rumen bacterial populations from cattle intoxicated with young oak (*Quercus pyrenaica*) leaves

A. Belenguer^{A,D}, G. Hervás^A, D. R. Yáñez-Ruiz^B, P.G. Toral^A, C. Ezquerro^C and P. Frutos^A

^A*Instituto de Ganadería de Montaña, CSIC - Universidad de León, Finca Marzanas s/n, 24346 Grulleros, León, Spain*

^B*Unidad de Nutrición Animal, Estación Experimental del Zaidín, CSIC, Camino del jueves s/n, 18100 Armilla, Granada, Spain*

^C*Instituto de Biología Molecular, Genómica y Proteómica, Universidad de León, Campus de Vegazana, 24071 León, Spain*

^DCorresponding author. E-mail: A.Belenguer@eae.csic.es

Short title: Rumen bacterial dynamics in cattle fed oak leaves

Abstract. Intoxication of grazing cattle occurs repeatedly when they consume large amounts of young oak leaves (OL), that are rich in hydrolysable tannins (HT), due to a shortage of other feed resources. The HT are antimicrobial, although some rumen bacteria can resist or degrade them into potentially toxic or harmless metabolites. To study the effect of the administration of HT-rich OL (*Quercus pyrenaica*) after a severe feed restriction on the rumen bacterial community and monitor the variations in some bacterial groups that are potentially able to resist or metabolize tannins, 3 ruminally cannulated bulls were initially fed grass hay and then subjected to a severe 8-day feed restriction period, before receiving OL for 6 days. Then, the animals were offered again grass hay for 12 more days. Rumen contents were sampled throughout the experiment. Quantitative real-time PCR and terminal restriction fragment length polymorphism (T-RFLP) were used to monitor the bacterial dynamics. Animal 1 was not intoxicated and showed lower relative abundances of *Streptococcus bovis* initially and after the OL administration than animals 2 and 3, which showed acute signs of intoxication. The genus *Prevotella* increased its abundance with the OL administration, whereas *Selenomonas ruminantium* was reduced. The bacterial T-RFLP profile of animal 1 clustered initially separately from the animals 2 and 3 and was less affected by the feed restriction period. These results showed that the effect of the consumption of HT-rich OL after a severe feed restriction is highly variable in cattle and might rely on the individual composition of the microbiota colonizing the rumen.

Additional key words: hydrolysable tannins, oak leaves, real-time PCR, rumen bacteria, T-RFLP.

Introduction

Leaves from oak (*Quercus* spp.) trees, shrubs and saplings are often consumed by grazing ruminants when there is a shortage of other feed resources. Cases of intoxication of beef cattle occur worldwide when the animals consume immature oak leaves (OL), rich in hydrolysable tannins (HT), which may cause poisoning if sufficiently large amounts are consumed (Fillipich *et al.* 1991; Garg *et al.* 1992).

This intoxication has been hypothesized to be related to their microbial metabolism in the rumen, which may result in the formation of absorbable low molecular weight metabolites, such as gallic acid, pyrogallol and other products that are potentially toxic to ruminants (Murdianti *et al.* 1992; Zhu *et al.* 1995). On the other hand, the phenolic ring may be cleaved and HT metabolized into harmless products by some rumen bacteria (Krumholz and Bryant 1986). Therefore, whether bacterial degradation of HT causes or prevents the intoxication remains to be resolved, and it might depend on the composition of the rumen microbiota.

Different bacterial strains, such as *Streptococcus* sp. (Nelson *et al.* 1995; Goel *et al.* 2005b) or *Selenomonas ruminantium* ssp. *ruminantium* (Skene and Brooker 1995) have been reported to be able to metabolize tannins, or even to use them as an energy source (*Selenomonas ruminantium* K2; Brooker *et al.* 2000). Although a number of microbes, including those previously mentioned and others, such as *Prevotella* spp. (McSweeney *et al.* 2000), are able to resist their toxic effect, tannins are generally regarded as inhibitory to the growth and activity of rumen microorganisms (Makkar *et al.* 1988; Martinez *et al.* 2006). Feeds rich in HT, such as OL, are supposed to alter the rumen microbial

populations, although the in vivo effect of HT on the rumen bacterial community structure and on specific bacterial groups remains unclear. Its study therefore might provide better understanding on the mode of action of such compounds and on the animal response (Firkins *et al.* 2008).

The aim of this work was to study, using modern molecular techniques, the effect of the consumption by cattle of young OL on the whole rumen bacterial diversity and on the abundance of some specific groups that are potentially able to resist or degrade HT.

Material and methods

Oak leaves

Very young OL (*Quercus pyrenaica*) were manually collected from saplings during the spring and frozen within 1 h at -30°C until the experiment was carried out. Samples were collected in the province of León in the northwest of Spain (Sahechores, León), at a mean altitude of about 900 m, where intoxications of beef cattle by OL consumption had occurred in previous springs. The tannin concentrations were 230 g of tannic acid equivalents/kg DM for total tannins and 19 and 11 g/kg DM for gallic and ellagic acid, respectively (Doce *et al.* 2008).

Animals and diets

Three young Brown Swiss bulls (about 1.8 years old, 498 ± 22.2 kg of BW at the beginning of the experiment), each equipped with a ruminal cannula of 10-cm internal diameter (Ankom Technology Corp., Macedon, NY, USA), were used. They were individually penned and fed approximately 5 kg of grass hay per animal and day for a 14-day adaptation period (see Fig. 1 for a diagram of the experimental schedule).

[Please insert Figure 1 near here]

It has been observed, under practical conditions, that a feed restriction previous to the OL consumption (which occurs due to a shortage of feed resources) favours the intoxications. Therefore, a very limited amount of the grass hay was then offered for a feed restriction period of 8 days (on average and on a fresh matter (FM) basis, 4 kg for 2 days, 3 kg for 1 day, 2 kg for the next 4 days, and 1 day of fasting). Afterwards, they were offered daily 14 g DM

of grass hay plus 14 g DM of OL/kg BW^{0.75} (on average 1.7 kg FM of hay and 5 kg FM of OL) for 6 days (days 1 to 6). The OL, defrosted and slightly chopped, were administered twice per day (at 0830 and 2000 h) through the rumen cannula to ensure that all animals received the established amount. Finally, all bulls received again the same amount of grass hay offered during the adaptation period (on average 5 kg/animal) for 12 more days (days 7 to 18) until they were euthanized. Clean water and vitamin-mineral blocks were always available.

The experiment was performed in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and other scientific purposes, within the framework of a research project on *Quercus pyrenaica* intoxications in cattle.

Rumen sample collection

Samples were taken from the rumen at approximately 0830 h (i.e., immediately before the feed supply), via the rumen cannula, throughout the experiment (see Fig. 1): before starting the severe feed restriction (day -10), after the restriction period (day 1), after 2 and 5 days of OL administration (days 3 and 6), and 4 and 12 days after the last OL administration (days 10 and 18). Rumen contents were collected and immediately frozen at -80°C for DNA extraction.

DNA extraction

Samples of rumen contents were freeze-dried and thoroughly mixed before DNA extraction, which was performed in duplicate from approximately 40 mg sample by physical disruption using a bead beater (Mini-bead Beater; BioSpec

Products, Bartlesville, OK, USA), following the protocol described by Yu and Morrison (2004), with the modification that a higher temperature (95°C) was used for lysis incubation. Duplicate DNA samples were combined, further purified to remove possible PCR inhibitors such as tannins (Krause *et al.* 2001) and used as templates for quantitative real-time PCR (qPCR) amplification and terminal restriction fragment length polymorphism (T-RFLP) analysis.

Quantitative real-time PCR

Real-time PCR was carried out using a ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The 16S rDNA targeted primer sets used are presented in Table 1. PCR reactions were performed in triplicate with SYBR Green Supermix (Takara Bio Inc., Otsu, Shiga, Japan). The reaction mixture in 20 µL of the final volume contained 10 µL of the Mastermix, 0.1 µL of each primer (10 µM), 0.4 µL of the ROX dye, 7.4 µL of sterile distilled water and 2 µL of template DNA. Amplification involved 1 cycle at 95°C for 30 s for initial denaturation and then 40 cycles of 95°C for 15 s followed by annealing at 60°C for 60 s. Detection of the fluorescent product was set at the last step of each cycle and analysis of product melting was performed after the amplification. The melting curve was obtained by slow heating with a 0.3°C/s increment from 60°C to 95°C, with fluorescence collection at 0.3°C intervals. Dilutions of samples and purified genomic DNA from control strains (*Streptococcus bovis* ES1, *Selenomonas ruminantium* Z108, *Prevotella ruminicola* 23) were used to construct species-specific calibration curves and check the PCR amplification efficiency for the relative quantification of specific DNA in total rumen DNA preparations, which was performed following Pfaffl

(2001). Briefly, for relative quantification the amplification of the target group is expressed relative to the amplification of reference primers utilizing an experimentally derived amplification efficiency to correct for possible biases. In this study a domain-level primer set that amplified all eubacterial species was used as the reference primer set. Negative controls without DNA template were run with every assay to assess the overall specificity.

[Please insert Table 1 near here]

Terminal restriction fragment length polymorphism analysis

A near-full length fragment of the 16S rRNA gene was amplified by PCR using the Bacteria-specific primer pair 27f (5'-6-FAM-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1389r (5'-ACGGGCGGTGTGTACAAG-3') (Hongoh *et al.* 2003). The 27f primer was labelled with 6-carboxy-fluorescein (FAM) and the fluorescently labelled PCR products were purified on GE Healthcare PCR purification kit column (GE Healthcare Life Sciences, Buckinghamshire, UK), and eluted in a final volume of 35 µL of sterile milli-Q water. The resultant purified PCR products were digested with *HhaI* (10 U), *HaeIII* (10 U) and *MspI* (10 U) (Takara Bio Inc., Otsu, Shiga, Japan) in single enzyme digestions in a total volume of 10 µL at 37°C for 12 h. The fluorescently labelled terminal restriction fragments (T-RF) were analyzed by capillary electrophoresis on an automatic sequence analyzer (MegaBace 500, GE Healthcare Life Sciences, Buckinghamshire, UK). Determination of the sizes of T-RF was performed with the size standard ET 400-R (GE Healthcare Life Sciences, Buckinghamshire, UK), and data were analyzed using the GeneMarker analysis software (SoftGenetics, PA, USA).

Sample data consisted of size (base pair, bp) and peak area for each T-RF. To standardize the DNA loaded, the sum of all T-RF peak areas in the pattern was used to normalize peak detection threshold in each sample. A new threshold value was obtained, following Kitts (2001), by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total area in the sample with the smallest total peak area) by the smallest peak area at the 50 detection threshold. For each sample, peaks with lower area were deleted from the data set. New total area was obtained by the sum of all the remaining peak areas in each pattern. The number of T-RF in each sample once standardized was used as an estimate of richness in the bacterial community.

To infer the potential bacterial composition in the samples, genomic DNA extracted from pure cultures of *Selenomonas ruminantium* Z108, *Streptococcus bovis* ES1 and *Butyrivibrio hungatei* JK615 was submitted to a restriction with the enzymes *HhaI*, *HaeIII*, and *MspI*, and *in silico* restriction for the major rumen bacteria with the primers and the enzymes used were obtained from the Ribosomal Database Project (<http://rdp.cme.msu.edu/>; Cole *et al.* 2009).

Statistical analysis

Some qPCR data did not meet the requirements of normality and were $\log_{10}$ transformed for the statistical analysis. These transformed qPCR data and the number of fragments generated by T-RFLP were analyzed by repeated measures, using the MIXED procedure of the SAS software package, version 9.1 (SAS Inst. Inc., Cary, NC, USA). The statistical model included the fixed effect of time and the animal was considered as a random effect.

Data from T-RFLP were analysed using hierarchical clustering with single linkage and Euclidean distances to build the dendrograms. The first dendrogram (Fig. 3) was performed using the GeneMarker analysis software (SoftGenetics, PA, USA) and a binary matrix with data obtained by *HhaI* digestion. The second dendrogram (Fig. 4) was built using the matrix with the square root of the areas obtained by the 3 single enzyme (*HhaI*, *HaeIII* and *MspI*) digestions with the Community Analysis Package 4 software (Pisces Conservation Ltd, Lymington, Hampshire, UK).

Results

Evolution of bacterial populations monitored by qPCR

Relative population sizes of the genus *Prevotella*, and *Selenomonas ruminantium* and *Streptococcus bovis* groups, estimated by qPCR, are presented in Fig. 2 (expressed as proportions of the eubacteria 16S rRNA gene copy number) and Table 2 (as $\log_{10}$ of the proportions). Amplification efficiencies were usually greater than 90% and always above 82%. The genus-level primer for *Prevotella* accounted for 9 to 41% of the total bacterial 16S rRNA gene copies (Fig. 2A). The highest proportion in the 3 animals was observed after 2 and 5 days of OL administration ($P = 0.017$; Table 2). However, the 16S rRNA gene copies of the *Selenomonas ruminantium* group generally accounted for a very small proportion (up to only 0.2%; Fig. 2B), showing the lowest value after 2 days of OL administration, followed by the proportion after 5 days of OL administration ($P = 0.003$). Likewise, the *Streptococcus bovis* group was not abundant before the OL administration ($< 0.7\%$; Fig. 2C), but its relative abundance increased significantly on day 3 and

remained in a similar level in animals 2 and 3 on days 6 and 10, whereas their percentage decreased considerably in animal 1. The lowest relative proportion of the *Streptococcus bovis* group was observed in the 3 animals on day 18 ($P = 0.006$).

[Please insert Table 2 and Figure 2 near here]

Bacterial community changes determined by T-RFLP

The similarity indices of the T-RFLP profiles illustrated in form of dendrograms are presented in Fig. 3 and 4. The bacterial profiles obtained before the OL administration from animal 1 are clustered together, showing a higher similarity between them compared to those from animals 2 and 3 in both dendrograms. Furthermore, in Fig. 3 all bacterial profiles from animals 2 and 3, except for the sample from animal 3 on day 18, are grouped separately from those from animal 1, and within the former profiles, those generated from animals 2 and 3 before the OL administration are shown to be in the same cluster and different from those obtained later.

The total number of peaks obtained by *HhaI* and *HaeIII* digestions were similar between animals and days, with average values of 34 ± 0.8 and 71 ± 2.7 peaks, respectively. On the other hand, with the enzyme *MspI* the initial number of peaks (day -10; 53 ± 3.5) was lower than those obtained on days 1 (60 ± 1.5), 3 (62 ± 1.5), 10 (65 ± 1.2) and 18 (63 ± 1.2). The data obtained on day 6 was not included in the analysis, because only 1 of the 3 samples could be amplified.

[Please insert Figures 3 and 4 near here]

The most interesting fragments generated in the T-RFLP profiles obtained from all the samples with the 3 enzymes are presented in Table 3. The percentages of the fragments obtained by *HhaI* digestion over the total peak area are given as an example, because the proportions of the matching fragments obtained by *HaeIII* and *MspI* digestions followed a similar pattern. Potential compatible bacterial spp. with these T-RF are also presented in Table 3. The major fragments generated in most samples fit with bacteria belonging to the phyla *Bacteroidetes* and *Firmicutes*, based on the *in silico* restriction performed. Other usually minor fragments that showed a remarkable increase in certain samples are also shown in the Table, such as the 368 (*Actinobacteria*-compatible peak) and 370 (*Proteobacteria*-compatible fragment) bp T-RF obtained by *HhaI* digestion. The latter consistently appeared in animals 2 and 3, showing the highest relative proportions after 2 (17 and 14% in animals 2 and 3, respectively) and 5 days of OL administration (15% in animal 3). The former peak (368 bp) showed considerable proportions in animals 2 and 3 on days 10 (10 and 5%, respectively) and 18 (30 and 4%, respectively). In animal 1 these T-RF only appeared on days 1 and 3 and with smaller relative proportions (3 and 1% for the 368 bp peak, and 2 and 8% for the 370 bp fragment), and disappeared afterwards. It is also remarkable that on day 3, a T-RF of about 373 bp was detected by *HhaI* digestion only in animal 1, with a considerable relative proportion (13%).

[Please insert Table 3 near here]

Discussion

Under practical conditions, notable individual differences have been observed in the response of grazing cattle to the consumption of OL (Jones *et al.* 1997).

Accordingly, in this study each bull experienced different consequences of OL administration after a period of feed restriction: animal 1 was not at all affected, animal 2 was acutely intoxicated, and animal 3 recovered after showing signs of acute intoxication (own data, to be published elsewhere).

This high inter-individual variation in response to OL consumption might be due to different rumen microbial communities, as suggested by the T-RFLP results. Thus, data from *HhaI* digestion show that all bacterial profiles from animal 1 were different from those obtained from animals 2 and 3, except the sample collected on day 18 from animal 3, when this bull was already recovered. Results after cleavage with the enzyme *HhaI* are shown independently because cleavage with *HaeIII* and *MspI* did not reveal exactly the same results, although they yielded a higher number of T-RF. These discrepancies may be accounted for by restriction sites in more conserved regions of the 16S rDNA with *HaeIII* (Braker *et al.* 2001) and *MspI*. Therefore, the *HhaI* digestion might distinguish bacterial populations better than *HaeIII* and *MspI* digestions, although the number of T-RF yielded was lower. When T-RFLP results from the three single digestions were combined, the initial profiles of animal 1 were still different from those generated from animals 2 and 3, the latter being more affected by the feed restriction period. This is consistent with the drastic reduction of the *in situ* ruminal degradation of grass hay (-43%) and OL (-28%) reported by Belenguer *et al.* (2008) in animals 2 and 3, after 4 days

of OL administration, compared to animal 1. All together, these results would agree with the hypothesis that the effect of HT on rumen and animal physiology is associated with the individual microbial composition.

Among the tannin-resistant and tannin-degrading bacteria identified in the rumen (Tjakradidjaja *et al.* 2000; Smith *et al.* 2005), *Selenomonas* and *Streptococcus* spp. have been often described as relevant bacteria involved in tannin metabolism (Skene and Brooker 1995; Goel *et al.* 2005a). In this study the relative proportion of the *Streptococcus bovis* group, which includes not only *S. bovis* but also strains of other *Streptococcus* species, increased in the three animals after 2 days of OL administration, although its relative abundance in animal 1, estimated by qPCR, was much lower later on (Fig. 2C), thus suggesting that other tannin-resistant bacteria were able to outcompete *Streptococcus* in the presence of HT or that the colonizing *Streptococcus* spp. (not detected by the primer) were different from those of the other animals. Furthermore, these bacteria showed a greater initial abundance in animals 2 and 3 (0.33 and 0.65% of the total bacterial 16S rRNA gene copy number in animals 2 and 3, respectively, vs 0.2% in animal 1), which might be related to a higher susceptibility to toxicity. No compatible peaks with *Streptococcus* spp. were detected by T-RFLP.

The *Selenomonas ruminantium* group did not show differences between animals and were not even stimulated by the OL administration (Fig. 2B), as it would be expected based on reports showing their ability to resist and degrade tannins (Skene and Brooker 1995). A 373 bp T-RF (obtained by *HhaI* digestion) and a 499 bp T-RF (obtained by *MspI* digestion), which appear with a

remarkable relative proportion only in animal 1 after 2 days of OL administration, might fit with *S. ruminantium*, but results from qPCR seem to rule out this possibility. It is more likely that the detected fragments are compatible with bacteria related to *Butyrivibrio hungatei*, tannin-resistant *Butyrivibrio*-like isolates from the rumen having been identified as bacteria able to metabolize tannins completely to volatile fatty acids and gas (Odenyo *et al.* 2001).

Concerning the genus *Prevotella*, which is a large group of ruminal bacteria (Stevenson and Weimer 2007), its greater relative abundances after 2 and 5 days of OL administration (Fig. 2A) would support its tannin-resistant nature (McSweeney *et al.* 2000).

The phylum *Firmicutes* is a very diverse and broad group of bacteria (Edwards *et al.* 2004), a few of which (e.g., *Butyrivibrio* sp., *Eubacterium oxidoreducens*) have been described as tannin-degraders (Krumholz and Bryant 1986; Odenyo *et al.* 2001). Notwithstanding, although different T-RF, showing considerable relative abundances, were generated by *HaeIII* and *MspI* digestions within this group, it was not possible to infer any possible relationship with the presence of tannins.

The analysis of T-RFLP data (shown in Table 3) revealed also two interesting peaks that are compatible with members of the phyla *Proteobacteria* and *Actinobacteria*. The *Proteobacteria*-compatible peak appeared always in animals 2 and 3, with remarkable proportions when large amounts of OL were present in the rumen. Members of the phylum *Proteobacteria*, closely related to *Ruminobacter amylophilus* (Jones *et al.* 1994), have been identified as tannin-resistant bacteria. The abundances of this peak and the *Actinobacteria*-

compatible fragment remained high in animal 2 on day 18, when this animal still showed an acute intoxication and had a high amount of OL in the rumen, and they were low or did not appear in animals 1 and 3. The *Actinobacteria* compatible with the detected fragment, such as the facultative anaerobic ureolytic bacteria belonging to the genus *Corynebacterium*, are generally present in the rumen in small populations (Wallace *et al.* 1997), but show tannase activity (Bhat *et al.* 1998).

To sum up, the results obtained showed a high inter-individual variation in the effect of the OL consumption on the rumen bacterial community. The bacterial T-RFLP profiles of the animals that showed signs of intoxication were different from those of the non-intoxicated animal. *Streptococcus* spp. might contribute to those differences, as the *S. bovis* group studied here showed higher relative abundances in the intoxicated animals. However, other bacteria that are potentially tannin-resistant, such as members of the genus *Prevotella*, which increased with OL, or tannin-degraders, such as *S. ruminantium*, which decreased, showed a similar pattern. Further research is therefore necessary to better understand the response of the bacterial community to the consumption of HT-rich feedstuff and to identify key tannin-degrading bacteria that might be related to the appearance or prevention of the intoxication.

Acknowledgments

This work was supported by the Ministry of Education and Science (MEC, project AGL2004-06076-C02-01). P. G. Toral gratefully acknowledges receipt of a predoctoral grant from the Spanish National Research Council (CSIC, I3P

Programme), and the authors wish to thank Drs. S. López and B. Rabanal, for kindly providing the DNA from pure cultures and technical assistance, respectively, and Dr. C. Walker for revision of the manuscript.

References

- Belenguer A, Hervás G, De Paz P, Ezquerro C, Frutos P (2008) Changes in rumen bacterial communities from cattle receiving immature oak (*Quercus pyrenaica*) leaves determined by fluorescence in situ hybridization and real-time PCR. *Proceedings of the Nutrition Society* (in press).
- Bhat TK, Singh B, Sharma OP (1998) Microbial degradation of tannins – A current perspective. *Biodegradation* **9**, 343-357.
- Braker G, Ayala-del-Río HL, Devol AH, Fesefeldt A, Tiedje J (2001) Community structure of denitrifiers, bacteria, and archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. *Applied and Environmental Microbiology* **67**, 1893-1901.
- Brooker JD, O'Donovan LO, Skene I, Sellick G (2000) Mechanisms of tannin resistance and detoxification in the rumen. In 'Tannins in Livestock and Human Nutrition: Proceedings of an International Workshop, Adelaide, Australia. ACIAR Proceedings No. 92'. Adelaide (Ed. JD Brooker) pp. 117-122. (Australian Centre for International Agricultural Research: Adelaide, Australia)
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The

- Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research* **37**, D141-D145.
- Doce RR, Hervás G, Belenguer A, Toral PG, Giráldez FJ, Frutos P (2008) Effect of the administration of young oak (*Quercus pyrenaica*) leaves to cattle on ruminal fermentation. *Animal Feed Science and Technology* **150**, 75-85.
- Edwards JE, McEwan NR, Travis AJ, Wallace RJ (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie van Leeuwenhoek* **86**, 263-281.
- Fillipich LJ, Zhu J, Oelrichs P, Alsalami MT, Doig AJ, Cao GR, English PB (1991) Hepatotoxic and nephrotoxic principles in *Terminalia oblongata*. *Research in Veterinary Science* **50**, 170-177.
- Firkins JL, Karnati SKR, Yu Z (2008) Linking rumen function to animal response by application of metagenomics techniques. *Australian Journal of Experimental Agriculture* **48**, 711-721.
- Garg SK, Makkar HPS, Nagal KB, Sharma SK, Wadhwa DR, Singh B (1992) Toxicological investigations into a (*Quercus incana*) leaf poisoning in cattle. *Veterinary and Human Toxicology* **34**, 161-164.
- Goel G, Puniya AK, Aguilar CN, Singh K (2005a) Interaction of gut microflora with tannins in feeds. *Naturwissenschaften* **92**, 497–503.
- Goel G, Puniya AK, Singh K (2005b) Tannic acid resistance in ruminal streptococcal isolates. *Journal of Basic Microbiology* **45**, 243-245.
- Hongoh YH, Yuzawa M, Okhuma M, Kudo T (2003) Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. *FEMS Microbiology Letters* **221**, 299-304.

- Jones GA, McAllister TA, Muir AD, Cheng KJ (1994) Effects of Sainfoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. *Applied and Environmental Microbiology* **60**, 1374-1378.
- Jones TC, Hunt RD, King NW (1997) Oaks. In 'Veterinary Pathology'. (Eds TC Jones, RD Hunt, NW King) pp. 704-705. (Williams and Wilkins: Baltimore, USA)
- Kitts CL (2001) Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Current Issues in Intestinal Microbiology* **2**, 17-25.
- Krause DO, Smith WJCS, McSweeney CS (2001) Extraction of microbial DNA from rumen contents containing plant tannins. *BioTechniques* **31**, 294-298.
- Krumholz LR, Bryant MP (1986) *Eubacterium oxidoreducens* sp. nov. requiring H₂ or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. *Archives of Microbiology* **144**, 8-14.
- Maeda H, Fujimoto C, Haruki Y, Maeda T, Koikeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, Takashiba S (2003) Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomicetescorritans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. *FEMS Immunology and Medical Microbiology* **39**, 81-86.
- Makkar HPS, Singh B, Dawra RK (1988) Effect of tannin-rich leaves of oak on various microbial activities of the rumen. *British Journal of Nutrition* **60**, 287-296.

- Martinez TF, McAllister TA, Wang Y, Reuter T (2006) Effects of tannic acid and quebracho tannins on *in vitro* ruminal fermentation of wheat and corn grain. *Journal of the Science of Food and Agriculture* **86**, 1244-1256.
- McSweeney CS, Palmer B, Krause DO (2000) Rumen microbial ecology and physiology in sheep and goats fed a tannin-containing diet. In 'Tannins in Livestock and Human Nutrition: Proceedings of an International Workshop, Adelaide, Australia. ACIAR Proceedings No. 92'. Adelaide (Ed. JD Brooker) pp. 140-145. (Australian Centre for International Agricultural Research: Adelaide, Australia)
- Murdiati TB, McSweeney CS, Lowry JB (1992) Metabolism in sheep of gallic acid, tannic acid and hydrolysable tannin from *Terminalia oblongata*. *Australian Journal of Agricultural Research* **43**, 1307-1319.
- Nelson KE, Pell AN, Schofield P, Zinder S (1995) Isolation and characterization of an anaerobic ruminal bacterium capable of degrading hydrolysable tannins. *Applied and Environmental Microbiology* **61**, 3293-3298.
- Odenyo AA, Bishop R, Asefa G, Jamnadass R, Odongo D, Osuji P (2001) Characterization of tannin-tolerant bacterial isolates from East African ruminants. *Anaerobe* **7**, 5-15.
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, 2002-2007.
- Skene IK, Brooker JD (1995) Characterization of tannin acylhydrolase activity in the ruminal bacterium *Selenomonas ruminantium*. *Anaerobe* **1**, 321-327.
- Smith AH, Zoetendal E, Mackie RI (2005) Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microbial Ecology* **50**, 197-205.

- Stevenson DM, Weimer PJ (2007) Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Applied Microbiology and Biotechnology* **75**, 185-174.
- Tjakradidjaja AS, Brooker JD, Bottema CDK (2000) Characterisation of tannin-resistant bacteria from the rumen fluid of feral goats and camels with restriction analysis of amplified 16S rDNA. In 'Tannins in Livestock and Human Nutrition: Proceedings of an International Workshop, Adelaide, Australia. ACIAR Proceedings No. 92'. Adelaide (Ed. JD Brooker) pp. 160-165. (Australian Centre for International Agricultural Research: Adelaide, Australia)
- Wallace RJ, Onodera R, Cotta MA (1997) Metabolism of nitrogen-containing compounds. In 'The rumen microbial ecosystem'. (Eds PN Hobson, CS Stewart) pp. 283-328. (Chapman & Hall: London, UK)
- Yu Z, Morrison M (2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* **36**, 808-812.
- Zhu J, Filippich LJ, Ng J (1995) Rumen involvement in sheep tannic acid metabolism. *Veterinary and Human Toxicology* **37**, 436-440.

Table 1. Primers used to detect the *Streptococcus bovis* and *Selenomonas ruminantium* groups, the genus *Prevotella* and total bacteria.

Name of primer	Sequence (5' → 3')	Target	Source of primer	Amplicon length (bp)
StrBov2F	TTCCTAGAGATAGGAAGTTTCTTCGG	<i>Streptococcus bovis</i> group	Stevenson and Weimer (2007)	127
StrBov2R	ATGATGGCAACTAACAATAGGGGT			
SelRum2F	CAATAAGCATTCCGCCTGGG	<i>Selenomonas ruminantium</i> group	Stevenson and Weimer (2007)	138
SelRum2R	TTCACTCAATGTCAAGCCCTGG			
PreGen4F	GGTTCTGAGAGGAAGGTCCCC	Genus <i>Prevotella</i>	Stevenson and Weimer (2007)	121
PreGen4R	TCCTGCACGCTACTTGGCTG			
TotBacF	GTGSTGCAYGGYTGTCGTCA	Total bacteria	Maeda <i>et al.</i> (2003)	147
TotBacR	ACGTCRTCCMCACCTTCCTC			

Table 2. Quantity of DNA, determined by real-time PCR, from the genus *Prevotella*, and *Selenomonas ruminantium* and *Streptococcus bovis* groups, expressed as log₁₀ of the proportion of the total bacterial 16S rRNA gene copy number, in rumen contents from 3 bulls before (day -10) and after (day 1) a feeding restriction period, after 2 (day 3) and 5 (day 6) days of administration of oak leaves (OL), and 4 (day 10) and 12 (day 18) days after the last OL administration.

Within each column, means followed by a different letter are significantly different at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$

Day	Genus <i>Prevotella</i>	<i>S. ruminantium</i> group	<i>S. bovis</i> group
-10	1.40 ^{ab}	-0.92 ^{ab}	-0.46 ^b
1	1.37 ^{ab}	-0.85 ^a	-0.50 ^b
3	1.47 ^a	-1.57 ^d	0.40 ^a
6	1.57 ^a	-1.27 ^c	0.10 ^{ab}
10	1.21 ^b	-1.19 ^{bc}	-0.21 ^{ab}
18	1.19 ^b	-1.17 ^{bc}	-1.39 ^c
s.e.d.	0.095	0.130	0.343
Significance	*	**	**

Table 3. Major terminal restriction fragments (T-RF; length in base pairs, bp) obtained from the terminal restriction fragment length polymorphism (T-RFLP) analysis of rumen contents from 3 bulls before (day -10) and after (day 1) a feeding restriction period, after 2 (day 3) and 5 (day 6) days of administration of oak leaves (OL), and 4 (day 10) and 12 (day 18) days after the last OL administration.

<i>HhaI</i>	Frequency ^A			<i>HaeIII</i>	<i>MspI</i>	Compatible bacteria ^B
T-RF (bp)	Animal 1	Animal 2	Animal 3	T-RF (bp)	T-RF (bp)	
66	8.3 - 30.7 (22.6, all)	14.1 - 35.6 (23.1, all)	6.0 - 32.1 (18.7, all)	257	91	<i>Bacteroidetes</i>
100	14.4 - 25.4 (19.1, all)	8.0 - 23.6 (17.6, all)	11.5 - 28.4 (19.2, all)	262	95	<i>Bacteroidetes</i>
191				237	93	<i>Firmicutes</i>
191	4.0 - 12.5 (6.9, all)	2.3 - 8.0 (3.8, all)	4.0 - 11.0 (7.0, all)	273	224	<i>Firmicutes</i> (<i>Roseburia</i> , <i>Ruminococcus</i>)
191				273	297	<i>Firmicutes</i> (<i>Butyrivibrio</i>)
368	1.3 - 2.9 (0.8, 1/3)	2.2 - 30.4 (8.7, 1/10/18)	3.7 - 4.9 (1.4, 10/18)	66	163	<i>Actinobacteria</i>
368				227	163	<i>Actinobacteria</i>
370	1.7 - 8.0 (1.9, 1/3)	0.6 - 17.4 (7.3, all)	1.4 - 14.5 (8.4, all)	228	165	<i>Proteobacteria</i>

373	0.6 - 12.9 (3.0, 1/3/10/18)	0.6 - 1.2 (0.5, -10/3/18)	0.7 - 1.3 (0.5, -10/1/18)	41	499	<i>Selenomonas ruminantium</i> , <i>Butyrivibrio hungatei</i>
-----	--------------------------------	------------------------------	------------------------------	----	-----	---

^A Range of abundances of the peaks obtained by *HhaI* digestion expressed as % of total area. In brackets the means followed by the days of the experiment when the peak was detected in the T-RFLP profile.

^B *In silico* restriction from the Ribosomal Database Project (<http://rdp.cme.msu.edu/>; Cole *et al.* 2009) and the real restriction of genomic DNA extracted from pure cultures of *Selenomonas ruminantium* Z108 and *Butyrivibrio hungatei* JK615 were used to infer the compatible bacteria with the T-RF obtained.

Figure captions

Fig. 1. Diagram of the experimental schedule.

Fig. 2. Relative abundances (proportion of the total bacterial 16S rRNA gene copy number) of the genus *Prevotella* (A), *Selenomonas ruminantium* group (B) and *Streptococcus bovis* group (C) from rumen contents obtained from 3 bulls before (day -10) and after (day 1) a feeding restriction period, after 2 (day 3) and 5 (day 6) days of administration of OL, and 4 (day 10) and 12 (day 18) days after the last OL administration. Shaded area indicates OL administration. (s.e. = 2.031, 0.011 and 0.306 for the genus *Prevotella*, *Selenomonas ruminantium* group and *Streptococcus bovis* group, respectively).

Fig. 3. Dendrogram from data generated by *HhaI* digestion, illustrating the effect of oak leaves (OL) administration after a feeding restriction period on T-RFLP bacterial profiles. The dendrogram represents results from rumen contents obtained from 3 bulls before (day -10) and after (day 1) a feeding restriction period, after 2 (day 3) and 5 (day 6) days of administration of OL, and 4 (day 10) and 12 (day 18) days after the last OL administration. The scale bar shows euclidean distances.

Fig. 4. Dendrogram from data obtained by 3 single enzyme digestions (*HhaI*, *HaeIII* and *MspI*), illustrating the effect of oak leaves (OL) administration after a feeding restriction period on T-RFLP bacterial profiles. The dendrogram represents results from rumen contents obtained from 3 bulls before (day -10) and after (day 1) a feeding restriction period, after 2 (day 3) and 5 (day 6) days

of administration of OL, and 4 (day 10) and 12 (day 18) days after the last OL administration. The scale bar shows euclidean distances.

Fig. 1

Fig. 2

A. Genus Prevotella

B. Selenomonas ruminantium group

C. Streptococcus bovis group

Fig. 3

Fig. 4