The Role of Protozoa in Feed Digestion*
- Review -

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ABSTRACT : Protozoa can represent as half of the total rumen microbial biomass. Around 10 genera are generally present on the same time in the rumen. Based on nutritional aspects they can be divided in large entodiniomorphs, small entodiniomorphs and isotrichs. Their feeding behaviour and their enzymatic activities differ considerably. Many comparisons between defaunated and refaunated animals were carried out during the last two decades to explain the global role of protozoa at the ruminal or animal levels. It is now generally considered that a presence of an abundant protozoal population in the rumen has a negative effect on the amino acid (AA) supply to ruminants and contribute to generate more methane but, nevertheless, protozoa must not be considered as parasites. They are useful for numerous reasons. They stabilise rumen pH when animal are fed diets rich in available starch and decrease the redox potential of rumen digesta. Because cellulolytic bacteria are very sensitive to these two parameters, protozoa indirectly stimulate the bacterial cellulolytic activity and supply their own activity to the rumen microbial ecosystem. They could also supply some peptides in the rumen medium which can stimulate the growth of the rumen microbiota, but this aspect has never been considered in the past. Their high contribution to ammonia production has bad consequences on the urinary nitrogen excretion but means also that less dietary soluble nitrogen is necessary when protozoa are present. Changes in the molar percentages of VFA and gases from rumen fermentations are not so large that they could alter significantly the use of energy by animals. The answer of animals to elimination of protozoa (defaunation) depends on the balance between energy and protein needs of animals and the supply of nutrients supplied through the diet. Defaunation is useful in case of diets short in protein nitrogen but not limited in energy supply for animals having high needs of proteins.
(Key Words : Rumen Protozoa, Carbohydrate Digestion, Protein Digestion, Ecosystem, Fermentation, Review)

INTRODUCTION

Around 50% of ingested organic matter is degraded in the rumen which is the first compartment of the digestive tract where feed arrives just after being ingested. No enzymes are supplied by rumen mucosa and, except a limited amount of lipase activity and a weak amylase activity derived from secretion in nasolabial glands of some animal species, all the enzymatic activities are of microbial origin in the rumen.

The rumen microbial ecosystem is characterised by an extreme variety and density of microbial cells. Bacteria are the most abundant micro-organisms (10^{10}, 10^{11} ml^{-1}). Protozoa are less numerous (10^5, 10^6 ml^{-1}) but have a larger size which explains why they can represent 50% of the total microbial biomass. Fungi are less abundant (10^3, 10^4 ml^{-1}) and their biomass is difficult to evaluate since they have a life cycle with alternation between a flagellate motile zoospore stage and a non-motile vegetative stage on digesta particles.

The impact of protozoa on the rumen digestion depends on their concentration and the generic composition of their population. Differences between genera in the uptake of feed particles and bacteria, as well as their enzymatic activities, have to be considered to explain variations observed on the effect of protozoa on digestion. Elimination of protozoa called defaunation was used to assess their global action in the rumen. Partial refaunation with single species or genera has been developed to identify the specific role of some protozoa. More recently, several authors tried to propose practical

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treatments to control the population of protozoa and studied their effects on milk and meat production. These different aspects will be developed here with the special emphasis of new possibilities offered by manipulation of rumen protozoal population in terms of its coneentration and generic composition.

PROTOZOA IN THE RUMEN MICROBIAL ECOSYSTEM

Protozoa has been identified in the rumen as flagellated and ciliates. In fact, there has been some confusion in the past between zoospores of chytridiomycete fungi and flagellates. Quins and Eadies ovals which are large bacteria were sometimes confused with protozoa.

Most of the rumen ciliates belong to the class Vestibuliferea (de Puytorac et al., 1974). They are divided in two orders with clear-cut differences in their digestive and metabolic activities : the order Trichostomatida and the family Isotrichidae on one hand, and the order Entodiniomorphida and the family Ophryoscolecidae on the other hand.

Protozoa and their metabolic activities

Although there are large variations in the number of protozoa and in the distribution of protozoal genera between animals fed on the same diet (table 1) and between days for the same animal, analysis of bibliographic data show clear evolution of protozoal population according to the diet. The number of protozoa or their biomass is related to the energy content of the diet (table 2).

The total concentration of protozoa increases with the amount of starch supplied in the diet (figure 1A) but the correlation established between dietary starch content and ophryoscolecid numbers from 25 experiments is weak ($r = 0.59$). A stronger correlation exists ($r = 0.86$) between the

Table 1. Variations in protozoal and fungal numbers between two sheep and their contribution to the duodenal flow of nitrogen (from Faichney et al., 1977)

	Grass hay diet		Grass hay/concentrate (3/2)	
	Sheep 1	Sheep 2	Sheep 1	Sheep 2
Anaerobic fungal zoospores ($\times 10^3$/ml)[a]	0.53	0.77	13	17
Protozoa ($\times 10^5$/ml)[a]				
Isotricha	0.03	0.04	0.08	0.14
Dasytricha	0.29	0.08	0.28	0.20
Entodinium	2.80	2.00	12.29	3.55
Eudiplodinium	NF	0.24	0.14	0.16
Epidinum	NF	0.40	1.40	0.61
Total	3.12	2.76	14.19	4.66
Protozoal N in rumen (g)	8.00	5.86	18.28	11.48
Protozoal N (% duodenal microbial N)	4.00	6.7	15.1	9.0
Bacterial N in rumen (g)	11.4	10.4	9.0	8.1
Bacterial N (% duodenal microbial N)	95.2	91.8	83.1	82.5
Fungal N in rumen (g)	0.21	0.60	0.42	0.69
Fungal N (% duodenal microbial N)	0.7	1.4	1.7	2.7

[a] determined in roll tubes (Joblin, 1981). NF : not found.

Table 2. Biomass of ciliates and liquid-associated bacteria in the rumen of steers fed different diets*

	Cellulose	Starch	Inulin	Sucrose
Biomass of protozoa (g DML^{-1})	3.7^a	8.9^b	12.6^c	9.3^b
Biomass of bacteria (g DML^{-1})	3.9^a	2.8^b	2.9^b	2.7^b

* The amount of dry matter (DM) intake of carbohydrates and crude proteins were similar in the different diets (1.5 kg day^{-1} and 800 g day^{-1} respectively).
Means with different superscript are significantly different ($p < 0.05$).

number of holotrichs and the content of sugars in the diet (figure 1B). This means that isotrich ciliates are primary users of soluble sugars, while ophryoscolecids are more susceptible to use a large variety of substrates as energy sources.

Except small entodinia, most of the ophryoscolecids are able to efficiently ingest small plant particles and use cell wall carbohydrates (Jouany and Martin, 1997). They can also take up soluble sugars by an active process at low concentration, or a passive process at high external concentrations (Williams and Coleman, 1992).

All entodiniomorphid protozoa engulf starch although the smaller in size can only take up the starch from rice. They have high amylase activity (Williams and Coleman, 1992). Both amylase and maltase are subject to product inhibition (Coleman, 1969). Ingested starch is degraded in the endoplasm and about 50% is released in the medium in form of maltose and glucose. The other part is stored in granules or in the skeletal plates inside the cells in form of amylopectin-like polysaccharide synthesized by a glycogen synthase from glucose 6-phosphate. The protozoal starch content is maximum 2-3 hours after feed

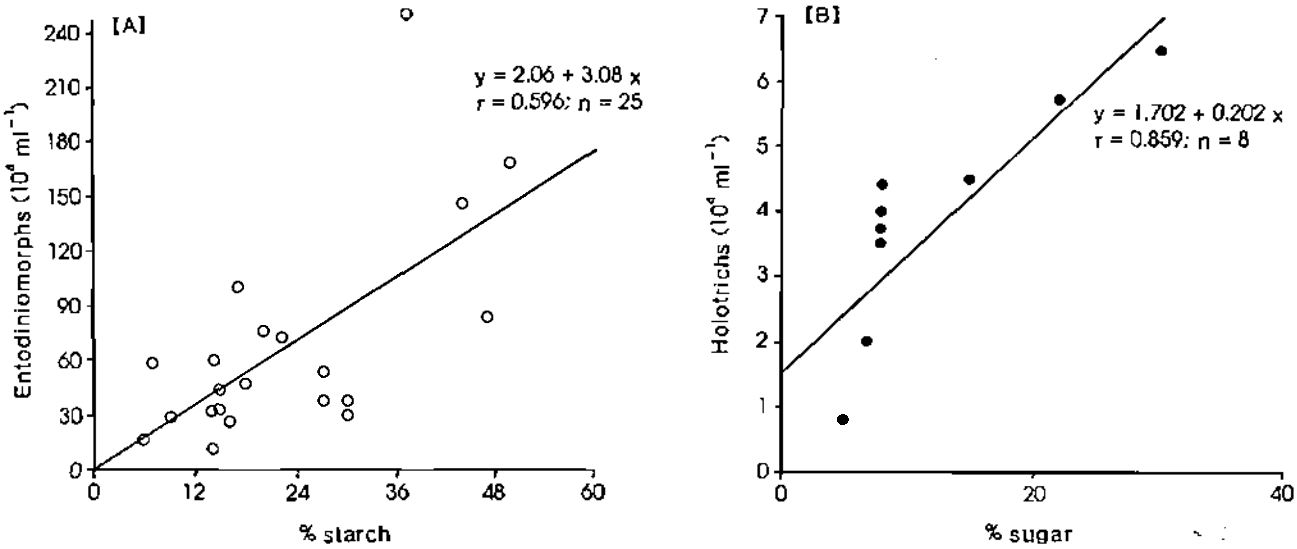

Figure 1. Relation between concentration of protozoa and starch content (A) or sugar content (B) in the diet.

intake and can reach as much as 37.7% of protozoal dry matter (Jouany and Thivend, 1972). In adult sheep weighing 70 kg fed on a mixed diet containing 60 % barley, the total pool of protozoal starch is 68 grams two hours after feeding. Taking in account that it turns over for about six hours, we calculated that more than 63% of dietary starch was engulfed and metabolised through the protozoa pool. In such a competition, amylolytic bacteria are obviously depressed.

Digestion of engulfed protein and bacteria occurs intracellularly. Protozoa release exo- and endo-peptidases into the medium, and produce peptides that become available to bacteria and to themselves (Ueda et al., 1975). Compared to bacteria, protozoa are less able to transport amino acids into the cytosol and deaminate them intracellularly (Forsberg et al., 1984). According to Ahuja and Sarmah (1979), intracellular amino acids (or small peptides) are present either in the cytosolic pool where they are used for protein synthesis, or in the non-cytosolic pool where they are catabolized or excreted. Excreted amino acids or small peptides can represent 50% of the total ingested protein nitrogen. Protozoa have high deaminase activities (Hino and Russell, 1987), and ammonia produced from deamination is excreted into the rumen medium. The contribution of ammonia coming from protozoa to the total rumen ammonia pool is high. It explains why rumen ammonia concentration decreases after elimination of protozoa.

Feeding behaviour of protozoa in the rumen

The strategy of protozoa for ingestion of plant tissues depends on the genus considered. Nice studies was done by French colleagues on this aspect. Grain and Senaud (1985) observed that *Epidinium caudatum* attach themselves to damaged plant fragments, sections of stems or leaves in 15 minutes by its apical region covered with a

thick glycoprotein coat. They have no ability to attach to plant epidermis. This attaching associated with ingestion of solid matter is called active as opposed to the passive phenomenon without uptake of matter. The passive attachment is the result of a weak joining between the cell coat of protozoa and plant cell walls. *E. caudatum* preferentially attach to the medullar parenchyma, and to the cortical parenchyma under the epidermis. As in all Vestibuliferea, ingestion by *E. caudatum* is made through a large cavity surrounded by vestibular lips opened on the funnel-shape vestibule located at the anterior end of the cell (figure 2).

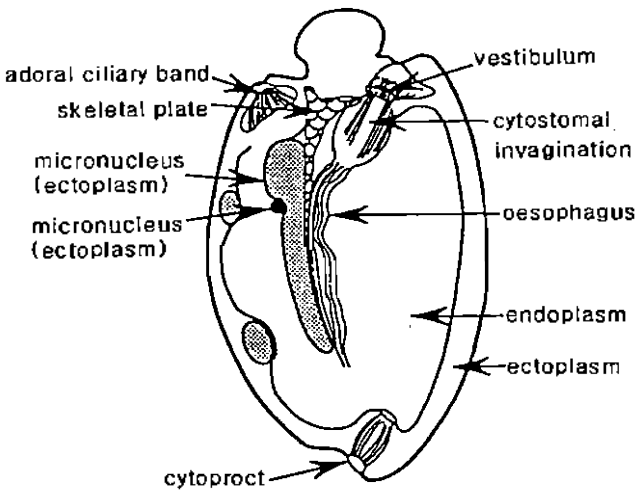

Figure 2. Schematic structure of vestibular and cytopharyngeal regions in ciliate.

Cilia located on the peristome are involved in motricity of ciliates and in creating liquid movements close to the vestibule to catch small particles. This vestibule penetrates deep into the cell by a cytopharynx. A spiral cilia band located on his wall plays an important role in uptake and migration of particles. The pharyngeal cytoplasm is limited by a puckered ring made of organized microtubules and microfibres. This ring is deeply opened on the vicinity of the macronucleus to allow the transit of digestive vacuoles from the pharyngeal cytoplasm to the deeper part of cytoplasm. Three modes of ingestion can happen : 1) When a small particle is taken, lips are opened and the vestibule is not distorted. Buccal cilia play the major role in the progression of particles towards the cytoplasm. 2) When the substrate is larger but flexible, the vestibule opens enough to surround the plant material. Lips grip tightly the substrates which are carried deep in the protozoa by cilia movements. 3) Rigid substrates are grasped by vestibular lips and the extended cuticular folds adhere strongly to ingested material. A thick microfibrillar layer reinforces these folds. Part of plant debris can therefore be detached by protozoa from large plant material in the rumen.

Immediately following ingestion, the cellulosic material is enclosed into a single vacuole (the primary vacuole) at the level of the vestibular wall. The ingested fibres are surrounded by a wide intravacuolar space preventing any contact with the membrane of vacuole. Digestive vacuoles migrate through the pharyngeal cytoplasm to the general endoplasm of the ciliate where most of the digestion occur (Senaud et al., 1986). Attached bacteria as well as free bacteria are taken up during ingestion of plant particles by *E. caudatum*. Pseudopodial tongues coming from the pharyngeal cytoplasm penetrate the digestive vacuoles and appear to setting apart bacteria and plant fragments. These tongues lead to fragmentation of primary vacuoles into smaller secondary vacuoles. Bacteria are at that time isolated in specialized small vacuoles. Products from the degradation of substrates are found in dense and compact vesicles. Most of the vesicles move from the endoplasm to the ectoplasm and insert between the cortical polysaccharide plates or granules. However, obvious anatomical relationships between vesicles and polysaccharidic structures have not yet been shown. This mode of intracellular cellulolysis is refered as cytotic (see review in Nilsson, 1979; Wichterman, 1986).

Contrary to *Epidinium* which is able largely to disrupt the vegetal tissues mechanically, *Polyplastron multivesiculatum* and the related genus *Eudiplodinium* are less firmly attach to plant particles and prefer to consume suspended particles. As in *Epidinium*, the phagocyted material is drawn into *Polyplastron* or *Eudiplodinium* through a digestive vacuole formed at the deep unciliated zone of the vestibular wall. This region lacks microtubules. If a large particle is ingested the vacuole expands considerably and may occupy the entire pharyngeal cytoplasm (Bohatier et al., 1990). Migration of the primary vacuole in the cytopharynx and formation of specialised vacuoles for bacteria are similar to those found in *Epidinium*. Cellulose degradation in vacuoles is marked by a change of texture and rearrangement of the fribrillar constituents. First, the texture becomes heterogenous. Then, the regular fibrillar organization becomes fuzzy. The filamentous constituents become denser and shorter, and form small bundles inside an electon translucent matrix which indicates degradation of some cell wall components. Later, the only few dense fibrils remaining are aggregated in compact pellets. At high

magnification, very fine filamentous material is observed around the aggregates within the clear matrix. Finally, the vacuoles open to the outside by the ciliates cytoproct, and undigested cellulosic or bacterial material is excreted. This cellulolysis process is described as permeative to distinguish it from the cytotic process (Bohatier et al., 1990). In *Polyplastron* and *Eudiplodinium*, it seems that the products of degradation of plant cell walls are transferred directly to the adjacent cytoplasm accross the primary vacuole membrane without participation of a secondary vesicular system.

Contrary to the entodiniomorphs, the isotrichs do not colonize the deep plant tissues. Therefore, they have no direct contribution to a mechanical degradation of cellulosic material in the rumen. Although isotrichs are mainly found in free form in the liquid phase of the rumen, they are attracted by soluble sugars released from the solid matrix immediately after feeding the animals. Microscopical observations show their presence at the cortical parenchyma level in plant tissues few minutes after feeding the animals. They possess an attachment organelle which appears as a longitudinal ridge, approximately 24-37 μm long and 5 μm wide, located close to the anterior extremity (Orpin and Hall, 1983). It is sometimes used to attach to plant material or to reticulum wall. The location of the vestibulum differs in the two species belonging to the genus *Isotricha* : the vestibulum of *I. prostoma* is at the posterior end of the cell, while it is on the side at approximately one-third of the body lenght away from the posterior end. The vestibulum has around 30 μm in lenght and is extended by the cytopharynx, the extremity of which is located close to the nuclear apparatus. Digestive vacuoles formed in the cytopharynx are then distributed within the endoplasm. Isotrichs possess granular microbody-like organelle 500 nm in diameter now identified as hydrogenosomes (Yarlett et al., 1983), located in the endoplasm close to the ecto-endoplasmic boundary, which makes *Isotricha sp.* less sensitive to oxygen. The larger *Isotricha sp.* is able to take up large amounts of starch and synthesise amylopectin granules from ingested soluble sugars. *Dasytricha sp.* use efficiently sugars but can take up only starch from rice because of its small size. Sugar uptake in isotrichs is not reduced by the presence of storage polysaccharide within the cell. The absence of any regulation mechanism explains bursting of numerous cells in the rumen when energy is supplied in excess.

The rumen protozoal population is able to degrade and ferment plant cell walls and this is confirmed by the widespread occurrence of cellulase in the entodiniomorphid ciliates (Coleman, 1985). The data relating to the holotrichs are however more ambiguous. All are able to ingest and digest proteins from the diet or bacteria, and to synthesise amino acids de novo and cell proteins.

The effect of protozoa on the other components of the rumen microbial ecosystem

Studies on the effect of protozoa on the concentration of bacteria and fungi are valid only if we consider that the rumen volume remains unchanged by defaunation or refaunation. In fact, the effect of protozoa on rumen volume is not consistent. According to Jouany et al. (1988), there are large differences between experiments. The evolution of the volume is closely related to the level of feed intake and the mean residence time which increases, decreases or remains stable depending of animals.

Studies such as those carried out by Ushida et al. (1986) support the contention that protozoal activity is the major contributing factor to intraruminal bacterial nitrogen cycling. As shown first by Eadie and Hobson (1962), most of studies done in this field indicate that inoculation of defaunated rumen with protozoa decreases the concentration of small bacteria. Also they observed that large bacteria like *Oscillospira* declined as did the flagellates which were probably fungi. Orpin and Letcher (1983/1984) found that defaunation increased the concentration of the liquid-associated bacteria while it had no effect on the solid-adherent bacteria. This indicates that protozoa chiefly prey on free bacteria. Studies carried out by Kurihara et al. (1978) on individual bacteria indicated that the number of cellulolytic bacteria increases from 2.4 $\times$ 10^9 ml^{-1} on faunation while that of amylolytic bacteria like *Bacteroides amylophilus* and *B. ruminicola* decreased from 6.5 to 0.59 $\times$ 10^9 ml^{-1}. Such an apparent selection for the predation of bacteria is explained by the higher rate of uptake of starch granules and their associated bacteria by ciliates compared to the uptake of cellulosic material and their adherent bacteria. Furthermore, *Ruminococcus albus* which has a higher cellulolytic activity than *R. flavefaciens*, was the main cellulolytic bacteria in faunated animals while they made up 33% of the cellulolytic bacteria in defaunated sheep. There is also some evidence that the population of bacteria fixed on particles is higher in faunated than in defaunated rumen (Argyle and Forster, 1989; Newbold et al., 1989). This could be due to a metabolic synergy between protozoa and adherent bacteria or by a physical contribution of protozoa to the disorganisation of the fibre structure then facilitating the attachment of bacteria on plant fibres. Although it was shown that protozoa predate on fungal zoospores or mycelium (Williams and Coleman, 1992),

the effect of protozoa on fungi remains still difficult to assess because, among other things, there is no reliable method to estimate the fungal population in the rumen. Use of chitin as internal marker has been abandoned and replaced by zoospore counts (Joblin, 1981) or by the evaluation of the number of sporangia fixed on pieces of agar containing cellulose, xylan, xylose, cellobiose and L-cystein inside nylon bags as described by Ushida et al. (1989), but both methods give different results. This could explain why the concentration of fungi increases or decreases sometimes after defaunation as indicated in the review written by Ushida et al. (1991). The real impact on fungi is probably low because their weight in the microbial population is weak, from 1.1 to 3.5% of total rumen microbial N (Faichney et al., 1997).

IMPLICATION OF PROTOZOA FOR THE ENERGY DIGESTION AND NUTRITION OF RUMINANTS

Metabolism of starch by protozoa

Starch granules which are greedily ingested by all the ciliates can account for 40% of their biomass for the 3 hours following the period of feeding the animals with a diet made of 53% barley and 47% wheat straw (Jouany and Thivend, 1972). Considering a protozoal outflow rate of 30% from the rumen, we can calculate that protozoal starch entering the duodenum is around 16 or 160 g per day in sheep or cow which represent nearly 4% of the total amount of starch fed to the animals. However, protozoa contribute to slow down the rumen digestion of starch and regulate the release of end products of fermentation as well as the pH (Ushida et al., 1991) and the osmotic pressure (Mendoza et al., 1993). This is due both to the sequestration of starch granules into protozoal cells as explained before, and to a decrease in amylolytic bacteria consecutively to their selective ingestion by protozoa together with starch granules to which they attach.

Using pure and decontaminated ciliate suspensions, we observed that *Polyplastron, Eudiplodinium, Entodinium* and *Isotricha* efficiently ferment starch used as sole substrate in small fermenters (Ushida and Jouany, 1994). *Isotricha* has the highest fermentative activity per cell compared to all the other ciliates. Although the volume of each cell of *Entodinium* is approximately 100 times lower than that of the large ciliates like *Polyplastron* and *Eudiplodinium*, they produce the same amount of gas per cell when fermenting starch. On the contrary, *Epidinium* has a very low activity against starch. Such a result is surprising and might be checked since *Epidinium* is able to engulf starch granules and produce α-amylases (Williams and Coleman, 1992). Starch and storage amylopectin granules are degraded inside the endoplasm by protozoa to give acetic and butyric acids, H_2, CO_2, with traces of formic, propionic and lactic acids as end products.

At the rumen level, the amylase activities are chiefly present in the liquid-associated protozoa and the solid-associated bacteria in rumen contents sampled from cattle fed 65% hay and 35% barley (Martin et al., 1993). They increase in the 2 subpopulations for 5 hours after feeding the animals and decrease thereafter untill the next meal (figure 3). The liquid-associated bacteria are almost free of amylase. Taking in account these results and the great ability of ciliates to engulf starch, it could be assumed that protozoa account for more than 50% of rumen starch digestion. This does not mean that digestion of starch could decrease in absence of protozoa since high-rate fermenting bacteria increase and all the starch becomes available to bacteria. According to the activity and the size of the bacterial or protozoal populations, the rumen digestion of starch can be positively or negatively influenced by the elimination of protozoa. Considering the few *in vivo* experiments which were carried out to study the effect of protozoa on starch digestion in the rumen, results are opposite. On one hand, Veira et al. (1983) noted a significant increase in the stomach digestibility of starch (89.2 vs 84.0%) in wethers fed corn and corn silage (50/50) after inoculation of protozoa into defaunated sheep. On the other hand, Mendoza et al. (1993) observed that protozoa significantly decreased the stomach digestibility of starch (84.2 vs 93.7%) in sheep fed high-moisture corn supplemented with grain sorghum. Starch digestibility in the whole digestive tract was not influenced by protozoa in these experiments, only some shift from rumen to the intestines can occur. Consequences of resulting changes in the blood supply of nutrients (VFAs vs glucose) and their metabolism in the animals could be accordingly significant. The absence of any significant influence of protozoa on apparent starch digestion in the rumen noted by Rowe et al. (1985) and by Meyer et al. (1986), adds some more confusion on this aspect. Obviously, more work must be undertaken on that topic to know the factors involved in the response of the rumen on the digestion of starch when the population of protozoa is manipulated.

Indirect effects of protozoa in relation with starch digestion have also to be considered. Less starch is available for less numerous high-rate fermenting bacteria when protozoa are present. Thus in animals given diets rich in starch, the postprandial pH drop is moderated by

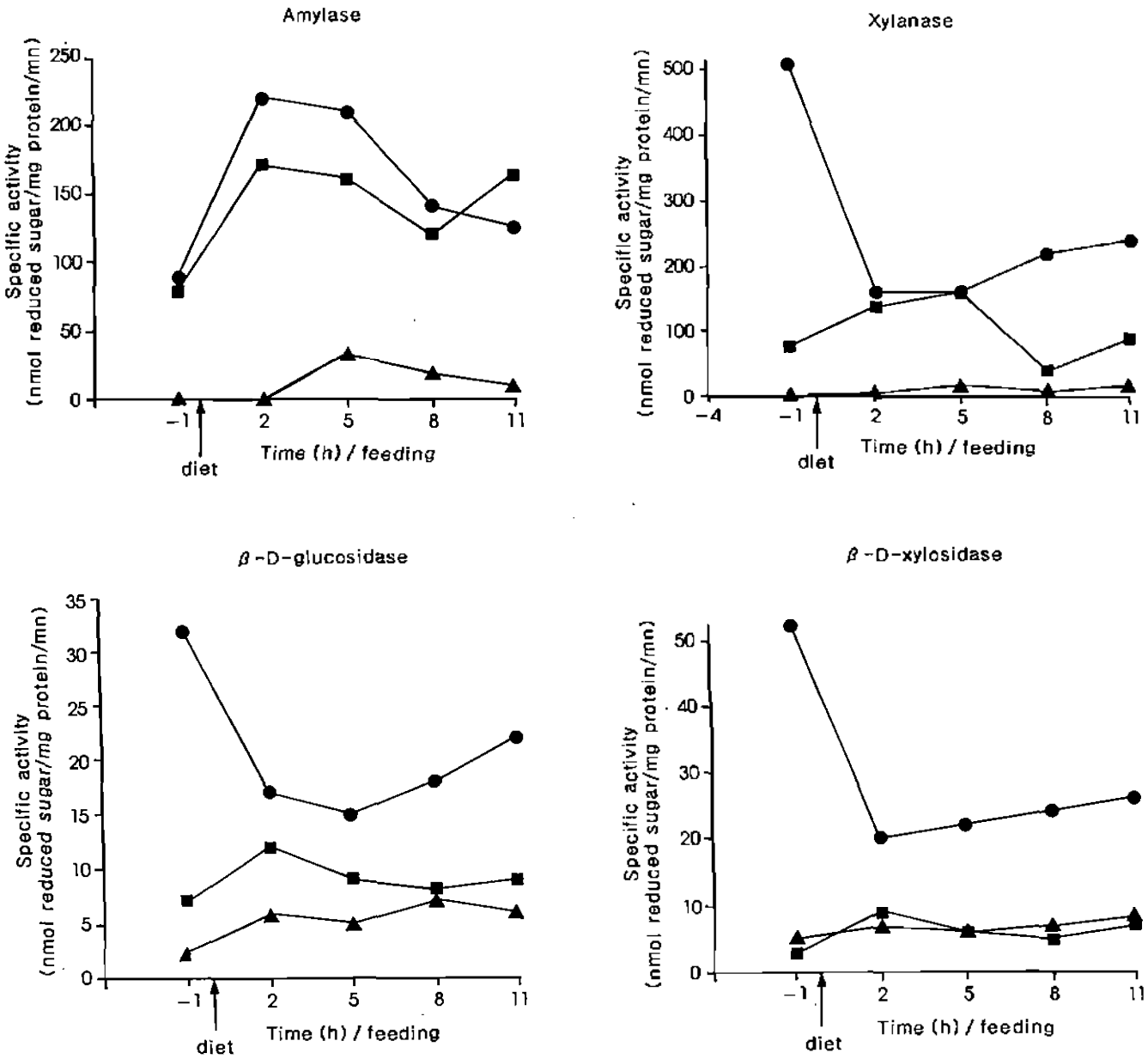

Figure 3. Enzymatic activities of the liquid-associated bacteria (▲), liquid-associated protozoa (■) and solid-associated bacteria (●) (from Martin et al., 1993).

ciliates (figure 4).

Such stabilised conditions are favourable for the growth of cellulolytic bacteria which limits the negative interactions between starch and plant cell wall digestion when animals are fed diets rich in cereals. It is now acknowledged that protozoa increase the ammonia level in the rumen liquid. This could stimulate the bacterial growth when degradable nitrogen is limiting in the diet. For instance, Mendoza et al. (1995) observed that addition of urea had no effect when protozoa were present while it improved in vitro starch digestion in defaunated rumen juice. The same effect could be taken in account to explain the higher growth rate of cellulolytic bacteria in presence of protozoa. A possible release of peptides by protozoa as discussed later may also interact with the activity of bacteria.

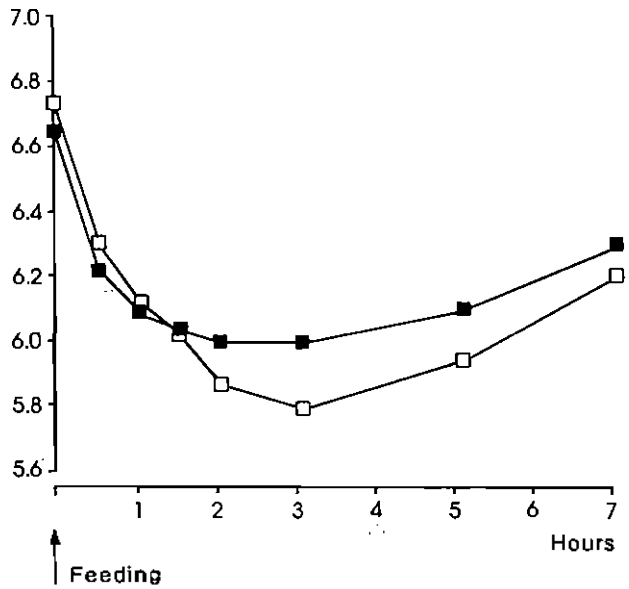

Figure 4. Evolution of pH in the rumen of defaunated (□) and refaunated (■) sheep (from Mathieu et al., 1996).

Effect of protozoa on the digestion of plant cell walls

Effect on microbial enzymes in the rumen

As shown before, large entodiniomorphs are able to ingest plant particles and digest them. Ushida and Jouany (1994) confirmed by manometric methods that *Polyplastron* and *Eudiplodinium* digest xylan and the crystalline cellulose avicel as well as the carboxy-methyl cellulose (CMC), indicating that both genera possess exo and endo 1, 4-β-glucanases. *Epidinium* is inefficient in digesting xylan and avicel but degrade CMC at the same rate as *Polyplastron* and *Eudiplodinium*. This agrees with the results of Bailey and Gaillard (1965) suggesting that *Epidinium* have no cellulases, and the observations of Coleman et al. (1976) that *Epidinium* do not grow *in vitro* on only dried grass as substrate. There has been debate on whether the enzymes are of bacterial or protozoal origin. The absence of any clear answer is due to the difficulty to grow rumen protozoa axenically. However, recent success in cloning of some endo-glucanase and xylanase genes of *Epidinium ecaudatum* and *Polyplastron multivesiculatum* indicates that protozoa have their own fibrolytic enzymes because the genes were retrieved from cDNA library constructed from mRNA pool in these protozoa (Takenaka and Itabashi, 1994). The enzymes in protozoa are believed to be concentrated in the protozoa cytoplasm and then released into vesicles where they act against the plant fragments. Holotrichs have no activity against xylan or avicel, but are able to degrade substituted celluloses like CMC or hexa-ethyl cellulose (HEC) (Ushida and Jouany, 1994) and pectin because of their own pectin esterase (Prins and Van Hoven, 1977).

The digestion of plant cell walls depends on the enzymatic activity of rumen microbes and the duration of contact with the substrates defined as the mean retention time of digesta in this compartment. Addition of protozoa into defaunated rumen stimulates the bacterial cellulolytic (Jouany and Senaud, 1979) and xylanolytic activities (Ushida et al., 1987). This result was observed either with cellulolytic ciliates like *Polyplastron* and *Eudiplodinium*, or when the non-cellulolytic genus *Entodinium* was added alone in the rumen. This explains why the total polysaccharolytic enzymes in the rumen digesta increase after refaunation (Williams et al., 1988). The same range of enzymes was found in rumen subpopulations, but their level of activity differs. Inoculation of protozoa increases the fibrolytic activity 4- to 8- fold on average, and the β-D-xylosidase activity 20-fold in the fraction of solid-adherent bacteria (Williams and Withers, 1990), the latter being the main component of the microbial biomass and the major fiber-degrading microbes in the rumen. It was shown that addition of several genera of cellulolytic protozoa to *Entodinium caudatum*, a non-cellulolytic protozoa, increased the total cellulolytic activity in the rumen by 60-100%, 19-44% being associated with bacteria (Kurihara et al., 1978). This confirms the stimulating effect of protozoa on cellulolytic activity of rumen bacteria on one hand, and the specific contribution of protozoa to the cellulolytic activity in the rumen on the other hand.

Effect on the dynamic of rumen digesta

The dynamic of rumen digesta determines the time of contact between the enzymes and substrates. It must therefore be taken in consideration to explain the evolution of the rumen cell wall carbohydrate degradation which is a slow process. The influence of protozoa on the mean retention time (MRT) of solid digesta in the rumen is inconstant (Faichney and Griffiths, 1978; Orpin and Letcher, 1983/84; Ushida et al., 1986; Jouany et al. 1988). Defaunation lowers the rate of rumen digestion (Kd) of cell walls and animals have two strategies to maintain their level of intake. Either they extend their rumen volume or they increase their outflow of digesta (Kp+Kl). Kp and Kl can be increased by rumination which induces

a smaller size of particles and a higher level of saliva secretion, but rumination is difficult to alter for defined feeding conditions. That is why an increase in the rumen volume is generally associated with a longer MRT and with a higher digestibility of plant cell walls as shown by Demeyer (1989). However there are great individual variations in the ability for animals to store more digesta in their rumen. This explains why data on the influence of ciliates on rumen volume and MRT are extremely variable between experiments and even inside an experiment.

Effect of protozoa on *in vivo* digestion of plant cell walls

Many experiments have been carried out around the world during the last 20 years to evaluate the impact of protozoa by comparing the digestion of cell walls in defaunated and refaunated animals. Comparisons made on rumen ADF digestion in 16 experiments (figure 5) clearly show that digestion of plant cell walls increases by 15% approximately when protozoa are present. Jouany et al. (1981) noted an opposite evolution when the rumen was inoculated with only *Isotricha*.

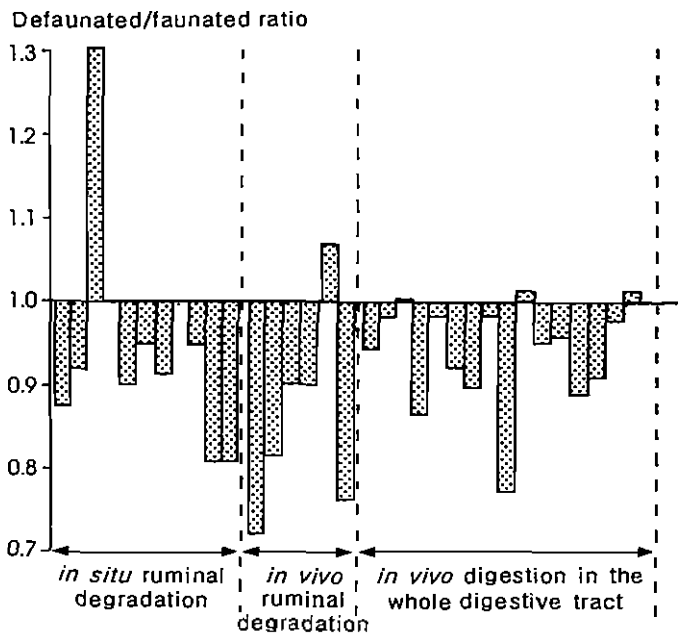

Figure 5. Effect of defaunation on ADF digestion in ruminants from bibliographic data (1 bar per experiment; see data in Jouany 1997).

Such a result could explain why defaunation stimulates cell wall digestion when animals are fed diets rich in molasses (Romulo et al., 1989) which favour the dominance of holotrichs among the dense ciliate population. The same tendency was noted when the rumen was refaunated with only small sized entodinia (Jouany et al., 1981), even if these ciliates were shown to stimulate the cellulolytic activity of bacteria as discussed before. The positive effect of large size entodiniomorphs on cellulolysis appears only in animals fed diets favouring their growth in the rumen (Ushida et al., 1989). This is due to the weight of protozoa in the rumen cellulolytic microbiota on one hand, and to the withdrawal of starch from the rumen medium by protozoa which limits the negative effect of digestive interactions between starch and cellulose in mixed diets on the other hand. For the same reason, no effect of refaunation is noted with a low digestible straw-based diet (Collombier, 1981) for which the ciliate population is very low.

Taking in account the complexity of the chemical structure of plant cell walls, Ushida and Jouany (1990) analysed the effect of protozoa on the digestion of cellulosic and hemicellulosic monosaccharides. According to Ushida et al. (1990), cell wall monosaccharides like xylose, mannose, galactose, arabinose and glucose are approximately 10% more digested in the rumen of refaunated sheep fed NH_3-straw based diet than defaunated ones. When corn substituted 20% for the treated straw, the positive effect of protozoa on rumen digestion of cell wall monosaccharides is magnified. Arabinose, xylose, glucose, mannose + galactose were respectively 14, 22, 40, 61% digested more. In another experiment carried out with sheep fed hay and barley (50/50), Jouany et al. (1997) noted that *in situ* rumen digestion of arabinose, xylose, glucose increased by 31, 48, 58% respectively. Although it is difficult to show any significant difference in the effect of protozoa between cellulose and hemicellulose digestion in the rumen, it appears here again that addition of starch amplifies the response of ciliates on this parameter. Mathieu et al. (1998) measured the pH and determined the protozoa inside the nylon bags (53 microns in pore size) and compared the values with those obtained in the rumen. Due to a deficit in exchange with the rumen liquid as indicated by Trabalza-Marinucci et al. (1992), pH values is 0.1 to 0.6 unit lower, and protozoa were less numerous inside the bags (table 3).

The distribution between the genera is also altered inside the bags. Large-size protozoa are 3 times less numerous, while small entodinia decrease by 30% and holotrichs show inconsistent modifications. The protozoal population establish in one hour inside the bags and remain then stable for 72 hours (table 3). Considering that large-size protozoa are chiefly involved in cell wall

Table 3. PH and protozoal numbers inside the rumen and in the liquid associated to nylon bags (LANB) according to their residence time in the rumen (from Mathieu et al., 1998)

Residence time (H)	Rumen pH		pH LANB		Rumen protozoa (10^5 ml^{-1})	Protozoa in LANB (10^3 ml^{-1})
	Defaunated	Faunated	Defaunated	Faunated		
0	6.70	6.67	ND	ND	ND	ND
1	6.16^{A}	6.27^{AB}	6.38^{B}	6.24^{AB}	642^{a}	425^{b}
3	5.99	6.00	5.98	5.93	ND	ND
6	6.31^{A}	6.24^{A}	6.02^{B}	5.97^{B}	708^{a}	423^{b}
12	5.66^{A}	5.65^{A}	5.50^{B}	5.41^{B}	789^{a}	483^{b}
24	6.60^{A}	6.67^{A}	6.46^{B}	6.35^{B}	629	553
48	6.64^{A}	6.63	6.59^{AB}	6.51^{B}	668^{a}	442^{b}
72	6.61^{A}	6.66^{A}	6.56^{B}	6.55^{B}	717^{a}	446^{b}

Means with different superscript are significantly different ($p < 0.05$) ; capital letters are used for differences in pH ; small letters are used for differences in protozoa numbers.
ND: not determined.

digestion, it can be concluded that such nylon bags are not appropriate to test the effect of protozoa in the rumen. Perhaps, the method could be improved by using bags with a larger pore size.

IMPLICATION OF PROTOZOA FOR THE PROTEIN DIGESTION AND NUTRITION OF RUMINANTS

Effect on the ruminal pool of bacterial proteins

Protozoa are not able to make de novo amino acid synthesis from ammonia. They need to use proteins or peptides to synthesise their own proteins. Protozoa actively prey on bacteria. Coleman (1975) reported that a single protozoon can take up 10^2-10^4 bacteria/h. Considering a protozoal population of 10^5 cells/mL and a mean concentration of 10^9 bacteria/mL, predation can almost renew the total bacterial biomass every 12 hrs. Bacterial proteins are degraded within protozoa into small peptides and free amino acids which are in turn incorporated into protozoal proteins without further interconversion (Coleman, 1975). Part of peptides as free amino acids coming from bacterial proteins are excreted in the rumen juice by protozoa (Denholm and Ling, 1984). Release of small molecular weight nitrogenous compounds may represent 50% of bacterial proteins ingested by protozoa (Coleman, 1975). As a consequence, the total concentration of bacteria is lower (Eadie and Hobson, 1962; Teather et al., 1984), and the concentration of free amino acids and peptides is higher in faunated than in defaunated rumen (Hsu et al., 1991; Itabashi and Kandatsu 1975; Itabashi et al. 1989; Ivan et al., 1991). This point is noteworthy to understand both the stimulating effect on the growth and the metabolism of bacteria, and the less efficient use of nitrogen after inoculation of defaunated animals.

Effect on the rumen degradation of dietary proteins

As discussed in a previous paper (Jouany, 1996), determination of enzymatic activity in autolysed or sonicated cells is not appropriate for quantifying the contribution of ciliates to rumen protein degradation since particles must be ingested first by protozoa to be then digested. Explained by the feeding behaviour of protozoa, the insolubility of proteins is the first factor to be considered for predicting the role of protozoa in their degradation. As a matter of fact, Michalowski (1989) tested different proteins on the growth of entodiniomorphid protozoa cultivated *in vitro* and found that these ciliates do not metabolise soluble proteins and do not grow unless insoluble proteins are supplied (Musynski et al., 1985). Ushida and Jouany (1985) also observed in an *in vitro* system that protozoa increased ammonia production when fish meal, soybean meal, lupin grains or peanut meal were added, while no effect of protozoa was detected when casein was used. Differences noted between *Eudiplodinium* and *Epidinium* + *Entodinium* are low. On the contrary, holotrichs seem more active against soluble proteins than entodiniomorphs (Onodera and Kandatsu, 1970) but they are less active against insoluble proteins (Jouany et al. 1992).

In situ, Ushida and Jouany (1985) showed that the potentially degradable fraction of the insoluble part of protein in soybean meal and its rate of degradation are both increased by 11% when a mixed A-type protozoa is inoculated into defaunated sheep. The theoretical degrad-

ation of the same fraction calculated with a passage rate = 0.06/h, is 13% higher in faunated animals.

The relative importance of protozoa on rumen protein degradation will therefore depend on the ratio between the particulate proteins and the soluble protein in the rumen. It is also related to the various protozoal genera and their concentration. Degradation of insoluble proteins is enhanced by large entodiniomorphs, while the soluble fraction is more degraded by the smallest and by holotrichs.

Effect of protozoa on the duodenal N flow in ruminants

With the exception of the results reported by Punia et al. (1987), most bibliographic data indicate that elimination of protozoa increases the flow of bacterial proteins at the duodenal level (figure 6).

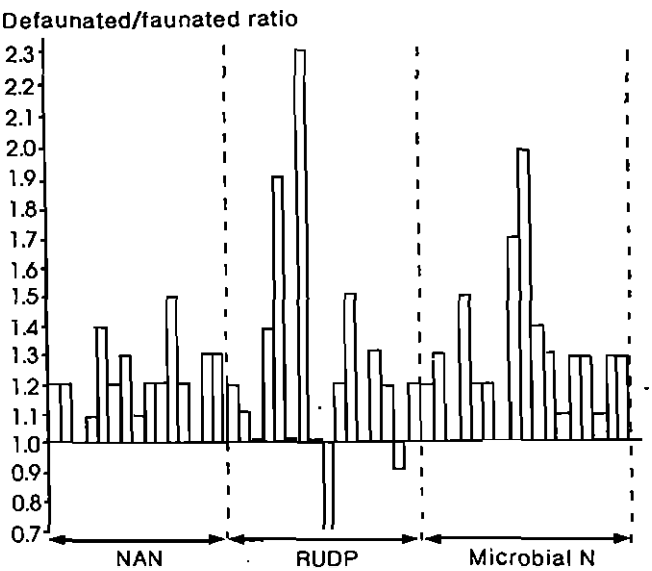

Figure 6. Effect of defaunation on the flow of different protein sources in the duodenum of ruminant (1 bar per experiment ; see data in Jouany 1994) NAN = non ammonia N ; RUDP = ruminally undegraded dietary proteins.

The absence of any effect of protozoa is noted when animals are fed low-energy diets like straw diets which do not favour the growth of ciliates in the rumen. In term of efficiency of bacterial protein synthesis expressed as bacterial N per unit of fermented organic matter, the improvement due to defaunation ranges from 40 to 125%. Such a large response is explained both by a higher net synthesis as discussed before and a decrease in the amount of ruminally fermented organic matter when protozoa are eliminated from the rumen. The direct contribution of protozoa to the total microbial protein flow in the duodenum calculated from 38 bibliographic data is 24.0% (figure 7).

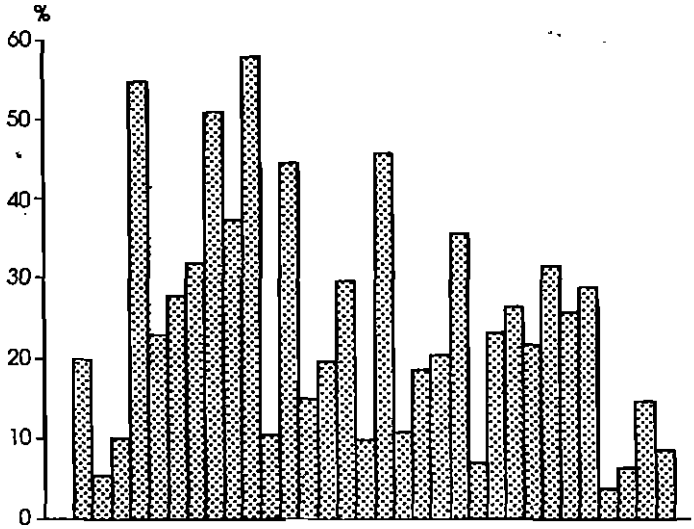

Figure 7. Duodenal contribution of protozoal N to microbial N flow (1 bar per experiment ; see data in Jouany 1996).

The coefficient of variation (58.7%) is high which indicates that the indirect methods used to estimate this parameter are not accurate. Perhaps molecular biology could be a good tool for a more precise determination in the future. According to Faichney et al. (1997), the flow of protozoa is closely related to the level of the rumen protozoal population; the highest values in the rumen give the largest contribution at the duodenal level (table 1).

The duodenal flow of ruminally undegraded dietary proteins (RUDP) also increased or remain sometimes unchanged after defaunation (figure 6). The absence of any effect is either due to the lack of sensitivity of the methods used to discriminate the different forms of nitrogen in the duodenum, or to a higher retention time in the rumen of insoluble proteins when protozoa are eliminated. The relative importance of the duodenal peptide pool resulting from the activity of protozoa in the rumen is unknown. However, Itabashi et al. (1989) found that the duodenal peptide flow decreased after addition of protozoa into defaunated rumen.

As a consequence of increases of both dietary and microbial proteins entering the duodenum, the intestinal supply of total protein nitrogen is nearly always significantly increased by defaunation (figure 6). This

effect is amplified with diets stimulating protozoal growth (Ushida et al., 1991). By altering the ratio between the microbial and feed protein sources flowing to the duodenum, defaunation can change the balance between essential and non-essential AA. A study of the first limiting EAA in goats suggests that proteins entering the small intestine are of lower quality when animals are defaunated (Onodera and Koga, 1987), while no difference was detected in the AA composition of duodenal digesta in faunated and fauna-free sheep by Veira et al., (1983). By Using ^{35}S- and ^{15}N-labelled mixed microbes, their intestinal digestibility was estimated to be 85% (Salter and Smith, 1977; Siddons et al., 1985). Storm et al. (1983) found similar results for the intestinal digestibility of bacteria isolated from the rumen. Although not measured in ruminants, Mc Naught et al. (1954) observed that the true digestibility of protozoal proteins was greater than bacterial proteins in rats, but protozoa like fungi represent a small proportion of the microbial matter entering the small intestine (table 1). The higher duodenal supply of AA following rumen defaunation was confirmed at the blood level (Itabashi et al., 1983; Itabashi et al., 1990, Itabashi and Matsukawa, 1979). Defaunation could therefore be a way of improving the protein status of productive ruminants.

Effect of protozoa on nitrogen excretion in ruminants

Fecal nitrogen losses increase after defaunation (table 4). The lower ruminal digestion of cell wall is compensated for by greater digestion in the large intestine (Ushida et al., 1991). More bacterial proteins are therefore produced in the hindgut and are then excreted in feces. Urinary nitrogen always decreases with defaunation (table 4). This results from a lower rumen ammonia production due to less degraded dietary proteins and less recycled microbial proteins, and a higher uptake of ammonia by more numerous bacteria for protein synthesis. The net balance of nitrogen excretion is not influenced by protozoa as indicated in table 4.

Table 4. Effect of protozoa on N excretion in feces and urine (from Mathieu et al., 1997)

Daily N excretion (g)	Defaunated	Refaunated
Urine	10.6[a]	11.9[b]
Feces	7.8[a]	6.0[b]
Total	18.4	17.9

Means with different letters are significantly different ($p \leq 0.05$).

Consequences on the microbial activities and the physico-chemical characteristics of the rumen digesta

In ruminants fed on low protein diets, nitrogen and sulphur availability may be limiting to support the potential growth of rumen bacteria. In such a situation, microbes in defaunated rumens will be in worse nutritional conditions and their activity will drop. According to Mathieu et al. (1997), the concentrations of ammonia measured 5 hours after feeding the animals were respectively 142 and 66 mg/L in faunated and defaunated rumens. As a consequence, animals must be fed more soluble nitrogen to optimise the bacteria synthesis when the population of rumen protozoa is low. Supply of peptides or free amino acids (FAA) by protozoa in the rumen medium must be considered to explain why some bacterial activities are decreased in defaunated animals. Here is a new way of research to know the real level of the peptide and AA pool resulting from the metabolism of protozoa and its impact on the digestive phenomena in the rumen.

Mathieu et al. (1996) observed that protozoa significantly decrease the redox potential of rumen contents by around 40 to 60 mV (figure 8).

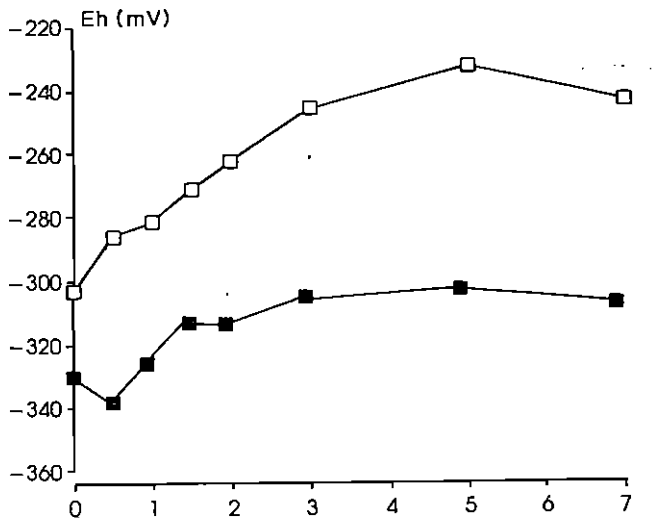

Figure 8. Evolution of redox potential (Eh) in the rumen digesta in defaunated (□) and refaunated (■) sheep (from Mathieu et al., 1996).

This could be due to a consumption of oxygen by anaerobic protozoa in their specialised hydrogenosomes (Yarlett et al., 1983; Williams and Coleman, 1992) and to a higher hydrogen production. As a result, strict

anaerobes like cellulolytic bacteria are stimulated. Furthermore, the stabilising effect of protozoa on rumen pH when animals have been fed diets rich in energy (figure 4) amplifies the stimulation of such pH-sensitive bacteria. Both these effects explain why inoculation of the non-cellulolytic genus *Entodinium sp.* into a defaunated rumen improved the cellulolytic activity in rumen digesta (Jouany and Senaud, 1979; Ushida et al., 1987).

Effect of protozoa on the end products of rumen fermentation

Defaunation either decreases or has no significant effect on total VFA concentration measured *in vivo*, and on VFA production measured *in vitro* (Jouany, 1994). This means that defaunation can sometimes decrease the amount of fermented organic matter with the associated consequences. In most cases, the molar proportion of acetate is not extensively altered, while the proportion of butyrate is decreased for the benefit of propionate after the protozoa have been eliminated. Entodiniomorphid ciliates which compose most of the protozoal population are able to metabolise large amounts of lactate into propionate, thus limiting the risk of acidosis and the subsequent fall in pH.

Comparing the rumen pools of VFAs five hours after feeding the animals, Mathieu et al. (1996) noted that the total VFA, acetate and butyrate pools are significantly higher when protozoa are present while the caproate pool is lower. The same authors observed that rumen gases enrich in methane and hydrogen at the expense of carbon dioxide in presence of protozoa.

Decrease of NH_3-N concentration in the rumen is a consistent result of the defaunation. This is due to a lower deamination of AA coming from the degradation of dietary and microbial proteins in the rumen as discussed reviourly.

Although protozoa are not able to produce methane, it has been shown that defaunation reduces methanogenesis by 30 to 45%. Ushida and Jouany (1996) indicated that protozoa are active hydrogen producers which are closely associated with archaea methanogens to generate methane.

CONCLUSION

Rumen protozoa are very sensitive to their environment. Large differences in their number are observed between diets and also between animals fed on the same diet. Their contribution to the total microbial ecosystem can be important. In such circumstances, their elimination induces large modifications in the main rumen functions. In general term, defaunation improves the protein status of animals but injures the energy supply as a consequence of a decrease in organic matter digestion of diets rich in forages. However, protozoa must not be considered as a whole, some genera having specific effects in the rumen. Furthermore, protozoa have also positive effects in the rumen and their elimination can have detrimental consequences on animals. For instance, it has been shown that protozoa can play a role against toxic compounds in foodstuffs (Kiessling et al. 1984; Ohta et al., 1978) which make the animals more sensitive after defaunation. Ivan et al. (1986) observed also that faunated rams are less sensitive to copper toxicity because protozoa stimulate formation of copper sulphide, making copper unavailable for absorption by the digestive tract. A control of the rumen protozoal population in terms of the total concentration or generic composition could be also a way to improve animal production on condition that the nutritional objective to be targeted is well known. In such a way, more research must be developed to recognise the specific role of different protozoal genera and how to control their population since no treatment is available to partially or totally defaunate the animals in practical conditions.

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