

Original article

Effect of anaerobic fungi on glycoside hydrolase and polysaccharide depolymerase activities, *in sacco* straw degradation and volatile fatty acid concentrations in the rumen of gnotobiotically reared lambs

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Summary — Four naturally born lambs were placed in sterile isolators 24 h after birth, before the natural establishment of the cellulolytic microorganisms. At the age of 4 weeks, a cellulolytic bacterial population of approximately 10^8 cells g^{-1} of rumen contents was established by inoculation with a 10^{-6} dilution of ruminal contents taken from an adult sheep. A pure culture of *Neocallimastix frontalis* MCH₃ and *Piromyces communis* FL was inoculated into the rumen 5 months after birth and a stable population of 10^3 – 10^4 zoospores g^{-1} developed; the cellulolytic bacteria and fungi established populations in the 4 lambs that were similar to those observed in conventional animals. The presence of fungi led to an increase in the activity of most of the glycoside and polysaccharide hydrolases of the particle-associated microbial populations. However, this effect was not accompanied by an increase in the *in sacco* degradation of wheat straw or an increase in the volatile fatty acid concentration in the rumen contents.

rumen / gnotobiotic lamb / anaerobic fungi / cellulolysis / microbial interaction

Résumé — Effet des champignons anaérobies sur l'activité des glycosides hydrolases et des polysaccharides depolymérasés, sur la dégradation *in sacco* de la paille de blé et sur la concentration en acides gras volatils dans le rumen d'agneaux gnotobiotiques. Quatre agneaux nés par mise bas naturelle ont été introduits en isolateurs stériles à l'âge de 24 h avant l'implantation naturelle des micro-organismes cellulolytiques. Ils ont ensuite été inoculés à l'âge de 3 sem avec la dilution 10^{-6} d'un contenu de rumen issu d'un mouton adulte puis vers l'âge de 5 mois avec une culture pure de *Neocallimastix frontalis* MCH₃ et de *Piromyces communis* FL. Les bactéries cellulolytiques

présentes dans l'inoculum 10^{-6} et les champignons se sont implantés dans le rumen des 4 agneaux à des niveaux comparables à ceux observés chez des conventionnels. L'implantation des champignons a induit une augmentation de l'activité de la plupart des enzymes impliquées dans la dégradation des parois végétales de la population adhérente aux particules végétales. Toutefois, cet effet n'a pas été accompagné d'une amélioration de la dégradation in sacco de la paille de blé ni d'une augmentation de la concentration en acides gras volatils du contenu de rumen.

rumen / agneau gnotobiotique / champignon anaérobie / cellulolyse / interactions microbiennes

INTRODUCTION

Lignocellulose compounds in the rumen are degraded by a diverse anaerobic microbial population comprising many species of bacteria, fungi and ciliate protozoa. Many interrelationships are known to exist between the different microbial groups. These interactions are essential for sustaining the stability of the rumen ecosystem and the collective activities of the microbial community (Wolin and Miller, 1988). Any attempt to optimize rumen function, notably experiments designed to improve degradation of the cell-wall polymers, cellulose and hemicellulose, will require a fuller understanding of the role and activity of the different hydrolytic microorganisms. There have been numerous studies on the activity of the main fungal and cellulolytic bacterial species *in vitro* (Fonty *et al*, 1988a; Orpin and Joblin, 1988; Stewart and Bryant, 1988; Fonty and Joblin, 1991) and their interactions in cellulolysis have been studied in cocultures (Bernalier *et al*, 1992, 1993; Roger *et al*, 1992, 1993; Stewart *et al*, 1992; Williams *et al*, 1994). In contrast, the role and activity of these species and their interactions *in vivo* are poorly characterized and are still incompletely understood. The only way to advance knowledge in this area is to use animals harbouring a defined and controlled rumen microbial population. However, it is difficult to rear ruminants gnotobiotically and it can be difficult to establish certain bacterial species, particularly the cellulolytic bacteria, in a rumen that has only a very simplified

microflora (Mann and Stewart, 1974; Lysons *et al*, 1976; Hobson *et al*, 1981; Fonty *et al*, 1983, 1988b). Nevertheless, the activity of the 3 main cellulolytic bacterial species, *Fibrobacter (Bacteroides) succinogenes*, *Ruminococcus flavefaciens* and *R albus*, alone or in association, and that of a fungal flora have been studied in newborn lambs (Fonty *et al*, 1988c; Fonty and Gouet, 1989). In these experiments, animals were placed in sterile isolators a few hours after birth, before the natural establishment of the cellulolytic bacterial, fungal and ciliate protozoa populations, so that they could be inoculated subsequently with a known cellulolytic flora.

The aim of the present work was use a similar animal model to study the effect of anaerobic fungi on cellulolysis and the level of activity of the enzymes involved in plant cell-wall degradation and the concentration of volatile fatty acids (VFA) in ruminal contents.

MATERIALS AND METHODS

Animals

Four lambs born naturally were left with their dams for 24 h and placed in sterile isolators before the natural establishment of cellulolytic microorganisms and then reared gnotobiotically (Fonty *et al*, 1987, 1989). Until 70 d of age they received cows' UHT-sterilized milk. From the age of 6 weeks they also received a ration of dehydrated lucerne hay in the form of 7 mm pellets sterilized

by γ -irradiation. When the animals were 2.5 months old, they were fitted with a permanent plastisoll rumen cannula (2.5 cm diameter). The lambs were reared gnotobiotically until the age of 8 months.

Inoculation of the animals

After verification of the absence of cellulolytic bacteria and anaerobic fungi in the rumen of the 4 lambs, the animals were inoculated *per os*, 4 weeks after birth, on 4 consecutive days with a 10^{-6} dilution of ruminal contents taken from an adult sheep fed a hay-based diet (*Period I*). It was confirmed microscopically and by cultivation that there were no anaerobic fungi in the inoculum. At the age of 5 months, the animals received 3 inocula of 48 h cellobiose grown cultures of *Neocallimastix frontalis* strain MCH₃ and of *Piromyces communis* strain FL (*Period II*) (from the collection of the Microbiology Laboratory of the INRA Research Station of Clermont-Ferrand-Theix).

Microbiological analysis

Weekly counts of the cellulolytic bacteria and the fungi were made throughout the experiment in all 4 animals according to previously described methods (Fonty *et al*, 1983, 1987). The rumen content samples were taken before the morning meal.

Isolation of microbial populations and enzyme preparation

During *Periods I and II*, the activities of enzymes involved in plant cell-wall degradation were measured in sub-populations of microorganisms isolated from the rumen fluid (liquid phase population) and from the solid digesta (particle-association populations).

Liquid phase population

Rumen digesta (100 g) were collected before feeding through the cannula and immediately transported to the laboratory in a capped bottle,

where they were strained in an anaerobic glove box through 2 layers of cheesecloth to remove large particles. The filtrate was centrifuged at 35 000 *g* for 30 min at 4°C. The cell pellets were resuspended in 10 ml of approximately 0.025 M MES (2-(*N*-morpholino)ethane sulfonic acid) buffer, pH 6.5 containing dithiothreitol (0.2 mg/ml). The cells were disrupted by sonication (MSE soniprep 150 disintegrator, MSE instruments, Crawley) for four 20 s periods at maximum power with 30 s intervals at 4°C under anaerobic conditions. The sonicate was centrifuged at 5 000 *g* for 15 min at 4°C and the supernatant was used as the enzyme preparation. Enzymes samples were stored at -20°C in 2 ml aliquots in screw-capped tubes with a CO₂ headspace before assay.

Particle-associated populations

One gram of chopped lucerne hay was placed in a nylon bag (5 x 10 cm; 50 μ m mesh size) and suspended in the rumen through the cannula. The bag was withdrawn after 24 h and placed in an anaerobic glove box. The digesta material was then resuspended in 100 ml of an anaerobic pre-cooled (4°C) buffer solution (Bryant and Burkey, 1953). The suspension was stirred very slowly for 20 min to separate plant material. The solid material was recovered by filtration through 2 layers of cheesecloth. The residue on the filter was washed in another 100 ml buffer. The washed residue contained the adherent population. The filtrate contained the trapped and loosely attached (readily detached) population, designated the 'semi-adherent' or 'loosely adherent' population. The washed residue containing the adherent populations was resuspended in 10 ml 0.025 M MES buffer (pH 6.5, 4°C) containing DTT (0.2 mg/ml) and homogenized for 4 min with a Stomacher 80 in the anaerobic cabinet. The cells in the adherent and semi-adherent populations were disrupted as described and the supernatant fractions obtained by centrifugation (5 000 *g*, 15 min, 4°C) were used as the enzyme preparations (Williams *et al*, 1989).

Enzyme assay procedures

Glycoside hydrolase and polysaccharidase activities were determined according to the methods described by Williams and Strachan (1984a) and Martin *et al* (1993).

Measurement of pH and VFA

pH values, composition and concentration of VFA in the ruminal contents were measured in both periods using samples taken before the morning meal and 2 h after the beginning of the feed consumption. The methods used have been described previously (Jouany, 1982; Fonty *et al*, 1983).

In sacco degradation of wheat straw

Fibrolytic activity against the wheat straw cell walls in ruminal contents of the lambs was measured by the nylon bag method (Michalet-Doreau, 1990). Each bag (of about 50 μ mesh size) contained 1.5 g (fresh weight) of ground wheat straw. The extent of straw degradation was determined in *Periods I* and *II* by measuring dry matter (DM) disappearance after 48 and 72 h of incubation in the rumen. For each time, DM disappearance was measured in at least 6 bags from each animal. Two bags were introduced simultaneously into the rumen.

Statistical analysis

Statistical tests were performed according to the students' *t*-test (Snedecor and Cochran, 1984).

RESULTS

Establishment of cellulolytic bacteria and anaerobic fungi

The cellulolytic bacteria in the 10^{-6} dilution of adult sheep rumen contents rapidly established in the lambs' rumens. One week after the final inoculation with diluted ruminal contents the cellulolytic population ranged between 5×10^6 and 5×10^7 bacteria g^{-1} of rumen contents in all 4 animals. Thereafter, the population varied between 0.5×10^8 and 1×10^9 g^{-1} .

The fungi also developed rapidly. The fungal population in the 4 animals, as estimated by the number of zoospores, fluctu-

ated between 5×10^3 and 5×10^4 zoospores g^{-1} of ruminal contents throughout *Period II*. Microscopic examinations of colonies that developed in the roll tubes showed the presence of the 2 species (*N frontalis* and *P communis*). Microscopic observations also confirmed that fungal colonization of the plant material had occurred in the nylon bags as well as in the rumen.

Polysaccharide-degrading enzymes in the liquid phase and particle-associated microorganisms

The activity of the glycoside hydrolases of the liquid phase microbial population before the inoculation with the fungi was higher than the activities present in the semi-adherent and adherent populations (table I). The activities of the polysaccharidases, with the exception of cellulase, were similar in the liquid phase and adherent populations. The activity of these enzymes was slightly lower in the semi-adherent population. The inoculation of the fungi into the rumen had no marked effect on the enzyme activity of the liquid phase population. In contrast, the activity of almost all the glycoside hydrolases and polysaccharidases was greater in the other 2 microbial populations after the establishment of the fungal populations. The increase was particularly pronounced in the strongly adherent population. For example, the activity of hemicellulase, xylanase, and CMCase increased threefold in the presence of chytrids while β -D-glucosidase, β -D-xylosidase and β -D-cellobiosidase activities were almost fourfold greater (table I).

pH, VFA

The pH of the ruminal contents was not modified by the inoculation of fungi. Before feeding the pH values were between 6.8 and 6.9 and 2 h after the beginning of feed

Table 1. The specific activities of some glycoside hydrolases and polysaccharide degrading enzymes in the liquid phase and particle-associated microbial populations in the rumen of gnotobiotically reared lambs in the absence or presence of anaerobic fungi.

Enzyme	Specific activities of enzymes					
	Liquid phase population		Semi-adherent population		Adherent population	
	Period I	Period II	Period I	Period II	Period I	Period II
α -L-Arabinofuranosidase	139 \pm 26.0	125 \pm 31.2	30.0 \pm 9.3	66.5 \pm 5.2 ^b	66.2 \pm 14.3	143 \pm 23.8 ^a
β -D-Xylosidase	49.6 \pm 13.9	61.0 \pm 35.7	16.6 \pm 9.6	41.5 \pm 6.6 ^a	25.5 \pm 13.7	160 \pm 11.5 ^b
β -D-Glucosidase	95.5 \pm 19.8	108 \pm 31.5	39.0 \pm 5.7	62.9 \pm 3.8 ^a	40.2 \pm 19.4	139 \pm 8.2 ^b
β -D-Cellobiosidase	29.0 \pm 9.0	35.4 \pm 9.8	6.4 \pm 2.0	13.0 \pm 7.4	15.6 \pm 10.5	56.4 \pm 11.4
α -D-Glucosidase	51.1 \pm 12.6	75.3 \pm 36.1	20.2 \pm 3.2	22.6 \pm 12.0	17.3 \pm 14.0	54.7 \pm 7.0
Hemicellulase	353 \pm 70.8	276 \pm 57.5	191.3 \pm 32.1	492 \pm 116.7 ^a	334 \pm 124	931 \pm 63.4 ^a
Xylanase	189 \pm 38.1	220 \pm 59.3	128 \pm 21.0	387 \pm 42.6 ^a	241 \pm 67.1	667 \pm 88.8 ^a
Carboxymethylcellulase	151 \pm 50.8	110 \pm 38.6	108 \pm 19.1	289 \pm 87.8 ^a	161 \pm 36.8	464 \pm 140 ^a

Specific activities (mean \pm SD of 8 determinations $- 2$ per lamb) are expressed as nmol *p*-nitrophenol released/mg protein/min for glycosidases and as nmol reducing sugar released/mg protein/min for polysaccharidases. ^{a, b} Activities significantly different at $P \leq 0.1$ and $P \leq 0.05$ respectively.

intake they ranged between 6.2 and 6.4 (table II). In the presence of chytrid-omycetes, VFA concentration was unchanged in animals that had not eaten but it was slightly lower in post-feed samples. The differences were not significant.

Degradation of wheat straw

Measurements made by the *in sacco* method showed no effect of the presence of fungi on wheat straw degradation. There was a slight but not significant decrease in DM disappearance after 48 and 72 h incubation in the rumen (table II).

DISCUSSION

The cellulolytic bacteria present in the 10^{-6} dilution of ruminal contents and the fungi established successfully in the animal model used, at levels close to those observed in

conventionally reared animals of the same age (Fonty *et al*, 1987; Grenet *et al*, 1989). Likewise, pH and VFA concentrations were comparable to those measured in conventionally reared animals receiving the same feed (Fonty, unpublished data). The cellulolytic bacterial microflora was not studied qualitatively but microscopic examination of partially degraded filter paper strips from culture tubes showed the presence of a diverse population composed of small coccoid bacilli similar in appearance to *F succinogenes* and of ruminococci-resembling cocci. Microscopic examination of the rumen contents also showed the presence of an abundant and diverse bacterial flora. Similar animal models have been used to study the activity of the main cellulolytic bacterial species alone or in combination (Fonty *et al*, 1988c).

All the glycoside hydrolases and polysaccharidases studied had detectable activity in the 3 microbial populations isolated from the liquid and solid phases of the ruminal

Table II. Wheat straw degradation and VFA concentrations in the rumen of gnotobiotically reared lambs in the absence or presence of anaerobic fungi.

<i>Ruminal parameter</i>	<i>Period I Fungi absent</i>	<i>Period II Fungi present</i>
Wheat straw digestion (% DM loss)		
48 h ^a	32.0 ± 1.7 ^b	30.5 ± 0.8
72 h	40.0 ± 2.1	37.8 ± 1.3
VFA concentrations (mM) before feeding		
Total VFA	49.9 ± 5.5 ^c	52.3 ± 2.4
Acetate	36.7 ± 4.1	38.3 ± 1.6
Propionate	11.9 ± 2.4	11.5 ± 0.6
Butyrate	1.2 ± 0.3	2.8 ± 0.5
VFA concentrations (mM) 2 h after feeding		
Total VFA	98.0 ± 7.2	90.5 ± 6.5
Acetate	70.6 ± 7.0	63.0 ± 6.1
Propionate	22.4 ± 3.4	21.9 ± 4.5
Butyrate	5.0 ± 1.0	4.0 ± 0.6

^a Period of incubation of nylon bags in the rumen of lambs; ^b mean ± SD of 24 bags (6 per lamb); ^c mean ± SD of 8 samples (2 per lamb).

digesta of the gnotobiotically reared lambs. In the period before the lambs were inoculated with fungi the polysaccharidases involved in the degradation of plant cell-wall polymers were as active in the liquid phase population as in the particle-associated populations. This finding contrasts with previously published observations with conventionally maintained animals (Williams and Strachan, 1984a,b; Martin *et al*, 1993). However, as observed elsewhere (Williams and Strachan, 1984a,b; Williams *et al*, 1989), the enzymes involved in the degradation of non-structural polysaccharides (eg, α -D-glucosidase) were more active in the liquid phase population. Inoculation of the fungi and the resulting increase in the enzyme activity of the established population modified this distribution pattern. In the presence of fungi all the enzymes with the exception of α -D-glucosidase were more active in the adherent population. This increase in enzyme activity after the establishment of the chytridiomycetes is consistent with what is known about their location in the rumen and their enzymic capabilities. Except during the mobile zoospore phase in rumen fluid, these microorganisms develop in close association with the plant fragments that they colonize immediately upon entering the rumen (Bauchop, 1979). The recalcitrant lignocellulosic structures that are the most difficult to degrade are generally those that are the most abundantly colonized (Akin, 1987; Bauchop, 1989; Grenet and Barry, 1988). In addition, anaerobic fungi have the necessary array of enzymes to degrade all plant cell wall polymers except lignin (Williams and Orpin, 1987; Hébraud and Fèvre, 1988). However, the increased enzyme activity of the adherent population was not accompanied by more extensive degradation of the wheat straw incubated in the rumen in nylon bags. The limiting factor was not therefore the enzyme activity of the microorganisms but probably the accessibility of the polysaccharide substrates (cellulose and xylan) to the microorganisms and

microbial enzymes. In straw, these 2 polymers closely interlock in a complex structure with lignin, which acts as a physical barrier and limits enzyme penetration (Chesson, 1993). Other studies have also shown that increased fungal enzyme activity does not necessarily result in enhanced fibrolysis (Joblin and Williams, 1991). The lack of effect of the chytridiomycetes on the degradation of straw *in sacco* may also have arisen as a consequence of the interactions between microorganisms competing for the same ecological niche in the rumen ecosystem. Antagonism is explained by the interaction between *R flavofaciens* and *N frontalis*, which has been observed *in vitro* in the degradation of xylan, cellulose and straw (Williams *et al*, 1991; Bernalier *et al*, 1992, 1993; Stewart *et al*, 1992; Roger *et al*, 1993). The species composition of the ruminal cellulolytic microflora harboured by the lambs was not determined, but it was probable that the ruminococci were members of this population. There is the possibility that fungal establishment displaced bacteria from certain niches. Previous studies using a similar animal model (Fonty *et al*, 1988c; Fonty and Gouet, 1988) indicated that straw degradation *in sacco* was as efficient in animals harbouring only one cellulolytic bacterial species as in animals inoculated with an association of 2 or 3 species, but cellulose degradation was lower in gnotobiotically reared animals harbouring only fungi (*N frontalis* and *P communis* alone or in association) as the sole cellulolytic microorganisms than in conventionally maintained lambs (Fonty and Joblin, 1991).

The increase in the activity of the polysaccharide depolymerases and the glycoside hydrolases did not lead to higher fermentative activity and elevated VFA concentrations in the ruminal contents. Calderon-Cortes *et al* (1989) observed that in sheep fed on barley straw the elimination of fungi resulted in an increase in the proportion of propionic acid and a significant decrease in the degrada-

tion of the straw. In a mixed bacterial fermentation that simulated the rumen (RUSITEC), the DM disappearance of wheat straw increased by approximately 15% when a strain of *N frontalis* was included. Inclusion of the fungus was also associated with a greater production of acetate at the expense of propionate. In contrast, the DM disappearance of sugar beet pulp was not significantly affected when the fungus was present (Hillaire *et al*, 1990). However, any comparison between these studies and the present one can only be tentative since the 2 experimental designs were entirely different. In the former, the rumen fungi were eliminated by feeding the sheep with wheat straw treated with sodium chlorite and glacial acetic acid; a chemical residue may have affected the composition of the microbial populations. It is apparent, however, that the presence of fungi in the ruminal microbial population results in enhanced polysaccharolytic enzyme activities, although the effects on DM digestion will be dependent on the nature of the fibre components of the forage.

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