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Impact of oxalic acid on rumen function and bacterial community in sheep

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Oxalic acid (OA) is a secondary compound occurring in a wide range of plants consumed by ruminants, especially in saline lands or in arid and semi-arid regions. However, its impact on the rumen microbial community and its changes over time, as well as the potential consequences on ruminal function, remain unknown. To examine this impact, five ewes fitted with a ruminal cannula and fed low-quality grass hay were dosed daily with 0.6 mmol of OA/kg body weight through the cannula for 14 days. On days 0 (before the start), 4, 7 and 14 of the administration period, samples of ruminal digesta were collected throughout the day (0, 3, 6 and 9 h after the morning feeding) for analysis of the bacterial community and fermentation parameters (pH, ammonia and volatile fatty acid (VFA) concentrations). In addition, two feedstuffs were incubated in situ *using the nylon bag technique to estimate ruminal degradation. Terminal restriction fragment length polymorphism was employed to monitor the dynamics of total bacteria, and quantitative real-time PCR was used to investigate the abundance of the oxalate-degrading* Oxalobacter formigenes*. Neither pH nor total VFA concentrations were affected. Nevertheless, OA dosing altered molar proportions of most individual VFA and ammonia concentrations ($P < 0.001$). The dry matter disappearance of alfalfa hay was reduced on days 7 and 14 and that of barley straw only on day 7 ($P < 0.01$). These slight changes were related to others observed in the relative frequency of a number of terminal restriction fragments. Variations in the ruminal microbiota occurred rapidly with OA administration, which did not modify the bacterial diversity significantly but altered the structure of the community. However, many of these changes were reversed by the end of the experiment, with no significant differences between days 0 and 14 of dosing. These results suggest a rapid adaptation of the rumen bacterial community linked to the estimated increase in the abundance of* O. formigenes *(from 0.002% to 0.007% of* oxc *gene in relation to the total bacteria 16S rDNA; $P < 0.01$), which is assumed to be responsible for oxalate breakdown.*

Keywords: *Oxalobacter formigenes*, oxalates, real-time PCR, ruminal microbiota, T-RFLP

Implications

This study shows that the rumen bacterial community adapts rapidly to the consumption of oxalic acid (OA), a potentially toxic secondary compound occurring in a wide range of plants. Oxalate-rich plants may become important forage resources for ruminants when other feedstuffs are in short supply, especially in saline lands or in arid and semi-arid regions. In such occasions, the adaptation of the ruminal microbiota to OA would most likely prevent animal poisoning and impairment of diet utilization. Understanding how rapidly the rumen microbial ecosystem responds to this compound suggests focusing on management during the first days of oxalate-rich plants introduction in the diet of ruminants.

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Introduction

Oxalic acid (OA) is an organic dicarboxylic acid occurring in a wide range of plants consumed by ruminants, especially in saline lands or in arid and semi-arid regions (Ben Salem *et al.*, 2010; El Shaer, 2010). Although most oxalate-rich feed resources present low levels of this secondary compound (around 10 to 20 g/kg dry matter (DM); Libert and Franceschi, 1987; Rahman and Kawamura, 2011), some of them, such as *Atriplex* spp., *Setaria* spp. or *Halogeton* spp., can accumulate oxalates to potentially toxic concentrations (up to 300 g/kg DM; Cheeke, 1995; Ben Salem *et al.*, 2010; Rahman and Kawamura, 2011). In such cases, the abrupt consumption of a sufficient quantity of these plants will most probably cause poisoning in livestock, the main signs including hypocalcaemia and renal toxicity (James, 1972; Cheeke, 1995).

However, gradual exposure to increasing levels of OA leads to an adaptation of the ruminal microbiota and a consequent greater breakdown of this compound, which protects the host animal from toxicity (Allison *et al.*, 1977; Duncan *et al.*, 1997). It has been assumed that OA is readily degraded in the rumen by the bacteria *Oxalobacter formigenes* (Daniel *et al.*, 1989), a specialist oxalotroph that uses oxalates as its sole or major carbon and energy source (Allison *et al.*, 1985). Thus, although other less specialized oxalate-degrading bacteria have been described in the gastrointestinal tract of non-ruminants, especially humans (Sahin, 2003; Abratt and Reid, 2010), very less work of a similar nature has been published concerning ruminants. Furthermore, available studies in the latter rely exclusively on traditional microbiology culture-based techniques (Dawson *et al.*, 1980; Daniel *et al.*, 1989), and, to our knowledge, the impact of OA on the structure of the rumen microbial community and its changes over time, as well as the potential consequences on rumen function, remain largely unknown.

Therefore, this study was conducted in sheep to examine the effect of OA on the rumen bacterial community, using cultivation-independent molecular techniques and paying particular attention to *O. formigenes*, and on ruminal fermentation and degradation of feedstuffs.

Material and methods

Animals, diets and experimental design

All experimental procedures were conducted in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental purposes.

Five individually penned Assaf ewes (mean BW, 80 $\pm$ 13.9 kg), fitted with a ruminal cannula (40-mm internal diameter), were used in this study. The animals were fed low-quality grass hay (929 g organic matter, 74 g CP, 592 g NDF and 337 g ADF per kg DM) at 1.2 times their estimated maintenance energy requirements (Agricultural and Food Research Council (AFRC, 1993)) for 18 days before commencing the assay and throughout the 14-day experimental period. The grass hay was offered in two equal meals at 0900 and 1800 h, and clean water was always available.

OA (75688; Sigma-Aldrich, Steinheim, Germany), at 0.6 mmol/kg BW per day, was dissolved in 100 ml of water and administered twice daily through the rumen cannula before each meal. The dose was gradually increased (from 20% to 100%) over the first 5 days. *In vivo* and *in situ* assays were performed on days 0 (immediately before starting the administration of OA, control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of the dosing period. Even though treatment may be confounded with experimental period, this approach was used, rather than a switchback or change-over design, to avoid possible residual carry-over effects of OA on ruminal microbiota. The same experimental design has been used previously to examine the rates of OA degradation in the rumen of sheep and goats (Duncan *et al.*, 1997).

Experimental procedures

In vivo studies. Samples of ruminal digesta (25 ml) were collected via the cannula at 0, 3, 6 and 9 h after the morning feeding and immediately frozen at -80°C. Digesta was then pooled to provide composite samples for each animal and sampling day, freeze-dried and stored again at -80°C until microbiology analysis. With the same frequency, rumen fluid was strained through two layers of muslin to collect samples for pH measurement and for ammonia (4 ml, acidified with 4 ml 0.2 M HCl) and volatile fatty acid (VFA; 0.8 ml, deproteinized with 0.5 ml of 20 g/l metaphosphoric and 4 g/l crotonic acids in 0.5 M HCl) determinations.

In situ studies. *In situ* rumen degradation of two structurally different feeds, alfalfa hay (132 g CP, 503 g NDF and 401 g ADF per kg DM) and barley straw (49 g CP, 739 g NDF and 430 g ADF per kg DM), were estimated using nylon bags (150 $\times$ 100 mm, pore size of 50 μm, Ankom Technology Corp., Macedon, NY, USA). Two bags containing 4 g of either alfalfa hay or barley straw, ground to pass 2-mm screen, were incubated in the rumen of each ewe for 12 and 24 h. After removal from the rumen, they were washed with cold tap water and frozen (-30°C) for 24 h to help remove microbial attachment to feed particles. Once defrosted, bags were washed again with cold water in a commercial washing machine, dried and weighed to determine DM losses.

Feed intake was monitored daily by weighing and drying the refusals.

Chemical analysis

The experimental diet and incubated feeds were analysed for DM (ISO 6496:1999), ash (ISO 5984:2002) and CP (ISO 5983-2:2009). NDF and ADF were determined by the methods described by Mertens (2002) and the Association of Official Analytical Chemists (Official Method 973.18), respectively, using the Ankom technology (Ankom Technology Corp.). NDF was assayed with sodium sulphite and α-amylase and expressed with residual ash (the latter also for ADF). Ammonia concentrations were determined by a colorimetric method (Weatherburn, 1967) and VFA by gas chromatography, with crotonic acid as internal standard (Ottenstein and Bartley, 1971).

Microbial community analysis

After thorough mixing and DNA extraction (Belenguer *et al.*, 2010), duplicates were combined and used as templates for quantitative real-time PCR (qPCR) amplification and terminal restriction fragment length polymorphism (T-RFLP) analysis. Sample DNA concentration was determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, DE, USA).

For T-RFLP analysis of 16S rRNA genes, a universal bacteria-specific primer pair set (Hongoh *et al.*, 2003) and three restriction enzymes (*Hha*I, *Msp*I and *Hae*III) were used (Belenguer *et al.*, 2010). The lengths of the fluorescently labelled terminal restriction fragments (T-RF) were determined with the size standard ET-900-R (GE Healthcare Life Sciences,

Buckinghamshire, UK) using the GeneMarker Analysis software (SoftGenetics, State College, PA, USA).

In silico restriction for the major rumen bacteria with the primers and enzymes used in the analysis, obtained from the Ribosomal Database Project II website (URL: http://rdp.cme.msu.edu/index.jsp; Cole *et al.*, 2009), were used to infer the bacterial composition of ruminal digesta.

The qPCR analysis was carried out using the Applied Biosystems StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) to investigate the abundance of *O. formigenes*. The primer sets used in this study included 16S rRNA-gene targeted oligonucleotides for total bacteria (McSweeney and Denman, 2007) and others that were designed on the basis of the sequence of the gene encoding for the oxalyl-CoA decarboxylase (*oxc*) for *O. formigenes* (Jiang *et al.*, 2011). The PCR reactions were performed in triplicate with SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA), using a 20 μl reaction mixture with 0.6 μl of each primer (10 μM) for total bacteria and 2.5 μl for *O. formigenes*, as described previously (Belenguer *et al.*, 2010). Dilutions of samples and purified genomic DNA extracted from a pure culture of the *O. formigenes* strain DSM 4420 (DSMZ Bacteria Collection, Braunschweig, Germany) were used to construct species-specific calibration curves and to check the PCR amplification efficiency for the relative quantification of specific DNA in rumen DNA preparations, as reported previously (Belenguer *et al.*, 2010). The qPCR efficiency ranged between 85% and 100%, and negative controls without DNA template were run with every assay to assess the overall specificity.

Calculations and statistical analysis

Data from T-RFLP (size, bp, and peak area for each T-RF) were analysed as outlined by Abdo *et al.* (2006), and used to determine the relative abundance of each fragment over the total peak area, as well as the diversity indices (number of T-RF or richness, and the Shannon–Wiener and Shannon evenness indices; Hill *et al.*, 2003). Hierarchical clustering analysis with the Ward's method on the basis of Jaccard or Bray–Curtis distances was performed using R-project software (www.r-project.org, version 2.13.1) to build a dendrogram. Analysis of similarity (ANOSIM) was also carried out with the same software, which produces a test statistic (*R*-value), indicating the extent to which the groups differ ($R > 0.75$ groups are well separated, $0.75 > R > 0.50$ groups are overlapping but clearly different, and $R < 0.25$ groups are barely separable; Frey *et al.*, 2009). The level of significance was based on the Bray–Curtis similarity matrix.

In addition to these statistical analyses of some microbiological results, T-RF relative abundances and all the remaining data (i.e. those from *in vivo* and *in situ* assays) were analysed by one-way analysis of variance (ANOVA), using the MIXED procedure of the SAS software package (version 9.2, SAS Institute Inc., Cary, NC, USA). As some qPCR results did not satisfy the assumptions of data normality, data were transformed to $\log_{10}$ before ANOVA. The statistical model included the fixed effect of OA administration (Control, Oxa4, Oxa7 and Oxa14) and the random effect of animal. Furthermore, *in vivo* data taken over time (hours post feeding) were analysed by repeated measures, using the MIXED procedure. The statistical model included the fixed effects of OA administration, hours post feeding and their interaction. Means were separated using the 'pdiff' option of the 'lsmeans' statement of the MIXED procedure. Differences were declared significant at $P < 0.05$ and considered a trend towards significance at $P < 0.10$. Least square means are reported.

Results

Rumen fermentation and degradation parameters

Variations due to hours post feeding were observed in rumen pH, and ammonia and VFA concentrations ($P < 0.01$); however, for clarity, only mean values are shown in Table 1. The administration of OA reduced ammonia mean concentration ($P < 0.001$), although this effect was not significant at all collection times (interaction, $P < 0.01$), and it did not affect ($P > 0.05$) the mean values of pH and total VFA, acetate and propionate concentrations. However, the molar proportions of acetic and propionic acids increased ($P < 0.001$), and both the concentrations and molar proportions of butyric acid and the sum of minor VFA (valeric, isobutyric, isovaleric and caproic acids) were reduced in response to OA ($P < 0.001$).

Table 1 also shows the effect of OA on ruminal degradation. DM disappearance of alfalfa hay was slightly reduced (−3%) after 12 h of incubation on Oxa7 and Oxa14 ($P < 0.01$), but this effect was not observed in the 24-h incubations. On the other hand, DM disappearance of barley straw was only transiently decreased ($P < 0.01$) on Oxa7 and after 24 h of incubation, with no significant differences by the end of the experiment (Oxa14).

The administration of OA had no effect on feed intake ($P > 0.10$).

Bacterial community analysis by T-RFLP

Rumen bacterial T-RFLP analysis of 16S rRNA genes generated on average 41.5 ± 0.89, 58.0 ± 1.15 and 63.0 ± 1.20 fragments with the enzymes *Hha*I, *Msp*I and *Hae*III, respectively. Hierarchical clustering indicated that OA altered only transiently the structure of the rumen bacterial community (Figure 1), as the analysis resulted in two major clusters, and samples obtained on days 0 and 14 of OA dosing were grouped together and separated from those collected on days 4 and 7. Although the diversity indices (richness, Shannon–Weiner and Shannon Evenness) were not modified by the treatment after either *Hha*I, *Msp*I or *Hae*III digestions ($P > 0.10$; Table 2), the ANOSIM (pairwise comparisons) indicates significant differences in the bacterial community between sampling days (Table 3). In this regard, the enzyme *Msp*I showed the most consistent results, with low and non-significant *R*-values for the comparison of initial (control) and final

Table 1 *Mean rumen fermentation characteristics (pH, ammonia concentration and VFA concentration and molar proportions), and DM disappearance from alfalfa hay and barley straw incubated for 12 and 24 h in the rumen of sheep on days 0 (Control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of OA dosing*

							P-value[1]		
		Control	Oxa4	Oxa7	Oxa14	s.e.d.	OA	H[2]	OA × H
pH		7.01	6.91	6.99	6.95	0.083	+	***	ns
Ammonia (mg/l)		108.2[a]	78.6[b]	67.1[b]	80.2[b]	15.27	***	***	**
Total VFA (mmol/l)		89.0	89.8	89.0	85.6	6.52	ns	***	ns
Acetate (%)		66.9[b]	67.5[b]	68.3[a]	68.6[a]	0.65	***	***	ns
Propionate (%)		18.9[c]	20.6[a]	19.6[b]	19.4[bc]	0.59	***	*	ns
Butyrate (%)		10.9[a]	9.2[c]	9.6[b]	9.4[bc]	0.39	***	***	ns
Minor VFA[3] (%)		3.21[a]	2.69[b]	2.45[c]	2.57[bc]	0.176	***	***	ns
DM disappearance (g/100 g incubated)									
Alfalfa hay	12 h	63.8[a]	63.6[a]	61.2[b]	62.2[b]	0.68	**	–	–
	24 h	67.0	66.8	65.9	66.3	0.63	ns	–	–
Barley straw	12 h	39.6	39.0	35.8	37.9	1.56	ns	–	–
	24 h	56.4[a]	55.7[a]	51.2[b]	55.0[a]	1.33	**	–	–

VFA = volatile fatty acid; DM = dry matter; OA = oxalic acid.
[1]Probability of significant effects because of OA administration (Control, Oxa4, Oxa7 and Oxa14), hours post feeding (H; 0, 3, 6 and 9) and their interaction (OA × H): ns = not significant ($P > 0.10$); $^{+}P < 0.10$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
[2]Although, as expected, variations because of hours post feeding (H) were observed in rumen pH, and ammonia and VFA concentrations ($P < 0.01$); for clarity, only mean values are shown.
[3]Calculated as the sum of valeric, isobutyric, isovaleric and caproic acids.
[a,b,c]Mean values with different superscripts in each row differ significantly ($P < 0.05$).

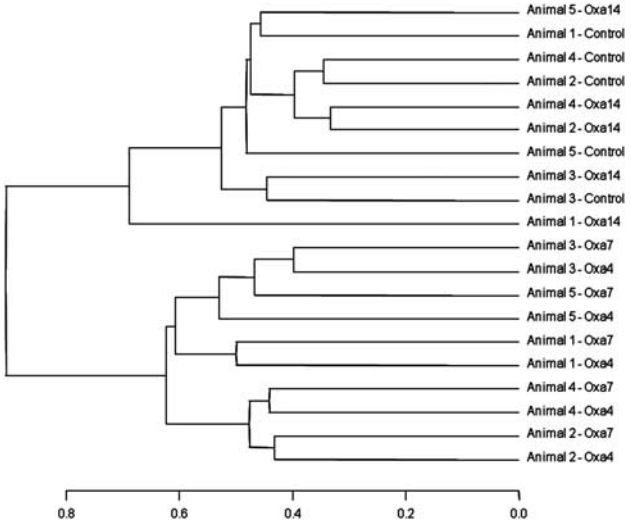

Figure 1 Cluster analysis of the terminal restriction fragment length polymorphism (T-RFLP) profiles, based on the Ward's method and the Jaccard distances, of total bacteria present in the ruminal digesta of sheep on days 0 (Control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of oxalic acid dosing.

(Oxa14) structures. Similar results were observed between bacterial profiles after 4 (Oxa4) and 7 (Oxa7) days on OA, whereas comparisons between control or Oxa14 and either Oxa4 or Oxa7 resulted in significant *R*-values higher than 0.5 ($P < 0.05$). Only the comparison control *v.* Oxa4 was significantly different ($R > 0.25$ and $P < 0.05$) with *Hha*I, and *R*-values were always low and not significant with *Hae*III.

Several different T-RF exhibited variations in their relative frequency out of the total peak area because of the presence of OA (Table 4). These changes were only transient in many cases, with the initial values being recovered by the end of the experiment (e.g. 91 and 579 bp with *Hha*I; and 96 and 297 bp with *Msp*I). The T-RF compatible with *O. formigenes* obtained with *Hha*I (567 bp) and *Hae*III (199 bp) showed no variations with OA ($P > 0.10$), although that obtained with *Msp*I (489 bp) tended to decrease its relative abundance on Oxa4 and Oxa7 ($P < 0.10$).

O. formigenes quantification by qPCR

The DNA abundance of the *oxc* gene of *O. formigenes* was very small relative to that of total bacterial 16S rRNA gene (mean $0.005 \pm 0.0007\%$); however, it was significantly increased over time with the administration of OA ($P < 0.001$; Table 5). Similar results were obtained when the abundance of the specific *oxc* gene DNA of these oxalate-degrading bacteria was expressed as pg/ng total DNA.

Discussion

Previous research has shown that adaptation of the rumen environment to the consumption of increasing levels of OA prevents poisoning of the host animal (Duncan *et al.*, 1997). However, little is known on the action of this secondary compound on the rumen bacterial community and its potential consequences on ruminal function. In this experiment, the level of OA was chosen on the basis of preceding studies to simulate moderate ingestion rates by ruminants under field conditions (Libert and Franceschi, 1987; Duncan *et al.*, 1997 and 2000).

Table 2 *Analysis of similarity (pairwise comparisons) of the total bacteria T-RFLP profile of ruminal digesta of sheep on days 0 (Control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of OA dosing*

	*Hha*I		*Msp*I		*Hae*III	
Pairwise comparison	*R*-value	*P*-value[1]	*R*-value	*P*-value[1]	*R*-value	*P*-value[1]
Control *v.* Oxa4	0.416	**	0.640	*	−0.036	ns
Control *v.* Oxa7	0.132	ns	0.612	**	0.064	ns
Control *v.* Oxa14	−0.068	ns	−0.012	ns	0.072	ns
Oxa4 *v.* Oxa7	0.08	ns	−0.156	ns	−0.056	ns
Oxa4 *v.* Oxa14	0.232	*	0.596	*	−0.048	ns
Oxa7 *v.* Oxa14	0.028	ns	0.572	**	−0.112	ns

T-RFLP = terminal restriction fragment length polymorphism; OA = oxalic acid.
[1]Level of significance of *R*-value: ns = not significant ($P > 0.10$); *$P < 0.05$; **$P < 0.01$.

Table 3 *Diversity indices (richness, R; Shannon–Wiener, H; and Shannon evenness, E) calculated from the total bacteria T-RFLP profiles of ruminal digesta of sheep on days 0 (Control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of OA dosing*

		Control	Oxa4	Oxa7	Oxa14	s.e.d.	*P*-value[1]
*Hha*I	R	41.4	39.8	42.6	42.0	2.64	ns
	H	3.17	3.16	3.26	3.16	0.123	ns
	E	0.85	0.86	0.87	0.84	0.025	ns
*Msp*I	R	59.2	56.0	56.8	59.8	3.36	ns
	H	3.73	3.50	3.50	3.64	0.146	ns
	E	0.92	0.87	0.87	0.89	0.027	ns
*Hae*III	R	64.4	61.6	62.6	63.2	3.63	ns
	H	3.76	3.65	3.69	3.65	0.101	ns
	E	0.90	0.89	0.89	0.88	0.016	ns

T-RFLP = terminal restriction fragment length polymorphism; OA = oxalic acid.
[1]Probability of significant effect due to oxalic acid administration (Control, Oxa4, Oxa7 and Oxa14): ns = not significant ($P > 0.10$).

Changes in the ruminal microbiota occurred rapidly with OA dosing (Allison *et al.*, 1977; Duncan *et al.*, 1997; Frutos *et al.*, 1998); however, interestingly, they were reversed on day 14, which suggests an adaptation of the rumen bacterial community along with the increase in the abundance of *O. formigenes* and therefore with the ability to degrade OA. This contrasts with the rumen degradation of other plant secondary compounds, such as tannins, involving a complex consortium of microorganisms (Belenguer *et al.*, 2010) and the consequent need of longer periods for adaptation of the whole rumen microbial community.

Changes in concentrations and molar proportions of specific VFA by OA administration suggest divergences in fermentation pathways probably because of a stimulation of specific microbial groups to the detriment of others (Russell and Wallace, 1997). This is supported by some T-RFLP results. For instance, T-RF compatible with uncultured bacteria of the family *Lachnospiraceae* (148 bp with *Msp*I and 244 bp with *Hae*III), which includes important ruminal butyrate producers, such as *Butyrivibrio* spp. (Mrazek *et al.*, 2006), decreased its relative abundance with OA, indicating a potential relationship with reductions in butyrate concentration.

The decrease caused by OA in ammonia concentration, together with the lower levels of minor VFA that originate mostly from deamination of specific amino acids, would be consistent with an inhibition of rumen proteolysis. This effect was initially related to variations of a T-RF (485 bp with *Msp*I) compatible with *Clostridium sticklandii*, which belongs to rumen hyper-ammonia-producing bacteria (Krause and Russell, 1996). However, changes in this fragment were inconsistent and prevented the establishment of a clear relationship.

OA reduced the ruminal DM disappearance of the two reference feedstuffs, but initial values were only recovered for barley straw. As both feedstuffs differ substantially in their fibrous fraction, these results would suggest a faster adaptation of hemicellulolytic bacteria to OA, probably because their presence was favoured by the low-quality forage offered to the animals (Tajima *et al.*, 2001). The lack of differences in alfalfa DM disappearance after 24 h of incubation was probably because of this feed reaching its maximum degradation before that time.

Shifts in ruminal fermentation could be speculated to be partially explained by the effect of OA administration on calcium and other cation levels in the rumen and the consequent impact on rumen function and bacterial community structure (Sahin, 2003).

Variations in ruminal degradation may also be related to changes in the relative frequencies of a number of T-RF showing significant differences on days 4 and 7 of OA administration, compared with the control, but recovering the initial values on day 14. Example of this are two fragments (94 and 96 bp with *Msp*I) that are compatible with *Bacteroidetes*, a large rumen microbial group (Edwards *et al.*, 2004), or another T-RF (297 bp with *Msp*I) compatible with species of the family *Ruminococcacea*, which includes some of the main cellulolytic bacteria (Krause *et al.*, 2003). It is probably worth mentioning here that, despite methodological biases and some remarkable differences in the relative abundances of particular T-RF (e.g. 94 and 96 bp with *Msp*I), the T-RFLP technique appears to be able to reliably detect the relative changes induced to individual components of complex microbial communities (Hartmann and Widmer, 2008).

Table 4 *Relative frequencies over the total peak area (%) of some T-RF identified by T-RF length polymorphism profiles in DNA samples of ruminal digesta of sheep on days 0 (Control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of OA dosing*

Compatible bacteria	T-RF (bp)	Control	Oxa4	Oxa7	Oxa14	s.e.d.	*P*-value[1]
Bacteroidetes							
*Hha*I	91	0.30^{b}	0.63^{b}	1.90^{a}	0.36^{b}	0.478	*
	100	19.8	16.2	16.1	15.1	3.86	ns
*Msp*I	87	0.50	2.47	3.96	1.02	1.319	$^{+}$
	94	0.00^{b}	10.51^{a}	11.94^{a}	0.48^{b}	3.933	**
	96	14.33^{a}	3.33^{b}	0.15^{b}	12.43^{a}	2.572	***
*Hae*III	263	5.78	9.29	6.30	7.18	1.390	$^{+}$
	264	8.88	8.81	7.82	7.14	1.628	ns
Lachnospiraceae							
*Msp*I	148	1.40^{a}	0.56^{b}	0.28^{b}	0.85^{ab}	0.370	*
*Hae*III	244	1.32^{a}	0.55^{b}	0.59^{b}	0.43^{b}	0.292	*
Clostridiales (Clostridium sticklandii)							
*Msp*I	485	2.05^{ab}	0.97^{c}	1.16^{bc}	2.37^{a}	0.437	*
*Hae*III	238	3.03	4.22	3.45	1.98	1.136	ns
Ruminococcaceae							
*Hha*I	577	5.69	0.97	3.78	5.81	1.901	$^{+}$
*Msp*I	297	2.71^{a}	1.44^{c}	1.59^{bc}	2.12^{ab}	0.305	**
*Hae*III	273	5.17	4.71	3.36	4.23	1.485	ns
	317	1.41	0.95	0.98	1.67	0.595	ns
Streptococcus							
*Hha*I	579	3.88^{b}	12.81^{a}	4.14^{b}	1.49^{b}	2.914	**
*Hae*III	263	5.78	9.29	6.30	7.18	1.390	$^{+}$
Oxalobacter formigenes							
*Hha*I	567	6.94	5.38	5.46	6.00	0.788	ns
*Msp*I	489	2.66	1.22	1.01	2.73	0.755	$^{+}$
*Hae*III	199	0.17	0.09	0.25	0.16	0.140	ns

T-RF = terminal restriction fragment; OA = oxalic acid.
[1]Probability of significant effect due to oxalic acid administration (Control, Oxa4, Oxa7 and Oxa14): ns = not significant ($P > 0.10$); $^{+}P < 0.10$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
[a,b,c]Mean values with different superscripts in each row differ significantly ($P < 0.05$).

Table 5 *Abundance of the oxc gene DNA of Oxalobacter formigenes quantified by real-time PCR (expressed as percentage of the total bacteria 16S rDNA, or as specific DNA concentration) in the ruminal digesta of sheep on days 0 (Control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of oxalic acid dosing*

	Control	Oxa4	Oxa7	Oxa14	s.e.d.	*P*-value[1]
%	0.002	0.003	0.007	0.007		
(transformed values)	(−4.917^{c})	(−4.509^{b})	(−4.186^{ab})	(−4.136^{a})	(0.199)	(***)
pg/ng total DNA	1.98	6.26	8.20	9.11		
(transformed values)	(0.175^{b})	(0.738^{a})	(0.905^{a})	(0.932^{a})	(0.168)	(**)

[1]Probability of significant effect due to oxalic acid administration (Control, Oxa4, Oxa7 and Oxa14): $**P < 0.01$; $***P < 0.001$.
[a,b,c]Mean values with different superscripts in each row differ significantly ($P < 0.05$).

Although different oxalotroph bacteria have been isolated from the digestive tract of humans (e.g. *Eubacterium lentum* or *Enterococcus faecalis*; Sahin, 2003; Abratt and Reid, 2010), rumen degradation of OA has been considered to rely only on *O. formigenes* (Dawson *et al*., 1980; Daniel *et al*., 1989). In this study, however, none of the three T-RFs that may correspond to *O. formigenes* (567 with *Hha*I, 489 with *Msp*I, and 199 with *Hae*III) was significantly increased by OA administration. Nevertheless, it is possible that other bacteria of the phylum *Firmicutes*, a broad and diverse rumen microbial group (Edwards *et al*., 2004), may result in similarly sized fragments and mask potential changes in *O. formigenes*. Moreover, T-RFLP may miss populations present in small proportions, unlike real-time PCR, which is more sensitive and accurate. In fact, results from the latter technique showed a low abundance of *O. formigenes* and a rapid increase with OA administration.

Real-time PCR analysis was based on the measurement of the abundance of the gene encoding oxalyl-CoA decarboxylase (*oxc* gene) in *O. formigenes* (Jiang *et al*., 2011). As these bacteria use that enzyme for the breakdown of OA (Allison *et al*., 1985), changes in the relative abundance of

the *oxc* gene would be a direct reflection of variations in their relative abundance in the rumen. As shown in Table 5, *oxc* gene represented $<$0.007% of total bacterial 16S rDNA, which was expected given the likely differences in the number of copies of both genes in the bacterial cell (Khammar *et al*., 2009), the dependency of *O. formigenes* on OA as source of energy, and the low intake of this compound in animals fed conventional diets. This agrees with earlier studies reporting very low proportions of oxalate degraders, likely *O. formigenes*, in the rumen (on average, 0.38% of total bacteria; Daniel *et al*., 1989). Furthermore, on the basis of previous measurements (Jiang *et al*., 2011) reporting that 1 ng DNA of *oxc* equals 4.82×10^5 cells of *O. formigenes*, the mean number of cells in the rumen of sheep was estimated to range between 0.2 and 4.1×10^8 cells/g after 0 or 14 days of OA administration, respectively. According to the rates of OA degradation in sheep reported by Duncan *et al.* (1997), probably a little proportion of OA escaped ruminal breakdown. Notwithstanding, if all the OA were metabolized by *O. formigenes*, the rates of degradation per cell would be up to 0.06 pmol/day, which is well below the maximum capacity estimated by Daniel *et al.* (1989) in these bacteria (0.94 pmol/day). This supports that rumen *O. formigenes* would be capable of detoxifying the oxalates consumed by sheep and may represent an example of a single organism taking an extremely specialized metabolic role in the presence of many other species (Wallace, 2008).

Conclusion

The administration of OA altered the rumen environment, including the bacterial community. However, the slight changes observed in certain ruminal fermentation and degradation parameters did not seem to compromise the rumen function. The modifications of the bacterial community structure were interestingly reversed in $<$14 days, suggesting a speedy adaptation of the ruminal microbiota to the consumption of this compound. This rapid adaptation was likely linked to the rapid increase in the abundance of *O. formigenes*, which is assumed to be responsible for oxalate breakdown.

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