Reprod Nutr Dev (1995) 35, 11-25

Original article

Effect of ruminal inoculation of *Isotricha* alone or a mixed B-type fauna in a defaunated rumen on the digestion of a hay–maize diet (70:30) in sheep

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(Received 19 October 1993; accepted 22 August 1994)

Summary — Two adult sheep (75 kg live weight) fitted with rumen cannulas were defaunated by the emptying method during the first period of the experiment. They were inoculated with the genus *Isotricha* alone during the second period, and with a mixed ciliate population (*Entodinium, Eudiplodinium, Epidinium*) during the third. They were fed a diet of grass hay (840 g) and pelleted maize grains (360 g) in 8 meals per day, every 3 h. Defaunation was successful and no accidental contamination occurred during the experiment. The protozoa had no significant effect on the volume of rumen digesta, nor on the turnover of the particulate phase. The addition of *Isotricha* and of the mixed fauna increased the ADF digestibility of the diet but, in the same animals, lowered the *in sacco* degradation of wheat straw. The ruminal pool sizes of dry matter (DM), organic matter (OM), nitrogen (N), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid lignin detergent (ADL) remained unchanged after protozoa inoculations. The concentration of total volatile fatty acids (VFA) was not altered by faunation with *Isotricha* or a mixed fauna. The molar proportion of acetate increased at the expense of all the other VFAs (mainly propionate with the mixed fauna). Correspondingly, the proportion of methane in the rumen gases increased and that of CO_2 decreased in inoculated animals. The ammonia concentration was highest in animals with a mixed fauna and lowest in those inoculated with *Isotricha* alone. This trend is explained in terms of the specific effect of the different genera of protozoa on nitrogen metabolism.

rumen / protozoa / digestion / kinetics of digesta

Résumé — Effet de l'inoculation d'*Isotricha* ou d'une faune conventionnelle de type B dans un rumen de mouton défauné sur la digestion d'un régime mixte foin-maïs (70/30). *Deux moutons adultes (75 kg de poids vif) porteurs de fistule permanente du rumen ont été défaunés par la*

méthode de vidage au cours de la première période. Leur rumen a été ensuite inoculé avec le seul genre Isotricha *pendant la deuxième période et avec une faune mixte de type B au cours de la troisième période. Ils ont reçu une ration composée de foin de Graminées (840 g) et de grains de maïs agglomérés (360 g) distribuée en 8 repas par jour, toutes les 3 h. La refaunation du rumen n'a pas modifié le volume total des digesta dans le rumen, ni la vitesse de renouvellement de la phase solide. La masse totale de MS, MO, N, NDF, ADF, ADL présente dans le rumen n'a pas non plus été affectée par la présence des protozoaires ciliés. La digestibilité de la lignocellulose de la ration (fraction ADF) a été accrue ($P < 0,05$) par l'addition d'*Isotricha *et d'une faune mixte dans le rumen bien que la dégradation* in sacco *d'une paille de blé ait été plus faible chez ces mêmes animaux. La concentration totale des AGV n'a pas été modifiée par l'introduction d'*Isotricha *ou d'une faune mixte dans le rumen ; la part de l'acétate dans le mélange des AGV a augmenté aux dépens de celle des autres acides. Corrélativement, la proportion de méthane dans les gaz a augmenté. La concentration en N-NH_3 a été la plus élevée chez les animaux à faune mixte et la plus faible chez les animaux mono-inoculés avec* Isotricha. *Des explications sur l'évolution de ce dernier paramètre sont données en relation avec l'action spécifique du genre* Isotricha *sur le métabolisme azoté dans le rumen.*

rumen / protozoaires / digestion / cinétique des digesta

INTRODUCTION

It is now generally agreed that ciliate rumen protozoa have a negative effect on nitrogen use by ruminants. In contrast, the results concerning their action on cellulolysis are conflicting (Bird and Leng, 1984; Romulo *et al,* 1986). This is an important issue since forage is the basis of ruminant diet. It can be of vital importance in countries where the nutritional value of roughage is low and where energy and nitrogen enrichment of the basic diet cannot be readily achieved on a wide scale.

The present study investigates the action of rumen protozoa in cellulolysis and in proteolysis; it is novel in that the main physiological, digestive and metabolic parameters were measured in the rumen of animals that were successively defaunated, monoinoculated with *Isotricha* sp and inoculated with a conventional mixed fauna. The overall effect of the protozoa was assessed by the differences recorded between the faunated and defaunated states. The intermediate monoinoculated state provided a means of studying the effects of *Isotricha* sp, which develop in the molasses-rich feed used by Bird and Leng (1978, 1984) and Bird (1989, 1991). It was with such a feed that these authors observed a negative effect of protozoa on cellulolysis, while most of the tests carried out in Europe, North America and Japan have yielded opposite results on animals harbouring a population rich in *Ophryoscolecidae* (Jouany *et al*, 1988). Furthermore, Jouany *et al* (1992) observed *in vitro* that addition of *Isotricha* alone in a defaunated rumen decreased the protein degradation while the degradation was increased with a mixed-fauna inoculation. It seems therefore that *Isotricha*, which has rarely been studied as the only protozoa *in vivo*, have a specific action in rumen digestion. In addition, Jouany *et al* (1981) showed that the introduction of *Isotricha* alone into defaunated sheep rumens decreased lignocellulose digestion in a mixed feed based on dehydrated alfalfa. It was thus decided to perform measurements of the dynamics of digestion as a complement to the standard measurements to know how *Isotricha* behaves in a grass–hay-based diet.

MATERIALS AND METHODS

Animals

Two castrated adult male Texel sheep weighing 73 kg (sheep A) and 76 kg (sheep B), fitted with

large rumen fistula (75 mm in diameter), were used for 3 measurement periods. Their rumens were defaunated according to the procedure of Jouany and Sénaud (1979a) during the first period, and measurements were started 60 d later. They were then inoculated with the single genus *Isotricha* as described by Jouany *et al* (1981) at the beginning of the second period. Measurements were started 30 d after stabilization of the *Isotricha* population. Finally they were inoculated with a mixed inoculum comprising the genera *Entodinium*, *Epidinium*, and *Eudiplodinium*, in addition to *Isotricha*. This inoculum was characterized as a B-type according to Eadie (1962). Measurements were started 30 d after stabilization of the ciliate population.

The sheep were housed in a building set apart from other animal facilities and specially designed so that was no inter-animal contact. In order to avoid contamination the staff were trained to handle the sheep that had been defaunated and subsequently refaunated with a single protozoal species or with a defined mixed protozoal population (see Jouany, 1978).

The welfare of the animals was strictly respected throughout the experiment, which was performed in accordance with the regulations laid down by French law.

Feed

The sheep were fed daily 840 g of chopped natural grass hay and 360 g of pelleted maize grains distributed semi-continuously by means of an automatic feeder (8 equal meals per day, every 3 h). Dietary concentrations of organic matter (OM), nitrogen (N), neutral detergent fibre (NDF), acid detergent fibre (ADF) and starch were 91.8, 1.3, 49.9, 29.6 and 21.2% of the dry matter (DM) respectively.

Measurements

Characterization of rumen digesta

The volume and weight of the rumen contents were measured after all rumen digesta were collected by completely emptying the rumen at 14.00 h, between 2 meals. Pools of DM, OM and cell-wall components were assayed twice per period for each sheep on a representative sample of digesta.

Breakdown of cell-wall components

The digestibility of the diet and cell-wall components was calculated by measuring feed intake and collecting faeces for 7 d.

In sacco degradation of wheat straw

Wheat straw is considered to be a substrate composed almost exclusively of pure cell walls. To avoid physical losses from bags it was ground in a mill fitted with 2 mm grating and then screened so as to retain only particles larger than 200 microns. Nylon bags 5.5 x 12 cm in size (Ankom Co, Fairport, NY, USA), made of a woven fabric 95 microns in mesh size and containing 3 g of straw, were left for 6, 12, 18, 24, 48, 72 and 96 h in the rumen. Two kinetic runs with 2 bags per time and per run were carried out in each sheep. The rates and the levels of straw degradation (parameters *a*, *b* and *c*) were estimated by plotting the curve of DM disappearance according to the model of Ørskov and Mc Donald (1979).

Measurement of feed retention time in the digestive tract

A fraction of the hay from the diet was washed in a domestic washing machine with a commercial washing soap to eliminate cytoplasmic contents and to obtain a residue containing about 90% NDF. It was then labelled with ytterbium (Yb) by soaking for 20 h in a solution of Yb acetate (50 mg Yb g^{-1} hay); it was then thoroughly washed with running water to eliminate unbound Yb. The hay thus treated contained about 25 mg Yb g^{-1} DM. The exact content of Yb was then determined.

Exactly 20 g of labelled hay was introduced *via* the cannula into the rumen and an aliquot of excreted faeces was taken every 4 h for 48 h, then every 8 h for the next 36 h, and finally every 12 h up to 168 h. The faecal Yb excretion curve was plotted and modelized according to the model of Grovum and Williams (1973), Faichney (1986), and Thielemans *et al* (1978) to determine the retention time of solid particles in the different digestive compartments.

The liquid phase was labelled by addition to the rumen of a 100 ml dose of a 20% (w/v) solu-

tion of polyethylene glycol (PEG 4000). A 200 ml sample of rumen content was taken with a 12 mm diameter tube connected to a rubber syringe 2, 4, 6, 8, 12, 16, 20, 24, 32, and 48 h afterwards. Twenty millilitres were used for PEG assay and the remainder was returned to the rumen. The retention time (T_h) and turnover rate ($k = 1/T_h$) of the liquid phase was calculated by the equatjion $C = C_o\ e^{-kt}$.

Measurement of fermentation end products and the number of protozoa and bacteria in the rumen

Samples of 200 ml rumen fluid were taken on 3 successive days in each of the sheep halfway between 2 successive meals, 5 times in the course of the day. About 15 ml was used to count protozoa and to estimate the bacterial population, to assay microbial metabolites, such as VFA, ammonia-N (NH_3-N), and to record pH; the remainder was returned to the rumen. Gases were sampled, at the same times as above, with a rumen cannula specially designed for this type of measurement (Jouany and Sénaud, 1979b) that was fitted the day before to avoid air contamination of the rumen gas phase.

Experimental design

Each sampling period started after rumen conditions were stabilized (60 d after defaunation was achieved for period 1; 30 d after the *Isotricha* sp population had become stable for period 2; 30 d after the mixed fauna had established for period 3). The periods lasted at least 4 weeks. The 2 *in sacco* kinetic runs were carried out in the first week. The distribution of transit markers and collection of faeces for the measurement of retention time and digestibility were made during the second week. pH measurements and microbial metabolite assays in the rumen were performed on 3 successive days during the third week. The rumen was emptied twice during the fourth week, on Tuesday and Friday.

Assays

DM was determined by oven drying at 80°C for 48 h. OM was measured after ashing in a muffle furnace (550°C for 6 h). Nitrogen was assayed by the method of Kjeldahl (AOAC, 1965) automated with the Kjeltec (Tecator, Paris, France). Cell-wall components were analyzed by the method of Van Soest and Wine (1967) automated with the Fibertec (Tecator, Paris, France) after starch had been eliminated by a glucoamylase treatment. The method of Thivend *et al* (1972) was used for starch assay. Ytterbium was analysed by flame spectrophotometry according to the method of Siddons *et al* (1985). PEG was determined by turbidimetry (Hyden, 1955).

Volatile fatty acids were assayed by the method of Jouany (1982). Samples of rumen fluid (5 ml) containing 5% (w/v) solution of orthophosphoric acid were kept at –15°C. Two milliliters of rumen fluid were mixed with 8 ml of a 12.5% (w/v) NaCl solution and stored at –15°C for determination of NH_3-N by a modified version of the method of Berthelot (Na salicylate – dichloroisocyanurate reactants were used instead of phenol – Na hypochlorite) with a Technicon autoanalyser (Van Eenaeme *et al*, 1969). Gases were analysed by gas-solid chromatography (Jouany and Sénaud, 1978).

The protozoa were counted under a binocular magnifier (x 80) in Dollfuss cells according to the method described by Jouany (1978) in rumen fluid obtained by filtration through metal gauze (1 mm mesh size). Total protozoa volume was calculated from an assumed volume of about $0.5 \times 10^6\ \mu m^3$ for each cell of *Isotricha*, *Epidinium* and *Eudiplodinium* and $2.0 \times 10^4\ \mu m^3$ for small entodinia (Warner, 1962). The corresponding mass of protozoa was established on the basis that 1.2×10^5 large ciliates (*Isotricha*, *Epidinium*, *Eudiplodinium*) contained approximately 1 mg N (Leng *et al*, 1981) and that the mean content of N in their DM was close to 6.0% (Jouany and Thivend, 1972). We also assumed that the volume, and therefore the weight, of small entodinia was 100 times less that of the large ciliates. According to these estimations, we calculated that 9 mg DM of ciliates was reached with 10^5 large ciliates and 10^7 small ciliates.

The total numbers of bacteria were determined by direct counts after staining with orange acridine under epifluorescent microscopy (Prevot *et al*, 1988).

Statistical analysis

As it is not possible to remove ciliates by mild treatment, the experimental design had to follow

the sequential order of defaunation, refaunation with only one species of protozoa, and faunation with a mixed fauna. A latin-square treatment was therefore inappropriate. Because of the difficulty of carrying out such an experiment with different fauna (including a single species inoculation), only 2 animals were used. Each was considered as its own control. Comparisons between the 3 different periods were made for each animal. In addition, samplings were several times to obtain more precise values of the means.

Data were treated by an Anova procedure (SAS, 1985). The model was as follows: $Y_{ij} = \mu + Ai + T_j + \sum_{ij}$, where Y_{ij} = trait observed, A_i = animal effect, T_j = protozoa effect and $\sum_{ij}$ = error, assumed to be the interaction between animal and protozoa effects.

RESULTS

No accidental contamination of the rumen fauna occurred during any of the 3 experimental periods.

There was no significant 'animal x protozoa' interaction ($p > 0.05$) in any of the digestive parameters tested. Hence, only one mean value is given for each period and comparisons were made between the 3 periods.

Establishment of Isotricha *sp and mixed fauna in sheep rumen*

The presence of *Isotricha* in the rumen of defaunated sheep was detected (minimal concentration = 50 cells ml^{-1}) 2 d after the introduction of 250 ml of rumen contents containing 4 x 10^3 ciliates ml^{-1}, sampled from a monoinoculated sheep previously prepared according to Jouany (1978). The concentration peaked by day 14 (3 700 *Isotricha* sp ml^{-1}), remained at this level for about 20 d, and then settled down at a value close to 2 000 ml^{-1} (table I).

At the beginning of the third period, animals were inoculated with 250 ml of mixed rumen contents sampled from sheep monoinoculated with *Entodinium* sp (75 000 ml^{-1}), *Epidinium* sp (3 500 ml^{-1}), and *Eudiplodinium* sp (30 000 ml^{-1}). The introduction of this mixed inoculum into the rumen of sheep monoinoculated with *Isotricha* spp resulted in a complex fauna whose population could be followed immediately. Peak values were reached between day 7 and day 14 for all the ciliates: 3 000 *Isotricha*, 2 000 *Epidinium*, 40 000 *Eudiplodinium*, 32 000 *Entodinium* ml^{-1} (fig 1). The *Entodinia* reached the highest number at about day 50, attaining a level of more than 10^5 ml^{-1}, which accounted for about 75% of the total population, while the numbers of *Epidinium* dropped from day 35 (50 – 400 *Epidinium* ml^{-1}). The population of *Eudiplodinium* fluctuated between 10^4 and 4 x 10^4 ml^{-1}. The population of *Isotricha* spp, when mixed with the other ciliates, stabilized at the same level (about 3 000 ml^{-1}) as it reached when alone in the monoinoculated sheep (figs 1, 2; table I).

Variations in protozoa populations during the day

Although the sheep were fed every 3 h (pseudo-steady state of the rumen), the concentrations of the protozoa varied over the 24 h cycle. The *Isotricha* sp population increased 1.5 h after each feeding and decreased just before feed was distributed.

Besides the variations linked to feeding, we observed nycthemeral variations in *Isotricha* concentration that may have been due to sequences of cell division. There was no significant variation between 9.00 h and 17.00 h, but a decrease occurred at 21.00 h and there was a significant increase at 1.00 h.

The concentrations of entodiniomorphs in the rumen of sheep inoculated with the mixed fauna slightly decreased immediately after feeding and reverted to their initial

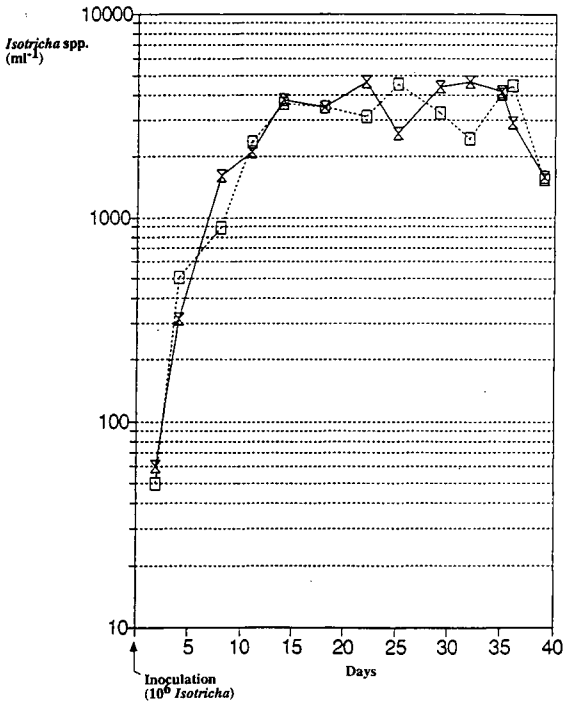

Fig 1. Establishment of *Isotricha* in a defaunated sheep rumen (sheep A: □ ; sheep B: ⧖).

values just before the following feeding. The general time course of *Isotricha* in animals inoculated with the mixed fauna was the same as that observed during the period when the sheep were monoinoculated with this genus.

Biomass of protozoa and concentration of bacteria according to the nature of the fauna

Both the volume and the mass of the ciliates in the rumen of the sheep with mixed fauna were about 10-fold greater than in the rumen of the monoinoculated sheep (table I). In the latter, the biomass of the ciliates ranged between 3.1 and 4.0 g l^{-1}.

The number of total bacteria was close to 3.4×10^9 ml^{-1} during the defaunated period. It stabilized at 1.7×10^9 ml^{-1} during monoinoculation with *Isotricha* or inoculation with a mixed conventional fauna. Given the large variations within each period, the differences between periods were not statistically significant.

Effect of protozoa on physiological and physical characteristics of rumen digesta

The volume and weight of total rumen contents were not significantly influenced by the rumen fauna (table II). Consequently, there was no difference in the specific weight of rumen content between periods. The

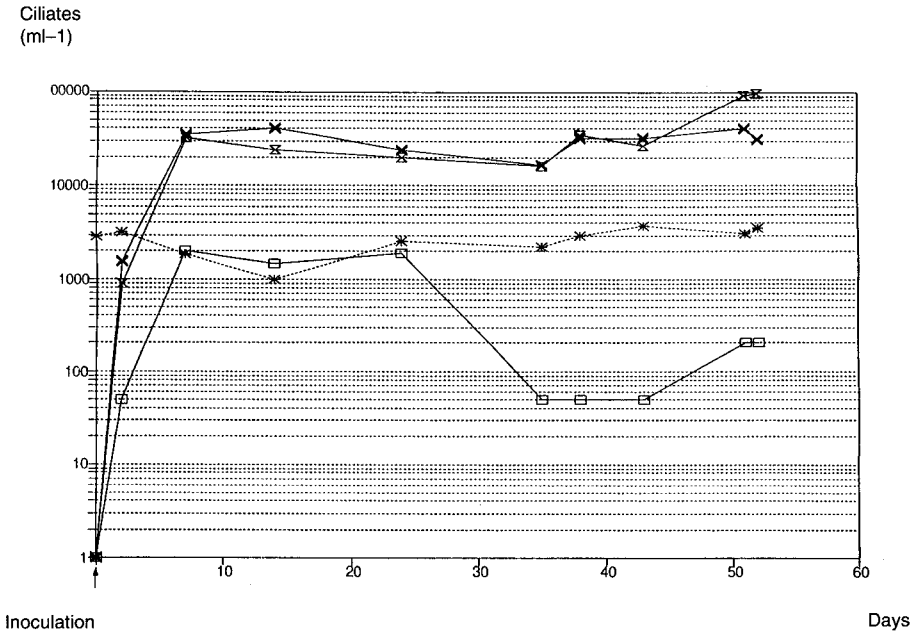

Fig 2. Establishment of mixed fauna in a defaunated rumen. Inoculation: 4 x 10^6 *Epidinium*; 5 x 10^6 *Entodinium*, 1 x 10^7 *Eudiplodinium*. *: *Isotricha*; □ : *Epidinium;* x *Eudiplodinium*; ⧖ : *Entodinium*.

Table I. Protozoal counts (x 10^3 ml^{-1}) and their approximate volume and dry weight (DW) in the rumen ($n = 2$).

	Sheep						*Animal effect*	*Protozoa effect*
	Defaunated		*Inoculated with* Isotricha		*Inoculated with mixed fauna*			
	A	*B*	*A*	*B*	*A*	*B*		
Isotricha sp	0	0	2.3	1.9	2.7	3.0	NS	0.001
Epidinium sp	0	0	0	0	0.37	0.15	NS	0.001
Eudiplodinium sp	0	0	0	0	30.0	40.0	NS	0.001
Entodinium sp	0	0	0	0	90	120	NS	0.001
Approximate volume of ciliates (ml l^{-1})	–	–	1.1	0.9	18.3	23.9	NS	0.01
Approximate DW of ciliates (g l^{-1})	–	–	0.21	0.17	3.1	4.0	NS	0.01
Rumen pool size of protozoal dry matter (g)	–	–	2.5	2.0	35.8	46.2	NS	0.01

NS: not significant.

Table II. Characterisics of rumen digesta determined by the emptying method according to the fauna ($n = 2$).

	Sheep [a]			Animal effect	Protozoa effect
	Defaunated	Inoculated with Isotricha	Inoculated with mixed fauna		
Total digesta (kg)	11.90	11.97	11.55	NS	NS
Density (gml^{-1})	0.908	0.906	0.894	NS	NS
Rumen pool size (g)					
Dry matter	1 202	1 268	1 251	NS	NS
Liquid	10 698	10 702	10 299	NS	NS
Organic matter	1 073	1 144	1 114	NS	NS
Nitrogen [b]	23.3 (31.0)	24.6 (31.8)	22.6 (34.3)	NS	NS
NDF	844	918	910	NS	NS
ADF	533	524	530	NS	NS
ADL	132	126	152	NS	NS

[a] Means are not statistically different ($P > 0.05$); [b] nitrogen was assayed on dried rumen content; values in brackets were obtained on fresh rumen content.

rumen pool sizes of DM, OM, N, NDF, ADF, acid detergent lignin (ADL) were unchanged after the inoculation of ciliates into the defaunated rumens.

End products of rumen fermentation

The pH of the rumen contents and total VFA concentrations were not significantly modified by the presence of either of the fauna types studied (table III). The increase in the proportion of acetate in the VFA mixture after the introduction of the mixed fauna (+9.4%) in the defaunated rumen was lower after inoculation of *Isotricha* (+4.5%). This tendency towards an increase is consistent with the greater amount of methane (+6%) present in the rumen gases. The proportion of propionate decreased in sheep inoculated with the mixed fauna (–40%). Addition of *Isotricha* to the defaunated rumens had no effect on propionate molar proportion. Correspondingly, the proportion of CO_2 in the rumen gases was slightly lower in the mixed faunated sheep (–4%). The butyrate proportion remained unchanged after the inoculation of protozoa in defaunated animals. The sum of branched VFA and valerate and caproate was lowest in *Isotricha*-inoculated animals.

Ammonia N concentration in the rumen fluid increased (+49%) after the addition of mixed fauna to the defaunated rumens and decreased (–29%) after inoculation of *Isotricha* alone.

In sacco *degradation of wheat straw*

The addition of *Isotricha* or a mixed fauna to the defaunated rumens did not modify the rapidly degradable fraction (fraction 'a') of the DM of wheat straw (table IV). The potentially digestible fraction (fraction 'b') was greatest in the defaunated sheep. It fell in the mixed-fauna animals while it reached an intermediate value in the presence of

Table III. End products of fermentation in the rumen according to the protozoa ($n = 2$).

	Sheep			Animal effect	Protozoa effect
	Defaunated	*Inoculated with* Isotricha	*Inoculated with mixed fauna*		
pH	6.48	6.43	6.50	NS	NS
Total V F A (mM)	82.1	69.3	65.9	NS	NS
Acetate (molar %)	67.0	70.1	73.3	NS	0.06
Propionate (molar %)	20.6	18.9	14.7	NS	0.01
Isobutyrate (molar %)	1.1	0.91	0.96	NS	NS
Butyrate (molar %)	8.9	8.2	8.5	NS	NS
Isovalerate (molar %)	1.3	1.1	1.3	NS	NS
Valerate (molar %)	0.78	0.56	0.72	NS	0.06
Caproate (molar %)	0.37	0.21	0.48	NS	0.01
N-NH_3 (mg l^{-1})	48.2	35.0	75.0	NS	0.05
Rumen gases					
CO_2 (% total volume)	69.4	66.7	66.9	NS	0.06
CH_4(% total volume)	30.5	33.3	33.1	NS	0.06

NS: not significant.

Isotricha. Compared to that of defaunated animals, the degradation rate (fraction 'c') was higher in the presence of a mixed fauna (+54%), and lower in the *Isotricha*-monoinoculated animals (–25%).

Point-by-point analysis of the kinetics shows that the positive effect of defaunation on DM breakdown of wheat straw was only significant after 12 h retention in the rumen.

Table IV. Parameters of *in sacco* degradation of wheat straw ($n = 2$) in defaunated rumens, rumens inoculated with *Isotricha* or a mixed fauna (type-B), from the model of Ørskov and McDonald (1979) [a].

Parameter	*Sheep*			*Animal effect*	*Protozoa effect*
	Defaunated	*Inoculated with* Isotricha	*Inoculated with a mixed fauna*		
a (%)	9.76	9.82	5.67	NS	0.06
b (%)	51.5	46.5	40.0	NS	0.04
c (h^{-1})	0.024	0.018	0.037	NS	0.04

[a] $D\ (t) = a + b\ (1 - e^{-ct})$; $D\ (t)$ = degradation at time t; a = fraction which is rapidly degraded in the rumen; b = fraction which is potentially degradable by rumen microbes. NS: not significant.

Kinetics of soluble and particulate markers

The volume of liquid in the rumen, as evaluated from the marker concentration at 'time zero' *(ie* the time the dose of marker was introduced) obtained by calculating the intersection of the decay curve of the marker concentration with the *y*-axis, was unaffected by the introduction of *Isotricha* in defaunated rumen, as confirmed by the emptying method (table V). In contrast, it was lowered (–15%) by the addition of the mixed fauna while with the emptying method this type of fauna had no discernible effect.

The retention time of the particulate phase in the different parts of the digestive tract calculated from the 2-mixing-compartment-one-delay model of Grovum and Williams (1973) indicated that the time delay, which reflects retention in the tubular part of the tract (omasum, abomasum, small intestine), was not influenced by the addition of the mixed fauna to the rumen of defaunated or monoinoculated sheep. There was no difference in the mean retention time of solid particles (34.2 ± 5.3 h) in compartment 1 (taken to be the rumen) between the 3 experimental periods, nor in that (16.6 ± 0.4 h) of compartment 2 (taken to be the caecum and proximal colon).

Digestibility of the diet throughout the digestive tract

Protozoa had no influence on the digestibilities of DM, OM, or N (table VI). Both types of protozoal inoculations (*Isotricha* alone or mixed fauna) increased the digestibility of the lignocellulose fraction (ADF) of the feed

Table V. Dynamics of digesta estimated by markers ($n = 2$).

	Sheep			*Animal effect*	*Protozoa effect*
	Defaunated	*Inoculated with* Isotricha	*Inoculated with a mixed fauna*		
Water pool size in the rumen (l)	8.1	10.2	6.9	NS	0.05
Turnover of liquid phase (% h^{-1})	5.8	6.1	7.8	NS	0.05
MRT of liquid phase in the rumen (h)	17.1	16.7	12.8	NS	0.04
MRT of particulate phase in the whole tract (h)					
Faichney model	ND	62.6	66.0	0.05	NS
Thielemans model	65.3	62.9	65.2	NS	NS
Grovum and Williams model	75.9	69.7	72.0	0.05	NS
Kinetics of particulate phase according to Grovum and Williams					
Time delay (TT) (h)	21.7	24.5	18.9	NS	NS
MRT in compartment 1 (h)	38.0	28.1	36.5	0.05	NS
MRT in compartment 2 (h)	16.2	17.1	16.6	NS	NS

MRT: mean retention time. ND: not determined, NS: not significant.

Table VI. Digestibility of the diet along the whole digestive tract of sheep ($n = 2$).

	Sheep			*Animal effect*	*Protozoa effect*
	Defaunated	*Inoculated with* Isotricha	*Inoculated with mixed fauna*		
Dry matter digestibility	61.7	62.3	63.2	NS	NS
Organic matter digestibility	64.6	65.6	66.4	NS	NS
Nitrogen digestibility	60.9	61.7	62.1	NS	NS
NDF digestibility	49.1	50.7	53.7	NS	0.06
ADF digestibility	46.7	51.9	53.4	NS	0.05

NS: not significant.

(+14% respectively). The positive effect of protozoa on the digestibility of the total cell-wall components (NDF) was only significant in the mixed faunated animals.

DISCUSSION

In agreement with previous studies of Sénaud *et al* (1980), Grolière *et al* (1980) and Grain *et al* (1980), our results showed that ciliates become rapidly established in a rumen that has been defaunated. The populations of *Ophryoscolecidae* and *Isotricha* reached their maximum size after 7 and 14 d, respectively. As observed by the previous authors, a stable plateau was obtained some 20 d after this peak, but at a lower level.

After the inoculation of the mixed fauna, the numbers of *Isotricha* did not decrease from their previous level, which proves that there was no interaction between the 2 types of ciliates. An absence of competition between *Isotricha* and *Entodinium* was previously observed by Grolière *et al* (1980) in a mixed diet similar to that used in our study. The only interaction observed by these authors was slight competition between *Isotricha* and a type-A fauna *(Polyplastron)*, which was unfavourable to the former. This suggests that *Isotricha* behaves differently towards type-B and type-A ciliates.

Although the animals were fed 8 equal meals during the day, the populations of protozoa were not in steady-state conditions. The decrease in *Isotricha* numbers noted before each meal is consistent with the results of Abe *et al* (1981) and Murphy *et al* (1985) obtained in cows and steers respectively, fed 2 meals per day. The authors explained that *Isotricha* rapidly migrate from the reticulum towards the rumen in response to a chemical stimulus originating from dietary soluble compounds. The decrease in entodiniomorphid ciliates after feed intake owing to their dilution in a higher volume of digesta is in good agreement with the previous results of Jouany *et al* (1973). The increase in these ciliates that occurred at 1.00 h could have been due to the high ruminating activity of animals from 21.00 h and during the beginning of the night which made more substrates available to rumen microbes even when animals were continuously fed (Sénaud *et al,* 1977).

The biomasses of protozoa in the rumen of mixed-faunated sheep (3.1 and 4.0 g l^{-1}) were close to those recorded by Jouany (1978) who used a method based on centrifugations to isolate protozoa: 1.5 g l^{-1}

for a diet based exclusively on grass hay; 3.6 g l^{-1} for a hay–barley diet; 5.0 gl^{-1} for a hay + Jerusalem artichoke diet, and 3.7 g l^{-1} for a diet of hay and sugar beet.

Although the statistical threshold was not reached, the tendency for the number of bacteria to decrease after ruminal inoculation of protozoa, whatever the type and level of the protozoa, confirms the negative correlation between bacterial and protozoal densities previously reported by Teather *et al* (1984). The variations in bacteria concentration we observed from one day to another and between animals were probably due to the method of direct counts used.

The variance analysis of kinetics of faecal output of markers used here to evaluate the dynamics of particles in the digestive tract indicated that animal effect was higher than protozoa effect. Differences between the models of Faichney and Thielemans were slight while the Grovum and William model gave higher values for digestive retention times of particles (+10 to +16%). The kinetics of liquid phase estimated by PEG dilution rate were influenced by protozoa, the highest rate being obtained with faunated animals which had also the lowest rumen volume of liquid. It must be noted that the emptying method showed no effect of protozoa on rumen volume.

The absence of any difference in the measured pool sizes of rumen digesta following the inoculation of protozoa is an indication that protozoa had no significant effect on quantitative aspects of ruminal digestion, since the retention time of solid particles was not changed by the inoculation of protozoa. According to Demeyer (1987), there are great variations in the dynamics of digesta in the rumen between animals in the response to defaunation; sometimes the outflow rate of digesta increases and the rumen volume decreases; sometimes opposite results are recorded. As in our experiment, Faichney and Griffiths (1978), Orpin and Letcher (1983-84), and Punia *et al* (1987) noted no effect of protozoa on the volume of the rumen nor on the mean retention time of particulate phase in the rumen.

There is still a wide debate about the real impact of refaunating a defaunated rumen on the digestion of plant cell walls. Most studies made during the last 15 years showed and increase in cell-wall carbohydrate digestion after inoculation of protozoa into defaunated rumens (see Jouany, 1989, 1991). A few inverse results were obtained in Australia (Bird and Leng, 1984; Romulo *et al,* 1986).

Our results clearly show that the inoculation of a single species or a mixed species of protozoa into defaunated rumens improved the digestion of the lignocellulosic fraction (ADF) of the diet more than the digestion of the total cell-wall fractions (NDF). This indicates that the role of protozoa was more important for the digestion of cellulose than hemicellulose fractions, which is in disagreement with the proposal of Ushida *et al* (1991). Consequently, we suggest that rumen ciliates have an effect on all the plant cell-wall fractions (hemicellulose or lignocellulose) and that the effects on the different components of cell walls observed depend on the chemical organization of the complex matrix of the cell-wall carbohydrates.

The positive effect of *Isotricha* or a mixed fauna on the digestion of the lignocellulose in the whole digestive tract we observed here is difficult to relate to the decrease in the potentially digestible fraction of wheat straw estimated *in sacco* in the rumen of the refaunated sheep. This indicates that the same substrate has to be used for *in situ* and *in vivo* digestion trials. Jouany (1989) discussed the 'substrate effect' on cell-wall digestion in relation to the response of protozoa. He observed that wheat straw, unlike other roughages, is not a suitable substrate to study the effect of protozoa on cellulolysis. Also, as suggested by Trabalza

Marinucci *et al* (1992), *in sacco* degradation may not reflect the real *in situ* degradation. However, if we suppose that *in sacco* trials were not biased, it follows that the lower digestion of cell-wall carbohydrates in the rumen of faunated animals was more than compensated for by a higher digestion in the hindgut of the same animals. However the reasons for such a shift in digestion which goes beyond mere compensation are difficult to explain.

The absence of any significant difference in VFA concentration and pH values after inoculation of protozoa in defaunated rumens indicates that protozoa had no great effect on the amount of fermented organic matter. It is noteworthy that no increase in the molar proportion of butyrate was observed after refaunation which is in contrast to findings reported in most previous reports (see Jouany, 1991). This trend is partly explained by the increase in the acetate proportion in faunated sheep since acetate is considered to be a major precursor of butyrate during rumen fermentations (Russell and Wallace, 1988).

The higher NH_3-N concentration in the rumen of animals faunated with the mixed B-type fauna can be accounted for by the involvement of the protozoa in feed and microbial protein breakdown, if we consider that the use of NH_3-N for microbial synthesis is not affected by the presence of ciliates as indicated by Itabashi and Kandatsu (1975). Interestingly, the presence of the genus *Isotricha* alone caused a fall (–30%) in rumen NH_3-N concentration. These results are in agreement with a previous *in vitro* experiment (Jouany *et al*, 1992) in which defaunation decreased the degradation of sparingly soluble protein in the rumen, and the addition of *Isotricha* sp to defaunated rumens resulted in an even greater decrease in protein degradation up to the NH_3-N stage, irrespective of protein solubility. This effect of *Isotricha* was confirmed by the decrease we observed in branched-chain VFA and caproate and valerate concentrations in the rumen of *Isotricha*-inoculated animals compared to the same concentrations in defaunated and mixed-faunated animals.

This study shows that when protozoa are added to the rumen, cellulolysis and proteolysis are affected. It confirms the greater digestibility of cell-wall carbohydrates in the presence of protozoa along the whole digestive tract and explains why the degradation *in sacco* of wheat straw is lower in the same animals. *Isotricha* had no specific effect on cellulolysis, which is at variance with a hypothesis we previously made from results of Bird and Leng (1984) and Romulo *et al* (1986). It induced changes similar to those observed with mixed fauna but to a lesser extent because it had a smaller biomass. The specific role of *Isotricha* in preserving protein degradation in the rumen must be underlined.

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