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Reassessing the manipulation of protein synthesis by rumen microbes

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Summary — Protein synthesis by rumen microbes plays an important role in ruminant nutrition. Microbial proteins that are not recycled flow out of the rumen and account for more than 50% of the total proteins entering the intestines. Determination of microbial nitrogen in digesta is based on the isolation of a representative sample of rumen microbes and on the use of specific markers (purine bases, RNA, ^{15}N incorporation). This review discusses the reliability of microbial markers and examines current methods for assessing differences in turnover rate and microbial composition among liquid and particulate compartments. Four groups of factors acting on microbial synthesis, which are also adaptable to farm conditions, are considered: I) modification of protozoal population; II) modification of liquid and solid phase kinetics in the rumen; III) balance in the components of the diet; and IV) probiotics. The effects of these factors are discussed, considering their feasibility and their usefulness for the host.

rumen / synthesis / microbe / protein

Résumé — Manipulation de la synthèse protéique par les microbes du rumen : actualisation des données. *La synthèse de protéines par les microbes du rumen est un processus capital dans la nutrition des ruminants. Les protéines microbiennes qui n'ont pas été recyclées sortent du rumen et représentent plus de la moitié des protéines arrivant à l'intestin grêle. La mesure de la teneur en azote microbien des digesta est fondée sur l'isolement d'un échantillon représentatif de microbes du rumen et sur l'emploi de marqueurs spécifiques (bases puriques, ARN, ^{15}N incorporé). Cette revue bibliographique évalue la validité de ces marqueurs microbiens et présente les méthodes récentes qui tiennent compte des différences de taux de renouvellement et de composition de la biomasse qui existent entre les phases liquide et particulaires dans le rumen. Puis 4 classes de facteurs susceptibles de modifier la synthèse de protéines microbiennes dans le rumen chez des animaux d'élevage sont considérées : i) une modification de la faune, ii) une modification de la cinétique de renouvellement des phases liquide et solide dans le rumen, iii) l'équilibre des constituants de la ration, iv) les probiotiques. Les effets de ces facteurs sont discutés en considérant les conditions d'application et l'intérêt pour l'animal.*

rumen / synthèse / microbe / protéine

INTRODUCTION

The rumen outflow of microbial matter with the liquid phase and undegraded feed components provides the host animal with well-balanced proteins, and accounts for more than 50% of the total proteins entering the intestines. As protein synthesis by rumen microbes plays an important role in ruminant nutrition, controlling this process is essential for optimizing the production performance of livestock. Several reviews have dealt extensively with this matter over the last few years (Orskov, 1982; Demeyer and Van Nevel, 1986; Van Nevel and Demeyer, 1988; Vérité *et al,* 1986; Durand, 1989; Hoover and Stokes, 1991; Komisarczuk-Bony and Durand, 1991a). Some objectives of this manipulation can be identified as follows: increasing the amount of protein synthesized on a high-roughage diet supplemented with cheap non-protidic nitrogen N sources; restoring normal protein synthesis efficiency on an easily fermentable diet; increasing dietary N retention by improving ammonia assimilation and urea recycling.

Manipulating the rumen protein synthesis means altering it in a controlled way to obtain a predictable response. Few means are at our disposal to achieve this at present. In this review, we consider mainly the modification of protozoal population, the action on rumen fluid kinetics, the balance between the components of the diet, and the use of probiotics. Some of these treatments can readily be used on an animal husbandry scale but others are still experimental and their use can only be recommended in a rather narrow range of breeding situations.

We begin by discussing the accuracy of current methods for estimating the ruminal protein production, since working out a reliable hierarchy between experimental treatments is still a major problem.

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MEASUREMENT OF MICROBIAL PROTEIN SYNTHESIS IN THE RUMEN

Protein synthesis is expressed by different means. The usual determinations *in vivo* or in continuous culture are: 1) the average output rate of microbial nitrogenous compounds or net protein synthesis, calculated in g N or g CP per day; and 2) the energy efficiency of this process or the efficiency of microbial protein synthesis (EMPS), determined by dividing the amount of protein synthesized by the amount of energetic substrate fermented. The divisor can be in fermentors, the theoretical amount of ATP or fermented hexoses estimated from the fermentation profile and metabolic pathways (Demeyer and Van Nevel, 1975) and *in vivo*, the amount of organic matter apparently (OMADr) or truly (OMTDr) digested in the rumen, sometimes after subtraction of lipids. However, the use of OMTDr leads to a less accurate determination of EMPS and does not separate the OM incorporated into microbes and the OM actually fermented to provide energy. Other ratios might be used, *eg*, microbial N synthesized upon dietary N truly digested in the rumen or upon urinary N excreted, if N retention through urea recycling involved environmental interest. The microbial N compounds in the rumen (or flowing out of it) can only be completely isolated from the feeds with diets containing no or pure proteins, *eg*, the synthetic diets used by Hume (1970b). In most studies, these must be estimated from the quantification of a marker in the digesta, followed by determination of the marker/N ratio in a microbial isolate assumed to be representative of the whole microbial bulk in the digesta. Besides the problems encountered in digesta flow measurements, this method relies on hypotheses of questionable valid-

ity. A survey of recent papers demonstrates that the most popular microbial markers are at present purine bases and ^{15}N incorporation. We did not consider other markers in this paper, such as DAPA (diaminopimelic acid), as they have been described elsewhere (Stern and Hoover, 1979; Harrison and McAllan, 1980; Arambel *et al,* 1987; Broderick and Merchen, 1992). The main characteristics of the markers are briefly summarized in table I.

^{15}N is used mostly as $(^{15}NH_4)_2SO_4$ and labels microbial nitrogenous constituents *via* the ammonia pool. Even if ammonia is the primary source of N for bacterial protein synthesis, preformed amino acids are also used as building blocks. ^{15}N enrichment of microbial matter thus depends on the availability of unlabelled, dietary or endogenous amino acids. ^{15}N enrichment appears to be up to 2-fold lower for protozoa than for bacteria (Pilgrim *et al,* 1970). In a recent study on sheep fed 5 different diets, Yang (1991) reported that ^{15}N enrichment was highest in liquid-associated bacteria (LAB) and regularly decreased in solid-associated bacteria (SAB) as the particle size increased. Purine bases are most frequently estimated according to Zinn and Owens (1982), or with a related method (Ushida *et al,* 1985). Under various dietary conditions, the RNA/protein ratio appears to be significantly lower in SAB than in LAB, although this difference is rather variable (Merry and McAllan, 1983; Bates *et al,* 1985). With pure cultures of major ruminal bacteria, Arambel *et al* (1982) reported RNA-N/total N ratios averaging 0.037 with a range of 0.027 to 0.062 for 6 Gram-positive strains, and averaging 0.062 with a range of 0.008 to 0.102 for 11 Gram-negative strains. This ratio is also lower in protozoa (Arambel *et al,* 1982; Bauchart *et al,* 1986).

LAB account only for 15–30 p100 of the microbial OM in the rumen (Craig *et al,* 1987; Olubobokun *et al,* 1988; Legay-Carmier and Bauchart, 1989). Using both markers, the over-representation of LAB in the microbial reference pool compared with SAB or protozoa leads in most cases to a variable underestimation of microbial N outflows. Since the critical reviews of Stern and Hoover (1979) or Harrison and McAllan (1980), several attempts have been made to include adherent bacteria in the microbial pool. The proportion of SAB can be increased by merely homogenizing the whole rumen contents in a blender before centrifugation of the filtrate. This technique gives poor control of the amount of SAB incorporated into the microbial pool but is

Table I. Characteristics of the methods generally used for measuring microbial protein synthesis in the rumen.

Method	*Main characteristics*
DAPA	Costly analysis, marks mainly the bacteria, tendency to overestimate the microbial N flow
Total purines	Simple, cost effective, marks all the microbes, differences in purine/N ratios among microbial pools
^{15}N incorporation	Stable isotope, marks all the microbes, microbial isolates more representative, costly analysis
^{35}S incorporation	Radioisotope of moderate radiotoxicity, costly waste disposal procedure

commonly used, probably for practical reasons. Several recent trials have calculated the rumen OM outflow of SAB origin, taking into account the turnover of the rumen liquid and solid compartments. In this case, a separate determination of the chemical composition of SAB is needed. SAB are desorbed by washing, chilling and then blending or pummeling a particulate fraction of the rumen contents (Merry and McAllan, 1983; Bates *et al*, 1985; Olubobokun *et al*, 1988; Legay-Carmier and Bauchart, 1989). The mean removal rate of SAB (attested by the reduction of microbial marker amounts) from the total bulk of particles is about 25–40 p100. However, taking into account the residual DAPA content of particles, Legay-Carmier and Bauchart (1989) calculated that the extraction rate decreased with decreasing particle size. Furthermore, the mechanisms involved in microbial attachment vary with species (Roger, 1990) and should induce specific sensitiveness to the above-mentioned techniques. Consequently, it can be seen that the ways of isolating SAB remain unsatisfactory. However, Whitehouse *et al* (1994) used microscopy and DAPA and purine removal, to compare 11 extraction methods combining homogenization, storage at 4°C, action of marbles, repeated washings, incubation with methylcellulose, low pH, addition of Tween 80, formaldehyde, methanol or tertiary butanol in the solutions. When applied to the washed ruminal digesta of cows fed 63% corn silage, 37% haycrop silage or 63% concentrate, 25% corn silage, 12% grass-legume silage, 2 treatments led to SAB removal rates of 79–82%. If confirmed for other diets, these methods could prove quite useful in helping to validate a satisfactory procedure for microbial N flux determination.

Beyond this stage, different methods of calculation have been proposed. Using purine bases, Doreau *et al* (1991) calculated the duodenal LAB flow from the LAB pool and from the fluid dilution rate in the rumen. They then estimated the duodenal SAB flow by assuming that the fraction of purine-based duodenal content not accounted for by LAB outflow was related to SAB matter. Using ^{15}N enrichment in different rumen microbial pools (LAB and SAB extracted from 3 particle pools (> 1 mm; > 0.1 mm; < 0.1 mm)) and in LAB and SAB from duodenal contents, Yang (1991) compared the 2 current methods based on the isolation of LAB from rumen or duodenal samples to 6 calculation methods using SAB. These differed 1) in the origin of microbial reference pool (rumen or duodenum); 2) in the nature of the solid fractions considered (particle size fractions or physiological pools according to Faichney (1986)); and 3) in the principle of microbial matter outflow calculation (summation of partial flows related to the fractions of digesta or composition of a microbial reference pool). The author concluded that taking into account SAB increased the estimation of rumen microbial N outflow by 17 p100 on average (within a range of 10–39 p100). This was mainly affected by changes in rumen particle kinetics. He recommended extracting the bacterial reference pool from duodenal digesta rather than from rumen contents when only LAB is isolated.

Another critical point is the isolation technique of the microbial sample. The digesta samples should be withdrawn on a regular basis over a period of several days because of changes in the chemical composition of microbes with respect to the time of feeding (Smith and McAllan, 1974; Czerkawski, 1976) and the day of sampling (Arambel *et al*, 1982). The centrifugation speed does not seem to matter but previous freezing of digesta should be avoided. Hsu and Fahey (1990) observed on samples from defaunated sheep that previous freezing lowered the amount of dry matter collected, its RNA content and the ratio RNA/N in the bacterial pellet, while a variation of the centrifu-

gation speed from 4 640 *g* for 30 min to 25 900 *g* for 20 min had no effect.

Some experiments on animals with no surgical preparation use the urinary amount of allantoin as a marker. This molecule is the end-product of purine degradation in herbivores and his excretion level is partly correlated to the intestinal absorption of microbial nucleobases (Antoniewicz *et al,* 1980; Giesecke *et al,* 1984). However, intermediate metabolites (hypoxanthine, xanthine and uric acid), which may be excreted in large and variable amounts, should be enzymatically converted into allantoin before the determination (Fujihara *et al,* 1987; Chen *et al,* 1993; Resines *et al,* 1993) or be separately quantified (Balcells *et al,* 1991, 1993). After these steps, the total amount of purine derivatives, after subtraction of endogenous excretion, is closely related to microbial nucleic acid inputs (Fujihara *et al,* 1987; Chen *et al,* 1990a; Balcells *et al,* 1991; Djouvinov and Todorov, 1994). One must note that according to Chen *et al* (1990b), this method should be corrected for ruminal degradation of salivary recycled allantoin and uric acid, which they estimate as 10 p100 of normal urinary excretion. However, its main flaw is also a possible shift in specific composition of microbial populations leading to different purine base/microbial N ratios between treatments.

MANIPULATION OF MICROBIAL SYNTHESIS IN THE RUMEN

Control of the rumen protozoal population

Techniques to defaunate the rumen or to obtain a controlled fauna

Different techniques for the complete elimination of ciliates from the rumen are now available to researchers. They have been widely used for the last 20 years to study the role of protozoa in the rumen digestion. They can be classified in 3 types: (i) the animals are separated from the dam 48 h after birth and are reared in rooms isolated from adult ruminants; (ii) chemicals are introduced into the rumen to kill the protozoa; several drugs have been proposed for this purpose (see Hobson and Jouany, 1988); and (iii) the rumen content is treated outside the animal and then, reintroduced after the protozoa are killed and the rumen wall has been cleaned (Jouany and Sénaud, 1979).

Contacts with conventional animals must be absolutely prohibited to avoid any risk of contamination. The staff manipulating the defaunated animals must avoid being a vector of living protozoa.

It has been shown that defaunating methods can have a direct effect on bacteria and fungi. It is, therefore, strongly recommended to reinoculate the rumen with thawed rumen contents, several weeks after it has been treated. Freezing kills protozoa but preserves bacteria and fungi which then develop in the animals reared in isolated conditions (Jouany, 1991).

Other methods to obtain and maintain protozoa-free animals on farms are now in progress. It has been shown that the addition of some fatty acids (Broudiscou *et al,* 1990) or some plants rich in saponins (Navas-Camacho *et al,* 1994) could be a solution. However, no 'natural' method for defaunating the rumen is available today that will achieve this aim in animals reared on farms.

Generic composition of the fauna can be controlled by inoculating a defaunated rumen with previously selected genera of ciliates. Selection of the ciliates is done with a magnifying glass and using a micromanipulator or a curved micropipette connected to a small bulb. The same precautions must be taken, as with defaunated animals, to avoid contamination of mono-

inoculated animals by undesirable protozoa. This technique has been successively carried out in our laboratory to obtain sheep inoculated with only *Entodinium*, *Epidinium*, *Polyplastron*, *Eudiplodinium* or *Isotricha*, or with a combination of 2 of the above (Jouany, 1978).

Effect of the elimination of protozoa on microbial protein synthesis

The total microbial protein synthesis is not significantly different between defaunated and faunated animals when the nutritional needs of the microbial population (NH_3, S, P) are supplied in both situations (Van Nevel and Demeyer, 1979). Net synthesis estimated by the duodenal supply of microbial proteins can be considerably increased by defaunation (table II). This means that the recycling of microbial N is significant in the presence of protozoa, for 2 main reasons: protozoa predate on bacteria, and they are partly sequestrated in the rumen.

Protozoa engulf bacteria and digest them to cover their nutritional needs. Bacterial proteins are degraded into peptides and amino acids inside the protozoa. Nearly half of the ingested amino acids are used by ciliates. The other half reappear in the medium where they are deaminated by bacteria (Coleman, 1967). Coleman (1975) calculated that as much as 90 g of bacterial dry matter can be engulfed by protozoa each day in a sheep rumen, which corresponds to a loss of 27 g of bacterial protein. The part of N retained by protozoa, corresponding roughly to the same amount of protein

Table II. Duodenal flow of microbial proteins and rumen ammonia nitrogen concentration in faunated (F) and defaunated (D) animals.

Reference	*Microbial N (g/d)*		*Protozoal N (% microbial N)*	*Rumen NH_3-N (mg/l)*	
	F	*D*		*F*	*D*
Lindsay and Hogan (1972)	12	14	–	271	220
	18	19	–	208	193
Jouany (1978)	–	–	10–55	170	110*
Harrison *et al* (1979)	–	–	23–28		
Colombier (1981)	14	18*	–	460	419
Jouany and Thivend (1983)	16	16	15	265	182
Ushida *et al* (1984)	12	18*	–	140	60*
	15	18	–	140	60*
Rowe *et al* (1985)	12	15	–	102	102
Kayouli *et al* (1986)	16	16	–	197	111*
	10	17			
Meyer *et al* (1986)	8	16*	36		
	12	17*			
Ushida *et al* (1986)	15	19	7	140	54*
	16	18	–	175	100
Ushida *et al* (1990)	6	8*	22	255	168
	7	9*	32	278	149
Hsu *et al* (1991)	34	40	–	268	190

NH_3-N: ammonia nitrogen; * significant difference among treatments ($P < 0.05$).

(27 g), is not totally available to the ruminant because protozoa are selectively retained in the rumen. As a consequence of the predation, the turnover of bacterial protein is increased by the presence of protozoa. It ranges from 0.003 to 0.027 h^{-1} in defaunated animals, and from 0.024 to 0.37 h^{-1} in faunated animals (Wallace and McPherson, 1987).

Several experiments have been performed in the last 20 years to estimate the contribution of protozoal proteins to the total microbial protein in the rumen and in the duodenum (table II). The results indicate that the specific outflow of protozoa is different from the specific outflow of the liquid phase and even from the specific outflow of solid particles in the rumen. The difference between protozoal concentrations in the rumen and in the duodenal digesta have been attributed to protozoal sequestration in the rumen. This sequestration is due to the 'filter bed' effect of solid particles and to the attraction they have on protozoa. This phenomenon is explained by the diffusion of soluble substrates from freshly ingested or ruminated particles, which attract protozoa by chemotaxism, mainly Isotrichs, and by adherence on large particles (ingestion of small pieces of these large particles) or on the reticulum wall (Abe and Iriki, 1989). This selective retention in the rumen enables protozoa, which have a high generation time, to remain in place, but then they lyse and recycle an important part of their proteins which are thus unavailable to the ruminant.

The increased recycling of microbial N in the rumen of faunated animals when compared to defaunated animals is one of the reasons for the greater concentration of N-NH_3 in the rumen of faunated animals, the other being the action of protozoa on dietary protein degradation. From bibliographic data (table II), one can observe that the concentration of N-NH_3 in defaunated animals (Itabashi *et al,* 1984; Jouany *et al,* 1995) is sometimes below the minimum recommended value for maximizing microbial growth yield, as evaluated by Komisarczuk-Bony and Durand (1991a). It is, therefore, strongly recommended to supply more fermentable N, in the form of urea for instance, in the diet of defaunated animals to optimize the microbial protein yield in the rumen and increase the positive effect of defaunation on animal production. Discrepancies between results on the effect of defaunation on animal growth could be partly explained by the differences in the N-NH_3 availability to microbes. This factor has been shown to play a decisive role in the response of rumen fibre degradation to defaunation (Jouany *et al,* 1992).

It is now well established that fat supplementation of the diet can strongly reduce protozoa numbers in sheep (Czerkawski *et al,* 1975; Knight *et al,* 1978; Ikwuegbu and Sutton, 1982; Broudiscou *et al,* 1990). The effect in dairy cattle is uncertain (Tamminga *et al,* 1983; Bauchart *et al,* 1986, 1988). Polyunsaturated C_{18} fatty acids (PUFA), which are present in great amounts in sunflower, safflower, soya and linseed oil, are particularly toxic to ciliates. However, the effects of PUFA supplementation on the EMPS and on the amounts of microbial N flowing out of the rumen appeared to be contradictory. Van Nevel and Demeyer (1981) suggested the presence of 2 antagonistic mechanisms: a direct inhibition of bacteria and a positive effect owing to partial defaunation. The balance between them depends partly on PUFA incorporation rate in the diet. This hypothesis was confirmed by Ikwuegbu and Sutton (1982), who supplemented sheep with increasing amounts of linseed oil and observed the greatest EMPS with the smallest PUFA supply rate, leading to a minimal ciliate density with no further decrease. An efficient use of PUFA requires an estimation of this optimal level with respect to the diet characteristics, as bacterial strains exhibit strong differences in

sensitivity to PUFA. The addition of long-chain fatty acids may also have an energy sparing effect on microbial growth, as rumen microbes tend to incorporate preformed fatty acids in their structural lipids rather than synthesizing them themselves (Hawke, 1971; Knight *et al,* 1977; Demeyer *et al,* 1978).

Bentonite, initially used for pH control, may also be beneficial to rumen protein production in reducing the number of ciliates by interfering with cilia motion (Wallace and Newbold, 1991).

It is tempting to try to reduce the protozoal population in ruminants supplemented with monensin to alleviate the negative effect of this antibiotic on microbial protein production. However, this manipulation can accelerate the chronic Cu poisoning of the animals, as both monensin and defaunation increase the liver concentration of Cu in sheep (Ivan *et al,* 1992).

Mineral buffers and slaframine

Van Nevel and Demeyer (1988) have extensively analysed the bibliography dealing with the use of buffers in ruminants. In the following, we shall only summarize the data relating to their main effects on microbial protein production. High-concentrate diets provide the rumen microbes with readily fermentable carbohydrates. They decrease salivary secretions by reducing chewing activity and increase the osmolarity of rumen contents. One of many consequences is that bacterial protein synthesis and its efficiency can be depressed, probably owing to a low pH level (Russell *et al,* 1979; Strobel and Russel, 1986) and a lowered turnover rate of rumen contents (Hoover *et al,* 1984). The use of mineral buffers (limestone, Na_2CO_3, $CaCO_3$ or $NaHCO_3$ in the form of salts or artificial saliva) has been proposed to prevent these negative effects. Several assays have justified their use in sheep (Mees *et al,* 1985) and in lactating cows (Herdman, 1988), but the mechanisms mainly involved in the observed improvement of bacterial N flow have not been clearly established. However, the total protein flow in the duodenum may remain unchanged since restoring a higher ruminal pH can enhance the breakdown of dietary proteins (Erfle *et al,* 1982; Mees *et al,* 1985). The influence of rumen fluid dilution rate or solid turnover rate on microbial proteosynthesis has been chiefly studied in continuous culture systems (Crawford *et al,* 1980; Hoover *et al,* 1984; Shriver *et al,* 1986). A similar relationship, however, has been reported by Harrison *et al* (1975) in sheep fitted with an intraruminal infusion of artificial saliva. The chemostat theory may explain part of the above-mentioned influence: the fraction of the total energy consumed by the microbes for maintenance decreases with the higher dilution rates and the faster specific growth rates that are related to them (Isaacson *et al,* 1975). However, the positive effect of dietary buffers on microbial protein synthesis through a change in rumen digesta kinetics only seems to occur when the digesta turnover rates with the basal diet are rather low (Mees *et al,* 1985). Furthermore, the choice of an inadequate level of incorporation may lead to excessive osmolarity that tends to depress microbial growth (Durand and Komisarczuk, 1988). An intramuscular injection of slaframine (a parasympathomimetic salivary stimulant) to steers increases the fluid dilution rate in the rumen (Froetschel *et al,* 1987), and tends to improve the microbial N outflow and production efficiency (Froetschel *et al,* 1989). Obvious practical reasons, however, limit the use of this pharmacological method to experimental situations.

Balanced diets

The extent to which a diet can facilitate microbial growth is determined by the avail-

ability of energy, N compounds, minerals and growth factors and by its physico-chemical characteristics.

Minerals

According to Durand and Komisarczuk (1988), the mineral requirements should be expressed in relation with the digestible OM or the OM fermented in the rumen (OMFr), rather than in terms of dietary DM content or ruminal concentration. Microbial protein synthesis is strongly dependent on an adequate supply of phosphorus, sulfur and magnesium (reviews of Komisarczuk-Bony and Durand, 1991a, b). In Rusitec, a P deficiency strongly decreased the efficiency of proteosynthesis, owing to energy uncoupling (Komisarczuk *et al,* 1987). Roughage, cassava and sugar beet pulp are known to be P deficient. Dietary P, which is poorly soluble in the rumen but which is solubilized in the abomasum, however, can be partly recycled through salivation. Therefore, the P availability in the rumen is determined by the amount of dietary P, its absorbability and the extent of salivary P secretion in relation to the physiological state of the host. *In vitro* experiments and calculations using a P/N ratio of 7 in microbes and an average EMPS of 30 g N incorporated/kg OMFr led to the same estimation of 4.3 g available P/kg OMFr to fulfill the microbial needs for proteosynthesis. However, cellulolysis seems to require a higher supply of available P. Komisarczuk and Durand (1991a) stressed that the available P requirement for optimal cellulose degradation should be about 7.7 g P/kg OMFr.

Sulfur should be added to diets composed of tropical forages, low quality roughages, such as straw, or feeds containing poorly available S, such as fescue, brome-grass or orchard-grass. In a Rusitec with urea as the N source, S supplementation of intact and alkali-treated straw increased the microbial protein outflow respectively by 19 p100 and 25 p100 with an enhanced cellulolysis and no effect on EMPS (Stevani *et al,* 1992). The available S requirement is about 2.5–3.0 g S/kg OMFr for both protein synthesis and cellulolysis. Cysteine and methionine can be readily incorporated into microbial matter. However dietary inorganic forms of S are partly reduced into sulfides and pass through the rumen wall. These losses must be taken into account as they represent up to 50–80 p100 of the actual microbial needs. Although high in most dietary conditions, the availability of dietary magnesium can be reduced by high pH, NH_3 and PO_4^{3-} concentrations or through binding to plant organic acids. According to Durand and Komisarczuk (1988), 2.3 to 3.8 g Mg/kg OMFr should be supplied for optimal rumen metabolism.

Growth factors

The growth factors required by numerous rumen bacteria strains in pure cultures are, under normal dietary conditions, provided in the rumen by the feeds, through cross-feeding or by microbial OM recycling.

Vitamins

Thiamine pyrophosphate is involved in aldehyde transfer or aldehyde removal reactions, such as decarboxylation of pyruvate into acetyl-coA and carbon dioxide. The addition of 4 mg/g thiamine to a protein-free diet increased the amount of proteins produced by 65 p100 in 3 h *in vitro* incubations by mixed rumen micro-organisms withdrawn from sheep fed the same purified diet (Candau and Kone, 1980). In actual breeding conditions, the effect of supplemental thiamine depends on the individual marginal deficiency for this vitamin, as shown by Grigat and Mathison (1982, 1983) on feedlot cattle. Nicotinic acid (or niacin) is the precursor of NAD and NADP, which act as hydrogen carriers in many oxido-reduction reactions. The effects of supplemental niacin

on microbial proteosynthesis *in vitro* are rather unclear. According to the review of Brent and Bartley (1984), the microbial growth efficiency was often improved, while no effect was observed in several essays. In cows adapted to a low niacin (oats) or a high niacin (barley) diet, Abdouli and Schaefer (1986) measured a free niacin rumen fluid content of 0.5 μg/ml, whatever the dietary intake, and this concentration appeared to greatly exceed the metabolic needs of the several niacin-requiring bacteria.

Isoacids

Major bacterial groups in the rumen, *eg*, cellulolytic species, are unable to incorporate preformed amino acids and must synthesize them from ammonia and carbon skeletons in the form of volatile fatty acids (Dehority *et al*, 1967). Valine, leucine, isoleucine derive from isobutyrate, isovalerate and 2-methylbutyrate. These branched-chain fatty acids (BCFA) are mainly provided by dietary protein breakdown and subsequent amino acid deamination, as reported by Von Kreuzer *et al* (1986). It is worth supplementing the ruminant diet with these growth factors when they are low in protein or when the deamination step is inhibited (*eg*, by the use of a chemical). Evidence for this favorable effect has been obtained *in vitro* (Chalupa and Bloch, 1983; Russell and Sniffen, 1984). Hume (1970a) increased by 14 p100 the daily amount of microbial protein synthesized in the rumen of sheep by supplementing a protein-free diet with 1.25 p100 DM of a mixture of isoacid sodium salts. Even in batch cultures on substrates with normal crude protein content (13 and 16 p100 CP), Cummins and Papas (1985) observed that the addition of mixed isoacids stimulated microbial growth. However, the interest of BCFA supplementation is still unclear. Varga *et al* (1988) did not observe any improvement in N degradation and microbial protein synthesis when BCFA or urea were infused in a continuous culture fed with formaldehyde treated soybean meal. Furthermore, supplying heifers fed low-quality grass hay, with various amounts of BCFA had no significant effect on the fermentation and microbial growth in the rumen (Gunter *et al*, 1990). Doré and Gouet (1991) stressed, with respect to pure culture results, that the effects of other growth factors should also be investigated, *eg*, aromatic compounds (phenylpropanoic and phenylacetic acids), heme compounds and quinones.

Form and amount of energy and N dietary sources

The influence of dietary characteristics, such as the nature and the amount of nitrogen and energy-producing compounds, on rumen protein synthesis has recently been reviewed (Hoover and Stokes, 1991). The intake level, particle size, forage content of the diet, forage maturity or availability of N compounds can alter both the amount of energy released by fermentation and available for growth, and the EMPS. They determine the rate of carbohydrate degradation, the occurrence of energy uncoupling, the rumen content kinetics and the pH level. Within a range of 5 to 30% CP, a CP content of 11–14% in the diet of sheep led to the maximal microbial activity for a minimal ammonia concentration in the rumen (Von Kreuzer *et al*, 1986). From the results of trials on dairy cows (Stokes *et al*, 1991a) or in continuous culture systems (Stokes *et al*, 1991b), Hoover and Stokes (1991) concluded that diets containing 10–13 p100 of 'degradable intake protein' and 56 p100 of total carbohydrate as non-structural carbohydrate, maximized microbial protein production and EMPS. However, an interaction between the forage/concentrate ratio and the type of grain (barley *vs* corn) on microbial protein yield has been reported by Rode and Satter (1988). Length of for-

age must also be considered. The feeding of chopped hay decreases the EMPS through a decrease in the turnover rate of particles (Rode *et al,* 1985; Rode and Satter, 1988).

Several investigators have reported minimal ammonia concentration of 50 mg/l for maximal bacterial protein production *in vitro* (Satter and Slyter, 1974; Russel and Strobel, 1987). This threshold seems to increase with easily fermentable substrates. According to Odle and Schaefer (1987), it should be of 60 mg/l with corn-based diets. *In vivo,* 80–90 mg/l appears to be an optimal concentration. The effect of ammonia level on rumen metabolism has been discussed by Komisarczuk-Bony and Durand (1991a) in relation to the different ammonia assimilation mechanisms in bacteria.

The optimal sequence of energy and N supply is still a matter for debate. On the one hand, in 12 h batch cultures of mixed microorganisms, asynchronous N and energy supply induced short-term N restriction but did not significantly change the final bacterial yield (Newbold and Rust, 1992). However, the authors pointed out that the effect of a temporary excess of N was poorly simulated. Henning *et al* (1993) reported no effect of energy and N supply synchronization on rumen microbial N outflow or EMPS in sheep fed near maintenance or at a higher nutritional level. On the other hand, Herrera-Saldana *et al* (1990) observed a greater rumen outflow of microbial protein and EMPS in dairy cows fed diets with starch and protein sources which were more degradable in the rumen than with unsynchronized or less degradable synchronized sources. Matras *et al* (1991) found that N retention in lambs was highest when the diet was composed of starch and N sources of similar rumen degradation rates (barley + urea; sorghum + 50:50 blood meal/corn gluten meal).

Unfortunately, most of the assays cannot lead to the design of predictive statistical models as the experimental designs will not detect curvature and evaluate possible models by additional combinations of experimental treatments. This must be done by collecting experimental results and analyzing the corresponding database.

Probiotics

The probiotics added in ruminant diets are mainly composed of live yeasts, *Saccharomyces cerevisiae* (SC), or fungi, *Aspergillus orizae* (AO) (Wallace and Newbold, 1992). They are added mixed with their growth medium. Some preparations are very poor in living cells ("Diamond V" yeast culture).

Several scientific papers published over the past 5 years indicate that probiotic supplementation alters microbial activities in the rumen and in the hindgut of horses (table III). It appears clearly that there is an important strain effect, some strains being more efficient than others. It also appears that the animal's response depends on the diet and requirements. The effects of probiotics in ruminants (feed intake, fibre degradation in the rumen, digestible or metabolizable energy, and use of trace elements) are not consistent from one experiment to another.

The few results which are available on microbial protein synthesis are in good agreement. Williams *et al* (1991) indicated that the flow of duodenal non-ammonia N increased by 15% when SC was added to sheep rumen (4 g/d), whereas the ileal flow was not modified. The gain in protein due to SC is therefore completely used by animals. Erasmus (1991) showed that the increase in duodenal protein flow was of microbial origin and that it was much higher than the daily supply of fungal proteins in the diet. Because SC does not grow in continuous fermentors simulating the rumen (Dawson *et al,* 1990) or *in vivo* (Newbold *et al,* 1990), it seems likely that the presence of SC stimulates bacterial growth by supply-

Table III. Effects of probiotics in the duodenal flow of microbial proteins and on rumen ammonia nitrogen concentration.

Reference	*Microbial N (g/d)*			*Rumen ammonia (mg/l)*		
	B	*+Ao*	*+YS*	*B*	*+Ao*	*+YS*
Gomez-Alarcon *et al* (1987)	355	635*				
Williams and Newbold (1990)	16.3	–	18.4			
Erasmus (1991)	254	–	294	106.8	–	96.2

B: basal diet; +Ao: addition of *Aspergillus orizae;* +YS: addition of yeast sacc; * significant difference among treatments ($P < 0.05$).

ing the bacteria with growth factors or by maintaining favorable conditions (pH, Eh, elimination of H_2) in the rumen. Dietary protein flow was not altered by SC. Edwards (1991) also observed that allantoin excretion, which is related to microbial N flow in the duodenum, was higher when young bulls received SC.

The supply of amino acids considered as limiting in dairy cattle (lysine, methionine) was increased in treated animals, which may explain the changes in milk protein production (Günther, 1989). Wanderley *et al* (1987) also reported an increase in duodenal protein flow when AO was given, although this fungus stimulates dietary protein degradation in the rumen (Boing, 1983).

Models and multifactorial approach

Although it has often been assumed that microbial growth in the rumen was mainly limited by energy availability, this phenomenon is likely to be under the simultaneous control of numerous factors, not only nutritional but also physico-chemical or ecological. Its energy efficiency is not constant. Furthermore, the existence of interactions between the factors of variation (*eg*, Rode and Satter, 1988) justifies a multifactorial approach for studies dealing with protein production manipulation. In addition, several models have been designed to simulate rumen functions and among them the production of microbial protein. The most frequent structure consists of a set of differential equations solved simultaneously by numerical methods. In the model of France *et al* (1982), microbial growth is only related to C and N substrates whose depletion induces the lysis of part of the microbial mass. The Baldwin *et al* (1987) model is based on a greater number of state variables (12 *vs* 9), and uses a more sophisticated numerical procedure (fourth-order Runge-Kutta *vs* Euler). It is intended to be part of a larger model of dairy cow metabolism. It divides the microbial bulk into 3 pools related to rumen fluid, and to small and large particles, with the intention of taking into account their differential outflow rates. Their model connects microbial growth to ATP release from fermentation, microbial maintenance needs, ammonia and preformed amino acids. The Australian protein unit relies on a less complex system, involving a set of empirical equations used in one-hourly iterative calculations. The microbes use the ATP produced by fer-

mentation, initially for maintenance. The occurrence of microbial growth or lysis depends on the ATP yield. In addition, the microbial growth can be limited by amino acids, ammonia, non-specific N or sulfur (Black *et al,* 1980–1981). Although the calculated and experimental responses followed the same pattern in many dietary situations, the predicted rumen total protein outflow appeared to be sensitive to changes in parameters still being evaluated, such as the fractional outflow rates of water and microorganisms (Beever *et al,* 1980–1981). One final approach is based on linear programming (Reichl and Baldwin, 1975, 1976; Sauvant and Giger, 1989). The simulation of microbial growth, however, remains unsatisfactory. In an evaluation of different models of rumen function in dairy cows, Sauvant and Ramangasoavina (1991) reported a poor relationship between a database of around 90 *in vivo* measures of microbial protein flow in the duodenum and the predicted values from the model by Baldwin *et al* (1987).

The microbial growth, and microbial N flows through the reticulo-rumen probably relies on an adequate description of the microbial population structure to be satisfactorily predictable. For instance, information on the quantity and species of entodiniomorphid protozoa appears to be essential in determining the extent of bacterial N recycling. On the one hand, these organisms greatly differ among species in rumen fluid clearance abilities, grazing preferences and relative digestion of bacteria. On the other hand, the composition of protozoal populations in ruminants can very widely according to the geographical area or even among neighbouring flocks (Williams and Coleman, 1992). The presence of bacteriophages may cause greater bacterial lysis (Styriak *et al,* 1991) but the extent of their potential influence is unknown. In the same way, conventional evaluation of feeds could be inadequate in predicting their influence on microbial growth.

With respect to rumen microbial proteosynthesis, the classical goals have been to improve the amount of protein produced, by increasing the amount of OM degraded or of the energy efficiency of proteosynthesis. The improvement of N retention has not received much attention so far. Nevertheless, this may turn out to be an important challenge as several European governments are encouraging farmers to reduce N-losses to the environment (Berentsen *et al,* 1992). A recent survey of N balance in 182 farms in north-west France indicated an average annual N surplus of 200–250 kg N/ha/year in dairy and intensive beef production (Simon and Le Corre, 1992). On intensive Dutch dairy farms, milk and meat production only accounts for about 14 p100 of the N-input (Korevaar, 1992). By simulating the effects of 4 different government environmental policies on Dutch dairy farms, Berentsen and Giesen (1994) showed that one of the most important factors acting on N use and N loss was the diet of cows, in addition to animal density and the method of manure storage and disposal. In an attempt to model N transactions in dairy cows, however, Dewhurst and Thomas (1992) clearly stressed the need for further knowledge of N flows in the rumen, while the feeding strategies for N retention improvement might prove to be highly inadequate.

CONCLUSION

Few practical ways of influencing rumen microbial protein synthesis have been reported so far. These have mainly been protozoa depletion and regulation of the supply of a nutrient identified as a limiting factor in specific nutritional conditions. All these methods produce rough alterations rather than actual manipulations of the

microbial processes. The more synthetic approaches based on rumen metabolism modelling are not effective, as yet, although they are very interesting for designing more environment-friendly cattle-raising practices in a number of European countries.

While a validated method for the measurement of rumen microbial protein production still has to be developed, one wonders if future studies on the manipulation of proteosynthesis would not be a poor compromise between both scientific aims, *ie* the study of microbial population dynamics, and practical objectives, *ie* the determination of the amount and nature of amino acids available in the duodenum.

It will be difficult to come to a final decision if one wishes to manipulate the rumen metabolism. One should have several nutritional aims in mind and they must be in keeping with the numerous other obligations of farming. The overall result must also increase farming incomes, even if the nutritional optimum is not reached.

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