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Effect of oxalic acid on rumen function and microbiota in sheep fed a low quality diet

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Abstract. Oxalic acid is a potentially toxic compound present in many plants that can be consumed by ruminants in some less-favoured areas. However, consequences of its consumption on the rumen function and microbiota remain unclear. Five ewes receiving low quality grass hay were daily dosed 0.6 mmol of oxalic acid/kg body weight through a rumen cannula for 14 days. On days 0 (before starting the dosing), 4, 7 and 14 of administration, alfalfa hay and barley straw were in situ incubated, and samples of rumen digesta were collected throughout the day (0, 3, 6 and 9 h after morning meal). The rumen bacterial community was studied using the terminal restriction fragment length polymorphism (T-RFLP) technique. Oxalic acid administration reduced the dry matter disappearance of alfalfa hay on days 7 and 14 and of barley straw on day 7. Neither pH values nor total volatile fatty acid concentrations were affected. Nevertheless, ammonia and butyrate concentrations were reduced and molar proportions of acetic and propionic acids were increased. Although oxalic acid did not modify rumen bacterial diversity, it altered the structure of the community and the relative frequencies of a number of TR-fragments over the total peak area. Most of these changes were reversed at the end of the experiment (on day 14). Therefore, despite the slight negative effect on ruminal degradation, the lack of a clear detrimental effect on rumen fermentation and the recovery of the initial values in some parameters suggest an adaptation of the ruminal microbiota within 2 weeks.

Keywords. Oxalate – Rumen fermentation – Secondary compound – T-RFLP.

Effets de l'acide oxalique sur le fonctionnement du rumen et le microbiote chez les ovins alimentés avec un régime de faible qualité

Résumé. *L'acide oxalique est un composé potentiellement toxique présent dans de nombreuses plantes qui peuvent être consommées par les ruminants dans des zones défavorisées. Cependant, ses effets sur la fermentation ruminale et le microbiote restent inconnus. Dans l'objectif d'étudier ces effets, cinq brebis canulées au niveau du rumen et alimentées avec une ration de faible qualité recevaient quotidiennement et durant 14 jours, 0,6 mmol d'acide oxalique/kg de poids vif administré à travers la fistule. Avant l'ajout de l'acide oxalique (jour 0) et durant la période d'administration (jours 4, 7 et 14), 3 aliments (foin de luzerne, paille d'orge et épinards) ont été incubés* in situ*, et une collecte* in vivo *des échantillons du contenu ruminal a été réalisée tout au long de la journée (0, 3, 6 et 9 heures après l'administration d'acide oxalique). La communauté bactérienne du rumen a été étudiée en utilisant la technique moléculaire d'étude du polymorphisme de longueur de fragments de restriction terminaux (T-RFLP). L'administration de l'acide oxalique a réduit la disparition de la matière sèche du foin de luzerne les jours 7 et 14, et celle de la paille le jour 7. Toutefois, ni le pH ni la concentration des AGV totaux ont varié significativement, bien que les concentrations en ammoniac et butyrate aient été réduites et les proportions molaires des acides acétique et propionique augmentées. Malgré que la diversité bactérienne n'ait pas varié significativement, l'administration de l'acide oxalique a causé une altération de la structure de la communauté bactérienne du rumen et des fréquences relatives de nombreux pics de fragments de restriction terminaux. Cependant, la plupart de ces changements n'ont pas été révélés en fin d'expérimentation. Ainsi, malgré le faible effet négatif sur la dégradation ruminale, l'absence d'un effet négatif clair sur les fermentations du rumen et le retour aux valeurs initiales de certains paramètres suggèrent que la communauté bactérienne du rumen serait capable de s'adapter à la présence d'acide oxalique en moins de 14 jours.*

Mots-clés. *Acide oxalique – Fermentation ruminale – Composé secondaire – T-RFLP.*

I – Introduction

Oxalic acid is a simple organic acid that is present in a range of plant species commonly consumed by ruminants in some less-favoured areas (Ben Salem *et al.*, 2010). Renal toxicity and hypocalcaemia may occur after the consumption of a substantial quantity of oxalic acid-containing plants (Von Burg, 1994). However, this plant secondary compound is known to be degraded in the rumen by microorganisms to yield formic acid and CO_2 (Allison *et al.*, 1985). *Oxalobacter formigenes* is considered as the main oxalate-degrading bacteria in the gastrointestinal tract, although other digestive microbes are also able to metabolise this compound (Sahin, 2003; Abratt and Reid, 2010). Gradual exposure to increasing levels of oxalic acid leads to an adaptation of the rumen microbiota, the breakdown of this compound preventing its detrimental consequences (Duncan *et al.*, 1997; Duncan *et al.*, 2000). Nevertheless, the action of oxalic acid on the ruminal function and microbial community composition remains unclear. Therefore, this work was carried out to study, in sheep, the effects of oxalic acid consumption on rumen fermentation, utilization of feedstuff and rumen bacterial structure.

II – Materials and methods

Five adult ewes (80 ± 13.9 kg body weight; BW), fitted with a rumen cannula, were individually penned and fed a low quality grass hay for a 2.5 week adaptation period. Then, animals received daily a dose of 0.6 mmol of oxalic acid (Sigma-Aldrich, Germany)/kg BW through the cannula for 14 days. The administration was gradually increased (from 20 to 100%) during the first 5 days. On days 0 (before starting the dosing; Control), 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) of administration, two different feeds (alfalfa hay and barley straw) were *in situ* incubated in nylon bags, for 12 and 24 h, to assess ruminal degradation, and samples of rumen digesta were collected throughout the day (*i.e.*, immediately before morning meal and 3, 6 and 9 hours later) to study *in vivo* ruminal fermentation parameters (pH, and concentrations of ammonia and volatile fatty acids, VFA) and the bacterial community structure. Samples were strained through two layers of muslin cloth and rumen fluid was used to measure the pH and for ammonia (4 mL, acidified with 4 mL 0.2 M HCl) and 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. All these samples were stored at -30 ºC until analysis. For microbial studies, the same volume of rumen contents were taken at the indicated times, composited daily for each animal and immediately frozen at -80 ºC.

Dry matter (DM) in feeds and in *in situ* incubation residues was determined at 100ºC to constant weight. Ammonia concentration was analysed by a spectrophotometric method and VFA by gas chromatography, using crotonic acid as an internal standard, as reported in Toral *et al.* (2009). For microbial studies, stored rumen samples were freeze-dried and thoroughly mixed before DNA extraction (Belenguer *et al.*, 2010). Duplicate DNA samples were combined and used as templates for terminal restriction fragment length polymorphism (T-RFLP) analysis, which was performed using a universal bacteria-specific primer pair set and the enzymes *HhaI* and *MspI*, as described in Belenguer *et al.* (2010). 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 900-R (GE Healthcare Life Sciences, Buckinghamshire, UK) and using the GeneMarker Analysis software (SoftGenetics, USA). Sample data, which consisted of size (base pair, bp) and peak area for each T-RF, were standardized and used to determine the number of T-RF (richness) and the Shannon-Weiner index (Hill *et al.*, 2003). In order to infer the potential bacterial composition in the samples, *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).

From the T-RFLP results, the relative abundance of each fragment over the total peak area was calculated. These data, the diversity indices, and the rumen degradation and fermentation parameters (pH, VFA and ammonia) were analysed by one-way ANOVA using the SAS software package version 9.2 (SAS Institute Inc., USA). The bacterial profiles obtained from T-RFLP were analysed by hierarchical clustering with the Ward's method based on Jaccard distances to build a dendrogram. This was performed with the Community Analysis Package 4 software (Pisces Conservation Ltd., UK).

III – Results and discussion

The effect of the administration of oxalic acid on DM disappearance of alfalfa hay and barley straw, and on the rumen fermentation parameters (daily means) are presented in Table 1. Dry matter disappearance of alfalfa hay, after 12 h of incubation, was reduced (P<0.01) on days 7 and 14, but this effect was not significant after 24 h incubation. The degradation of straw was significantly decreased (P<0.01) on day 7 in 24 h incubation and the initial values were recovered by the end of the study (day 14). The reduction in the degradation of both feeds might be related to an effect of oxalic acid on rumen microbiota (James *et al.*, 1967), although fibrolytic bacteria seemed to have adapted to the presence of oxalic acid at the end of the experiment, as reflected by the recovery of the initial values of DM disappearance of straw.

Table 1. Dry matter disappearance (DMD) of alfalfa hay and barley straw incubated *in situ* for 12 and 24 h, and rumen fermentation parameters (pH, ammonia and VFA) in cannulated sheep fed a low quality diet before (day 0; Control) and after 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) days of administration of 0.6 mmol oxalic acid/kg BW

		Treatment				SED†	P
		Control	Oxa4	Oxa7	Oxa14		
DMD 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
DMD 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	**
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
Molar proportions (%)							
Acetic		66.9^{b}	67.5^{b}	68.3^{a}	68.6^{a}	0.65	***
Propionic		18.9^{c}	20.7^{a}	19.6^{b}	19.4^{bc}	0.59	***
Butyric		11.0^{a}	9.2^{c}	9.6^{b}	9.4^{bc}	0.39	***
Minor VFA††		2.81^{a}	2.36^{b}	2.12^{b}	2.13^{b}	0.176	***

†Standard error of the difference.
††Calculated as the sum of valeric, isobutyric, isovaleric and caproic acids.
NS, non significant (P<0.10); **, P<0.01; ***, P<0.001. Values in a row with different superscripts are significantly different (P<0.05).

Oxalate reduced the ammonia concentrations (P<0.001), whereas the variations in pH and total VFA concentrations were not significant (P>0.05). However, some specific VFA were significantly altered, with increases in the molar ratios of acetic and propionic acids and reductions in the concentrations (data not shown) and molar proportions of butyric and minor VFA (sum of valeric, isobutyric, isovaleric and caproic, P<0.001). These minor VFA originate mostly from deamination of specific amino acids, so their decrease, together with the lower ammonia concentration, is consistent with an inhibitory effect of oxalic acid on rumen

proteolysis. The lack of effects on the total VFA would indicate that rumen fermentation was not negatively affected, although the changes in molar proportions of VFA reflects divergences in fermentation pathways, probably due to stimulation of specific microbial groups to the detriment of others or to a shift in microbial degradation pathways (Russel and Wallace, 1997).

Even though diversity indices (richness and Shannon-Weiner) were not altered significantly (P>0.10), the administration of oxalic acid modified the bacterial structure in the rumen, as observed in Fig. 1. T-RFLP profiles in samples from days 0 and 14 were grouped together and separated from those from days 4 and 7, indicating that the bacterial community was able to adapt to the presence of oxalic acid within 14 days.

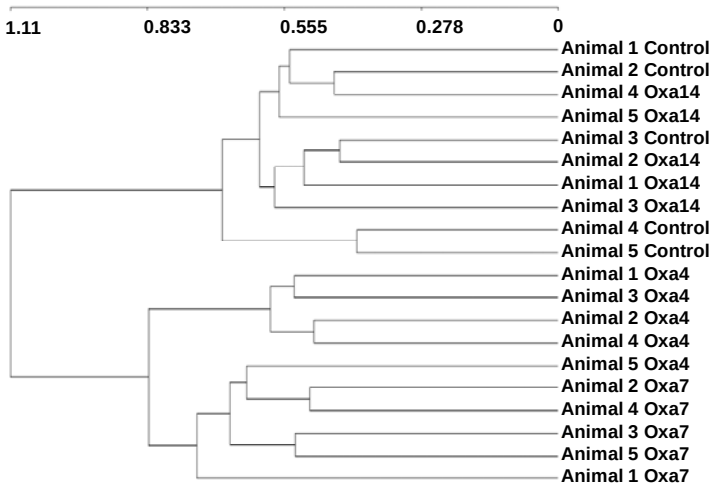

Fig. 1. Cluster analysis of the T-RFLP profiles of total bacteria present in the rumen contents of cannulated sheep fed a low quality diet before (day 0; Control) and after 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) days of administration of 0.6 mmol oxalic acid/kg BW.

The relative frequencies over the total peak area of several T-RF were significantly affected by the treatment (Table 2). Surprisingly, the T-RF compatible with *O. formigenes* obtained with *HhaI* (567 bp) showed no significant variations and those obtained with *MspI* (486 + 489 bp) decreased their relative abundance on days 4 and 7. Nonetheless, it is important to consider that other bacteria belonging to the orders *Bacteroidales* and *Clostridiales,* which are abundant in the rumen (Edwards *et al.*, 2004), may also match those fragments. Therefore, concomitant variations in these microorganisms might have masked the potential variations in *O. formigenes*. Although the latter is the only functional oxalotrophic bacteria isolated from the gastrointestinal tract (Sahin, 2003), other digestive bacteria are also able to degrade oxalates (Abratt and Reid, 2010). Unfortunately, the methodology used does not allow discerning which bacterial populations may or may not be responsible for the degradation of the oxalic acid. The initial abundance of some other detected T-RF (*e.g.*, 579 bp with *HhaI* and 91 bp with *MspI*) was recovered by the end of the experiment, supporting the hypothesis that the bacterial community would be adapted to oxalic acid consumption within 14 days.

IV – Conclusion

Overall, the results show that oxalic acid alters the pattern of ruminal fermentation and the bacterial community structure. Nonetheless, despite the slight negative effect on feed

degradation, the lack of detrimental consequences on ruminal fermentation and the recovery of the initial values in some parameters suggest that the rumen microbiota was able to adapt to the presence of this secondary compound in less than 14 days.

Table 2. Relative abundances (%) over the total peak area of several fragments obtained by T-RFLP analysis of microbial DNA samples extracted from the rumen contents of cannulated sheep fed a low quality diet before (day 0; Control) and after 4 (Oxa4), 7 (Oxa7) and 14 (Oxa14) days of administration of 0.6 mmol oxalic acid/kg BW

Enzyme	Length (bp)	Treatment				SED†	P
		Control	Oxa4	Oxa7	Oxa14		
HhaI	321	1.61^{a}	0.19^{b}	0.00^{b}	0.16^{b}	0.200	***
	567	5.63	5.38	5.60	6.12	1.239	NS
	577	5.97	1.12	3.88	6.00	2.000	T
	579	3.94^{b}	12.83^{a}	4.13^{b}	1.53^{b}	2.938	**
MspI	91	3.87^{a}	0.78^{c}	2.04^{bc}	3.34^{ab}	0.767	**
	95	15.01	4.26	12.5	11.76	3.760	T
	261	2.31	7.09	4.25	2.36	1.908	T
	486+489	3.92^{ab}	2.54^{b}	2.53^{b}	5.45^{a}	0.806	**

†Standard error of the difference.
NS, non significant (P>0.10); T, P<0.10; **, P<0.01; ***, P<0.001. Values in a row with different superscripts are significantly different (P<0.05).

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References

Abratt V.R. and Reid S.J., 2010. Oxalate-degrading bacteria of the human gut as probiotics in the management of kidney stone disease. In: *Advances in Applied Microbiology*, 72, p. 63-87.

Allison M.J., Dawson K.A., Mayberry W.R. and Foss J.G., 1985. *Oxalobacter formigenes* gen. Nov., sp. Nov.: Oxalate-degrading anaerobes that inhabit the gastrointestinal tract. In: *Archives of Microbiology*, 141, p. 1-7.

Belenguer A., Toral P.G., Frutos P. and Hervás G., 2010. Changes in the rumen bacterial community in response to sunflower oil and fish oil supplements in the diet of dairy sheep. In: *Journal of Dairy Science*, 93, p. 3275-3286.

Ben Salem H., Norman H.C., Nefzaoui A. *et al.,* 2010. Potential use of oldman saltbush (Atriplex mummularia Lindl.) in sheep and goat feeding. In: *Small Ruminant Research*, 91, p. 13-28.

Cole J.R., Wang Q., Cardenas E. et al., 2009. The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis. In: *Nucleic Acids Research*, 37, p. D141–D145.

Duncan A.J., Frutos P. and Young S.A., 1997. Rates of oxalic acid degradation in the rumen of sheep and goats in response to different levels of oxalic acid administration. In: *Animal Science*, 65, p. 451-456.

Duncan A.J., Frutos P. and Young S.A., 2000. The effect of rumen adaptation to oxalic acid on selection of oxalic-acid-rich plants by goats. In: *Britich Journal of NutrItion*, 83, p. 59-65.

Edwards J.E., McEwan N.R., Travis A.J. and Wallace, R.J., 2004. 16S rDNA library-based analysis of ruminal bacterial diversity. In: *Antonie van Leeuwenhoek*, 86, p. 263-281.

Hill T.C.J., Walsh K.A., Harris J.A. and Moffett B.F., 2003. Using ecological diversity measures with bacterial communities. In: *FEMS Microbiology Ecology*, 43, p. 1-11.

James L.F., Street J.C. and Butcher J.E., 1967. In vitro degradation of oxalate and of cellulose by rumen ingesta from sheep fed *Halogeton glomeratus*. In: *Journal of Animal Science*, 26, p. 1438-1444.

Russell J.B. and Wallace R.J., 1997. Energy-yielding and energy-consuming reactions. In: Hobson P.N and Stewart C.S. *The Rumen Microbial Ecosystem*. London, UK: Chapman and Hall, p. 246-282.

Sahin N., 2003. Oxalotrophic bacteria. In: *Research in Microbiology*, 154, p. 399-407.

Toral P., Belenguer A., Frutos P. and Hervás G., 2009. Effect of the supplementation of a high-concentrate diet with sunflower and fish oils on ruminal fermentation in sheep. In: *Small Ruminant Research*, 81, p. 119–125.

Von Burg, R., 1994. Toxicology update. Oxalic acid and sodium oxalate. In: *Journal of Applied Toxicology*, 14, p. 233-237.