Effects of defaunation on microbial activities in the rumen of rams consuming a mixed diet (fresh Digitaria decumbens grass and concentrate)

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Original article

Effects of defaunation on microbial activities in the rumen of rams consuming a mixed diet (fresh *Digitaria decumbens* grass and concentrate)

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Abstract – The effects of defaunation on microbial activities in the rumen of sheep given mixed diets (fresh *Digitaria decumbens* and concentrate) were studied. Eight faunated rams and eight defaunated rams, with ruminal cannula were used. Each animal was fed with 4 different mixed diets, *Digitaria decumbens* grass supplemented with 4 mixtures of Soya meal and ground maize. The protein digestible in the intestine (French feeding system, PDI) to energy (French feeding system, forage unit UFL) levels (P/E) in the diets were 80 (D1), 100 (D2), 120 (D3) and 140 (D4). The major cellulolytic bacteria were enumerated by specific 16S rRNA targeted hybridisation probes, some microbial activities of the rumen were estimated by enzymatic assays and by the in sacco degradation technique. The diet (P/E ratio) and defaunation had no effect on the percentage of the cellulolytic bacteria, represented by three species: *Ruminococcus albus*, *Ruminococcus flavefasciens* and *Fibrobacter succinogenes*. The diet had a significant effect ($P < 0.05$) on the total activity of the fibrolytic enzymes (Carboxymethyl cellulase, xylanase) and glycosidases (β-D glucosidase, β-D xylosidase). Defaunation decreased ($P < 0.05$) the total activity of fibrolytic polysaccharidase (Carboxymethyl cellulase, xylanase) and glycosidase (β-D glucosidase, β-D xylosidase) enzymes. Neither defaunation nor the diet had any effect on α-amylase activities. Defaunation decreased ($P < 0.05$) total α-D glucosidase activity. The decrease of the total activity of carboxymethyl cellulase after defaunation was more marked ($P < 0.05$) with the D3 diet. The diet had a significant effect ($P < 0.01$) on the in sacco DM degradation (96 h). Defaunation decreased the in sacco DM degradation (96 h, $P < 0.05$) and this decrease was negatively correlated with the P/E ratio. Defaunation significantly decreased the total volatile fatty acid concentration ($P < 0.05$) and the molar proportion of butyrate ($P < 0.01$). From these results, despite reduced fibrolytic and amylolytic activities, a lower proteolysis and a more advantageous ruminal VFA profile occurred in defaunated animals. As a consequence, a better use of nutrients (protein, energy) can be expected by defaunated animals.

enzymatic activities / microbial population / defaunation / fibre degradation / sheep / rumen

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Résumé – Les effets de la défaunation sur les activités microbiennes du rumen de moutons ingérant des rations mixtes (*Digitaria decumbens* en vert complétée avec du concentré). Huit béliers faunés et 8 béliers défaunés munis de canules ruminales ont été utilisés. Chaque animal était alimenté par 4 rations mixtes différentes composées d'herbe de *Digitaria decumbens* complémentée avec 4 mélanges de tourteau de soja et de maïs broyé. Le niveau de protéine (PDIN) / énergie (UFL) de la ration (P/E) était de 80 (D1), 100 (D2), 120 (D3) et 140 (D4). Les principales populations de bactéries cellulolytiques ont été dénombrées à l'aide de sondes d'hybridation spécifiques ciblant l'ARNr 16S, certaines activités microbiennes du rumen ont été estimées par des dosages d'activités enzymatiques, et par la technique de dégradabilité in sacco. La ration (rapport P/E) et la défaunation n'ont pas eu d'effet sur le pourcentage de bactéries cellulolytiques, représentées par trois espèces : *Ruminococcus albus*, *Ruminococcus flavefasciens* et *Fibrobacter succinogenes*. La ration a eu un effet significatif ($P < 0,05$) sur l'activité totale des polysaccharidases fibrolytiques (Carboxyméthyl cellulase, xylanase) et des glycosidases (β-D glucosidase, β-D xylosidase). La défaunation a diminué ($P < 0,05$) l'activité totale des polysaccharidases fibrolytiques (Carboxyméthyl cellulase, xylanase) et des glycosidases (β-D glucosidase, β-D xylosidase). Ni la ration, ni la défaunation n'ont eu d'effet significatif sur les activités de l'α-amylase. La défaunation a diminué ($P < 0,05$) l'activité totale de l'α-D glucosidase. La diminution de l'activité totale de la carboxyméthyl cellulase après défaunation a été plus marquée ($P < 0,05$) avec la ration D3. La ration a eu un effet significatif ($P < 0,01$) sur la dégradation in sacco (96 h) de la matière sèche (MS). La défaunation a diminué la dégradation in sacco (96 h) de la matière sèche ($P < 0,05$), de plus cette diminution était négativement corrélée avec le rapport P/E. La défaunation a significativement diminué ($P < 0,05$) les concentrations moyennes en acides gras volatils (AGV). La proportion molaire de butyrate a été significativement diminuée par la défaunation ($P < 0,01$). A partir de ces résultats, en dépit d'une réduction des activités des enzymes fibrolytiques et amylolytiques, une moindre protéolyse et un profil d'AGV plus avantageux sont observés dans le cas des animaux défaunés. En conséquence, une meilleure utilisation des nutriments (protéines, énergie) peut être attendue pour les animaux défaunés.

activités enzymatiques / population microbienne / défaunation / dégradation des fibres / ovin / rumen

1. INTRODUCTION

The effects of defaunation of ruminants have been investigated for many decades now, as an attempt to enhance animal production and valorise local resources or by-products [12, 20]. Many digestion trials were conducted to find out the factors of digestion that may explain the results observed on animal production. Numerous data were obtained on the effects of defaunation on the characteristics of the rumen (VFA, NH_3, pH) and on nitrogen balance (review by Eugène et al. [8]). However, the effect of defaunation on the digestion of the plant cell wall is still under debate. Ruminal degradation of plant fibre has mainly been investigated with the in sacco method or by the in vivo digestion balances, but few studies have shown the effect of defaunation on the bacterial population. To our knowledge, only two studies of enzymatic activities have been published [43, 44], and no modern tool such as molecular probes has been used to analyse the effect of defaunation. As Bird and Leng [2], we hypothesise that the nature of the diet may be the major factor of variation of the effect of defaunation on animal performance, especially through the resulting nitrogen to energy balance of the absorbed nutrients. Thus, the interaction between the nature of the diet and the fauna initially present in the rumen may play a key role. The aim of this study was to investigate the effect of defaunation and its interaction with the diet on the evolution of the cellulolytic bacterial population, the fibrolytic and amylolytic activities of microbial enzymes and the plant material degradation in the rumen. The effects of defaunation on the growth and digestion in sheep receiving the same mixed diets were published in a first article [9]. The use of oligonucleotide probes for the determination of bacterial species has generated increasing interests.

Indeed, this technique permits a rapid identification of the microorganisms, at a large variety of taxonomic levels. Moreover, this technique is culture independent.

2. MATERIALS AND METHODS

2.1. Location

The research was carried out at the animal experimental station of the National Agricultural Research Institute of the French West Indies, Guadeloupe (latitude 16° 16', longitude 61° 30'). Average temperatures during the experiment ranged from 26 °C to 31 °C. The rainfall on the experimental site is 3000 mm a year.

2.2. Animals, diets and experimental design

2.2.1. Animals

Sixteen "ovin Martinik" rams (average live weight: 45.1 ± 7.7 kg; 2 years old), each containing surgically implanted rumen cannula, were used in this trial. Half of them were defaunated using a milk diet, adapted from a technique described by Fujihara [11]. During the defaunation process, the diet was gradually changed from a fresh young grass to milk powder diet within 10 days. Then the animals were fed milk alone during 7 days. The diet was gradually changed from milk to fresh grass forage. The milk powder (200 g) was mixed with water (400 mL, 37 °C), and poured into the rumen via a rumen cannula, once a day.

2.2.2. Diets

The animals were fed four experimental diets. The D1 diet was a *Digitaria decumbens* grass (28 days age of regrowth) distributed ad libitum with 1000 g of ground maize. In the D2, D3 and D4 diets, the *Digitaria decumbens* (28 days age of regrowth) was complemented with 700 g (100 g Soya meal + 600 g maize), 460 g (160 g Soya meal + 300 g maize) and 200 g (200 g Soya meal) of concentrate respectively. The chemical composition of the diets is shown in Table I. The protein to energy ratio, expressed as PDI / UFL, is an easy criterion in the French feeding system to determine the animal nutritional requirements. The PDI (Protéines Digestibles dans l'Intestin grêle) estimates the amount of amino nitrogen N × 6.25 absorbed in the small intestine from the dietary protein which has escaped fermentation in the rumen, and the microbial protein arising from that fermentation [40]. The feed unit (UFL) represents the energy value of the feeds. One UFL is equivalent to the energy value of 1 kg of standard barley [38]. In the text we refer to the PDIN, protein digestible in the intestine from nitrogen origin, and to the UFL, "Unité Fourragère Lait". The fermentable protein to fermentable energy index (FP/FE) of the diets was

Table I. Organic matter (OM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents ($g \cdot kg^{-1}$ DM) of the diets[1].

Diets	D1	D2	D3	D4
Chemical composition (%DM)				
OM	96	95	94	93
CP (N × 6.25)	9.8	12.1	13.9	14.2
NDF	34.8	46.7	53.4	63.8
ADF	15.2	21.7	25.5	31.4
ADL	1.9	2.5	2.8	3.4
PDIN/UFL ($g \cdot UFL^{-1}$)	80	100	120	140
FP/FE index (%)[2]	8.8	11.9	14.4	18.1

[1] Mixed diets composed of fresh *Digitaria decumbens* grass (28 days age of regrowth) and 4 concentrate mixtures (Soya meal + ground maize); the diets were formulated to provide 4 protein energy ratios of 80, 100, 120 and 140 g PDIN/UFL on diet D1, D2, D3 and D4, respectively.

[2] FP/FE is the fermentable protein fermentable energy index estimated as the protein truly digested in the rumen/organic matter truly digested in the rumen.

estimated as the protein truly digested in the rumen/the organic matter truly digested in the rumen. Mineralised salt blocks were available ad libitum. The blocks' composition was the following ($g \cdot kg^{-1}$): Ca (60.0), P (20.0), Mg (10.0), Na (280.0), Zn (17.5), Mn (5.5), Fe (1.5), I (0.03), Co (0.03) and Se (0.01).

2.2.3. Experimental design

The 16 rams were conducted according to two parallel Latin Square designs (faunated vs. defaunated). Defaunated and faunated rams, placed in individual metabolism cages and housed in the same room, were separated physically with a punch cloth. Each period was composed of 14 days of adaptation to the diet, 5 days of intake measurements, 4 days of ruminal content sampling and 4 days for nylon bag incubation in the rumen. Care was taken to prevent accidental inoculation of the rams with protozoa. Each week, the ruminal fluid of the defaunated animals was examined, and they remained free of protozoa during the experiment.

2.3. Experimental procedures

2.3.1. Ruminal samples

Samples of rumen liquid were taken from the sheep, immediately before the morning meal, and at 3, 6 and 12 hours after food was first available, during two consecutive days. The pH was measured immediately after sampling. Ruminal content was strained through a nylon filter (100 µm). The samples of rumen fluid (10 mL) were frozen (–20 °C) after adding a mixture of H_3PO_4 and $HgCl_2$ (1 vol./10 vol.) prior to VFA determination. During five days, 9 mL of rumen fluid were collected before and 3 hours after the morning meal. They were added to 1 mL of a preservative solution (50% glycerol, 48% distilled water and 2% formaldehyde) before protozoa counting. On two consecutive days, representative samples of rumen contents (300 g) were collected 3 hours after the morning meal from faunated and defaunated animals fed with the D1 and D4 diet and then freeze-dried, before bacterial population determination. Two representative samples of rumen contents (300 g) were collected, one just before the morning meal and the second 3 hours after, in order to measure enzyme activities from the solid-adherent microorganisms. The measurements were carried out on two consecutive days, on 4 defaunated and 4 faunated animals. The samples were then strained through a 100 µm nylon filter to collect the solid phase, under anaerobic conditions (CO_2 flows).

2.3.2. In sacco measurements

The degradation of the forage was measured by the nylon bag technique. The surface of the nylon bags was 50 cm^2, with a pore size of 50 µm (Blutex, Tissage Tissus Technique, Villeneuve La Garenne, France). The nylon bags were filled with 15 g of chopped (0.5 cm long) fresh 28 day *Digitaria decumbens* grass. The same forage, stored at – 20 °C, was used during the four periods. The nylon bags were placed in the rumen after feeding and the times of incubation were 24, 48 and 96 hours. Three nylon bags were placed in 5 defaunated and 5 faunated animals at each incubation time, during the 4 periods. After removal from the rumen, the bags were rinsed under cold running water and were frozen (–20 °C) until the end of the experiment. Then, the bags were washed (3 times, 10 min every time) in water using a small automatic washing machine (Alternatic Calor, capacity: 1 kg) to minimise contamination of diet residues by the microbial population. A fourth washing (10 min) took place in an ultrasonic bath to remove adherent bacteria associated with the diet residues.

2.4. Chemical analysis

Dry matter was determined by drying to a constant weight at 60 °C in a forced

Table II. Characteristics of the oligonucleotide probes used to identify three cellulolytic bacterial species (*Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*).

Reference	Probes	Target	Sequence (5'-3')	Tw (°C)
Sthal et al. [34]	S-D-Bact-0338-a-A-18	Eubacteria	GCTGCCTCCCGTAGGAGT	54
Sthal et al. [34]	S-S-F.suc-0650-a-A-20	*F. succinogenes*	TGCCCCTGAACTATCCAAGA	48
Odenyo et al. [30]	S-S-R.albus-0196-a-A-18	*R. albus*	GTCATGCGGCTTCGTTAT	46
Odenyo et al. [30]	S-S-R.fla-1269-a-A-20	*R. flavefaciens*	TTCTCTTTGTTAATTGCCAT	45

Tw: specific wash temperature.

draught oven. The samples were ground (1 mm) prior to chemical determination. The OM content was determined as the difference between dry and ash weight. The samples were burnt for 10 h at 550 °C. The Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and Acid detergent lignin (ADL) were estimated according to the method of Van Soest et al. [37]. Nitrogen concentration was determined using the Dumas combustion method [3]. Ruminal volatile fatty acids were measured in the rumen liquid by gas chromatography [31]. The protozoa population was determined with a Dolfüss counting cell (Elvetec Services, Clermont-Ferrand, France) according to the method described by Jouany and Sénaud [16].

The structure of the cellulolytic bacterial community (*Ruminococcus albus,* R*uminococcus flavefaciens* and *Fibrobacter succinogenes*) was investigated using specific oligonucleotide probes targeting 16S rRNA, on ruminal total digesta. The Stahl et al. method [34] was adapted. Rumen digesta (50 mg) were mixed with 1 mL of RNAzol (Bioprobe System, Montreuil, France) and with 0.3 g zirconium beads (Biospec products, INC). Mechanical disruption for 2 min, at maximal speed (bead beating) was applied. A chloroform, isopropanol, ethanol extraction was performed in order to recover total microbial RNA [27]. Total microbial RNA was then conserved in water treated with DEPC (Diethyl pyrocarbonate) and stored at –20 °C. Oligonucleotide probes were labelled with gamma ATP (^{32}P) (Eurogenetec) with a specific activity of 4000 μCi·mmole^{-1} at the 5'P end using a T4 kinase (Eurogenetec). Four oligonucleotide probes were used (Tab. II). After denaturation with glutaraldehyde (2% phosphate buffer 50 mM, pH = 7), RNA extracts from the samples (between 25 and 100 ng RNA) and DNA standards (Eurogenetec) used as the control (10 ng) were blotted on a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Saclay, France) using a dot blot apparatus (Biorad). The membrane was air dried, then pre-incubated as described by Michalet-Doreau et al. [27]. Then labelled probes were added to each membrane (at about 2×10^5 cpm per sample dot) and were incubated for 5 hours at 45 °C in a hybridisation oven. The membranes were washed for 20 minutes with SSC 1X buffer (150 mM NaCl, 15 mM Sodium Citrate, pH = 7) and 1% SDS at temperatures specific to each probe, at which duplex DNA-RNA is dissociated (Tab. II). The membranes were then air dried and exposed to a screen (Storm, Molecular Dynamics) before bound probes were quantified using Image Quant Software (Molecular Dynamics). The results of each quantity (ng RNA) of cellulolytic bacterial species were expressed as a percentage of the total quantity (ng RNA) of bacterial 16Sr RNA (obtained with eubacteria probe EUB338). The cellulolytic bacterial population was estimated by the sum of the three major culturable cellulolytic bacterial species (*R.a, R.f, F.s*).

Enzymatic manipulations were performed under anaerobic conditions. Enzyme extractions from the solid adherent microorganisms were achieved according to the procedure detailed by Martin and Michalet-Doreau [25]. The samples of the solid phase (70 g) were washed with 350 mL of a prewarmed (39 °C) salt solution (Coleman buffer [5]) and then were strained through a nylon filter (100 µm). A sample of the washed digesta was cut (0.5 cm) and then suspended in 25 mL MES buffer (2-(N-morpholino) ethane sulfonic acid) and stored at –80 °C. After centrifugation (15000 × *g*, 15 min, 4 °C), the microbial cells present in the homogenate were disrupted by defrosting and sonication (Labsonic U, B. Braun Biotech International) during 4 × 30 s periods at 4 °C, and the supernatant used as the enzyme preparation. Enzymes from solid-adherent microorganisms were measured using the assay procedures detailed by Nozière et al. [28] for the glycosidase and Nozière and Michalet-Doreau [29] for the polysaccharidase. Polysaccharide activities were determined by measuring spectrophotometrically at 410 nm [23] the amount of reducing sugars released from the purified substrate (Carboxymethyl cellulose, xylan and starch) incubated (60 min at 39 °C) with the enzyme preparation. Glycosidase activities were obtained by spectrophotometrically measuring at 420 nm the rate of *p*-nitrophenol released from the appropriate *p*-nitrophenyl glycoside (pNP β-D xylanopyranoside, pNP β-D glucopyranoside and pNP α-D glucopyranoside) after incubation for 30 to 60 min at 39 °C with enzyme preparation. Enzyme and substrate controls were also performed simultaneously by replacing substrate and enzymes by the MES buffer. Control values corresponding to non-specific cleavage were then withdrawn from the amounts of reducing sugars and were *p*-nitrophenol detected. The protein content of the enzyme preparations was determined according to Pierce and Suelter [32]. Enzyme activities were expressed as micromoles of reducing sugars (for polysaccharides) or micromoles of *p*-nitrophenol (for glucosidase) released per milligram of protein per hour (specific activity) or per gram of rumen DM content per hour (total activity).

2.5. Statistical analysis

The ANOVA procedure (GLM) was used in all the statistical analyses [33]. For the enzymatic activities, nylon bag degradation, VFA and pH, the statistical model used was:

$$Y_{ijkl} = \mu + Def_j + Diet_k + Per_l + (Def \times Diet)_{jk} + Animal(Def)_{i(j)} + e_{ijkl}.$$

Y_{ijkl} is the observed character for animal i, µ the global mean, Def the defaunation effect (faunated vs. defaunated, 1 DF), Diet the diet effect (3 DF), Per the period effect (3 DF), (Def × Diet) the interaction between the defaunation effect and the diet effect (3 DF), Animal(Def) the animal effect and e_{ijkl} represents the residual error.

The number of bacteria was analysed using the previous model with defaunation (faunated vs. defaunated, 1DF), diet (1 DF, only two diets were tested), period (3 DF) and the interaction between defaunation and the diet effect, considered as the fixed effects.

The means between defaunated and faunated animals on the same diet were compared by the Student test; significance was declared at $P < 0.05$. The defaunation effect was tested using the Animal(Def) as the error term.

3. RESULTS

3.1. Protozoa and cellulolytic bacterial populations in the rumen

Before the morning meal, the mean number of protozoa in faunated animals raised up from $1.9 \times 10^5 \cdot mL^{-1}$ ($\pm 1.8 \times 10^5 \cdot mL^{-1}$) on the D4 diet to $9.4 \times 10^5 \cdot mL^{-1}$ ($\pm 1.8 \times 10^5 \cdot mL^{-1}$) on the D2 diet. Then it dropped to $6.0 \times 10^5 \cdot mL^{-1}$ ($\pm 1.8 \times 10^5 \cdot mL^{-1}$) on the D1 diet. The same trend was observed three hours after the morning meal.

Table III. Effect of defaunation on some cellulolytic bacteria, in the rumen of rams consuming mixed diets composed of *Digitaria decumbens* grass (28 days age of regrowth) and concentrate mixture (Soya meal + ground maize). The two extreme diets, D1 and D4 were chosen to enumerate cellulolytic bacteria.

PDIN/UFL Maize intake (%DMI)	D1 63		D4 0			Effect		
	F	D	F	D	s.e.	Def	Diet	Def × Diet
% Ruminococcus albus (R.a)	1.8	3.1	1.9	1	0.89	NS	NS	NS
% Ruminococcus flavefaciens (R.f)	–	1.32	–	–		–	–	–
% Fibrobacter succinogenens (F.s)	2.3	1.4	3.5	2	0.72	NS	NS	NS
% Celluloytic bacteria	4.1	4.9	5.7	3.1	1.31	NS	NS	NS

F: faunated animals, D: defaunated animals, Def: defaunation effect, Diet: diet effect, Def × Diet: interaction effect; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: $P > 0.05$.
[a, b] Different letters in a same row for the same diet correspond to a significant difference ($P < 0.05$).
% Cellulolytic bacteria: sum of the three major cellulolytic species *R.a*, *R.f* and *F.s*.

We decided to choose the two extreme diets, D1 and D4, for bacteria enumeration, since these two diets were thought to induce the major effect on the bacterial populations. The amount of *Ruminococcus flavefaciens* in some ruminal samples was too low to be detectable, although the control was always positive.

The diet (P/E ratio) and defaunation had no effect on the percentage of the cellulolytic bacterial species (*Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogenes*) (Tab. III). The percentage of the cellulolytic bacterial population, estimated by the sum of the percentage of the three major cellulolytic bacterial species (*R.a, R.f, F.s*) was not affected by the diet effect. This latter also remained unchanged after defaunation (4.45 ± 0.87%). No significant interaction between defaunation and the diet appeared.

3.2. Enzymatic activity

The main polysaccharidases (Carboymethyl cellulase (CMCase) and Xylanase) that participate in fibre degradation and starch degradation (α-amylase) are presented in Table IV.

The increased P/E ratio of the diet significantly increased the total activity (μmol reducing sugars·g^{-1}DM·h^{-1}) of the CMCase ($P < 0.05$) and the xylanase ($P < 0.05$), measured before (0 h) and 3 hours after the morning meal (3 h). Defaunation decreased the total activity of CMCase (0 h, 3 h) ($P < 0.05$) and xylanase (0 h, 3 h) ($P < 0.01$). The decrease of CMCase was more marked for the D3 diet ($P < 0.05$). The increased P/E ratio of the diet significantly increased ($P < 0.05$) the specific activity (μmol p-nitrophenol·mg^{-1}protein·h^{-1}) of CMCase (0 h, 3 h) and of xylanase (0 h, 3 h) (Tab. IV). Defaunation decreased the specific activity of CMCase (0 h) ($P < 0.05$) and of xylanase (0 h, 3 h) ($P < 0.01$). Concerning the amylolytic enzyme, neither the diet nor defaunation had a significant effect on the total and specific activities of α-amylase (0 h, 3 h).

The main glucosidase enzymes that participate in fibre and starch degradation are presented in Table V. The increased P/E ratio of the diet significantly increased ($P < 0.05$) the glycosidases that exhibit the highest activities, β-D-glucosidase total activity (0 h, 3 h) and the β-D xylosidase (0 h, 3 h). Defaunation significantly decreased ($P < 0.01$) both β-D-glucosidase total activity (0 h,

Table IV. Effect of defaunation on enzymatic activity (polysaccharidases) in rumen solid-associated microorganisms, in rams consuming mixed diets[1].

PDIN/UFL Maize intake (%DMI)	D1 63		D2 38		D3 20		D4 0			Effect		
	F	D	F	D	F	D	F	D	s.e.	Def	Diet	Def × Diet
Item and time after feeding												
Total activity (μmol reducing sugar·g^{-1}DM·h^{-1})												
CMCase												
0h	3.5	1.1	20.5	13.5	51.9^{a}	4.2^{b}	9.5	6.0	7.6	*	*	*
3h	9.6	2.1	14.2	4.6	47.2^{a}	15.3^{b}	24.8	11.2	7.6	*	*	NS
Xylanase												
0h	20.5	0.7	144.0	42.4	265.0^{a}	93.7^{b}	243.0^{a}	86.0^{b}	40.2	**	**	NS
3h	28.8	6.7	130.0	17.4	174.0	41.4	217.0^{a}	51.7^{b}	27.2	***	**	NS
Amylase												
0h	51.5	–2.9	15.4	9.0	19.8	15.9	47.4	12.5	19.0	NS	NS	NS
3h	59.5	5.4	37.0	27.6	30.0	32.9	66.0	36.6	18.7	NS	NS	NS
Specific activity (μmol reducing sugar·mg^{-1}protein·h^{-1})												
CMCase												
0h	0.3	0.3	3.0	2.2	5.2^{a}	0.8^{b}	2.0	1.0	0.9	*	*	NS
3h	1.7	2.3	2.2	1.1	4.3	2.4	3.1	2.8	1.0	NS	NS	NS
Xylanase												
0h	–3.2	0.9	23.8	10.3	35.8	16.0	39.4	21.2	6.9	*	**	NS
3h	0.5	1.8	21.6	10.1	24.7	8.2	25.7	17.9	5.3	*	**	NS
Amylase												
0h	7.5	–0.1	2.7	2.2	2.1	2.0	5.7	1.0	2.5	NS	NS	NS
3h	7.4	6.2	6.1	15.5	5.9	8.9	6.2	12.8	3.5	NS	NS	NS

[1] Mixed diets composed of fresh *Digitaria decumbens* grass (28 days age of regrowth) and 4 concentrate mixtures (Soya meal + ground maize); the diets were formulated to provide 4 protein/energy ratios of 80, 100, 120 and 140 g PDIN/UFL on diet D1, D2, D3 and D4, respectively.
F: faunated animals, D: defaunated animals, Def: defaunation effect; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: non significant.
CMCase: Carboxymethyl Cellulase.
[a, b] Different letters in a same row for the same diet correspond to a significant difference ($P < 0.05$).

3 h) and the β-D xylosidase (3 h). The diet had no effect on the specific activity of β-D-glucosidase (0 h, 3 h). On the contrary, the specific activity of the β-D xylosidase (0 h) was significantly increased by the increased P/E ratio of the diet ($P < 0.05$). Defaunation had no effect on the specific activity of β-D-glucosidase, nor on the β-D xylosidase. The diet had no effect on the total activity of α-D-glucosidase, whereas this enzyme was decreased after defaunation ($P < 0.05$, 0 h and 3 h). The increased P/E ratio of the diet significantly increased the specific activity of α-D-glucosidase (0 h), whereas it remained unchanged after defaunation.

Generally speaking, specific activities of both β-D-glucosidase and β-D-xylosidase followed the same variations as their respective total activities.

Table V. Effect of defaunation on enzymatic activity (glycosidases) in rumen solid-associated microorganisms, in rams consuming mixed diets[1].

PDIN/UFL Maize intake (%DMI)	D1 63		D2 38		D3 20		D4 0			Effect		
	F	D	F	D	F	D	F	D	s.e.	Def	Diet	Def × Diet
Item and time after feeding												
	Total activity (µmol *p*-nitrophenol·g^{-1}DM·h^{-1})											
β-D glucosidase												
0h	35.6	16.3	67.6	39.5	89.9^{a}	50.3^{b}	74.2	48.5	9.9	**	**	NS
3h	37.2	10.6	60.7^{a}	27.1	75.2^{a}	34.9^{b}	101^{a}	46.3^{b}	9.3	***	***	NS
β-D xylosidase												
0h	26.1	21.2	88.8	71.9	125	79.2	112	87.6	15.4	NS	***	NS
3h	22.5	13.7	76.3	45.5	89.4	57.2	124^{a}	74.1^{b}	13.2	**	***	NS
α-D glucosidase												
0h	7.9	4.5	11.0	6.1	10.9^{a}	4.2^{b}	5.5	3.6	1.9	**	NS	NS
3h	8.9	4.3	12.4	5.8	14.1	5.4	9.9	6.6	3.2	*	NS	NS
	Specific activity (µmol *p*-nitrophenol·mg^{-1}protein·h^{-1})											
β-D glucosidase												
0h	8.0	9.9	9.5	9.3	11.2	10.5	11.5	10.1	1.2	NS	NS	NS
3h	11.3	8.8	9.4	10.0	9.7	8.8	11.3	11.9	1.6	NS	NS	NS
β-D xylosidase												
0h	5.7	11.1	13.1	16.1	16.3	15.0	18.1	20.2	2.8	NS	*	NS
3h	8.7	7.6	11.5	13.6	11.6	12.9	13.8	18.9	3.4	NS	NS	NS
α-D glucosidase												
0h	1.8	2.1	1.6	0.8	1.2	0.4	0.8	0.2	0.4	NS	**	NS
3h	2.7	3.3	1.9	2.3	1.8	1.4	1.0	2.6	0.9	NS	NS	NS

[1] Mixed diets composed of fresh *Digitaria decumbens* grass (28 days age of regrowth) and 4 concentrate mixtures (Soya meal + ground maize); the diets were formulated to provide 4 protein/energy ratios of 80, 100, 120 and 140 g PDIN/UFL on diet D1, D2, D3 and D4, respectively; F: faunated animals, D: defaunated animals, Def: defaunation effect; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: non significant.
[a, b] Different letters in a same row for the same diet correspond to a significant difference ($P < 0.05$).

3.3. In sacco DM degradation

Whatever the nylon bag incubation time in the rumen, the DM degradation increased with the increasing P/E ratio in the diet ($P < 0.01$) (Tab. VI).

Defaunation tended to reduce in sacco the nylon bag DM degradation of the forage, when nylon bags were incubated for 24 hours in the rumen (47.8 vs. 46.9, $P = 0.24$) and for 48 hours (61.0 vs. 63.7, $P = 0.18$) but the defaunation effect was not significant. The defaunation effect became significant after 96 hours of incubation of the bags (74.0 vs. 70.3, $P = 0.01$). Differences in DM degradation (96 h) between faunated and defaunated animals was more marked for the D1 diet ($P < 0.05$).

3.4. pH and concentrations of VFA in the rumen

The increased P/E ratio of the diet significantly increased the pH mean values of ruminal fluid ($P < 0.001$) (Tab. VII). The

Table VI. Effect of defaunation on in sacco dry matter (DM) degradation in the rumen of rams consuming mixed diets[1].

PDIN/UFL Maize intake (%DMI)	D1 63		D2 38		D3 20		D4 0			Effect		
	F	D	F	D	F	D	F	D	s.e.	Def	Diet	Def × Diet
Item and time after feeding												
In sacco degradation of DM												
24h	40.6	34.2	47.8	44.6	51.4	50.1	53.4	54.7	2.5	NS	***	NS
48h	59.4	51.5	63.1	60.5	66.0	64.0	66.5	67.9	2.7	NS	**	NS
96h	72.5[a]	60.9[b]	73.2	71.9	75.5	72.8	75.0	75.7	1.8	*	**	*

[1] Mixed diets composed of fresh *Digitaria decumbens* grass (28 days age of regrowth) and 4 concentrate mixtures (Soya meal + ground maize); the diets were formulated to provide 4 protein/energy ratios of 80, 100, 120 and 140 g PDIN/UFL on diet D1, D2, D3 and D4, respectively. F: faunated animals, D: defaunated animals, Def: defaunation effect; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: non significant.
[a, b] Different letters in a same row for the same diet correspond to a significant difference ($P < 0.05$).

Table VII. Effect of defaunation on fermentation parameters in the rumen of rams consuming mixed diets[1].

PDIN/UFL Maize intake (%DMI)	D1 63		D2 38		D3 20		D4 0			Effect		
	F	D	F	D	F	D	F	D	s.e.	Def	Diet	Def × Diet
Item and time after feeding												
Mean pH	5.89	6.01	5.97	6.08	6.13	6.20	6.28	6.46	0.08	*	***	NS
Mean total VFA	61.2	55.1	67.5	63.0	62.4	59.7	59.4[a]	55.1[b]	2.3	*	*	NS
Molar proportions												
Acetate (C2, molar %)	59.6	60.8	62.0	64.4	63.7	64.9	66.9	66.8	1.49	NS	**	NS
Propionate (C3, molar %)	20.7	21.3	22.1[a]	18.7[b]	19.9	21.7	18.3	20.5	0.86	NS	NS	*
Butyrate (C4, molar %)	13.6[a]	10.7[b]	11.9	12.2	12.7[a]	9.0[b]	10.5	8.5	0.78	***	*	NS

[1] Mixed diets composed of fresh *Digitaria decumbens* grass (28 days age of regrowth) and 4 concentrate mixtures (Soya meal + ground maize); the diets were formulated to provide 4 protein/energy ratios of 80, 100, 120 and 140 g PDIN/UFL on diet D1, D2, D3 and D4, respectively. F: faunated animals, D: defaunated animals, Def: defaunation effect; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: non significant.
[a, b] Different letters in a same row for the same diet correspond to a significant difference ($P < 0.05$).

pH mean values of ruminal fluid remained lower with faunated rams than with defaunated rams ($P < 0.05$).

The increased P/E ratio of the diet significantly increased the VFA mean concentrations in the ruminal fluid ($P < 0.05$) and these latter decreased ($P < 0.05$) after defaunation (Tab. VII). No significant interaction between defaunation and the diet appeared. The diet had a significant effect on acetate ($P < 0.01$) and butyrate ($P < 0.05$) mean concentration in the rumen. Defaunation tended to decrease ($P = 0.06$) acetate and propionate mean concentrations and significantly decreased ($P < 0.001$) butyrate mean concentrations (7.6 vs. 5.9 ± 2.6 mM).

The increased P/E ratio of the diet significantly decreased the molar proportion of acetate ($P < 0.01$) and the molar proportion of butyrate ($P < 0.05$). Defaunation significantly decreased the molar proportion of butyrate (12.2 vs. $10.1 \pm 0.39\%$, $P < 0.01$).

Defaunation significantly increased ($P < 0.05$) the acetate to propionate ratio with the D2 diet. The acetate to butyrate ratio increased significantly ($P < 0.01$) after defaunation (5.33 vs. 6.58 ± 0.24). The propionate to butyrate ratio increased significantly ($P < 0.01$) after defaunation (1.69 vs. 2.09 ± 0.06), whereas the ratio decreased after defaunation with the D2 diet. The (acetate + butyrate) to propionate ratio increased after defaunation with the D2 diet ($P < 0.05$), data not shown.

4. DISCUSSION

The present study indicates that defaunation decreased hydrolytic and fibrolytic microbial activities in the rumen, but no significant effect of defaunation on bacterial counts could be detected.

Several authors have observed that defaunation increases the number of total bacterial population in the rumen by classical bacterial viable counts [19, 22]. Our results, though not significant, suggest that total bacteria in the rumen tended to increase after defaunation and were in agreement with previous results. This can be explained since protozoa are the major predator of bacteria in the rumen and consequently are mainly responsible for the recycling of bacteria in the rumen [4, 17].

It is admitted that the three major cultivable cellulolytic bacteria in the rumen are composed of *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*, that is why we chose to study only these 3 species [4, 10].

Despite their major role in fibre degradation, our results indicate that this cellulolytic bacteria only represent 3 to 5% of the total bacteria in the rumen. This result is in agreement with previous reports [34, 41]. However, these microbial population estimations are low and may suggest that the technique used can be improved. Several limits exist including a high biological variability (animal, RNA distribution), the availability of only a few species specific probes, and the low accuracy of the specificity of the probes.

There is a wide diversity of microorganisms present in the rumen. The results on microbial ecology provided with the same techniques indicate that bacterial microorganisms account for 60 to 90% of total microorganisms in the rumen, and that 3 to 30% are eukaryotes, while 0.5 to 3% are archaea populations [24]. Moreover, interactions between microbial species are crucial [13]. These results illustrate the wide diversity of microbial microorganisms in the rumen, and may explain the rather low proportion of the cellulolytic bacterial population [13]. These results suggest that although our results on cellulolytic bacterial counts may seem low, we cannot exclude that the cellulolytic bacteria in the rumen only represent 3–5% of the total bacteria in the rumen.

Our results demonstrate that the hydrolytic capacity of the rumen decrease after defaunation. Indeed, in our study, defaunation decreased in sacco DM degradation, but similar results have been observed by other authors [18, 36]. The decrease of the in sacco DM degradation could be explained on the one hand, by the decrease in enzymatic activities and on the other hand, by the modification of the structure of the microbial population, after defaunation. Defaunation decreased the total activity of the fibrolytic enzymes of the solid adherent microorganisms. Similar results were obtained by Kurihara et al. [21]. This decrease could be attributed to the decrease of the specific activity of these enzymes and/or to the decrease of the number of fibrolytic microorganisms that produce these enzymes. Indeed, our results indicate that both carboxymethyl cellulase and xylanase specific

activity decrease after defaunation. Another factor that may explain the decreased hydrolytic activity in the rumen is the modification of the structure of the microbial ecosystem after defaunation [14, 35]. This decrease may mainly be attributed to the disappearance of the fibrolytic activity of protozoa after defaunation. Indeed protozoa are reported to have the highest fibrolytic activity in the rumen compared to bacteria or fungi [6, 15]. Moreover, no variation of the main cellulolytic bacterial population was observed after defaunation in our results. The effect of defaunation on cellulolytic bacteria is still unclear; some studies indicate an increased cellulolytic bacterial population after defaunation [15, 43], whereas others indicate a decreased [1, 7] or unchanged one [42]. Hence, in our study since no effect was observed, decreased fibrolytic activities of protozoa in the rumen may not be counterbalanced by an increased cellulolytic bacterial population.

Another factor that is reported to explain the decrease of fibrolytic activity is the decrease of the pH under 6.0 units [15, 26]. Our results indicate that defaunation increased the ruminal pH above 6.0 units. Increased pH after defaunation is coherent with previous results which indicate that the defaunation effect on the pH varied according to the diet [8]. Hence, when the percentage of concentrate in the diet was inferior to about 50%, defaunation increased the pH. Indeed, in our study the percentage of concentrate was always inferior to 50%, except with the D1 diet. When the percentage of the concentrate increases above 50%, the protozoa intake large amounts of starch grain, thus contributing to stabilise the pH of the rumen. In our study, the effect of defaunation on pH may not induce a decrease of fibrolytic activities in the rumen.

The relative proportion of fermentation end products can change according to the structure of the microbial ecosystem, the sizes of the population and the activities of the microbial species [41]. Defaunation decreased VFA concentration. Nevertheless the decrease of OM degradation in the rumen may partly be compensated for by an increased digestibility of OM, in the lower gut, thus limiting the decrease of energy availability for the animal after defaunation [8]. This result was in agreement with the literature data with such diets [14]. Defaunation also modified the VFA profile in the rumen, inducing a significant decrease of the butyrate concentration. As a consequence, higher C3/C4 and C2/C4 ratios were observed after defaunation in our study. The decreased molar proportion of butyrate observed after defaunation is coherent with the elimination of protozoa after defaunation, which are important butyrate producers [4, 14]. Moreover, amylolytic bacteria, which produced succinate which is then decarboxylated by *Selenomonas ruminantium* to give propionate, are reported to increase after defaunation [7, 21]. Indeed, protozoa ingest a high number of starch grains and amylolytic bacteria are associated with them [15]. This may lead to increase the use of absorbed energy in defaunated animals, since the propionate metabolic used is more favourable than the acetate or butyrate ones [39]. Furthermore, the decrease of VFA concentration (7%) could mostly be explained by a decreased OM degradation in the rumen that may occur since the in sacco DM degradation in the rumen decreased after defaunation.

5. CONCLUSION

In our study, the diet had important effects, nevertheless it did not strongly interfere with the defaunation effect, on the contrary to what was expected. The most striking interaction indicated that the highest decrease of the in sacco DM degradation appeared when the P/E ratio was the lowest, on the D1 diet.

Our results on the effect of defaunation on some enzymatic microbial activities confirm previous studies [15, 21]. Despite a high variability of the values, the determination of the evolution of a microbial

population after defaunation, with modern tools can give good results. Nevertheless, as an attempt to determine more predominant bacterial species in the rumen, more specific probes should be elaborated.

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