Effects of forage species, grass water soluble carbohydrates and red clover polyphenol oxidase activity on the *in vitro* rumen microbial ecosystem

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Introduction Novel varieties of perennial ryegrass with high water soluble carbohydrate (WSC) concentrations have been bred to increase energy availability in the rumen. In red clover (RC) the activity of polyphenol oxidase (PPO) can help protect protein in the rumen by decreasing or delaying proteolysis. Both factors (WSC and PPO) offer potential to improve the synchronization or balance between energy and nitrogen availability for rumen microorganisms and consequently to optimise rumen microbial synthesis. However, it remains unclear if these effects can be attributed to differences in diet composition or to changes in the rumen microbial ecosystem. The objective of this *in vitro* experiment was to study how the rumen microbiota was affected by diet WSC content and PPO activity.

Material and methods Two ryegrass varieties, bred to express high and normal (control) WSC concentrations (HWSC *vs.* LWSC), and two strains of RC, wild type with normal PPO activity (PPO+) and a PPO knockout strain with undetectable PPO activity (PPO-), were used. The experiment was carried out using sixteen continuous culture rumen simulation technique (Rusitec) systems allocating 4 vessels per treatment that were inoculated with fresh rumen contents. Ryegrass was cut from outdoor plots daily (at approximately 09:00h) and RC had been previously grown in pots in growth chambers to collect sufficient material; it was then defrosted to help to activate the PPO enzyme. Each day new plant material (50 g fresh matter/d) was added in one of two nylon bags in each Rusitec vessel, where it remained for 48h. Artificial saliva was infused continuously to obtain a liquid dilution rate of 3.65%/h. After 9 days of adaptation the fermentation vessels were sampled by aspiration for 3 consecutive days at 2, 4, 8 and 24h after 'feeding'. DNA was extracted from lyophilised samples and the bacterial biodiversity was analysed by the terminal restriction fragment polymorphism (TRFLP) procedure using 4 different restriction enzymes (HAE3, RSA1, MSP1 and HHA1). A dendrogram was created using the Bray-Curtis average linkage clustering method. Absolute bacterial and protozoal DNA concentrations were determined by quantitative PCR (qPCR), while the relative abundance of different species was estimated using the $\Delta\Delta C_T$ method. Samples from 2 and 4h (<4h) or from 8 and 24h after feeding (>8h) were considered representative of early or later rumen fermentation respectively. Data were analyzed by ANOVA with blocking by machine and using three orthogonal contrasts to separately determine the effect of forage (C1; grass *vs.* RC), WSC content (C2; HWSC *vs.* LWSC) and PPO activity (C3; PPO+ *vs.* PPO-).

Results Forage species had a significant effect on rumen bacterial biodiversity and clustering, with RC promoting a higher diversity than ryegrass. No differences were attributed to WSC content or PPO activity. Quantitative PCR agreed with the TRFLP results, with significant forage effects on the concentrations of most of the microorganisms studied. Within the ryegrasses, WSC concentration had no effect on the rumen ecosystem, and RC PPO activity only increased bacterial concentrations in the later fermentation times, possibly because of a higher proportion of slowly available N in PPO+ diets.

Table 1 Abundance of different microbial groups determined by qPCR

		Ryegrass		Red clover		SED	Significance		
		HWSC	LWSC	PPO+	PPO-	*n=4*	C1	C2	C3
Abundance (µg/g DM)									
Bacterial	<4h	808	829	610	558	64.5	***	NS	NS
	>8h	531	516	561	436	42.9	NS	NS	*
Protozoa	<4h	9.0	8.3	7.3	10.2	3.40	NS	NS	NS
	>8h	10.1	7.2	10.2	10.3	3.32	NS	NS	NS
Relative abundance (%)									
R. albus	<4h	0.22	0.24	9×10^{-3}	0.01	0.04	***	NS	NS
	>8h	0.19	0.19	7×10^{-3}	7×10^{-3}	0.02	***	NS	NS
F. succinogenes	<4h	2.69	2.96	1×10^{-3}	1×10^{-3}	0.36	***	NS	NS
	>8h	2.02	1.91	8×10^{-6}	4×10^{-6}	0.28	***	NS	NS
B. fibrisolvens	<4h	2.17	2.33	1.94	1.87	0.31	NS	NS	NS
	>8h	2.02	2.01	1.66	1.67	0.35	NS	NS	NS
S. ruminantium	<4h	9.76	9.90	1×10^{-6}	1×10^{-5}	1.87	***	NS	NS
	>8h	6.22	6.52	2×10^{-7}	2×10^{-8}	1.18	***	NS	NS
Anaerobic fungi	<4h	1.22	1.27	3×10^{-3}	4×10^{-3}	0.20	***	NS	NS
	>8h	0.80	0.79	4×10^{-4}	1×10^{-3}	0.17	***	NS	NS
Biodiversity (TRFs/enz.)		55.0	55.4	59.4	60.3	2.27	**	NS	NS

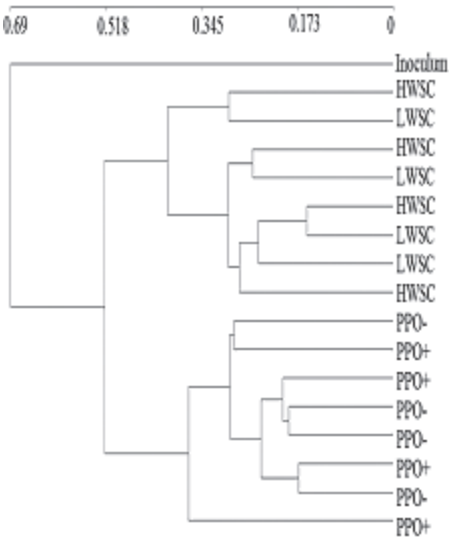

Figure 1 Effect of diet on bacterial biodiversity determined by TRFLP.

* P<0.05; ** P<0.01; *** P<0.001; Contrasts, effects of: C1 = type of forage; C2 = WSC concentration; C3 = PPO activity.

Conclusion Type of forage (ryegrass *vs.* RC) led to significant differences in the rumen microbial population. No effects of WSC concentration, and only minor effects of PPO activity, were observed under our experimental conditions.

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