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Original article

Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents

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Abstract — Four sheep were fed an alfalfa hay diet. Rumen content samples were collected three hours after feeding in order to total microorganism population (TP), solid attached population (SAP) and solid attached firmly population (SAFP). Fibrolytic specific activities (xylanase, CMCase and β-glycosidases) were estimated by the amount of reducing sugars or *p*-nitrophenol released from the appropriate substrate. The distribution of the three main cellulolytic bacterial species (*Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*) was quantified by dot-blot hybridisation using specific 16S-rRNA-targeting probes. Specific activities of polysaccharidase enzymes were higher in SAP than in TP, and in SAFP than in SAP. The sum of RNA of the three cellulolytic bacterial species represented on average 9% of the total bacterial RNA, and increased after filtration. In all samples, the relative population size of *F. succinogenes* was higher than that of *R. albus* and of *R. flavefaciens*. These results demonstrate that the most active enzymes are secreted by the particle-associated microorganisms. The differences in composition of the microflora between the solid and liquid phase suggest that bacteria are not equally distributed throughout the rumen content: the cellulolytic species are present in a higher proportion in the solid phase of rumen contents.

rumen content / fibrolytic activity / cellulolytic bacteria / oligonucleotide probe

Résumé — **Répartition de l'activité fibrolytique microbienne et des bactéries cellulolytiques entre phases solides et liquides du contenu ruminal.** Quatre moutons, munis d'une canule du rumen, ont reçu un régime à base de foin. Des échantillons de contenus ruminaux étaient prélevés 3 h après la distribution du repas afin d'isoler la population totale de micro-organismes (TP), la population de micro-organismes associés à la phase solide du contenu (SAP) et celle fermement associée à cette phase solide (SAFP). Les activités fibrolytiques spécifiques (xylanase, CMCase et β-glycosidases) étaient estimées

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à partir de la quantité de sucres réducteurs ou de *p*-nitrophenol libéré. La distribution des 3 principales espèces bactériennes cellulolytiques (*Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens*) était déterminée par hybridation sur membrane en utilisant des sondes spécifiques de la région ciblant l'ARNr–16S. Les activités spécifiques des enzymes polysaccharidasiques étaient plus élevées dans TP que dans SAP, et dans SAFP que dans SAP. La somme des 3 espèces bactériennes cellulolytiques représentaient en moyenne 9 % de l'ARN bactérien total, et augmentaient après filtration. Dans tous les échantillons, *F. succinogenes* était mieux représenté que *R. albus* et que *R. flavefaciens*. Ces résultats montrent que les enzymes les plus actives sont sécrétées par les microorganismes associés à la phase particulaire du contenu ruminal. Les différences de composition de la microflore ruminale entre phase solide et phase liquide suggèrent que les espèces cellulolytiques sont également en proportion plus importante dans la phase solide.

contenu du rumen / activité fibrolytique / bactérie cellulolytique / sonde oligonucléotide

1. INTRODUCTION

Knowledge of the mechanisms of digestion in the rumen, in particular those leading to the degradation of plant cell wall polysaccharides, requires a study of the microbial ecosystem. The ruminal microbial fraction consists largely of bacteria and protozoa, and the solid attached microbial population (SAP) seems to play a major role in ruminal forage digestion in terms of mass [3] as well as in terms of enzymatic activity [13, 24]. The bacterial contribution to nutrient flows at the duodenum has mainly been calculated from a ruminal free-floating bacteria sample, or more recently from SAP [7, 8] or from a mix of liquid- and solid-associated bacteria [2]. There were large differences in chemical composition [3, 10, 17] and enzymatic activities [13] between protozoa and bacteria, liquid- and solid-associated bacteria. Do these differences correspond to variations in bacterial community structure or not?

To provide some answers to this question, in this experiment we studied the distribution of microbial fibrolytic activities in the rumen digesta ecosystem in relation to the composition of the cellulolytic bacterial community involved in the polysaccharide degradation process.

2. MATERIALS AND METHODS

2.1. Animals and feeding

Four Texel sheep fitted with ruminal cannulae were fed an alfalfa hay diet (1 500 $g \cdot d^{-1}$). The animals were fed twice daily at 08:00 and 17:00 h. They also received a mineral-vitamin block and were given free access to water. An adaptation period of three weeks was allowed before the measurements were made.

2.2. Isolation of the solid-associated microorganisms

Approximately 300 g of a representative sample of rumen content was collected manually via the cannula 3 h post-feeding, and separated into three parts for the isolation of total microorganism population (TP), solid attached population (SAP) and solid attached firmly population (SAFP). The first fraction, corresponding to TP, was not treated. The two other fractions were strained through a 100 µm nylon filtre, under a streamed CO_2, and the solid phase sample, obtained by filtration, was either directly used to study SAP, or washed with anaerobic salt solution (pH 6.5; 39 °C, 1 g fresh weight of solids in 5 mL diluent [4]) to remove the loosely adherent microbial population, and then recovered by filtration (100 µm) to study SAFP.

2.3. Enzyme preparation and activity measurement

In each fraction, enzyme extraction from microorganisms was performed under anaerobic conditions according to the method detailed by Martin and Michalet-Doreau [12]. A sample (5 g) of digesta containing microorganisms was chopped under anaerobic conditions and suspended in 25 mL of anaerobic buffer containing 2- (N-morpholino) ethane sulfonic acid (MES) buffer (0.025 mol·L^{-1}, pH 6.5, 4 °C) and stored at –80 °C. The microbial cells present in the homogenate were disrupted by defrosting and sonication (Labsonic U, B. Braun Biotech Inc, Bethlehem, PA, USA) for four 30-s periods at 4 °C. The remaining solids were removed by centrifugation (15000 × *g* for 15 min at 4 °C) and the supernatant used as the enzyme preparation.

Fibrolytic activities (polysaccharidase and glycosidase) were measured using the assay procedures detailed by Nozière and Michalet-Doreau [15]. Polysaccharidase activities were determined by measuring the amount of reducing sugars released from the polysaccharide purified substrates (Birchwood-xylan, Sigma X-0502; carboxymethylcellulose, sodium salt, low viscosity, Sigma C-5678; 2 mg·mL^{-1} in 0.025 mol·L^{-1} MES, pH 6.5) during a 60 min incubation at 39 °C. Reducing sugars were quantified spectrophotometrically at 410 nm, using the *p*-hydroxybenzoic acid hydrazide method [11] with xylose and glucose as standards for xylan and CMC assays, respectively. Glycosidase activities were assayed by measuring the rate of *p*-nitrophenol released from the specific substrate (Sigma, *p*-nitrophenol-β-glucopyranoside N-7006; -xylopyranoside N-1232; 5 mmol·L^{-1} in 0.025 mol·L^{-1} MES; pH 6.5) after incubation for 30–60 min at 39 °C. The amount of *p*-nitrophenol released was quantified spectrophotometrically at 420 nm. Control assays were performed simultaneously on the substrate and enzyme preparation to adjust for spontaneous substrate breakdown or formation of non-specific products. The protein content of the enzyme preparation was determined by the Pierce method [18] using bovine serum albumin standards. Specific activity was expressed in μmol of reducing sugars (polysaccharidases) or mmol of *p*-nitrophenol (β-glycosidases) released per mg protein per h.

2.4. Nucleic acid extraction, hybridisation and labelling of the oligonucleotide probes

The structure of the ruminal cellulolytic bacteria community in the microbial ecosystem was studied with specific oligonucleotide probes targeting specific 16S-rRNA [20].

In each fraction of rumen digesta, RNA was extracted from 50 mg of freeze-dried sample after the mechanical disruption of microorganisms with zirconium beads [20]. The cell pellet was taken up in 1 mL of RNAzol (Bioprobe Systems, Montreuil-sous-Bois, France). The mixture was shaken for 2 min, incubated for 2 min at 100 °C, and centrifuged (14000 *g*, 20 min). After two extractions with an equal volume of $CHCl_3$, RNA was recovered by isopropanol precipitation.

Hybridisations were conducted using synthetic HPLC-purified oligonucleotide probes (Eurogentec, Seraing, Belgium) that are 5' end labelled with ^{32}P using a T4 kinase (Eurogentec). Four γ-^{32}P-labelled oligonucleotide probes were used: the universal probe S-D-Bact-0338-a-A-18 targeting the bacterial domain used as a positive control, probe S-S-F.suc-0650-a-A-20 targeting *Fibrobacter succinogenes*, probe S-S-R.alb-0196-a-A-18 targeting *Ruminococcus albus*, and probe S-S-R.fla-1269-a-A-20 targeting *Ruminococcus flavefaciens* (Tab. I). The temperature used for the stringent washes are also shown.

RNA extractions were blotted on nylon membranes (Hybond N+, Amersham Pharmacia Biotech, Saclay, France) using a dot-blot apparatus (Schleicher and

Table I. Oligonucleotide probes used in this trial.

Reference	Probes	Target	Sequence (5'–3')	Tw (°C)
Stahl et al. [20]	S-D-Bact-0338-a-A-18	Eubacteria	GCTGCCTCCCGTAGGAGT	54
Stahl et al. [20]	S-S-F.suc-0650-a-A-20	*F. succinogenes*	TGCCCCTGAACTATCCAAGA	48
Odenyo et al. [16]	S-S-R.alb-0196-a-A-18	*R. albus*	GTCATGCGGCTTCGTTAT	46
Odenyo et al. [16]	S-S-R.fla-1269-a-A-20	*R. flavefaciens*	TTCTCTTTGTTAATTGCCAT	45

Schuell Co., Dassel, Germany). Membranes were incubated for 1 h at 40 °C with 3 mL of hybridisation buffer [0.9 M NaCl; 50 mM $NaPO_4$, pH 7.2; 5 mM EDTA, pH 7.0; 10× Denhart solution and 0.5 mg of poly (A) per mL] before the addition of labelled oligonucleotide to about 2×10^5 cpm per sample dot. Incubation was continued for 3 h at the same temperature, and filtres were washed in SSC 1× (150 mM NaCl, 1.5 mM sodium citrate) –1% sodium dodecyl sulfate for 15 min, at the temperature indicated in the Table I. Membranes were dried and exposed to a screen (Storm, Molecular Dynamics, Z I Les Bordes, CE 2318, 11 rue Madiot, 91923 Bondoufle, France). Bound probe was quantified by Image Quant software (Molecular Dynamics).

2.5. Statistical analysis

Microorganism enzyme activities and concentrations of the rumen content were analysed as a factorial model using the GLM procedure [19], with two main factors: the nature of digesta and the animal. The sums of squares were further partitioned with orthogonal contrasts to compare the crude rumen content with the filtered samples (TP/(SAP + SAFP)), and the effect of washing (SAP/SAFP).

3. RESULTS

3.1. Fibrolytic activities

The specific activities of polysaccharidase and glycosidase involved in the degradation of structural polysaccharides were determined in TP, SAP and SAFP.

The specific activities of polysaccharide depolymerase enzymes (xylanase and CMCase) varied considerably with the nature of the sample (Tab. II). Xylanase activity increased from 9.16 to 19.73 µmol reducing sugars released per mg protein per h after filtration (TP/(SAP + SAFP)). This tendency was statistically confirmed with CMCase ($P < 0.001$). The washing thus significantly increased the specific activities of xylanase and CMCase. The highest specific activity was found with the two enzymes in the filtered washed residue (SAFP).

Levels of enzymes hydrolysing glycosidic substrates – β-D-xylosidase and β-D-glucosidase – were significantly higher in the microbial population of the solid residue remaining after filtration (SAP + SAFP) than in that of crude digesta (TP) (Tab. II). The washing of the solid residue revealed two distinct activity profiles for the glycoside hydrolases. The glycosidase enzymes involved in the degradation of hemicelluloses (β-D-xylosidase) had the same activity in the solid residue after filtration, before and after washing. The glycosidases more directly involved in cellulose breakdown (β-D-glucosidase) had a different distribution on washing. Their specific activities were increased in the treated digesta by the washing process.

3.2. Bacterial populations

Examination of the microbial community collected 3 h post-feeding revealed that

Table II. Effect of nature of the population (total population, TP, solid attached, SAP, and solid attached firmly population, SAFP) on polysaccharidase- and glycosidase-specific activities.

Treatment	TP	SAP	SAFP	SEM	Contrast	
					TP/(SAP + SAFP)	SAP / SAFP
polysaccharidase activities (µmol RS per mg protein per h)						
Xylanase	9.16	15.53	23.94	3.51	**	*
CMCase	0.91	1.27	5.61	0.22	**	***
glycosidase activities (µmol *p*-NP per mg protein per h)						
β-D-xylosidase	2.47	4.66	4.49	1.32	*	NS
β-Dglucosidase	1.32	1.83	3.14	0.34	**	**

NS: non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; RS: reducing sugars; *p*-NP: *p*-nitrophenol.

the amounts of total bacteria 16S-rRNA detected per g of DM in the solid phase were higher in the filtered rumen contents (TP/(SAP + SAFP)), but this difference was not significant (Tab. III). The sum of the RNA of the three cellulolytic bacterial species was also significantly higher after filtration (TP/(SAP + SAFP)). The total amount of RNA was respectively 36.7 and 63.7 $\mu g \cdot g^{-1}$ DM digesta (Tab. III), and its relative proportion of the total bacterial community increased from 7.5 to 9.6% (Tab. IV). This difference between filtered and digesta was mainly due to a modification in the *F. succinogenes* population (Tabs. III and IV) that was much greater than that of either *R. albus* or *R. flavefaciens*.

For any of the bacterial populations considered, no significant difference was found between the filtered residue and the filtered washed residue (SAP/SAFP) (Tabs. III and IV).

4. DISCUSSION

The amount of polysaccharidases and glycosidases in a particle-associated microbial population, isolated by filtration, was higher than in the total microbial population of the rumen. The results reveal the heterogeneity of the distribution of carbohydrate-degrading enzymes. The location and level of activity of the main polysaccharidases and

Table III. Effect of nature of the population (total population, TP, solid attached, SAP, and solid attached firmly population, SAFP) on the amount of RNA ($\mu g \cdot g^{-1}$ DM).

Treatment	TP	SAP	SAFP	SEM	Contrast	
					TP/(SAP + SAFP)	SAP / SAFP
Total bacteria	543.26	657.3	672.7	175.9	NS	NS
R. albus	4.4	6.1	4.9	1.6	NS	NS
R. flavefaciens	8.6	16.6	18	3.7	*	NS
F. succinogenes	23.7	39.1	42.7	6.2	***	NS
Sum of the 3 cellulolytic bacteria	36.7	61.8	65.6	11.9	***	NS

NS: non-significant; * $P < 0.05$; *** $P < 0.01$.

Table IV. Effect of nature of the population (total population, TP, solid attached, SAP, and solid attached firmly population, SAFP) on the proportion of the ruminal cellulolytic bacteria, expressed as a fraction of total bacteria RNA.

Treatment	TP	SAP	SAFP	SEM	Contrast	
					TP/(SAP + SAFP)	SAP / SAFP
R. albus	0.6	0.9	0.75	0.2	NS	NS
R. flavefaciens	2.1	2.5	2.2	0.4	NS	NS
F. succinogenes	4.6	6.6	6.3	1.3	*	NS
Sum of the 3 cellulolytic bacteria	7.5	10.0	9.3	1.6	*	NS

NS: non-significant; * $P < 0.05$.

glycosidases involved in the degradation of cell wall components were different in microbial populations associated with the liquid and the solid phases of rumen content. These enzymes were the most active in the particle-associated microorganisms, thus our results are in agreement with those of Williams and Strachan [23]. In the liquid phase, the specific activities of bacterial polysaccharide-degrading enzymes were lower than those of the protozoal community [13, 23]. In this trial, rumen digesta is a mixture of a complex solid mass suspended in a liquid phase. Total population activities correspond to mean activities; they are the weighted sum of enzyme profiles of different microbial populations isolated from the liquid and solid phases. Thus the fibrolytic enzyme activities in total digesta are lower than those in filtered digesta.

Polysaccharide-degrading enzyme activities in SAP were lower than in SAFP and higher than in TP, so the organisms released from the digesta by washing had lower fibrolytic activities than particle-associated microorganisms and higher than liquid phase microorganisms. Entodiniomorphs, which represent the main population of ciliates, are present in a high proportion in the rumen dorsal sac [14], and they are weakly attached and (or) sequestered within the fibrous dorsal pad [25]. As stated, enzymatic activities of protozoa were lower than those of particle-associated microorganisms and higher than those of the liquid phase bacterial population. After washing, the loosely adherent population of protozoa was almost completely eliminated, with only 1–2% of the protozoa in the original fluid being retained in the solid fraction of digesta [5], and this removal induced an increase in polysaccharidase activities in filtered washed digesta.

In our trial, the amount of bacteria associated with the rumen liquid phase did not differ from that in the solid phase of the rumen content. In contrast Briesacher et al. [1], using the same technique, found that the bacterial rRNA concentration associated with the mat phase was lower than that in the fluid phase before feeding, but that three hours after feeding, there were no differences between the solid and liquid phases of the rumen content. The three predominant cellulolytic species, quantified using 16S-rRNA-targeting probes, represented on average 9% of the amount of total bacterial 16S-rRNA. This value was low, but higher than that reported by Weimer et al. [22] obtained with the same technique from rumen content sampled 3 h after feeding, that is 1.5% of the total bacterial RNA. In studies comparing different methods of evaluating relative cellulolytic population size, rRNA probes have showed results close

to those obtained with traditional cultural methods, direct counting and most-probable-number estimates [6, 9]. The sum of the RNA of the three cellulolytic bacterial species studied in this trial, in terms of amount and proportion of the total bacterial community, differed according to the nature of the sample, as it was higher in filtered digesta. These results suggest differences in the composition of microflora between the liquid and solid phases. Bacteria may not be equally distributed throughout the rumen content, with the fibrolytic species being present in a higher proportion in the rumen solid phase, and this heterogeneous distribution of bacteria could explain the higher fibrolytic activity of the solid-associated microorganisms. In contrast, the washing of rumen samples had no effect on the sum of the three cellulolytic bacterial species. These results support the previous hypothesis; the washing of digesta might facilitate the elimination of protozoa that would be entrapped in the mat of solid digesta which would explain the higher fibrolytic activity in SAFP vs. SAP. And the distribution of bacterial species in SAFP vs. SAP would not be modified.

In our trial, the relative population size of *F. succinogenes* was always higher than that of ruminococci. In dairy cows receiving mixed diets including alfalfa or corn silage and concentrate, *R. albus* was the most abundant species [22]. Krause et al. [9], with experimental material similar to that used in our experiment, reported approximately equal proportions of ruminococci and *F. succinogenes* in sheep fed a forage-based diet. Comparison of population sizes based on the proportion of species-specific RNA in the rumen content must be interpreted with care because individual strains within a given species can display marked variations in response to probes specific to their species [16]. The oligonucleotide probe used in this trial had a wide profile, identifying different strains of *F. succinogenes*. Hence the differences in the relative populations of ruminococci and *F. succinogenes* between experiments might have been due to the inter-strain differences in response to the probes. Another hypothesis is based on the possible effect of diet. Large differences between animals in the proportions of the cellulolytic species were reported by Van Glyswyk [21] and Weimer et al. [22], and individual animals would be responsible for larger differences than the changes that were brought about by diets [22]. Therefore, it is difficult to attribute differences in the cellulolytic bacterial community structure between experiments to digestive factors, animal species or the nature of the diet.

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