Rumen Ecology Research Planning

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Edited by

R.J. Wallace

A. Lahlou-Kassi

THE INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

BOX 30709, NAIROBI, KENYA • BOX 5689, ADDIS ABABA, ETHIOPIA

The International Livestock Research Institute (ILRI) began operations on 1 January 1995. The institute incorporates the resources, facilitites and major programmes of two former CGIAR centres founded about two decades ago—the International Laboratory for Research on Animal Diseases, in Nairobi, Kenya, and the International Livestock Centre for Africa, in Addis Ababa. The research and outreach programmes of ILRAD and ILCA have been consolidated, streamlined and reoriented to support an expanded mandate. ILRI will conduct strategic research in the biological, animal and social sciences to improve livestock productivity in sustainable agricultural systems throughout the developing world.

The objectives of the new institute's research programme are to improve animal health, nutrition and productivity (milk, meat, traction) in ways that are sustainable over the long term, to characterize and conserve the genetic diversity of indigenous tropical forage species and livestock breeds, to promote sound and equitable national policies for animal agriculture and natural resource management, and to strengthen the animal husbandry research programmes of developing countries.

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Preface

There is increasing evidence that manipulation of microbiological activities in the rumen and bioengineering of rumen organisms could lead to the development of technologies to improve animal productivity from the available feed resources.

The merging of the International Livestock Centre for Africa (ILCA) and the International Laboratory for Research on Animal Diseases (ILRAD) expertise in animal nutrition, physiology and animal biotechnology open to the International Livestock Research Institute (ILRI) a new perspective in developing a research agenda on rumen ecology.

However, there is a need to assess the possible uses of available technology, identify applications of direct relevance to ILRI research, define areas where ILRI would have a comparative advantage, and identify partners for collaborative research projects.

The planning workshop is proposed to bring together experts from the rapidly advancing field of rumen microbiology to assist ILRI staff to identify these opportunities and set priorities.

Workshop objectives

- Identify and prioritise areas of rumen ecology which are promising for their potential impact on improving nutrition status of tropical ruminants.
- Develop a rumen ecology research programme for ILRI based on relevance to developing countries and ILRI's comparative advantage vis-a-vis other institutions.
- Identify potential collaborators in advanced research institutes and define mode(s) of collaboration.

Workshop organising committee

A. Lahlou-Kassi *(Coordinator)*

P. Fajersson

K. Getahun

Opening address

H.A. Fitzhugh
Director General, International Livestock Research Institute

On behalf of the Institute, Dr Lahlou-Kassi, who organised this workshop, and all ILRI staff, I welcome you who have come such long distances to help us develop a plan for research in rumen ecology.

I would like to start by introducing you to this new international research institute: the International Livestock Research Institute. The buildings may not be new, but that is because this new institute is something of a chimera, being built on the foundation of two international livestock research institutes.

The International Laboratory for Research on Animal Diseases was established in 1973 with a global mandate to develop effective control measures for livestock diseases that seriously limit world food production. ILRAD's research programme focused on animal trypanosomiasis and tick-borne diseases, particularly theileriosis (East Coast fever).

The International Livestock Centre for Africa (ILCA) was established in 1974 with a mandate to focus on livestock production systems in Africa. ILCA followed an interdisciplinary approach combining social and environmental sciences with the animal sciences. The founders of ILCA believed that technologies to improve traditional animal production systems were available from the laboratories and research stations of Australia, Japan, North America and Europe and that an understanding of those livestock production systems and their constraints would reveal how modern technologies might be best fitted to meet the needs for improving livestock production in the developing world. However, those traditional farming systems were evolving very rapidly, especially during the 1980s, along with social, political and cultural changes throughout Africa. Technologies available off the shelf from stations and laboratories elsewhere were often not suitable, and were sometimes inappropriate, for livestock producers in sub-Saharan Africa. The special characteristics of livestock production in tropical environments provided new challenges to research.

ILRAD and ILCA were supported by a consortium of donors known as the Consultative Group for International Agricultural Research, or the CGIAR. This Group was established in the early 1970s. The number of CGIAR institutes increased through the 1970s and more were added in the early 1990s. A few years ago, this consortium, which had increased in funding from 20 or so million dollars to about 300 million dollars, went through a crisis of confidence that all of us involved in research felt. Many donors lacked confidence that research was the way toward solving important development environmental problems. The CGIAR, therefore, began to reassess whether international agricultural research was worth supporting. Fortunately the answer was 'yes'. In 1995 we believe that we have regained the confidence of the donors. But during this time of reassessment some changes were made within the system. One was the establishment of the International Livestock Research Institute to combine animal health and production research.

Both ILRAD and ILCA were focusing on African problems. Studies conducted by the donor community that supported these international research institutes indicate that there is a strong demand and need for livestock research in Asia and, to a somewhat lesser extent, in Latin America, West Asia and North Africa or the Mediterranean region. One priority for livestock research in Asia are the major economic developmental and environmental problems that have arisen due to intensification of agriculture.

Because of the location of our campuses and our access to the problems of developing countries, ILRI is able to serve as a transfer agent, not only by transferring technologies but also by providing access and contacts between the laboratories of developed countries, which are substantially better endowed, and the laboratories and research centres of developing countries. Perhaps more important is the research-based information and knowledge that are being transferred and the increasing opportunities to apply this knowledge and experience to the problems of developing countries.

International research centres make up only a small component in the whole spectrum of research on problems of agriculture in developing countries. The largest players are the national research institutes in the developing countries the world over. Many of these institutes are underfunded but their scientists are increasingly well trained. Through collaborative research, we can employ the large scientific capacity in the national institutes.

Most research at ILRI is done in collaboration with other scientists and institutes in both developing and developed countries. We plan to expand our collaborative activities and that is one of the reasons why we brought you here, so that we can become better acquainted. This will help us to identify useful collaborations. This institute has a very broad set of programme activities ranging from work in the laboratories in molecular biology to work in the field. We are able to cover this very broad base of research activities only because of our ability to work and collaborate with a host of other scientists and institutions.

I remind you that the appropriate type of research for an international institute lies at the upper end of the applied spectrum rather than that of adapting technologies to meet the needs of farmers and their livestock. Our research must be development- oriented. We must be clear as to how our research will serve development needs. Ninety-eight percent of our funding comes from the development vote. And so even though our research is fundamental in character, we must be able to articulate a clear use of the research products to further development.

One of our research areas is utilisation of feed resources. Any analysis of the constraints to livestock production in developing countries consistently identifies feed resources as a primary constraint. The primary constraint to livestock production may be the production of feed resources or their utilisation, depending on the specific production system and the particular environment.

The goal of the feed utilisation programme is to improve efficiency of use of feed resources by tropical ruminants. We look to our sister centres to focus their work on production which leaves us to focus on utilisation. It is usually the intersection between production and utilisation that is the major problem. Forage production, for example, will often be facilitated by genes that promote within the plant characteristics that

protect it from pests or predators. Sometimes selecting for rapid growth rate will generate qualities that are anti-nutritional in character. There is, therefore, need to match what is good for production of the plant with what is good for utilisation of the products of plant growth.

There were opportunities for real synergies in the types of research conducted in Nairobi and Addis Ababa. ILCA was carrying on some work in the area of rumen ecology while ILRAD was carrying on work at the microbial and molecular level. So we felt that there would be good opportunities for synergisms by putting these two institutions together and we quite deliberately delayed having this workshop until we could have scientists from both former ILCA and former ILRAD working together.

The objectives of this workshop are very straightforward. We will look for a very specific recommendation coming out of this workshop as to whether or not ILRI as an international institute has a comparative advantage in conducting research in this broad field of rumen ecology. If at the end of the week you decide ILRI does have a comparative advantage for this type of research, we will ask that you be specific as to exactly where this comparative advantage lies. While doing this, we ask you not to think of ILRI as just a new institute, but as a set of resources and opportunities that can be developed as part of a much larger global network of institutes, many of which you yourselves represent.

Improving fibre utilisation and protein supply in animals fed poor quality roughages: ILRI nutrition research and plans

P.O. Osuji[1], *S. Fernández-Rivera*[2] *and A. Odenyo*[1]

1. International Livestock Research Institute, P.O. Box 5689, Addis Ababa, Ethiopia

2. International Livestock Research Institute, ICRISAT Sahelian Centre, BP 12404, Niamey, Niger

Abstract

Inadequate nutrition is the main cause of low productivity of livestock in sub-Saharan Africa. The primary feed resources include fodder trees, natural pastures and crop residues. This paper presents results from studies conducted at ILRI in evaluations of these resources. The effect of supplementing teff straw with lablab or cowpea in cattle on microbial protein supply was evaluated using urinary purine derivatives. There was no effect of type of supplement but the level of supplementation significantly improved microbial protein supply. Similarly, supplementing maize stover with oilseed cake increased total microbial protein flow in sheep. Teff straw supplemented with *Sesbania sesban* increased dry matter degradation and liquid passage rates. Degradation of *Sesbania, Lucaena, Chamaecytisus* and *Vernonia* foliage was also evaluated. *Sesbania* was degraded rapidly and *Vernonia* slowly. The effects of anti-nutrient extracts from *Vernonia amygdalina, Chamaecytisus palmensis, Sesbania sesban* and *Acacia angustissima* were tested on pure cultures of cellulolytic rumen microbes and *A. angustissima* prolonged the lag phase. *A. angustissima* killed sheep and *Tephrosia* caused massive rumen stasis. Studies conducted in semi-arid West Africa indicated that the microbial digestive activity, as measured by the disappearance *in sacco* of a standard forage, varies both seasonally and across animal species. Genetic variation in feeding value of crop residues, forage legumes and fodder trees were assessed. The variation among varieties of sorghum and pearl millet were relatively small and inconsistent across years. Relatively larger differences were observed in forage legumes. Preliminary results suggest possibilities for identifying geographical areas that produce better quality fodder trees. Future work should include the biochemical basis of the interactions between rumen microbes and the chemicals contained in fodder trees, and the seasonal variations of microbial populations in various ruminants across agro-ecological zones.

Introduction

Inadequate year round nutrition is a major smallholder production constraint. Ruminants owned by smallholders in all ILRI mandate regions will continue to subsist, for the

foreseeable future, on unimproved native pastures and crop residues. These poor quality roughages are bulky, high in fibre, poorly degraded in the rumen, low in nitrogen and minerals (Table 1) resulting in very low intakes. Population pressure and urbanisation, particularly in Africa, will limit the quantity of grain available for animal feeding. However, these deficiencies could be corrected by the addition of fodder trees, herbaceous legumes or multipurpose trees (MPTs) (Tables 2 and 3).

Table 1. *Nitrogen (N) and neutral detergent fibre (NDF) concentrations of low quality roughages used in ILRI research.*

	g/kg dry matter (DM)	
Roughage	N	NDF
Maize stover	5.4	755
Teff straw	4.9-7	798
Oat straw	3.8	727
Oat hay	7.9	815
Barley straw	5.4	829
Debre Zeit hay	12.0	722
Pea straw	7.5	768
Sorghum stover	8.4	773
Sululta hay	9.4	738
Cynodon hay	11.8	737
Wheat straw	4.6	772
Napier grass	4.8	720
Pearl millet	8-11	704

Table 2. *Characteristics of MPTs: Degradation rates of N for the fresh leaves (FL) and dry leaves (DL) of two accessions of* Sesbania sesban *and* Acacia siberiana *pods*.

Sesbania ILCA Acc. No.	Readily soluble fraction (g/kg)	N degradation rate (/h)
10865 DL (dry leaves)	298	0.065
10865 FL (fresh leaves)	468	0.110
15021 DL	314	0.075
A. siberiana (pods)	433	0.055

Several factors affect the utilisation of poor quality roughages by ruminants. These include rumen environment (where conditions should be pH > 6.2, NH_3 > 3.5 mmol/l), microbial adhesion, particle size reduction, passage rates of both particulate and liquid digesta, roughage degradation rate and volatile fatty acid production, adequate supply of iso-acids for microbial protein production and the availability of by-pass protein. ILRI nutrition research has focused on how to enhance fibre degradation and microbial protein supply by rumen manipulation aimed at optimising the above conditions. The use of MPTs as a means to correct nutrient imbalances and improve the conditions in the rumen has been a priority area of research (ILCA 1995; Osuji 1994). This paper describes some of the nutrition research work done at ILRI. First, results obtained in

Table 3. *Chemical characteristics of MTPs.*

	Low	High
(g/kg DM)		
Nitrogen	*Dichrostachys cinerea (13.9)*	*Leucaena (40.5)*
Fibre (NDF)	*Sesbania (206)*	*D. cinerea (498)*
NDF-N	*Sesbania (2.4)*	*Tagasaste (9.2)*
Soluble tannins	*Carissa edulis (21.5)*	*A. siberiana (327)*
Condensed tannins (absorbance units/g NDF at 500nm)	*Sesbania (13)*	*Tagasaste (56)*
Phosphorus	*Vernonia (0.7)*	*Sesbania (2.7)*
Calcium	*Tagasaste (10.5)*	*Leucaena (20.0)*
Sulfur	*Tagasaste (1.5)*	*Leucaena (2.3)*
(mg/kg)		
Iron	*Sesbania (360)*	*Tagasaste (520)*
Manganese	*Leucaena (66)*	*Tagasaste (200)*
Copper	*Leucaena (13)*	*Vernonia (20)*
Zinc	*Leucaena (19)*	*Tagasaste (39)*

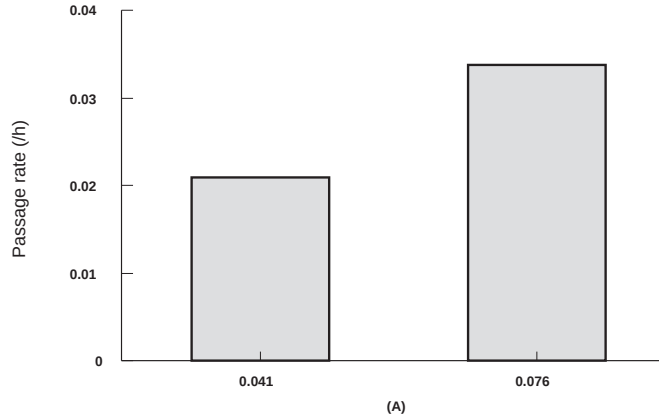
NDF: Neutral detergent fibre.
NDF-N: Nitrogen bound to NDF.

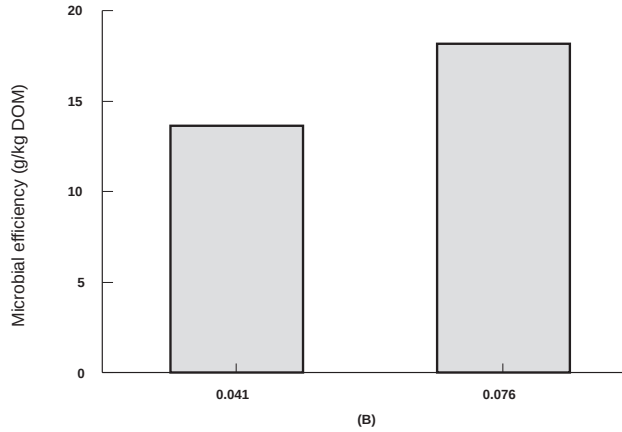
the use of fodder or multipurpose trees (MPTs) and herbaceous legumes in animal feeds are presented. Research aimed at identifying and overcoming constraints to rumen function in grazing animals is discussed. The selection of crops and trees for improved feeding value is addressed. Finally, research plans on rumen ecology are presented.

Supplementation with MPTs and herbaceous legumes

Ruminants depend on microbial protein production to meet their N requirements. In ruminant production systems where poor quality roughages constitute the principal source of feed there are two major objectives aimed at optimising their use. There is need to enhance the fermentation of the roughage to ensure adequate energy supply, mainly as VFA. Secondly, given the scarcity of protein in such systems, there is a need to maximise the supply of microbial protein. Several strategies are available to manipulate the rumen. The strategy of choice depends on the desired output. One strategy that has often been used, particularly when poor quality forages are fed, is supplementation. The principal objective of supplementation is to increase the supply of nutrients, mainly energy and protein, such as to create favourable conditions in the rumen which result in better fermentation and microbial protein supply (Figure 1). Supplementation with MPTs can do this in several ways—MPTs can increase the amount of energy supplied by the basal feed by:

1. Alleviating a deficiency hampering microbial fermentation of the basal feed e.g. N or S.





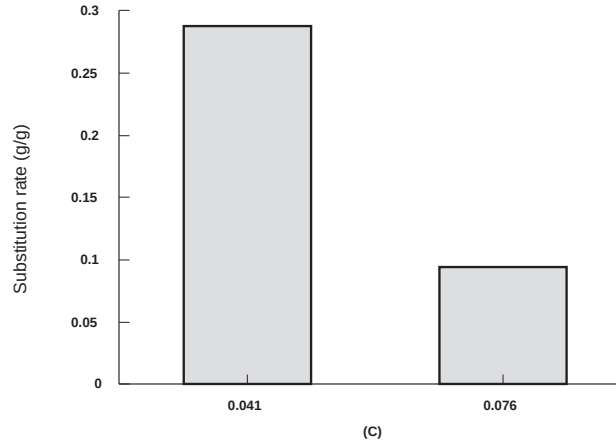


Figure 1. *Effect of rate of degradation of browses on (A) rate of passage of particulate matter, (B) efficiency of microbial N supply and (C) substitution rate.*

2. Improving the rumen environment (e.g. pH, rumen NH_3 or rumen-degradable protein) to ensure increased fermentation of the basal roughage diet. For example, increased numbers of cellulolytic bacteria will increase the invasion of and adhesion onto the fibrous feed.
3. Improving the rate and extent of particle size reduction and thus increasing the passage rates of both liquid and particulate matter, leading to increased feed intake.
4. Supplements such as MPTs are themselves sources of energy. In fact some MPTs are fed at certain times of the year as the sole feed.

Thus MPTs, as supplements, can increase protein supply to the host animal by increasing the supply of both degradable and undegradable protein, and by creating a favourable rumen environment resulting in enhanced fermentation of the basal roughage and thus increased microbial protein synthesis.

Nutrition research at ILRI has involved *in vivo*, *in sacco* and *in vitro* studies. *In vivo* work has looked into the effects of various factors on microbial protein supply. In one trial (Abule 1994), supplementation of teff straw with graded levels of cowpea or lablab significantly increased microbial N supply in calves. The type of supplement had no significant effect on microbial N supply but the level of inclusion did (Table 4). When sheep were given a basal diet of maize stover supplemented with oilseed cakes, total microbial protein flow into the intestine was significantly affected ($P < 0.01$) and was increased further by maize supplementation (Osuji et al 1993). When poor quality roughages were supplemented with MPTs, the rate of degradation of the basal feed, the fractional outflow rate of liquid matter from the rumen and the efficiency of microbial protein supply were increased (Figure 2). Rapidly degraded MPTs like *Sesbania* increased passage rate and the efficiency of microbial protein supply and are better supplements. The feeding of rapidly degraded MTPs has resulted in reduced substitution of the basal roughage diet (Bonsi et al 1995).

Table 4. *Microbial N supply of calves fed a basal diet of teff straw supplemented with lablab hay*[†].

Inclusion rate (g/kg LW)	Microbial N (g/day)
0	4.6
5	12.4
10	20.4
15	24.9

[†]No differences were observed between cowpea and lablab.

In another set of trials sheep were given a basal diet of teff straw supplemented with 0, 175, 245 or 315 g of *Sesbania* to create different rumen environments. Dry matter degradation and liquid passage rates were estimated. Figure 2 shows clearly that both dry matter degradation and liquid passage rates increased with increasing level of *Sesbania* up to 245 g/head per day and then declined. Improved or faster liquid outflow rate may result in increased intake of the basal roughage as new material is eaten to replace what has left the rumen (Bonsi et al 1995).

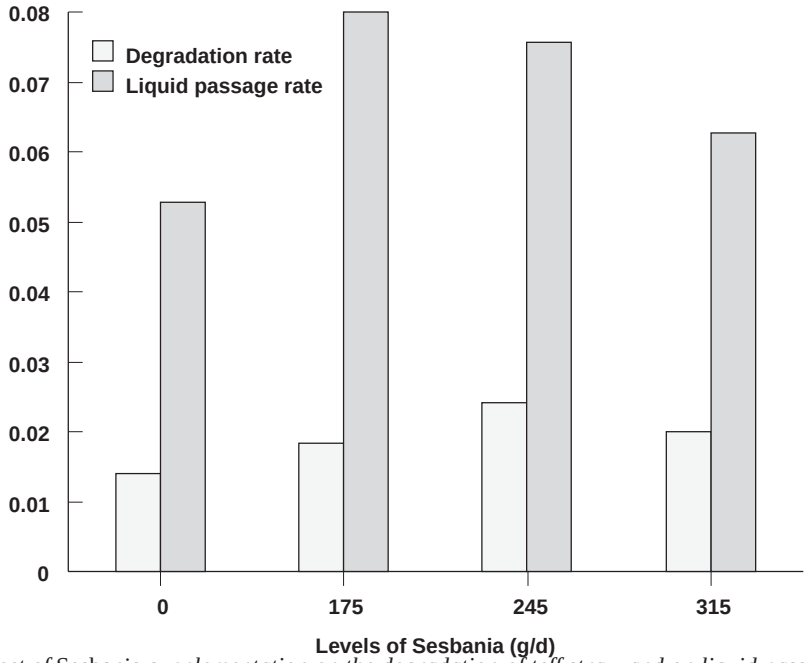


Figure 2. *Effect of* Sesbania *supplementation on the degradation of teff straw and on liquid passage rate.*

Effect of plant parts

With most MPTs there is the choice of feeding the leaves alone or the leaves plus the fruits or the fruits alone. *Sesbania sesban* 10865 leaves were compared with *Acacia albida* pods. The rate of degradation of fresh leaves was much higher than that for the albida pods. Bulkiness of the MPT seems to be an important additional factor affecting the intake of fibrous feeds (Osuji 1994).

Degradation properties of MPTs and interaction with rumen microbes

In sacco degradation techniques (Ørskov and MacDonald 1979) have been used at ILRI to study factors which affect the interaction of MPTs with rumen microbes. The results of these studies indicate that several factors affect the interaction of the MPT with the rumen microbes. The type of MPT, its form and the quantity fed affect roughage utilisation. When MPTs of the genera *Sesbania, Leucaena, Chamaecytisus* and *Vernonia* were evaluated using the *in sacco* method, the rates of both DM and N degradation varied significantly among the four MPTs (Figure 3). *Sesbania* was degraded most rapidly while *Vernonia* was degraded relatively more slowly. It has been found that the rate of degradation of MPTs varies with the basal diet (Figure 4). Results from several trials, where different MPTs were incubated in the rumens of sheep, showed that the form of the MPT affected the rate of DM degradation. Fresh forms of the MPTs were degraded much faster than the dry forms (Figure 5). Both the fast-degrading and the slow-degrading MPTs could be harnessed to nutritional advantage (Bonsi et al 1995; Nsahlai et al 1994; Siaw et al 1993; Umunna et al 1995).

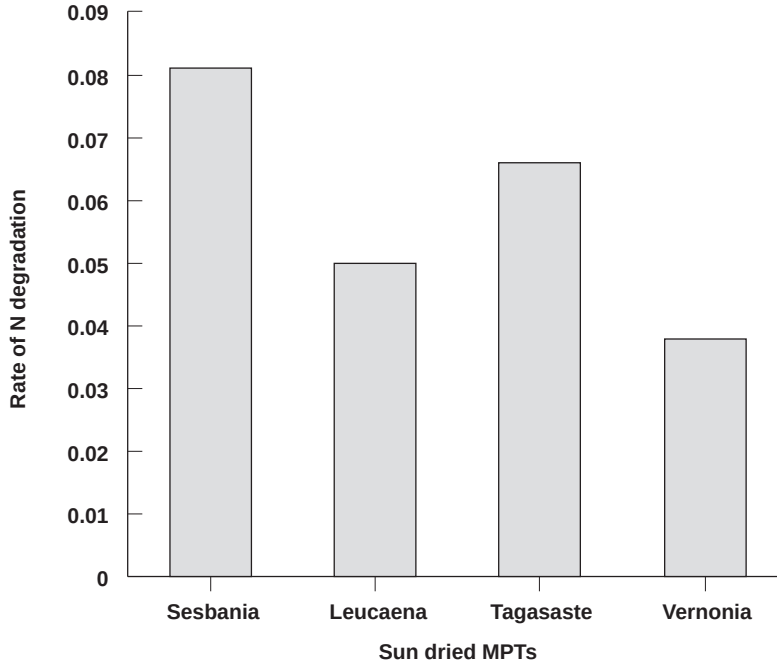


Figure 3. *Effect of the type of MPT (sun dried) on rate of nitrogen degradation*.

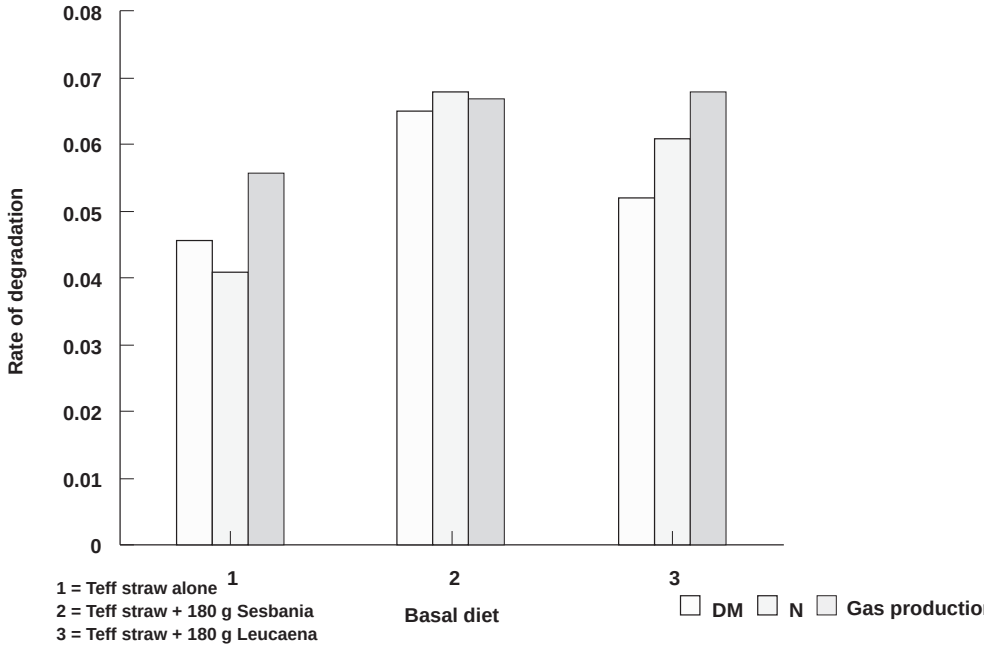
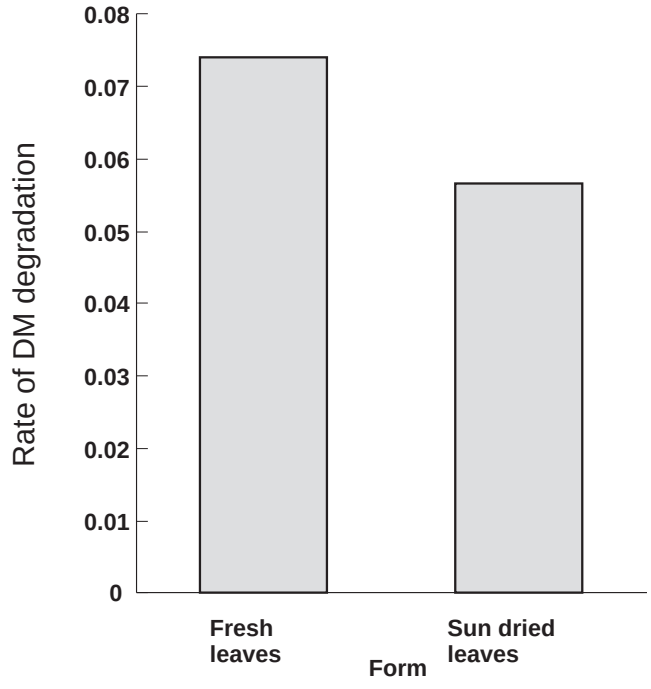


Figure 4. *Effect of different basal diets on rate of degradation of dry matter and nitrogen, and gas production (per hour) of four MPTs* (Sesbania, Leucaena, Tagasaste and Vernonia).



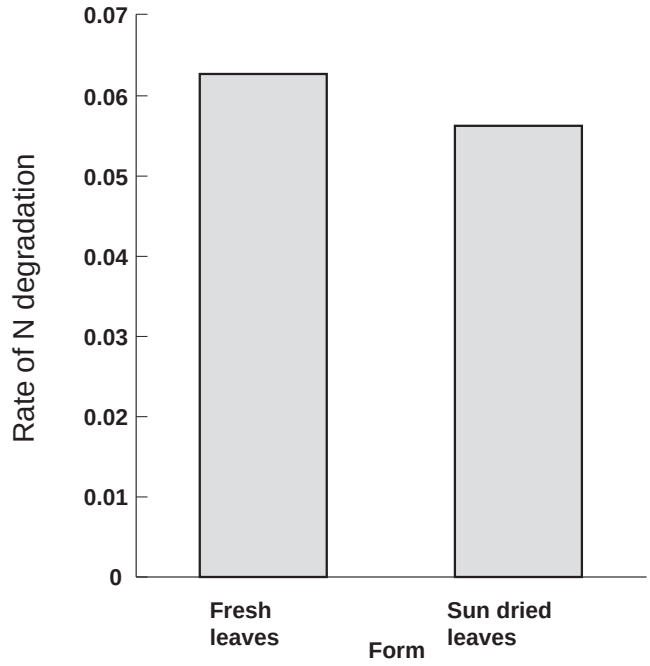


Figure 5. *Effect of different basal diets on rate of dry matter and nitrogen degradation*.

Effect of quality of basal roughage

For roughages of different N content supplemented with MPTs, the degradation rate of the unsupplemented basal roughage increased with the N content and the effect of the MPT supplement on roughage degradation rate was most marked in the basal roughage with the lowest N content. This clearly demonstrates that smallholders who depend on poor quality roughages (native pastures and crop residues) will benefit most by supplementing their animals with fodder trees. Therefore a farmer with limited MPT supplement would be better off feeding it to animals on maize stover or teff straw rather than to animals fed better quality Napier grass.

Anti-nutritional factors

MPTs could also contribute to creating an unfavourable rumen environment, e.g. by lowering rumen pH which would result in a reduction in the number of rumen fibrolytic organisms and thus reduce cellulolysis (Table 5).

There is a wide range of anti-nutritional factors found in MPTs (Table 5) though the phytochemistry and mode of action of these anti-nutritional factors are not fully understood. There are indications, however, that some of them have defaunation qualities, some are bactericidal, others bacteriostatic. Furthermore, poisons from MPTs can act either on the rumen microbes or on the host animal itself. This makes the evaluation of MPTs rather difficult. Figure 6 demonstrates the effect of anti-nutritional factors on dry matter intake. *A. angustissima* is very high in proanthocyanidins compared to *S. sesban* 10865. Dry matter intake was depressed to such an extent in sheep fed *A. angustissima* that many of the animals died (Kurdi 1994).

Attempts have been made to harness anti-nutritional factors (e.g. tannins) to nutritional advantage. In this regard, oilseed cakes were fed in association with tanniferous browses (1994). Only minor depressions in the rate of fermentation of oilseed cake were observed. The changes due to this associative feeding of oilseed cakes and browses on growth rate were equally small. Attempts to match roughages with protein supplements have not demonstrated any benefits that could be attributed to the interaction between the tannin content of sorghum stovers and the oilseed cakes (Osafo 1993). What is clear is that cottonseed cake promoted better gains than noug cake.

The consumption of tannins caused a shift in the paths of nitrogen excretion from the urine to faeces. In addition, there was an increase in the insoluble fraction of faecal nitrogen. These effects of tannins can lead to decreased volatilisation losses and mineralisation rates, which may have important effects in the process of nutrient cycling and crop production (Powell et al 1994).

Effects of MPTs on microbes

The effects of MPTs on rumen microbes have been studied at ILRI using the gas production technique (Menke et al 1979). The effects of MPTs on mixed rumen microbes seem to be different from their effects on pure cultures (El Hassan 1994). *In vitro* trials suggested that *S. sesban* reduced the number of protozoa, suggesting that *S. sesban*

Table 5. *Toxic substances found in some MPTs*

MPT	Toxic component	Part of plant	Concentration	References
Sesbania	Tannins	Foliage	0.62%	Reed (1986)
				National Academy of Sciences (1979)
	Saponins	Foliage	—	Kinghorn
	Alkaloids	Foliage	—	and Smolenski
	Amines	Foliage	—	(1981)
Acacia	Fluoroacetic acid	Foliage/pods	—	Topps (1992)
	Tannins	Foliage/pods	3.12%	Ahn et al
	Cyanide	Foliage/pods		(1989)
	Oxalate	Foliage/pods	—	Goodchild
	Saponins	Foliage/pods	—	and McMeniman (1987)
Gliricidia	Tannins	Foliage	2.05%	Ahn et al (1989)
	Dicoumarol	Leaves	—	ILCA (1991)
Albizia	Dicoumarol	Leaves	2.25%	ILCA (1991)
Calliandra	Dicoumarol	Leaves	7.91	ILCA (1991)
Tagasaste	Tannins	Foliage	—	Borens and
	Alkaloids	Foliage	—	Poppi (1990)
Leucaena	Mimosine	Leaf	1–12%	Tangendjaja
		Seed	3.3–14.5	et al (1990)
	Tannins	Leaf	3–14%	D'Mello and
		Seed	7.1%	Fraser (1981)
	3-Hydroxy-4(1H)-	Leaf	5.1–8.2%	D'Mello and
	pyridone (DHP)	Seed	ND	Taplin (1978)
	Trypsin inhibitors	Leaf	—	Acamovic and
		Seed	—	D'Mello (1984)
	Saponins	Leaf	2–11%	Tangendjaja
		Seed	2–11%	and Lowry (1984)
	Flavonols	Leaf	3–6%	Lowry
		Seed	2–11%	et al (1984)

10865 may have defaunating properties. However this was not confirmed in an *in vivo* trial where *S. sesban* 10865 was compared to *Medicago sativa* (El Hassan 1994). No particular efforts were made in this trial to isolate the animals used as the numbers of rumen microbes were similar for both treatments. Cross contamination might have taken place.

Mueller-Harvey et al (1988) found that extracts of Ethiopian browses increased the lag time and reduced the growth rate of *Streptococcus bovis*. They found that extracts from *Acacia nilotica* were particularly detrimental to rumen bacteria. Alternatively, the rumen population may have adapted to metabolise the anti-protozoal agent.

The effects of extracts of *Vernonia amygdalina, C. palmensis, S. sesban* 10865 and *A. angustissima* on the growth of pure cultures of *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *R. albus* were investigated (El Hassan 1994). *Vernonia amygdalina, C. palmensis* and *S. sesbania* 10865 extracts affected the growth of only *R. albus* by prolonging the lag phase. On the other hand *A. angustissima* at lower

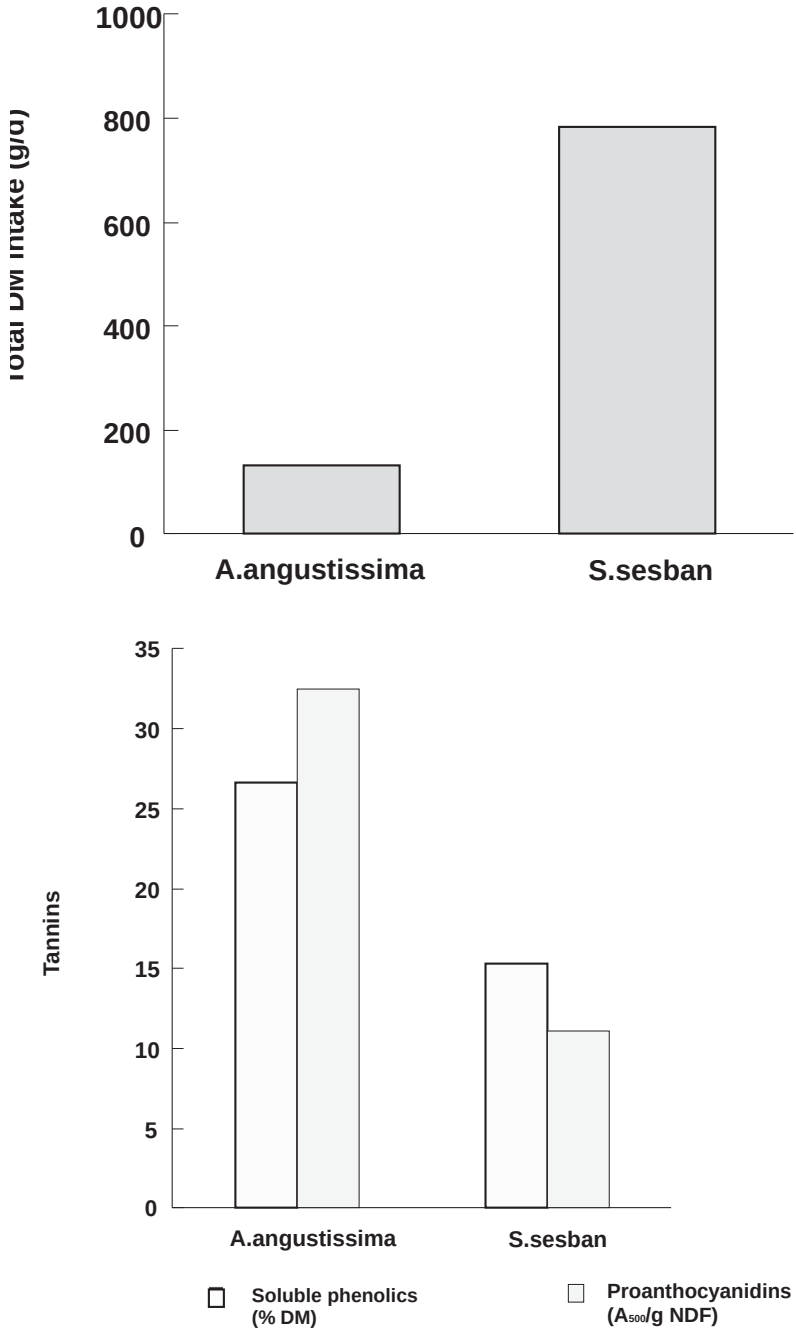


Figure 6. *Effect of tannin content on total DM intake.*

inclusion rates greatly reduced the growth of *Ruminococcus* species and slowed the growth of *F. succinogenes.* Extracts of *L. leucocephala* prolonged the lag phase of cellulolytic bacteria. *Ruminococcus flavefaciens* and *R. albus* are very important for fibre digestion.

Using the gas production technique, it was found that gas production rates decreased substantially when some MPTs were incubated with rumen fluid *in vitro*. *Acacia angustissima* suppressed fermentation and the extent of suppression depended on the amount of MPT included (El Hassan 1994) (Figure 7). The reverse was true for leucaena. These effects were illustrated *in vivo* when animals fed *A. angustissima* died and those fed *Tephrosia* had massive rumen stasis.

More work on the utilisation of fodder trees as livestock feed is needed. This should aim at refining rapid methods of evaluation, expansion of the work on (1) anti-nutrition factors to other compounds other than tannins and (2) the effects of various fodder trees on specific rumen microbes. These would add to the tools needed to use fodder trees effectively in rumen manipulation aimed at enhancing ruminant production.

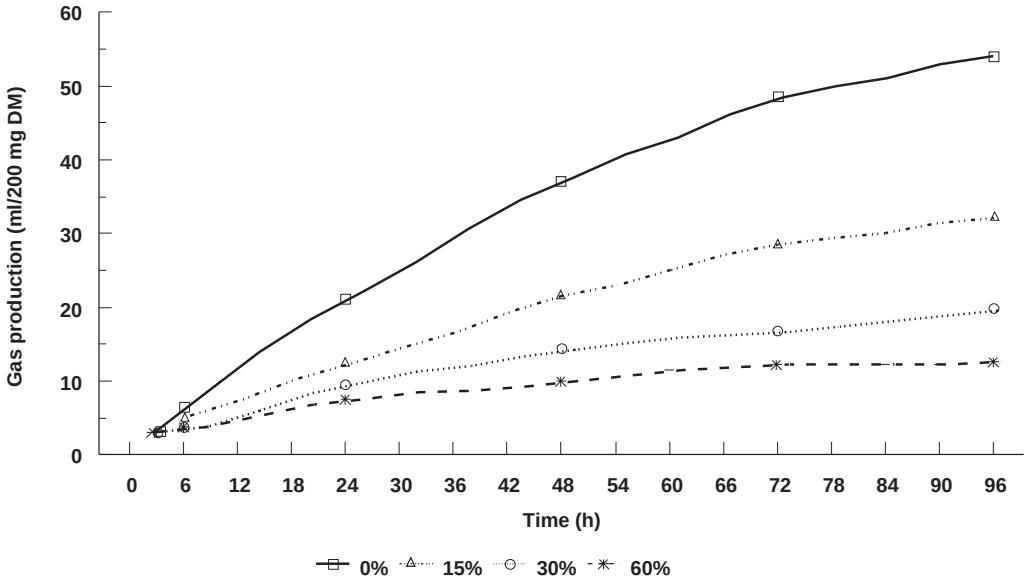


Figure 7. *Gas production (ml)* in vitro *from different inclusion levels of* Acacia angustissima *with teff straw.*

Seasonal variation in the rumen environment under traditional feeding systems

Fibre digestion and microbial protein synthesis in the rumen are greatly affected by the conditions that prevail in the rumen. In general, when low quality forages are consumed, the conditions needed for efficient digestion of fibrous feeds and microbial growth are not met. In addition, the ingestion of feeds containing anti-nutritional factors is

associated with decreased microbial activity. Studies on the environmental conditions in the rumen in traditional feeding systems can help identify the nature of the constraints to rumen function. Their alleviation can result in substantial improvement in the efficiency of use of the feed resources available. If nutrient imbalances are also overcome, the economic response to supplementation can be improved.

Studies on environmental conditions in the rumen of grazing animals are under way at the zonal programmes of ILRI in Niger and Nigeria. In these studies, the nylon bag technique is used to evaluate the quality of the ruminal conditions as judged by the disappearance rate of a standard forage. Some results obtained with cattle, sheep and goats in Niger (Fernández-Rivera 1994) are presented here. Six intact females, four ruminally fistulated males and four oesophageally fistulated males of each species were used in the study. All animals grazed pearl millet residue fields (December 1992 to March 1993) and Sahelian rangelands (March 1993 to December 1993). A standard forage (pearl millet stover leaves) was incubated monthly in the rumens of the fistulated animals to determine the rate and extent of disappearance of organic matter (OMD). Rumen fluid samples were taken monthly (mean of am and pm values) from the intact animals and analysed for NH_3-N. Extrusa samples were collected monthly from the oesophageally fistulated animals and analysed for crude protein (CP) and OMD.

All variables were influenced by species and month of measurement. During most of the year, sheep and goats selected a diet with similar CP, but the NH_3-N concentration in the ruminal fluid was lower in goats than in sheep, which reflects differences in solubility of the nitrogen consumed by these species (Figure 8). Cattle selected a diet lower in CP and had a lower concentration of NH_3-N in the rumen than small ruminants. The concentration of NH_3-N in the rumen fluid of cattle was less than 70 mg/l for most of the dry season, whereas in small ruminants NH_3-N did not appear to limit rumen function at any of the sampling times. In spite of the lower concentration of NH_3-N in the rumen fluid of cattle, the potentially digestible insoluble fraction of the standard forage was higher and less variable than in small ruminants (Figure 9). Large seasonal variations in the magnitude of the potentially digestible insoluble fraction were observed. The rate of OM digestion and the lag time were affected by month of measurement but not by species ($P = 0.08$). The lag time was longer in the dry season, whereas no clear trends were observed for the monthly variation in digestion rate. The reduction in potentially degradable fraction (B) with advancing dry season probably reflects increasing cell wall and thus decreased soluble carbohydrates. However, it is not clear to what extent the reduction in the B fraction may have been compensated by the increased rate of degradation of this fraction resulting from increased N supply from browses available at this time.

These results suggest that the environmental conditions and the digestive microbial activity in the rumen of cattle, goats and sheep grazing year-round on crop residue fields and Sahelian rangelands vary seasonally. This variation appears to be associated with the dietary selectivity of the three species. The potentially digestible fraction is generally thought to depend on the nature of the feed. However, these results suggest that anti-nutritional factors may decrease the potential extent of digestion of fibrous feeds. Since the different animal species differ in dietary selectivity, the constraints to rumen

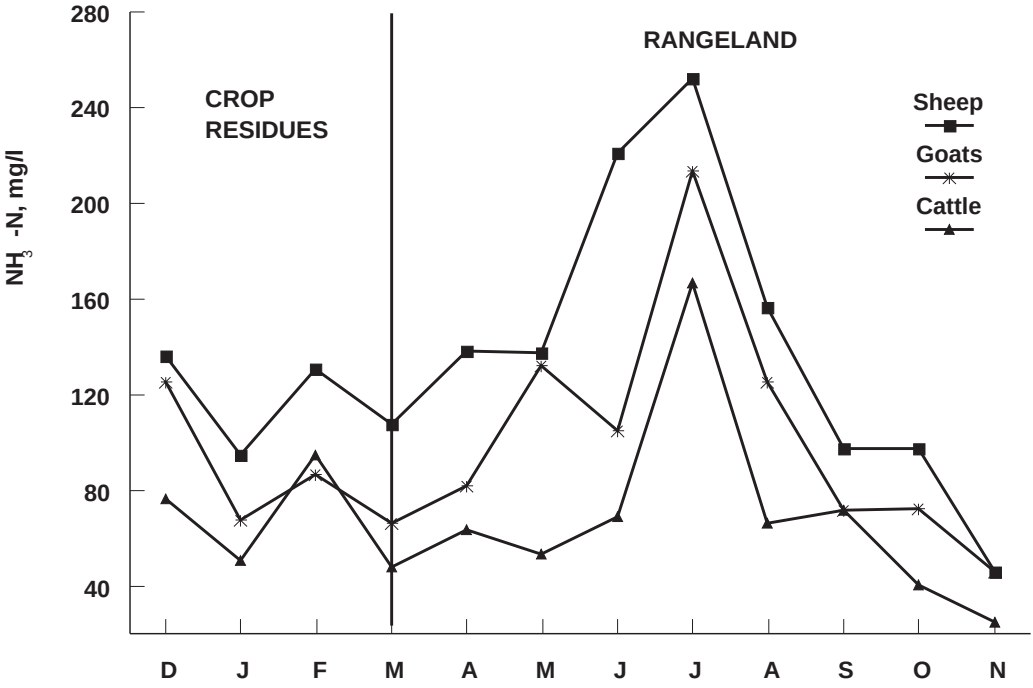


Figure 8. *Ammonia concentration in rumen fluid of cattle, goats and sheep grazing crop residues and rangeland in Niger.*

function appear to be different across species. The variation in the ruminal environment observed under traditional feeding regimes in the tropics must be considered when attempting to manipulate the rumen microbial population. Special consideration should be given to the influence of toxic substances and the competition among microbes or their ability to tolerate periods of starvation.

These results also have implications for defining supplementation priorities. For instance, during crop residue grazing, high levels of intake and diet quality were observed only during the first three or four weeks of the season, when the amount of above-ground leaf exceeded 400 kg/ha (ILCA 1993). After the first month of grazing the amounts of protein- and energy-yielding nutrient appears to be insufficient for efficient microbial activity and the maintenance of the animals. Therefore a response to rumen degradable (RDP) and undegradable (RUDP) protein and energy could be expected. In a supplementation experiment, the response to metabolisable energy (ME) followed a diminishing return pattern and decreased as the initial live weight of the animals increased. The sheep grew faster when supplemented with RUDP and this response rose as ME intake increased, which suggests that the microbial protein

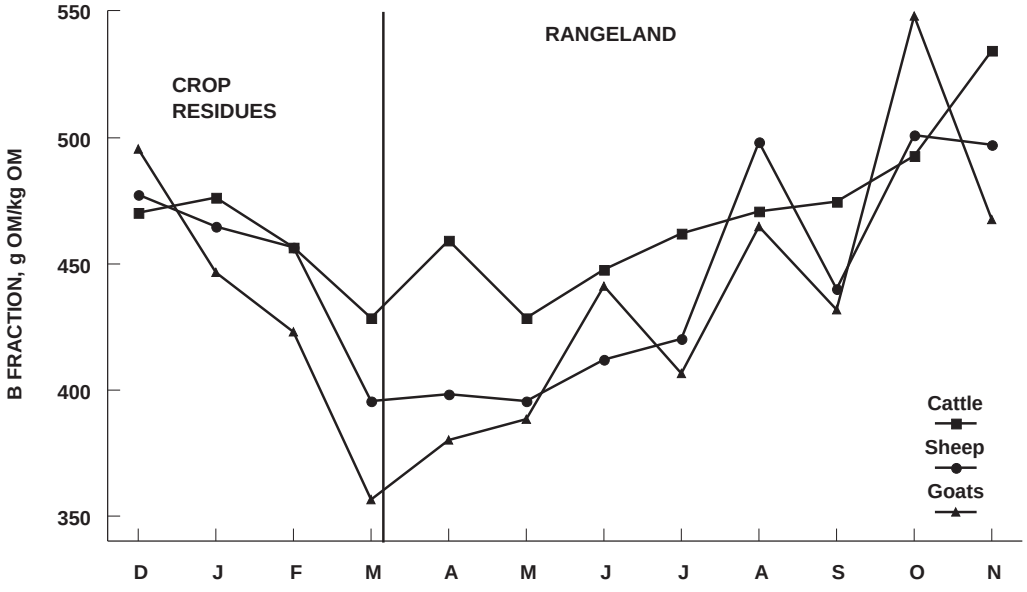


Figure 9. *Degradation (b fraction)* in sacco *of a standard forage incubated in the rumen of cattle, goats and sheep grazing crop residues and rangeland in Niger.*

synthesis was not sufficient to meet sheep protein requirements. Similarly, the influence of ME was also dependent on the consumption of RUDP. Further studies should determine the economically optimum levels of supplementation.

Improving fibre digestion through genetic selection of crops and trees

Fibre digestion in the rumen is constrained by retention time, digestion rate and the magnitude of the indigestible fraction (Allen and Mertens 1988; Ørskov 1991). These constraints could be alleviated by increasing rumen volume, improving the environmental conditions in the rumen or increasing the fibrolytic capacity of the rumen microbes. The constraints to fibre digestion in the rumen are determined by the chemical and physical nature of the fibre, which in turn depends on the genetic make-up of the plant and the environment where it grows. Therefore the selection of cultivars that produce high levels of grain and better quality residues offer possibilities for improving fibre breakdown in the rumen (Bartle and Klopfenstein 1988; Ørskov 1991).

Three different avenues for improving fibre digestion through plant selection have been followed by ILCA/ILRI, in collaboration with crop-oriented institutions. The first is the exploitation of variation across varieties in cereal crops (sorghum and millet) and forage legumes (cowpea and groundnut). The second is the introduction of genetically controlled quality related traits, as the low-lignin brown mid-rib (bmr) and trichomeless

(tr) traits in pearl millet. The third is the identification of geographic locations in the semi-arid zone that could produce higher quality trees. In the last approach, studies are under way in Niger to evaluate the variation in feed potential of several species.

Influence of genotype on feeding value of cereal crops

Bird-resistant and non-bird-resistant varieties of sorghum grown in Ethiopia did not differ in neutral detergent fibre (NDF) concentration of leaves (Reed et al 1987, 1988). However, leaves from non-bird-resistant varieties were more digestible. Most of the variation in leaf NDF digestibility was accounted for by differences in lignin or insoluble proanthocyanidins. Further studies by Osafo (1993) showed that varieties differed only in the potentially degradable fraction (Ørskov 1991) of leaves but not in the readily soluble fraction. Since no differences were observed in other plant parts, the degradation rates and the potential degradation of the whole stover were similar across varieties.

In 12 millet varieties grown in Niger, Reed et al (1988) found differences in NDF and lignin of leaf and stem, although the range in variation appeared to be smaller than the inter-varietal differences that they had observed for sorghum. However, these differences do not appear to be consistent over the years. In a three-year collaborative study with the International Crop Research Institute for the Semi-arid Tropics (ICRISAT), the forage quality of the stover from nine pearl millet varieties grown under three phosphorus fertiliser levels (0, 30, 60 kg P_2O_5/ha) was evaluated. In the three years of the study, the millet varieties differed ($P < 0.01$) in stover yield. These differences were not affected by phosphorus fertiliser and were consistent across years. The concentration of NDF in leaf and stem was affected by variety in two of the three years, whereas digestibility of leaf was influenced by variety ($P < 0.01$) in one year and that of stem ($P < 0.01$) in two years. The annual means of OM digestibility of leaf were 442, 447 and 490 g/kg and those of stem were only 235, 227 and 217 g/kg. Digestibility was lowest in the highest grain yielding year, but no correlation between grain yield and digestibility was detected within years. This study indicated that differences among varieties are large for residue yield, but small for quality traits. Grain yield is highly positively correlated with stover yield but appears not to be correlated with digestibility or NDF of the stover.

In another collaborative study with ICRISAT, the effects of the brown mid-rib (bmr) and trichomeless (tr) traits on the quality of millet stover were studied (ILCA 1994a). At grain harvest, stover leaves and stems from 120 progenies of either bmr or normal millet and 20 progenies of either tr or normal millets were collected and sequentially analysed for ashless NDF, ADF and lignin, as well as for organic matter (OM) and *in sacco* OM disappearance. Leaf from bmr millet had less lignin (31 versus 44 g/kg DM, standard error of the mean, sem = 2.0) and was more digestible than normal millet. Digestible OM (g/kg DM, i.e. D value) was 602 for bmr and 572 for normal millet (sem = 3.8). Similarly, millet bearing the bmr trait produced stems with less lignin (62 versus 87 g/kg DM, sem = 5.5) and a higher D value (479 versus 419 g/kg, sem = 4.9) than their normal counterparts. Although leaf yield (g DM/plant) was not different

between genotypes (88.8 g for bmr and 85.2 g for normal millet, sem = 3.9), bmr millet produced less stem than normal millet (85.6 versus 102.1 g DM/plant, sem = 4.4).

Pearl millet plants bearing the tr trait tended ($P = 0.09$) to produce more leaf (123.0 versus 95.3 g DM/plant, sem = 12.2) and stem (191.7 versus 152.4 g DM/plant, sem = 16.4) than their normal counterparts. No differences were observed in fibre constituents of leaves from both millet types, but tr millet leaves had a higher OM concentration (918 versus 902, sem = 3.8, $P < 0.01$) possibly owing to the chemical nature of the trichomes, and a higher OMD (670 versus 645 g/kg, sem = 6.1, $P < 0.01$) than normal millet leaves. Stems from tr millet had less ($P < 0.01$) NDF (667 versus 726 g/kg DM, sem = 17.6), ADF (463, versus 537 g/kg DM, sem = 19.8) and lignin (87 versus 103 g/kg, sem = 5.4) and a higher OMD (521 versus 441 g/kg, sem = 9.1) than stems from normal millet.

These results confirmed that the effects of the bmr trait on lignin and digestibility of residues from millet grown for grain production and harvested at advanced stages of maturity are similar to those found in forage millet. An increased residue quality of bmr genotypes could be obtained at the expense of decreased stem yield. They also suggest that the tr trait might improve the feed quality of millet stover without compromising stover yield.

In summary, in the semi-arid zone the differences in feeding value of the stover among sorghum and pearl millet varieties appear to be small and variable over the years. However, there are possibilities for introducing quality-related genes that can modify the composition of the fibre and increase digestibility. Further studies must evaluate the influence of such traits on the adaptability, grain yield and disease resistance of the crops. Preliminary data suggest that the bmr trait reduces grain yield and promotes lodging in pearl millet. However, these effects appear to vary across parent lines (A. Kumar, personal communication), which offer additional possibilities for selection.

Influence of genotype on feeding value of forage legumes

In collaboration with the International Institute for Tropical Agriculture (IITA) and ICRISAT, the differences in feed quality of hay from cowpea varieties grown as sole crop (20 entries) or inter-cropped with millet (16 entries) were studied.

Varieties grown as sole crops differed in leaf yield (range = 134 to 1115, sem = 145 kg DM/ha), stem yield (range = 157 to 1131, sem = 177 kg DM/ha) and litter leaf as proportion of leaf produced (range = 0.221 to 0.772, sem = 0.09). No differences were observed in OMD (mean = 720 g digestible OM/kg DM, $P > 0.48$) or in crude protein (mean = 197 g/kg DM, $P > 0.16$) of leaf. However, high variation among varieties was observed in OMD (range = 459 to 611, sem = 20 g/kg) and CP concentration of stems (range = 108 to 190, sem = 6.6 g/kg DM). Similar trends were observed in the study with varieties grown intercropped with millet, but in this experiment differences among entries were also observed for CP of leaf.

Similar on-going trials with groundnut varieties show large differences across varieties in feed value of the stem but little or no differences in feed quality of the leaf. In

summary, these studies suggest that the selection of cowpea and groundnut varieties for feed quality and yield of hay is promising. This may result in important income or production benefits, since these are the most important cash crops of the semi-arid zone and are used widely in the more intensive feeding systems.

Influence of genotype on feeding value of multipurpose trees

In a collaborative study with the International Center for Research in Agroforestry (ICRAF) the genetic variation in fodder quality of *Combretum aculeatum,* among other species, was studied in 60 half-sib families. Seeds were collected in a 100 km long and 20 km wide area along the Niger River. The collection zone was divided into provenances. From each provenance several half-sib families were collected, grown in the nursery and transplanted to the fields (J. Weber, personal communication).

During the dry season, the concentration (g/kg DM) of N in *C. aculeatum* foliage varied from 15.7 to 36.2 (mean = 22.4, SD = 3.1), that of phosphorus from 0.7 to 2.7 (mean = 1.5, SD = 0.4), and that of organic matter from 798 to 994 (mean = 918, SD = 29). Although the samples were taken in early physiological stages, the results suggest that *C. aculeatum* is a valuable source of protein for dry season feeding. Preliminary analyses suggested that variation exists among families of *C. aculeatum* for concentration of N, P and OM. Nitrogen concentration was positively correlated with that of P ($r = 0.41$, $P < 0.01$) and OM ($r = 0.25$, $P < 0.01$). Digestibility data are not yet available. Although preliminary, these results support the hypothesis that geographical areas that produce superior genotypes of trees could be selected for fodder yield and quality. Studies with other species of importance in the semi-arid zone are on-going.

Suggested activities for ILRI rumen ecology research

Based on the results of ILRI research summarised above, future ILRI rumen ecology work is planned:

1. To evaluate other *in vitro* methods (Odenyo et al 1991; Theodorou et al 1994) as procedures for early assessment of MPTs for negative effects.
2. To extract and partially purify anti-nutrients from MPTs. These semi-purified extracts will be used to isolate microbes that can degrade such substances.
3. To eventually identify MPTs that are not toxic to the rumen microbes and may be safely used as supplements when basal roughage diets are fed. Both classical and molecular methods will be used to evaluate effects of MPTs on rumen microbes.
4. To follow the microbial population dynamics according to seasonal variations.
5. To identify and characterise microbes from indigenous ruminants that effectively degrade fibre, and those that can tolerate or degrade anti-nutrients from fodder trees by classical and molecular methods. These microbes may be transferred to ruminants not adapted to such diets. It is also possible to manipulate these microbes using recombinant DNA technology to enhance fibre-degrading capabilities of already established rumen microbes.

A collaborative programme among ILRI, Australia and Indonesian scientists is planned. The work will focus mainly on isolation of tannin tolerant or degrading microbes from indigenous ruminants. The microbes will also be characterised genetically to make it possible to develop nucleic acid probes for future persistence studies in the new host.

Conclusion

Results of ILRI work summarised above strongly suggest that the focus of future work should include attempts to explain the role of rumen microbes in the utilisation of high fibre feeds supplemented with multipurpose trees. Furthermore it is important to explain the biochemical basis of the interactions between rumen microbes and the chemicals contained in MPTs, especially anti-nutritional factors. There is need to do more research on the seasonal variations of microbial populations in various ruminants across agro-ecological zones. Improving fibre digestion through genetic selection of crops and trees is another promising area of research.

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Potential application of rumen ecology manipulation to animal nutrition in developing countries

Nitrogen metabolism in the rumen: biotechnological problems and prospects

R.I. Mackie[1] *and M. Morrison*[2]

1. Department of Animal Sciences and Division of Nutritional Sciences
University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

2. Department of Animal Sciences and Center for Biotechnology, University of Nebraska-Lincoln, Lincoln, NE 68583-0908, USA

Abstract

Ruminant nutritionists have recognised the central importance of ammonia in nitrogen metabolism in the rumen for many years. This was emphasised in studies in which growth, reproduction and lactation were obtained from dairy cows on protein-free, purified diets and were thus solely dependent on the products of ruminal fermentation. The ruminant mode of digestion in which microbial fermentation precedes the hydrolytic digestive process allows ruminants to utilise dietary non-protein nitrogen efficiently, to up-grade low quality dietary protein to high quality microbial quality protein, and to survive on low intakes of dietary nitrogen by efficiently utilising nitrogen recycled to the rumen via the saliva. Since ruminal ammonia not utilised for microbial growth is absorbed through the ruminal walls and converted to urea in the liver, it follows that nitrogen economy of ruminant animals is dependent on proper balances of degradable and bypass protein.

If nitrogen supply to the rumen bacteria is inadequate, rumen function is affected in a number of ways. Digestion of starch and cellulose and other polysaccharides can be depressed. Bacterial synthetic effort can be diverted from protein to storage polysaccharide and adenosine triphosphate (ATP) can be diverted to uncoupled fermentation. Death and lysis of bacteria will probably be increased and the reduced growth rate means that a larger proportion of ATP is likely to be used for maintenance and less for protein synthesis. In the rumen ecosystem the rate and efficiency of microbial protein synthesis is determined by assimilation of the key enzymes of ammonia or the energy required for this process. The three primary enzymes of ammonia assimilation are glutamate dehydrogenase, glutamine synthetase and glutamate synthase and the synthesis and activity of these enzymes are central to the regulation of the intracellular nitrogen pool and the control of ammonia assimilation. These enzymes of ammonia assimilation play a critical role in the rate and efficiency of bacterial protein synthesis in the rumen. The recent biotechnology initiative on the genetic manipulation of the ruminal ecosystem has focused entirely on fibre degradation. The enzymes cloned from ruminal bacteria into *Escherichia coli* encode enzymes involved in the hydrolysis of plant cell wall material.

Introduction

Feedstuffs consumed by ruminants are all initially exposed to the fermentative activity in the rumen prior to gastric and intestinal digestion. Dietary polysaccharides and protein are generally degraded by the ruminal microorganisms into characteristic end products, which in turn provide nutrients for metabolism by the host animal. The extent and type of transformation of feedstuffs thus determines the productive performance of the host. Fermentation of feedstuffs in the rumen yields short-chain volatile fatty acids (VFA) (primarily acetic, propionic and butyric acids), carbon dioxide, methane, ammonia and occasionally lactic acid. Some of the change in free energy ($\Delta G^{0\prime}$) is used to drive microbial growth, but heat also is evolved. Ruminants use the organic acids and microbial protein as sources of energy and amino acids, respectively, but methane, heat and ammonia can cause a loss of energy and nitrogen (N). The quality and quantity of rumen fermentation products is dependent on the types and activities of the microorganisms in the rumen. This, in turn, will have an enormous potential impact on nutrient output and performance of ruminant animals. It is only with a thorough understanding of the mechanisms involved that this system can by successfully manipulated and fully exploited (Mackie and White 1990).

Ammonia plays a central role as an intermediate in the degradation and assimilation of dietary nitrogen by rumen bacteria. Ammonia is the major end-product of digestion of dietary protein and non-protein nitrogen (urea and amino acids) as well as the major source of nitrogen for synthesis by ruminal bacteria. A comparison of peptide and amino acid utilisation showed that peptides were more effectively incorporated into bacterial protein while a greater proportion of amino acids were fermented to VFA. Ruminal digestion results in the production of VFA and bacterial cells which are used as the major energy and protein sources, respectively, for metabolism by the host animal. As a result, nitrogen metabolism in the rumen is intimately related to the metabolism and utilisation of nitrogen by ruminant tissues. Growth and production in ruminants is dependent on bacterial protein synthesised in the rumen and ammonia is of central importance in this process. The integration of metabolism demands that consideration be given to protein and energy interactions in the rumen in order to achieve a balanced supply of nutrients at the duodenum. For the ruminant animal, the quantity of microbial plus undegraded dietary protein arriving at the duodenum has a great influence on productivity. The potential of enhancing the productivity and lean tissue growth of ruminants by growth promotants and hormone manipulations is determined by a balanced supply of protein and energy to the animal (MacRae and Lobley 1986).

Nitrogen metabolism in the rumen

Proteases, proteolysis and protein degradation

In ruminants, the amino acid requirements are provided by microbes synthesised in the rumen and from dietary protein that is not degraded in the rumen but is intestinally digestible (bypass or escape protein). A large but variable proportion (60% to 90%) of

the dietary protein is degraded by rumen microorganisms and it is the rate at which the different proteins can be hydrolysed that controls the extent of their degradation before they pass out of the rumen (Leng and Nolan 1984; Mackie and Kistner 1985; Tamminga 1979). This has an important influence on the proportions of undegraded dietary protein and microbial protein that are presented to the small intestine for digestion by the host animal. Furthermore, this forms the basis of all modern systems for evaluating and predicting protein utilisation by ruminants. Although much attention has been focused on physical and chemical methods of controlling the rate of protein degradation in the rumen, little research has been done on the factors influencing the proteolytic activity of ruminal bacteria despite the nutritional significance of this activity.

For ruminants receiving diets containing protein, the change in quantity and pattern of amino acids, which results from the conversion to microbial protein in the rumen, can be an advantage or a disadvantage, depending on the composition of the food protein. If the latter is of good quality, biological value is reduced because the microbial protein is of lower digestibility and is accompanied by nucleic acids. Under these conditions, it would be advantageous to limit degradation of dietary protein, provided this does not lead to a reduction of the microbial population and their activities, such as fibre digestion and VFA production (Smith 1979). However, proteolysis during ruminal fermentation may benefit the host animal if the microbial protein synthesised from the products is of higher biological value than the feed proteins (Tamminga 1979).

Recent work confirms that the proteolytic activity in the rumen is almost entirely associated with bacterial cells and that cell-free rumen fluid and protozoa have little activity toward soluble proteins (Nugent and Mangan 1981). However, the protozoa play an important role in the engulfment of bacteria and particulate matter and hence degradation of insoluble proteins (Coleman 1979). Proteolytic activity in the rumen is not confined to a single bacterium but is a variable property possessed by many different bacteria that may be active in the degradation of other feed constituents, mainly carbohydrates. Furthermore, the predominant proteolytic bacteria will differ depending on diet (Mackie 1982). Although extracellular enzymes are usually produced by Gram-positive bacteria, the most important protease-producing bacteria in the rumen are Gram-negative, including species of *Prevotella*, *Selenomonas*, and *Butyrivibrio* (Cotta and Hespell 1986). Other species, probably of less significance, are *Megasphaera elsdenii*, *Streptococcus bovis*, *Clostridium* spp., *Eubacterium* spp., *Lachnospira multiparus*, *Succinivibrio dextrinosolvens*, and the Spirochaetes (Allison 1970). Russell et al (1981) demonstrated that the Gram-positive *S. bovis* played a predominant role in ruminal proteolysis, especially on high concentrate diets.

Rumen microbial proteolytic activity has a broad optimum in the neutral range (Blackburn and Hobson 1960; Brock et al 1982; Kopecny and Wallace 1982). In general, rumen bacteria have a mixture of proteases based on the chemistry of their active site and the specificity of their hydrolytic activity (Brock et al 1982; Kopecny and Wallace 1982; Prins et al 1983; Wallace 1983; Wallace and Brammall 1985). This activity is predominantly of the cysteine protease type with large contributions from the serine- and metallo-protease type. Aspartic acid proteases were of minor importance in rumen bacteria. Protozoal proteases were mainly of the cysteine type and aspartic activity was

significant (Forsberg et al 1984). In contrast, the proteases of *Neocallimastix frontalis*, a prominent member of the anaerobic rumen fungi, had significant metallo-protease activity with trypsin-like specificity (Wallace and Joblin 1985).

Proteases have been studied in few pure cultures of rumen bacteria. *Prevotella ruminicola*, which can utilise peptide N instead of ammonia, had proteolytic activity in batch culture that was maximal and mostly (>90%) cell-associated during the midexponential phase of growth (Hazlewood and Edwards 1981; Hazlewood et al 1981). The proteolytic activity comprised a mixture of serine, cysteine and aspartic acid proteinases with the possibility that some of the activity is dependent on the presence of metal ions (Mg^{2+}). *Ruminobacter amylophilus* produces both cell-bound and cell-free proteolytic activity, which consistently amounts to 80 and 20%, respectively, of the total activity during exponential growth, but the proportion of cell-free activity increases in stationary phase cultures (Blackburn 1968). This cell-free activity may be truly extracellular or it might indicate that a proportion of bacteria are lysing during growth.

In contrast, proteolytic activity of *Butyrivibrio fibrisolvens* was essentially all extracellular, regardless of stage of growth. The number and approximate molecular weight (MW) of extracellular proteases produced by *Butyrivibrio fibrisolvens* H17c were determined by gelatin-PAGE. Nine bands of protease activity with apparent MW of approximately 101,000, 95,000, 87,000, 80,000, 76,000, 68,000, 63,000, 54,000 and 42,000 were found in supernatants from exponential phase sultures (Strydom et al 1986). The proteases were stable and survived unchanged in stationary phase cultures. The activity of all 10 exoproteases was optimal between pH 6.0 and 7.5 and a temperature of 55°C. The activities of all 10 protease fragments were inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Their activities were not affected by inhibitors of trypsin-like enzymes or metallo-, sulfhydryl- and carboxylproteases, thus confirming that they are all serine proteases. Production of the 10 exoproteases was not subject either to nitrogen or carbohydrate catabolite repression, and all 10 exoproteases were produced under conditions of nitrogen and carbohydrate excess. Production of serine exoproteases by *Butyrivibrio fibrisolvens* H17c did not require a specific inducer and exoproteases were constitutively produced in various media. Production of exoproteases was positively correlated with growth and would therefore be growth-rate dependent.

However, additional strains of these organisms and other proteolytic species require urgent examination before a clear understanding of the proteolytic activity in the rumen will be achieved.

Peptide transport and utilisation

Proteolysis results in oligopeptide production. These oligopeptides then undergo degradation to smaller peptides and amino acids. A comparison of peptide and amino acid used showed that peptides were more effectively incorporated into bacterial protein while a greater proportion of amino acids were fermented to VFA (Cotta and Hespell 1986; Wright 1967). Thus, free amino acids are not incorporated into microbial protein per se but undergo rapid deamination providing ammonia for bacterial growth. The

deamination and degradation of specific amino acids are of special relevance to bacterial growth in the rumen. The most important of these is the conversion of leucine, isoleucine and valine to isovalerate, 2-methylbutyrate and isobutyrate, respectively. These branched-chain fatty acids are either required or highly stimulatory to the growth of many ruminal bacteria, particularly the fibrolytic species (Bryant and Robinson 1962, 1963).

It has been well documented that many rumen bacteria can utilise peptides as a source of amino acids; for several bacterial systems, peptide transport into the cell followed by intracellular cleavage has been demonstrated. Because transport mechanisms for free acids appear to be absent in many rumen bacteria, the transport of peptides into the cell represents an important means of obtaining N for the synthesis of cellular components, especially those lacking the ability to utilise ammonia. Pittman and Bryant (1964) showed that *Prevotella ruminicola* was able to utilise peptide and ammonia N but not amino N for growth. In further studies, Pittman et al (1967) showed that the same strain of *Prevotella ruminicola* did not transport [^{14}C]proline or glutamic acid and very little valine, whereas peptides of MW up to 2000 were transported. Russell (1983) also showed that the addition of Trypticase to cultures of *Prevotella ruminicola* B_14 significantly improved growth yields, although peptides alone could not support the growth of this organism. However, the actual transport of peptides by rumen bacteria has only been demonstrated using a radiolabelling technique that is unable to distinguish between possible extracellular hydrolysis of labelled proteins, or possible efflux of labelled amino acids from the intracellular pool.

Studies on the transport of peptides of varying chain length and amino acid composition were carried out with *Streptococcus bovis* using a dansylation procedure (Westlake and Mackie 1990; Westlake et al 1987). Under these conditions the transport of the majority of components of Trypticase, Leu-Trp-Met-Arg-Phe, and Phe-Arg has been demonstrated, although the defined structure peptides were completely hydrolysed by extracellular peptidases to smaller units before transport. The pentapeptide was hydrolysed to eight different components in the transport buffer and all were present within the cell after 2 min of incubation. The Phe-Arg was hydrolysed extracellularly to phenylalanine and a second compound, but not alanine. However, both alanine and phenylalanine were present within the cell after 2 min of incubation. These studies emphasise the limitations in the use of ^{14}C-labelled substrates in transport experiments and the need to account for possible extracellular hydrolysis of labelled compounds, which in the case of *Streptococcus bovis* appears to be considerable. *Streptococcus bovis*, a Gram-positive organism, has been shown to play a major role in ruminal proteolysis on high concentrate diets (Russell et al 1981).

Ammonia assimilation in rumen bacteria

Depending upon the diet, 60–90% of the daily nitrogen intake by the ruminant is converted to ammonia and 60–80% of bacterial N is derived from ammonia, with the balance derived from N-containing compounds (peptides mainly) which do not equilibrate with the NH_3-N pool (Pilgrim et al 1970; Mathison and Milligan 1971; Nolan and

Leng 1972). Bryant and Robinson (1961, 1962, 1963) found that 92% of ruminal bacterial isolates could utilise ammonia as the main source of N, while it was essential for the growth of 25% of all isolates tested. Little amino acid-nitrogen is available for microbial growth and amino acids are rapidly deaminated to ammonia, VFA and CO_2 before being utilised for microbial protein synthesis. The deamination and degradation of specific amino acids are of special relevance to bacterial growth in the rumen. The most important of these is the conversion of leucine, isoleucine and valine to isovalerate, 2-methylbutyrate and isobutyrate, respectively. These branched-chain fatty acids are either required or highly stimulatory to the growth of many ruminal bacteria, particularly the fibrolytic species (Bryant and Robinson 1961, 1962, 1963). Although our knowledge about other mammalian gastrointestinal ecosystems is not as extensive or definitive as that of the rumen, results indicate that ammonia is the major N source supporting bacterial growth in the cecum and colon of pigs, horses and humans (Allison et al 1979; Herbeck and Bryant 1974; Maczulak et al 1985; Robinson et al 1981; Takahashi et al 1980; Varel and Bryant 1974; Wozny et al 1977). Thus, the rumen provides an ideal model for the study of these processes since it has been extensively characterised. Ammonia produced in the rumen that is not incorporated into microbial cells is largely absorbed through the wall of the reticulo-rumen and converted into urea by the liver. This is excreted in the urine leading to environmental pollution in the vicinity of intensive animal production operations. Between 60 and 75% of the N in excreted manure is converted into NH_3, of which 25 to 40% is lost during storage, and an additional 20 to 60% is lost during spreading (Tamminga and Verstegen 1992).

The concentration of ammonia in the rumen varies widely ranging from 2–40 mM. Satter and Slyter (1974) found that when the concentration of NH_3-N is lower than 3.5 mM, microbial growth decreased significantly. Levels of ammonia in dairy cows were found to range from 7–13.5 mM (Wohlt et al 1976). Thus, under adequate feeding regimens, prevailing ammonia concentrations should always be adequate for optimal growth of rumen bacteria. Earlier research with sheep fed low-protein (2.9% CP) *Eragrostis tef* hay showed NH_3-N concentrations of 1.8 mM immediately after feeding which went down to 0.05 mM 8 h after feeding (Schwartz and Gilchrist, Internal Progress Report, Digestion and Metabolism in the Ruminant Unit, Veterinary Research Institute, Onderstepoort, South Africa). Grazing ruminants could be expected to have similar low ammonia levels during winter or dry periods not only in the USA but elsewhere in the world. It is worth noting that most of the world's ruminant population live and produce under grazing conditions that exist in the subtropics and tropics (Gilchrist and Mackie 1984). When environmental ammonia concentrations are greater than 1 mM, the glutamate dehydrogenase pathway of ammonia assimilation predominates, whereas when concentrations of ammonia are less than 0.5 mM the high affinity glutamine synthetase pathway is more important. Schaefer et al (1980) reported ammonia saturation constants for 10 species of predominant ruminal bacteria which ranged from 5 to 50 uM which suggests that these organisms could attain 95% of their maximal growth rate in the presence of 1.0 mM ammonia and an unlimited energy supply. Although many species of ruminal bacteria are efficient scavengers of ammonia, both pathways may be used simultaneously in different microorganisms. Thus, irrespective

of the prevailing environmental ammonia concentration, enzymes of ammonia assimilation are critical in procuring this essential nutrient for bacterial cell growth.

Theoretically, optimal ruminal NH_3 concentrations required for maximal microbial growth are in the range of 2–3 mM; a concentration below 1 mM is limiting. However, this assumes that there is no other limiting nutrient and is an oversimplification since many other factors need to be considered. Thus bacteria adherent on surfaces may be exposed to environmental NH_3 concentrations that are less than optimal. Additionally, NH_3 concentrations that facilitate maximal levels of fibre degradation may be lower than those that maximise microbial protein synthesis and feed intake. It is also possible that, in grazing ruminants consuming low protein forages where ruminal microbes are dependent mainly on recycled NPN sources, fermentation uncoupled from bacterial growth may occur. This area of research needs further focus.

Enzymes of ammonia assimilation

Since ammonia is the preferred source of N for most rumen bacteria, enzymes of ammonia assimilation are essential to the growth of most rumen microorganisms. Glutamate dehydrogenase (GDH) and the dual enzyme system glutamine synthetase (GS) and glutamate synthase (GOGAT) are the two most important routes by which ammonia may be assimilated.

Glutamate dehydrogenase (GDH; EC 1.4.1.2 and 1.4.1.4) catalyses the following reaction:

$$\text{2-Ketoglutarate} + NH_3 + NAD(P)H + H^+ \overset{\text{GDH}}{\leftarrow \rightarrow} \text{Glutamate} + NAD(P)^+$$

High levels of both NADH- and NADPH-linked GDH activity have been observed in ruminal contents (Chalupa et al 1970) and in continuous culture of mixed ruminal bacteria (Erfle et al 1977). NADH-linked GDH has been reported in *Ruminococcus albus* and *Megasphaera elsdenii,* whereas most other ruminal bacteria possess NADPH-linked activity (Joyner and Baldwin 1966). *Selenomonas ruminantium* was shown to possess both types of activity in a relatively constant activity ratio (0.25–0.35) under both glucose- and ammonia-limited growth conditions (Smith et al 1980). In general, NADP-linked GDH is assumed to have a biosynthetic role and functions efficiently at high ammonia concentrations as evidenced by its high K_m for ammonia. The NAD-linked enzyme has a catabolic function associated with limiting levels of ammonia and growth on amino acids (glutamate) (Brown 1980; Dalton 1979). Smith et al (1980) showed that NADP-linked GDH activity was optimal at *ca* 400 mM monovalent salt concentration (Na^+, K^+ or NH_4^+) in *S. ruminantium.* Apparent K_m values for this enzyme of 6.7 and 23 mM were estimated from a double-reciprocal plot of the enzyme activity as a function of NH_4Cl concentration. It is also of interest that GDH activity in *Bacteroides thetaiotaomicron* was influenced by salt concentration. NADP-linked activity increased by 73% and NAD-linked activity decreased by 50% in the presence of 0.1 M NaCl (Glass and Hylemon 1980).

The second major pathway for ammonia assimilation in ruminal bacteria is mediated by the concerted action of two enzymes. Glutamine synthetase (GS; EC 6.3.1.2) fixes ammonia in the amide of glutamine by an ATP-dependent reaction. Then glutamate

synthase (GOGAT; EC 1.4.1.13), the second enzyme of this system, catalyses the reductive transfer of the glutamine amide to 2-ketoglutarate (Meers et al 1970).

$$\text{Glutamate} + \text{ATP} + NH_3 \xrightarrow{\text{GS}} \text{Glutamine} + \text{ADP} + \text{P}$$

$$\text{2-Ketoglutarate} + \text{Glutamine} + \text{NADPH} + H^+ \xrightarrow{\text{GOGAT}} 2\ \text{Glutamate} + NADP^+$$

Glutamine synthetase activity can be assayed by several different methods (Hespell 1984) which makes the observation of GS activity in whole rumen contents (Chalupa et al 1970) and during continuous culture of mixed ruminal bacteria (Erfle et al 1977) difficult to interpret since each organism has different conditions for optimal enzyme activity. In *S. ruminantium,* GS activity was measured using the biosynthetic assay since γ-glutamyltransferase activity was absent (Smith et al 1980). Furthermore, GS activity was not under covalent control by the adenylylation-deadenylylation reaction and feedback inhibition commonly found in other bacteria. In contrast, both *Ruminobacter (Bacteroides) amylophilus* (Jenkinson et al 1979) and *Succinivibrio dextrinosolvens* (Patterson and Hespell 1985) contain γ-glutamyltransferase activity which was under control of adenylylation-deadenylylation *Escherichia coli* type of regulation for GS.

Glutamate synthase activity has been demonstrated in only one strain of the gut anaerobe *S. ruminantium* strain D (Smith et al 1981) and was unusual in that neither NADPH or NADH were suitable as electron donors for the reaction. The enzyme was reductant-dependent and used dithionite-reduced methyl viologen as the electron carrier system in the assay. Thus some unidentified low-potential electron carrier is required and may help to explain problems involved in measuring GOGAT activities in other species of rumen bacteria.

The Ruminococci are mesophilic, Gram-positive bacteria which play an important role in ruminal cellulose digestion. *Ruminococcus albus* and *R. flavefaciens* are among the predominant cellulolytic bacteria isolated from the rumen of animals fed a variety of diets and ammonia is essential for their growth since they are unable to utilise nitrogen from amino acids or peptides (Bryant and Robinson 1961, 1962, 1963). Reports of GDH activity in Ruminococci are scarce. Joyner and Baldwin (1966) reported the presence of NADH-linked GDH activity in *R. albus.* This was confirmed in *R. albus 7* grown under glucose limitation in chemostat (Kistner and Kotze 1973). Cell associated NADP-linked GDH activity in *R. flavefaciens* 67 was approximately three-fold higher in cells grown under ammonia limitation and was independent of growth rate (D = 0.06–0.15 h^{-1}) (Pettipher and Latham 1979).

We have focused our studies on ammonia assimilation by *R. flavefaciens* FD-1 in both batch and continuous culture (Duncan and Mackie 1989; Duncan et al 1991, 1992). Initially the principal routes of ammonia assimilation and the activities of the corresponding enzymes were examined. Results from batch culture confirm that GS and GDH are active routes of NH_3 assimilation and that their relative activities depend on the concentration of NH_3 in the growth medium. GS activity increased under N-limiting conditions but activity was inhibited 50% by NH_3 shocking. The adenylylation form of

GS modification common in many bacteria (Magasanik 1982; Merrick 1988) does not occur in *R. flavefaciens* (Duncan et al 1992).

All GS and most of the GDH activity present in whole cells was sedimented by ultracentrifugation (328,000 × g; 37 min; 4°C). Streicher and Tyler (1980) reported a rapid purification of GS from a variety of bacteria based on differential centrifugation which depends on the integrity of the GS-DNA complex in cell extracts. However, in contrast to their findings, prior treatment of the cell extracts of *R. flavefaciens* with DNAse or RNAse had no effect on sedimentation. However, harvesting and fractionation of cells in the presence of a detergent (CTAB) reduced the amount of sedimentable activity of both GS and GDH and the balance was recovered in the supernatant. This provides preliminary evidence that GS and some of the GDH activity may be membrane-associated. The nature of the interaction with the membrane fraction is being studied using different detergents and salt washes combined with PAGE and activity gel assays to locate activity of GS and GDH.

Reaction kinetics of NADP-linked GDH were assayed in cell fractions (membrane pellet and supernatant) to address the possible existence of multiple forms of GDH with different physiological roles and kinetic characteristics. Michaelis-Menten kinetics were demonstrated and Lineweaver-Burk plots were linear for the substrate concentration range (1–50 mM). The affinity for ammonia was higher in the membrane pellet fraction (K_m = 14.2mM) than supernatant (K_m = 47.9 mM). Further research is required to determine if these are different enzymes or different forms (isozymes) of the same enzyme.

Glutamate dehydrogenase (L-glutamate: $NADP^+$oxidoreductase, deaminating, E.C. 1.4.1.4; GDH) from *R. flavefaciens* FD-1 has been purified and characterised in our laboratory (Duncan et al 1992). The native enzyme and subunits are 280 and 48 kDa, respectively, suggesting that the native enzyme is a hexamer. This is characteristic of bacterial and fungal NADP-linked GDHs (Britton et al 1992). The enzyme requires 0.5 M KCl for optimal activity, and has a pH optimum of 6.9–7.0. The K_m's for ammonia, α-ketoglutarate and glutamate are 19, 0.41 and 62 mM, respectively. The sigmoidal NADPH saturation curve revealed positive cooperativity for the binding of this coenzyme. The first residue in the N-terminal sequence was alanine, suggesting that the protein may be modified post-translationally. Comparison of the N-terminal sequence with those of *E. coli*, *S. typhimurium* and *Clostridium symbiosum* revealed only 39% amino acid homologies. Sequence comparisons for GDHs from a diverse range of sources show that the hexameric enzymes are structurally similar. However, the similarity is poor for the N-terminal 50 residues and then rises significantly with long stretches of highly similar sequences over the next 350 residues (Britton et al 1992).

Regulation of ammonia assimilation in bacteria

A thorough understanding of the process of ammonia assimilation by bacteria requires identification of the possible pathways of assimilation for different N sources in different organisms and characterisation of the enzymes involved in these pathways. This knowledge must then be integrated with information on the physiological state of

cells growing under different conditions. Finally, a genetic analysis is required both of the structural genes which encode the enzymes of ammonia assimilation and of the regulatory genes which are essential in the control and coordination of the level of these enzymes in the cell. Such a complete description has been achieved in very few systems but the most comprehensive description available thus far is in the enteric bacteria, namely *E. coli, S. typhimurium, Klebsiella pneumoniae* and *K. aerogenes* (Kustu et al 1986; Magasanik 1982, 1988; Merrick 1988). However, in rumen bacteria we have no information on the mechanisms controlling expression of the primary ammonia assimilation enzymes (GS, GOGAT and GDH). Rapid developments in these systems can be expected due to the availability of gene probes and promoter sequence information from the enterics.

Of the three primary enzymes of ammonia assimilation only GS expression is markedly regulated in response to changes in the availability of ammonia. GS from enteric bacteria is comprised of 12 identical subunits each with 50 kDa molecular weight. In *E. coli,* GS activity is regulated by covalent modification involving reversible adenylylation of a specific tyrosine residue on each subunit. Maximum biosynthetic activity is obtained when the enzyme is completely unadenylated. The adenylation of GS is carried out by an adenylyl transferase (ATase) encoded by the *glnE* gene. ATase can act in reverse to deadenylate GS and the activity of ATase is determined by its interaction with a small regulatory protein P_{II}. If ammonia is abundant, ATase acts to adenylylate GS and reduces its activity, while under ammonia limitation GS is deadenylated by ATase. The regulatory protein P_{II} is the product of the *glnB* gene. The unmodified P_{II} stimulates adenylylation of GS by ATase while uridylylated P_{II} enhances the deadenylylation reaction. The covalent attachment of a UMP residue to P_{II} is caused by uridylyl transferase (UTase), the product of the *glnD* gene. Regulation of GS activity is therefore achieved by a complex cascade of events which are ultimately controlled by the relative levels of glutamine and 2-ketoglutarate inside the cell. A high glutamine:2-ketoglutarate ratio causes UTase to deuridylylate P_{II} which in turn stimulates adenylylation of GS by ATase. The reverse sequence occurs when the ratio is low. GS levels are also regulated transcriptionally such that in ammonia-limited cultures, the level of GS is higher than that measured in cells grown with excess ammonia. The system which mediates this control is known as the nitrogen regulation *(ntr)* system. The gene which encodes GS *(glnA)* has been cloned from the enterics as well as several other bacteria (Merrick 1988; Southern et al 1986; Usdin et al 1986).

In contrast GOGAT and GDH are apparently subject to far less control and even in the enterics, the precise nature of their regulation is presently unknown (Merrick 1988). However, recent research on the *nac* gene suggests a second tier of nitrogen regulation which intercedes between *ntr* and nitrogen-controlled systems including GOGAT and GDH. It is possible that *nac* is specifically involved in the regulation of systems which can provide the cell with carbon as well as nitrogen (Bender 1991). Cloning and sequencing of *nac* and the analysis of promoters under *nac* control have recently been published (Maculuso et al 1990). This type of system may be important in rumen bacteria where catabolite regulatory mechanisms appear to be involved in regulation (Russell and Baldwin 1978; Russell et al 1990).

Bacterial ammonium transport

With few exceptions, the metabolism of a compound starts with its transport across the cell membrane, mediated in most cases by specific transport proteins or carriers. This uptake is frequently strictly regulated. However, regulation of transport is almost impossible if a compound crosses the membrane by non-specific diffusion. With respect to bacterial membranes, there is ample evidence that transport systems for most ions and large polar molecules (M_r approx >100) exist. If the cell membrane was not basically impermeable, it would be difficult for cells to retain ionic nutrients as these could also diffuse out into the surrounding dilute aqueous environment. However, the exception may be those ions that are in equilibrium with a non-ionic, relatively lipid soluble form such as the NH_4^+-NH_3 pair. The important question is which form of ammonia passes across the membrane, since the pKa for the dissociation

$$NH_4^+ \leftrightarrow NH_3 + H^+$$

is 9.25 at 24°C. It is obvious that at physiological pH, the vast bulk of substrate is present as NH_4^+. However, it is possible that under conditions of high substrate repression and inhibition of scavenging active transport systems, passive diffusion across the cell membrane could still occur. A case could also be made for a facilitated diffusion mechanism, although net flux would only occur if the solute passes down a concentration gradient.

The occurrence of NH_4^+ transport systems is widely distributed amongst many species of bacteria with varying physiology and ecology. In general, the affinity of the NH_4^+ carriers is high, with K_m values of 5–50 μM (Kleiner 1985). Most of the ammonium transport systems have been detected with $^{14}CH_3NH_3^+$. With the exception of the cyanobacteria, the NH_4^+ gradient is similar to the methylamine gradient and ranges from 50 to 200 fold. The cyanobacteria are able to generate NH_4^+ gradients as high as 3000 fold (Boussiba et al 1984). Another important feature apart from concentrative uptake is energy dependence. Energy dependent NH_4^+ transport has been inferred from studies which demonstrate dependence on an energy source such as ATP, inhibition by inhibitors of energy metabolism, and inhibition by compounds which decrease PMF. Taken together, the available evidence favours a component of the PMF, the membrane potential ($\Delta\Psi$), as the driving force for NH_4^+ transport in most bacterial carriers (Kleiner 1985).

The synthesis of the bacterial NH_4^+ carriers is repressed when grown in media with high levels of NH_4^+. This is similar to the high affinity GS enzyme system for ammonia assimilation described earlier. These enzymes are under the nitrogen control *(ntr)* regulatory system (Magasanik 1982; Merrick 1988) which comprises three regulatory genes *ntrA* (or *glnF), ntrB (glnL)* and *ntrC (glnG).* The first indication that the *ntr* system may also regulate ammonium transport (Amt) was derived from a regulatory mutant of *Klebsiella pneumoniae* (Kleiner 1982) which was unable to transport methylamine. This mutant was also defective in glutamine synthetase (Gln^-), nitrogenase (Nif^-) and histidine utilisation (Hut^-). A more detailed study carried out with *E. coli* strains containing mutations in different *ntr* genes showed that both the *ntr A (glnF)* and the *ntrC (glnG)* gene products were required to activate synthesis of the NH_4^+ carrier, while

the *ntrB (glnL)* gene product played a role in its repression (Servin-Gonzalez and Bastarrachea 1984). Similar effects were observed on the synthesis of GS so that the Gln^- phenotype also was always Amt^-.

Under conditions of derepression (synthesis) of the NH_4^+ carrier, bacteria generally assimilate NH_4^+ via the GS/GOGAT pathway (Magasanik 1982; Merrick 1988). The putative regulator of NH_4^+ transport is thought to be glutamine since intracellular levels respond rapidly to external NH_4^+ concentrations (Kleiner 1976, 1979; Kleinschmidt and Kleiner 1981). It serves as a feedback inhibitor of several enzymes strongly affecting the modification of GS (Kleiner et al 1981; Tyler 1978) and acts as a key regulator of the *ntr* system (Magasanik 1982; Merrick 1988) . Recently, the regulatory effect of glutamine on Amt was studied in *glnL* mutants of *E. coli* which constitutively express Amt (Castroph and Kleiner 1984; Jayakumar et al 1987). These results showed that Amt was regulated by the internal glutamine pool via feedback inhibition. Both $CH_3NH_3^+$ and NH_4^+ transport of many bacterial strains is strongly inhibited by methionine sulfoximine, a potent inhibitor of GS, indicating the existence of a regulatory glutamine binding site at the carrier (Kleiner 1985). Recent cloning and sequencing of the *E. coli amtA* gene, which codes for one of the soluble components of the ammonium transport system, should lead to rapid progress in the field (Fabiny et al 1991).

However, repeated attempts in our laboratory to demonstrate active transport of NH_4^+ using $^{14}CH_3NH_3^+$ as the probe have been unsuccessful in *Ruminococcus flavefaciens* FD-1. These experiments involved cells grown under N-limitation from both batch and chemostat grown cultures (P.A. Duncan and R.I. Mackie, unpublished results). Validation of the transport assay showed that *R. flavefaciens* exhibited energy dependent, concentrative uptake of ^{14}C-labelled 2-deoxyglucose. Further experiments with $^{14}CH_3NH_3^+$ showed that *E. coli* actively transported this ammonium probe. We have had most success monitoring NH_4^+ depletion from the external medium over time in 30-min assays with *R. flavefaciens* but this technique lacks sensitivity and is unsuitable for determining bioenergetic aspects of ammonium transport. However, initial rates of ammonia depletion were clearly different between cells grown under ammonium-limited as compared to carbon-limited growth (11.2 versus 0.8 nmol/min/mg cell protein, respectively). This is consistent with the higher specific activity or GDH and GS found for this organism when grown under ammonium limitation.

Biotechnological problems and prospects

Protein and peptide catabolism

Protein and peptide metabolism have been studied extensively and proteolytic enzymes from bacteria, fungi and protozoa have been demonstrated. However, our knowledge and understanding of the biochemistry and regulation of proteinase, peptidase and deaminase enzymes which are involved in the sequential catabolism of protein in the rumen is superficial. This process is complicated by the wide distribution of these activities between the different microbial groups and also that activities are predominantly cell associated. Much attention has been focussed on the association of rumen

microbes with plant surfaces and cereal grains mainly from the standpoint of fibre degradation or carbohydrate utilisation but not from the protein utilisation and degradation standpoint. Similarly, our understanding of the molecular basis for recognition and adhesion to specific sites remains largely unknown although these studies have been initiated in relation to cellulose binding by fibre degrading bacteria. This microbial association with surfaces is a logical ecological adaptation and could possibly be exploited to manipulate the kinetics of protein degradation in the rumen.

Quantitative estimates of intraruminal recycling indicate that as much as 30 to 50% of the daily N intake may be recycled. These values may be underestimates since turnover of peptide-N independent of the NH_3-N pool may not be accounted for by this technique. Further studies of this nature are warranted under a variety of feeding conditions which are known to provide different ruminal microbial populations. An obvious target is protozoal activity which is thought to be a major contributing factor to intraruminal N recycling. Defaunation invariably results in improved protein flow from the rumen although most protocols for this procedure are suitable experimentally but not practically. Novel approaches to control protozoal activity using the 'smugglin' concept, secretion of antiprotozoal antibodies or delivery of such compounds by bacteria specifically engineered for this purpose using recombinant DNA technology are potential methods. Further ecological studies which describe bacterial and fungal population changes accompanying defaunation are an integral component of this approach.

It has long been recognised that a diverse population of bacteriophage is maintained in the rumen and some research has been carried out on this neglected group of microbes. However, our knowledge of their biology and molecular bases of infection, lysogeny and lysis are limited. Studies on quantitating phage DNA to estimate fluctuations in phage populations will provide useful information relating to intraruminal nitrogen recycling and also to bacterial population changes associated with phage blooms. Bacteria also produce a range of antibacterial compounds which are active in the gastrointestinal tract. The first report of a bacteriocin-like compound produced by *Ruminococcus albus* 8 has recently been published. Documentation of these intimate relationships is dependent on utilisation of modern molecular ecology techniques.

Although methods exist for controlling protein degradation, most of these strategies are applicable to dietary supplements and intensive production systems but probably have little current application to extensive, forage-based production systems. Ionophores such as monensin increase peptide and amino-N flow from the rumen but its inhibitory spectrum is probably too broad for application in grazing and forage fed ruminants. Pretreatment of protein sources, and more recently amino acids, have provided improvements in protein supply to the animal but are expensive and require increased management inputs. The interaction between dietary tannins, particularly in browse species, and protein requires further study. Most improvements with high tannin levels have been documented in temperate, highly degradable forages where protein degradation is retarded by this association.

Future research efforts should focus on highly selective and specific methods for inhibiting microorganisms responsible for proteinase, peptidase and deaminase activity in the rumen.

Conclusions

Two quotes from pioneers in the field of rumen microbial ecology and metabolism are still pertinent today. In 1960, Hungate proposed that an analysis of an ecological habit required elaboration of the numbers and kinds of microorganisms present, an analysis of the activities of these microbes *(in vitro),* and finally examination of the extent to which these activities are expressed *(in vivo).* Allison (1970) concluded his review on nitrogen metabolism of ruminal microorganisms as follows: 'Exciting possibilities for improving the nitrogen economy of the ruminant may be ahead if measures for protecting protein and amino acids from degradation in the rumen are perfected. It may be, however, that more will be done towards alleviation of the protein shortages in the world by imaginative exploitation of the ability of ruminants to employ ruminal microbes for synthesis of good quality protein from non-protein nitrogen.'

Modern analytical, biochemical and molecular techniques applied at both the organismal and whole animal level will afford scientists an excellent opportunity to provide the first complete description of this system and not only an extension of our knowledge and understanding. Further improvements in ruminant nitrogen metabolism will require innovative approaches to manipulate the degradative and bisynthetic activities of the rumen microorganisms.

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Antimicrobial factors in African multipurpose trees

S.M. El Hassan[1,2], *A. Lahlou-Kassi*[1], *C.J. Newbold*[2] *and R.J. Wallace*[2]

1. International Livestock Research Institute, P.O. Box 5689, Addis Ababa, Ethiopia

2. Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, UK

Abstract

Samples and extracts of foliage from African multipurpose trees (MPT) were screened for their effects on rumen protozoa and bacteria. The MPT species tested were *Acacia aneura, Acacia angustissima, Chamaecytisus palmensis, Brachychiton populneum, Flindersia maculosa, Sesbania sesban, Leucaena leucocephala* and *Vernonia amyedalina. S. sesban* was highly toxic to rumen protozoa from sheep reared in Aberdeen. The toxic factor was associated with the saponin-containing fraction of the plant. *S. sesban* may therefore be useful in suppressing protozoa and thereby improving protein flow from the rumen; however, protozoa from the rumen of sheep raised in Ethiopia were resistant to *S. sesban*, indicating that resistance, either endogenous or acquired, may be a problem in exploiting this antiprotozoal effect. The *Acacia* species were toxic to rumen bacteria, particularly the cellulolytic species of *Ruminococcus.* The cause of this toxicity was not identified.

Introduction

In order to meet the increasing demand for animal products in Africa, it is necessary to enhance the utilisation of available fodder and agricultural byproducts and also to look for alternative feed resources. Multipurpose trees (MPT) and shrubs may help in this respect and in addition may provide other benefits, in terms of increasing soil fertility by nitrogen fixation (Woodward and Reed 1989), providing green manure and mulch (McKell 1980), protecting soil from erosion by stabilising surface soil, control of desert encroachment (McKell 1980; Woodward and Reed 1989) and providing fuel and shelter (McKell 1980; Woodward 1988). MPT are a rich source of protein, carotenoid precursors of vitamin A and many minerals, but generally have a low phosphorus content (Le Houérou 1980; Kumar and Vaithiyanathon 1990; Olasson and Welin-Berger 1991). In many areas of Africa they provide an available supplement to grasses in the dry season (Le Houérou 1980), lessening the dependence on expensive protein supplements. However, the presence of antinutritional compounds in MPT limits their utilisation as feedstuffs.

Plants produce a large number of chemicals, arbitrarily categorised as primary or secondary metabolites, that are essentially defence mechanisms for the plant against animal and insect predators and microbial infection (Deshhande et al 1986; Feeny

1970). Several deleterious compounds have been identified in forages and fodder trees, including alkaloids, non-protein amino acids, mycotoxins, terpenoids, steroids, lectins and protease inhibitors (Cheeke and Shull 1985; Lowry 1990; D'Mello 1992). Many of the African browse species contain high levels of polyphenolics and insoluble condensed tannins (proanthocyanidins) that bind to neutral detergent fibre (El Hassan 1994; Reed 1986; Reed et al 1990; Woodward and Reed 1989). Some phenolic compounds are toxic to rumen bacteria (Akin 1982; Borneman et al 1986; Chesson et al 1982; Martin and Akin 1988), rumen fungi (Akin and Rigsby 1987) and rumen protozoa (Akin 1982).

Oxalate and the alkaloid perloline inhibited cellulose degradation by the rumen microbial population (Hemken et al 1984; James et al 1967). Thus MPT may have components that are toxic to the micro-organisms of the rumen as well as to the host animal.

The aim of the work described here was to determine if seven potentially useful MPT contained factors that were antimicrobial and therefore detrimental to rumen fermentation. One particular MPT, *A. aneura*, proved toxic to rumen bacteria and therefore inhibitory; however, *S. sesban* contained an antiprotozoal factor that may be useful in enhancing rumen productivity by suppressing wasteful breakdown of bacterial protein by rumen protozoa.

Antiprotozoal effects of the foliage of multipurpose trees

Protozoa are responsible for over 90% of bacterial protein turnover in the rumen (Wallace and McPherson 1987). Thus they cause a major decrease in the microbial protein flowing from the rumen (Weller and Pilgrim 1974). The degradation of labelled bacterial protein by protozoa can be used as a measure of protozoal activity (Wallace and McPherson 1987). This method was used here as an assay of the antiprotozoal activity of MPT, using samples of rumen fluid taken from sheep in Aberdeen, UK, which received a mixed grass hay/concentrate diet, and from sheep in Debre Zeit, Ethiopia, receiving teff straw.

Effects of MPT on protozoa from sheep in Aberdeen

The breakdown of the rumen bacterium *Selenomonas ruminantium*, in the presence and absence of added MPT, was measured in rumen fluid taken from sheep in Aberdeen (Table 1). The rate of breakdown of *S. ruminantium* was measured on different samples of rumen fluid, each with a slightly different protozoal population, thus accounting for the variation in the rate of breakdown in the absence of MPT, which ranged from 7.3 to 13.3% h^{-1}. All of the MPT caused a progressive decrease in the observed rate of release of ^{14}C-leucine from *S. ruminantium*, most likely due to a physical interference with the predatory activity of the protozoa as well as a degree of toxicity. However, the effect with *S. sesban* was particularly pronounced, causing a 59% decrease at 6 mg ml^{-1} and complete abolition of predatory activity at 24 mg ml^{-1} (Table 1).

Table 1. *Influence of MPT on breakdown of* ^{14}*C-labelled* S. ruminantium *in rumen liquor taken from sheep in Aberdeen.*

MPT	Rate of degradation (% h^{-1}) at inclusion level of †				
	0 mg ml^{-1}	6 mg ml^{-1}	12 mg ml^{-1}	24 mg ml^{-1}	SED
Acacia aneura	8.6^{a}	7.5^{ab}	6.8^{ab}	4.6^{b}	1.80
Brachychiton populneum	11.7^{a}	10.1^{ab}	8.8^{bc}	7.5^{c}	0.96
Chamaecytisus palmensis	7.3^{a}	6.4^{ab}	5.6^{bc}	4.3^{c}	0.72
Flindersia maculosa	13.3^{a}	12.7^{a}	11.6^{a}	9.4^{b}	0.84
Leucaena leucocephala	7.8^{a}	6.9^{a}	6.3^{a}	3.7^{b}	0.90
Sesbania sesban	11.4^{a}	4.7^{b}	0.2^{c}	0^{c}	1.14
Vernonia amyedalina	8.6^{a}	7.6^{a}	6.2^{a}	3.9^{b}	1.03

† The balance was made up by wheat straw to give a total substrate concentration of 40 mg ml^{-1}.
$^{a, b, c}$ Values with different superscripts are different ($P < 0.05$).

Identification of the active component of *S. sesban*

All of the MPT species contained phenolics (El Hassan 1994), so the possibility that tannins or phenolics were responsible for the antiprotozoal effect of *S. sesban* was tested.

Tannins can be removed from solution by polyethylene glycol or polyvinyl pyrrolidine (PVP; Garrido et al 1991). When PVP was added to the incubation containing *S. sesban* and ^{14}C-labelled *S. ruminantium*, the precipitation of tannins had no effect on the antiprotozoal property of *S. sesban* (Figure 1). Thus it was concluded that tannins were not responsible for the observed antiprotozoal property of *S. sesban.*

Butanol extraction of plant material causes the removal of saponins (Headon et al 1991; Wall et al 1952). When an aqueous extract of *S. sesban* was extracted with n-butanol and the butanol extract and remaining aqueous phase were tested for their effects on the degradation of *S. ruminantium*, the antiprotozoal activity was removed to the butanol phase (Figure 2; Newbold et al 1994). The remaining aqueous extract had no effect (Figure 2).

The effects of *S. sesban* were compared in rumen liquor obtained from sheep in Aberdeen, UK, and rumen liquor collected from sheep at ILRI's Debre Zeit Research Station, Ethiopia. It is not known if the latter group had previously been exposed to *S. sesban.* As before, the degradation of ^{14}C-labelled *S. ruminantium* by rumen protozoa from Aberdeen sheep was completely inhibited in the presence of *S. sesban*, but *S. sesban* had no effect on the activity of the lower numbers of protozoa in the Ethiopian sheep (Figure 3). Either the protozoa in Ethiopian sheep were intrinsically resistant to the toxic material, or the bacterial population had adapted to metabolise the material and thus remove its toxicity.

It can be concluded that the component of *S. sesban* toxic to rumen protozoa is butanol extractable and therefore likely to be part of the saponin fraction. Wallace et al (1994) showed that other sources of saponins were toxic, but less so than extract from *S. sesban*, suggesting that it is a specific type of saponin or saponin-like substance in *S. sesban* that is responsible for antiprotozoal effect. The observation that saponins are toxic to

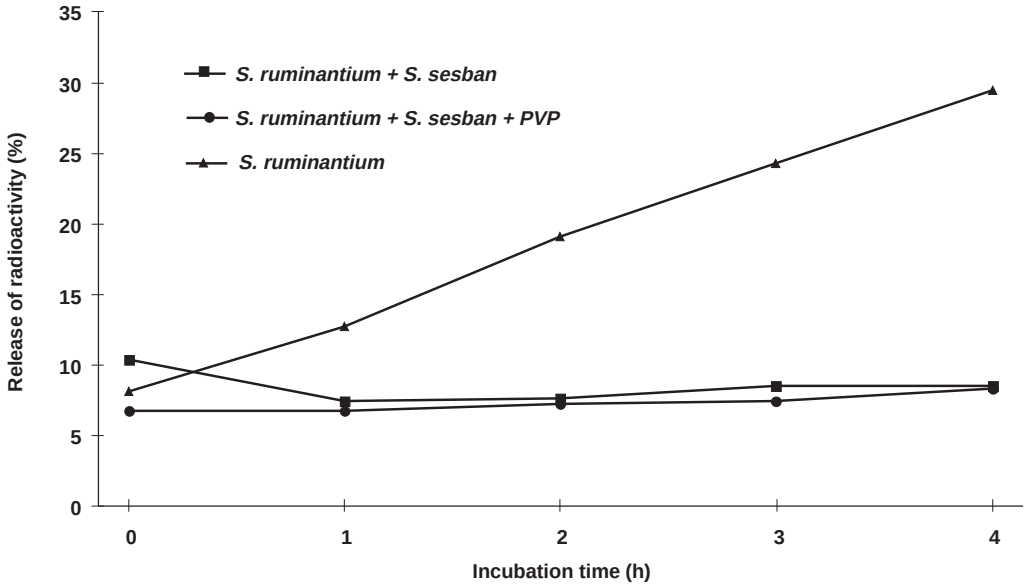

Figure 1. *Influence of removal of tannins from* S. sesban *on breakdown of* Selenomonas ruminantium *by rumen protozoa. The rumen bacterium,* S. ruminantium, *was labelled with* ^{14}*C-leucine and incubated with rumen liquor removed from sheep in Aberdeen as described previously (Wallace and McPherson 1987). The radioactivity released into acid-soluble material gives a measure of protozoal activity.* S. sesban *was present at a concentration of 20 mg ml*$^{-1}$*. PVP, which precipitates tannins, was added to the incubation mixture at a concentration of 20 mg ml*$^{-1}$.

rumen protozoa is consistent with earlier studies in dairy cows (Valdez et al 1986). Recent results from Colombia indicate that the antiprotozoal effects of other plants, including *Sapindus saponaria,* are similarly due to saponins (Navas-Camacho et al 1994). Other antiprotozoal factors in plants have been known for a long time. Eadie et al (1956) showed that certain terpenes and other substances present in plant material had marked toxic properties toward rumen protozoa. Warner (1962) found that minor plant constituents such as terpenes or alkaloids may have specific effects on individual species of rumen micro-organisms. It is unlikely that terpenes or alkaloids would be extracted into butanol by the method used here. Akin (1982) observed that p-coumaric acid reduced the motility of entodiniomorphid protozoa, but again it is unlikely that phenolic acids would be extracted from *S. sesban* into butanol.

Thus the indications are that it is the saponin fraction of *S. sesban* that is toxic to rumen protozoa, although more work is required to identify the precise component. Selective elimination of protozoa, if it were to persist, would enhance the flow of microbial protein from the rumen and improve the nutrition of the animal. Defaunation has been reported to increase microbial outflow (Leng et al 1981) and increase the efficiency of feed utilisation and hence growth rate of cattle on diets low in bypass

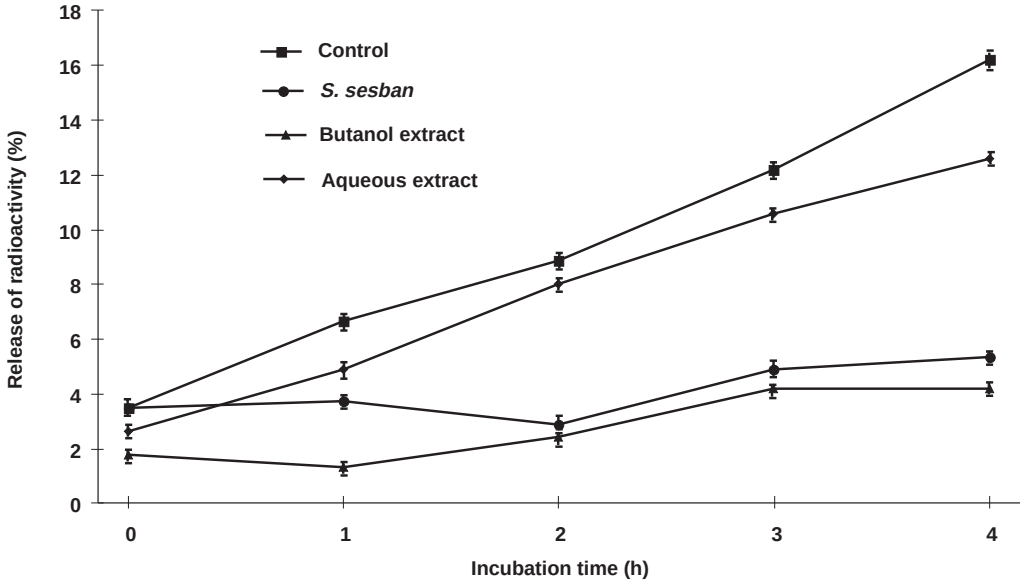

Figure 2. *Influence of butanol extraction of an aqueous extract of* S. sesban *on its inhibition of the breakdown of* Selenomonas ruminantium *by rumen protozoa. Protozoal activity was measured as described in Figure 1.*

protein (Bird and Leng 1978). Similarly, the rate of wool growth was increased in defaunated lambs (Bird et al 1979). Provided the loss of protozoa does not compromise fibre breakdown, which can occur under some circumstances (Ushida et al 1991), the antiprotozoal effects of some MPT maybe useful in enhancing feed utilisation over and above the nutrient content of the feed. There may even be scope for exporting appropriate plant extracts for use in other countries. Possible problems in the use of these materials must be investigated. Indications are that toxicity to the host animal will not be a problem (El Hassan 1994; Navas-Camacho et al 1994), but more work is required. The finding that Ethiopian sheep were resistant may mean that the method has limited applicability in regions where the MPT are indigenous species. It also has implications for their use elsewhere, depending on the nature and time-scale of the development of resistance. These factors should be investigated.

Antibacterial effects of the foliage of multipurpose trees

In contrast to rumen protozoa, there is no assay that can be applied specifically to rumen bacteria. To give a broad view of the effects of MPT on mixed rumen bacteria,

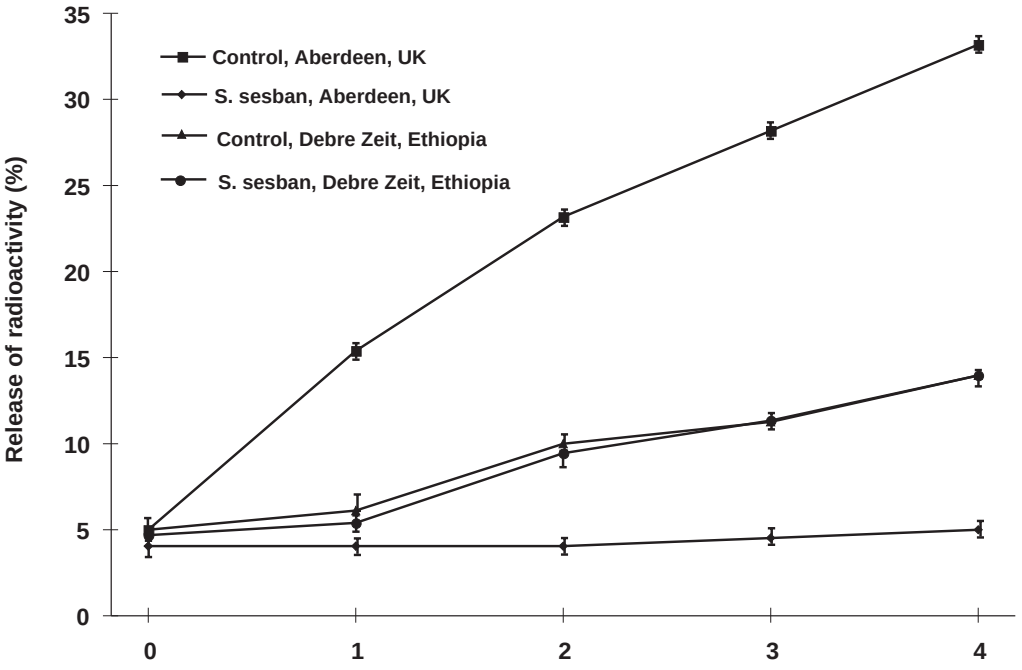

Figure 3. *Comparison of the effects of* S. sesban *on rumen protozoa from rumen fluid taken from sheep in Aberdeen and Ethiopia.*

rumen liquor from which protozoa had been removed by centrifugation was assayed for adenosine 5′-triphosphate (ATP) as an indication of live biomass (Wallace and West 1982). Individual species of rumen bacteria were then examined to determine if the toxicity of MPT was selective with respect to different species of bacteria.

Influence of MPT on ATP concentrations in mixed rumen bacteria

The effect of MPT on the mixed bacterial population were investigated in protozoa-free rumen liquor (PFRL) prepared from sheep in Aberdeen. ATP was analysed in acid extracts as described by Wallace and West (1982) after 4 and 24 h incubation *in vitro* of a mixture of PFRL with MPT and wheat straw. The ATP pools of the different samples of PFRL without addition of MPT were variable and tended to decline between 4 and 24 h, presumably reflecting some cell death over the longer incubation (Table 2). Toxic effects of MPT were seen at 24 h, but not 4 h. Indeed, *C. palmensis* increased ATP at 4 h, most likely because it contained fermentable substrate, giving rise to a greater bacterial biomass. The only evidence of toxicity was after 24 h, when the ATP pool was significantly decreased by *A. aneura*, *S. sesban* and *V. amyedalina* (Table 2).

Table 2. *Influence of MPT on ATP content of mixed rumen bacteria incubated* in vitro.

MPT	Incubation time (h)	ATP (mM) at inclusion level of † 0 mg ml^{-1}	6 mg ml^{-1}	12 mg ml^{-1}	24 mg ml^{-1}	SED
Acacia aneura	4	21.1	22.1	21.6	20.1	2.07
	24	10.0^{a}	8.6^{ab}	6.6^{b}	3.7^{c}	1.78
Brachychiton populneum	4	34.7	32.5	31.8	30.8	10.02
	24	28.2	25.8	25.7	23.9	7.74
Chamaecytisus palmensis	4	29.1^{a}	30.4^{a}	35.1^{ab}	41.1^{b}	4.55
	24	21.1	18.7	17.5	16.5	2.64
Flindersia maculosa	4	35.6	34.3	33.3	36.2	8.59
	24	25.2	21.2	20.4	17.2	3.78
Leucaena leucocephala	4	29.0	28.8	30.7	29.8	7.73
	24	23.9	20.6	20.3	16.3	4.63
Sesbania sesban	4	24.9	28.8	29.4	27.1	3.97
	24	27.1^{a}	21.9^{b}	20.4^{b}	15.4^{c}	2.14
Vernonia amyedalina	4	32.3	32.7	32.5	32.2	6.35
	24	26.9^{a}	24.3^{ab}	21.9^{ab}	18.6^{b}	2.61

†The balance was made up by wheat straw to give a total substrate concentration of 40 mg ml^{-1}.
^{abc}Values with different superscripts are different ($P < 0.05$).

These effects of MPT on bacterial ATP are indicative only, because they represent a balance between ATP increases caused by fermentable materials in the MPT and decreases resulting from toxicity. Nevertheless, some indication of toxicity was obtained, which was greatest with *A. aneura.*

Influence of MPT on growth of individual species of rumen bacteria

The influence of MPT on the growth of nine major species of rumen bacteria was examined by adding aqueous extracts of the plants to pure cultures of bacteria and determining the effects on growth by monitoring optical density. Only two examples are given here, illustrating typical differences between effects observed with cellulolytic and non-cellulolytic species. Full details are given elsewhere (El Hassan 1994).

A. angustissima and *L. leucocephala* were most toxic to non-cellulolytic bacteria, with the other species having little or no effect; the effect was relatively minor, however, as illustrated here with *Streptococcus bovis* (Figure 4). Relatively minor effects were seen with cellulolytic bacteria as well, except for *A. angustissima,* which abolished growth of *Ruminococcus flavefaciens* (Figure 5) and *R. albus.* The third major cellulolytic species commonly found in the rumen, *Fibrobacter succinogenes,* was much less sensitive to *A. angustissima,* and responded more like the other non-cellulolytic species (data not shown).

A toxic effect of *A. angustissima* against two of the three cellulolytic rumen bacteria would be expected to have major consequences for fibre breakdown in ruminants consuming this MPT. The results presented here were consistent with results obtained with the Menke gas production technique, in which *A. angustissima* produced the most

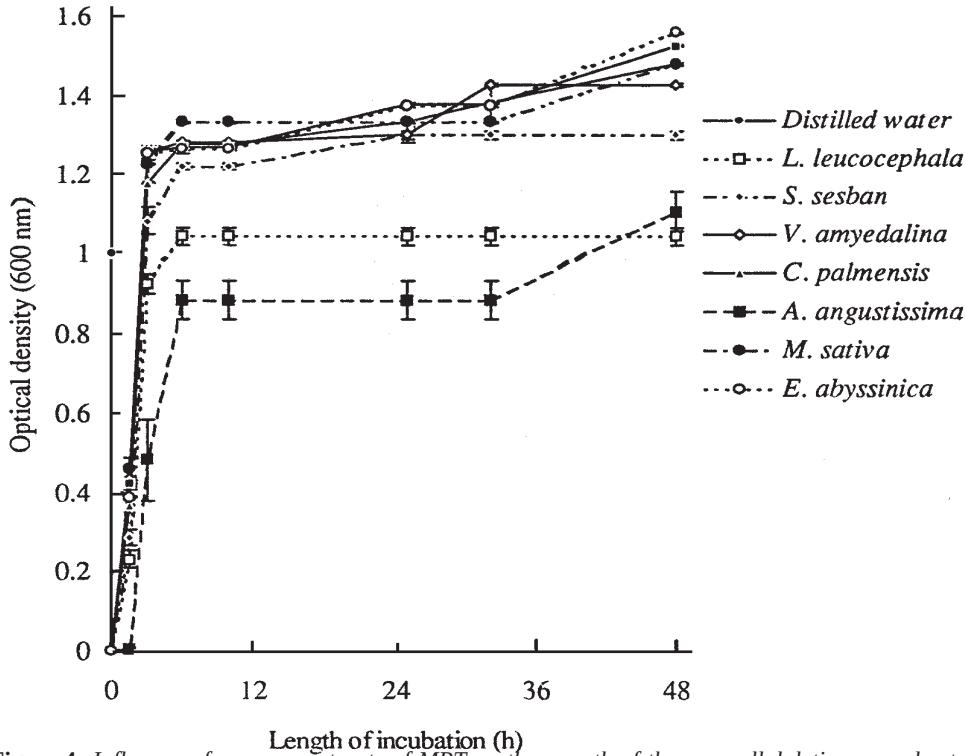

Figure 4. *Influence of aqueous extracts of MPT on the growth of the non-cellulolytic rumen bacterium,* Streptococcus bovis. *Foliage from the MPT (5 g) was extracted with 70% aqueous acetone and the residue after drying resuspended in 50 ml of water, filtered, sterilised, and 0.3 ml was added to 10 ml of culture medium. Growth was determined turbidimetrically.*

pronounced effect on gas production among the MPT examined (El Hassan 1994). Mueller-Harvey et al (1988) noted that all extracts from Ethiopian browse increased the lag time and reduced biomass yields of *S. bovis* and they observed differences in the extent of these antimicrobial effects that were consistent with the concentration of phenolics from browse. The toxicity of phenolics has been demonstrated in a wide range of bacteria, including cellulolytic and xylanolytic species (Akin 1982; Chesson et al 1982; Stack and Hungate 1984). However, no species specificity in the antibacterial effects were noted previously. The cause of the inhibition of *Ruminococcus* species caused by *A. angustissima* is not clear. They may have been due to soluble polyphenolics, which may contain simple phenolics and proanthocyanidins (Reed 1986). *A. angustissima* contained the highest content of soluble polyphenolics among MPT examined (El Hassan 1994). However, much work remains to be done to identify the anti-*Ruminococcus* factor present in *A. angustissima.*

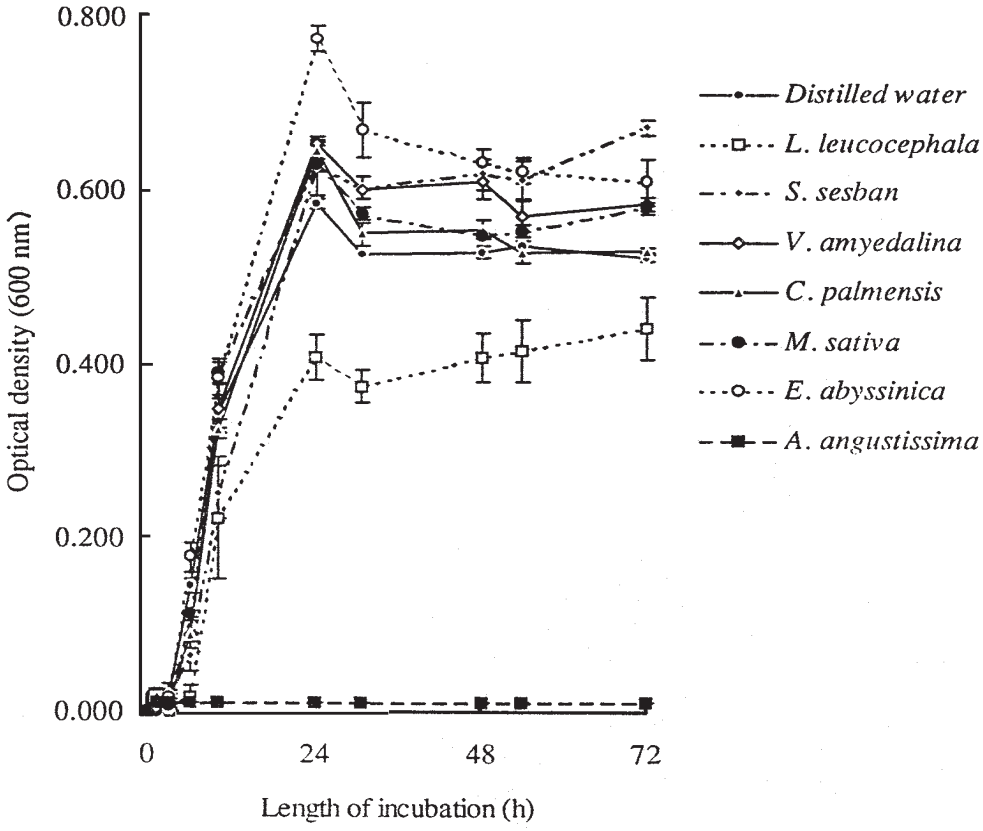

Figure 5. *Influence of aqueous extracts of MPT on the growth of the cellulolytic rumen bacterium,* Ruminococcus flavefaciens. *Foliage from the MPT (5 g) was extracted with 70% aqueous acetone and the residue after drying resuspended in 50 ml of water, filtered, sterilised, and 0.3 ml (or 0.2 or 0.1 ml of* A. angustissima) *was added to 10 ml of culture medium. Growth was determined turbidimetrically.*

Conclusions and implications

Some MPT, such as *S. sesban*, may be useful as protein supplements and as defaunating agents in ruminants in sub-Saharan Africa. These MPT may have some commercial value as defaunating agents in other regions, including Europe and North America. Other MPT had relatively minor effects on rumen micro-organisms, except for *A. angustissima*, which had a specific toxic effect on *Ruminococcus* species. Unless a means can be found of removing the toxic factor from *A. angustissima*, its use is not recommended due to likely detrimental effects on fibre breakdown.

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Australian Meat Research Corporation's rumen bacteria manipulation key programme: rationale and progress 1990–1995

I.D. Johnsson

Meat Research Corporation, P.O. Box 498, Sydney South NSW, Australia 2000

Abstract

In 1990, the Meat Research Corporation contracted four Australian research teams to develop genetically modified rumen bacteria with enhanced cellulolytic activity. The goal was a sustained increase in digestibility by at least 5 percentage units in ruminants grazing on poor quality forage typical of the northern Australian grasslands, and more generally of 90% of Australia's pastoral country. With an A$4.9 million (US$3.7 m) budget, the programme planned to explore four alternative research options over three years (mutagenesis of rumen bacteria, bacteria-bacteria gene transfer, fungi-bacteria gene transfer, cleavage of ligno-cellulose bonds), then choose the most promising methodologies for refinement and *in vivo* testing in years 4 and 5. After a review in year 3, additional funding of A$1.3 (US$1.0 m) million was allocated to extend the research phase for two years to June 1995. Difficulty in successfully transforming rumen bacteria has been the major stumbling block for two years. A genetically modified *Butyrivibrio fibrisolvens* expressing a highly active cellulase derived from the rumen fungus *Neocallimastix patriciarum* is now being tried in cattle under quarantine, but no significant impact on digestibility has been demonstrated to date. Genes for a number of other cellulases have been isolated and characterised for transformation into suitable host bacteria. It is likely that further funding will be needed to optimise expression and survival in the rumen, and to achieve transfer of more than one desirable gene into multiple strains of host bacteria, in order to achieve the programme goal. The industry outcome is low cost bacterial strains that spread naturally between animals in the same herd and survive through fluctuating seasons, conferring an advantage in fibre digestibility across a large range of grass-based diets. Their application may be limited to management systems where nitrogen and other limiting factors can be supplied to ruminants in the dry season.

Introduction

The Meat Research Corporation was established by the red meat industries and the Government of Australia to invest industry research levies, which are matched by the

government, in research and development (R&D) of benefit to the industries involved. The MRC's current portfolio covers 200–300 projects ranging from aspects of meat production (new plant varieties, animal genetics, stock health, grazing management) and processing (new abattoir equipment, carcass measurement, meat hygiene, packaging, storage) to human nutrition, work-related issues, retailing practices and strategic market research.

In 1988/1989, the corporation decided to take a more pro-active approach to R&D, and established its 'key programme' methodology for identifying, commissioning and managing large, integrated programmes of R&D to address agreed industry priorities. Now more than half of our annual A$50 million (US$38 m) budget is used in key programmes, as opposed to supporting single research proposals submitted by individual research groups.

In 1989, MRC had been supporting Dr K. Gregg, University of New England, for five years to improve the cellulolytic capacity of rumen bacteria, and Dr J. Brooker, University of Adelaide, to improve nitrogen efficiency in rumen bacteria. The University of New England work included limited screening of rumen microflora in other species (camel, buffalo etc.) for highly cellulolytic, naturally occurring bacteria that could be readily transferred to cattle but this was unsuccessful. Given the complexity of the science involved and the perception that technological developments were beginning to accelerate, MRC decided to draw together the major research groups involved in Australia to establish a 'critical mass' across the necessary disciplines to address the issue of improved fibre digestion in grazing ruminants.

Over 90% of Australia's pastoral industry relies on moderate-poor quality native grasslands which are unlikely to be improved other than by broadcasting low-phosphorus tolerant legumes on better soil types and by seasonal supplementation of energy, protein and minerals.

With highly variable rainfall, and pronounced wet/dry seasons in the tropical north, vast areas of grassland are too low in nutritive value to support animal liveweight for a significant part of each year, unless supplemented with nitrogen and phosphorus. Animals can literally lose weight and die in a 'sea' of dry feed. Even in more favoured temperate zones, with improved pasture species, livestock traditionally only maintain or lose weight over summer/autumn, and extensive use is made of crop stubbles and conserved hay. In assessing the economic benefits of an R&D programme to increase fibre digestion in grazing ruminants by up to about 5 percentage units, a conservative benefit cost analysis indicated maximum annual net benefits of up to A$180 million (US$135 m) per year. This analysis assumed a technology that was low cost to graziers, based on local experience with the naturally occurring rumen bacteria that was imported into Australia to detoxify mimosine ingested by cattle grazing the tropical legume, leucaena.

The major factors in the decision to establish the key programme were:

- Low-cost of final 'product' to end user.
- Unsophisticated, robust product (cf highly sophisticated research).
- Likely to be compatible with current management systems.
- Potential for rapid adoption by target end-users.

- Potentially applicable to a large proportion of the production sector.
- 'Enabling' technology with potentially useful spin-offs e.g., nitrogen efficiency, detoxification of plant toxins etc.

Major concerns were, and still are:

- Can genetically modified bacteria thrive in the rumen (are they advantaged or disadvantaged by carrying novel genes)?
- Can we identify and manipulate, spatially and temporally, the key enzymes that are limiting within the complex of enzymes responsible for cellulolysis?
- If the modified host bacteria can spread between animals, will they colonise feral pest species (camels, goats, horses, rabbits etc.) and increase environmental impact?
- Will increased fibre digestion in ruminants lead to increased over-grazing and 'desertification'?
- Will the Australian public allow the release of genetically-modified rumen bacteria?

Alternative technologies were examined but rejected for the following reasons:

- Genetic engineering of pasture plants: relatively more complex, much slower and far less total adoption in the target sector.
- Genetic engineering of ruminants themselves: more complex, greater ethical 'problems', very much slower adoption.
- Nutritional supplementation: mature technology, already widely known/used, delivery difficult in more extensive areas.
- Manipulation of rumen fungi: role and potential to manipulate unknown, molecular genetics unknown, current basic work in progress funded by 'sister' corporation (being done by Dr G. Gordon).

Key programme structure

In 1990, MRC contracted four research teams to examine various methodologies to develop rumen bacteria with enhanced cellulolytic activity. The methodologies were proposed by the team leaders, and the plan was to fund four 'competing' methodologies for three years to 1993, and to then choose the most promising for optimisation and field testing in years 4 and 5.

The overall programme objective was 'to obtain a sustainable increase in fibre digestibility of at least 5 percentage points by establishing modified bacteria in the rumens of sheep and cattle grazed under controlled conditions by June 1995'. Two subprogramme objectives and the major thrust of each research team are shown in Figure 1. After an external review in December 1992, a more integrated programme structure was adopted, with greater emphasis on sharing of knowledge, pooling of ideas and exchanging components of transformation systems. Although the 1993 target deadline was obviously not going to be achieved, the MRC agreed to support the research phase for an additional two years.

In addition to the core projects, two ancillary projects which will impact on the progress have also been supported by MRC and are shown in Figure 1. At UNE, a team led by Dr K. Gregg has been examining a solution to fluoroacetate toxicity in grazing ruminants by modification of rumen bacteria. At CSIRO Division of Tropical Animal

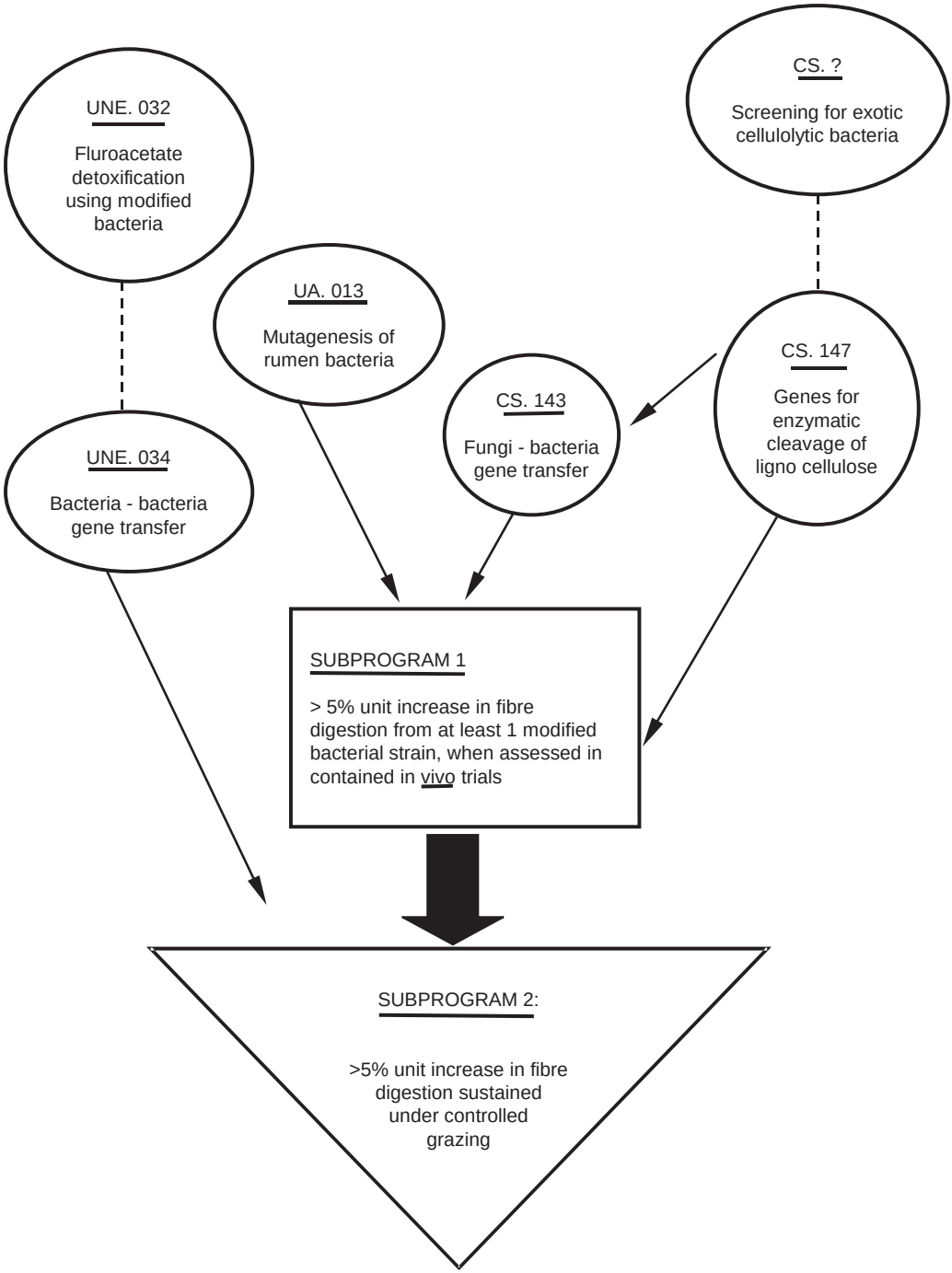

Figure 1. *Key programme structure.*

Production, a new project will screen 'exotic' ruminants (particularly antelope and deer species, such as oryx, addax, gemsbok) for novel strains of rumen bacteria. These animals are being run under rangeland conditions at the Tipperary Sanctuary for Endangered Wildlife in the Northern Territory.

Progress to date

Project UA.013 (Dr J. Brooker) attempted to develop highly cellulolytic bacteria by transposon-mediated mutagenesis of naturally cellulolytic bacteria. This proved unsuccessful, although transposon-marking of specific strains (Tn 916 in *B. fibrisolvens* E14) was initially useful to track populations in the rumen. A naturally occurring *Streptococcus* spp. with some ability to degrade plant tannins was isolated from feral goats and work was initiated on the cloning and enzymology of *Clostridium* spp. with the hope of providing useful cellulase genes. This project was not extended after the mid-term review.

Project UNE.034 (Dr K. Gregg) continued previous work to introduce cellulase genes from highly cellulolytic rumen bacteria into other predominant, but poorly-cellulolytic rumen bacteria. Major progress has been:

- The cloning and sequencing of genes from *Ruminococcus albus* AR67 (Gregg et al 1993).
- Transformation achieved for *Prevotella ruminicola AR20* to reasonable efficiency (K. Gregg, personal communication).
- Transformation achieved for *Butyrivibrio fibrisolvens* OB156 (Beard et al 1994).
- Development of qualitative and quantitative PCR systems to track *B. fibrisolvens* and *P. ruminicola* strains in cattle and sheep (K. Gregg, personal communication).
- Demonstration of long-term persistence of introduced laboratory-grown strains of *B. fibrisolvens* (E14, AR10) and *P. ruminicola* (AR20, AR29) in the rumens of sheep or cattle, and of their ability to transfer naturally between hosts in some cases.

Project CS.143 (Dr J. Aylward) aimed to transfer genes for highly active fungal cellulases secreted by *N. patriciarum* to cellulolytic rumen bacteria to enhance their capacity to degrade cellulose. Major progress has been:

- Cloning of five genes of varying activities from *N. patriciarum* (celA, celB, celC and celD, plus xynA), after preparation of a comprehensive gene library. (Xue et al 1992; Orpin and Xue 1993).
- Development of a transformation system for five strains of *B. fibrisolvens* (K.S. Gobius, personal communication).
- Quantification of relative activities of various enzymes *in vitro*, alone and in combination, using standard forage substrates (J. Aylward and P. Kennedy, personal communication).

Project CS.147 (Dr P. Kennedy) aimed to find enzymes which are capable, under anaerobic conditions, of at least partial hydrolysis of ligno-cellulose bonds. Significant progress has been:

- The development of a range of model substrates for assaying enzymes which cleave various linkages.

- Demonstration of higher lignin content in tropical grasses than indicated by the traditional acid detergent lignin technique, and of higher lignin digestion than previously recognised (Lowry et al 1994).
- With CS.143, the cloning of acetyl xylan esterases from the *N. patriciarum* gene library (B. Dalrymple, personal communication).
- A study of cinnamoyl ester hydrolases isolated from the *B. fibrisolvens* E14 gene library (D. Cybinski and B. Dalrymple, personal communication).
- Development of techniques (with UNE.034) to study persistence of *B. fibrisolvens* strains in cattle and characterisation of their fibre degrading capacity *in vitro* (C.S. McSweeney, personal communication).

The most significant breakthroughs with regard to the attainment of the programme goal have occurred in the last 12 months:

- In a closely related MRC-funded project, K. Gregg at UNE developed the first potentially useful recombinant rumen bacterium, *B. fibrisolvens* OB156 expressing a dehalogenase enzyme which degrades toxic fluoroacetate (Gregg et al 1994). This recombinant strain has been shown to persist for at least five months in penned sheep, and its efficacy *in vivo* is currently being tested.
- First transformation of several *B. fibrisolvens* strains with cloned fungal genes from *N. patriciarum* and secretion of cellulase/xylanase enzymes (K.S. Gobius and G.P. Xue, personal communication).
- First evidence that transformation of a rumen bacteria with a xylanase gene can enhance the ability of the native strain to digest fibre when incubated in pure cultures *in vitro* (C.S. McSweeney, personal communication).

The future

On the basis of these results, the MRC is likely to contribute further funding to take the research through to 'proof of concept', namely the demonstration of an increase of at least 5 percentage units in digestion of poor quality grasses in ruminants inoculated with at least one genetically-modified bacterial strain, when assessed in containment facilities.

This should be achieved by December 1995, most probably using a suite of modified bacterial strains, each expressing one of a range of novel enzymes of bacterial or fungal origin.

Subsequent molecular biology research could then be undertaken to optimise expression, stability (is chromosomal integration essential?) and persistence, to explore combinations of recombinant genes in the one host strain and to extend the library of useful genes and candidate hosts, in addition to the detailed rumen microbiology and nutrition studies needed to quantify the commercial value of the modified strains in diverse commercial grazing situations. One can thus envisage 'waves' of new recombinants being produced for animal evaluation as the scientists involved become more experienced in the manipulation of rumen bacterial species and find new sources of novel enzymes.

However, from an industry viewpoint, this research is of no value if Australian society decides not to condone release of these recombinants for commercial use. Thus a priority

for the MRC programme, once the concept is proven, will be to assess the likely environmental impact of highly cellulolytic recombinant bacteria, in terms of transfer to non-target species (feral rabbits, horses, camels and goats, in particular, as well as native macropods) and of effects on grazing animal intake, and therefore grazing pressure, on fragile landscapes.

The ultimate decision will be made on the basis of risk assessment, based on an evaluation of the benefits and risks. Thus comprehensive efficacy data will be essential, probably across a range of management systems. If the concerns regarding feral animal competitiveness can be addressed, it seems likely that approval will be granted, given that the ultimate outcome is similar to the impact of increasing stocking rate, changing from *Bos taurus* to *B. indicus* cattle (with consequently higher survival rates) in tropical areas or changing from a smaller breed to a larger breed of cattle. In these cases, changes in grazing management practices have been required to ensure sustainable land use, and these new practices are being increasingly adopted by Australian pastoralists.

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State of art in manipulation of the rumen microbial ecosystem

Ruminants and rumen microorganisms in tropical countries

H. Kudo1, S. Imai2, S. Jalaludin3, K. Fukuta4 and K.-J. Cheng5

1. Japan International Research Center for Agricultural Sciences, Ibaraki, 305 Japan
   Present address: National Institute of Animal Industry, Inashikigun, Ibaraki, 305 Japan
2. Nippon Veterinary and Animal Science University, Tokyo, 180 Japan
3. Universiti Pertanian Malaysia, Selangor, Malaysia
4. National Institute of Animal Health, Ibaraki, 305 Japan
5. Agriculture and Agri-Food Canada, Alberta, Canada T1J 4B1

Abstract

Crop residues and by-products can be utilised for animal production. Some by-products contain toxic components and may require supplementation or modification to reduce the toxicity.

Mimosine/DHP poisoning by Leucaena may be reduced by introducing rumen bacteria that can degrade mimosine and DHP. The comparative study on ciliate fauna strongly indicated the presence of geographical distribution. For example, Eudiplodinium giganteum, Eudiplodinium kenyense, Enoploplastron stokyi, Diplodinium africanum, Diplodinium nanum and Ostracodinium iwawoi have been found only in Africa. In the percentage composition of genera, the numbers of Entodinium, which normally predominate in rumen in other areas, were very low in African ruminants. The presence of Metadinium ypsilon and Ostracodinium trivesiculatum is characteristic of Malaysian cattle and water buffaloes. The level of Metadinium and Eudiplodinium, considered to be fermenters of cellulosic materials, was higher in Malaysian cattle and water buffaloes. Cellulolytic bacteria isolated from water buffaloes possess a higher ability to degrade cellulose than those from cattle.

From the viewpoint of rumen microbiology, rumen flora and fauna of lesser mousedeer (Tragulus javanicus), the smallest ruminant (1.3 to 1.7 kg at adult size), are unique and very interesting. Fairly large motile bacteria, similar to Oval and Oscillispira, are present in large numbers, contributing to a higher microbial mass. The natural occurrence of mono-fauna protozoa or the total absence of protozoa have also been observed. So far, no fungus has been detected or isolated from the rumen contents. For the successful development of rumen microbiology in the field of biotechnology, it is important to study and include more basic characteristics of rumen microorganisms, including some ecosystems which have so far not received attention.

Introduction

Today's ruminants receive an increasing variety of feeds. In extreme cases, farmers feed ruminants like non-ruminant animals, ignoring the fact that they are ruminants, because of the availability of feed and the adaptability of the animals. As the human population increases, we produce a greater variety and quantity of waste products which can be consumed by animals but consequently the land available for both housing and agriculture is reduced. The nutrient energy in most of these by-products is not readily available and is not sufficient, even for the ruminant digestive system. This is especially the case for cellulosic materials which have slower rates of digestion because the cellulose is in a highly organised and complex form, and because this kind of feed generally lacks the nitrogen necessary to balance the large amounts of available carbon. We are now trying to manipulate the rate of digestion of various feeds to favour both humans and ruminants. We are trying to accelerate those rates that are slow, while delaying those with a high initial rate of nutrient release which can cause digestive disturbances, such as acidosis, bloat and liver abscesses (Cheng et al 1991). The former objective is more interesting and more important to tropical countries. Theoretically it is conceivable that up to 90% of ruminant feed could be made up of by-products which have been modified to improve digestibility.

In practical terms, the benefits from the successful attainment of the objectives would be (1) to make underutilised by-products available as ruminant feed, (2) to improve the digestibility and efficiency of utilisation of these by-products for animal feed, and (3) to reduce the requirement for hay and animal feed products—thus freeing land for more valuable crops. In Asia, ruminants are kept by small farmers on a small scale and the production systems are closely integrated with farming systems. One of the limiting factors for the expansion of production is the available feed resources, as under such farming systems the farmers do not have adequate feed resources or the capacity to increase available feed. Usually agricultural by-products are utilised for feeding domestic ruminants. Given the present decline in the area available for forage production, ruminants will have to depend more and more on fibrous residues and by-products for their energy sources. These by-products still have problems to be solved and many of them cannot be used without some detoxifying treatments. However, considering the quantities of the by-products and the fact that tropical ruminants are better converters of low quality feed into milk and meat (Abdullah 1987), the by-products are likely to play an important role as potential feed sources in ruminant nutrition.

Ruminants in Southeast Asia

Southeast Asia is rapidly expanding its manufacturing industry but agriculture continues to be the mainstay of the rural economy. Agriculture in Southeast Asian is crop-based, producing abundant feedstuffs (conventional and non-conventional) which can be utilised for animal production. Demand for animal products has increased due to rapid urbanisation and improved living standards and local production is inadequate.

The slow growth of ruminant production in Southeast Asia, in spite of readily available resources (especially feeds), may be attributed to the inefficiencies in their utilisation—the consequence of inappropriate technological development and application (Jalaludin et al 1992).

The lesser mousedeer *(Tragulus javanicus)*

Successful breeding in the laboratory

The lesser mousedeer is the smallest ruminant, weighing 1.3 to 1.8 kg, and is considered a 'living fossil' which escaped from extinction and remains in its original morphology after 25 million years. The lesser mousedeer is found in Southeast Asia and all the Sunda Islands. The family Tragulidae comprises the three Asian species of mousedeer or chevrotain, genus Tragulus and the water chevrotain of Africa, Hyaemoschus aquaticus (Anderson and Jones 1967).

The study of the nutritional physiology of herbivorous domestic animals such as the ruminant is very important in the field of veterinary and zootechnical sciences (Goto and Hashizume 1978; Kudo and Oki 1981, 1982, 1984). However, small herbivorous laboratory animals are still not established for comparative purposes. From the viewpoint of conservation and protection of wildlife, it is also very important to establish a breeding colony. With permission from the Department of Protected Wild Life and National Park, Malaysia, August 1990 the rumen microbiology laboratory of Universiti Pertanian Malaysia has started to establish a breeding colony of mousedeer and has initiated some studies on the reproduction, nutrition and microbial population and activity in the rumen of mousedeer. Ten (five males and five females) mousedeer were introduced from the jungles of Selangor and Pahang, Malaysia. We feed them with rabbit pellets, sweet potatoes, carrots, kangkong (Ipomoae aquatica) and long beans. Hand feeding, mainly on sweet potato, is used for better reproductive results. Despite the fact that they are all from the wild, they take feed from hands on the same day they are introduced into the laboratory. Foster nursed mousedeer can be toilet trained like dogs and cats. We kept a pair of mousedeer in half of a stainless steel cage of 46 Hÿ×ÿ116Wÿ45 cm D with a divider.

Mortality of captured lesser mousedeer is usually quite high (60–70%; National Zoo, Malaysia, and Institute of Medical Research, Malaysia, personal communication). We recorded about 10% mortality in our laboratory. The behaviour of the tragulids is of particular interest because of the remarkable convergence in morphology and ecology between the tragulids and the caviomorph rodents of South America. There are many features that differ from the ruminants (Fukuta et al 1991; Ralls et al 1975). It ruminates while standing, does not use its tongue for eating but instead uses it for drinking (like a dog or a cat), sits dog-like and can scratch its head with the hind leg. Rabbit-like stamping is often observed. Thus, they behave more like dogs and cats rather than like ruminants. Males do not have antlers but instead they have well-developed canines which extend beyond the upper lip. Their feeding style and habit are very selective, very often eating only particular parts of plants. Therefore, this animal may not be

suitablefor feeding experiments. On the other hand, this feeding style may have protected the animals from plant poisoning. For example, they like lundai leaf (Sapium baccatum, a tropical shrub tree) very much but the appetite for this leaves is not consistent. The lundai leaf is thought to be one of the important components of the diet of the Malaysian mousedeer in its natural rain forest habitat but it contains a very high level of a toxic substance, mimosine. Their small mouths permit them to select parts of plants in a way that the larger ungulates are not able to do. The teeth type is not particularly suited for grinding (Vidyadaran et al 1981). The rumen of the adult lesser mousedeer is fairly large (¾ of the total abdominal cavity) and the ratio of reticulo-rumen volume is larger than that of cattle and sheep (Vidyadaran et al 1982). The omasum is vestigial.

Mousedeer reach sexual maturity at about four to five months of age and adult size at five months. The earliest age of the first parturition in our laboratory was 258 days. The canines of the young male do not extend beyond the upper lip until they are about nine to ten months old. Sexual cycle of female lesser mousedeer is estimated to be 16 days from mounting action, the duration of mating season is two days where copulation occurs several times and they resume estrus unless they are pregnant. In mousedeer, pregnancy is not apparent but females tend to sit all the time for a few days before parturition. The female mousedeer are known to have a postpartum estrus (Cadigan 1972; Davis 1965) and copulation is observed often after 30 min to a few hours after the parturition. There is no breeding season in the laboratory. All five pairs from jungles and the subsequent offspring reproduce well and gave relatively regular continuous parturition which may compensate for their relatively long gestation period. Although there are numerous reports that the gestation period of mousedeer is 140 to 177 days (Anderson and Jones 1967; Lekagul and McNeely 1977; Tubb 1966), we observed a shorter gestation period. The apparent gestation period is 136 to 164 days, but actual gestation is estimated to be 134 (SEÿ=ÿ2) days, judging from the intervals of continuous parturitions. A delay in implantation due to lactation probably occurs. It was believed that reproduction in the laboratory was quite difficult but we did not have a major problem, unlike other wild animals introduced into the laboratory. The size of new offspring is fairly large (120 to 190 g) in relation to the mother animal (about 10%). Infant mortality among the mousedeer at the zoo was unusually high (¾; Ralls et al 1975). Infant mortality at our laboratory was about 14% (6/43, until weaning). Our reproductive data at Universiti Pertanian Malaysia for 3½ years is summarised in Table 1.

Rumen content of mousedeer is very much like a thick slurry with a pH range of 5.5 to 6.5. From the viewpoint of rumen microbiology, the rumen flora and fauna of mousedeer are unique and very interesting in that they differ totally from other domestic ruminants. A number of bacterial isolates (e.g. Fibrobacter succinogenes, Ruminococcus albus and R. flavefaciens) are highly cellulolytic. Fairly large motile bacteria, similar to Oval and Oscillospira, are present in large numbers, hence contributing to a higher microbial biomass. The occurrence of mono-fauna protozoa or a total absence of protozoa have also been observed. The reasons for these phenomena are not known. Six species of protozoa belonging to two genera were detected in our laboratory (Table 2). Ciliate composition is poor as compared with other domestic ruminants (Table 3)

Table 1. *Reproductive data of captured lesser mousedeer at Universiti Pertanian Malaysia.*

Breeding season	All year round
Estrous cycle	14 to 16 cyclic days
Mating period	2 days
Earliest age of copulation (male)	166 days
Earliest age of conception (estimated)	125 days
Earliest age of the first parturition	258 days
Shortest gestation period	132 days
Estimated actual gestation period	134 (SE = 2) days
Earliest copulation after parturition (postpartum estrus)	30 min (Yes)
Delay of implantation of fertilised egg	Yes
Maximum continuous parturition within 136 days of interval	3 times (136, 133 and 136 days)
Litter size	1
Infant mortality before weaning	6/43

Table 2. *Ciliate protozoa in the rumen of lesser mousedeer at Universiti Pertanian Malaysia.*

Total ciliate number	45.5×10^4/ml
Species identified	*Isotricha jalaludinii* *Entodinium simplex* *E. dubardi* *E. anteronucleatum* *E. nanellum* *E. convexum*

but the total counts are comparable (4.6ÿ×ÿ105/g of rumen contents) to other domestic ruminants (Imai et al 1995). So far, no fungus has been detected and isolated from the rumen contents.

Feeding systems for ruminants in Southeast Asia

Pasture utilisation

The standard of feeding should be improved along with the increase of the genetic potential of the ruminants in Southeast Asia. Southeast Asia has a sizeable land area covered with natural pastures, especially in Indonesia where it is more than twice the area under cropping systems. Fresh fodder is also produced at a rate of 5–10 ton/ha from land under crops. A major part of the roughage intake for ruminants in Southeast Asia is provided by about 20ÿ×ÿ106 ha of permanent pastures. A conservative yield estimate of 5 ton dry matter per year would be sufficient to supply the requirements of all large ruminants in the region. There are at least 60 species of naturally grown forages, of which 70% are palatable. Due to poor soil quality and rapid maturity, tropical forages are low in nutritive value. However, with phosphorus dressing, a stocking rate of four zebu cattle per ha is possible. Individual liveweight gain ranges from 281–448 g/day

Table 3. *Comparative host distribution of rumen protozoa*.

	Lesser mousedeer	Water buffalo	Cattle	Goat
Entodinium	+	+	+	+
Isotricha	+	+	+	+
Diplodinium	+	+	+	+
Eodinium		+	+	
Eudiplodinium	+	+	+	+
Metadinium		+	+	+
Polyplastron		+	+	+
Elytroplastron		+	+	+
Ostracodinium		+	+	+
Enoploplastron			+	+
Epidinium	+	+	+	+
Epiplastron			+	
Ophryoscolex		+	+	+
Opisthrotrichum		+		

Source: Ogimoto and Imai 1981.

but in terms of annual liveweight gain per ha, the highest yield, at 855 kg, was obtained on mixed grass-Leucaena pasture with a stocking rate of 7.3 animals per ha. The grazing intensity can be raised to ten animals per ha on fertilised grass pasture, yielding 1100 kg/ha per year, if accompanied by moderate supplementation of concentrate (Jalaludin et al 1992).

Non-conventional feeds in Southeast Asia

Non-conventional feeds, such as crop residues and by-products, are fast emerging as an alternative source of nutrients for intensive ruminant production. In Southeast Asia, rice cultivation and oil palm production produce the largest quality of residues and by-products. The total quantity of fibrous crop residues available in Malaysia is more than 300ÿxÿ103 tons (comprising mainly rice straw and palm press fibre (PPF). Rice straw utilisation has been widely reported but information on the potential use of oil palm by-products as animal feed is lacking.

In Malaysia, research to promote a viable, low cost ruminant production system based on oil palm by-products as the main source of nutrients is being undertaken. By-products such as PPF, oil palm trunk (OPT) and oil palm fronds (OPF) are very fibrous but potentially rich in energy, while palm kernel cake (PKC) contains high protein. The fibrous residues, e.g. PPF and OPT, are characterised by poor digestibility due to a high lignin content. They are also low in crude protein. Treatment of PPF with 8% sodium hydroxide increased the dry matter digestibility from 43.2 to 58.0%. Physical treatment with steam at 12 kg/cm2 for 5–10 min increased the digestibility from 26 to 28% (Oshio et al 1990). The response of OPT to chemical and physical treatment is similar to that obtained for PPF.

Palm kernel cake is widely used as animal feed in Malaysia and can be a good feed. However, its high concentration of copper and imbalance of Ca/P cannot be over-

Table 4. *Mineral composition of palm kernel cake (solvent extracted).*

	Palm kernel cake (PKC)							
	1	2	3	4	5-1	5-2	6-1	6-2
Ca (%)	0.28	0.25	0.28	0.27	0.25	0.24	0.24	0.25
P (%)	0.60	0.62	0.57	0.61	0.64	0.64	0.62	0.62
Mg (%)	0.23	0.30	0.31	0.30	0.29	0.28	0.27	0.28
K (%)	0.76	0.78	0.79	0.79	NT	NT	NT	NT
S (%)	0.19	0.24	0.23	0.23	NT	NT	NT	NT
Cu (ppm)	28.50	28.90	27.50	26.40	19.80	21.30	19.30	19.70
Zn (ppm)	40.50	43.60	48.30	48.40	41.60	42.30	40.40	40.70
Fe (ppm)	6130	5666	844	835	817	3486	665	1125
Mn (ppm)	201	196	204	197	38.40	147.90	79.60	73.6
Co (ppm)	—	—	—	—	—	—	—	—
Se (ppm)	0.30	0.32	0.23	0.24	NT	NT	NT	NT
Pb (ppm)	NT	NT	NT	NT	—	—	—	—
Ni (ppm)	NT	NT	NT	NT	—	—	—	—
Cd (ppm)	NT	NT	NT	NT	—	—	—	—
Mo (ppm)	NT	NT	NT	NT	—	—	—	—
Na (ppm)	NT	NT	NT	NT	96.00	82.30	89.60	

A dash (—) represents values below detectable limits; NT not tested.
PKC 5-1 and 5-2 were duplicates, as were 6-1 and 6-2; PKC 1 to 4 were analysed by Dr M. Ivan, Agriculture Canada; PKC 5 and 6 were analysed by Dr T. Kawashima, Japan International Research Centre for Agricultural Sciences.

looked. Table 4 shows the mineral composition of PKC. Although few negative papers regarding PKC as animal feed have been published, the author (H.K.) has observed abnormalities of rumen flora and fauna of ruminants fed on PKC. Feeding less than 30% PKC showed no effect. Palm oil is the major agricultural industry in Malaysia and the residues are usually burned. As oil palm bunch ash (OPBA) is strongly alkaline, Murayama and Zahari (1993) studied OPBA as a correctant for soil acidity. Low germination rate and abnormal growth were observed in corn grown on OPBA-applied soil.

Characteristics of the forage material

Forages differ greatly in their composition and structure. Temperate and tropical forages are covered on the outer surface by a thin waxy cuticle, which contains a tough basal polyester layer. Immediately below this is the plant cell, in which the cell walls account for the bulk of the aerial portion of the plant. The cell wall is composed primarily of cellulose and hemicellulose, with a small portion of lignin (van Soest 1982).

The cuticular surface layer presents a formidable barrier to invasion by rumen microorganisms. The cutin layer appears to be totally resistant to microbial digestion within the rumen, except for some rumen fungi which may penetrate it (Ho et al 1988a). Cutin forms the structural component of the plant cuticle and is a polyester C-16 and C-18 hydroxy- and hydroxyepoxy fatty acid. The cutin fraction ranges from 0.2% of the cell wall of wheat straw to 2.4% for mature alfalfa (van Soest 1982). The ester

linkage of cutin is hydrolysed by some pathogenic fungi and some aerobic bacteria. Most of the usable plant nutrients are internal. External plant tissues are only poorly colonised by rumen microorganisms while the inner tissues are heavily colonised. However physical disruption (e.g. chewing) is necessary to allow optimal microbial access to the inner tissues which are then avidly colonised (the inside-out digestion concept; Cheng et al 1990b).

Characteristics of rumen microorganisms

Rumen bacteria

Ruminants posses a complex stomach system, in which the stomach is divided into three or four compartments, the first and the largest of which is the rumen. It is here that continuous anaerobic fermentation takes place by a complex community of microorganisms. The rumen is an extremely complex community of many microorganisms, protozoa, bacteria, fungi and probably other unknown microorganisms (Figure 1). When ruminants are born their rumen is germ-free, the unique flora and fauna start to establish after birth. Once established, the rumen microbial community is very stable and will change only when the nutrients are changed (Cheng and Costerton 1980). This may be the reason that feed efficiency in ruminants has stayed the same in recent decades with the exception of some success using feed additives such as the ionophores, monensin and lasalocid while that of pigs and chickens has greatly improved. Although a definite reason has not been found, it is known that some ruminants, notably the buffalo and the yak, can utilise poor quality feed more efficiently. Our experimental results indicated that the cellulolytic bacteria, Fibrobacter succinogenes (formerly Bacteroides succinogenes; Montgomery, 1988) and Ruminococcus flavefaciens strains isolated from water the buffalo possess a more active cellulase activity than strains isolated from cattle (Tables 5 and 6). This is probably because these animals have been kept on low quality roughage feeds for a long time. There was no difference in numbers of cellulolytic bacteria between water buffaloes and KK cattle in Malaysia. We often detected and isolated antibiotic-resistant strains of rumen bacteria from Indonesian and Malaysian domestic ruminants.

Rumen protozoa

Tropical countries are known to have a wider diversity of flora and fauna, including many toxic plants and highly active microorganisms. As it is known that the ciliate composition varies according to the feeding, geographical distribution and/or physical condition of the host, ciliate composition has been used as an index of the conditions in the rumen. However, the classification of these ciliates is not easy when we study tropical ruminants, because the taxonomy of these ciliates is still inconsistent and furthermore most reports on rumen ciliates have described only those of the ruminants in temperate zones. Tropical ruminants have many characteristics and varieties of ciliates.

Table 5. *Fermentation products produced by strains of* Fibrobacter succinogenes *isolated from steers and water buffaloes.*

Strain		Fermentation products (mM)†							
		Acetate		Butyrate		Succinate		Lactate	
Steer	Water buffalo	Steer	Water buffalo	Steer	Water buffalo	Steer	Water buffalo	Steer	Water buffalo
a	112	5.0	10.1	—	1.3	9.2	11.5	—	—
Z	116	5.2	7.3	—	0.6	8.7	12.7	—	1.2
b	123	4.9	6.1	—	1.2	8.8	12.6	—	1.1
M	127	5.5	7.1	—	0.6	7.9	11.9	—	1.2
C	126	5.5	5.6	—	—	7.7	12.7	—	1.7
A	114	5.0	8.0	—	—	7.9	10.2	—	1.7
D	104	5.2	8.1	—	—	7.5	11.5	—	—
o	121	3.9	7.4	—	1.2	8.7	10.1	—	0.8
O	128	4.8	4.8	—	—	7.5	11.1	—	2.8
d	120	4.6	4.4	—	—	7.6	12.3	—	1.8
V	107	4.2	7.0	—	—	7.9	11.5	—	—

†Cultures were incubated for five days at 39°C in 10 ml of Scott and Dehority's medium with 30 mg of Whatman No. 1 cellulose filter paper.

Table 6. *Fermentation products produced by strains of* Ruminococcus flavefaciens *isolated from steers and water buffaloes.*

Strain		Fermentation products(mM)†					
		Acetate		Lactate		Succinate	
Steer	Water buffalo	Steer	Water buffalo	Steer	Water buffalo	Steer	Water buffalo
1	102	2.9	6.5	2.2	—	4.5	8.5
2	106	3.4	6.9	2.2	—	4.4	6.8
3	115	2.8	6.2	2.0	2.2	4.4	7.8
4	136	3.3	8.7	2.3	0.6	4.1	9.4

†Cultures were incubated for five days at 39°C in 10 ml of Scott and Dehority's medium with 30 mg of Whatman No. 1 cellulose filter paper.
None of the strains produced ethanol.

Protozoa in African ruminants

From the comparative study of ciliate fauna, the presence of a geographical distribution was strongly indicated. For example, Eudiplodinium giganteum, Eudiplodinium kenyense, Enoploplastron stokyi, Diplodinium africanum, Diplodinium nanum and Ostracodinium iwawoi have been found only in Africa (Imai et al 1992). In the percentage composition of genera, the numbers of Entodinium, which normally predominates in the rumen in other areas, were very low in African ruminants. In contrast, the values of the genera belonging to the subfamily Diplodiniinae, such as Eudiplodinium, Ostracodinium and Diplodinium, were high in African ruminants. The ratio of genera is affected by the diet of the host (Hungate 1966). The Diplodiniinae were observed to ingest many fragments of plants. These ciliates have been considered

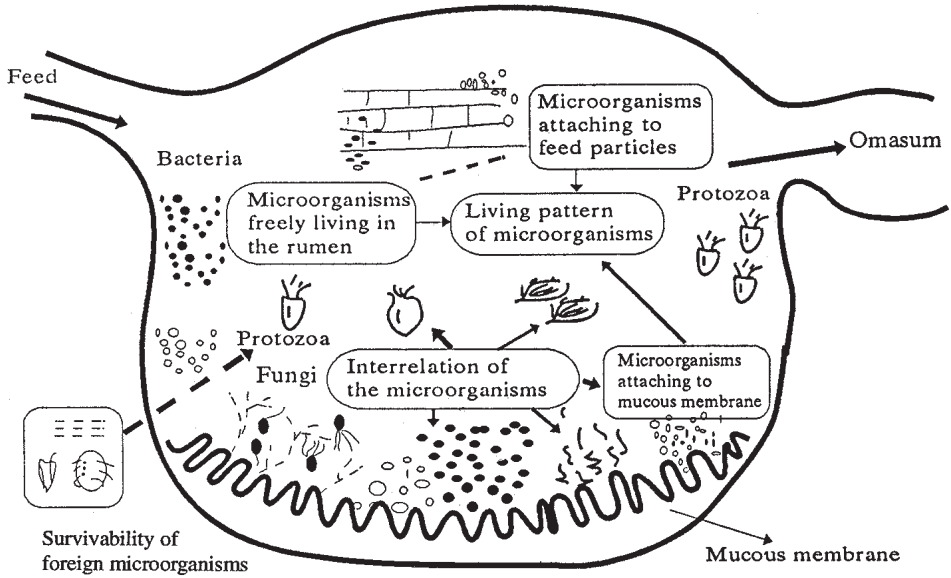

Figure 1. *Social structure of rumen microbial consortia*

to possess cellulolytic activity (Hungate 1978). Thus, the ratio of ciliate genera obtained in African study may reflect the fact that the zebu cattle examined had been fed roughage. The very low ratio of Entodinium might indicate the low amount of starch in the ration since it is known that the entodinia principally utilise starch grains as their energy source (Abou-Akkada and Howard 1960). When comparing the ciliate compositions in East African zebu cattle with those in India (Banerjee 1955; Kofoid and MacLennan 1930, 1932, 1933) and Sri Lanka (Imai 1986; Kofoid and MacLennan 1930, 1932, 1933), a high similarity is found, suggesting a close phylogenetic relationship between East African zebu cattle and Indian zebu cattle (Imai 1988). A similar relation has been found in the distribution of haemoglobin-beta alleles (Namikawa 1980).

Protozoa in Southeast Asia

The ciliate fauna of zebu cattle and water buffaloes in Thailand resembled that of zebu cattle and water buffaloes in Malaysia and the Philippines rather than that of zebu cattle in the Indian area. Although most of the protozoan species detected from water buffaloes and KK cattle in Malaysia have been detected from domestic ruminants and temperate areas, the presence of Metadinium ypsilon and Ostracodinium trivesiculatum is characteristic of Malaysian cattle and water buffaloes (Imai et al 1995). The level of Metadinium and Eudiplodinium, considered to be fermenters of cellulosic materials, was higher in Malaysian cattle and water buffaloes. This suggests that Malaysian cattle and water buffaloes possess a ciliate protozoal composition favourable for digestion of

cellulosic feedstuffs. Of the species which had low incidence, Entodinium longinucleatum spinolobum, E. parvum monospinosum, E. tsunodai, E. bubalum, E. fujitai and E. javanicum have been reported only from ruminants in Southeast Asia (Imai 1985).

Rumen fungi

All the species of rumen fungi so far isolated are capable of fermenting structural carbohydrates of plant cell walls. Rumen fungi in the rumen also contribute significantly to the prime function of the rumen, which is the digestion of plant cell walls to provide fermentation products for the nutrition of the host animal (Orpin and Ho 1991). One of the characteristics of fungi is their penetration into the plant material. Microscopic study indicates that the rhizoid penetrates the tissue, colonising the schlerenchyma and vascular tissues, eventually degrading the schlerenchyma (Ho et al 1988a). The rhizoidal system attaches to the more recalcitrant vascular tissue, resulting in the fungus remaining attached to this tissue despite the degradation of surrounding cells. In this way, the fungus remains attached to the tissue and is not washed out of the rumen with the liquid phase of the rumen contents. Attachment to plant cell walls within the digesta fragments is, in some species, by way of appressoria (Ho et al 1988b).

Comparison of fungal strains between those from temperate areas and tropical areas has been difficult, as the practical transportation of fungal strains has not yet been established. Although we do not yet have a reliable method to determine fungal numbers, in general, the higher the fibre content of the diet, the is higher the population density of rumen fungi (Orpin and Ho 1991). It is interesting that as the digestibility decreased and lignin content increased, fungal populations increased (Orpin 1977).

Plant toxins and rumen microorganisms

Leucaena is widely grown in tropical and subtropical countries. The plant is potentially an excellent source of crude protein. However, its use as a feed has been limited because it contains mimosine, a toxic amino acid that causes many undesirable problems (low weight gains, poor health condition and hair loss, etc.) in both ruminants and non-ruminants. Mimosine toxicity is more acute in non-ruminants owing to the absence of endogenous microorganisms capable of enzymatic detoxification. Mimosine is hydrolysed in the rumen by microbial enzymes to 3,4 DHP, a potent goitrogen, and sometimes to 2-hydroxy-3-(1H)-pyridone (2,3 DHP), a structural isomer of 3,4 DHP which is probably as goitrogenic as 3,4 DHP.

Previous Canadian (Kudo et al 1984, 1986) and Malaysian (Kudo et al 1989b, 1989c) studies on the in vitro metabolism of mimosine showed that degradation occurred in the microbial fraction and that concentrate diets increased the microbial population that degraded mimosine, but probably not sufficient to completely escape from mimosine poisoning. When we examined the rate of mimosine degradation in the rumen fluid of cattle fed five different diets, significant (Pÿ<ÿ0.01) differences were seen between the highest rate on a blue grass-molasses diet and the lowest rate on corn silage. The highest rate obtained with bluegrass (*Poa prantensis)*-molasses suggests

that high rates of metabolism can also be associated with inocula from diets other than concentrates. Intermediate rates were obtained with alfalfa hay, fresh alfalfa herbage and orchard grass hay (Dactylis glomerata) but these rates were not significantly different. In vitro rates for mimosine and DHP degradation using ruminal contents were conducted in Malaysia during 1988 (Kudo et al 1989b). All rumen samples from cattle, sheep and water buffaloes produced no disruption of the heterocyclic ring, suggesting a complete absence of DHP detoxification. In 1989, the active rumen fluid from Indonesian goats fed on and adapted to Leucaena was infused into one of these cattle. Not only the infused animal but also the untreated neighbouring animals gained the ability to detoxify DHP (Kudo et al 1989c, 1990c). During this study, we established that the ability to detoxify DHP is very rapidly passed from treated to untreated neighbouring animals. To date, only the rumen microorganisms in Hawaii (Henke 1958; Jones 1981) and Indonesia (Kraneveld and Djaenoedin 1947) have been shown to detoxify mimosine in vivo and protect the animals from any ill effects caused by the plants. Thus, ruminants in some parts of the world possess microorganisms capable of mimosine detoxification. It was demonstrated that Australian cattle could be protected from mimosine poisoning by transferring rumen fluid obtained from Hawaiian goats that can degrade DHP (Jones and Megarrity 1986). A rumen bacterium capable of DHP degradation was recently isolated from the rumen contents of a goat from Hawaii (Allison et al 1992). Recent grazing trials with Leucaena pastures have also shown that transfers of DHP-degrading bacteria can protect cattle in both Australia and North America (Hammond et al 1989; Pratchett et al 1991; Quirk et al 1988). We should note that in tropical countries the quality of feed supplied, including that supplied to laboratory animals, is unsatisfactory, varying from batch to batch. Some feeds contain toxic substances, which cannot be degraded even with the special capabilities of rumen microorganisms.

Factors affecting feed degradation

Microbial attachment

Recent studies have demonstrated that, for the digestion of cellulose (Figures 2 and 3) and starch, attachment to these insoluble substrates is a pre-condition for both pure cultures and the natural mixed population if digestion is to proceed. Cellulolytic species differ in the nature of their attachment to insoluble substrates and in the nature of their enzymatic attack (Kudo et al 1987b; McAllister et al 1990; Minato and Suto 1978). When these substrates are placed in the rumen, they attach to substrates very rapidly (< 15 min). Among the major cellulolytic bacteria in the rumen, F. succinogenes attaches very closely to the substrate while R. flavefaciens attaches at a small distance and R. albus remains at a much greater distance. This spatial distribution appears to be dictated by the width of the glycocalyx structure that these species use to attach to cellulose but, in all three of these bacterial species, their cellulases reach and digest the colonised cellulose substrate. Similarly, the digestion of starch is affected by the structure of the glycocalyx of the amylolytic organisms (Cheng et al 1990a). Amylase

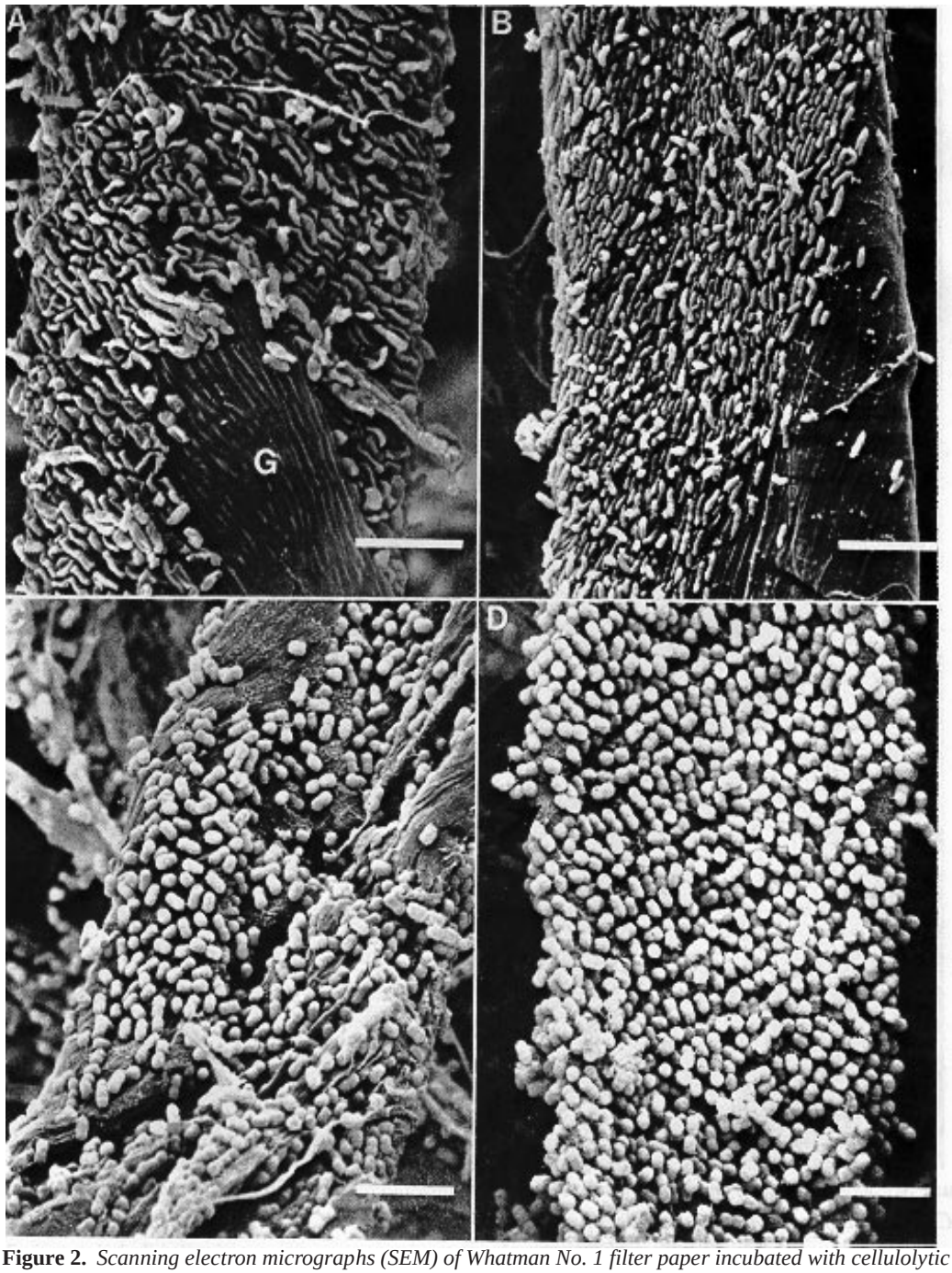

Figure 2. *Scanning electron micrographs (SEM) of Whatman No. 1 filter paper incubated with cellulolytic bacteria at 39°C for 20 h showing extensive bacterial adhesion to cellulose fibres. (A)* Fibrobacter succinogenes *BL 2. Note that grooves (G) have formed as a result of cellulose digestion and that some deformation of cells is evident. (B)* Fibrobacter succinogenes *E. Note that the bacterial cells attach avidly to the fibre and are oriented in the same direction as the cellulose subfibres. (C)* Ruminococcus flavefaciens *1. (D)* Ruminococcus flavefaciens *2. Note that the extensive secretion of exopolysaccharide glycocalyx material by these organisms has produced a fibrous network (C and D), arrows seen here after dehydration during preparation of SEM. (Bars = 5* μm*; source: Kudo et al 1987b).*

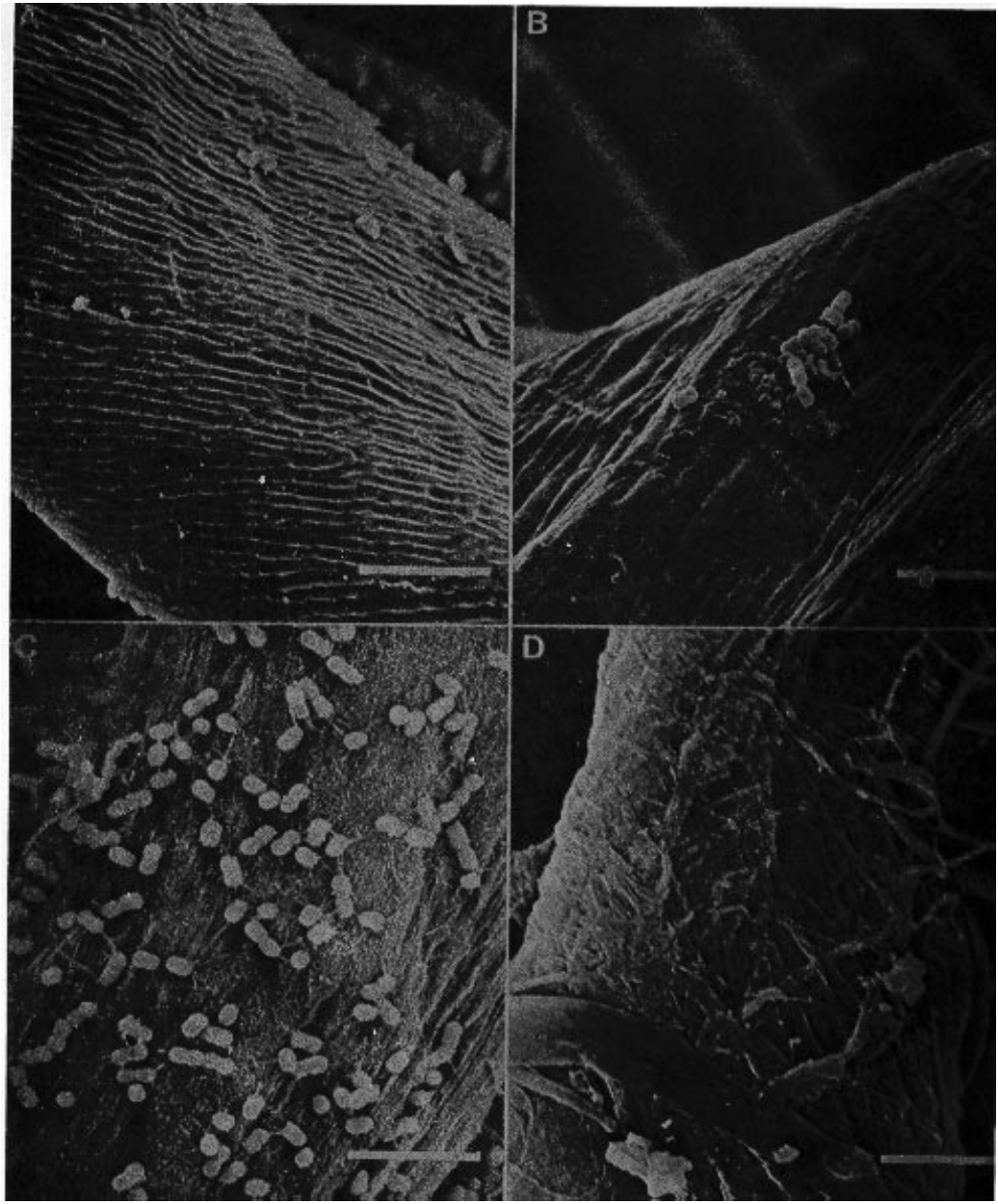

Figure 3. *Scanning electron micrographs (SEM) of bacterial detachment from Whatman No. 1 filter paper. (A–C) Filter paper was first incubated with cellulolytic bacteria at 39°C for 20 h and then treated with 0.1% methylcellulose at 39°C for 1 h. These SEM show the very effective detachment of cellulolytic bacteria from their cellulosic substrata that is caused by methylcellulose. (A) Fibrobacter succinogenes BL2. Note very regularly formed parallel grooves, as evidence of fibre degradation, and the complete absence of adherent bacteria. (B) Ruminococcus albus SY3. There is some irregular evidence of digestion, in the form of grooves or pits, and only a few adherent cells remained. (C) Ruminococcus flavefaciens 1. Note that some cells are still attached to the fibre by means of fibrous extracellular material that formed a network over the cellulose surface and that no grooves or pits are formed as a result of digestion. (D) Filter paper was incubated with cells of Fibrobacter succinogenes at 39°C for 20 h, in the presence of 0.1% (w/v) methylcellulose, and no bacterial adhesion or evidence of digestion can be seen on the cellulose fibre surfaces. (Bars = 5 μm; source: Kudo et al 1987b.)*

producers adhere to starch but not to cellulose, while cellulose decomposers adhere to cellulose but not to starch (Minato and Suto 1978). Cheng and Costerton (1980) postulated that rumen microorganisms can be classified into three groups: (i) microorganisms attaching to the rumen wall; (ii) microorganisms living freely in the rumen; and (iii) microorganisms attaching to feed particles. In the rumen, as much as 75% of these microorganisms are attached to feed particles. Microorganisms in the rumen have a variety of surfaces to which they may attach, and a distinct population of microorganisms adheres to each different surface. From an ecological viewpoint, bacteria with the ability to attach to feed particles have a great advantage over non-attaching microorganisms, which can flow more quickly from the rumen. In addition to adhesion, ingestion of feed particles was observed in protozoa (Figure 4). The importance of the adhesion of microorganisms to substrates suggests that there is a possibility for better feed efficiency if we can increase the attachment.

Digestive consortia

Pure cultures of cellulolytic bacteria and fungi digest cellulose in vitro but digestion does not proceed at a similar rate to that seen in the rumen unless consortia are formed with non-cellulolytic Treponema bryantii, Butyrivibrio fibrisolvens and methanogenic bacteria. Electron microscopy of partly digested plant material shows that consortia of F. succinogenes, R. flavefaciens and R. albus are in direct contact with the cellulose fibres, while cells of Treponema species, Butyrivibrio species and methanogenic bacteria are more loosely associated. Morphological examination, by electron microscopy, showed that the cells of T. bryantii associate with the plant cell wall materials in straw, but that cellulose digestion occurs only when these organisms are present with cellulolytic species such as F. succinogenes. These results show that cellulolytic bacteria interact with non-cellulolytic Treponema to promote the digestion of cellulosic materials (Kudo et al 1987a). Recent studies have shown that both protozoa and fungi may be active members of consortia (Ho et al 1988b; Imai et al 1989; Kudo et al 1990a). We have recently discovered that mycoplasma tend to be associated with most rumen fungal cultures (Kudo et al 1990b). We have confirmed that the rumen fungal cultures, reportedly pure, were also contaminated with these cell wall-deficient bacteria. Thus, the microbial ecology of the rumen is obviously very complex, including many interactions of microbial species and even though primary cellulolytic organisms may be present in a system, many other factors may be required to facilitate actual cellulose digestion.

Practical applications in animal production

The results of up to date ruminant production indicate that immediate improvements in feed efficiency in ruminants may not be expected. However, there is some potential. Some of the forages and by-products, for example Leucaena and palm oil by-products, may require manipulations to reduce the toxicity. PKC is used widely as animal feed but its high concentration of copper and imbalance of Ca/P cannot be ignored.

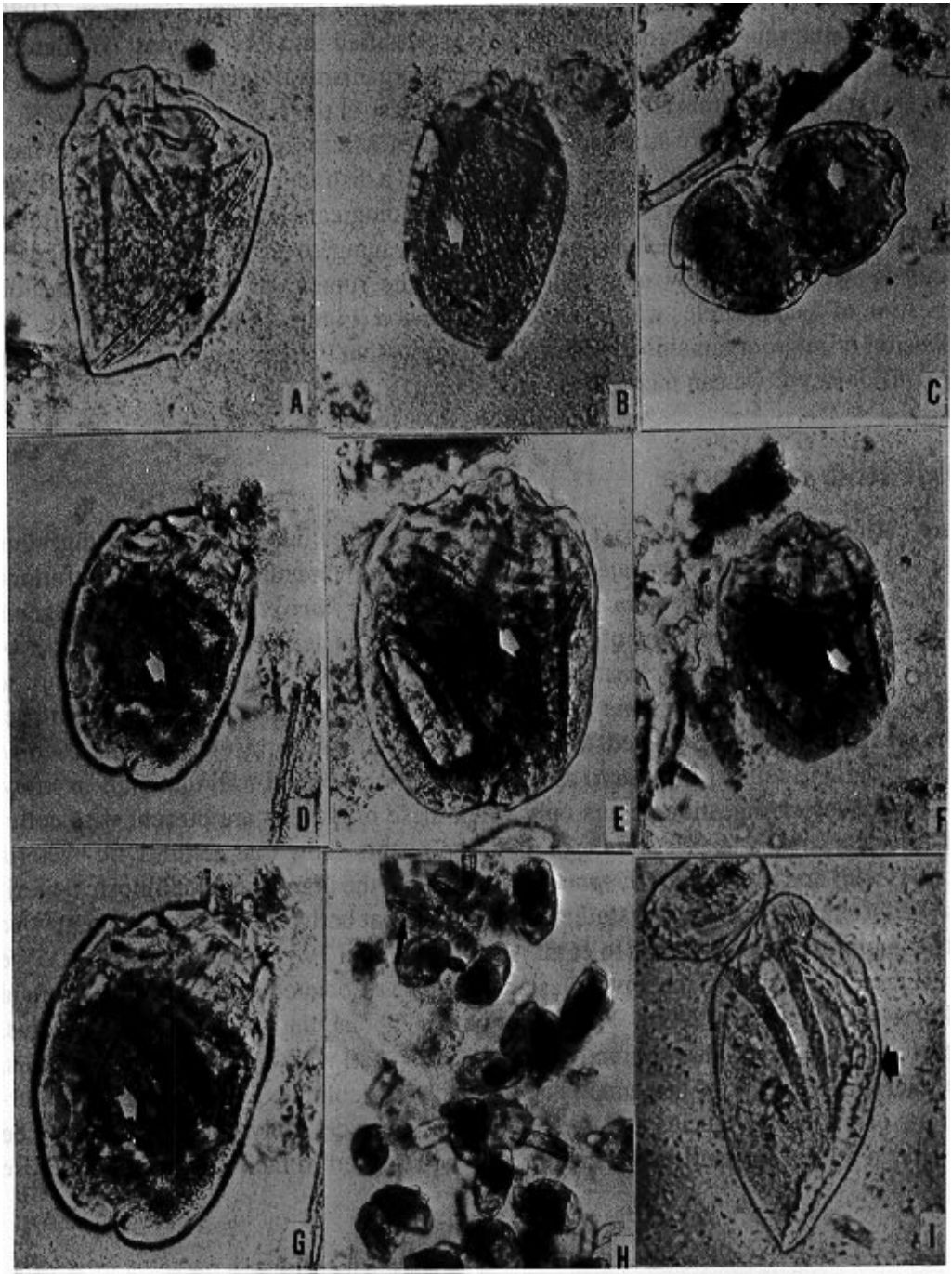

Figure 4. *Light photomicrographs showing rumen ciliates that are uniquely found in cattle fed only barley straw. All ciliates contain fragments of barley straw (arrows) inside the cell. (A)* Ostracodinium clipeolum. *(B)* Ostracodinium obtusum. *(C)* Polyplastron multivesiculatum, *in binary fission. (D–G)* Polyplastron multivesiculatum. *(H) Group of* Diploplastron *and* Diplodinium. *Structures surrounding the protozoa are considered to be molds. (I)* Polyplastron multivesiculatum. *Transformation of the shape of cell outward due to the plant material ingested is noted (source: Kudo et al 1990a).*

Alfalfa (Medicago sativa L.) is a valuable leguminous forage crop with a high yield and an excellent nutritive value. However, the risk of bloat caused by the high initial rates of microbial digestion and nutrient release from the more digestible leaves often limits its use. We (Kudo et al 1985) have shown that through plant breeding programmes this adverse effect can be limited in order to reduce the high initial rate of digestion and provide an engineered forage that is equivalent to the original plant but does not have an adverse effect on the ruminant. This concept could be applied to low-quality forages in tropical countries by similar plant breeding programmes to increase the initial rate of digestion. Tropical ruminants are known to be better converters of poor quality feed. Therefore, breeding based on this fact and the better chewing characteristics of certain individual animals may improve animal production in tropical countries.

Problems in rumen biotechnology

The present approach in biotechnology has many problems if we expect the genetic engineered microorganisms to survive in the rumen. For the successful development of rumen microbiology, it is very important to study and to include more basic characteristics of rumen microorganisms including the ecosystem. Otherwise, further development in rumen and ruminant studies would not be expected (Ling 1994).

Electron microscopy revealed that when grass leaves (Akin 1976a, 1976b; Akin and Amos 1975; Akin et al 1974) or straw (Cheng et al 1984) were exposed to natural populations of rumen bacteria, the thick highly structured cellulosic cell walls of these plant materials were seen to be colonised by pleomorphic cells of F. succinogenes. Attempts to isolate F. succinogenes from the rumen and to maintain active cellulolytic activity under laboratory conditions have not always been successful (van Gylswyk and Schwartz 1984; Stewart et al 1981). These difficulties limited the number of F. succinogenes used in in vitro studies to only a few strains (Bryant and Doetsch 1954; Bryant et al 1959; Dehority 1963; van Gylswyck and Schwartz 1984; Mackie et al 1978; Stewart et al 1981). Many of the cellulolytic bacteria were isolated a long time ago. For example, F. succinogenes S85, which is one of the most popular cellulolytic bacterium in the laboratory, was isolated over at least 40 years ago (Bryant and Doetsch 1954). Growth of fresh isolates of F. succinogenes in liquid glucose medium is very good but some strains would not grow after eight to ten subcultures in this medium. F. succinogenes S85 can survive in glucose medium although great loss of cellulolytic activity is evident. Most strains of fresh isolates of Ruminococcus sp. cannot utilise glucose (Hungate 1966) but most ‘laboratory strains’ of Ruminococcus sp. can utilise glucose. Continued growth of N. patriciarum in vitro, with glucose as carbon source, resulted in a loss of ability to utilise cellulose for growth, but little loss of activity was evident when grown on plant tissues or cellulose (Orpin and Ho 1991). We have confirmed this with other rumen fungi, Neocallimastix, Piromyces, Sphaeromonas and Orpinomyces. In addition, cellulolytic microorganisms do not belong to the population freely living in the rumen but they belong to the population attached to feed particles. Therefore, we should use only insoluble cellulosic materials when subculturing cellulolytic microorganisms.

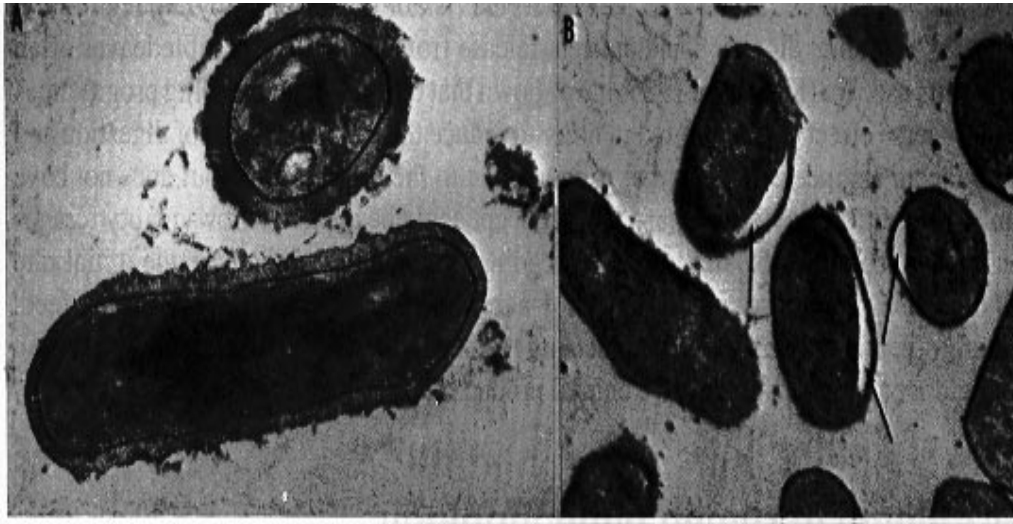

Figure 5. *Transmission electron micrographs (TEM) of ruthenium red-stained pure culture of* Bifidobacterium. *(A) Very thick irregular Gram-positive cell wall* (ca. *0.2* μm*). (B) The thin regular Gram-positive cell wall* (ca. *0.05* μm*) and the formation of spaces between the cell and the cytoplasm (arrows). This naked bacterial strain is probably a mutant strain produced and adapted under laboratory conditions (source: Kudo et al 1989a).*

The bacteria that comprise the wall-associated biofilm population produce very large amounts of exopolysaccharide glycocalyx material when grown in culture immediately after isolation from the rumen (McCowan et al 1980). However, this elaboration of slimy material was seen to decrease sharply upon subculture and almost entirely after five to ten subcultures. We have shown the possible mutation of some strains of Bifidobacterium sp. which lacks extracellular glycocalyx material (Kudo et al 1989a; Figure 5). This 'naked' Bifidobacterium sp. is probably a mutant strain produced and adapted under laboratory conditions. To generate and maintain a glycocalyx, a bacterial cell must expend energy, and in the protected environment of pure culture the glycocalyx is a metabolically expensive luxury conferring no selective advantage. Cells that fabricate these elaborate coatings are usually eliminated from pure cultures by uncoated mutants that can devote more of their energy budget to proliferation. The bacterial glycocalyx was ignored because the familiar pure laboratory strains do not need it and therefore do not fabricate it (Costerton et al 1978). Therefore, the 'naked' laboratory strains of bacteria have an advantage in laboratory conditions as an energy saver but not in natural conditions. Another example of mutation in the laboratory is Streptococcus bovis. On initial isolation the rumen strains of this species are sensitive to oxygen. Later, this sensitivity diminishes, and the cells grow aerobically (Hungate 1966).

Most researchers subculture fungi at five-day intervals. Fresh isolates of fungi require three-day intervals of subculture if we do not want to lose any of the cultures. Practically this is difficult and sometimes the subcultures are done at four- to six-day intervals and we lose the strains. So finally we have the cultures which can survive

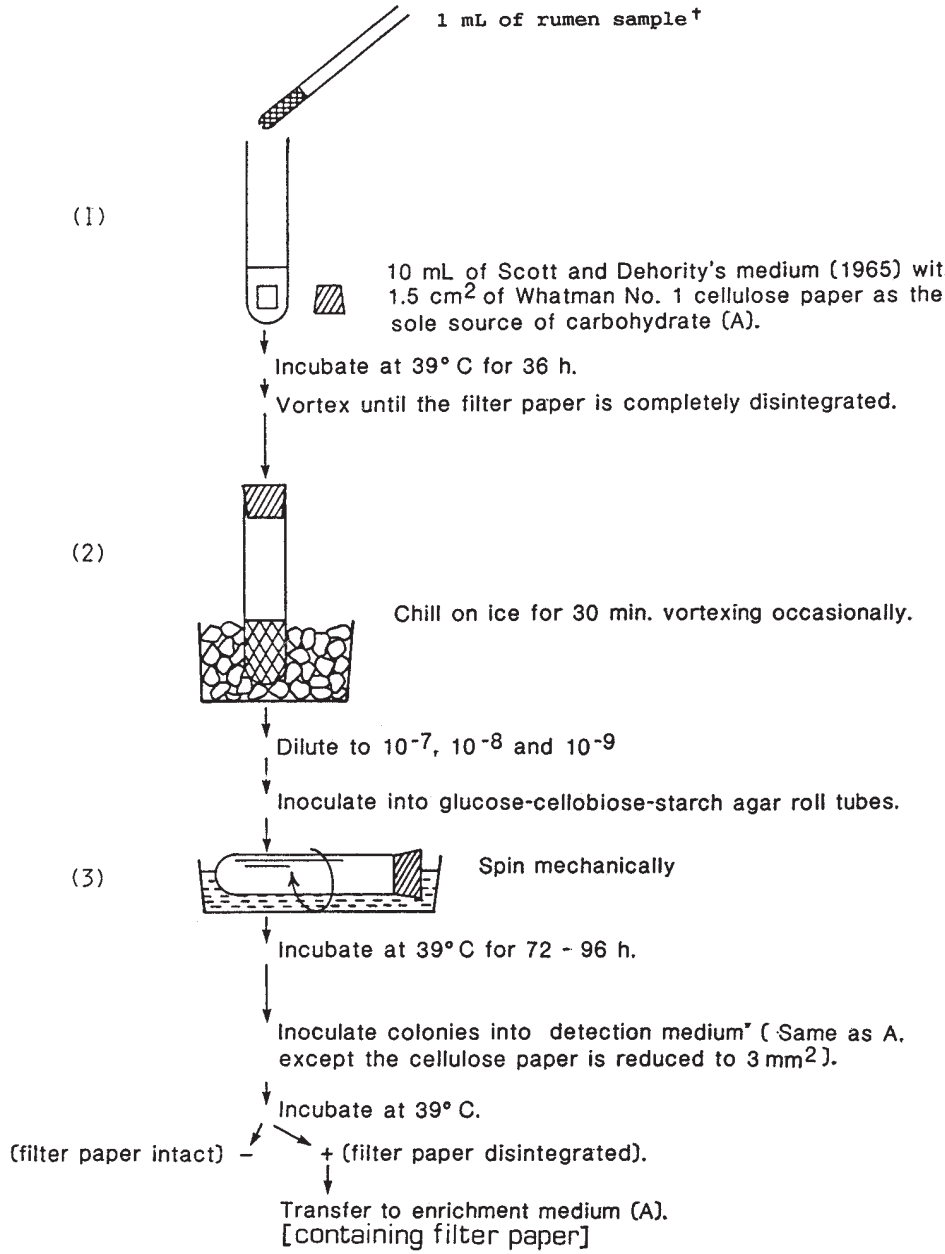

Figure 6. *Simple isolation method for rumen cellulolytic bacteria. Rumen sample*† *: Take one part of rumen fluid and one part of solid contents (preferably taken from the upper one-third of rumen contents), blend for 30 s at low speed, 30 s rest, 30 s at high speed, squeeze through two layers of cheese cloth or gauze and use as inoculum. Incubation time may vary. If* F. succinogenes *is desired use Whatman No. 5 or 6 cellulose paper (crystalline cellulose) and use amorphous (e.g. ball-milled) cellulose for* Ruminococcus albus, *which cannot digest crystalline cellulose. Use only solid cellulosic materials in the medium for subculture and never use soluble carbohydrates (especially glucose) in the medium as these dramatically reduce cellulase a ctivities.*

five-day intervals of subculture. Thus, laboratory strains, especially cellulolytic bacteria, that are currently used have many problems. Therefore, we have developed a simple isolation method for cellulolytic bacteria using medium enriched with Whatman cellulose filter paper (the type of the papers should be chosen according to the target cellulolytic bacteria) as the sole selective substrate (Figure 6). Once isolated, soluble carbohydrates (especially glucose) should not be used as carbon sources in the medium as these reduce the enzyme activities.

The author (H.K.) criticised genetic engineering but this does not mean the denial of biotechnology. We should study and include more basic characteristics of rumen microorganisms, including ecosystems which have been ignored totally. Genetically engineered microorganisms from 'laboratory strains' might not survive in the rumen if we introduce them back into the rumen. Even laboratory strains might not survive in the rumen any more. Generally, in case of aerobic microorganisms, substrates are utilised effectively towards production of microbial cells and metabolic products are mostly released as carbon dioxide and water. In contrast, anaerobic bacteria produce less microbial cells and large amounts of organic materials (ethanol, methane, volatile fatty acids, non-volatile fatty acids, etc.) are accumulated. Thus, rumen microorganisms have a potential for practical applications in other industries such as food and fermentation industries, although up to now they and other related microorganisms have not been used in other areas.

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An important role for ruminal anaerobic fungi in the voluntary intake of poor quality forages by ruminants

G.L.R. Gordon1, C.S. McSweeney2 and M.W. Phillips1

1.ÿDivision of Animal Production
Commonwealth Scientific and Industrial Research Organisation (CSIRO)
Prospect, Sydney, New South Wales 2148, Australia

2.ÿDivision of Tropical Animal Production
Commonwealth Scientific and Industrial Research Organisation (CSIRO)
Long Pocket, Brisbane, Queensland 4068, Australia

Abstract

Anaerobic fungi are prevalent in the rumen of grazing ruminants where they are closely associated with plant particles. An important role for these unusual microorganisms in ruminant nutrition has been proposed over the course of the past decade or so. The significance of this role was based largely on in vitro studies of anaerobic fungi. However, there was very little direct evidence from studies with animals to support this position until recently. It is now known that anaerobic fungi make a significant contribution to the intake of poor quality pasture. There may also be a contribution to forage digestion in the rumen, although much of the fermentation of structural polysaccharides in the rumen is attributed to anaerobic bacteria, either alone or in association with ciliate protozoa. Fungal populations in the rumen are known to be limited by a low sulphur content of both rough pastures and poor quality crop residues. Where appropriate, fertilisation of the growing pasture with sulphur improves the sulphur content of feed which subsequently is eaten in greater quantities by ruminants with ruminal populations of anaerobic fungi returned to 'normal'. Alternatively, supplementing the feed with a suitable sulphur compound is also effective in improving the voluntary intake of digestible feed both with crop residues and harvested rough pastures. In addition, considerable potential exists for the selection and intra-ruminal inoculation of superior strains of anaerobic fungi obtained from either domesticated or wild herbivorous mammals. These possible mechanisms for improving forage intake by ruminants grazing rough pastures through the manipulation of anaerobic fungi in the rumen have implications for ruminant production in Africa.

Introduction

Anaerobic chytridiomycete fungi have been known to be a component of the rumen microbiota for 20 years (Orpin 1975). Together with the anaerobic bacteria and ciliate

protozoa, they are responsible for the production of hydrolytic enzymes which degrade dietary polysaccharides and other carbohydrates and the fermentation of the resulting monosaccharides. An important role for anaerobic fungi in the rumen has long been proposed (Akin et al 1983; Bauchop 1979; Orpin 1977). Until recently, there was little or no direct evidence for the magnitude of the contribution made by anaerobic fungi to digestion in the rumen. However, it is now known that anaerobic fungi are extremely important for the voluntary feed intake of poor quality, mature herbage by sheep (Gordon and Phillips 1993).

In our paper we present some of the evidence which supports this conclusion and discuss some of the possible reasons for it. Also, we consider some ways in which the benefits obtainable from anaerobic fungi could be harnessed in domesticated ruminants in semi-arid regions of Africa. There are a number of recent reviews (Fonty and Joblin 1991; Li and Heath 1993; Teunissen and Op Den Camp 1993; Trinci et al 1994; Wubah et al 1993) which should be consulted for detailed information on the biology of anaerobic fungi.

Contribution of anaerobic fungi to voluntary feed intake and the nutrition of ruminants

A definite positive relationship exists between the presence of anaerobic fungi in the rumen and the voluntary intake of herbage diets of low digestibility (Akin et al 1983; Gordon 1985; Gordon and Phillips 1993; Morrison et al 1990; Weston et al 1988). This is quite possibly a result of fungal attack of lignified plant tissues (Akin 1987; Akin and Borneman 1990) with the resultant weakening of these tough plant components (Akin et al 1983, 1989).

Table 1 shows that the removal of anaerobic fungi from the rumen of sheep reduced the voluntary intake of poor quality feed by about 30%, with little effect on the populations of bacteria and ciliate protozoa. Ruminal fungal activity is often accompanied by increased feed digestibility in vivo (Gordon and Phillips 1993; Weston et al 1988). An oral inoculum of anaerobic fungus stimulated hay intake in early weaned calves (Theodorou et al 1990). A plan for selecting appropriate strains of anaerobic fungi for inoculation into the rumen of mature ruminants at pasture has been proposed (Gordon 1990).

Anaerobic fungi have the potential of contributing to the protein supply of the host animal. They are proteolytic and may contribute to ruminal protein degradation (Wallace and Munro 1986), although the extent of this contribution remains to be conclusively determined (Bonnemoy et al 1993). Fungal cells are composed of proteins with a well balanced combination of amino acids (Gulati et al 1989, 1990; Kemp et al 1985) which are highly digestible and available to the ruminant host (Gulati et al 1989, 1990). Until an accurate method of measuring the biomass in the rumen is developed (Faichney et al 1991), the possible extent of the fungal contribution to protein supply is largely conjecture. However, should an increase in the biomass of ruminal fungi prove to be feasible, it is unlikely that the supply of high-quality microbial protein to the host ruminant would be diminished.

Table 1. *The effect of the removal of anaerobic fungi from the rumen of sheep fed on a straw-based diet and the subsequent reinoculation of a* Neocallimastix sp. into the rumen on voluntary feed intake, feed digestion, rumen parameters and microbial populations.

Parameter	A. Pretreatment	B. No fungi	C. With fungi added
Intake (g/d)			
OM	894 (30.8)	628 (36.4)	877 (52.6)
ADF	390 (9.3)	264 (16.4)	373 (22.6)
Digestibility (%)			
OM	53.2 (1.30)	50.3 (1.38)	57.3 (1.02)
ADF	51.2 (1.03)	46.5 (1.28)	55.0 (1.64)
Rumen pH	6.8 (0.05)	7.0 (0.05)	6.6 (0.07)
Rumen ammonia (mg N/l)	46 (4.9)	50 (10.8)	32 (9.0)
Rumen VFA (mM)	69 (5.1)	55 (10.8)	78 (2.4)
molar proportions:			
acetic	0.69	0.56	0.72
propionic	0.19	0.34	0.19
butyric	0.09	0.07	0.07
branched chain	0.03	0.03	0.02
Anaerobic fungi			
Rumen (zoospores/ml; $\times 10^3$)	7.6 (4.0)	UD	19 (5.7)
Faeces (zoospores/g; $\times 10^3$)	5.2 (2.9)	UD	11 (5.6)
Bacteria (cells/ml)			
total viable ($\times 10^9$)	0.8 (0.18)	1.4 (0.37)	1.6 (0.33)
cellulolytic ($\times 10^8$)	0.4 (0.19)	2.6 (1.48)	1.1 (0.29)
Ciliate protozoa			
(cells/ml; $\times 10^5$)	3.8 (0.92)	3.0 (0.53)	4.8 (0.49)

Data are mean (SE) for four sheep.
ADF: acid-dergent fibre; OM: organic matter; VFA: volatile fatty acid; UD: undetectable.
Data from Gordon and Phillips 1993.

Effect of defaunation of the rumen on populations of anaerobic fungi

The treatment of ruminants with defaunating agents (usually ionic detergents) to remove ciliate protozoa from the rumen (Bird 1989) has a secondary effect; it results in increased populations of ruminal fungi (Soetanto et al 1985). Therefore an inverse relationship between the sizes of the fungal and the ciliate populations in the rumen is apparent, at least in herbage-fed animals. This relationship has been confirmed in several studies (Hsu et al 1991; Romulo et al 1986, 1989). Defaunation can also be accomplished through non-chemical methods and the same general inverse relationship between anaerobic fungi and ciliates is frequently (Newbold and Hillman 1990; Ushida et al 1990) but not always (Ushida et al 1990; Williams and Withers 1991) observed. The underlying mechanism by which defaunation increases the fungal population is possibly a reduction in the turnover of fungal protein in the rumen (Newbold and Hillman 1990). Therefore, it is likely that the nutritional benefits due to defaunation of animals being fed poor-quality, low-nitrogen herbage is at least in

part a result of increased fungal numbers with their associated degradative activity against plant fibre.

Effect of dietary sulphur on anaerobic fungi in the rumen

Early in the study of anaerobic fungi, it was recognised that the sulphur content of hay diets or pasture was a significant factor governing the fungal population in the rumen (Akin et al 1983; Gordon 1985). When sulphur was present in the diet at levels of around 1.0 g S per kg organic matter or less, anaerobic fungi were apparently absent from the rumen of sheep fed on hay made from the tropical pasture grass Digitaria pentzii (Akin et al 1983). The size of the anaerobic fungal populations in the rumen increased dramatically after either an application of a sulphur fertiliser to the pasture used to make the hay (Akin et al 1983) or a sulphur supplement to the low-S hay (Gordon et al 1984). Fertilisation of the pasture resulted in an average increase of 38% in ad libitum feed intake (Akin et al 1983; Gordon 1985). A diet of another tropical grass hay (speargrass, Heteropogon contortus), which had a low sulphur content, also resulted in an undetectable fungal population in the rumen (Morrison et al 1990). On the other hand, diets of cereal straw (usually wheat straw) which were low in sulphur supported a low, but detectable, population of anaerobic fungi (Gordon et al 1983; Gulati et al 1985; Weston et al 1988). Some of the results of these studies are summarised in Table 2. The apparent relationship between the declining sulphur content of a pasture and a declining ruminal fungal population did not apply to a ryegrass pasture in Scotland where anaerobic fungi increased with increasing pasture maturity which was accompanied by a declining sulphur content (Millard et al 1987). Therefore, the form of sulphur in the feed (e.g. inorganic-S, non protein-S, protein-S) may be as important as its total sulphur content in determining the ruminal fungal population.

Herbage diets with a low content of sulphur can have a negative effect on the size of the fungal population in the rumen. In some cases the fungi are apparently absent from the rumen (Akin et al 1983; Gordon 1985; Morrison et al 1990), whereas in other cases the fungal populations are greatly reduced (Gulati et al 1985; Weston et al 1988). In all cases, supplementation of these straws with several different types of sulphur allowed fungi to proliferate in the rumen and resulted in increased voluntary feed intake. At the same time, there was little or no change in the ruminal populations of bacteria and ciliate protozoa due to dietary supplementation (Akin et al 1983; Gulati et al 1985; Morrison et al 1990). Diets of low-sulphur Digitaria have been successfully supplemented with methionine and elemental sulphur (each about 1 g S/d per head; Gordon 1985; Gordon et al 1984), and cereal straw supplemented with either methionine (Gordon et al 1983; Gulati et al 1985) or sulphate (Weston et al 1988) supported a greatly increased number of anaerobic fungi in the rumen. Heteropogon (speargrass) supplemented with sulphate supported a higher fungal population in the rumen compared with the same hay when unsupplemented (Morrison et al 1990). Anaerobic fungi grown in vitro require reduced forms of sulphur (Orpin and Greenwood 1986; Phillips and Gordon 1991) indicating the need for reduction of supplementary sulphate in the rumen before it can be available

Table 2. *Influence of sulphur in the diet on feed intake of poor quality forages by sheep.*

Low S feed	S in feed[†] (g S/kg OM)	S added to diet as:	Increased ruminal population of of fungi after S addition	Change due to S addition		Reference
				Voluntary OM intake	Digestible OM intake	
Digitaria pentzii (grass hay)	0.8–1.1	fertiliser	presumed	+40%	+31%	Rees et al (1982)
		on pastur	known	+36%	ND	Akin and Hogan (1983)
		methionine	known	+11%	ND	Gordon (1985)
		sulphur	presumed	+6%	–4%	Rees et al (1982)
Wheat straw (alkali-treated)	0.7	methionine	known	+6%	+23%	Gulati et al (1985)
		sulphate	known	+7%	+16%	Weston et al (1988)
Heteropogon contortus (speargrass hay)	0.5	sulphate	known	+75%	+96%	Morrison et al (1990)

OM: organic matter; ND: no data.

[†]S content of diets increases to 1.3-1.7 g S/kg OM after either fertilisation of the pasture or use of supplement to diet.

for anaerobic fungi. However, a sulphur supplement which is either specific for anaerobic fungi in the rumen or relatively so is still to be discovered.

Influence of dietary phenolics on anaerobic fungi

Early studies showed that anaerobic fungi preferentially colonised the sclerenchyma patches of tropical grass leaves (Akin and Hogan 1983; Akin et al 1983) which suggested an affinity for lignified tissues. Subsequently, selected strains of anaerobic fungi were found to solubilise about 35% of the label from a 14C[lignin]lignocellulose preparation (Gordon 1987; Gordon and Phillips 1989b) whereas the consistent loss of acid-detergent lignin from wheat straw was not demonstrated (Gordon and Phillips 1989a). However, around 34% of the lignin component of sorghum stem was removed by the anaerobic fungus Neocallimastix patriciarum (McSweeney et al 1994) confirming that 'core' lignin can be attacked by at least some anaerobic fungi. Pure cultures of anaerobic fungi are unable to mineralise lignin-derived phenolics to CO2 (Bernard-Vailh, et al 1995; Gordon 1987).

Phenolic compounds, derived from lignocellulose within the rumen, are a potential barrier to the degradation of structural polysaccharides in the rumen (Wilson 1994). Ferulic and p-coumaric acids inhibited fibre degradation by mixed anaerobic fungi in vitro (Akin and Rigsby 1985; Tanaka et al 1991). These phenolic compounds also inhibited fibre degradation by pure cultures of both monocentric and polycentric anaerobic fungi, with polycentric strains generally being less sensitive than the monocentric strains (G. Gordon, H.K. Wong and M. Phillips, unpublished observations). The inhibition by plant phenolics is potentially significant because anaerobic fungi produce powerful hydrolytic enzymes for releasing ferulic and p-coumaric acids from plant cell walls (Borneman et al 1990). Even though the infusion of free phenolic acids into the rumen of sheep had no effect on digestion of herbage by sheep (Lowry et al 1993), the localised concentrations of phenolics liberated by fungal enzymes in the rumen may be high enough to hinder fungal growth and activity.

The deleterious effects of another form of dietary phenolics, the plant tannins, on rumen function have been broadly characterised but the ability of the rumen microbial population to interact with and adapt to the presence of tannins has received little attention. A better understanding of interactions between tannins and the rumen microbial population will enable us to capitalise on the beneficial attributes of tannin-containing plants.

The minimum inhibitory concentration of tannin appears to be much higher for fungi than bacteria (Scalbert 1991). This would imply that anaerobic ruminal fungi may be more tolerant of tannin than the bacteria which compete with them in the rumen. The tolerance of rumen fungi to tannins was demonstrated by the ability of *Neocallimastix patriciarum* to degrade cellulose effectively in the presence of 100 ug per ml condensed tannin (CT; from birdsfoot trefoil, *Lotus corniculatus;* McAllister et al 1994).

Also, it is thought that the protein complexed with tannin is in a form unavailable to the microorganisms of the rumen. Cleavage of a proportion of these tannin-protein complexes may be achieved by modifying the microbial population. Protein availability in the rumen would then be optimised while allowing sufficient complexed plant protein to bypass the

Table 3. *Growth of several strains of ruminal fungi in the presence of condensed tannins (CT) purified from* Calliandra calothyrsus *(C.S. McSweeney, unpublished data). Growth was strong (+++), moderate (++), weak (+), or absent (–) compared with controls that lacked free CT or CT-protein complexes.*

	Free CT[†]		CT-protein complex[‡]	
Fungus	150 μg/ml	300 μg/ml	$+NH_3$	$-NH_3$
Neocallimastix frontalis	++	–	+++	++
N. hurleyensis	++	–	++	++
N. patriciarum	++	++	++	+++
Piromyces communis	++	+	–	–
Caecomyces communis	–	–	+++	–

[†]Basal medium contained minerals, glucose and ammonia.
[‡]Basal medium contained minerals, glucose and CT-protein complexes (8% w/v).

rumen for intestinal digestion. Therefore some rumen anaerobic fungi have been screened for their tolerance to tannin and their ability to utilise protein from tannin-protein complexes. Condensed tannin from the shrub legume *Calliandra calothyrsus* was solvent extracted, purified by gel chromatography (Terrill et al 1990) and used as a model tannin in the screening procedure. *Calliandra* CT was included in media which lacked protein so that tolerance to free CT could be tested. Stable complexes of Calliandra CT and bovine serum albumin were prepared in acidic buffer and dried before addition to media to examine the ability of fungi to utilise the complexed protein (Table 3). *Neocallimastix patriciarum* appeared to grow better in the presence of free CT compared with two other related species and *Piromyces communis* and *Caecomyces communis.* All three *Neocallimastix* spp. grew when nitrogen was present only as a CT-protein complex while growth of the other fungal genera was inhibited under these conditions.

Conclusions

There have been a large number of studies on the biology of anaerobic fungi, some of which have dealt with the contribution, both as measured and as perceived, that is made by these unusual microorganisms to the availability of nutrients for the host animal. Some of the essential features of these studies have been given in the present paper. From these studies, it is apparent that anaerobic fungi are extremely important to ruminants that are consuming diets of poor-quality, mature herbage through the mechanism of increasing the voluntary intake of feed. This influence of ruminal fungi is most apparent when additional sulphur is added to tropical pasture grasses that have a low content of sulphur. The high lignocellulose content of tropical grasses (Wilson 1994) together with the relatively high tolerance of anaerobic fungi to plant phenolics may be determining factors in the effectiveness of these microorganisms. Therefore, a considerable potential exists for the manipulation of fungal numbers and activity in the rumen to benefit the utilisation of poor-quality herbage by domesticated ruminants (cattle, goats, sheep, buffalo and camels) in the semi-arid regions of Africa and other continents for the improved production of milk, meat, hair, wool and hide.

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Rumen fungi in domestic and wild herbivores

J. Kopečný

Institute of Animal Physiology and Genetics, Czech Academy of Sciences
Prague 10, Uhlínęves, 104 00, Czech Republic

Abstract

Anaerobic fungi play an important role in fibre degradation in herbivorous animals. Therefore, 35 fungal strains were isolated from the digestion tract of domestic ruminants (3 species) and 86 strains from wild herbivores kept in Czech zoos (29 species). In total 44 herbivorous animal species were tested. Some of the isolated fungal strains were tested for cellulolytic, hemicellulolytic and pectinolytic activities. Pectinolytic activity was studied in detail. In addition to the pectin lyase described, pectate lyase and polygalacturonase activities were found in some isolates. No pectin esterase was detected in any isolate tested. Generally, relatively few of the isolates (ca. 20%) were able to degrade pectin to any extent.

Development of the fungal population may be limited in the rumen ecosystem. A negative effect of ruminococci has been reported (Stewart et al 1992). We found another inhibiting—an action of chitinolytic bacteria in the rumen. These bacteria can reduce fungal growth and fungal cellulose degradation in pure cultures. Their highest effect was observed in presence of *Caecomyces*. Positive interactions of fungi with rumen bacteria have been studied. In the presence of intermediate-utilising *(Megasphaera elsdenii* and *Eubacterium limosum)* bacteria, the polycentric rumen fungus *Orpinomyces joyonii* A_4 was able to increase cellulose degradation and biomass production. There was a shift in the fermentation pattern too. Systematics of rumen fungi is based on spore and mycelium morphology. Unfortunately, the shape of mycelium and sporangium is dependent on growth conditions. An attempt to distinguish different genera by their DNA fingerprints was made. DNA fingerprints of three fungi were tested. In our view, analysis of genetic information is the only reliable approach to rumen fungal systematics.

Introduction

Anaerobic fungi represent a special group of microorganisms inhabiting the rumen ecosystem and possess a life cycle alternating between a motile reproductive flagellated form (zoospore) and a non-motile vegetative form (thallus). The history of their discovery is interesting. The motile form was first observed in 1912 in the fresh water crustacean *Cyclops stenuus* and *Callimastix cyclopis* was described as a parasitic polyflagellate protozoan by Weissenberg (1912). The next year a very similar organism was observed by Braune (1913) in the rumen of sheep and was described as a

Callimastix frontalis. In the mid-1960s, it was found that *Callimastix cyclops* had a cell ultrastructure similar to fungi and the species was transferred to chytrid fungi in the Blastocladiales (Vavra and Joyon 1966). The rumen '*Callimastix*' species remained in Protozoa in a new genus, *Neocallimastix*, with *Neocallimastix frontalis* as the type species. In 1975, Orpin described the life cycle of *N. frontalis* consisting of motile and non-motile stages. The genus was transferred to fungal class Chytridiomycetes (Heath et al 1983). On the basis of the zoospore ultrastructural characteristics, a new family, Neocallimasticaceae (Barr 1988), was established, now with six subdivisions of the family.

	monocentric	*Neocallimastix*
		Piromyces
		Caecomyces
and	polycentric	*Orpinomyces*
		Ruminomyces
		Anaeromyces

The most important feature of these fungi is their plant fibre degrading ability. They are attracted to fibre which they degrade and expose plant material to bacterial attack. All isolates produce cellulases and hemicellulases. In very few isolates is either of these activities suppressed (our observation). The fibrolytic activity produced by pure strains of rumen fungi is in generally comparable or higher than the fibrolytic activity of pure cultures of rumen bacteria (Orpin and Joblin 1988; Wood et al 1986). Fungi colonise even lignified cell walls which are not attacked by rumen bacteria (Akin and Rigsby 1987). This is probably facilitated by their high activity of p-coumaroyl- and feruloyl-esterases (Borneman et al 1992).

This study focuses on the occurrence of anaerobic fungi in the digestive tract of different herbivorous animals, their hydrolytic properties and on interactions with other rumen microbes with the aim of increasing their importance in rumen fermentation.

Materials and methods

Chemicals

Citrus pectin (GENU Pektin, Denmark) with 65% esterification was used for all experiments. Pectin was washed twice with 80% (v/v) ethanol to remove soluble sugars and freeze dried. It contained 3.50% (w/w) galactose, 0.41% (w/w) rhamnose and 0.27% (w/w) mannose. Content of arabinose, xylose and glucose was lower than 0.05% (w/w). Polygalacturonic (pectic) acid was prepared from the same pectin by the method of Kertesz (1957).

Colloidal chitin used as a substrate for all experiments was prepared from crab shell chitin (Sigma) according to Shimahara and Takiguchi (1988). Chitin suspension was autoclaved and stored at 4°C.

Microorganisms (interactions with chitinolytic bacteria)

A strain of rumen fungus *Orpinomyces joyonii* A_4 was isolated from the rumen fluid of a camel according to Joblin (1981).

Microorganisms (microbial interactions)

Megasphaera elsdenii L_2 was isolated in our laboratory from rumen fluid of a sheep on PY medium with lactate. The strain L_2 utilised fructose, glucose, maltose, mannitol and lactate. *Eubacterium limosum ATCC 8486* is a type strain from The American Type Culture Collection (Rockford, MD, USA). Of the sugars tested, only fructose and glucose were utilised by this strain.

Microorganisms (pectin degradation)

Four fungal strains showing pectinolytic activity were selected from 24 isolates obtained in our laboratory according to Joblin (1981). *Orpinomyces joyonii* A_4 was isolated from rumen fluid of a camel, *Neocallimastix* sp. JL3 from faeces of a red deer, and *Neocallimastix* sp. OC and H15 from sheep rumen.

Cultivation methods

Rumen bacteria and fungi were grown anaerobically at 38°C in 100 ml flasks with butyl-rubber stoppers in modified medium M10 (Caldwell and Bryant 1966). Medium was enriched with 0.1% (v/v) vitamin mixture (pyridoxine 200 mg, riboflavin 200 mg, thiamine 200 mg, nicotine amide 200 mg, pantothenic acid 200 mg, p-aminobenzoic acid 1 mg, biotin 0.5 mg, cobalamin 0.5 mg in 100 ml of water), 0.1% (v/v) microminerals ($NiCl_2$, 25.3 mg; H_3BO_3, 33.3 mg; $Na_2MoO_4.2H_2O$, 50 mg; $FeSO_4.7H_2O$, 1.1 mg; $MnSO_4.4H_2O$ 50, mg; $ZnSO_4.7H_2O$, 50 mg; $CuSO_4.5H_2O$,25.3 mg; $CoCl_2.6H_2O$, 25.3; EDTA, 10 mg; $KAl(SO_4)_2.12H_2O$, 10 mg; NaOH, 5.06 g in 100 ml H_2O, pH 7), 20% (v/v) clarified rumen fluid and 4 g of substrate (chitin, glucose, microcrystalline cellulose, amorphous cellulose or cellobiose instead of a mix of glucose, cellobiose and starch). Medium was boiled and after cooling 0.5 g of cysteine and 4 g of Na_2CO_3 was added (Holdeman et al 1977). Reduced medium was transferred into flasks in an oxygen-free atmosphere (70% N_2, 25% CO_2, 5% H_2) and autoclaved.

Isolation of anaerobic fungi

Anaerobic fungi were isolated according to Kudo et al (1990) from serial dilutions (10^{-1}–10^{-3}) of rumen fluid or faeces in medium M10 with agar, cellobiose and antibiotics (penicillin, streptomycin and chloramphenicol) in an anaerobic atmosphere. After three days colonies were reisolated and then transferred into liquid medium. Culture purity was checked microscopically. Animal faeces were transported under a CO_2 atmosphere at a temperature of 30 to 40°C.

Isolation of chitinolytic rumen bacteria

Chitinolytic rumen bacteria were isolated on plates with the same medium M10 containing 0.5% colloidal chitin and 1.1% agar. Samples of rumen fluid were diluted in anaerobic flasks and inoculated into agar. Cultivation was done at 40°C under anaerobic atmosphere for 48 h. Cleared zones indicated chitinolytic strains.

Total counts

Anaerobic bacterial counts were estimated on PYG medium with 15% rumen fluid under anaerobic conditions.

Analytical methods

Volatile fatty acids (VFA) were determined by gas chromatography after diethylether extraction on a column of DB-Wax Megabore (J and W, USA). Succinate was estimated on the same column but after esterification with methanol and extraction with chloroform. Ethanol was measured directly by GLC on a column with 10% SP1200 with 1% phosphoric acid on Chromosorb WAW. Formate was estimated colorimetrically (Sleat and Mah 1984). Lactate was oxidised to acetaldehyde and measured in microdiffusion chambers (Marounek and Bartos 1987). Protein concentration was measured according to Lowry et al (1951) and Herbert et al (1971).

Enzyme activity (chitin)

N-acetyl-β-glucosaminidase was assayed with p-nitrophenyl-β-N-acetyl-glucosaminide (pNAG, Sigma) according to Bidochka at al (1992). The reaction mixture contained 100 µl of enzyme solution, 100 µl of 4 mM pNAG and 200 µl of 100 mM phosphate buffer pH 6.5. Reaction was stopped with 800 µl of 2% Na_2CO_3. After centrifugation, released p-nitrophenol was measured at 410 nm in the supernatant.

Chitinase was determined by a colorimetric assay with p-dimethylaminobenzaldehyde (DMAB) (Boller and Mauch 1988) or with p-hydroxybenzoic acid hydrazide (Lever 1977).

Enzyme activity (pectin)

Activity of pectin esterase (EC 3.1.1.11) was measured by continuous titration with NaOH (Shejter and Marcus 1988). Pectate (EC 4.2.2.2) and pectin lyase (EC 4.2.2.10) activity were measured by recording absorbance at 232 nm during incubation with polygalacturonic acid and pectin (Tagawa and Kaji 1988). Polygalacturonase (EC 3.2.1.15) activity was estimated according to Tagawa and Kaji (1988) by release of reducing sugars. Sample (0.5 ml) was incubated with 0.5 ml of 0.5% polygalacturonic acid and 1 ml of 100 mM phosphate buffer pH 6.0 with 6 mM EDTA for 10–60 min at 40°C. Reaction was stopped in a boiling water bath for 5 min. After cooling and centrifugation, reducing sugars were estimated in the supernatant (Lever 1977).

Results and discussion

Isolation of anaerobic fungi from the digestion tract of herbivores

Anaerobic fungi were isolated from rumen fluid and faeces of different animals over a period of two years (Tables 1 and 2). The distribution of fungal species changed during

Table 1. *Occurrence of anaerobic fungi in rumen fluid of domestic and wild animals.*

Animal	Number of samples	Fungi isolated	Fungal strains
Domestic animals			
Sheep	12	12×	*Caecomyces* *Neocallimastix*
Cattle	15	15×	*Caecomyces* *Neocallimastix*
Goat	8	8×	*Neocallimastix*
Wild animals			
Red deer *(Cervus elaphus)*	3	3×	*Neocallimastix*
Mouflon *(Ovis musimon)*	1	—	
Hungarian Steppe Cattle	3	3×	*Neocallimastix*
Swamp Buffalo *(Bos bubalis)*	3	3×	*Orpinomyces*
Chamois *(Rupicarpa rupicarpa)*	1	—	
Cattle	1	—	
Sheep	2	2×	*Neocallimastix*
Deer *(Pseudaxis sika)*	1	—	
Goat *(Carpa aegargus hircus)*	1	—	
Roe-deer *(Capreolus capreolus)*	1	1×	*Neocallimastix*

the year and even within one animal species individuals did not bear the same types of fungal strains. Some unknown types of anaerobic fungi were observed and we are sure there exist several undescribed species. Such a wide spectrum of fungi was not observed in other animals kept in a zoo (Teunissen et al 1991).

Metabolic tests were done with some isolated fungi (Table 3). Only alditols, mannose, sucrose and inulin were of diagnostic importance. Fibrolytic enzymes were measured in most isolates. The range of activities varies up to three orders (Table 4). Since extracellular β-xylosidase and β-glucosidase activity were not measured in many samples we did not include the results in Table 4. However, these enzymes were always detected in the medium. The most important is the activity of cellulases and hemicellulases. The efficiency of the cellulolytic and hemicellulolytic complex is so high that the fungi are able to degrade soft wood (Joblin and Naylor 1989).

Pectin degradation by anaerobic fungi

Pectinolytic strains isolated in our laboratory belong to two genera of anaerobic fungi. Strains *OC, H15, JL3* shared characteristics similar to the monocentric members of the genus *Neocallimastix* (Borneman et al 1989; Mountfore and Asher 1989).

The strain A_4 is a polycentric fungus and had properties typical of *Orpinomyces joyonii* (Li et al 1991). Similar to other anaerobic fungi, all pectinolytic isolates utilised glucose, cellobiose, cellulose, fructose, xylose and lactose. Other sugars tested were metabolised by some strains only. No growth was observed in the presence of galactose, galacturonic acid or arabinose. Fermentation products of these pectinolytic isolates grown on glucose and pectin were similar (Table 5). Amounts of individual fermenta-

Table 2. *Occurrence of anaerobic fungi in faeces of domestic and wild animals.*

Animal	Number of inocula	Fungi isolated	Fungal strains
Wild animals Prague Zoo			
Adax *(Addax nasomaculatus)*	3	3×	*Orpinomyces*
Antelope bongo *(Boocercus eyryceros)*	5	5×	*Orpinomyces*
Bison *(Bibos bison)*	8	8×	*Caecomyces* *Orpinomyces*
Porcupine *(Hystrix cristata)*	3	—	
Hippopotamus *(Hippopotamus amphitius)*	3	—	
Hippopotamus *(Choeropsis liberiensis)*	3	—	
Yak *(Bibos gruniens)*	3	2×	*Caecomyces* ???
Deer *(Rucervus duvanceli)*	4	4	*Caecomyces*
Deer *(Rucervus eldi)*	5	5×	*Caecomyces* *Piromyces*
Deer *(Elaphurus davidianus)*	3	1×	*Caecomyces*
Red kangaroo *(Macropus rufus)*	2	—	
Kiang *(Equus hemionus kiang)*	4	2×	*Neocallimastix*
Goat *(Capra falconeri)*	3	3×	*Neocallimastix*
Goat *(Capra caucasica)*	3	1×	???
Kulan *(Equus hemionus-asianus)*	4	2×	*Caecomyces*
Przewalski horse *(Equus cabal.przewalskii)*	6	6×	*Orpinomyces* *Neocallimastix*
Llama pako *(Lama vicugna pacos)*	4	4×	*Orpinomyces*
Llama guanaco *(Lama guanicoe glama)*	9	9×	*Caecomyces* ???
Rhinoceros *(Diceros bicornis)*	4	3×	*Piromyces* *Neocallimastix*
Somalian ass *(Equus somaliensis)*	1	1×	???
Sheep *(Ammotragus lervia)*	3	2×	???
Chilean pudu *(Pudu pudu)*	1	1×	???
African elephant *(Loxodonta africana)*	3	3×	*Caecomyces*
Indian elephant *(Elephas maximus)*	3	2×	*Orpinomyces*
Bactrian camel *(Camelus bactrianus)*	4	4×	*Caecomyces*
Indian cattle *(Hemitragus* sp.)	3	1×	*Orpinomyces*
Urson *(Urson dorsatus)*	2	—	
Grevy zebra *(Equus grevyi)*	6	6×	*Orpinomyces*
European bison *(Bibos bonasus)*	4	4×	*Neocallimastix* *Orpinomyces*
Rothschild giraffe *(Giraffa rotchildi)*	4	4×	*Orpinomyces* *Neocallimastix* *Ruminomyces*

tion products except formate were greater with glucose as growth substrate. No measurable amount of methanol was detected in any media tested.

Pectinase activities found in intracellular and extracellular fractions of *Neocallimastix* sp. H15, JL3, OC and *O. joyonii* A_4 are given in Table 6. In all strains the highest

Table 3. *Carbohydrates utilised by isolated rumen fungi.*

	Isolate													
	N. frontalis								*C. communis*				*O. joyonii*	
Substrate	OC	H15	JL2	JL3	CB3	G1	B1	ZU4	JB1	SC2	M8	B5	A4	A1
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	N	+	+	+	+	+	?	–	–	+	+	+	–	+
Galactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	N	–	+	+	?	+	–	–	–	–	–	–	?	+
Dulcitol	N	–	+	+	?	?	–	–	–	?	–	?	–	+
Sorbitol	N	–	+	+	–	–	+	–	–	–	+	–	–	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	N	+	+	+	+	+	+	+	–	+	N	?	+	+
Mannose	N	–	–	–	+	+	+	+	–	–	–	–	–	–
Sucrose	+	–	+	+	–	?	–	+	–	–	+	–	+	+
Xylose	+	+	–	+	+	+	+	+	+	+	+	+	+	+
Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xylan	+	+	–	–	+	–	–	–	–	+	+	+	+	+
Hemicellulose	N	+	+	+	+	+	+	+	–	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	–	+	+	–	+	+
Starch	+	–	+	+	+	–	–	+	–	+	+	–	+	+
Pectin	+	+	–	+	–	+	–	+	–	–	–	–	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	+	+	+	–	–	–	–	+	–	–	–	–	+	+

Source of fungal strains: Sheep H15; OC; SC2; Bison ZU4; Red deer JB1; JL2; JL3; Mouflon M8; Cattle B1; B5; CB3; Camel A1; A4 Goat G1.

activities observed were intracellular pectin lyase (EC 4.2.2.10) and polygalacturonase (EC 3.2.1.15). Enhanced polygalacturonase activities were recorded when EDTA replaced $CaCl_2$ in the assay, indicating the absence of catalytic requirements for divalent ions (Table 7). The pH optima of these enzymes were tested in intracellular and extracellular fractions (Table 8). All extracellular polygalacturonases had a pH optimum at pH 6.0, except in strain H15 which had another peak at pH 7.5. On the contrary, in intracellular fraction this enzyme possess other optima beside the main one at pH 6. These data are in agreement with observations reported by Collmer at al (1988).

Our pectinolytic isolates also produced pectate lyase (EC 4.2.2.2). In comparison with the two enzymes mentioned above, the level of this activity was lower in the intracellular fraction (Table 2). No detectable pectinesterase (EC 3.1.1.11) was observed (data not shown). Several species of bacteria and protozoa are responsible for the degradation and fermentation of pectin in the rumen (Orpin 1984; Szymanski 1981). These microbes produce mainly exo-polygalacturonase, endo- and exo-pectate lyase and pectin esterase

Table 4. *Range of cellulolytic and hemicellulolytic activities of anaerobic fungi from wild herbivores.*

Enzyme	Substrate	Activity—mg hexose or pentose/h per ml	
		Intracellular	Extracellular
CBH	MC	42–942	31–280
EG	CMC	63–1360	16–293
β-GLU	PNP-β-1,4-glu	561–5107	ND
XYL	Xylan	10–1465	157–1360
β-XYL	PNP-β-1,4-xyl	738–9450	ND

ND: not determined.

Table 5. *Fermentation products of rumen fungi grown on pectin and glucose*[†].

Fermentation Products[‡]	Substrate (4 g/l)	A_4	Fungal strain OC	H15	JL_3
Formate	glucose	32.9	29.7	24.5	26.3
	pectin	26.0	35.0	27.8	27.5
Acetate	glucose	6.4	14.0	12.3	17.6
	pectin	9.0	8.8	9.6	8.7
Propionate	glucose	0	0	0.2	0.6
	pectin	0.2	0.4	0.5	0.7
Butyrate	glucose	0	0	0.5	0.9
	pectin	0.8	0.7	0.4	0.7
iso-Butyrate	glucose	0	0	0	0.1
	pectin	0.1	0.2	0.1	0.4
iso-Valerate	glucose	1.0	1.9	1.1	1.5
	pectin	0	0	0	0
Ethanol	glucose	6.8	4.7	3.4	3.3
	pectin	2.4	1.6	1.4	1.1
Lactate	glucose	17.1	20.8	14.2	18.1
	pectin	1.7	3.8	3.8	3.1
Succinate	glucose	0.2	0.5	0.4	0.5
	pectin	0.2	0.2	0.3	0.2
Reducing sugars	glucose	239	429	1449	757
	pectin	877	897	877	1034

[†]Fungi were grown for three days in modified M10 medium with glucose (4 g/l) and pectin (4 g/l) as the only energy source. After cultivation, fermentation products were measured in centrifuged (10000 xg, 10 min, 4°C) medium. Reducing sugars remaining in the medium after three days of incubation (mg hexose l^{-1}).
[‡]Concentration of short chain fatty acids (SCFA) in mmol l^{-1}.

(Paster and Canale-Parola 1985). Contrary to the former findings of Phillips (1989), extracellular pectinase activity was observed in mono- and polycentric rumen fungi (Gordon and Phillips 1991). The predominant pectinolytic activity in the monocentric fungus *Neocallimastix* sp. was represented by an endoacting pectin lyase with pH optimum at 8.0 (Gordon and Phillips 1992). We found that this enzyme was located mainly in the intracellular fungal fraction. The mixture of pectin degrading enzymes (pectin and pectate lyase and polygalacturonase) should be able to degrade natural pectins in a cooperative manner in the absence of pectin esterase (Tsuyumu and

Table 6. *Activity of enzymes involved in pectin degradation produced by rumen fungi grown on pectin.*

Enzyme activity	Fungal strain			
	A_4	OC	H15	JL_3
Polygalacturonase[†]				
Extracellular	46 ± 9	29 ± 6	10 ± 3	27 ± 10
Intracellular	251 ± 62	43 ± 3	435 ± 50	167 ± 11
Pectate lyase[†]				
Extracellular	110 ± 39	83 ± 7	29 ± 17	1 ± 2
Intracellular	69 ± 2	31 ± 1	91 ± 4	49 ± 5
Pectin lyase[†]				
Extracellular	142 ± 4	185 ± 89	166 ± 41	93 ± 24
Intracellular	258 ± 17	274 ± 5	294 ± 17	908 ± 22

[†]Enzyme activity is expressed in μg galacturonic acid.h^{-1}.mg $protein^{-1}$.

Table 7. *The effect of calcium ions on fungal extracellular polygalacturonases.*

Fungal strains	Polygalacturonase activity[†] μg galacturonic acid.ml^{-1}.h^{-1}	
	10 mM $CaCl_2$[‡]	10 mM EDTA[‡]
A_4	56.3 ± 10.1	61.1 ± 2.1
OC	38.6 ± 0.3	61.1 ± 3.7
H15	37.0 ± 0.1	136.7 ± 10.8
JL3	ND	125.4 ± 5.8

[†]Activity of extracellular polygalacturonase was estimated in the presence of 100 mM Tris HCl buffer pH 7 with polygalacturonic acid by measuring reducing sugars.
[‡]Final concentration of reagents.
ND = not determined.

Table 8. *Optimum pH of rumen fungal polygalacturonases*[†].

Fractions	Fungal strains			
	A_4	OC	H15	JL_3
Extracellular	6.0	6.0	6.0, 7.5	6.0
Intracellular	6.0, 8.1	5.05, 6.0	6.0, 8.1	6.5, 8.55

[†]Activity was estimated with 100 mM phosphate-citrate buffer in range of pH 3–7.9 and with 100 mM borate buffer from pH 8.1–9.0.

Miyamoto 1986). Thus the results of this study confirmed the possibility of complex utilisation of pectin and pectin-like substances by anaerobic fungi. Nevertheless, the determination of the activity of specific pectic enzymes in crude pectolytic preparations is considerably complicated by the partial suppression of the activity value due to simultaneous effect of these enzymes on the same substrate. There is still a need for a more detailed description of isolated pectic enzymes produced by anaerobic rumen fungi.

Table 9. *Counts of chitinolytic bacteria in the bovine rumen.*[†]

	Chitinolytic strains[‡]	Total counts
Animal 1	2.32×10^8	8.01×10^9
Animal 2	9.10×10^7	9.22×10^9
Animal 3	5.23×10^4	1.98×10^{10}
Animal 4	0	7.21×10^9
Animal 5	0	5.43×10^9

[†]Cows were fed with a silage-concentrate diet.
[‡]Chitinolytic strains were detected on agar medium 10 with colloidal chitin (4 g/l).

Table 10. *Characteristics of chitinolytic strain* Cl. tertum *ChK5.*

General features			
G ± straight to curved rods			
motile,			
spores oval and terminal, often released			
gelatin not hydrolysed, catalase and lipase negative			
Metabolic profile of the strain ChK5[†]			
Adonitol	+++	Lactose	+
Amygdaline	++	Lactate	—
Arabinose	—	Maltose	++
Cellobiose	++	Mannitol	+++
Cellulose wheat	—	Mannose	++
Dulcitol	—	Mellibiose	+++
Esculine	++	Raffinose	+
Fructose	+++	Rhamnose	+
Galacturonic acid	+	Ribose	—
Galactose	+++	Salicin	+++
Glucose	+++	Sorbitol	—
Glycerol	+	Starch	—
Inositol	+	Trehalose	+++
Inulin	++	Xylose	++

[†]Cultures were grown anaerobically on PY medium with different carbohydrates at 24°C for 36 h.

Interaction of rumen fungi with anaerobic bacteria

Isolation of chitinolytic bacteria

Chitinolytic bacteria were screened in rumen fluid of sheep and faeces of herbivorous animals from the Prague Zoo. From 14 animal species, chitinolytic bacteria were present in sheep, American bison, deer milu, przewalski horse, llama paco, Somalian ass and European bison.

Different chitinolytic strains were isolated from the rumen fluid of five cows (Table 9). Their counts were in the range 5×10^4 to 2×10^8 per ml. Many chitinolytic strains were examined. Most of them were spore-forming rods. One of the isolated strains, ChK5, was fully characterised (Table 10) and it bore all features of *Clostridium tertium* (Cato et al 1984).

Properties of *Cl. tertium* ChK5

The growth rate of ChK5 differed significantly on glucose and chitin. Exponential growth on glucose was complete in 4–6 h in comparison with more than 30 h in case of colloidal chitin. Chitin significantly stimulated sporulation. Fermentation products also changed. The main product in both cases was acetate (Table 11). The other main fermentation product on glucose was propionate. When chitin was used as a substrate, there was almost no propionate production and increased levels of acetate, butyrate and lactate were observed.

Production of chitinolytic enzymes was measured in the culture medium of ChK5 grown on chitin (Table 12). Endochitinase activity was usually much higher than N-acetylglucosamidase, which is cell-associated.

Table 11. *Fermentation end-products of strain* Cl. tertium *ChK5 grown on glucose and chitin.*[†]

mmol/l	glucose	chitin
Acetate	16.8	23.7
Propionate	19.5	0.8
Butyrate	1.4	7.4
Lactate	0.1	8.8
Succinate	0.6	0.1

[†]Cultures were grown anaerobically on medium 10 with 10% rumen fluid, 4 g glucose/l or 4 g colloidal chitin.

Table 12. *Activity of chitinolytic enzymes in culture medium of* Cl. tertium *ChK5.*

	Activity (ug N-acetylglucosamine/h/ml)
Endochitinases	131.7 + 29.7
N-Acetylglucosamidases	42.0 + 2.9

The effect of *Cl. tertium* on rumen fungi

Cell walls of anaerobic fungi represent a substrate for chitinase that is normally present in the rumen. A lot of chitinase activity is produced by fungi themselves. It is necessary to maintain hyphal growth and branching in fungi. Therefore, the effect of the chitinolytic bacterium on rumen fungi was studied.

The polycentric fungus *Orpinomyces joyonii* A_4 was cultured on microcrystalline cellulose. SCFA production was measured for 10 days (Figure 1). Exponential growth of the fungus was observed up to the fifth day of incubation. Strain *Cl. tertium ChK5* was inoculated at different stages of the fungal culture and cultivated for five more days (Figure 1). In co-cultures SCFA production was decreased. The inhibitory effect was related to the time of *Cl. tertium* inoculation. The sooner the chitinolytic strain was added the bigger the decrease of SCFA that was found.

In the same experiments chitinolytic activity in the medium and cell fraction was measured. Addition of *Cl. tertium* to the fungal culture decreased total chitinolytic activity (Figure 2). Cellulolytic activity was decreased in a similar manner (data not

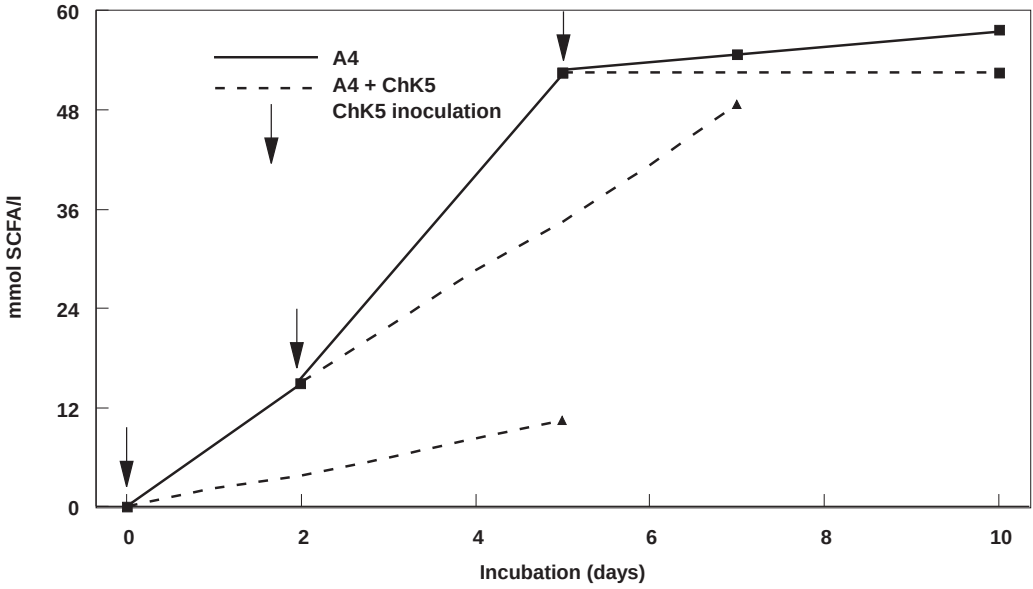

Figure 1. *The effect of* Cl. tertium *ChK5 on SCFA production in co-culture with* O. Joyonii A_4 *grown on microcrystalline cellulose. Fungus was grown alone (solid line) and* Clostridium *was added at different intervals (broken line).*

shown). It would be interesting to compare the effect of rumen ruminococci and chitinolytic bacteria on anaerobic fungi.

Fungal interactions with intermediate utilising bacteria

The polycentric fungus A_4 was assigned to the genus *Orpinomyces joyonii* on the basis of its morphological and biochemical characteristics (Breton et al 1989).

In monoculture of strain A_4, cellulose was degraded extensively and metabolised to yield mainly formate, acetate, ethanol and lactate (Table 13). High levels of glucose and cellodextrins released by the fungus were observed from the second day of cultivation. The association of the fungus A_4 with *E. limosum* or *M. elsdenii* clearly increased cellulose degradation. It was also significant that in these associations the free sugar concentration (glucose and cellodextrins) was much lower than in the fungal monoculture (Table 13). Besides the fact that bacteria profited from the association with the fungus, the low sugar concentrations positively influenced fungal enzyme activities (data not shown).

The end products of cellulose fermentation for the two co-cultures (Table 13) were qualitatively similar but there were variations in the amounts of the individual products. Association of the fungus with *E. limosum* as a H_2-consuming bacterium resulted in a shift

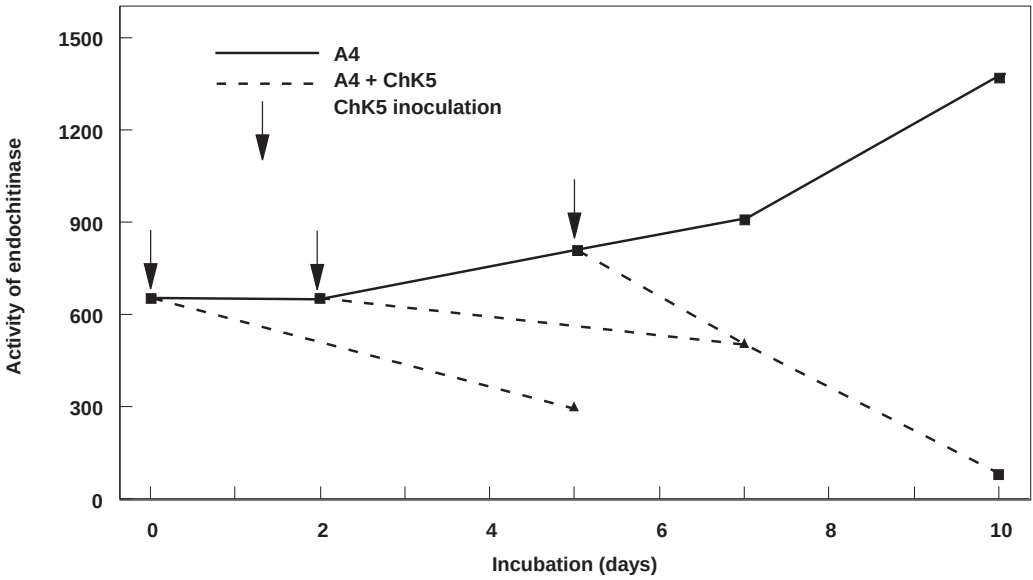

Figure 2. *The effect of* Cl. tertium *ChK5 on total endochitinase activity in co-culture with* O. joyonii *A_4 grown on microcrystalline cellulose. Fungus was grown alone (solid line) and* Clostridium *was added at different intervals (broken line).*

of fermentation pattern toward more acetate and butyrate. The shift was similar to changes observed with monocentric fungi (Bernalier et al 1993). Since the utilisation of hydrogen by *E. limosum* is repressed in the presence of glucose (Genthner and Bryant 1987), cellulolysis in our case would be enhanced mainly by consumption of free glucose by the bacterium. Utilisation of formate by *E. limosum* might have some effect as well (Loubière et al 1992). Although the strain did not grow on formate as a sole source of energy, it was able to utilise it in presence of other ATP-generating mechanisms (Loubière et al 1987). Formate is used for acetate and butyrate production (Genthner et al 1981). Utilisation of lactate by *E. limosum* for the production of butyrate is in agreement with results of Loubière et al (1992). The decrease in ethanol concentration in co-culture could be caused by bacterial degradation (Genthner et al 1981) or by lower fungal production.

The association of the fungus strain A_4 with *M. elsdenii* led to a greater extent of cellulose degradation in comparison with *O. joyonii* plus *E. limosum* co-culture. *M. elsdenii* L_2 is a ruminal species with a nutritional strategy based on the utilisation of lactate and glucose. Increases in butyrate and caproate production associated with the disappearance of acetate allowed us to assume that the main substrate utilised by strain L_2 was glucose. This assumption is supported by the findings of Forsberg (1978) that acetate was essential for growth of *M. elsdenii* in the presence of glucose but not in the presence of lactate. The conversion of acetate to butyrate and caproate is an energy

Table 13. *Cellulose degradation and fermentation product patterns of* O. joyonii *alone and in co-cultures with* E. limosum *and* M. elsdenii *grown on microcrystalline cellulose.*

	O. joyonii	*O. joyonii* + *E. limosum*	*O. joyonii* + *M. elsdenii*
Cellulose	degraded in glucose units		
%	77.07 ± 1.05	85.03 ± 0.33	87.19 ± 0.61
mmol l^{-1}	44.60 ± 0.60[a]	46.31 ± 0.18[b]	47.47 ± 0.33[b]
Unfermented oligosaccharides (mmol glucose units.l^{-1})			
Glucose	6.53 ± 0.56[a]	0.93 ± 0.86[b]	1.62 ± 0.07[b]
Cellodextrins	9.77 ± 1.14[a]	3.16 ± 0.56[b]	2.60 ± 0.67[b]
Cellulose fermented (mmol glucose units.l^{-1})			
	28.30 ± 0.79[a]	42.20 ± 0.65[b]	43.28 ± 0.45[b]
Metabolites[†] (mol/100 mol glucose units fermented)			
Formate	98.59 ± 8.13[a]	4.00 ± 3.57[b]	78.09 ± 4.02[c]
Acetate	64.66 ± 4.59[a]	91.47 ± 6.22[b]	–3.93 ± 3.97[c]
Propionate	–0.07 ± 0.74[a]	0.81 ± 0.32[b]	–2.77 ± 0.09[c]
iso-Butyrate	0.11 ± 0.18[a]	2.61 ± 0.28[b]	1.96 ± 0.10[b]
Butyrate	0.78 ± 0.71[a]	14.50 ± 2.93[b]	39.51 ± 6.72[c]
iso-Valerate	0.28 ± 0.25[a]	3.46 ± 0.16[b]	3.40 ± 0.19[b]
Valerate	0.18 ± 0.28[a]	0.45 ± 0.05[a]	4.07 ± 0.26[b]
Caproate	0.32 ± 0.04[a]	ND	7.97 ± 1.21[b]
Lactate	3.50 ± 0.39[a]	0.18 ± 0.05[b]	0.16 ± 0.05[b]
Succinate	2.23 ± 0.32	1.21 ± 0.14	1.43 ± 0.12
Ethanol	43.82 ± 4.13[a]	22.82 ± 2.24[b]	21.97 ± 3.30[b]
CO_2	142.37 ±12.08	102.09 ± 2.93	55.36 ± 1.33
Biomass($g.l^{-1}$) 1.48 ± 0.35	2.36 ± 0.42 (100%)	2.81 ± 0.46 (159.4%)	(189.9%)
$Y_{cellulose}$ (g dry matter/mol glucose unit)	52.29	55.92	64.93
Carbon recovery (%)	107.9%	109.5%	99.3%

[†]Bacteria were inoculated into medium 10 with 1% of microcrystalline cellulose 24 h after the rumen fungus. The co-cultures were incubated for a further 7 days at 40°C. Concentration of VFA (mmol.l^{-1}) in original medium was the following: acetate 8.38, propionate 1.51, isobutyrate 0.56, butyrate 0.96, iso-Valerate 0.40, valerate 0.14, succinate 0.05, formate 0.05, lactate 0.21, ethanol 0.49. These values were subtracted from co-culture and monoculture final products.
ND = Not determined.
[a, b, c]Mean + SD ($P < 0.05$).

consuming reaction in which the acetate serves as an electron sink (Hino et al 1991). Acetate alone never supported growth of our strain without glucose.

M. elsdenii utilises lactate via the acrylate pathway (Ladd and Walker 1965). This metabolic pathway is associated with ATP production because propionate is the main end-product of lactate fermentation. In contrast, in our co-culture with the fungus, valerate but not propionate production was observed.

Mixed cultures had a stimulating effect on specific biomass production. Co-culture with *M. elsdenii* was more efficient ($Y_{cellulose}$ = 64.9 g dry matter (mol glucose)$^{-1}$ than with *E. limosum* ($Y_{cellulose}$ = 55.3 g dry matter (mol glucose)$^{-1}$ in specific production of total dry matter. The yield of the fungus alone was $Y_{cellulose}$ = 52.3 g dry matter (mol

glucose)$^{-1}$. Carbon recovery higher than 100% was probably due to utilisation of compounds from the rumen fluid.

Microbial interactions in the rumen are highly complex. Any type of improvement in cellulose degradation will lead to a higher efficiency of anaerobic fermentation in the rumen. Detailed studies on microbial interactions with more microbial species and more substrates will be necessary for a better understanding of this type of fermentation.

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Factors limiting proliferation of desirable groups of bacteria in the rumen of animals fed poor quality feeds of high fibre content

N.O. van Gylswyk

Department of Animal Nutrition and Management
Swedish University of Agricultural Sciences
Kungsängen Research Centre, S-753 23 Uppsala, Sweden

Abstract

Animal production in Africa is very reliant on natural pastures as well as on other plant material often of poor quality. The role of fibre-digesting flora in the rumen of animals feeding on such material is particularly important in releasing fermentable carbohydrate both for themselves and other microbes. The nutritional requirements of the important fibre-digesting bacteria is examined and related to deficiencies in poor quality feeds. Low protein content implies deficiencies of ruminal ammonia and branched chain volatile fatty acids (BC-VFA) essential for the cellulolytic bacteria. The fate and possible role of the BC-VFA is considered. An additional factor contributed by protein and favouring fibre digestion is reduced sulphur. Methionine and peptides are of importance in stimulating certain hemicellulolytic bacteria. Foremost among mineral deficiencies on such poor feeds are those related to phosphorus and sodium. The major cellulose-digesting bacteria, as well as others, have absolute requirements for a range of vitamins. Low concentrations of these in the rumen could limit fibre digestion but little is known about this. The results of two experiments with sheep on the effects of supplementing low-protein hays with urea and BC-VFA are summarised. In one of these the numbers and types of cellulolytic bacteria were determined and their relation to fibre digestion in the rumen and animal performance is discussed. Mechanisms for the 'protein-sparing' action of readily fermentable carbohydrate are also considered.

Introduction

In many parts of Africa natural grazing is often the most important or even the sole source of nutrition for ruminants. Where available, cereal straws or stubble are often also used as well as any type of plant material (shrubs and trees) including crop residues. For large parts of the year the grazing can be dry and of very poor quality, characterised by high fibre content of low digestibility and very low protein content. Such material cannot maintain the condition of ruminants. In periods of drought even this material is in short supply. The purpose of this paper is to look at some ways by which such low

quality material has been upgraded and attempt to relate the results to the rumen ecology, particularly the rumen bacteria.

Important fibre-digesting bacteria of the rumen

It is natural to assume that fibre-digesting bacteria in the rumen are of primary importance when ruminants feed on poor quality material of which cellulose and hemicellulose can comprise about 80% in roughly equal proportions. The cellulolytic bacteria provide fermentable energy sources both for themselves and other bacteria. Table 1 shows the types of greatest significance.

Table 1. *Some characteristics of important rumen bacterial species active in digesting cellulose (C) and hemicellulose (H).*

Species	Type of fibre digested	Main fermentation products	Comments
Fibrobacter succinogenes	C, H	Acetate, succinate.	Can not utilise pentose released from H. Ferments glucose but few other carbohydrates.
Ruminococcus flavefaciens	C, H	Acetate, succinate, hydrogen.	Most strains do not utilise pentose. Some strains may ferment glucose and a few other sugars.
Ruminococcus albus	C, H	Formate acetate, ethanol, hydrogen.	Strains may or may not ferment glucose and a few other sugars. Many strains (perhaps 30%) are not cellulolytic. These ferment many sugars.
Butyrivibrio fibrisolvens	C, H or H alone	Formate, (acetate), butyrate, lactate.	Most strains non-cellulolytic. Approx. 5% are weakly cellulolytic. Many carbohydrates fermented. Proteolytic.
Eubacterium cellulosolvens	C	Formate, butyrate, lactate, n-valerate.	Many sugars are fermented.
Prevotella ruminicola	H	Acetate, propionate, succinate.	Many sugars are fermented. Proteolytic.

All the species listed ferment cellobiose. These and many non-cellulolytic rumen bacteria can ferment soluble and even poorly soluble hydrolysis products of cellulose (Russel 1985), thus demonstrating the reliance of the non-fibre digesting bacteria on fibrolytic ones. *Fibrobacter succinogenes* and the ruminococci are considered to be the most active in cellulose digestion. The hemicellulolytic activity of *F. succinogenes* and *Ruminococcus flavefaciens* appears to be directed mainly towards exposing cellulose through removal of hemicellulose because the mono- and oligosaccharides of this fibre component cannot be utilised by these bacteria and are available for others. In particular,

F. succinogenes appears to occur in high numbers (10% or more of 'total culturable' bacteria) in the rumen of animals feeding on material containing highly crystalline forms of cellulose such as wheat straw (Byrant and Burkey 1953) or maize straw (van Gylswyk and van der Toorn 1986). *Butyrivibrio fibrisolvens* can comprise more than half of the more active hemicellulolytic bacteria in the case of the latter diet (van der Linden et al 1984).

F. succinogenes and the ruminococci have an absolute requirement for ammonia. Amino acids and other N sources are of little importance in their nutrition (Byrant 1973). These species also require certain branched chain (BC) and straight chain volatile fatty acids (VFA) for growth. They are shown in Table 2.

Table 2. *Branched and straight chain volatile fatty acids required by the actively cellulolytic rumen bacteria (Bryant and Doetsch 1954; Wegner and Foster 1963).*

Species	Isobutyrate		2-Methyl-butyrate		Isovalerate		2-Ketoiso-valerate	5 to 8 carb. straight chain (e.g. n-valerate)
Fibrobacter succinogenes	+	or	+		–		–	+
Ruminococcus flavefaciens	+		–	or	+		–	–
Ruminococcus albus	+	or	+	or	+	or	+	–

Protein is rapidly broken down in the rumen to amino acids and peptides and it was shown (El-Shazly 1952; Menahan and Schultz 1963) that the BC-VFA originated from the BC amino acids valine, leucine and isoleucine after deamination or, more usually, deamination followed by decarboxylation. n-Valeric acid may be derived by deamination of γ-aminovaleric acid which is probably formed by means of a Stickland-type reaction involving alanine and proline. n-Valeric acid is also produced by *Eubacterium cellulosolvens* (Table 1) as a product of carbohydrate fermentation.

The cellulolytic bacteria incorporate the straight and BC-VFA mainly into n-C_{13} to n-C_{17} straight and BC fatty acids and aldehydes as part of the lipid component of bacterial cells (Allison et al 1962; Wegner and Foster 1963). Only in the case of *R. flavefaciens* are significant amounts of BC-VFA used in amino acid synthesis (Allison and Byrant 1963). However, the total ruminal flora tends to incorporate BC-VFA predominantly into the corresponding BC amino acids (Allison and Byrant 1963). It has been suggested (D. Palmquist, personal communication) that the long BC acids and aldehydes lend fluidity to the lipids of the cellulolytic rumen bacteria as is the case for unsaturated long chain straight acids in aerobic organisms. In the rumen and other anaerobic environments there is a strong tendency towards saturation of double C bonds due to reducing conditions (Harfoot and Hazlewood 1988). The special need for fluidity in the lipids of the cellulolytic bacteria could indicate that it is concerned with cellulolysis, perhaps in the transport of the large molecules of cellulolytic enzymes

across membranes. Cellulases tend to be excreted in membranous vesicles (Chesson and Forsberg 1988). In this context lipid fluidity may also be of significance.

The cellulolytic rumen bacteria have requirements for vitamins but all of the vitamins listed in Table 3 for a particular species are not necessarily required by all strains of that species. The growth and cellulolytic activity of *R. albus* is stimulated by 3-phenylpropionic acid (Stack and Hungate 1984). The subspecies *ruminicola* of *Prevotella ruminicola* (hemicellulolytic) requires heme for growth (Caldwell et al 1965) because it contains cytochromes. Strains of this species require methionine when ammonia is the major N source (Pittman and Bryant 1964). *P. ruminicola* does not appear to require vitamins.

Table 3. *Vitamin requirements of important fibre-digesting rumen bacteria.*

Species	Biotin	Folate	PABA†	Pyridoxine	Thiamin	Riboflavin
F. succinogenes	+	–	+	–	–	–
R. flavefaciens	+	+	+	+	+	+
R. albus	+	–	+	+	–	–
B. fibrisolvens	+	+	–	+	–	–

†p-Aminobenzoic acid.
Source: Wolin and Miller (1988).

Rumen bacteria require a range of macro- and micro-elements. In tropical regions, the elements most likely to be deficient for grazing cattle are P followed by Cu and Co. Deficiencies in Na and I are widespread (McDowell et al 1984). Deficiencies in P and Na are likely to have the greatest effect on rumen bacteria. Wheat and barley straw have very low P contents, not sufficient for the bacteria (Hungate 1966). Rumen bacteria are somewhat halophilic, e.g. the Na concentration must be at least 100 mM to support the growth of *R. albus* (Mackie and Therion 1984). Sulfur can be expected to be limiting in diets low in protein. Sulfate is a good source of S for rumen bacteria, although not for the protozoa (Hungate 1966). However, reduction of sulfate for utilisation in biosynthesis requires energy.

Feeding experiments

Hemsley and Moir (1963) conducted experiments with sheep. Milled oat hay (0.7% N) was supplemented as shown in Table 4 which also gives some of the results. The addition of BC-VFA increased intake and digestion of the hay over that obtained by supplementing only with urea. In another experiment with sheep (van Gylswyk 1970) a poor quality teff hay (0.5% N) resulted in increased intake on supplementation with 3% urea and a further increase occurred when BC-VFA were added (Table 5). With the latter diet the sheep often ate the complete ration and intake was therefore restricted. This part of the experiment was subsequently repeated with unrestricted intake. It showed that the urea/VFA supplement raised the quality of the hay to achieve more than maintenance. Numbers and types of cellulolytic bacteria were determined and these are shown in Table 6.

Table 4. *Effects of supplementing a low protein oat hay with urea and other additions when fed to sheep .*

Treatment	Daily intake of hay (g)	DMD† (%)	MRT of solids‡ (h)	Digestion of cotton thread§ (h)
Basal (hay + mineral)	613	54.5	48	84
Basal + 3% urea	839	62.2	46	38
Basal + 3% urea + 10% molasses	896	61.7	41	38
Basal + 3% urea + 6.6% sucrose	906	62.8	43	43
Basal + 3% urea + 0.56% VFA mix	989	62.0	38	38

†Dry-matter digestibility.
‡Mean retention time of coloured particles in the rumen.
§Time taken to achieve a 50% loss in weight of cotton thread bundles suspended in the rumen.
Source: Hemsley and Moir (1963).

The number of cellulolytic and total culturable bacteria did not change when the hay was supplemented with urea. However, the increased rate of passage of feed particles plus bacteria would mean that the bacteria had grown more rapidly and cellulolytic activity increased to account for greater digestibility (Table 5). Further addition of a BC-VFA mixture increased the numbers of both cellulolytic and non-cellulolytic bacteria. The percentage of cellulolytic bacteria remained the same indicating the dependance of the total bacterial flora on the activity of the fibre-digesting ones. Together the ruminococci were the predominant cellulolytic species on all three diets followed by *B. fibrisolvens* and *E. cellulosolvens*. The proportion of ruminococci increased with supplementation of urea and even more so when the VFA were included. Unfortunately the method used for the primary culture of cellulolytic bacteria was not suitable for the growth of *F. succinogenes*. It is most likely that this species was an important component of the cellulolytic population because poor quality roughages such as wheat straw (Byrant and Burkey 1953) and maize straw (van Gylswyk and van der Toorn 1986) fed to cows and sheep, respectively, support rumen populations containing several-fold more *F. succinogenes* than ruminococci.

In vitro experiments

Hemsley and Moir (1963) found that molasses and sucrose could partly replace BC-VFA. Other workers also found that even small amounts of easily fermented carbohydrate such as starch, added to poor quality roughage, could enhance its utilisation. It was suggested (Brüggemann and Giesecke 1967) that lysis of bacteria not requiring BC-VFA could release BC amino acids which are broken down to BC acids. Evidence of this was obtained *in vitro* when *Ruminobacter amylophilus* grew in a simple medium containing starch (Miura et al 1980). After growth had ceased, BC-amino acids were released. When it was co-cultured with the amino acid-dependent *Megasphaera*

Table 5. *Effects of supplementing low protein teff hay with urea and a mixture of branched and straight chain VFA when fed to sheep.*

Treatment	Daily intake of hay (g)	Cellulose digestibility (%)	Hemicellulose digestibility (%)	Loss in body weight over 12 weeks (%)
Basal (hay + minerals)	717[†]	57	45	19.0
Basal + 3% urea	902[†]	72	64	8.2
Basal + 3% urea + VFA mixture	1077[‡]	72	67	3.8
	>1500[†]	ND[§]	ND	gain

[†]Unrestricted intake. [‡]Restricted intake. [§]Not done.
Source: van Gylswyk (1970).

elsdenii in a similar medium, also containing glucose, the BC-amino acids were converted to BC-VFA required for the growth of *R. albus.* A small amount of starch in medium containing glucose and cellobiose gave successive growth of the three species (when mixed) in the order *R. amylophilus, M. elsdenii* and *R. albus.* A second type of mechanism was also demonstrated (Stevenson 1978). A range of pure cultures of different rumen bacteria were grown separately in medium containing glucose, cellobiose, starch and $(NH_4)_2SO_4$. All strains excreted amino acids during active growth. The BC amino acid, valine, was generally excreted in relatively large amounts. These two mechanisms could explain the BC-VFA-sparing action of starch or other readily fermentable materials added to poor quality roughages. It would, of course, also be a protein-sparing action. However, protein is a source of peptides and the growth of the important hemicellulolytic bacterium, *P. ruminicola,* as well as others, is stimulated by these. Furthermore, protein is a source of reduced sulfur.

Table 6. *Mean counts and proportions (%) of cellulolytic bacteria in the rumen of sheep fed low protein teff hay supplemented with urea and a mixture of branched and straight chain VFA.*

Treatment	Cellulolytic counts ($\times 10^{-7}$/g *ingesta)*	*R. albus (%)*	*R. flavefaciens (%)*	*B. fibrisolvens (%)*	*E. cellulosolvens (%)*
Basal (hay + minerals)	4.0	34	26	39	1
Basal + 3% urea	3.5	42	28	22	8
Basal + 3% urea + VFA mixture	9.8	46	46	5	4

Source: (van Gylswyk 1970).

Vitamins

Vitamins may be limiting for growth of bacteria in the rumen (van Gylswyk et al 1992). Pure cultures of six species of common rumen bacteria were grown separately in filter-sterilised rumen fluid from cows and sheep fed good diets. Glucose was the only added energy source. Parallel cultures were grown with yeast extract as the only other additive. It was found that *P. ruminicola,* the most abundant species in the rumen of

animals from which rumen fluid was taken, grew almost as rapidly in rumen fluid free of yeast extract as in that containing yeast extract. *B. fibrisolvens,* the second most numerous bacterium, grew more rapidly with yeast extract than without but at a greater rate than species occurring in much lower numbers in the rumen. Growth stimulating factors were only slowly released from ruminal microbes and feed particles. *P. ruminicola* appears to have low or no requirements for vitamins. A *B. fibrisolvens* strain was subsequently found to be stimulated by pyridoxine and folic acid and peptides when grown in rumen fluid containing glucose (Wejdemar 1994). It is considered possible that the vitamin supply in the rumen is one of the factors that regulate the proportions of different species in the rumen. The possibility of bloating when vitamins, together with other nutrients, are in plentiful supply was examined recently (van Gylswyk 1994).

Minerals

In the animal experiments cited above, mineral supplements were provided, but low quality roughages are usually deficient in minerals for rumen bacteria. Recently, the effects of P, Ca and Mg concentrations on the growth of *F. succinogenes* and *R. flavefaciens* were examined (Komisarczuk-Bony et al 1994). Concentrations below 15 mg/l for P and 5 mg/l for Mg reduced growth and cellulose degradation by *R. flavefaciens* while the corresponding concentrations for *F. succinogenes* were 5 mg/l and close to zero, respectively. The response to Ca concentrations was similar for both species with inhibitions below 1 mg/l. While the Ca content of a straw was adequate for optimal degradation by *F. succinogenes*, the P content was so low that dry matter digestibility was decreased appreciably (>25%). These results suggest that the proliferation of *F. succinogenes* is favoured more than that of *R. flavefaciens* in the rumen of animals feeding on poor quality hay or straw; at least with respect to P and Mg. *P. ruminicola* subsp. *ruminicola,* which possesses cytochromes, will require Fe. This element is unlikely to be lacking in roughages as all plant material contains cytochromes. Although the iron content of rumen fluid is low it is also quite constant. As mentioned earlier the Na content of such diets could also limit growth and cellulose degradation by rumen bacteria. The intake of soil will contribute to the alleviation of mineral deficiencies.

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Exploitation of rumen microbial enzymes to benefit ruminant and non-ruminant animal production

L.B. Selinger[1], *K.-J. Cheng*[1], *L.J. Yanke*[1], *H.D. Bae*[1], *T.A. McAllister*[1] *and C.W. Forsberg*[2]

1. Lethbridge Research Centre, Lethbridge, Alberta, Canada T1J 4B1

2. Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Abstract

As competition in the livestock industry increases, producers are actively seeking technological advances through which to increase their production efficiency. Supplementation of diets of non-ruminant animals with fibrolytic enzymes, such as cellulases, xylanases and β-glucanases, increases their feed conversion efficiency and growth rate. These enzymes enhance the release of sugars from plant cell wall polymers, thereby increasing their availability to the animal and eliminating some of their naturally occurring anti-nutritional effects. Recent research indicates that enzyme supplementation can also produce similar improvements in ruminant production, even though the rumen microbial population is known to produce an extensive array of potent endogenous fibrolytic enzymes. Widespread adoption of this promising technology has been hampered in the past by the cost and inconvenience of enzyme production and delivery. However, over the last decade, advances in recombinant DNA technology have significantly improved microbial production systems. In addition, the rumen has been recognised as a rich, alternative source of genes for industrially useful enzymes and novel strategies are being developed for effective delivery of these gene products. Thus, the biotechnological framework is in place to achieve substantial improvements in animal production through enzyme supplementation.

Introduction

There is a growing trend in the non-ruminant livestock industry to supplement diets with fibrolytic enzymes. Amending rations with cellulases and/or xylanases increases the feed efficiency of livestock. Enzymatic hydrolysis of cellulose and xylan to simple sugars (e.g. glucose and xylose) provides the non-ruminant animal with carbon sources that are normally not made available by intestinal enzymes. Furthermore, enzymes eliminate certain forms of these polymers (e.g. arabinoxylan, found in wheat and rye; β-glucan, in barley and oats) that may have deleterious effects on nutrient absorption and promote intestinal disturbances by pathogenic enteric microorganisms.

Unlike non-ruminant animals, ruminants have an extensive array of microbial fibrolytic enzymes produced in the rumen, and these enzymes play an important role in the ruminant digestive process. Fibrolytic activity in the rumen arises primarily from the activities of three bacterial species: *Fibrobacter succinogenes, Ruminococcus albus* and *R. flavefaciens* (Forsberg and Cheng 1992). Enzymes produced by other microorganisms, including fungi and protozoa, also contribute to fibre degradation. Ruminal fungi are noted for their production of potent fibrolytic enzymes and their ability to degrade the most recalcitrant of plant cell wall polymers (Forsberg and Cheng 1992; Forsberg et al 1993; Trinci et al 1994; Wubah et al 1993). Nonetheless, recent evidence suggests that enzyme supplementation can also dramatically increase the average daily gain of ruminants (T.A. McAllister and K.-J. Cheng, unpublished data).

Fibrolytic activity in the rumen is estimated to be 10 times higher than in any other known fermentation system. The microflora of the rumen represents a rich and underutilised source of superior fibrolytic enzymes. In this review, the current status of the inclusion of enzymes and microorganisms in animal feeds is outlined briefly, and some of the recent advances in enzyme technology are highlighted. From this perspective, we examine the microflora of the rumen as a source of enzymes and discuss the application of these enzymes in the livestock feed industry.

Feed enzymes

Silage

Some commonly ensiled plants (e.g. grasses and, in particular, alfalfa) are low in soluble carbohydrate and therefore often ensile poorly. The inclusion of cellulase and endoxylanase enzyme mixtures from a variety of fungi or bacteria during ensiling increases the amount of free soluble carbohydrate available for fermentation (van Vuuren et al 1989). Consequently, conversion of carbohydrate to lactic acid is more extensive, resulting in a lower pH in enzyme-treated silage as compared to untreated silage (Chen et al 1994; Selmer-Olsen et al 1993). In some instances, enzyme treatment has increased the intake (DM basis) of silage by dairy cows and improved milk production, but these responses are less pronounced for cereal silages (Chen et al 1994) or silages with a high dry matter content (Fredeen and McQueen 1993). Enzyme treatment of grass silages has increased (Jacobs et al 1992), decreased (Jacobs and McAllan 1991) and had no effect (Jacobs and McAllan 1992) on the apparent whole-tract digestibility of organic matter. In a single study, different enzyme preparations were found to increase and decrease the digestibility of organic matter in sheep (Chamberlain and Robertson 1989). These inconsistencies emphasise the need for more precise definitions of enzyme preparations and of the forage properties for which a given enzyme treatment is most likely to elicit a positive response in animal performance.

Poultry and swine diets

The addition of glucanases and pentosanases (endoxylanases) to poultry diets containing barley, oats, wheat and rye has been shown to increase feed efficiency and to enhance

growth rates by 5 to 17% (Campbell and Bedford 1992; Classen et al 1991; Marquardt et al 1987; Rotter et al 1987). Protein sources such as oilseed meals (e.g. canola meal, soybean meal), commonly incorporated into these rations, contain substantial amounts of non-starch polysaccharides. The addition of cell-wall digesting enzymes to canola-based diets for poultry increases the digestibility of non-starch polysaccharides from 3 to 37% (Slominski and Campbell 1990). The benefits of enzyme supplementation on growth performance of swine are more variable with improvements in average daily gain ranging from 0 to 15% (Inborr 1990). This variability can be partially attributed to the age of the pigs, since in older pigs (> 15 weeks) considerable degradation of mixed-link β-glucans by the ileal microflora and greater endogenous enzyme secretions may negate the beneficial effect of enzyme supplements on digestion (Johnson et al 1993).

The mechanisms by which glucanases and xylanases enhance animal performance have been elucidated. Barley and oats contain mixed-linkage (1→3,1→4)-β-D-glucans as a major constituent of the endosperm cell wall whereas the endosperm cell walls of rye and wheat are rich in arabinoxylans (Pettersson and Aman 1989). During digestion these polymers are released from cereal cell walls, become hydrated and dramatically increase the viscosity of the digesta. An increase in viscosity impairs the activity of digestive enzymes, reduces digestive flow and nutrient absorption and contributes to the formation of 'sticky' faeces which is especially problematic in poultry operations, as it adheres avidly to feathers and to eggs (Classen et al 1988, 1991; Pettersson and Aman 1989).

Small quantities of purified β-glucanase (Edney et al 1989) and/or endoxylanase (GrootWassink et al 1989) in poultry diets cleaves these polymers and lowers the viscosity of the digesta (Almirall et al 1993; Hesselman and Aman 1986; Pettersson and Aman 1989; Rotter et al 1990). The associated improvements in nutrient utilisation and decreases in digestive upset reduces the need for antibiotics in poultry and swine diets (Inborr 1990; Johnson et al 1993).

The addition of microbial phytase to poultry feed increases phosphorus availability by more than 60% and reduces phosphorus in the droppings by 50%, resulting in significant increases both in growth rate and feed conversion (Simons et al 1990). Similarly, the addition of phytase to diets for growing pigs increases the apparent digestibility of phosphorus by 24–53% and lowers the amount of phosphorus in the faeces by 35% (Ketaren et al 1993; Simons et al 1990). The inclusion of phytase in the diet induces hydrolysis of plant phytate (inositol hexaphosphate), releasing dietary phosphate in the gastrointestinal tract. Phytate constitutes approximately 1 to 2% (wt/wt) of cereals and oil seeds, and phosphorus as phytate accounts for 60 to 90% of the total phosphorus present in cereal diets (Cheryan 1980; Swick 1991). Phytate is a strong chelating agent and binds with proteins and minerals, reducing the bioavailability of these nutrients in feeds (Erdman 1979; Cheryan 1980). Consequently, supplementation of poultry and swine diets with phytase not only improves phosphorus retention, but often enhances the digestion of dry matter and crude protein, and the utilisation of calcium and zinc (Lei et al 1993; Mroz et al 1994; Zyla and Koreleski 1993).

Ruminant diets

In the 1960s, several studies were conducted to examine the efficacy of adding enzyme preparations to diets for ruminants (Burroughs et al 1960; Clark et al 1961; Perry et al 1966; Rovics and Ely 1962). Clark et al (1961) found that enzyme supplementation improved average daily gains by as much as 20%. However, responses to enzyme preparations were inconsistent (Perry et al 1966; Rust et al 1965; Theurer et al 1963), possibly because of differences in diet composition, method of enzyme application and the stability and activity of the enzyme preparations.

Improvements in fermentation technology and the biotechnological development of more defined enzyme preparations have prompted a renewed interest in the use of enzymes in ruminant diets. More recent studies have attempted to define the conditions in a given feed that are most likely to result in a favourable animal response to enzyme supplementation (Feng et al 1992a, 1992b; Judkins and Stobart 1988). Factors such as substrate specificity of the enzymes, moisture level of the feed, the time required for enzyme-substrate interaction and temperature at time of treatment are all likely to influence the extent to which enzymes enhance the utilisation of feeds by ruminants. The binding of enzymes to their appropriate substrates is an absolute prerequisite for the digestion of plant cell walls in the rumen (McAllister et al 1994). It follows, then, that treatment methods enabling adequate interaction between enzyme and substrate prior to feeding are most likely to improve animal performance.

In light of the exceptional fibre-digesting capacity of the rumen, it is difficult to explain why pretreatment of forages with fibrolytic enzymes prior to consumption would further improve the utilisation of these feeds by ruminants. The enhanced degradation in enzyme-treated forages may be related to the increase in passage rate and reduction in the retention time of forage particles in the rumen of cattle as observed by Feng et al (1992b). Scanning electron microscopy has shown that the surface of plant cell walls is occasionally colonised by a single bacterial morphotype (Cheng et al 1981, 1984). Complete digestion of plant cell walls, however, requires an array of enzymes and therefore a single morphotype may not produce the diversity of enzymes required to effect complete cell wall digestion. Furthermore, these primary colonisers may limit the access of complementary bacteria to the surface of the plant cell walls. Under such circumstances, prior treatment of forages may contribute to the digestive process the precise enzymes which would otherwise be limiting the rate and/or extent of plant cell wall digestion. Ultimately, enzyme cocktails should be designed to overcome the specific constraints limiting digestion of a particular type of forage. Component enzymes in such cocktails might vary even for a given forage, targeting particular maturity levels or dry matter concentrations. Developments in biotechnology make it feasible to engineer such enzyme cocktails for xylanase and β-glucanase, but the technology for specific production of many of the other enzymes (e.g. ferulic acid esterase, acetylxylan esterase, arabinofuranosidase) required for cell wall digestion is lacking. In the short term, then, improvements in ruminant performance arising from enzyme treatment of forages will most likely be the result of treatment of the forage with broad spectrum crude enzyme extracts from cellulolytic microorganisms (e.g. *Aspergillus* spp., *Trichoderma reesei).*

Recent advances in the development of enzymes to enhance digestion of feedstuffs

A major factor limiting the widespread use of enzymes in the livestock industry is the expense of traditional large-scale fermentations and downstream processing. This has prompted manufacturers and researchers to seek more powerful enzymes and to examine alternative systems for enzyme production and delivery. Recently, application of recombinant DNA technology has enabled manufacturers to increase the volume and efficiency of enzyme production, and to create new products (Hodgson 1994; Ward and Conneely 1993). The original source organism need no longer limit the production of commercial enzymes. Genes encoding superior enzymes can be transferred from organisms such as anaerobic bacteria and fungi, typically impractical for commercial production, into well characterised industrial microbial production hosts (e.g. *Aspergillus* and *Bacillus* spp.). These genes may also be transferred to novel plant (Pen et al 1993; van Rooijen and Moloney 1994) and animal (Hall et al 1993) expression systems.

The rumen as a source of enzymes

The rumen is increasingly being recognised as a particularly promising source of superior fibrolytic enzymes. Cellulases and xylanases produced by ruminal fungi are among the most active fibrolytic enzymes described to date (Gilbert et al 1992; Trinci et al 1994). The quest to elucidate the mechanisms of fibre digestion and to find more efficacious enzymes for industrial applications, and technological developments which now allow the genetic manipulation of rumen microorganisms have inspired the cloning of a growing number of genes from ruminal bacteria and fungi. At least 75 different genes, the majority of which encode enzymes with a role in fibre digestion, have been cloned thus far from ruminal microorganisms. Most of these have been isolated from a small number of bacterial species, including *R. albus, R. flavefaciens, F. succinogenes, Butyrivibrio fibrisolvens* and *Prevotella ruminicola* (reviewed by Flint 1994; Forsberg et al 1993; Hespell 1989; Wallace 1994). Several of the bacteria possess a multiplicity of genes coding for different cellulases and xylanases. For example, *F. succinogenes* appears to possess nine non-homologous glucanases (Malburg and Forsberg 1993; McGavin et al 1989) and four unrelated xylanases (Malburg et al 1993; Paradis et al 1993; Sipat et al 1987) in addition to a gene coding for cellodextrinase activity (Gong et al 1989). *R. flavefaciens* has also been found to produce multiple xylanases (Flint et al 1994). The presence of numerous glycanases in a single bacterium suggests that they all may have a role during growth on glycan substrates. Verification of this hypothesis will have to wait until mutant strains of cellulolytic and xylanolytic bacteria with null mutations in each gene have been isolated and tested for growth on cellulose and xylan.

Researchers have only recently turned to the study of the genetics of anaerobic fungi isolated from the rumen. Enticed by their powerful fibrolytic activity and ability to utilise the most recalcitrant of plant cell wall polymers, researchers have cloned at least 27 genes from four fungal species. The cloned genes include five cellulases (Black et al 1994; Selinger et al 1994; Xue et al 1992a, 1992b; Zhou et al 1994) and three

xylanases (Black et al 1994; Gilbert et al 1992; Tamblyn Lee et al 1993) from *Neocallimastix patriciarum,* an endoglucanase from *Orpinomyces joyonii* (Selinger et al 1994), eight cellulases, four xylanases and five mannanases from *Piromyces* sp. (Ali et al 1995) and a phosphoenolpyruvate carboxykinase from *N. frontalis* (Reymond et al 1992). A notable report (Gilbert et al 1992) of a ruminal fungal xylanase (XynA from *N. patriciarum)* possessing the highest specific activity currently known (6000 μmol of xylose/min per milligram of XynA) will likely stimulate a more rigorous examination of these microbes.

Gene expression and delivery systems

The cost of livestock performance-enhancing enzymes can be reduced through the use of more effective expression and delivery systems. Recombinant bacterial and plant systems are being examined for their efficacy in improving the ensiling of low quality forages, and in producing and delivering enzymes on a continuous basis to livestock. Ultimately, researchers envisage eliminating the need to use the feed as a 'middle man' in enzyme delivery altogether by enabling transgenic animals to secrete endogenous fibrolytic enzymes from the pancreas into the small intestine.

Silage inoculants

The cost and inconvenience of adding enzymes prior to the ensiling of forages low in carbohydrates could be avoided if the bacterial species in the inoculant were also capable of producing the enzymes required to release soluble sugars from plant cell walls. However, attempts at isolating such microorganisms have proven unsuccessful, and as a result attempts have been made to develop genetically engineered strains of lactobacilli which produce fibrolytic enzymes. Genetically modified *Lactobacillus* strains have now been developed which express cellulase and xylanase genes isolated from other organisms (Baik and Pack 1990; Bates et al 1989; Scheirlinck et al 1989, 1990) and which have, in at least one case, been shown to exhibit competitive growth in silage (Sharp et al 1992). Furthermore, those researchers observed that pM25, a pSA3 derivative containing a *Clostridium thermocellum* cellulase gene, was maintained at high levels by the rifampin-resistant host cells (by 100% of host cells when pM25 was present as a chromosomally integrated element and by 85% when present as an autonomously replicating plasmid element). We have observed similar stabilities of recombinant plasmids when modified *Lactobacillus plantarum* constructs were applied to the forage during the ensiling of alfalfa. Plasmid constructs pSAG (pSA3::*Bacillus subtilis* endoglucanase gene) and pSAX (pSA3::*B. circulans* xylanase) were found to persist in 83% and 62% of rifampin-resistant *L. plantarum* cells, respectively.

Persistence of newly developed recombinant lactobacilli beyond their target environments is an important, but under-studied phenomenon. In a single study, Sharp et al (1994) demonstrated the rapid loss of both unmodified and recombinant *L. plantarum* silage inoculants from the rumen. Elimination of these strains was attributed to predation by protozoa and other undefined factors (e.g. turnover rate, cell death). These findings are also relevant to the use of these genetically engineered microorganisms (GEMs) as

probiotic feed additives, in which case it is essential that they establish and persist in the gastrointestinal tract. Factors influencing environmental persistence must be identified and taken into consideration during selection of microbial strains for genetic manipulation.

Two major drawbacks of plasmid constructs are their instability in the absence of antibiotic selective pressure and environmental concerns arising from the potential for horizontal transfer of antibiotic resistance genes carried on plasmids. Consequently, methods have been sought to integrate recombinant genetic material into the *L. plantarum* chromosome. Progress in this direction was achieved when the pSA3 plasmid was integrated into the *L. plantarum* chromosome (Rixon et al 1990; Sharp et al 1992), but inclusion of the erythromycin resistance gene was still of environmental concern. Another method, more suitable for GEMs destined for environmental release, permits the integration of select DNA fragments into the chromosome through a two-step procedure involving gene inactivation and replacement (Biswas et al 1993; Hols et al 1994). In the first step, the entire plasmid construct is forced to integrate, via homologous recombination, into a predetermined locus. The process is completed by select recombinational events between tandemly repeated elements which result in the loss of the vector and maintenance of the desired gene.

A number of researchers (Baik and Pack 1990; Bates et al 1989; Scheirlinck et al 1989, 1990; Sharp et al 1992) have reported the introduction of cellulase and xylanase genes into *L. plantarum*, but the development of efficacious recombinant silage inoculants has been hampered by low levels of heterologous gene expression (Hols et al 1994; Oosting et al 1995). The construction of hybrid genes, consisting of homologous expression-secretion signals fused with the structural component(s) of heterologous genes encoding fibrolytic enzymes, may provide an improvement in the levels of gene expression. Hols et al (1994) demonstrated the effectiveness of this technique by isolating promoter signal sequence regions from *L. plantarum* and using them to drive expression and secretion of foreign amylase and levanase genes in *L. plantarum.* The heterologous expression of levanase enabled the modified *L. plantarum* isolates to utilise inulin as a carbon source. Future research employing recently developed advances in gene expression and integration technology should lead to the development of genetically modified *Lactobacillus* strains as silage inoculants.

Probiotics

Probiotics have been defined by Fuller (1989) as live microbial feed supplements which benefit the host animal by improving the intestinal microbial balance. Bacteria commonly fed to domestic animals as probiotics include *L. acidophilus, L. bulgaricus, L. plantarum, L. casei, Enterococcus faecium* and other enterococcal species (Jones 1991).

Lactobacillus spp. are naturally occurring inhabitants of the gastrointestinal tract, especially of poultry, but their role in the ecology of the tract is not well understood (Fuller 1989; Juven et al 1991). *L. acidophilus* is the most common lactic acid-producing bacterium colonising the epithelial surfaces of the gastrointestinal tract. Its beneficial effects are thought to be exerted in a number of ways, including the secretion of

bacteriocins or bacteriocin-like substances which inhibit both (presumably pathogenic) Gram-positive and Gram-negative bacteria, the production of hydrogen peroxide and the production of lactic acid, which has a strong germicidal action at low pH (Gilliland 1990). Alternate mechanisms by which various species of *Lactobacillus* inhibit other microorganisms include the deconjugation of bile acids, thereby enhancing their antimicrobial nature, and the production of antimicrobial compounds such as acidolin and reuterin (Juven et al 1991). In addition to inhibiting the growth of harmful bacteria, *Lactobacillus* spp., as primary colonisers, also provide digestible nutrients or digestive enzymes beneficial to the animal (Fuller 1989). Adding fibrolytic agents to the enzymes currently produced by the lactobacilli would no doubt confer upon them a competitive advantage for nutrients in the gut, and secondarily would benefit the host animal by making available more of the dietary energy contained in plant polymers. Progress toward this goal has been made. Baik and Pack (1990) reported high extracellular glucanase activity from *L. acidophilus* in which they had induced expression of a *B. subtilis* endoglucanase gene. To date, however, there are no reports of the effect(s) of genetically modified lactobacilli on animal health.

Feeding probiotics has been more prevalent in non-ruminant livestock industries than in ruminant feeding, although opportunities may exist for improving the health and growth of ruminants in this way (Asby et al 1989; Aseltine 1991). Other reports, however, have indicated no response (Harrison et al 1988; Mir and Mir 1994) in ruminants fed these agents. Future studies must identify the mode of action of these feed additives if uniform and reproducible animal responses are to be obtained.

Microflora of the gut

The manipulation of digestion in ruminants through genetic modification of anaerobic bacteria is currently being investigated in many laboratories, and numerous reviews have been written on this subject (Armstrong and Gilbert 1985; Flores 1989; Forsberg and Cheng 1992; Forsberg et al 1986, 1993; Russell and Wilson 1988; Wallace 1994). Strategies proposed for the manipulation of ruminal bacteria include expression of heterologous genes coding for fibrolytic enzymes, artificial peptides, bacteriocins and for detoxification or antiprotozoal factors.

The lack of functional gene transfer systems has in the past impeded progress toward achieving expression of foreign genes in anaerobic bacteria. However, conjugal and electroporation gene transfer systems have now been developed and refined for a number of ruminal bacterial isolates, including *P. ruminicola* (Béchet et al 1993; Flint et al 1988; Russell and Wilson 1988; Shoemaker et al 1991; Thomson and Flint 1989; Thomson et al 1992), *Streptococcus bovis* (Hespell and Whitehead 1991a; Whitehead 1992), *B. fibrisolvens* (Beard et al 1995; Clark et al 1994; Hespell and Whitehead 1991b; Teather 1985; Ware et al 1992; Whitehead 1992), *R. albus* (Cocconcelli et al 1992), *Escherichia coli* (Scott and Flint 1995) and *Selenomonas ruminantium* (Kopecny and Fliegerova 1994). Gene expression in the above studies was limited to the expression of vector-specific functions and antibiotic resistance markers. Application of gene transfer technologies to construction of recombinant anaerobic bacteria, designed for modification of the rumen environment, is illustrated in the following examples.

Whitehead and Hespell (1989) cloned an endoxylanase gene from the hemicellulolytic ruminal bacterium *B. ruminicola* 23 and, using the vector pVAL-l, subsequently introduced it into *B. fragilis* and *B. uniformis*, where enzyme activity was increased dramatically (Whitehead and Hespell 1990). Endoxylanase activities of the newly constructed xylanolytic strains were up to 50 times higher than that measured in *B. ruminicola* grown on xylan. The xylanase gene has since been integrated into the chromosomal chondroitin lyase II gene of *Bacteroides thetaiotaomicron* to obtain stable endoxylanase production in the absence of selective agents (Whitehead et al 1991). The activity of the enzyme in *B. thetaiotamicron* was still 17 times higher than that produced by *B. ruminicola* 23, and was stable when the modified *B. thetaiotamicron* was grown in a carbon-limited chemostat culture for over 60 generations. Introduction of the new strain into the rumen to increase xylan degradation was proposed, but it remains to be determined whether or not the genetically modified organism will persist in sufficiently high numbers to substantially increase hemicellulose digestion, especially since *B. thetaiotaomicron* is not a naturally occurring species in the rumen. Animal trials with this novel GEM will provide preliminary information on its persistence in the rumen and its contribution to ruminal fibre digestion.

Many shrubs and trees native to Australia accumulate monofluoroacetate in their shoots and are therefore poisonous to domestic livestock (Gregg and Sharpe 1991; Gregg et al 1994; Jones and Megarrity 1986). Jones and Megarrity (1986) attempted to detoxify fluoroacetate by introducing bacteria capable of degrading the toxin into the rumen. An unsuccessful search for microbes indigenous to the rumen and capable of detoxifying fluoroacetate prompted the introduction of this phenotype into *B. fibrisolvens* (Gregg et al 1994). A transcriptional fusion between the fluoroacetate dehalogenase gene from *Moraxella* sp. strain B and the *erm* promoter from pAMβ1 was ligated to pBH*erm* (Beard et al 1995), a *B. fibrisolvens/E. coli* shuttle vector. *B. fibrisolvens* OB156 isolates carrying the dehalogenase expression plasmid (pBHf) were able to detoxify fluoroacetate *in vitro* and, under non-selective conditions, pBHf was maintained for up to 500 generations. It was estimated that at the level of dehalogenase expression observed in *B. fibrisolvens* OB156 (pBHf), toxin protection for the ruminant would be provided if *B. fibrisolvens* OB156 (pBHf) cells were present in the rumen at 10^6 to 10^7 cells/mL (Gregg et al 1994). The stability and dehalogenase activity of *B. fibrisolvens* OB156 (pBHf) *in vivo* has yet to be tested. Attempts are under way to introduce the gene into a number of bacteria from the rumen and improve gene expression, to increase theoretical maximum dehalogenase activity levels in preparation for whole animal ruminal inoculation trials (Gregg et al 1994).

In the rumen microbiology laboratory at the Lethbridge Research Centre, *B. fibrisolvens* has also been selected as a host for heterologous gene expression. As a naturally occurring, ubiquitous resident in the rumen, this bacterium is an ideal candidate for the introduction of new genetic information to the ruminal environment. Our preliminary experiments were successful in the introduction and expression of a *S. bovis* amylase gene in *B. fibrisolvens* H17c (Clark 1994; Clark et al 1992, 1995). The amylase gene was cloned onto an *E. coli/B. fibrisolvens* shuttle vector, and the resulting construct (pUBLRSA) was introduced into *B. fibrisolvens* H17c by electroporation. Expression of the *S. bovis* amylase was detected

by zymogram analysis. The amylolytic activity of *S. bovis* (pUBLRSA), grown on glucose as the sole carbon source, was 2.5 times higher than that of wild type *B. fibrisolvens* H17c. This is the first report of the expression of a foreign polymer-degrading enzyme in a bacterium indigenous to the rumen. Research on construction of a more effective expression system and introduction of additional genes encoding fibrolytic enzymes into native ruminal species is now under way in our laboratory.

Transgenic plants

Recent advances in plant biotechnology may revolutionise the commercial enzyme industry by offering alternative, cost-effective methods of enzyme production and delivery. Large quantities of plant biomass can be produced inexpensively through the use of existing agricultural infrastructure. Expression of enzymes in plant species commonly used for animal feed will minimise downstream processing as the whole or parts of the producing plants are fed directly to livestock. The expression of a xylanase in tobacco plants has been reported (Herbers et al 1995), as has the expression of a phytase in tobacco and soybean (Pen 1994; Pen et al 1993; Russell 1994).

Herbers et al (1995) achieved the constitutive expression of a truncated *Clostridium thermocellum xynZ* gene (Grepinet et al 1988) encoding a thermostable, high specificity xylanase in tobacco via the cauliflower mosaic virus (CaMV) 35S promoter. The proteinase inhibitor II signal sequence was used to target the xylanase into the apoplastic space. *C. thermocellum* truncated XynZ comprised up to 4.1% of the total protein in leaf extracts and 50% of the apoplastic proteins. The thermal stability of XynZ was exploited to achieve a 31-fold purification through a 20 min, 60°C incubation.

In similar experiments, transgenic tobacco plants were made to express phytase encoded by the *Aspergillus niger phyA* gene (van Hartingsveldt et al 1993; Pen 1994; Pen et al 1993). Constitutive expression was driven by the CaMV 35S promoter and targeted for the apoplast using the tobacco PR-S signal sequence. In the seeds of transgenic tobacco plants, the expressed *A. niger* phytase comprised 1% of total soluble protein. Feeding trials demonstrated that transgenic tobacco seeds were as effective at promoting growth of broilers as was a commercial *A. niger* phytase product or inorganic phosphorus (Pen 1994).

At the Lethbridge Research Centre, we are combining the utility of plant expression systems with superior fibrolytic enzymes produced by ruminal fungal isolates. In collaboration with researchers at the University of Calgary, we have constructed oleosin (oil body membrane protein)-*N. patriciarum* xylanase gene fusions in preparation for *Agrobacterium*-mediated transfer of the xylanase gene to canola. A number of peptides, including β-glucuronidase from *E. coli,* hirudin and interleukin 1-β (amino acids 163–171), have already been expressed in this way (van Rooijen 1993; van Rooijen and Moloney 1994). Yields of recombinant protein (in excess of 1% of total seed protein) at significantly reduced cost are possible with this system.

In addition to providing an efficient alternative to traditional microbial systems, transgenic plants offer the added advantage of a safe and stable formulation system in the

form of seeds (Pen 1994). Recombinant enzymes were stable in seeds stored for up to one year at 4°C and room temperature (Pen et al 1993; van Rooijen and Moloney 1994).

Transgenic animals

Technological developments enabling introduction of genetic material into domestic animals (Briskin et al 1991; Ward et al 1989) have validated the concept of direct expression of these glucanases and xylanases in the animal itself as an option to adding microbial enzymes to the feed. To be of benefit to the animal, the enzymes should be expressed in the appropriate tissue, secreted into the lumen of the gastrointestinal tract, resistant to proteases, and active in environmental conditions (e.g. pH, temperature, osmolarity) prevailing in the lumen (Forsberg et al 1993; Hall et al 1993).

Expression of fibrolytic enzymes in a non-ruminant animal was first demonstrated by Hall et al (1993). A truncated endoglucanase E gene from *Clostridium thermocellum*, under the control of the elastase I gene promoter, was expressed in the exocrine pancreas of transgenic mice. Carboxymethylcellulase activity was detectable in small intestinal contents, demonstrating that the recombinant enzyme was secreted from the pancreas. Work is under way to develop transgenic mice in our laboratories as well. Twenty genes have been screened for their suitability for introduction into non-ruminant animals. To date, gene fusions of three selected genes with the elastase I gene or amylase *amy* 2.2 gene promoters have been constructed. The obvious potential of this approach is a powerful incentive for continued efforts in this field of research.

Conclusions

The potential for improvement of the efficiency of non-ruminant and ruminant livestock feed utilisation by enzyme supplementation is widely recognised. However, the cost of this technology has inhibited its widespread application in the industry. Reducing the cost of feed enzymes through improved enzymes and more efficient production systems is the focus of much research in this area. Microbial isolates from the rumen produce a wide array of enzymes and comprise an immense and under-utilised gene pool. As exploration of this enzymological and genetic resource progresses from domestic ruminants to those adapted for survival in diverse habitats worldwide, the isolation of even more potent enzymes is certain. The technology required for practical and safe modification and application of these powerful agents is in place.

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Clostridium paraputrificum var. *Ruminantium:* colonisation and degradation of shrimp carapaces *in vitro* observed by scanning electron microscopy

M.A. Cobos[1] and M.T. Yokoyama[2]

1. Programa de Ganaderia, Colegio de Postgraduados, Montecillo, Edo. de México, 56230

2. Department of Animal Science, Michigan State University, East Lansing MI 48824.

Abstract

Previously a spore-forming bacterium identified as *Clostridium paraputrificum* var. *ruminantium* was isolated from the rumen of dairy cows. Compared with other clostridial species in the rumen, this bacterium is unable to degrade cellulose, but rapidly degrades chitin, shrimp carapace and crab shell waste meal. The present study was undertaken to characterise the morphology of *C. paraputrificum* var. *ruminantium* and to improve our understanding of the process of shrimp carapace degradation by this bacterium using scanning electron microscopy. Bacteria growing on shrimp carapace showed vegetative cells 3 to 5 μm in length with rounded ends. Cells with terminal spores were larger, averaging 7.3 μm in length. The spores were prominent between 1 to 2 μm in length and 0.7 to 1 μm in width. Filamentous forms were observed only when the substrate had been depleted. Incubation of *C. paraputrificum* var. *ruminantium* with pieces of shrimp carapace was characterised by three major events. First, colonisation and growth was only observed on the inner surface of the shrimp carapace. This activity was observed within the first 3 h after incubation and extended through 24 h of incubation. Between 24 and 72 h of incubation, bacterial growth was characterised by the degradation of the different multiple layers of chitin that comprise the shrimp carapace to finally reach the outer surface. After 96 h of incubation, when the shrimp carapace had been almost totally degraded, the bacteria entered a death phase and latency, marked by the predominance of free spores and lysed bacteria. Since chitin degradation is initiated only after bacterial attachment occurs, a study of factors that influence this adhesion may result in more efficient use of chitinous waste as a feedstuff for ruminants.

Introduction

Shellfish waste is the generic name used to describe the by-products generated by the fishing industry after meat extraction from marine crustaceans such as crab, shrimp, lobster

and prawn, and freshwater crustaceans such as crawfish. Shellfish waste consists of shells, viscera and unextracted meat. Most of the shellfish waste, which approximates 85% of the fresh catch, is largely disposed of by its return to the environment either on landfill sites or by direct return to the sea and presents an environmental pollution problem.

A potential use of large quantities of shellfish waste is as a feed for livestock. For example, shrimp carapaces dry matter contains in excess of 37% crude protein (Husby et al 1981). The main limitation of shrimp carapaces as a feedstuff is its chitin content (21% of DM), which cannot be efficiently digested by most productive animals. However, ruminant animals, because of the symbiotic microorganisms living in their rumen, are considered better adapted than non-ruminants to assimilate the nutrients contained in shellfish waste.

The potential of chitinous waste as a feed for ruminants relies on the capacity of some ruminal microorganisms to degrade chitin (Brundage et al 1981, 1984; Laflamme 1988; Velez et al 1991), but the ruminal microorganisms involved in chitin degradation have never been isolated and identified.

Recently, a spore-forming bacterium was isolated from the rumen of dairy cows (Cobos and Yokoyama 1993). The clostridial isolate was classified as *C. paraputrificum*, with 99% confidence, using the RapID II ANA system. However, at least 50 different strains of this *Clostridium* have been isolated from very diverse environments such as from soil, marine sediments, human infant faeces, and porcine, avian and bovine faeces (Cato et al 1986). Therefore, we proposed that the ruminal strain isolated be designated *C. paraputrificum* var. *ruminantium*. The metabolic profile of the clostridial isolate indicates that this bacteria is highly specialised for chitin utilisation. It is unable to degrade cellulose, a common characteristic of other clostridial species isolated from the rumen of cattle and sheep such as *C. polysaccharolyticum* (van Gylswyk et al 1980), *C. chartatabidum* (Kelly et al 1987) and *C. longisporum* (Varel 1989).

Although the intimate association of the bacterium with chitin suggested that it attaches to chitin before degradation, similar to the attachment of other bacteria to cellulose and starch, little was known about the actual process.

Materials and methods

Organism and culture conditions

A chitinolytic bacterium previously isolated from the rumen of dairy cows, *C. paraputrificum* var. *ruminantium*, was used in this study. The bacterium was grown at 39°C in chitin (CH) medium (CH-medium components described in Table 1). A 48 h culture of the bacterium growing in CH-medium was used as inoculum. The bacterial concentration in the inoculum, as estimated by the roll tube technique, was about 10^{10} cells per ml.

Shrimp carapace preparation for colonisation study

Shrimp carapace (SC) were used as substrate to observe bacterial colonisation rather than crab shell meal or pure chitin (Sigma) because in initial attempts the last two substrates

Table 1. *Composition of the chitin medium used for growing* C. paraputificum *var.* ruminantium.

Item	Chitin medium (per 100 ml)
Deionised water	51.6 ml
Clarified ruminal liquid[†]	30.0 ml
Sodium carbonate 8% solution	5.0 ml
Mineral solution 1[‡]	5.0 ml
Mineral solution 2[§]	5.0 ml
Cystein-sulfide solution[¶]	2.0 ml
Resazurin 0.1% solution	0.1 ml
Trypticase-peptone (LLB)®	0.2 g
Yeast extract (LLB)®	0.1 g
Chitin (Sigma)	1.0 g

[†]Fresh ruminal fluid previously filtered through a cheese cloth is centrifuged at 23,420 g for 15 min at 4°C, autoclaved 20 min at 15 psi-121°C.
[‡]Contains (per 1000 ml) K_2HPO_4, 6.0 g.
[§]Contains (per 1000 ml) KH_2PO_4, 6.0 g; $(NH_4)_2SO_4$, 6 g; NaCl, 12 g; $MgSO_4$, 2.45 g; and CaCl° 2 H_2O, 1.6 g (Bryant and Robinson 1962).
[¶]2.5 g L-cysteine (dissolved in 15 ml of 2N NaOH); $Na_2S.9H_2O$, 2.5 g and resazurin, 0.1 ml (1% sol. in H_2O) in a final volume of 100 ml heated until the resazurin indicator is colourless, and autoclaved.

did not provide a good enough contrast with the scanning electron microscope. The SC were washed in tap water to separate residues of meat, cut into pieces of about 0.5 cm^2, and boiled in tap water for 10 min. Five small pieces of the boiled SC were placed into eight Dacron bags (5 × 12.5 cm) with 20–35 μm pore size (Ankom, Fairport, NY). Also, in order to observe for possible cellulose colonisation and/or degradation, one small piece of Whatman paper No. 4 (0.5 × 2 cm) was added to each bag. The Dacron bags were placed into a 1 litre Erlenmeyer flask, containing 500 ml of anaerobic SC medium. The SC medium components are given in Table 2, and was prepared anaerobically according to the method of Hungate (1969) as modified by Bryant (1972).

The SC medium in the Dacron bags was inoculated with 5 ml of a 48 h culture of *C. paraputrificum* var. *ruminantium*, then incubated at 39.7°C. The Dacron bags were recovered aseptically and consecutively after 0, 3, 6, 12, 24, 48, 72 and 96 h of incubation. The Dacron bags were previously found to be very useful for a fast and aseptic recovery method for multiple samplings and that is the main reason for their use.

Scanning electron microscopy

At appropriate time intervals, Dacron bags were removed aseptically and the residual shrimp carapace were recovered by gently rinsing with distilled water and immediately fixed for 1 h in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). After fixation the samples were rinsed with 0.1 M phosphate buffer. Samples were then dehydrated with a graduated ethanol series (25, 50, 75, 90 and 100% ethanol), and critical point-dried with CO_2. Specimens were mounted on aluminum stubs, sputter-coated and examined with a JEOL JSM-35C scanning electron microscope at the Center for

Table 2. *Composition of the SC anaerobic medium used for observing bacterial attachment to and degradation of shrimp carapace.*

Item	Quantity per 100 ml
Deionised water	52.6 ml
Clarified ruminal liquid	30.0 ml
Sodium carbonate, 8% solution	5.0 ml
Minerals solution 1[†]	5.0 ml
Mineral solution 2[‡]	5.0 ml
Cysteine-sulfide solution[§]	2.0 ml
Resazurin, 0.1% solution	0.1 ml
Trypticase-peptone (LBB®)	0.2 g
Yeast extract (LBB®)	0.1 g

[†]Contains (per 1000 ml) K_2HPO_4, 6.0 g.

[‡]Contains (per 1000 ml) KH_2PO_4, 6 g; $(NH_4)_2SO_4$, 6 g; NaCl, 12 g; $MgSO_4$, 2.45 g and $CaCl_2$° 2 H_2O, 1.6 g (Bryant and Robinson 1962).

[§]2.5 g L-cysteine (dissolved in 15 ml of 2N NaOH); $Na_2S.9H_2O$, 2.5 g and resazurin, 0.1 ml of a 1% solution in H_2O in a final volume of 100 ml heated until the resazurin indicator is colourless, and autoclaved.

Electron Optics of Michigan State University, following the procedures described by Klomparens et al (1986). The same procedures were followed with the pieces of Whatman filter paper added to the same Dacron bag for each period.

Results

Figure 1 shows structural characteristics of the shrimp carapace (SC) as to its outer surface, inner surface and lateral view, at time zero of inoculation. The outer surface (Figure 1a) is an even surface with the eventual appearance of small appendages about 700 μm in length which look like the rhizoid structure of a fungus. In contrast, the inner surface (Figure 1b) is rough with honeycomb-like compartments present in the curved parts of the exoskeleton. Between the outer and inner surfaces, there appear to be several layers of chitin (Figure 1c), which together with the outer and inner surface layers make up the shrimp carapace.

Figure 2 is a series of scanning electron micrographs representing the major events which take place during the process of colonisation and degradation of SC by *C. paraputrificum* var. *ruminantium* at different incubation periods. The results show that the bacterium attaches onto the SC inner surface within the first 3 h of incubation (Figure 2a), while the outer surface does not show any evidence of bacterial colonisation (Figure 2b). After 6 h of incubation, colonisation at the inner surface has spread considerably (Figure 2c). In contrast, the outer surface still remains uncolonised at the same time, even after degradation of the external appendages is apparent (Figure 2d). Strong evidence of carapace digestion was observed after 9 h of incubation, as indicated by the presence of typical digestion grooves formed on the inner surface of the shrimp carapace (Figure 2e). At the same time, colonisation and degradation was also present on the different multiple layers of chitin at the exposed sides (Figure 2f). The same photo

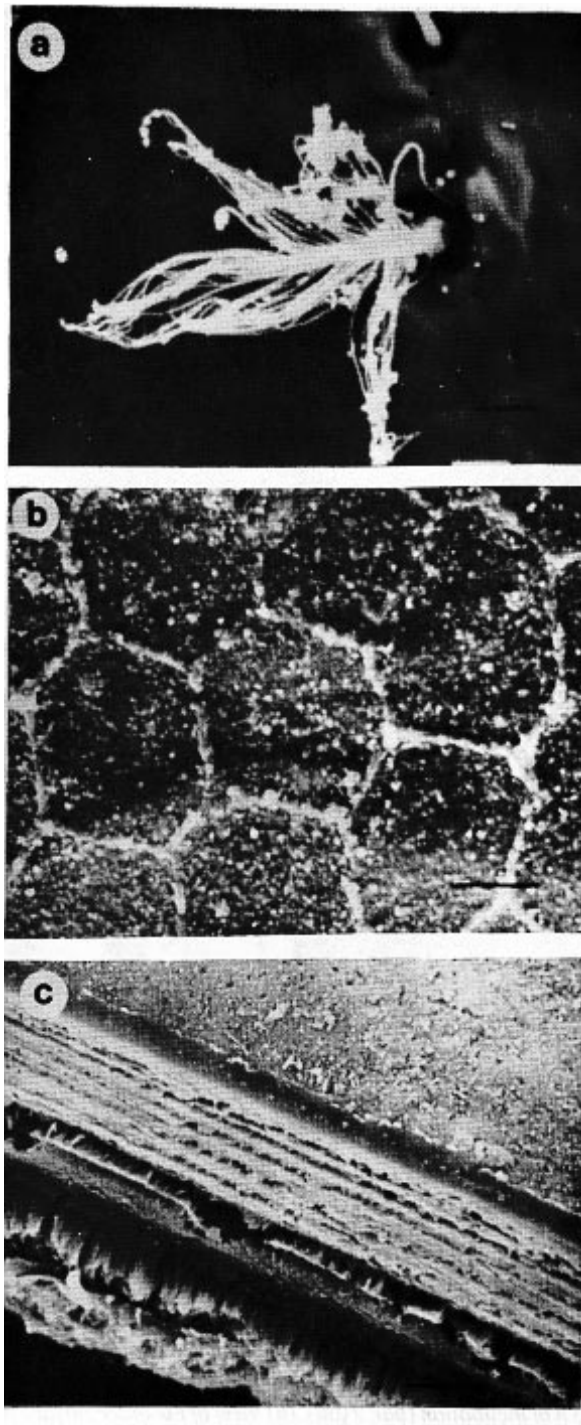

Figure 1. *Structural characteristics of the shrimp carapace at its (a) outer surface (bar, 10 μm), (b) inner surface (bar, 10 μm) and (c) a lateral view showing the different chitin layers that comprise its structure (bar, 5 μm).*

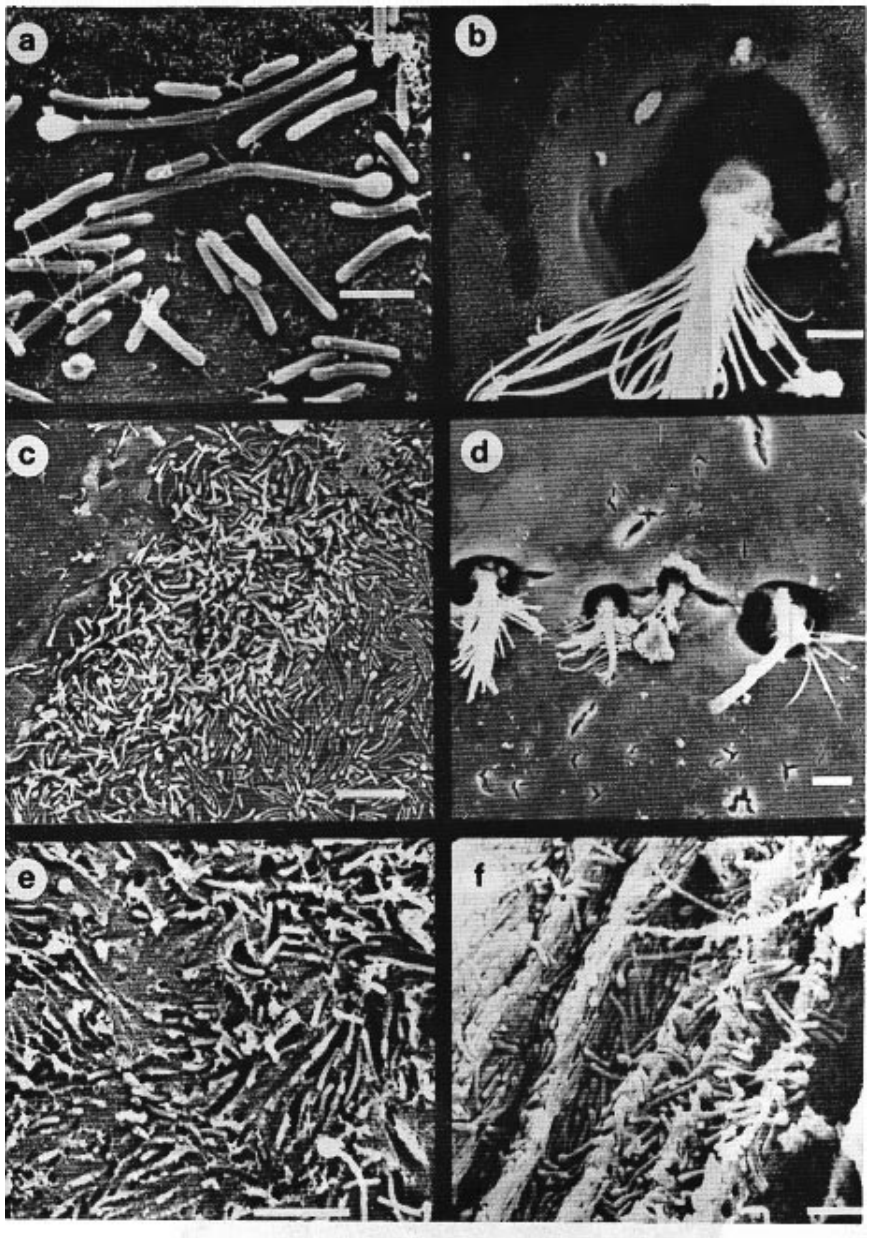

Figure 2. *Scanning electron micrographs of shrimp carapace incubated with* C. paraputrificum *var.* ruminantium. *(a) Morphological details of vegetative and spore-forming forms attached to the inner surface of the shrimp carapace after 3 h of incubation (bar, 2* μm*). (b) View of the outer surface at the same incubation period (3 h) with no signs of bacterial growth on its surface (bar, 5* μm*). (c) Degree of colonisation after 6 h of incubation (bar, 10* μm*). (d) Outer surface of the shrimp carapace still uncolonised after 6 h of incubation (bar, 10* μm*). (e) Digestive grooves formed on the inner surface of the shrimp carapace after 9 h of incubation (bar, 5* μm*). (f) Strong colonisation at the different layers that comprise the shrimp carapace after 9 h of incubation. Degradation of chitin is indicated by the presence of digestion pits that penetrate into the interior of the chitin layers (bar, 5* μm*).*

illustrates that the bacteria have formed digestion pits that penetrate into the interior of the chitin layers.

After 24 h of incubation, the ruminal clostridium showed profuse growth and colonisation on the inner surface of the shrimp carapace (Figure 3a). At the outer surface near cracked areas, bacterial growth was also evident just behind the outer layer, while the external surface remained unaltered (Figure 3b). Between 48 and 72 h of incubation, the presence of digestion pits that penetrate into the interior of the chitin layers was observed (Figure 3c). By 72 to 96 h of incubation, most of the shrimp carapace had been digested with the exception of the thin outer layer, which looked undigested, but very fragile, due to the lack of support (Figure 3d). By 96 h of incubation, most of the substrate was depleted, and the bacteria entered a distinct phase marked by the appearance of lysed bacteria and released spores indicating the senescence of the culture (Figure 3e and f).

When most of the shrimp carapace appeared to be degraded after 4 days of incubation, the culture was checked to see if the bacteria would switch their enzymatic system to cellulose degradation as an alternative source of energy. However, the Whatman paper added was neither colonised nor degraded even by the end of the incubation period (7 d).

Cellular morphology

Vegetative cells were straight or slightly curved rods about 0.3 μm wide and 3 to 5 μm long with rounded ends, and occurred singly or in pairs. Rods carrying spores were larger than the vegetative cells averaging 7.3 μm in length. Spores were oval, terminal and caused the cells to swell. The spores were about 1 to 2 μm long and 0.7 to 1 μm wide. Sporulation seemed to be triggered by either the lack of substrate for growth or by the adverse build up of toxic metabolites in the medium. Also filamentous forms were observed, mainly when the shrimp carapace had been degraded.

Discussion

The results of this study confirm the ability of *C. paraputrificum* var. *ruminantium* to bind to chitin. This characteristic is not particularly surprising if we consider the strong adhesive characteristic of chitin (Gooday 1990), and the necessity for this attachment. The adhesion of *C. paraputrificum* var. *ruminantium* to chitin may be essential in aqueous environments such as the rumen ecosystem, where the substrate must be degraded to dimers or monomers before their efficient absorption can occur. Attachment would optimise contact between the chitinolytic enzymes and the chitin molecules. In addition, the N-acetylglucosamine and/or chitobiose produced from chitin degradation would be more efficiently absorbed. Without attachment, there is also the possibility that the chitinolytic enzymes may be degraded and assimilated by other ruminal bacteria, or the N-acetylglucosamine released during chitin hydrolysis may be taken up by competing bacteria.

The mechanisms of adhesion are not known, but Montgomery and Kirchman (1993) reported that the specific attachment to chitin particles appears to be mediated by

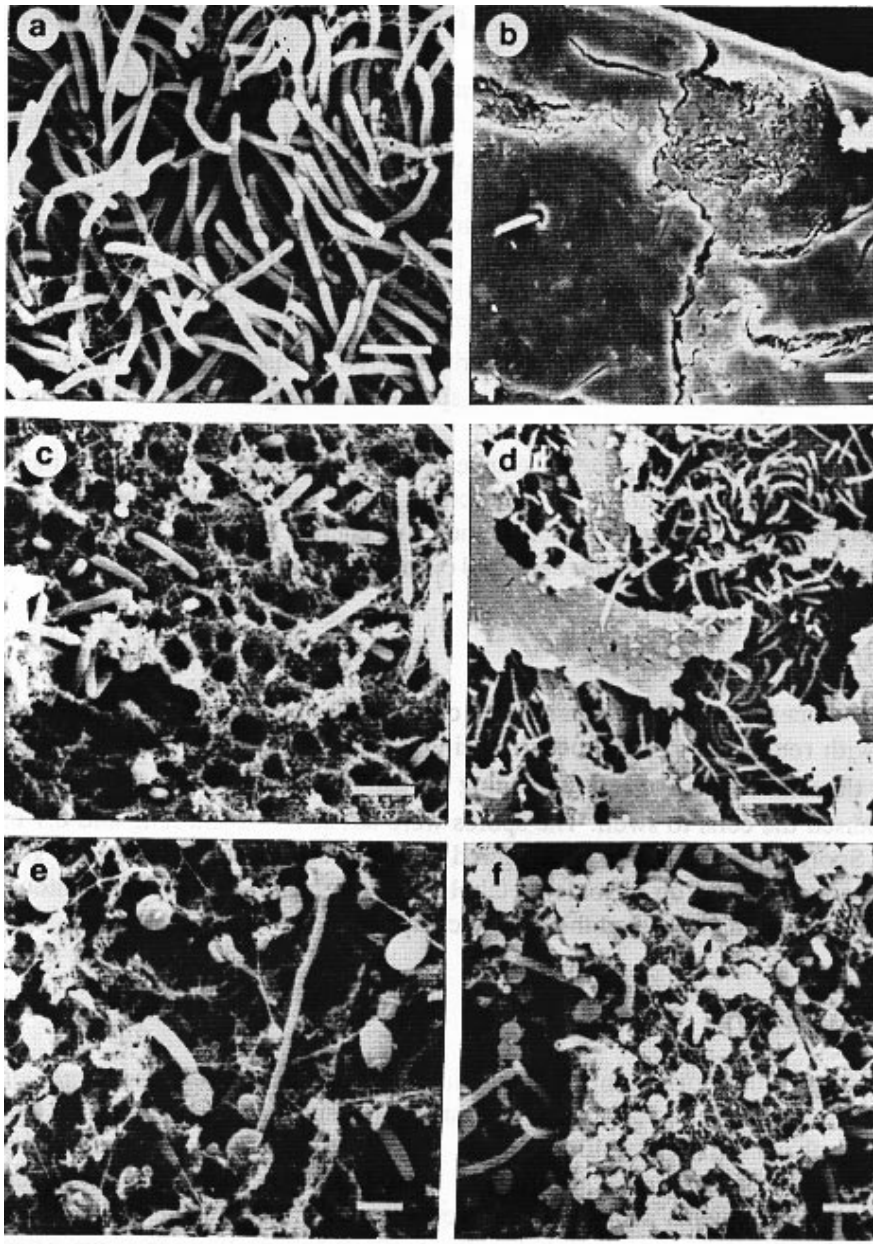

Figure 3. *Scanning electron micrographs of shrimp carapace incubated with* C. paraputrificum *var.* ruminantium *representing the major events that take place between 24 and 72 h of incubation. (a) Profuse bacterial growth and colonisation of the inner surface was observed by 24 h of incubation (bar, 1*μm*). (b) At the same period of time there is evidence of prodigal bacterial growth in cracked areas behind the outer surface; the external surface remains undigested (bar, 10* μm*). (c) Between 48 to 72 h of incubation the degree of digestion is evident by the presence of digestion pits in most of the inner surface of the shrimp carapace (bar, 1* μm*). (d) By 72 to 96 h of incubation, most of the chitin layers have been digested with the exception of the outermost layer (bar, 5* μm*). (e and f) represent the senescence of the culture as indicated by the appearance of lysed bacteria and the high proportion of free spores (bar, 1* μm*).*

chitin-binding proteins associated with the bacterial cell membrane. Another suggested mechanism is that lateral and polar flagella are responsible for cell attachment to binding sites on the chitin surface (Belas and Colwell 1982).

The external surface of the shrimp carapace seems to have a protective film of unknown structural complexity which prevents bacterial attachment and is recalcitrant to degradation. This protective film may play a role in the defense of shrimps against bacterial or fungal infections. It is unknown if this protective film is also present in other shellfish wastes, but it would seem advisable to grind shellfish waste when it is used as a feed for ruminants, to increase the attachment surface and the efficiency of degradation.

Although not well documented, it is believed that the rate of chitin degradation is enhanced by the adherence of chitinolytic microflora (Gooday 1990). Ou and Alexander (1974) observed that separation of chitin and chitinolytic bacteria by layers of microbeads greatly reduced the rate of mineralisation of chitin particles by pure cultures of a soil *Pseudomonas* species. This phenomenon has also been observed in *C. thermocellum,* an anaerobic bacterium which degrades α-crystalline cellulose after the cell adheres very strongly to the insoluble substrate (Bayer and Lamed 1986).

Since bacterial attachment to chitin seems to be essential for chitin degradation to occur, attention has been placed on possible plant components that inhibit or promote bacterial binding and therefore the digestion of chitinous compounds. For example, wheat germ agglutinin (WGA), a chitin-binding lectin from wheat embryos, exhibits binding specificity toward chitin (Bloch and Burger 1974). WGA lectin and other chitin-binding lectins isolated from stinging nettle rhizomes are potent inhibitors of fungal growth. Chitin chains in the fungal cell wall appear to be cross-linked and the hydrolysis of preformed chitin chains in the process of apical growth of fungal hyphae is inhibited (Broekaert et al 1989).

It is unknown if chitin-binding lectins are regular components of forages used as feedstuffs. Also, it is not known if lectins are easily degraded by ruminal proteolytic bacteria. However, it is known that the binding of lectins to chitin blocks proteolytic and β-N-acetyl-glucosaminidase activity against lectins and chitin, respectively (Knorr 1991). Therefore, if lectins are present in the rumen of cattle fed diets rich in chitin by-products like shellfish waste, they may have a negative effect on the digestion of chitinous compounds and animal performance.

Not much is known about the factors which affect the animal response to the intake of chitinous compounds, especially those factors which affect the efficiency of the rumen chitinolytic microorganisms. However, the observation that bacterial attachment is essential for chitin hydrolysis opens up a new research area concerning the possible factors that affect bacterial attachment to chitin and its consequences in the rumen ecosystem.

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Comparative abilities of feed intake and microbial digestion in the forestomachs of camelids and ruminants

J.P. Jouany[1], *J.P. Dulphy*[1] *and C. Kayouli*[2]

1. I.N.R.A., Station de Recherches sur la Nutrition des Herbivores, Centre de Recherches de Clermont Ferrand-Theix, 63122 Saint Genès Champanelle, France

2. INAT, 43 Avenue Charles Nicolle, 1002 Tunis Belvédère, Tunisie

Abstract

There are large differences in anatomy and digestive physiology between the forestomachs of camelids and ruminants. The most voluminous compartment corresponding to the rumen is named compartment 1 in camelids. Its ventral part is composed of a cranial sac and a caudal sac separated by a transverse muscular pillar. Each sac is covered by a band of several rows of glandular sacs made of musculomembranous cells. Compartment 2 is small and opens widely onto compartment 1. Compartment 3 is pear-shaped and composed of two distinct parts. Only the pyloric area secretes acid and enzymes. The ingestion time, the length of the main meals and the number of meals are not different between llamas and sheep. Llamas have fewer but longer ruminating periods and are more efficient masticators than sheep (2 g vs 1.3 g DM/min). Rumination occurs mainly at night in camelids. The liquid phase turnover is higher in dromedaries and llamas than in ruminants.

Camelids have lower water requirements and excrete less water and nitrogen in urine than ruminants. Both dromedaries and llamas have a high capacity for recycling nitrogen. Bicarbonates and phosphates are extensively secreted by the mucosa of the forestomachs and in the glandular sacs. This explains the higher buffering capacity of the digesta in camelids. Also, these secretions are probably responsible for the higher and more stable osmolality of the digesta of llamas compared to ruminants. Ammonia concentration is lower and more variable in compartment 1 than in the rumen. Total VFA concentration is more difficult to characterise. Butyrate concentration is frequently higher in camelids. The microbial ecosystems are basically the same in camelids and ruminants, but their glycolytic activities are different. The *in sacco* degradation of plant cell walls is higher in camelids than in ruminants. The amount of digested cell wall carbohydrate is much larger in camelids than in ruminants. The lower temperature of the digesta in the forestomachs of camelids could indicate that microbes produce less extra heat and that more ATP is available for microbial growth. These characteristics of ingestive and digestive abilities of camelids show that their rumen microbiota has a higher digestive activity.

Introduction

Ruminants and camelids both belong to the order Artiodactylae in the mammalian class. The family Camelidae belongs to the suborder Tylopoda which appeared 50 million years ago. The family Bovidae (cow, sheep, goat) in the suborder *Ruminantia* developed more recently, only 3 million years ago (Romer 1966). The family Camelidae is composed of 6 species: 2 of the genus *Camelus*, 1 of the genus *Vicugna* and 3 of the genus *Lama* (llama, guanaco, alpaca).

Camelids, like ruminants, developed large forestomachs harbouring a dense and complex microbial ecosystem which makes them efficient in the use of forages as a main source of nutrients. However, there are large differences between camelids and ruminants in the anatomical organisation of their forestomachs and the histology of their mucosa which give them some specificities in the digestive abilities of their microbial ecosystem. Our paper will compare feeding abilities and microbial digestive abilities of camelids and ruminants. Dromedaries and South American camelids will be considered in these comparisons.

Anatomical and physiological differences in the forestomachs of camelids and ruminants

The ruminant stomach is composed of four compartments (rumen, reticulum, omasum, abomasum) located between two sphincters: the cardia at the end of the oesophagus and the pylorus at the beginning of the duodenum. Following ingestion, solid feeds are submitted to an intense microbial digestion in the first two compartments. Large exchanges of digesta occur between rumen and reticulum. A sphincter located between reticulum and omasum controls the retention time of feed particles in the main fermentation chamber, the reticulo-rumen. The mucosa of the first three compartments is keratinised and covered by papillae. The omasum contains numerous lamellae which act as a filter for large particles and absorb the end products of microbial fermentations.

The camelid stomach is composed of three compartments. Compartment 1 could be compared to the rumen while compartment 2 could be compared to the reticulum. Compartment 3 is completely different from the omasum in its function and organisation. It is pear-shaped and internally covered by a mucosa which secretes hydrochloric acid and enzymes only in the pyloric region.

In contrast to the rumen, which is composed of six separate sacs, compartment 1 is made of a cranial and a caudal sac separated by a muscular transverse pillar. In contrast to true ruminants, the epithelium of the camelid forestomach is devoid of papillae and is made of a smooth stratified epithelium. Two rows of glove-shaped glandular sacs are located on the wall of compartment 1; one row is found on the cranial sac and the other on the caudal sac. They are composed of glove-shaped cavities opening onto the inside of compartment 1. Each cavity has a muscular sphincter opening at its fundus. They have been described as a place for large bicarbonate secretions and a subsequent absorption of volatile fatty acids (VFA) as shown by Cummings et al (1972) and Luciano et al (1979). Papillae are absent on the wall of the first two compartments, but glandular

cells cover the entire ventral part of compartment 1, compartment 2 and the upper part of compartment 3. These secretive cells produce a bicarbonate-rich mucus and could be involved in VFA absorption as indicated previously for glandular sacs.

Differences in ingestive capacities between camelids and ruminants

Richard (1989) reviewed the available data on the voluntary intake of dromedaries in natural conditions. The mean consumption varied from 14–15 g DM/kg liveweight (LW) for straw and poor roughages to 23–24 g DM/kg LW for good quality forages. Data concerning straw are comparable to those noted for cattle in tropical areas, and slightly higher than for cattle in Europe (12 g/kg DM with heifers).

Experiments on direct comparisons between animals show that the camelid intake was lower than sheep intake (El-Shaer 1981; Gauthier-Pilters 1979; Gihad et al 1989; Kandil 1984; Shawket 1976). Kayouli et al (1995) found that mean intake for three diets was 12 g/kg LW for dromedaries and 25 g/kg for sheep. Gihad et al (1989) also noted that dromedaries ingested 11.4 g DM of three different diets per kg LW while the mean consumption by sheep was 17.9 g/kg. In a comparison carried out on four dromedaries, four rams and two goats fed on oat-vesce hay, H. Rouissi, D.I. Demeyer, C. Kayouli and van C.J. Nevel (unpublished data) showed that goats ate twice as much as dromedaries (25 vs 13 g DM/kg LW respectively), while rams had an intermediary intake (Table 1).

Few data involving llamas are available. From 11 direct comparisons, we observed that intake of dry forages, supplemented or not with concentrate, was 15.9 g DM/kg LW (49.3 g/kg $LW^{0.75}$) in llamas and 18.4 g DM/kg LW (51.7 g/kg $LW^{0.75}$) in sheep (Cordesse et al 1992; Dulphy et al 1994a, 1994b; Lemosquet et al 1995; Warmington et al 1989). When data are computed in a figure, it is noteworthy that llamas ingest less good quality forage, on a metabolic liveweight basis, than sheep (Figure 1).

Comparisons of feeding activities

Ingestion and rumination times during the day are not different between camelids and ruminants. According to Gauthier-Pilters (1979) and Khorchani et al (1992), it took 400–500 min/d for ingestion at pasture. At trough, Abdouli and Kraiem (1990) noted that ruminating time was 500–600 min/d. Kaske et al (1989) observed that rumination occurs principally during the night in camels. In a comparative trial, Lemosquet et al (1995) noted that ingestion time was the same for llamas and sheep (368 vs 389 min/d, respectively) while the former had a shorter ruminating time (468 vs 538 min/d). The lengths of main meals were similar (160 min and 151 min for llamas and sheep respectively) as well as the number of meals (7.0 and 7.9/d, respectively). However, llamas had fewer (7.5 vs 12.1) but longer ruminating periods which took place mainly during the night. Llamas masticated 2 g/min while sheep were less efficient (1.3 g/min). Such behaviour could explain why there are more large particles (>1 mm) in the faeces of llamas than in sheep (Warmington et al 1989). Also, Lechner-Doll et al (1991) observed that the critical size for particles to move out of the rumen is 3 mm in llamas

Table 1. *Comparative ingestive capacities of adult camelids and ruminants.*

Authors	Animals	Liveweight (kg)	DM intake (g/kg LW)					DM intake (g/kg LW$^{0.75}$)				
			Straw	Hay				Straw	Hay			
Dulphy et al (1994a)	Sheep	60	11.2	15.5	18.0	20.5	23.0	31	43	50	57	64
	Llamas	100	13.0	14.5	15.5	16.5	17.5	41	46	49	52	55
Rouissi (1994)	Dromedaries	220	13.0	11	—	—	—	53	63	—	—	—
	Sheep	46	19.0	20	—	—	—	49	51	—	—	—
	Goats	34	25.0	17	—	—	—	60	44	—	—	—

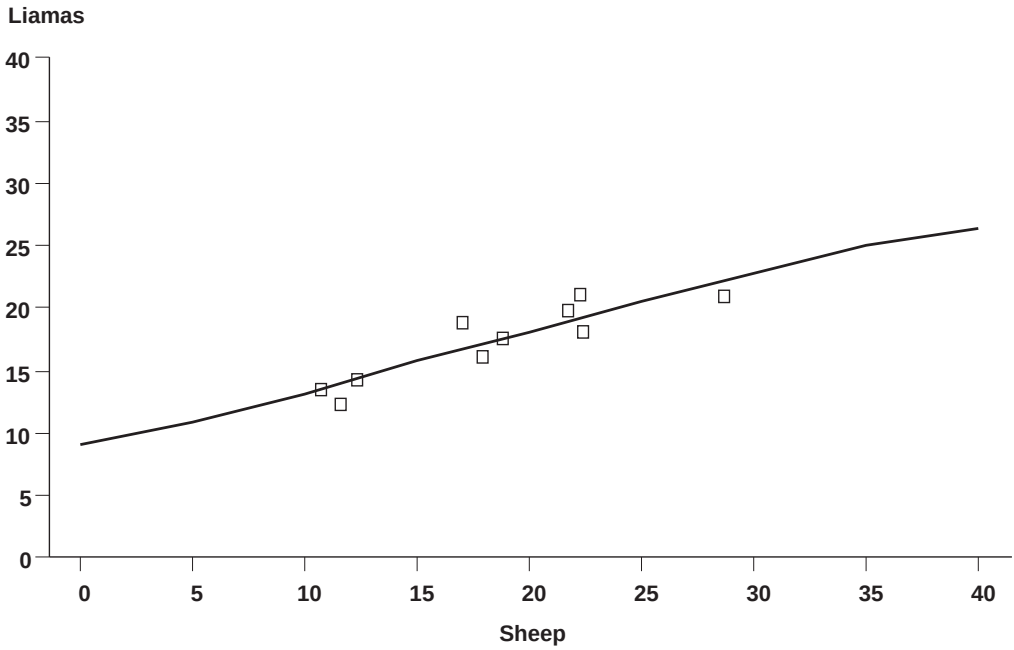

Figure 1. *Comparisons of DMI (g/kg LW) in llamas and sheep (n = 11 comparisons; from Dulphy et al 1994a).*

while it is 1 to 2 mm in sheep and cattle. This could be due to the absence of the filter effect of omasal lamellae in llamas.

Turnover rates of digesta in forestomachs

Mean retention time of feed particles is longer in camelids than in ruminants (Lechner-Doll et al 1991; Kayouli et al 1993a) and this could explain the lower ingestion capacity of camelids. Such differences are not explained only by the differences in animal body weights. Using llamas and sheep with similar LW, Dulphy et al (1994b) and Lemosquet et al (1995) found a mean retention time of 44 h and 27 h respectively for solid particles in the rumen.

These differences are mainly due to forestomach anatomy, to the motility of the digestive tract and to the feeding behaviour of animals. For instance, the low ruminating activity during the light part of the day will limit the comminution of solid particles and maintain them in compartment 1 for a longer period of time in camelids. As described by

Engelhardt and Höller (1982), Heller et al (1984) and Engelhardt et al (1986a), forestomach motility is different in dromedaries and llamas to that described in ruminants. There are more type B contractions in camelids, which could explain the efficiency of the mixing effect inside compartment 1 and the lower propulsive effect outside the compartment (Gregory et al 1985). According to Engelhardt et al (1986b), such a motility is favourable to a better separation of the liquid and solid parts of digesta. As a consequence, the turnover of liquids and solutes is greater in camelids compared to ruminants (Kayouli et al 1993a). From the results of Maloiy (1972), Farid et al (1979) and Kayouli et al (1993a), the mean retention time of the liquid phase in the rumen is 6 h in dromedaries and 8 h in sheep. In a direct comparison, Lemosquet et al (1995) noted that the liquid remained for 11 h in compartment 1 of llamas vs 14 h in the rumen. The higher turnover rate of liquids and the longer retention time of solids both explain the higher DM content of digesta in compartment 1 of camelids as observed by Dardillat et al (1994a).

Farid et al (1979), Lechner-Doll et al (1990) and Kayouli et al (1993a) observed that the weight of fresh rumen content is higher in dromedaries than in ruminants; it represented on average 14.7 and 10.5% of the LW of dromedaries and sheep, respectively, fed the same diets. However, according to Dulphy et al (1994b) and Lemosquet et al (1995), the weight of ruminal contents of digesta was not different between llamas and sheep and represented 18.7% of LW when fed inside, at trough.

Water intake, salivation and secretions in the forestomachs

Dromedaries, and llamas to a lesser extent, drink and urinate less than ruminants. According to Gihad et al (1989), water intake per kg DM intake in dromedaries equals 65% that of sheep. On the same basis, Warmington et al (1989) also noted that llamas ingest 57% of water intake by sheep.

Kay and Maloiy (1989) showed that dromedaries secrete larger amounts of saliva than cattle and ovines. According to Engelhardt and Höller (1982), camelid saliva is richer in bicarbonates and phosphates, although Engelhardt et al (1984) considered, in another paper, that the production and composition of saliva is not very different between ruminants and camelids.

Camelids have a high capacity for recycling nitrogen through their forestomach mucosa (Ali et al 1977; Emmanuel et al 1976; Engelhardt and Höller 1982; Engelhardt et al 1986b; Farid et al 1979), thus limiting urinary losses. The low rate of glomerular filtration by kidneys saves both water and urea (Yagil 1985), and explains why urea concentration can be lower in urine than in blood (Schmidt-Nielsen et al 1957; Wilson 1984). This originality makes the camelids more sensitive to the toxicity of diets with a high content in soluble nitrogen. Similar observations were made on llamas (Hinderer and Engelhardt 1975). Camelids therefore have a greater ability to recycle nitrogen in their forestomachs than ruminants.

Bicarbonates and phosphates are secreted inside compartment 1 via the mucosa and more intensively in the glandular sacs. This mechanism contributes efficiently to the stabilisation of physico-chemical conditions in compartment 1 of camelids and maintains the activity of microbes in extreme feeding situations (Jouany and Kayouli 1989;

Lemosquet et al 1995). Engelhardt and Höller (1982) consider that the secretion of bicarbonates greatly improves the absorptive capacity of VFA by mucosa of the camelid forestomach. They estimated that the rate of absorption is two to three times higher in camelids than in ruminants.

Physico-chemical conditions in the forestomach

Physico-chemical conditions are more stable in the forestomachs of camelids as indicated by Kayouli et al (1991, 1993b), Dardillat et al (1994a), Smacchia et al (1995) and Lemosquet et al (1995). Compared to ruminants, pH remained higher in dromedaries and llamas after feeding as previously indicated by Maloiy (1972) and Vallenas and Stevens (1971). The substitution of 25% of hay by barley had no effect on pH in llamas while it decreased the pH in sheep (Lemosquet et al 1995), indicating a higher buffering capacity of digesta in camelids. Even when fed a poor hay, pH remained higher and more stable in dromedaries than in sheep or goats (H. Rouissi, D.I. Demeyer, C. Kayouli and van C.J. Nevel, unpublished data; Figure 2). NH_3-N concentration was lower and less variable in dromedaries as confirmed by Farid et al (1979) and H. Rouissi, D.I. Demeyer, C. Kayouli and van C.J. Nevel (unpublished data) (Figure 3). However, Lemosquet et al (1995) noted that the concentration of NH_3-N is higher in llamas than sheep at the end of the night, before the morning meal. VFA concentrations are more difficult to characterise since they are sometimes higher (Kayouli et al 1993a; H. Rouissi, D.I. Demeyer, C. Kayouli and van C.J. Nevel, unpublished data) or lower (Farid et al 1979) in dromedaries when compared to sheep. On a molar proportion basis, the amount of butyrate is higher in camelids than in ruminants. This indicates that a shift in metabolic pathways occurs in camelids. As a consequence, VFAs are more efficiently absorbed since rate of absorption is related to the length of acid chains (Rémond et al 1995). According to Smacchia et al (1995), the buffering capacity of digesta in the forestomachs of camelids disappears when digesta are transferred into *in vitro* fermenters. It means that this capacity is a direct effect of animals. Secretion of bicarbonate by the wall of compartment 1 and the subsequent higher rate of VFA absorption explain the major part of this effect. The higher turnover rate of liquid associated with digesta in camelids also contributes to the elimination of the end products of fermentations. Such stable conditions are favourable to the growth of cellulolytic bacteria and fungi which are both sensitive to high acidic conditions.

Because of the high recycling capacity of NH_3-N through saliva and rumen wall, camelids are able to supply rumen microbes with their needs in soluble nitrogen even in low nitrogen feeding conditions. Cellulolytic bacteria which have high needs in soluble nitrogen can thus maintain their activity when animals are fed poor roughage diets with a low nitrogen content. This also means that camelids excrete small amounts of nitrogen in urine which makes them very sensitive to overdosages of soluble nitrogen in diets and limits their contribution to soil pollution.

Considering these results, it can be assumed that camelids are better adapted than ruminants to using diets supplemented with energy concentrates. Such complementary energy will be fermented in compartment 1 to give higher production of VFA available for animals without any negative effect on cellulolytic bacteria and fungi. The subsequent

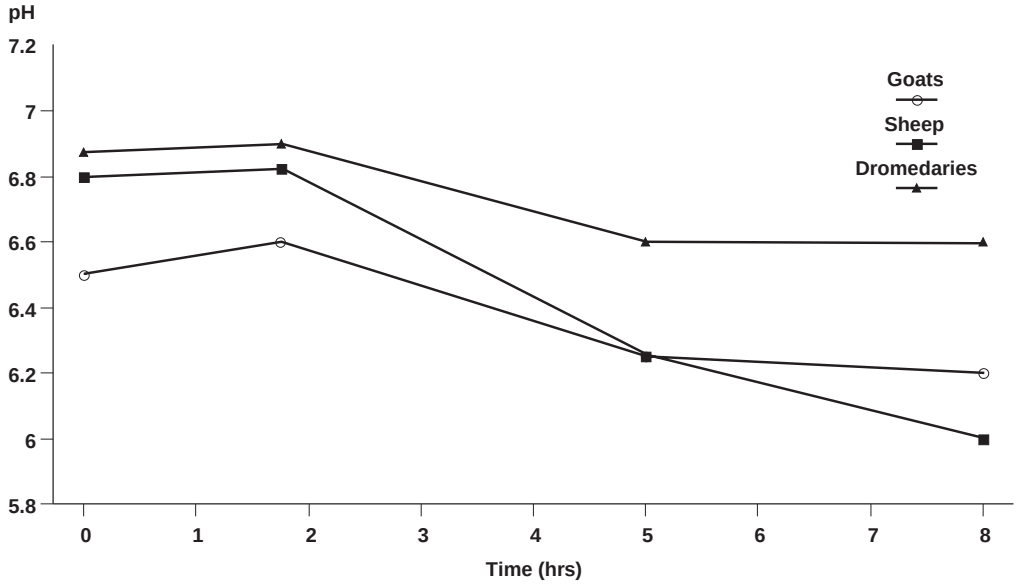

Figure 2. *Evolution of pH in the forestomachs of camelids and ruminants.*

increase in ATP production associated with higher NH_3-N availability will serve protein synthesis and therefore, supply camelids with more amino acids.

Lemosquet et al (1995) showed that osmolality of the fluid in the digesta of forestomachs was significantly higher in llamas (277 mOsm l^{-1}) than in sheep (259 mOsm l^{-1}) fed three different diets. This result confirms the higher content of electrolytes due to secretions in the digesta of camelids. Also, osmolality was more stable in camelids all throughout the day.

Microbial ecosystems and their activities in the forestomach

Only a few differences could be shown by scientists in the microbial population found in the forestomachs of camelids and ruminants. According to Williams (1963) and Ghosal et al (1981), the dominant species of bacteria are the same and their numbers are not different between camelids and ruminants. Several observations made by Jouany and Kayouli (1989) and Kayouli et al (1991, 1993a) showed that the total population of protozoa tended to be lower in llamas and dromedaries, while the distribution of the different genera is the same. We found that entodinia represented 90% of the number of ciliates and that the large *Entodiniomorphida* protozoa are only of type B in camelids (see the classification of Eadie 1962), whereas some animals harbour type A and others type B in ruminants. Also, the fungal population seems to be higher in camelids (Fonty, personal communication).

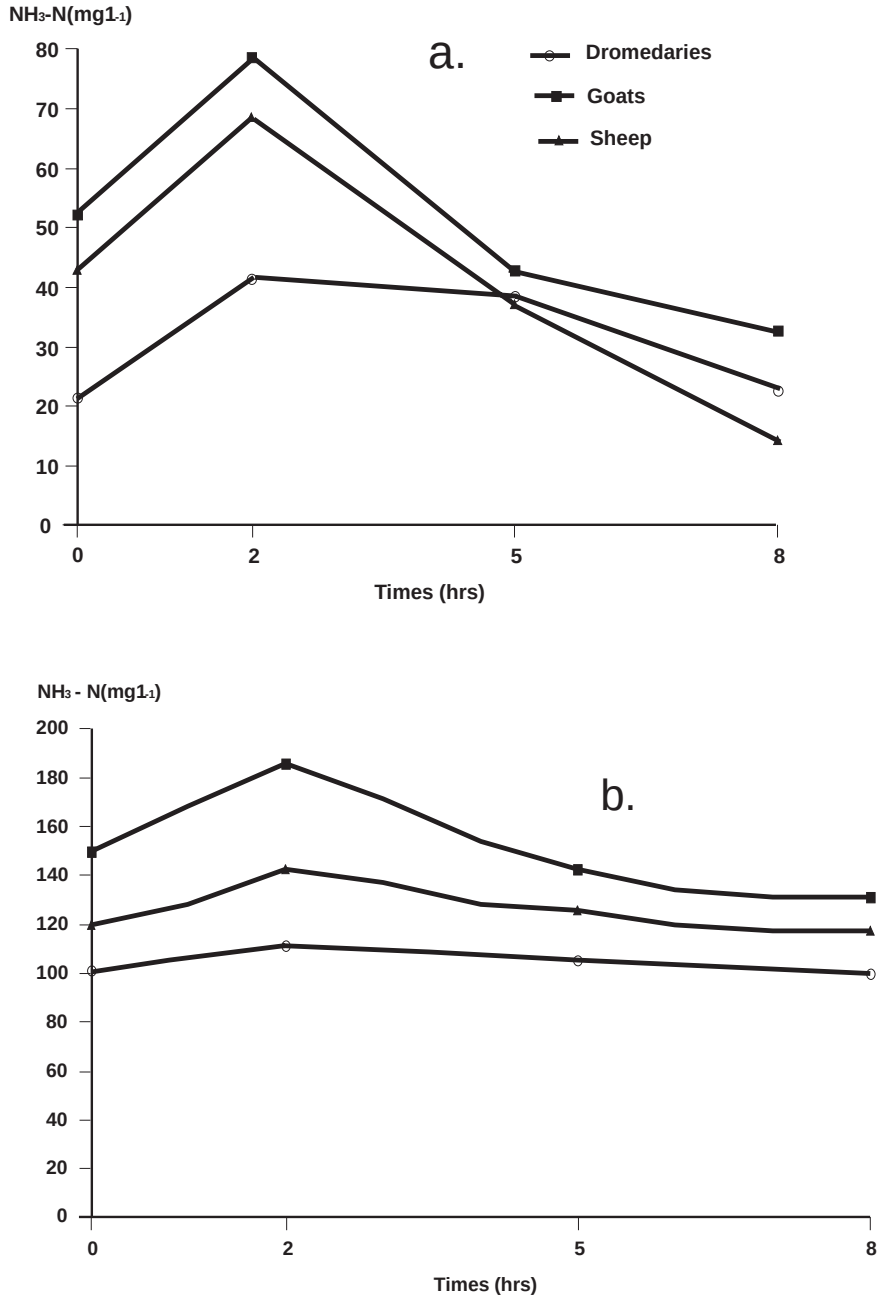

Figure 3. *NH_3-N concentrations in the forestomachs of camelids and ruminants—a) animals were fed oat-vesce hay; b) animals were fed a mixed diet.*

However, these slight differences in the microbial ecosystems could not explain the higher cellulolytic activities noted in the microbial population of camelids.

Kayouli and Jouany (1990), Kayouli et al (1991, 1993a, 1993b) and Dardillat et al (1994a) observed that the *in sacco* degradations of the cell wall part of several roughages

was higher in camelids after 24 h of retention time in the rumen than in ruminants. The more indigestible the substrate, the higher the difference in favour of camelids (Kayouli et al 1991). To confirm these results, Kayouli et al (1993b) and Dardillat et al (1994b) showed that the indigestible *in sacco* residue of straw from the sheep was digested again when introduced into compartment 1 of llamas. The rate of cell wall degradation was not different between animals. Using the same technique, H. Rouissi, D.I. Demeyer, C. Kayouli and van C.J. Nevel (unpublished data) showed that the percentage of ADF degradation of oat-vesce hay at 72 h was not different in goats and dromedaries (37 and 39%, respectively); it was significantly higher in these animals than in sheep (27%). No differences were noted in proteolytic activity of microbes in the forestomachs of camelids and ruminants.

Comparisons on digestibilities and microbial protein synthesis

The data obtained by Farid et al (1979) and Gihad et al (1989) suggests that the digestibility of the organic matter (dOM) of five different diets was on average 56.2% in dromedaries vs 52.4% in sheep. Using data from Hintz et al (1973), Warmington et al (1989), Cordesse et al (1992) and Lemosquet et al (1995), we showed that the dOM of 11 diets was 58.5 in llamas vs 54.3% in sheep. Differences between llamas and sheep (4 points) remain constant regardless of the nature of the diet (Figure 4). It is noteworthy that differences are much higher when the digestibility of plant cell walls is considered, 8.7 points on average in favour of dromedaries from five comparisons and 7.1 points in favour of llamas from eight comparisons. These results clearly indicate that camelids are far more efficient than ruminants in the use of cell wall carbohydrates. This is explained both by a higher cellulolytic activity of microbes due to the physico-chemical conditions and to the higher retention time of feed particles in compartment 1 as discussed before. Addition of 25% barley to a hay diet had no negative effect on the cellulolytic activity in the forestomach of camelids (Lemosquet et al 1995). The absence of disturbance of the microbial ecosystem could explain why llamas reduce hay intake less than ruminants when additional concentrate is offered. This ability of camelids has to be confirmed with higher starch additions.

Differences between camelids and ruminants in the digestibility of nitrogen are negligible (Cordesse et al 1992; Farid et al 1985; Gihad et al 1989; Kayouli et al 1993a; Maloiy 1972). Only Hintz et al (1973) found that the digestibility of nitrogen was 5 points higher in llamas than in sheep. There is no available information in the literature on the efficiency of microbial protein synthesis, but there are some indications that camelids show a greater efficiency. They recycle more nitrogen in the fermentative chamber, they produce more VFA and make more ATP available for microbial growth, pH is maintained in a range favourable for maximum growth, and the increase in the turnover rate of liquids in the fermenter stimulates the growth rate (Harrison et al 1975). The temperature in the fermenter is 2°C lower than in the rumen (Lemosquet et al 1995) indicating smaller losses in extra heat by microbes and therefore a better use of energy for growth.

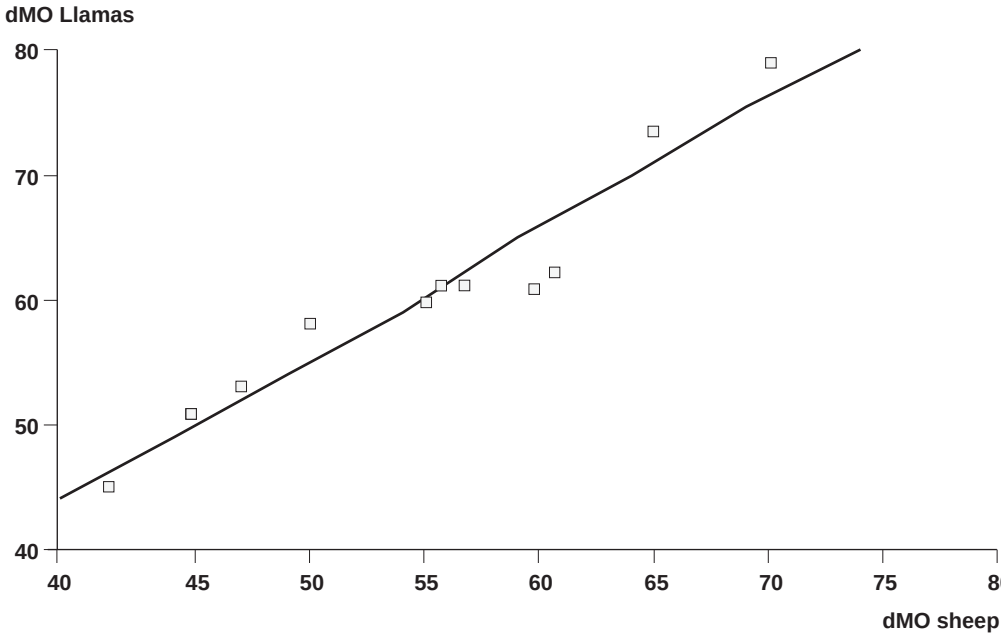

Figure 4. *Comparisons of dMO (%) in llamas and sheep (n = 11 comparisons).*

These comparisons show that camelids are able to use poor roughages better than domesticated ruminants in temperate climates. They use energy supplements efficiently without any negative effect on the digestion of the basic diet. However, some care has to be taken when diets rich in soluble nitrogen are given.

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Optimising rumen environment for cellulose digestion

E.R. Ørskov

Rowett Research Institute, Bucksburn, Aberdeen, Scotland, AB2 9SB

Abstract

The most common constraints in the rumen ecosystem which prevent fermentation of cellulosic feeds from accruing as at maximal low rate are: deficiencies of N and to a lesser extent S; number of bacteria in solution in the rumen fluid to invade new substrate; antimicrobial factors; and low rumen pH. The latter occurs almost exclusively with high concentrate feeding which is seldom economical in less industrialised countries.

Deficiency of N is common and can be alleviated by adding a source of NH_3-yielding material, usually urea. This can be added in the form of molasses blocks for rangeland and in other suitable packages for stabled animals. The number of bacteria in solution can be enhanced by feeding small amounts of easily digestible fibre where poor quality roughages are used. Examples are brans, sugar beet pulp, grass and leaves from MPT. Antimicrobial factors contained in some MPT can in small quantities have a positive effect on the rumen ecosystem, they can also be complexed by protein and other phenolic binding agents. An optimal rumen environment generally ensures maximal microbial yield and energy utilisation.

Introduction

Regardless of animal product, be it meat, milk, draught power or maintenance, as many livestock serve an important function for security and risk aversion (Ørskov and Viglizzo 1994), it is important that the feed resources are utilised as efficiently as possible. It can also be said that it is important that the potential nutritive value of roughages are extracted. Since most of the ruminant livestock in developing countries feed mainly on cellulosic crop residues, pasture, trees and bushes, it is important that the fermentation process be unimpeded.

Effect of ammonia concentrations

In many crop residues, the NH_3 generated in the rumen from degraded protein is often too low to ensure an efficient digestion process. In such cases addition of NH_3-yielding compounds can greatly enhance both intake and digestibility. Since straw ferments slowly, it is important that the N supplement is also provided at a slow rate. If, for

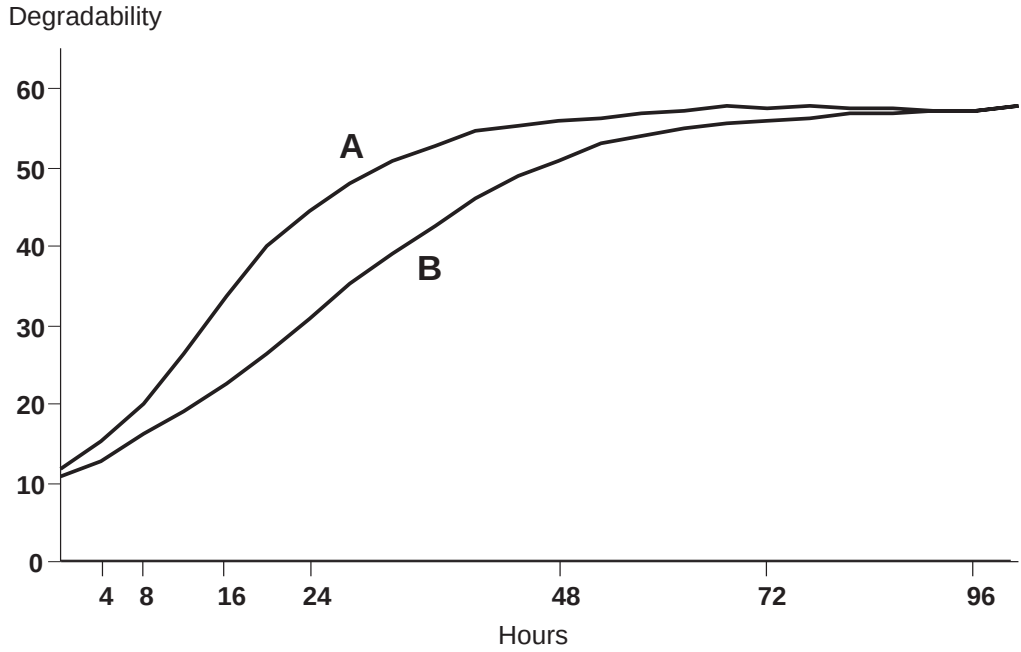

Figure 1. *Effect of rumen environment on degradation rate of the same feed. A represents rumen environment in which cellulolysis is maximal; B represents a rumen environment less than maximal. Note that the intercepts and the asymptotes are similar.*

instance, urea is used for pasture grazing it is common to include it in blocks for the animals to lick. For stall-fed animals it is generally more convenient and cheaper to spray the solution on the straw so that it is consumed at the same rate as straw. Apart from poor utilisation of urea when it is given rapidly, it can also result in urea poisoning in extreme cases. Urea is also provided from recycling and there is evidence that some breeds of cattle recycle more urea to the rumen than others due to difference in renal reabsorption.

The problem of inhibition of degradation is illustrated in Figure 1, which shows how the same feed in an optimal environment (A) ferments faster than in a less than optimal rumen environment (B). Where feeds are described by the exponential model $p = a + b(1 - e^{ct})$ (Ørskov and McDonald 1979), it can be clearly seen that the potential (a + b) is the same. In both environments the asymptote will be reached but in the impeded environment (B) the rate constant is less than in A. As a result, for instance, if a normal rumen retention is 48 h there could be substantial differences in digestibility leading to substantial reductions in feed intake.

Effect of number of microbes in solution

This phenomenon has only recently been given some prominence and is as yet poorly understood. One observation that intrigued us and led to a greater understanding on use

Table 1. *The effect of feeding untreated but urea-supplemented or ammoniated straw to fistulated animals on degradation of untreated straw in two rumen environments.*

Diet	Rumen pH	Rumen NH_3 (mg/litre)	Degradation at 48 h. (%)
Untreated straw + urea	6.9	268	45.3
Ammoniated straw	6.8	244	53.1

of supplements was a trial in which rumen-fistulated sheep were given *ad libitum* untreated straw + urea or ammoniated straw (Silva and Ørskov 1988).

The urea was added to the untreated straw at feeding time in order to ensure that the degradation rate was not impeded by rumen ammonia concentrations. Subsequently untreated straw and ammoniated straw was incubated in their rumens. The most significant aspect was that in spite of optimal pH conditions and ammonia concentrations the degradation rate of untreated straw proceeded more rapidly in the rumens of animals receiving ammoniated straw than in the rumens of animals eating untreated straw. This is illustrated in Table 1 with the 48 h degradability. Subsequent to this observation, Silva et al (1989) attempted to find supplements which when given to the untreated straw gave a rumen environment similar to the ammoniated straw and found that it was generally supplements containing a source of easily fermentable fibre which elicited a response in degradation rate. Sugar beet pulp, for instance, was successful as shown in Table 2. Inclusion of 15% of sugar beet in the diet had the effect of increasing straw intake by about 25%. There was no increase in intake of ammoniated straw indicating that the process of ammoniation itself made available a certain amount of easily fermentable fibre so that no further improvement came about.

Table 2. *Effect on straw intake when urea-supplemented, untreated or an ammonia-treated straw diet was supplemented with sugar-beet pulp.*

	Sugar beet pulp (g/kg straw)	Straw intake (g/d)	Total intake (g/d)	Live-weight gain (g/d)
Untreated straw	0	414	414	–65
Untreated straw	150	505	589	–6
Ammoniated straw	0	729	729	1
Ammonia-treated straw	150	717	852	51

Silva et al (1989).

In the tropics and subtropics there is a great interest in understanding how best to utilise multipurpose trees (MPT). MPT contain an excess of N for rumen microbes so that they can be used as a source of N. They also contain easily fermentable fibre. An interesting experiment was carried out by Pathirana et al (1992) when they fed untreated or urea-supplemented rice straw to sheep and supplemented with different increments of *Gliricidia* (Table 3). *Gliricidia* is a leguminous tree which grows well in many areas. It contains

Table 3. *Effect of supplementing a rice straw diet with* Gliricidia *leaves on straw intake with or without supplement of urea.*

Urea level (%)	*Gliricidia* levels (%)	Intake of straw (g/d)	Total intake (g/d)
0	0	574	574
0	5	673	708
0	15	848	998
0	30	721	1030
2	0	690	690
2	5	727	765
2	15	880	1035
2	30	715	1021

Pathirana et al (1992).

about 20% crude protein. Here it can be seen that the animals ate considerably more when *Gliricidia* forage was added to unsupplemented than to urea-supplemented straw. It would appear that the response to the untreated straw was mediated both by relieving an N deficiency and by providing a source of easily fermentable fibre while the response to the supplemented rice was mediated only by the source of fermentable fibre. This is of course speculative and more work is needed to clarify these issues. If no supplements are available it is possible to make some, for instance by ammoniation. In many parts of Africa crop residues are grazed *in situ* and not gathered. If a small amount is gathered, ammoniated and fed at night, it might benefit utilisation of grazed crop residues.

Manyuchi et al (1992) carried out a small trial to look at the effect of adding a small amount of ammoniated straw to untreated but urea-supplemented straw. Here again the results (Table 4) indicate that intake of untreated straw was considerably enhanced when a supplement of treated straw was provided. It is apparent that there is a great deal to learn about supplements and how they can best support utilisation of basal feeds as well as being utilised as energy and protein sources in their own right.

Table 4. *Effect of supplementing untreated straw with ammoniated straw on total intake by sheep.*

Treatment	Intake of untreated straw (g/d)	Intake of NH_3 straw (g/d)	Total intake (g/d)	Digestible dry-matter intake (g/d)
Untreated straw	641	0	641	324
Untreated straw + 200 g NH_3 straw	814	187	1001	452
Untreated straw + 400 g NH_3 straw	667	352	1019	563
NH_3 straw	—	1152	1152	673

Effect of low rumen pH

Problems in the rumen caused by low pH due to feeding of large quantities of grains or starch-based diets, often aggravated by over-processing and intermittent feeding, is a

problem generally associated with industrialised countries where intensively fed livestock are given large grain supplements. Low rumen pH not only in part but sometimes totally abolishes cellulose fermentation; it also exposes the animals to problems of acidosis, acetonaemia, laminitis and other feed-associated problems. Since this problem does not occur with mainly roughage diets, which is by far the prevalent feed in developing countries, it will not be discussed here in more detail.

Problems of anti-nutritive factors

Anti-nutritive factors are in effect plant defence mechanisms against attack by animals, insects or microbes. Some of the anti-nutritive factors are targeted to host animals and some are targeted to the microbial population in the rumen. Most of the microbial anti-nutritive factors are phenol related or tannins.

A higher understanding of issues relating to microbial anti-nutritive factors is needed. Many chemical determinants of tannins have proved inadequate to quantify the severity of phenolic anti-nutritive factors. In some instances a high tannin content seems to be tolerated while in others a small amount can cause a large depression in intake (see Makkar et al 1989; Mueller-Harvey and Reed 1992). New biological methods making use of the gas-production techniques with or without a tannin-complexing compound such as polyvinylpyrrolidone or polyethylene glycol appear promising (Khazaal and Ørskov 1994) and need more development. There is also a need to identify accurately the active compounds so that they can be targeted or eliminated by genetic manipulation or other means.

Conclusion

An increase in understanding of rumen ecology has the potential to make a major impact on feed utilisation in general and in particular in the parts of the world where cellulosic roughages are likely to be the most important feed for ruminants and in areas where higher quality supplements, e.g. brans and oilseed cakes, are scarce. It is also of particular interest in areas where trees and bushes form important feed ingredients.

A better understanding of rumen ecology can lead to:

1. A better strategic use of scarce supplements so that they can be utilised not only as supplements in their own right but also provide support for basal feeds.
2. Greater production of rumen microbial protein so that animals can grow better on basal feeds alone without a need for protein supplements. A change in the ratio of volatile fatty acids to microbial biomass has the further advantage that it will increase capture of fermented energy and reduce methane production.

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Forage legumes as protein supplements to poor quality diets in the semi-arid tropics

J.H. Topps

Department of Animal Science, University of Zimbabwe
P.O. Box MP167, Mount Pleasant, Harare, Zimbabwe

Introduction

In the semi-arid tropics, the natural pasture has a low nutritive value during the dry season when the standing grass is mature and dormant. After the first two or three months of the dry season, crop residues become available following the harvesting of grain, but most of the residues are from cereal crops which have a nutritional value similar to that of the mature natural grass. Residues from leguminous crops are also available but in small quantities. They have a quality better than that of cereal residues and may be used as protein supplements to the poorer quality residues and to the mature pasture. In addition there is now increasing interest in, and use of legumes, either herbaceous or shrub species, from which the foliage can be harvested and used as protein supplements. When ruminants consume poor quality diets, food intake is low so the supply of nutrients is insufficient to meet requirements for maintenance.

The main objective of providing a forage legume as a protein supplement is to increase both the total food intake and that of the basal poor quality diet. Early work on the use of forage legumes was mainly concerned with the need to improve the nitrogen content of diets based on poor quality roughages in order to overcome a deficiency of nitrogenous substrates for the rumen microorganisms. More recent evidence indicates that other changes occur in this supplementation situation which result in an enhancement of intake and digestibility of the diet. This review will assess the likelihood of such changes and the effects they may have on animal production.

Food intake

In the experiments reviewed (Minson and Milford 1967; Siebert and Kennedy 1972; Mosi and Butterworth 1985; Smith et al 1990; Kitalyi and Owen 1993), the forage legumes were fed in discrete amounts and separately from the poor quality basal diets, which were given *ad libitum*. In all experiments, the legume supplement increased total food intake but the response varied widely between experiments. According to published observations, the legume supplements were consumed without any refusals, so the differences in dietary intake were mainly due to differences in the intake of the basal diet.

In addition, there are large differences between experiments in the change in intake of the poor quality forage in response to supplementation. In general, if the level of supplementation was less than 30 to 40% there was an increase in the intake of the basal

diet, but in some experiments the intake was unchanged or slightly reduced. A very large increase in intake of mature pangola grass *(Digitaria decumbens)* was found in the study by Minson and Milford (1967). Above 40% forage legume, the intake of the basal diet fell below that seen with the unsupplemented control diet, except for that observed by Siebert and Kennedy (1972) when there was no change when lucerne contributed 45% of the total dry matter intake. In contrast, Mosi and Butterworth (1985) found that the intake of the basal diet on the supplemented treatments was always less than that of the unsupplemented control diet. The reasons for such differences appear to be related to differences in quality of the forage legume and of the basal diet and other factors.

Substitution of the basal diet

Before these factors are considered, it is worth examining the extent of substitution of the basal diet by the forage legume and its practical implications. As indicated earlier, substitution usually occurs when the legume supplement contributes at least 30 to 40% of the total dry matter intake, and it is due to the bulk effect of the forage legume. However, assessing substitution on a 1:1 basis is misleading since the two components of the diet will have different digestibilities and different bulking effects in the reticulo-rumen. Since the forage legume is likely to be more digestible, it will occupy less space than the poor quality roughage. The poor quality roughage equivalent of the forage legume can be calculated by multiplying the intake of the forage legume by the ratio of the digestibility of the poor quality roughage given alone to the calculated digestibility of the forage legume in the mixed diets. This calculated digestibility is obtained as the intercept of the linear regression of diet digestibility on the proportion of the poor quality roughage in the diet. Anticipated intake of the poor quality roughage can be obtained by subtracting the poor quality roughage equivalent of the forage legume from that of the poor quality roughage when not supplemented. Any change in intake is then calculated as the difference between anticipated and observed intakes of the poor quality roughage. According to the work of Manyuchi (1994), when this condition for differences in digestibility is made the observed is nearly always greater than the anticipated intake. Although this derivation is informative and reassuring, in practice any substitution on a 1:1 basis should be avoided by keeping the inclusion rate of forage legume to a third or less of the total dry matter. This recommendation has the merit of recognising the nutritional quality of the forage legume, especially if it has been grown on a small farm where the growing of the legume may be limited by the land and labour available.

Quality of the basal diet

If the basal diet has a low nitrogen content, the addition of a forage legume will increase the nitrogen content of the total diet, which is likely to increase food intake and the rate of degradation of the basal diet in the rumen. Such positive associative effects are well known and at one time were thought to be either the main or only effect of providing protein-rich supplement. A number of recent reports have shown that responses to forage

supplements are not entirely due to an increase in dietary nitrogen (Smith et al 1990; Getachew et al 1994; Manyuchi 1994).

However, when different basal diets are compared any difference in quality, of which the nitrogen content is a part, will be reflected in the size of the effect of the legume supplement on intake. Mosi and Butterworth (1985) carried out a study in which *Trifolium tembense* hay was used as a supplement to four different cereal straws, maize, oat, teff and wheat. The straws differed appreciably in crude protein content (23 to 62 g/kg) and in acid detergent fibre (710 to 780 g/kg). It was found that the extent of substitution of the basal diet tended to be greater with the apparently better quality straws (oat and maize). Part of this difference was probably due to overcoming a deficiency of nitrogen in the straws.

Quality of the forage supplement

Very few comparisons have been made between forage legumes that differed in their nutritional characteristics. From the early work on the need for nitrogen supplementation (Elliott and Topps 1963) it may be deduced that forages with a high content of rumen degradable nitrogen will elicit greater responses in food intake than those with a low content. The results of a study by Smith et al (1990) support this hypothesis. Three forage legumes, pigeon pea *(Cajanus cajan),* cowpea *(Vigna unguiculata)* and lablab *(Lablab purpureus)* hay which differed in nitrogen content and *in sacco* DM degradability were compared as supplements to maize stover. Cowpea, which had the highest nitrogen content, promoted the greatest intake of maize stover. However, the extent of substitution was inversely related to the 48 h degradability of the legumes measured *in sacco*. In a similar study, Getachew et al (1994) compared three other legumes *(Desmodium intortum, Macrotyloma axillare* and *Stylosanthes guianensis)* as supplements to maize stover. *S. guianensis* had the highest nitrogen and lowest neutral detergent fibre content and promoted the greatest intake of maize stover. Recently in the author's laboratory, Masama et al (1995) found differences between four shrub legumes in the response produced as supplements. Although the nitrogen content appears to be a dominating factor, it should be recognised that legumes with a high nitrogen content are likely to contain less fibre and the total organic matter may be more easily fermented. It may be that the energy available to the microorganisms is a factor equal in significance to the supply of degradable nitrogen. In fact with certain tropical legumes the presence of anti-nutritional factors may obviate any causal relationship between nitrogen content and increase in food intake (Topps 1992).

Digestibility and rumen degradability

Forage legumes invariably increase the digestibility of the total diet which is somewhat expected. The digestibility would probably increase as the proportion of the forage legume is increased, which reflects the contribution of the legume with its higher digestibility. In most studies, the proportional change in digestibility is equal

to or less than the fractional increase of the high quality forage in the diet. This indicates the absence of any positive associative effect of the forage legume on the digestibility of the basal diet. Only a few studies have recorded a positive associative effect of the forage legume e.g. Minson and Milford (1967) and Mosi and Butterworth (1985).

If the presence of a forage legume increases the activity of rumen microorganisms, a concomitant increase in degradation of fibre should be observed. Several reports have described such an effect, but it may not occur with certain recalcitrant poor quality roughages. McMeniman et al (1988) studied five legumes as supplements to rice straw and observed that degradation of the straw was increased by the presence of the legume. Similarly, Ndlovu and Buchanan-Smith (1985) found that lucerne increased the rate of degradation of barley straw, brome grass and maize cobs. In contrast, Manyuchi (1994) found that groundnut hay did not alter the *in sacco* degradation of poor quality grass hay. It is likely that any change in the degradation of the basal diet as a result of an increase in microbial activity may depend on the number of available sites for microbial attachment (Akin 1989). With some roughages the cuticule layer and extent of lignification are barriers to microbial colonization, so that an increase in rumen microbial population may not be manifested in an increase in rate of degradation.

Rumen environment

Very few studies have been carried out in which changes in the rumen environment have been measured when forage legumes are fed with poor quality basal diets. Inevitably poor quality forages provide insufficient degradable nitrogen and fermentable energy to sustain optimum digestion of fibre. Forage legumes are relatively good sources of degradable nitrogen and fermentable energy so their inclusion in the diet is expected to increase the rumen population of cellulolytic microbes. Such an effect was seen in a related study by Kolankaya et al (1985) when a higher growth of selected cellulolytic microorganisms was observed with ammonia-treated compared with untreated straw.

Concentrations of rumen ammonia increase following supplementation with a forage legume (McMeniman et al 1988; Getachew et al 1994; Manyuchi 1994), the increase being a function of the degradability of the nitrogen in the forage legume. In a study by Said and Tolera (1994), the legume with the lower nitrogen content *(M. axillare)* gave higher rumen ammonia concentrations than *D. intortum,* which had more crude protein but with a lower degradability. For certain forage legumes, especially certain species of shrubs, the availability of the nitrogen compounds would be limited by tannins (Mangan 1988). Forage legumes increase the total concentration of volatile fatty acids (VFA) without affecting the relative proportions and the rumen pH. These results indicate that forage legumes are likely to maintain a stable fermentation pattern. Ndlovu and Buchanan-Smith (1985) observed that the feeding of a lucerne supplement increased the proportion of branched chain VFA and suggested that this increase may stimulate the growth of cellulolytic microorganisms.

Kinetics of digesta

A potential increase in digestibility when forage legumes are added to poor quality basal diets may be offset by a reduction of retention time of digesta. Such an effect was recorded by Ndlovu and Buchanan-Smith (1985) when lucerne was fed with maize cobs, and by Vanzant and Cochran (1994) when lucerne was fed at different levels with low quality prairie forage. Similar results were obtained by Manyuchi (1994) (Table 1). The addition of groundnut hay increased the fractional outflow rate of rumen solids without altering the pool size of the rumen digesta. Manyuchi concluded that the increase in food intake following supplementation with a forage legume was largely facilitated by an increase in rate of passage of digesta. The mechanism by which this occurs is not fully known. Changes in osmotic pressure of the rumen may be implicated but the results of Manyuchi (1994) do not confirm the presence of a relationship.

Table 1. *Intake, digestibility and kinetics of rumen digesta in sheep fed on veld hay plus 1% urea alone (Control) or veld hay supplemented with 100, 200 or 300 g of groundnut hay (100 Gnut, 200 Gnut, 300 Gnut) or groundnut hay alone.*

	Control	100 Gnut	200 Gnut	300 Gnut	Gnut	SED
DM intake (g/d)						
Groundnut hay	0	90	180	270	1656	
Veld hay	912	896	905	785	0	
Total	912	986	1085	1055	1656	58.0
DM digestibility	0.48	0.52	0.53	0.55	0.64	0.02
Rumen liquid						
Volume (l)	12.48	13.14	12.60	12.54	11.70	1.68
Fractional outflow (%/h)	6.53	6.49	6.45	6.64	6.70	0.67
Rumen solids						
Pool size (g)	2036	1763	2324	2272	2378	410
Fractional outflow (%/h)	2.59	2.90	3.14	2.98	3.79	0.24

Animal performance

Increases in food intake and in digestibility of the total diet arising from supplementation with forage legume should be manifested in a significant increase in animal performance. There are very few reports of a marked improvement of production when tropical legumes are fed. Work carried out in Kenya with *leucaena leucocephala* and Napier grass (Bana grass) by Muinga et al (1992, 1993) has established that sizeable responses in milk yield can occur (Tables 2 and 3). In both experiments there was a small increase in the intake of Napier grass when leucaena was given as a supplement and a significant increase ($P < 0.05$) in total DM intake. These enhanced intakes resulted in significant increases ($P < 0.05$) in milk yields and some curtailment of weight losses. In the work published in 1992 it is interesting to note that the response with the poorer quality was greater than that with the higher quality Napier grass.

Table 2. *Intake and milk production of Jersey cows given Napier fodder* ad libitum *without (Control) and with (Treated) 5 kg of fresh leucaena per day.*

	Control		Treated	
	Mean	SE	Mean	SE
DM intake (kg/d)				
Napier	5.20	0.27	5.70	0.24
Total	5.20	0.27	6.90	0.24
Milk yield (kg/d)	4.20	0.20	5.20	0.18

Table 3. *Intake of dry matter and milk production (kg/d) of crossbred dairy cows given* ad libitum *Napier grass cut at either 1.0 or 1.5 m height and supplemented with 0, 4 or 8 kg fresh leucaena per day from day 15 to 112 of lactation*

	Napier harvest height (m)						
Leucaena	1.5			1.0			
supplementation (kg)	0	4	8	0	4	8	SED
Intake							
Napier	6.5	6.6	7.1	9.1	9.6	9.3	0.51
Total	6.5	7.8	9.3	9.1	10.7	11.5	0.51
Milk yield	6.1	6.9	7.8	8.5	8.5	8.8	0.55

Conclusions

Forage legumes can be used as a supplement to poor quality diets but the responses produced are unpredictable. Much of the nature of the response relates to changes that occur in the rumen environment. It is axiomatic that substantial improvement in the rumen microbial activity especially that of the cellulolytic organisms will lead to greater food intake and increases in digestibility of the diet. These changes in turn will improve animal performance. A knowledge of the factors controlling the changes in rumen microbial activity is essential.

In Zimbabwe, the work of Manyuchi (1994) has shown that groundnut hay is an effective supplement when given with either Napier grass hay or poor quality grass hay. Groundnut hay may therefore be a useful model for future studies. In addition, groundnuts are a dual purpose crop in that both the nuts and the forage are harvested, which is attractive to farmers. To successfully encourage small farmers to grow forage legumes such considerations are important. Acceptability of crops such as cowpeas, pigeon peas and groundnuts means that the widespread establishment of these crops is more likely than that of legumes which provide forage only. For this reason intensive studies are needed on certain legumes to ascertain the best way of harvesting, storing and using the forage as a supplement to poor quality roughages.

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State of art in bioengineering of rumen microorganisms

Application of molecular biology in the production of food from ruminants

R.M. Cook

Michigan State University, East Lansing, Michigan 48824, USA

Abstract

Several methods are available to alter end-products of the rumen fermentation. These include glycopeptides which increase propionate and amino acids and decrease methane and ammonia nitrogen production. Monensin increases propionate and decreases methane and acetate production. Isoacids (e.g. isobutyrate) increase acetate and decrease ammonia production. Virginiamycin increases propionate production and, in some studies, decreases butyrate, acetate and lactate. The changes in rumen fermentation products brought about by these chemicals can have positive effects on animal production depending on whether growth, fattening or milk production is the desired product. The effect of these chemicals at the molecular level on the rumen ecosystem is poorly understood. Understanding their mechanism of action can lead to better methods for controlling rumen fermentation. Of equal importance in improving food production by ruminants is a knowledge of utilisation of rumen fermentation products at the molecular level. Each metabolic event requires transcription of genes. It is critical to understand gene regulation in adipose, muscle and the mammary gland.

Identifying the important genes regulating metabolism in food producing animals is in its infancy. It may be that the activity of some genes should be increased while for others it should be lowered. Some current genes of interest are fatty acid synthase (fat deposition) and calpastatin (muscle metabolism). The cloning of Holstein mammary gland acetyl CoA synthetase gene is an example of the molecular approach to studying utilisation of rumen fermentation products for milk production. The product of this gene catalyses the initial step in the utilisation of rumen acetate for energy production and for fat synthesis. Acetyl CoA synthetase is believed to play a key regulatory role in ruminants. Understanding molecular mechanisms regulating the rumen fermentation and utilisation of rumen fermentation products will provide a means to enhance food animal production.

Introduction

An important problem in rumen ecology is to define conditions required for optimum fermentation of feedstuffs, especially low protein, highly fibrous plant material. There are several chemicals that alter end-products of the rumen fermentation. These include

glycopeptide antibiotics, isoacids and monensin. Our laboratory has conducted both *in vitro* and *in vivo* studies of the effects of glycopeptides, isoacids and monensin on rumen fermentation (Brondani et al 1991; Quispe et al 1991). Acetate is a major end-product of the rumen fermentation. Currently we are studying the acetyl CoA synthetase gene whose product catalyses the initial step in acetate utilisation. These studies will be described.

Glycopeptide antibiotics

Teichomycin A2 is an antibiotic produced by *Actinoplanea teichomyceticus.* Avoparcin is an antibiotic produced by a strain of *Streptomyces candidus* (Kunstmann et al 1968). Their antimicrobial properties (growth and bacteria) stem from their ability to interfere with cell wall synthesis (Parenti et al 1978; Redin and Dornbush 1969).

Cattle fed avoparcin have lower feed intake and improved daily gains and feed efficiency (DeLay et al 1978; Johnson et al 1979). Changes in the rumen include increases in propionate and decreases in acetate (Chalupa et al 1981; Froetschel et al 1983) and butyrate (Froetschel et al 1983) molar percent and α-amino-N.

In vitro studies with teichomycin A2 indicate that this glycopeptide alters rumen fermentation similar to avoparcin (Brondani 1983; D.J. Phillips and J.M. Tadman, unpublished data). However, studies directly comparing the two antibiotics under *in vivo* conditions have not been conducted. Additionally, most studies measured only propionate production rates. We determined the effects of teichomycin A2 and avoparcin on *in vivo* production of acetate, propionate and butyrate, and rumen nitrogen variables (Brondani 1986).

In each of two trials, six rumen-cannulated crossbred ewes were kept in individual pens. Average body weight for animals in Trials 1 and 2 were 40.6 and 39.2 kg, respectively. In Trial 1 (high roughage diet), animals were fed 1000 g/d of alfalfa hay and had free access to trace mineral salt. In Trial 2 (low roughage diet), animals received 900 g/d of a ration composed of 43% alfalfa meal, 10% ground corn cobs, 20% ground shelled corn, 20% ground oats, 5% H_2O, 1% trace mineralised salt, 0.5% dicalcium phosphate and 0.5% of a vitamin premix (vitamin A, 4400 IU/g; vitamin D, 500 IU/g; and vitamin E, 44 IU/g). Daily feed was provided in two portions (0800 and 1600) with free access to water.

In each trial, animals were divided into three groups of two sheep each and randomly allocated to one of the treatments: control, 30 ppm teichomycin A2, or 30 ppm avoparcin (dry matter basis). Antibiotics were injected directly into the rumen through a fistula immediately following feeding. On days 28 and 29, samples for the determination of rumen VFA and rumen nitrogen constituents were collected. Rumen fluid samples were collected prior to morning feeding and then at hourly intervals for the next 8 h.

Measurements of rates of acetate production and liquid flow were performed on days 31 and 37. Three hours after the morning feeding, 100 ml of water containing 10 g of polyethylene glycol (PEG, M.W. 4000) and 100 μCi of Na-[1-^{14}C]acetate were added to the rumen. Serial rumen fluid samples were collected every 20 min for the next 4 h. The rates of production of propionate and butyrate were measured on days 32 and 39

and days 33 and 40, respectively. The procedure was essentially the same as that used for acetate (Cook 1966; Knox et al 1967).

Volatile fatty acid concentrations in rumen fluid were determined by gas liquid chromatography according to the procedure of Ottenstein and Bartley (1971a, 1971b).

Fractionation of rumen fluid for the determination of nitrogen components was according to the procedure of Bergen et al (1968) and Isichei (1980). α-Amino-N and protein were determined using a Technicon Auto-Analyser System (Technicon Instrument Corp., Terrytown, New York). Ammonia-N analysis was according to the method of Okuda et al (1965) as modified by Kulasek (1972).

The effects of teichomycin A2 and avoparcin on concentrations of rumen VFA in sheep fed high and low roughage diets are summarised in Table 1. Volatile fatty acids as a percent of total moles are shown in Table 2. In the high roughage ration, glycopeptides did not change the concentrations of acetate ($P < 0.10$) but increased propionate. Increases over control were 26.0 and 30.8% for teichomycin A2 and avoparcin ($P < 0.10$), respectively. Glycopeptides tended to lower butyrate concentrations. Total VFA concentrations were not changed by the treatments ($P < 0.10$). Teichomycin A2 decreased the concentrations of isobutyrate and isovalerate in rumen fluid ($P < 0.05$), whereas avoparcin decreased isovalerate ($P < 0.05$). The glycopeptides did not affect the molar percent of acetate and butyrate (Table 2). Propionate molar percent was increased by the glycopeptides relative to control ($P < 0.05$). As a result, the acetate to propionate ratio was lower for the treated groups in the high roughage diet ($P < 0.05$).

For the low roughage ration, the trends for VFA concentrations were similar to those in the high roughage ration. Propionate concentrations increased ($P < 0.10$) and butyrate concentrations decreased ($P < 0.10$) due to glycopeptide supplementation. Concentrations of acetate and total VFA were not affected by treatments ($P < 0.10$). The lower concentrations of isoacids caused by the glycopeptides in the high roughage ration were not detected in the low roughage ration. Teichomycin A2 and avoparcin increased propionate ($P < 0.01$) and decreased butyrate ($P < 0.05$) molar percent with respect to

Table 1. *Effects of teichomycin A2 (TE-A2) and avoparcin (AVO) on concentrations of rumen VFA in sheep fed high and low roughage diets (mmoles/dl)*[†‡].

	High roughage				Low roughage			
Variable	Control	TE-A2	AVO	SE[§§]	Control	TE-A2	AVO	SE[§§]
Acetate	9.94	9.75	6.51	0.57	6.31	5.89	6.12	0.63
Propionate	1.77[††]	2.23[‡‡]	2.31[‡‡]	0.12	1.74[††]	2.38[‡‡]	2.75[‡‡]	0.13
Isobutyrate	0.19[§]	0.11[¶]	0.14[§¶]	0.02	0.21	0.19	0.17	0.03
Butyrate	0.81	0.73	0.65	0.14	0.97[††]	0.63[‡‡]	0.71[‡‡]	0.07
2-M-butyrate	0.16	0.11	0.13	0.03	0.18	0.14	0.19	0.04
Isovalerate	0.14[§]	0.09[¶]	0.07[¶]	0.01	0.16	0.15	0.13	0.03
Valerate	0.17	0.14	0.16	0.04	0.19	0.21	0.18	0.04
Total VGA	10.26	10.16	9.97	0.96	9.76	9.59	9.75	1.13
Total higher and isoacids	0.66[††]	0.45[‡‡]	0.50[‡‡]	0.04	0.74	0.69	0.67	0.06

[†]36 observations per mean.

[‡]Means in a row within a ration without a common superscript differ: [§,¶] $P < 0.05$; [††,‡‡] $P < 0.10$.

[§§] Standard error of each mean in a row within a ration.

Table 2. *Effects of teichomycin A2 (TE-A2) and avoparcin (AVO) on molar percent distribution of rumen VFA in sheep fed high and low roughage diets*[†‡].

	High roughage				Low roughage			
Variable	Control	TE-A2	AVO	SE[§§]	Control	TE-A2	AVO	SE[§§]
Acetate	68.0	66.3	65.2	0.89	64.6	61.4	62.8	0.82
Propionate	17.3[C]	22.0[¶]	23.2[¶]	0.61	17.8[§]	24.8[¶]	23.1[¶]	0.59
Isobutyrate	1.9	1.1	1.5	0.11	2.1	2.0	1.7	0.16
Butyrate	7.9	7.2	6.5	0.49	9.9[††]	6.6[‡‡]	7.3[‡‡]	0.51
2-M-butyrate	1.6	1.1	1.3	0.13	1.8	1.5	1.9	0.09
Isovalerate	1.4	1.4	1.7	0.11	1.9	2.2	1.8	0.13
Valerate	1.9	1.4	1.7	0.11	1.9	2.2	1.8	0.13
A:P ratio	3.9[††]	3.0[‡‡]	2.8[‡‡]	0.13	3.6[§]	2.5[¶]	2.7[¶]	0.17

[†]36 observations per mean.
[‡]Means in a row within a ration without a common superscript differ: [§,¶] $P < 0.05$; [††,‡‡] $P < 0.10$.
[§§] Standard error of each mean in a row within a ration.

control. Acetate molar percent was slightly lower in the treated groups but differences were not significant. This resulted in the lower acetate to propionate ratios in the treated groups ($P < 0.01$).

The effect of teichomycin A2 and avoparcin on the rates of production of acetate, propionate and butyrate in sheep fed high and low fibre diets are shown in Tables 3–5. Animals fed the high roughage diet had a higher rumen fluid turnover rate than animals fed low roughage. These results are in line with those reported for sheep (Froetschel et al 1983). Froetschel et al (1983) reported an increase in rumen volume in animals fed avoparcin. However, the present study did not show increases for either the avoparcin or the teichomycin A2 groups.

In the high roughage ration, there was a trend for lower acetate production in the treated groups (Table 3). However, differences were not significant ($P > 0.10$). Although acetate pools were not affected by treatments ($P > 0.10$), the lower rate of acetate

Table 3. *Effects of teichomycin A2 (TE-A2) and avoparcin (AVO) on acetate production and rumen fluid variables in sheep fed high and low roughage diets*[†].

	High roughage				Low roughage			
Variable	Control	TE-A2	AVO	SE[¶]	Control	TE-A2	AVO	SE[¶]
Acetate								
Pool size (moles)	0.36	0.34	0.39	0.02	0.31	0.27	0.29	0.03
Turnover time (min)	110.3[‡]	112.4[‡]	134.1[§]	6.9	103.5	97.6	102.8	9.1
Production (moles/d)	4.74	4.41	4.12	0.27	4.39	3.89	4.18	0.36
Rumen fluid								
Volume (litres)	5.1	4.4	4.6	0.47	4.4	3.8	4.0	0.31
Turnover time (h)	8.7	9.2	8.5	1.39	14.3	16.1	13.1	0.98
Turnover rate (%/h)	11.1	10.5	11.9	1.03	6.7	6.3	7.7	0.62

[†]Four observations per mean.
[‡,§]Means in a row within a ration without a common superscript differ ($P < 0.10$).
[¶]Standard error of each mean in a row within a ration.

Table 4. *Effects of teichomycin A2 (TE-A2) and avoparcin (AVO) on propionate production, rumen volume, and rumen fluid turnover rate in sheep fed high and low roughage diets*[†].

	High roughage				Low roughage			
Variable	Control	TE-A2	AVO	SE[¶]	Control	TE-A2	AVO	SE[¶]
Propionate								
Pool size (moles)	0.12	0.16	0.14	0.02	0.13[‡]	0.17[‡§]	0.21[§]	0.03
Turnover time (min)	136.80	123.50	115.20	9.73	121.70[‡]	119.80[‡]	114.4[§]	7.12
Production (moles/d)	1.24[‡]	1.84[§]	1.72[§]	0.12	1.55[‡]	2.09[§]	2.12[§]	0.14
Rumen fluid								
Volume (litres)	4.40	5.10	4.70	0.36	3.50	3.90	4.40	0.42
Turnover time (h)	9.30	8.40	10.10	1.24	15.20	13.40	14.30	0.86
Turnover rate (%/h)	10.80	12.10	9.10	0.97	6.40	7.30	6.80	0.74

[†]Four observations per mean.
[‡,§]Means in a row within a ration without a common superscript differ ($P < 0.10$).
[¶]Standard error of each mean in a row within a ration.

production due to avoparcin resulted in significantly less acetate pool turnover times relative to control ($P < 0.10$). In the low roughage ration, trends were the same as those found in the high roughage ration. Acetate production and acetate pool sizes tended to be lower in the treated groups, but differences were not significant ($P > 0.10$).

Propionate production rates were increased by glycopeptide supplementation in both rations (Table 6). In the high roughage diet, increases in propionate production relative to controls were 48 ($P < 0.05$) and 38.7% ($P < 0.05$) for teichomycin A2 and avoparcin, respectively. In the low-roughage ration, increases in propionate production due to teichomycin A2 and avoparcin were 34.8 ($P < 0.10$) and 36.8% ($P < 0.10$), respectively. The increase in propionate production resulted in higher propionate pool sizes for both antibiotics in both rations. However, significant differences from control were detected only for avoparcin in the low roughage diet ($P < 0.10$). Butyrate production (Table 5) was not affected by treatments in either ration ($P > 0.10$).

Table 5. *Effects of teichomycin A2 (TE-A2) and avoparcin (AVO) on butyrate production, rumen volume, and rumen fluid turnover rate in sheep fed high and low roughage diets*[†‡].

	High roughage				Low roughage			
Variable	Control	TE-A2	AVO	SE[§]	Control	TE-A2	AVO	SE[§]
Butyrate								
Pool size (moles)	0.051	0.044	0.047	0.004	0.067	0.071	0.059	0.003
Turnover time (min)	150.60	138.90	143.80	13.10	213.20	214.60	199.30	17.50
Production (moles/d)	0.475	0.453	0.461	0.06	0.443	0.469	0.429	0.09
Rumen fluid								
Volume (litres)	5.40	4.80	5.10	0.49	4.30	5.10	3.80	0.39
Turnover time (h)	8.10	8.50	9.20	1.19	12.20	14.40	12.90	0.96
Turnover rate (h)	12.10	11.40	10.20	0.97	8.50	6.80	7.50	0.71

[†]Four observations per mean.
[‡]Effects of treatment are not significant in either ration ($P < 0.10$).
[§]Standard error of each mean in a row within a ration.

Table 6. *Effects of teichomycin A2 (TE-A2) and avoparcin (AVO) on rumen nitrogen constitutents in sheep fed high and low roughage diets (mg/dl)*[†].

	High roughage				Low roughage			
Variable	Control	TE-A2	AVO	SE[‡‡]	Control	TE-A2	AVO	SE[‡‡]
Soluble protein	46.19	52.1	39.43	4.76	37.09	44.03	49.12	4.11
Bacterial protein	57.29	65.8	61.20	5.12	63.14	54.28	63.19	4.63
Protozoal protein	61.34	72.13	65.23	6.17	265.19	234.29	251.12	19.25
Alpha-amino-N	2.94[‡]	5.34[§]	6.41[§]	0.48	3.19[¶]	4.81[††]	4.74[††]	0.42
Ammonia-N	16.59[‡]	13.21[‡§]	11.19[§]	1.19	17.35[‡]	10.24[§]	11.86[§]	1.28

[†]36 observations per mean.
[‡,§]Means in a row within a ration without a common superscript differ, $P < 0.05$; [¶,††]$P < 0.05$.
[‡‡] Standard error of each mean in a row within a ration.

Changes in VFA production rates found in the present study followed similar trends for both glycopeptides. In general, supplementation with teichomycin A2 and avoparcin resulted in significant increases in propionate concentrations and production rates. For acetate and butyrate, there were trends toward lower concentration and production rates but differences were not significant ($P > 0.10$). This is in contrast with results from studies in sheep (Froetschel et al 1983) and cattle (Chalupa et al 1981). Froetschel et al (1983) reported higher propionate but lower acetate, butyrate and total VFA concentrations in sheep fed high and low fibre diets supplemented with avoparcin. Chalupa et al (1981) reported that avoparcin supplementation to cattle increased propionate and butyrate and decreased acetate. Total VFA concentrations were not affected by the antibiotic. However, our results are in line with those obtained in feedlot trials by Johnson et al (1979). In these experiments, avoparcin consistently increased propionate without affecting acetate or butyrate concentrations.

One point of importance is the time required for the patterns of rumen VFA to return to pre-treatment values when feeding of antibiotics is discontinued. In contrast with ionophores, the effects of glycopeptides seem to be persistent (Brondani 1983; D.J. Phillips and J.M. Tadman, unpublished data). Ten days after glycopeptide supplementation was discontinued, the molar proportion of rumen VFA had not returned to normal. These results question the validity of using Latin squares or reversal designs to study the effects of glycopeptides on rumen metabolism unless there are long adaptation periods to the new treatment to avoid carryover effects.

The effect of teichomycin A2 and avoparcin on rumen nitrogen constituents in sheep fed high and low fibre diets is shown in Table 6. Soluble, bacterial and protozoal protein fractions were not affected by treatments ($P > 0.10$).

In the high roughage ration, increases in α-amino-N relative to control were 81.6 ($P < 0.05$) and 118% ($P < 0.05$), respectively, for teichomycin A2 and avoparcin. In the low roughage diet, teichomycin A2 and avoparcin increased α-amino-N concentrations by 50.1 ($P < 0.05$) and 48.6% ($P < 0.05$), respectively. Ammonia-N in the high roughage diet was decreased by 10 ($P < 0.05$) and 23.8% ($P < 0.05$) by teichomycin A2 and avoparcin. In the low roughage diet, decreases in ammonia-N due to teichomycin A2 and avoparcin were 33.2 and 22.7%, respectively (Table 6).

Results for ammonia-N and α-amino-N indicate that teichomycin A2 and avoparcin exert a marked effect on rumen nitrogen metabolism. These results are in agreement with *in vitro* results for teichomycin A2 (Brondani 1983) and *in vivo* results for avoparcin (Chalupa et al 1981; Froetschel et al 1983). The decrease in ammonia-N concentration concomitant with increases in α-amino-N suggests that teichomycin A2 and avoparcin reduce deamination. Additional evidence is provided by the decline in the concentration of isoacids in treated animals (Table 1). Although some rumen bacterial species can synthesise branched chain acids *de novo* (Bryant and Doetsch 1955; Miura et al 1980), the majority of isoacids in the rumen comes from the degradation of dietary protein. Therefore, the decline in isoacids suggests that amino acid breakdown was depressed by the glycopeptides.

Both compounds increase propionate production but do not affect acetate and butyrate production. Both glycopeptides increase α-amino-N and decrease ammonia-N concentrations in rumen fluid, suggesting less protein and/or amino acid degradation in the rumen.

Isoacids

Ruminal bacteria require isoacids (isobutyrate, 2-methylbutyrate, isovalerate and valerate), NH_3 and hydrogen sulfide for growth (Brondani et al 1991). Little information is available concerning the interaction of these three nutrients, particularly when high fibre diets are fed. To study the interaction of isoacids, NH_3 and sulfur, a 2^3 factorial crossover (three factors each of three levels) conducted in two 4×4 Quasi-Latin squares was used (Brondani et al 1991).

Double blocking criteria were animals and time (non-random repeated measurements were obtained from each subject assigned to a sequence of treatment combinations). In each trial, eight rumen-fistulated adult Tabasco sheep were divided into two groups. Sheep in Groups 1 and 2 weighed 27 and 36 kg, respectively, and were assigned randomly to rows of 1 square, corresponding to a predetermined sequence of treatment combinations. The three factors were isoacids, N, and S each at two levels. Composition of the basal diets for Trials 1 and 2 is given in Table 7. Corn stover was chopped with a silage chopper to lengths of 1.26 cm. Both sugar cane bagasse and stover were air-dried.

Based on results of previous experiments, an equal weight mixture of isoacids (isobutyrate, 2-methylbutyrate, isovalerate and valerate) was administered at 0.1 and 0.2 g/kg body weight (BW) per day. To achieve two levels of NH_3-N in the rumen (about 5 and 15 mg/dl), the basal diet was fed either alone or supplemented with urea (high N treatment) at 1.5% of DM. To achieve two levels of sulfide in the rumen (4 and 8 μg/ml), the basal diet was fed alone or supplemented with elemental S at 0.2% of DM. Sulfur and N supplementation was designed to provide four different N:S ratios. Final N:S ratios were approximately 3:1, 5:1, 8:1 and 12:1. Isoacids, urea and S were premixed weekly with part of the sorghum and then incorporated into the totally mixed diet. The daily ration was fed at 0700 and feed intake was recorded daily for each animal. Water

Table 7. *Composition and analysis of basal diets*[†].

Item	Trial 1	Trial 2
Ingredient (%)		
Corn stover	—	25.0
Corn cobs	—	25.0
Sugarcane bagasse	44.0	—
Sorghum grain	55.0	49.0
Bone meal	0.5	0.5
Trace-mineralized salt[‡]	0.5	0.5
Analysis[§]		
CP (%)	7.2	7.7
Crude fibre (%)	22.0	18.2
Digestable energy (mcal/kg)	2.6	2.9
Total N (%)	1.15	1.23
Total S (%)	0.14	0.18
N:S	8.0	6.8

[†]DM basis.
[‡]Composition: Zn = 0.35%; Mn = 0.20%; Fe = 0.20%; Cu = 0.03%; Co = 0.005%; I = .007%; and NaCl = 96%.
[§]Calculated NRC values.

was provided *ad libitum.* Animals were adapted to a given diet for 14 days prior to measurements.

In vivo production rates of acetate were measured by a single injection radioisotope technique (Cook 1966; Rogers and Davis 1982). On day 15, 3 h after morning feeding, each animal received an intraruminal injection of 100 μCi of Na-[1-^{14}C]acetate with 100 ml of a 10% solution of polyethylene glycol (molecular weight 3350). Following infusion, ruminal fluid samples were collected every 20 min for the next 3 h. Samples were strained through four layers of surgical gauze and stored frozen at –20°C. Ruminal hydrogen sulfide was determined using a hydrogen sulfide-sensing electrode (Lazar model GS-136). Isolation of ^{14}C-acetate from rumen fluid was as described above under glycopeptides. Determinations of acetate production, ruminal NH_3-N and acetate were described previously (Cook 1966).

In a third trial, sheep in Groups 1 (25 kg) and 2 (35 kg) were each fed 1215 and 1700 g/d of pineapple tops and 20 and 27 g/d of minerals, respectively (Quispe et al 1991). Pineapple tops were 6.6 and 0.13% CP and S, respectively.

Pineapple tops were chopped in a silage cutter, air-dried to 20% moisture, and finally ground for formulating rations. Isoacids (equal weights of isobutyrate, 2-methyl-butyrate, isovalerate and valerate) were mixed into rations at two levels to provide 0.07 and 0.14 g kg BW^{-1} d^{-1}. In order to achieve ruminal NH_3-N levels of approximately 5 and 15 mg/dl, pineapple tops were fed without or with urea at 0.43 g kg BW^{-1} d^{-1}. Similarly, either 0 or 0.086 g S kg BW^{-1} d^{-1} was provided by adding elemental sulfur. Experimental periods were one week. Acetate production was measured on the last day of each period.

For Trials 1 and 2, results are presented in view of the existence of two significant two-way interactions, i.e. the interactions between N with S and N with isoacids. The

Table 8. *Effects of N and S on feed intake and ruminal fermentation variables in sheep fed high fibre diets.*

	DMI (g/d)	Feed N:S	NH_3 N (mg/dl)[†]	Sulfide-S (μg/ml)[‡]	Acetate production (mol/d)
Trial 1 (sugarcane bagasse diet)					
Low N, low S	387	8.1	6.11	2.43	2.18[¶]
High N, low S	454	12.7	12.19	2.16	1.94[¶]
Low N, high S	442	3.1	5.14	5.84	2.25[¶]
High N, high S	462	5.0	13.11	5.51	3.16[§]
S.E.	16	—	0.84	0.33	0.24
Trial 2 (corn stover diet)					
Low N, low S	430	7.0	5.28	2.97	2.24[¶]
High N, low S	412	10.7	14.19	3.34	2.82[¶]
Low N, high S	461	3.2	6.93	5.26	2.31[¶]
High N, high S	442	4.7	13.11	4.81	3.67[§]
S.E.	15	—	0.71	0.28	0.21

[†]Significant effect of N ($P < 0.01$).
[‡]Significant effect of S ($P < 0.01$).
[§,¶]Means in the same column within a trial with different superscripts differ ($P < 0.10$).

interaction of isoacids and S was not significant. Therefore, there was no specific examination of combination means involving those two factors. When two factors interact, it is appropriate to examine the means of two-way combinations (averaged over other factors, if any) to determine the nature of the interaction. For the two-way combinations between N and S (Table 8), DMI did not differ among treatment groups in either trial. Urea increased ($P < 0.01$) ruminal NH_3-N in both trials. Similarly, S supplementation resulted in higher ($P < 0.01$) levels of sulfide-S in ruminal fluid of sheep on both trials. In both trials, acetate production was not changed by either urea or S when fed separately. Supplementation of both resulted in a 44% increase in acetate production in Trial 1 and a 63% increase in Trial 2. Although the lack of an increase in acetate production in the group with low N and high S may be explained by the low level of ruminal NH_3-N (Table 8), the results for the group with high N and low S were not expected. The calculated N:S ratio and the percentage of S in the basal diets (Table 8) were within the ranges commonly recommended for adult sheep (Bray and Till 1975; Ørskov 1982). However, S intake by animals in the group with high N and low S may have been limiting. Hume and Bird (1970) reported that S intake of 1.95 g/d supported maximal ruminal microbial protein synthesis in sheep. In the present study, intake of total S in the group with high N and low S averaged 0.65 and 0.74 g/d, respectively, for Trials 1 and 2. For the group with high N and high S, average daily intake of S for sheep in Trials 1 and 2 was 1.66 and 1.80 g/d, respectively. The precise level at which ruminal sulfide concentration limits microbial growth and fermentation has not been defined clearly, but 1 μg of sulfide-S/ml of ruminal fluid has been suggested to be the lower limit for optimal fermentation (Harrison and McAllen 1981). Concentrations of sulfide-S in the groups with high N and low S were greater than 2 μg/ml but may not have been high enough for maximal fermentation. These results suggest that S supplementation

Table 9. *Effects of isoacids (Iso) and N on feed intake and ruminal fermentation variables in sheep fed high fibre diets.*

	DMI (g/d)	NH_3 N (mg/dl)[†]	Total isoacids (mmol/dl)[‡]	Acetate production (mol/d)
Trial 1 (sugarcane bagasse diet)				
Low Iso, low N	384	6.82	0.28	1.91[§]
Low Iso, high N	421	15.10	0.29	2.86[¶]
High Iso, low N	438	7.14	0.49	1.97[§]
High Iso, high N	417	14.36	0.53	3.74[††]
S.E.	16	0.64	0.03	0.17
Trial 2 (corn stover diet)				
Low Iso, low N	431	8.31	0.26	2.64[¶]
Low Iso, high N	456	17.11	0.28	2.95[¶]
High Iso, low N	471	7.34	0.47	2.88[¶]
High Iso, high N	448	15.21	0.48	3.87[††]
S.E.	15	0.71	0.02	0.21

[†]Significant effect of N ($P < 0.01$).
[‡]Significant effect of isoacids ($P < 0.01$).
[§,¶,††]Means in the same column within a trial with different superscripts differ ($P < 0.05$).

based solely on the N:S ratio or on the percentage of S in DM may not be adequate when high fibre diets containing low N are fed. The total amount of S that would allow maximal microbial protein synthesis should be considered first. Once that is provided, supplementing N to attain an N:S ratio of about 10 (Ørskov 1982) should result in maximal fermentation efficiency.

Increasing the level of isoacids in the diet resulted in higher ($P < 0.01$) concentrations of these acids in the rumen for both trials (Table 9). In Trial 1, acetate production was higher when N was supplemented along with low isoacids ($P < 0.05$). Increasing the amount of isoacids in the diet, without adding N, did not change acetate production. When both were present at the high level, acetate production was further increased ($P < 0.05$) by 30%. These results show that rates of fermentation in the rumen can be only as high as the availability of the most limiting nutrient. In Trial 1, ruminal NH_3-N provided by the basal diet was more limiting than isoacids. Once N supply was increased by the addition of urea to the diet, isoacids became limiting.

Trends in acetate production found in Trial 2 were similar to Trial 1 (Table 9). However, increases ($P < 0.05$) in acetate production were found only when both N and isoacids were fed at a high level. This suggests that ruminal NH_3-N provided by the basal diet in Trial 2 was adequate to support microbial fermentations as efficiently as the diet that was supplemented with N. However ruminal NH_3-N was not sufficiently high to permit utilisation of the higher amounts of isoacids supplied by the high isoacid treatment.

In Trial 3, two different ruminal levels of H_2S and NH_3-N were achieved by supplementing pineapple tops with elemental sulfur and urea (Table 10). Ruminal H_2S averaged 7 to 9 μg/ml when S was added compared to 0.7 to 4 μg/ml with no S. Similarly, ruminal NH_3-N was 13 to 16 mg/dl when urea was added compared to 5 to 7 mg/dl without urea.

Table 10. *Effects of interaction of isoacids, urea, and sulfur, each at 2 levels, on ruminal acetate production, H_2S, and NH_3-N in sheep fed pineapple tops.*

Treatments[†]	H_2S (μg/ml)[‡]	NH_3-N (mg/dl)[‡]	Rumen (NH_3-N:H_2S)	Ration (N:S)	Acetate (mol h^{-1} per sheep)
$A_LB_LC_L$	0.7	6.7	90	8	0.21
$A_HB_HC_L$	4.0	14.8	36	12	0.22[††]
$A_HB_LC_H$	7.8	7.4	9	3	0.33[§]
$A_LB_HC_H$	7.3	12.8	18	5	0.25[¶]
$A_LB_LC_H$	7.0	7.0	10	3	0.17[¶]
$A_LB_HC_L$	2.9	15.9	54	12	0.27
$A_HB_LC_L$	3.5	4.8	14	8	0.28[††]
$A_HB_HC_H$	9.1	13.9	16	5	0.44[§]

[†]A, B and C are levels of isoacids, urea, and sulfur, respectively. L and H are low and high levels.
[‡]S.E. for H_2S and NH_2-N is 0.6 and 1.2, respectively.
[§,¶,††]Two pooled treatment contrasts were different: § vs. ¶ ($P < 0.05$), positive effect of isoacids when sulfur was present and § vs. †† ($P < 0.1$), positive effect of sulfur when isoacids were high. All other treatment comparisons were not different ($P > 0.01$).

Ruminal concentrations of isoacids were not increased with high levels of isoacids. This may be due to rapid utilisation in the rumen. The ruminal N:S ratio (Table 10) reflected the dietary N:S ratio.

For acetate production, interactions were only significant between isoacids and S. Therefore, the Bonferroni t-test was used to test differences among treatment means within each level of isoacids or S. These results show that acetate production was increased ($P < 0.05$) with S supplementation. Similarly, acetate production was higher when adding S and high isoacids ($P < 0.01$).

Acetate production was higher with a dietary N:S ratio of 5:1 relative to 3:1, 8:1 or 12:1 (Figure 1). This ratio is narrower than the ratio usually considered adequate for sheep (Bray and Till 1975). The dietary N:S ratio of 5:1 resulted in ruminal NH_3-N to H_2S ratio of 17:1, which is close to the N:S ratios for ruminal bacteria of approximately 13:1 proposed by Bray and Till (1975).

Acetate production was more correlated to N:S ratios (r^2 = 14.6%) than to ruminal NH_3-N concentration (r^2 = 3.2%). There was a trend towards more acetate production as ruminal NH_3-N increased, but differences were not significant.

In general, acetate production was 42% greater when higher levels of isoacids and S were added to the feed ration. Adding urea to diets containing high levels of isoacids and S increased acetate production by another 33%. It is concluded that 0.14 g of isoacids, 0.43 g urea and 0.086 g S kg BW^{-1} gave the maximum fermentation of pineapple tops. These treatments gave a dietary N:S ratio of 5:1.

Because production of VFA from carbohydrates in the rumen is coupled with microbial growth, maximal microbial yield can be attained only if precursors for protein synthesis are made available to the microbiota simultaneously and in adequate quantities. This study suggests that high fibre diets low in N are utilised better when all three factors (N, isoacids and S) are adequate.

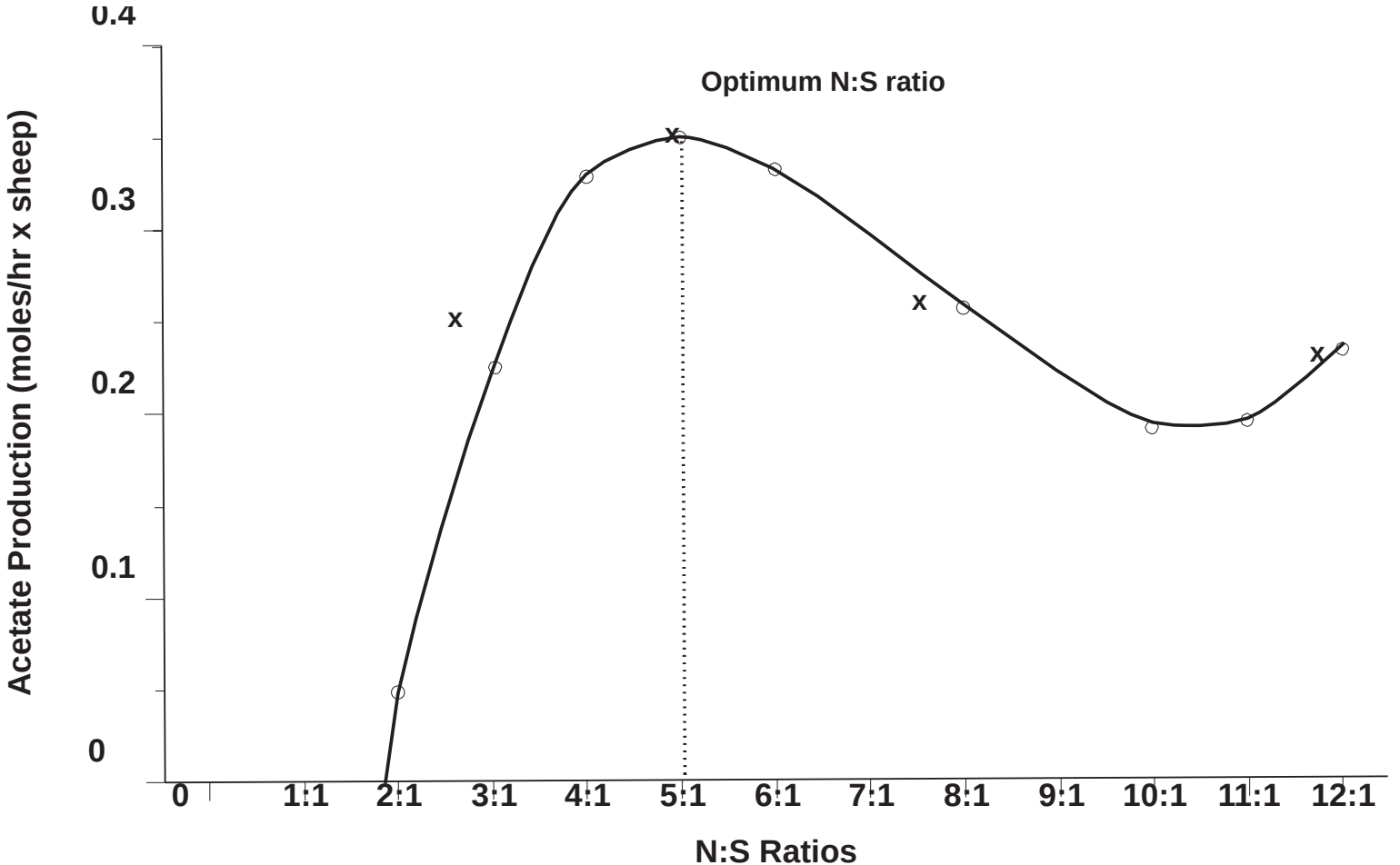

Figure 1. *Effects of N:S ratios in the ration on mean rumen acetate production in sheep (SE = 0.05).*

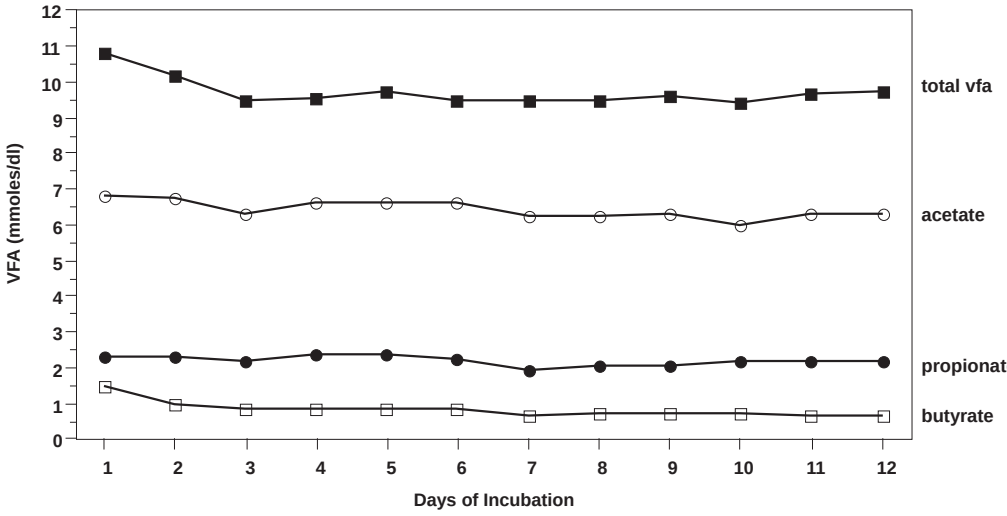

Figure 2. *Daily rumen VFA concentration* in vitro *using timothy hay as substrate*.

Monensin

Monensin has been used extensively in diets for feedlot cattle for several years. Isoacids have been used as a nutritional supplement for lactating dairy cattle. Both monensin and isoacids affect rumen fermentation. However, little is known about the effects of the combination of the two compounds in the rumen. *In vitro* rumen fermentations have shown that monensin decreases the acetate:propionate ratio (Chalupa 1977; Richardson et al 1976). These results have been attributed to a toxic effect of the ionophore on ruminococci (Henderson et al 1981). Unlike monensin, isoacids (isobutyric, isovaleric, 2-methylbutyric and valeric acids) increase rumen acetate production, probably due to an enhanced growth of ruminococci (Allison and Bryant 1958; Bryant 1973; Felix et al 1980).

An *in vitro* system was used to study the effects of monensin and isoacids on the rumen fermentation (Kone et al 1989). Ruminal fluid was obtained 3 h post-feeding from a mature non-pregnant and non-lactating rumen-fistulated Holstein cow that weighed 550 kg. Samples from different parts of the rumen were strained through two layers of surgical gauze into 1 litre glass bottles kept at 40°C.

The semicontinuous culture technique of Short (1978) was used with minor modifications (Figure 2). The culture was maintained in the same flask throughout the experiment. The substrate was timothy hay.

Isobutyric, 2-methylbutyric, isovaleric and valeric acids were mixed at equimolar concentrations and neutralised with sodium hydroxide. The final concentrations of the

Table 11. *Effect of isoacids on rumen VFA concentration and gas composition* in vitro *using inoculum from a cow fed timothy hay.*

Variable	Isoacid concentration, mg/dl				SEM
	Control	10	15	20	
	mmol/dl				
VFA	7.87[§]	8.72[†]	8.93[†]	8.41[‡]	0.08
Acetate	5.48[‡]	6.07[†]	6.17[†]	5.61[‡]	0.07
Propionate	1.55[‡]	1.69[†]	1.63[†]	1.52[‡]	0.02
Butyrate	0.60	0.58	0.66	0.65	0.03
Isoacids	0.25[¶]	0.40[§]	0.49[‡]	0.59[†]	0.01
Isobutyrate	0.03[¶]	0.06[§]	0.07[‡]	0.08[†]	0.001
2-methylbutyrate	0.06[¶]	0.10[§]	0.13[‡]	0.15[†]	0.003
Isovalerate	0.04[¶]	0.10[§]	0.12[‡]	0.13[†]	0.003
Valerate	0.12[§]	0.14[§]	0.16[‡]	0.22[†]	0.006
	Percent				
CH_4	35.15	36.15	34.38	33.08	1.99
CO_2	49.02	54.94	49.03	47.37	3.15
H_2	3.83	4.00	3.67	3.83	0.2

[†,‡,§,¶] Means in a row with different superscripts differ ($P < 0.05$).

isoacid mixture in the incubation flasks were 10, 15 and 20 mg/dl of media. Monensin (Sigma Chemical Co., S. Louis, MO) was first dissolved in 10 mg of methanol and then diluted with water. The final medium concentrations were 100, 150 and 200 μg/dl.

Experiment 1 investigated the effect of isoacids at 10, 15 and 20 mg/dl of final incubation medium. Experiment 2 investigated the effect of monensin at 100, 150 and 200 μg/dl of final incubation medium. For Experiment 3, monensin was fixed at 150 μg/dl, and isoacids were added to the incubation medium at 10 or 15 mg/dl of the final incubation.

Isoacids at 10 and 15 mg/dl increased acetate, total VFA and propionate concentration, but the increase was not as great at 20 mg/dl. As expected, branched-chain fatty acid concentrations increased in proportion to the amounts added in the medium. In contrast to VFA production, CH_4, CO_2 and H_2 were not affected by isoacids (Table 11).

The effects of different concentrations of monensin are in Table 12. In contrast to isoacids, acetate concentration decreased at all concentrations of monensin tested. Propionate concentration was significantly increased, whereas butyrate concentration decreased at all concentrations tested. Monensin decreased methane production. Neither CO_2 nor H_2 was affected. The results from the monensin experiment agree with similar studies conducted by Chalupa (1977). Monensin consistently decreased acetate and butyrate but increased propionate production.

The addition of monensin caused a decrease in acetate and propionate after 24 h of incubation, but after 48 h, propionate was higher than control values, whereas acetate remained lower than the controls. The addition of isoacids at 10 and 15 mg/dl to flasks containing monensin increased acetate concentration compared with monensin alone ($P < 0.05$; Figure 3). However, the addition of isoacids to flasks containing monensin did not alter propionate concentration (Figure 4).

Table 12. *Effect of monensin on rumen VFA concentration and gas composition in vitro using inoculum from a cow fed timothy hay.*

Variable	Monensin, µg/dl				SEM
	Control	100	150	200	
	mmol/dl				
VFA	8.54[†]	7.22[‡]	6.84[‡]	6.88[‡]	0.14
Acetate (A)	6.02[§]	4.28[‡]	3.74[†]	3.60[†]	0.11
Propionate (P)	1.73[§]	2.27[‡]	2.28[‡]	2.42[†]	0.03
Butyrate	0.52[‡]	0.46[‡]	0.42[†]	0.35[†]	0.02
Isoacids	0.18	0.16	0.16	0.19	0.01
A:P	3.50[†]	1.90[‡]	1.75[‡§]	1.61[§]	0.07
	Percent				
CH_4	36.33[†]	27.07[‡]	26.77[‡]	26.00[‡]	0.91
CO_2	49.83	48.33	42.50	47.50	3.2
H_2	3.50[‡]	3.5[‡]	3.5[‡]	4.00[†]	

[†,‡,§]Means in a row with different superscripts differ ($P < 0.05$).

It is interesting to speculate on the mode of action of the combination of isoacids and monensin. According to Wolin and Miller (1983), monensin affects the rumen fermentation by selecting for organisms that participate in the production of relatively more propionate and against those that contribute to the production of acetate, butyrate and precursors of methane. The growth of ruminococci and butyrivibrios is inhibited by very low concentrations of monensin. These genera are important producers of acetate, butyrate and the substrate for methanogens, H_2 and CO_2. Selenomonads are very insensitive, whereas bacteroids, although sensitive, rapidly become resistant to the ionophore. Both organisms are important in the production of propionate. The addition of isoacids to the culture containing monensin may cause an outgrowth of bacteroids and perhaps ruminococci and methanogenic bacteria, which require isoacids, resulting in more acetate, succinate and formate. Succinate would be decarboxylated to propionate by selenomonads and formate would be used as an energy source by methanogens. The end result would be an increase in acetate and probably more substrate degraded as indicated by the higher VFA in Experiment 3 (Figures 3 and 4).

The combination of isoacids and monensin may be of practical application in cattle rations. The addition of monensin alone to the diet of growing steers increases the efficiency of growth by increasing propionate production. Because of the importance of propionate in glucose metabolism and insulin secretion in ruminants, the increase in ruminal propionate production will promote growth. Isoacids increase acetate, total VFA and microbial synthesis. Because the addition of isoacids to cultures containing monensin does not alter the effect of monensin on propionate production, the additional energy and microbial protein may further improve growth.

The changes in rumen fermentation products brought about by glycopeptides, monensin and isoacids can have positive effects on animal production. In addition, there are other chemicals such as virginiamycin and lasolacid that affect rumen VFA production. These chemicals provide a tool to study regulation of microbial growth at the molecular level. Knowledge in this area is critically needed to provide a basis for

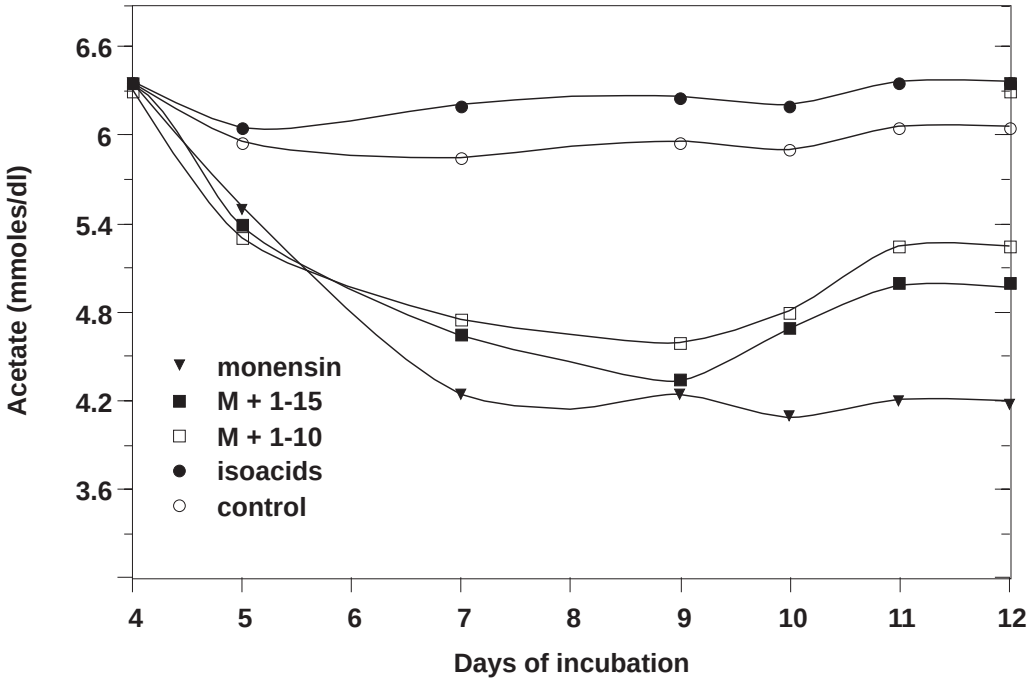

Figure 3. *Effect of the combination monensin/isoacids on daily rumen acetate concentration by rumen microbes* in vitro *using timothy hay as substrate.*

developing methods to control the rumen ecosystem under a variety of feed management systems.

Utilisation of rumen VFA

In addition to developing better methods to control the rumen ecosystem, it is equally important to understand utilisation of rumen VFA for production of meat, milk and fibre. Of course, molecular mechanisms regulating utilisation of substrates in ruminants are not known. Our laboratory is engaged in a study of the regulation of acetate for milk synthesis at the molecular level. Acetate, as acetyl coenzyme A (acetyl-CoA) is a major substrate for milk synthesis and occupies a central position in metabolism of Holstein mammary gland as well as in both prokaryotic and eukaryotic cells. Acetyl-CoA is generated via the acetate activation reaction catalysed by acetyl coenzyme A synthetase (ACS).

Our work on characterisation of ACS in ruminants has determined the tissue distribution, intracellular localisation, physical and catalytic properties and changes in mammary gland throughout lactation. In addition, a bovine mammary gland ACS cDNA has been cloned (Raafat 1994). ACS is most active in heart, kidney, adipose tissue and mammary gland at peak lactation (Cook and Qureshi 1972; Cook et al 1969, 1975). There is marginal activity in brain and skeletal muscle. Unlike non-ruminants, acetate

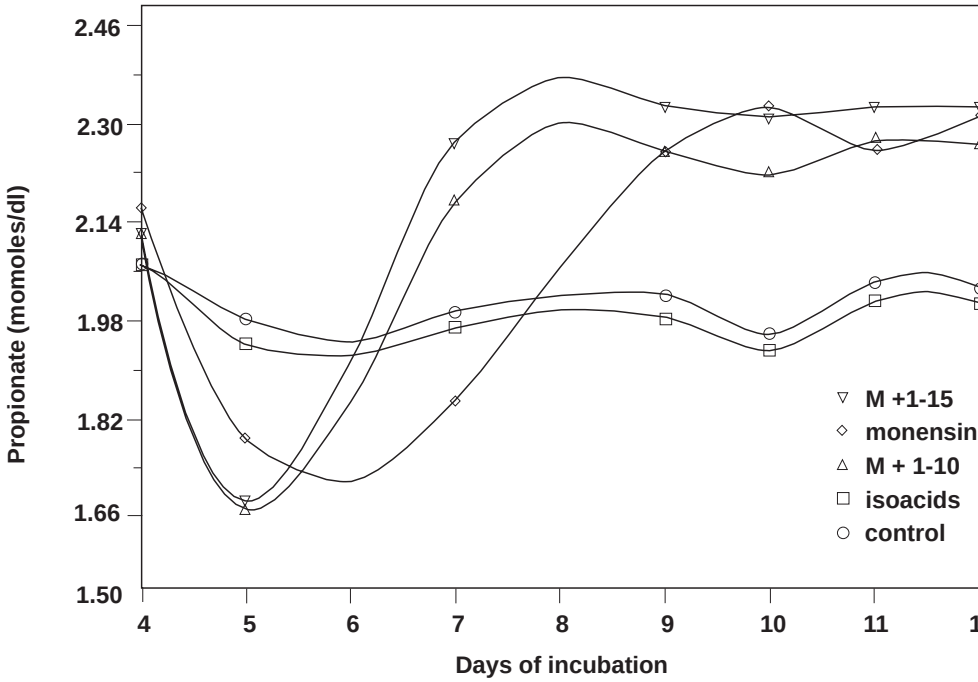

Figure 4. *Effect of the combination monensin/isoacids on daily rumen propionate concentration by rumen microbes* in vitro *using timothy hay as substrate.*

is not utilised by ruminant liver (Cook 1970; Cook and Miller 1965) and ACS activity is not found in liver (Ricks and Cook 1981a). However, *in vitro,* acetate can be oxidised by liver because propionyl CoA synthetase can activate acetate at a low rate (Ricks and Cook 1981a). The apparent molecular weight of the mammary gland enzyme is 63,000 (Qureshi and Cook 1975). ACS is a glycoprotein and the carbohydrates have been identified (Ricks and Cook 1981b; Stamoudis and Cook 1975).

In early lactation, blood growth hormone is relatively high and insulin is low. As lactation advances and milk production declines, blood levels of growth hormone decrease and insulin increases. Also, high producing cows have lower blood insulin and higher growth hormone than do low producing cows (Ghirardi and Cook 1987). Insulin inhibits and growth hormone stimulates ACS activity in lactating goat mammary gland. As lactation advances, ACS activity decreases sharply but can be partially reinstated by injecting a combination of growth hormone, prolactin and dexamethasone (Marinez et al 1976). The existence of 5′ regulatory site hormonal response elements (e.g. glucocorticoids) in the ACS gene might explain these coordinated regulatory patterns.

In cattle, ACS activity is marginal in a dry mammary gland, increases from parturition to peak milk production and then declines in activity as lactation advances (Marinez et al 1976). ACS activity is directly correlated with milk production. Thus, as lactation advances, less acetate can be utilised.

Given the central role of ACS in ruminant metabolism and milk production, cDNA cloning of the ACS gene was initiated using poly(A+)RNA isolated from bovine mammary tissue, taken at peak lactation. Mammary gland mRNA was reverse transcribed with AMV reverse transcriptase (Promega Inc., Madison, WI), according to the manufacturers' instructions. Following second strand synthesis and EcoRI adaptor addition, cDNAs were cloned into the EcoRI site of lambda gt11. Initial screening with rabbit anti-ACS sera, prepared by injection of purified bovine ACS, yielded a truncated cDNA clone, AR8. AR8 remained positive through several rounds of plaque purification. Subsequent screening of 750,000 plaques using AR8 yielded 1 clone, designated ATC5, which remained positive through several rounds of plaque purification. ATC5 contains a 4.2 Kbp insert, more than sufficient to encode the complete ACS protein. The insert from ATC5 was excised by EcoRI digestion and subcloned into the EcoRI site of pUC19 (generating pATC5) for further analysis and sequencing.

Prior to initiation of full-scale DNA sequence analysis, several steps were taken to ensure pATC5 represented a true copy of ACS gene coding sequences.

1. Rabbit anti-ACS was affinity purified using the fusion protein produced by induction of pATC5 with IPTG. Purified anti-ACS removed over 90% of ACS activity from a preparation of partially purified bovine mammary ACS. In contrast, preimmune sera did not adversely affect ACS activity.
2. Anti-ACS, affinity purified using LacZ-ACS fusion protein from clone pAR8, was used in Western blots of mitochondrial extracts from heart, liver, kidney, mammary gland and spleen. Proteins with the expected molecular weight and tissue distribution as ACS were recognised.
3. Preliminary DNA sequencing of pAR8 and pATC5 revealed an open reading frame with 25% homology to other synthetases (ATP and HSCoA binding proteins). No homology to common structural proteins was observed.
4. The pattern of RNA expression detected in bovine tissues is consistent with known protein expression patterns of ACS. Specifically, heart and mammary gland contain significant amounts of ACS RNA while liver and kidney contain very little.

A restriction map of the 4.2 Kbp insert of pATC5 has been prepared and the insert partially sequenced. To date, over 1200 bp have been sequenced, revealing a single long open reading frame. Northern blots of poly(A+) RNA from bovine heart, liver, mammary gland and kidney were probed with [alpha-^{32}P]-ATC5. Northern analyses clearly show multiple forms of ACS mRNA in most tissues. For example, three distinct transcripts were detected in heart and mammary gland while two transcripts were detected in kidney and one in liver. ACS transcripts range in size from 0.8 to 5.2 kb. Multiple forms of ACS mRNA were not unexpected, based on results from other synthetases, and may be the result of multiple promoters and/or alternative splicing. It is curious, however, that a faint ACS mRNA (4.2 kb) would be present in liver where no ACS activity can be detected. This suggests that either the liver transcript is not translated, leads to translation of an inactive molecule, or is regulated at the post-translational level. It is also possible that our ACS probe hybridised with another closely related mRNA (e.g. propionyl CoA synthetase).

The expression of ACS activity in ruminants is unique. ACS activity is constitutive in heart, controlled by nutrition and physiological state in the mammary gland but not expressed in liver (Marinez et al 1976; Mellenberger et al 1973; Ricks and Cook 1981a). Ruminant liver is not lipogenic (Emery 1980). Therefore, acetate is not utilised by liver. The liver utilises propionate as the major source of glucose (Amaral et al 1990; Reynolds et al 1988). Since there is a paucity of glucose in ruminants, this pattern of tissue utilisation of acetate spares glucose for other vital metabolic functions (Ricks and Cook 1981a).

ACS is the first committed step in acetate oxidation which is a major source of energy for milk synthesis. Also, in the ruminant mammary gland, ACS is the first committed step in fatty acid synthesis. The activity of ACS is marginal in a dry gland, increases to peak lactation and then declines in activity as lactation advances (Marinez et al 1976). In rat mammary gland, ACC is generally considered to be the first committed step in fatty acid synthesis (Ponce-Castaneda et al 1991). Acetyl-CoA in rat mammary tissue is from glucose via the ATP citrate lyase reaction and this source of acetyl-CoA is generally considered to be relatively constant.

Since we find multiple forms of ACS mRNA, acetyl-CoA synthetase could be regulated by tissue-specific promoters. This would explain why the enzyme is active in heart (three forms of mRNA) but not liver (one form of mRNA). Also, there may be an alternative promoter in the mammary gland that is activated by nutrition and/or physiological state. However, tissue specific alternative splicing mechanisms for acyl-CoA synthetases that produce protein isoforms specific for individual fatty acids cannot be ruled out at this point. Understanding regulation of ACS is critical because the reaction catalysed by this enzyme is the initial step in fatty acid synthesis and acetate oxidation in ruminants.

It is important to target other genes regulating metabolism. In ruminants, this endeavour is in its infancy (Bergen et al 1995). However, the genomic regulation of protein and fat deposition promises to be the next advance in food producing animals. The genes encoding fatty acid synthase and calpastatin are of current interest and a knowledge of their regulation can provide better methods to regulate nutrient composition and tenderness of meat products.

In summary, there are several chemical methods available to regulate the rumen ecosystem such that end-products of feed fermentation can be altered to improve growth and fattening or milk production. A knowledge of the mechanism of action of these chemicals at the molecular level will provide new and better methods to regulate the rumen ecosystem. However, this is just one aspect of improving feed utilisation by ruminants. A knowledge of utilisation of rumen fermentation products by extraruminal tissue is paramount if improved efficiency in food producing animals is to be achieved. This will require detailed knowledge of the regulation of genes important in substrate utilisation. Also, the availability of probes for these important genes will provide additional opportunities for genetic modification of ruminants using marker-assisted selection programmes.

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Engineering the rumen for enhanced animal productivity

J.D. Brooker

Department of Animal Science, University of Adelaide, Glen Osmond, 5064, Australia

Abstract

The rumen can be manipulated in several ways. Plasmid vectors that can transform rumen bacterial species have been developed and some that shuttle between different species have been described. One of these, pMU1328, has been used to transform *Streptococcus bovis* and to form the basis of construction of a suicide vector for integration into the *S. bovis* chromosome. This vector, pJDB9, replicates in *Escherichia coli* but not in *S. bovis* due to removal of the *ori-S.* Insertion of a 0.47 kb fragment of Tn916 into pJDB9 generated pJDB0.5 that can integrate into its chromosomal Tn916 target sequence in *S. bovis* and be maintained in a stable fashion. The amylase gene promoter has been inserted in these plasmids to promote expression of various genes, including β-1,4-endoglucanase, xylanase, pea albumin and tannin acyl hydrolase in *S. bovis* and related species.

Population control in the rumen has also been demonstrated. Transfer of lactic acid utilising bacteria from grain-adapted animals to livestock just prior to the introduction of a grain-based feeding regime reduced the potential lactate concentrate from 100 mM to less than 10 mM and avoided the onset of acidosis. Bacterial transfers from feral goats to domestic sheep and cattle also demonstrated the viability of bacterial transfers in improving the N balance of animals feeding on tannin-containing *Acacia*. Improved weight gain and wool growth in sheep is reported. These examples demonstrate the feasibility of rumen manipulation to improve productive performance of domestic ruminants browsing fibrous forages.

Introduction

Limitations to ruminant production include poorly digestible fibrous plant material, nitrogen losses and toxic compounds present in the feed. In tropical and arid regions, these limitations can be so great as to become critical to the survival of ruminant animals in that environment. The fibre content of many tropical grasses is high and digestibility of the feed is low compared with improved Mediterranean-type pastures (McMeniman et al 1981). Feed supplements are often unavailable or expensive. Anti-nutritive components are also very prevalent in tropical plants and these restrict the forage range for domestic livestock. Many of these limitations may be overcome, or at least alleviated by manipulation of rumen microbial populations.

The rumen can be manipulated in two fundamental ways: firstly by the application of genetic techniques to modify the functional capacity of specific bacterial species; secondly, through modification of rumen ecology by introducing novel bacterial species or by selectively enhancing populations of existing species.

Considerable advances have been made in developing techniques for the genetic manipulation of rumen bacteria and the predominant species have been transformed, albeit at low frequencies in some cases. Various plasmid vectors have been designed, and for some species such as *Streptococcus*, recombination of genes into the chromosome of the host has been demonstrated. Recombinant strains of rumen bacteria expressing various foreign genes have been produced and some recombinant strains have been returned to the rumen and have been shown to persist in the absence of direct selection.

Modification of microbial ecology by population control has also been demonstrated to be an effective way to improve rumen function. Lactic acidosis in feedlot animals can be controlled by ruminal inoculation of lactate-utilising bacteria. Tannin toxicity can be alleviated and productivity of animals browsing tannin-containing tropical shrubs has been enhanced by the cross inoculation of rumen fluid from wild to domestic ruminants.

There is now ample evidence that animal production, especially in marginal areas, can be improved by manipulation of the rumen. Further work on the isolation of novel bacterial species (Brooker et al 1994) and genes that may be transferred between bacterial species will be valuable additions to studies on animal nutrition that will ultimately lead to increased productivity in domestic ruminants in tropical and arid regions.

Methods and materials

Bacterial strains

Bacterial strains used were *E. coli* K12, strain DB11 (B. White, University of Illinois, USA) and *S. bovis* strain WI-1 (a local ruminal isolate). Plasmid pMU1328 was obtained from B. Davidson, Melbourne University, Australia. All subcloning and plasmid manipulation steps were carried out using pUC19. *E. coli* was grown in LB medium. *S. bovis* WI-1 was grown in nutrient medium (YTGS) containing 5 g yeast extract, 15 g trypticase, 10 g glucose and 15 g starch per litre of water. *S. bovis* WI-1::Tn*916* was obtained by conjugation between *Enterococcus faecalis*::Tn*916* and *S. bovis* WI-1 (Brooker and Lum 1993). Transformation of *E. coli* and *S. bovis* was by electroporation using the Biorad Gene Pulser under conditions previously described (Whitehead 1992) except that cells were grown to A_{660} of 0.8, washed in sterile distilled water and suspended in 10% glycerol. Most genetic techniques are as described by Sambrook et al (1989).

Preparation of anaerobic medium

Anaerobic medium was prepared by boiling for 5 min then bubbling with O_2-free CO_2 (passed over copper turnings at 300°C) for 15 min. Solutions were dispensed into Hungate tubes or 100 ml crimp-top bottles under CO_2 before autoclaving. Media were stored in a Coy anaerobic hood under an atmosphere of 95% CO_2 and 5% H_2.

Virginiamycin (Stafac 500) was kindly supplied by Dr. J.B. Rowe (University of New England, Australia).

Fermenter conditions

A Bioflow IIC microprocessor-controlled laboratory scale fermenter (New Brunswick Scientific Co., Inc.) with a 600 ml working volume was used to develop an *in vitro* model for acidosis. The pH was maintained at 6.0 by the automatic pumping of either 1 M HCl or 1 M KOH and the fermenter was sparged at a rate of 20 ml/min with CO_2 and N_2 gases in a ratio of 3:1. The temperature was maintained at 39°C. Cultures were mixed continuously at 100 rpm for pure culture studies and at 50 rpm for crude rumen fluid incubations.

Metabolism studies in rumen fluid-inoculated sheep

Twelve three- to four-year-old merino wethers and two female domestic angora goats were treated with a broad-spectrum anthelmintic (Valbazen: Smith Kline and French, Australia) and placed in individual metabolism cages. During the first seven days, the animals were allowed to acclimatise to their surroundings and were fed 1 kg lucerne pellets daily. Thereafter *Acacia* (mulga) was harvested daily from trees 4–6 m in height, the leaves were separated as described by McMeniman et al (1981) and fed *ad libitum* at 0800 h each morning.

Two days after commencing the mulga diet the first of four ten-day metabolism studies (period 1) was conducted. Daily urine and faeces output were measured and samples were collected and bulked for N analysis. Daily dry matter intake (DMI) was measured and liveweight (LW) change was recorded periodically. The second metabolism study (period 2) commenced 25 days after starting the mulga diet.

Results

Genetic manipulation in the rumen

Various vectors have been designed for rumen bacteria. Some of these are based on endogenous plasmids, e.g. pJDB216 for *Selenomonas ruminantium* (Attwood and Brooker 1992). Others are based on compatible plasmids from other bacterial species, e.g. pBS42 from *Bacillus subtilis* that functions in *Butyrivibrio fibrisolvens*. Transformation systems have been developed for several ruminal species. This work has now been extended to the development of a recombination system to insert foreign genes directly into the host chromosome.

Development of an expression system for *Streptococcus* spp

To develop an expression vector capable of mediating expression of foreign genes in *Streptococcus* spp, firstly we cloned the non-secreted α-amylase gene from *S. bovis* into

E. coli (DB11). The gene was isolated from a pUC19 plasmid library by screening for starch degradation on nutrient agar plates using the iodine reaction to localise positive colonies. The cloned DNA was mapped and 500 bp of the DNA was sequenced and compared with previously published data. The gene corresponded to that recently described in *S. bovis* by T.R. Whitehead (personal communication). Using PCR primers, constructed so that a synthetic *Hin*dIII site was present on either end, the promoter region and ribosomal binding site for the gene was synthesised. The promoter fragment was cloned in pMU1328 and sequenced to verify its structure and orientation. This fragment has been placed in the mcs of the suicide vector, pJDB9 to drive expression of new genes inserted nearby. Genes to be expressed include a β-1,4-endoglucanase, xylanase, tannin acyl hydrolase and pea albumin.

Construction of vectors for recombination in *S. bovis*

Plasmid pMU1328 was digested with *Pst*I to delete the streptococcal replicon *(ori-S)*, self ligated and electroporated into *E. coli* DB11. This plasmid, pJDB9, was isolated from single recombinant colonies and mapped. The *ori-S* deletion was confirmed although a minor rearrangement involving inversion of the *ori-C*-containing fragment had also occurred (Figure 1). This rearrangement appeared to have no adverse effect on subsequent manipulations.

To construct a vector capable of integration into the *S. bovis* WI-1::Tn*916* chromosome, we inserted a PCR-generated 0.47 kb fragment of transposon Tn*916* into the multiple cloning site of pJDB9. This was achieved by cloning the tet M-containing 4.8 kb *Hinc* II fragment of Tn*916* into the *Sma* I site of pUC19 to form pJDB4.8. A 0.47 kb fragment of the tet M gene, between nucleotides 70 and 540, was PCR amplified using primers synthesised from the published sequence (Burdett 1990) but with an *Eco*RI restriction site added to each end. The fragment was digested with *Eco*RI and inserted into the *Eco*RI site of pJDB9 to form pJDB0.5 (Figure 1).

To establish homologous recombination of pJDB0.5 in *S. bovis* WI-1::Tn*916*, cells were electroporated with pJDB0.5 and the cells were then incubated for various times on agar containing 5 mg/ml erythromycin. Plasmids pJDB9 and pMU1328 were electroporated into *S. bovis* WI-1 cells as controls. *S. bovis* WI-1 cells were only transformed by pMU1328. *S. bovis* WI-1::Tn*916* cells were transformed by pJDB0.5 and pMU1328; pJDB9 failed to transform either host. Erythromycin resistant *S. bovis* WI-1::Tn*916* transformants generated with pJDB0.5 were tetracycline sensitive, consistent with integration of pJDB0.5 into the tet M target site. pMU1328 transformants of *S. bovis* WI-1::Tn*916* were resistant to both erythromycin and tetracycline. The frequency of recombination was estimated to be 3×10^{-8}/cell.

Analysis of *S. bovis*::Tn*916* recombinants

To demonstrate chromosomal integration of pJDB0.5, total DNA from recombinant *S. bovis* WI-1::Tn*916* was digested with *Bgl* II or *Pvu* I, enzymes for which there are no sites within pMU1328 or the tet M gene, and analysed by gel electrophoresis and

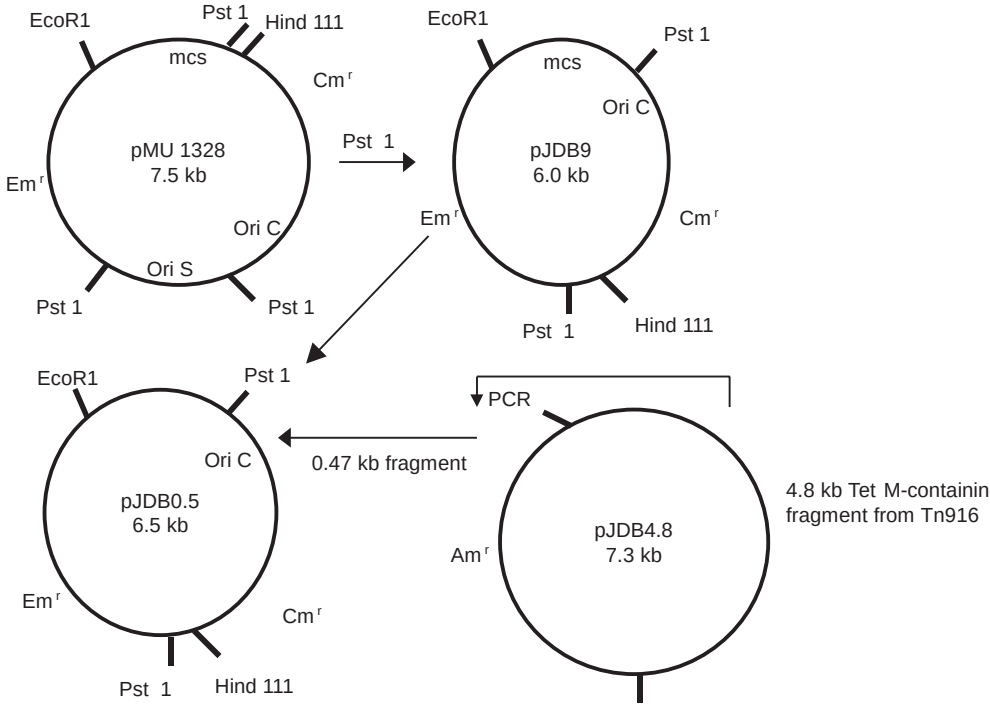

Figure 1. *Derivation of suicide vector pJDB9 and recombination vector pJDB0.5 from pMU1328.*

Southern blot. A single high molecular weight (> 23 kb) band was observed that hybridised with pMU1328-specific probe, and, after washing and reprobing, with the tet M-specific probe. Control *S. bovis* WI-1::Tn*916* DNA hybridised with the tet M-specific probe but not pMU1328.

To examine the organisation of the integrated DNA, chromosomal DNA from two *S. bovis* WI-1::Tn*916* recombinants, JDB-2 and JDB-3, was digested with *Hin*dIII, *Eco*RI or *Hin*dIII + *Eco*RI. Fragments were analysed by gel electrophoresis and Southern blot using radiolabelled pMU1328 as a probe. The results (Figure 2) show that in each digest, several positive bands were detected, and in the single digests, one band in each track displayed a stronger hybridisation signal than the other bands and was the same size as the original linearised plasmid. The same pattern was observed for both recombinants tested.

Stability of recombinants

Due to regeneration of the Tn*916* target sequence during homologous recombination of pJDB0.5, spontaneous excision of the vector sequence may occur in the absence of erythromycin selection. To assess the stability of recombinants, a culture of JDB-2 was passaged for approximately 100 generations in the absence of erythromycin. Analysis

of total and erythromycin-resistant viable cell counts showed that even after extensive passaging in the absence of selection, between 75 and 84% of total viable cells still maintained the pMU1328 em^r sequence.

Population control in the rumen

Bacterial transfer to control acidosis

Acidosis in ruminants is caused by rapid fermentation of cereal starch and the production of excess lactic acid in the rumen. This can be avoided by slow adaptation to the cereal diet. During adaptation to this diet *Sel. ruminantium* subsp *lactilytica* was the predominant lactic acid utilising bacterium present. We therefore tested the effectiveness of a fresh isolate of this species to prevent the short-term development of acidosis when the animals were acutely fed with wheat rations.

In control sheep, ruminal pH fell to 4.7 ($P < 0.001$) within 8 h of grain feeding and remained around this value for the duration of the experiment. In contrast, sheep inoculated with *Sel. ruminantium* subsp *lactilytica* strain JDB201 maintained a stable ruminal pH which did not drop below pH 6.3. Total ruminal VFA in control animals fell to less than 20 mM in 8 h and to less than 10 mM by 16 h. In inoculated animals, total ruminal VFA increased significantly ($P < 0.001$) from 50 to 92 mM within 8 h, but this value declined to 65 mM by 24 h. These changes in inoculated sheep were the result of a twofold increase in propionate and a fourfold increase in butyrate compared with a decrease in propionate and butyrate in control sheep (Table 1). In control sheep, ruminal lactate increased to 109 mM within 8 h of grain engorgement whereas lactate was undetectable in inoculated animals. Blood lactate concentrations were similar for both groups of animals and varied between 1.7 and 2.6 mM.

Over the 24 h experimental period, sheep inoculated with *Sel. ruminantium* subsp *lactilytica* strain JDB201 appeared to suffer no ill effect from acutely administered grain whereas control sheep exhibited symptoms of physiological distress, diarrhoea, listlessness and lack of appetite. However, in treated sheep, if grain feeding was continued past 24 h without further bacterial treatment, symptoms of acidosis (distress, lack of appetite) became evident. At the completion of the 24 h experiment, inoculated sheep immediately resumed normal feeding on the original lucerne diet, whereas control sheep took another 3 days before they resumed feeding.

Ruminal inoculation with a bacterial mixture

Since *Sel. ruminantium* subsp *lactilytica* was only effective in controlling acidosis for 24 h, we tested a combination of *Sel. ruminantium* subsp *lactilytica* and *Megasphaera elsdenii.* To help monitor the introduced bacteria in one of the three treated sheep, the bacterial inoculum was contained within a dialysis bag with a pore size of approximately 10,000 Daltons. Control sheep were not tested beyond one day to avoid acute acidosis in these animals.

Within one day of intraruminal administration of wheat slurry, ruminal pH in control sheep decreased from 6.0 to 4.6. In contrast, all inoculated sheep maintained a ruminal

Table 1. *Effect of* Sel. ruminantium *subsp* lactilytica *inoculation on individual VFA in wheat-fed sheep.*

VFA (mM)	Sampling time (h)	Animal treatments Control (SED)[†]	Inoculated (SED)[‡]
Acetate	0	31.0 (3.65)	35.9 (3.21)[ns]
	8	10.7 (1.13)	53.8 (8.06)[¶]
	16	5.1 (0.96)	42.6 (8.39)[¶]
	24	10.7 (2.50)	31.7 (20.0)[§]
Propionate	0	8.8 (0.92)	9.2 (1.03)[ns]
	8	3.4 (0.38)	21.4 (2.39)[††]
	16	0.7 (0.42)	21.1 (2.53)[††]
	24	0	17.0 (0.90)
Butyrate	0	3.2 (0.50)	3.4 (0.24)[ns]
	8	0.5 (0.03)	13.6 (1.95)[††]
	16	0.28 (0.05)	13.7 (1.13)[††]
	24	0.47 (0.04)	14.5 (1.36)[††]

[†]Mean (standard error mean of four replications).
[‡]Mean (standard error mean of three replications).
[§]Significant ($P < 0.05$) difference.
[¶]Significant ($P < 0.01$) difference.
[††]Highly significant ($P < 0.001$) difference.
[ns] not significant.

pH ($P < 0.05$) greater than 5.5 throughout the four-day experiment. Grain administration also significantly ($P < 0.05$) elevated the ruminal L-lactic acid concentration of control sheep from 0 to 99.0 mM within 24 h. In inoculated sheep, L-lactic acid remained at less than 1 mM for at least four days. Total VFA values were greater than 100 mM in all animals before administration of grain. Within one day of grain feeding, total VFA values in control sheep fell sharply to 21 mM. In animals inoculated with *Sel. ruminantium* subsp *lactilytica* strain JDB201 and *M. elsdenii* strain JDB301, total VFA decreased transiently to 65–73 mM and then increased to greater than 80 mM.

In vitro model for lactic acidosis

In a continuous culture experiment *in vitro, S. bovis* was grown to produce lactic acid. The effectiveness of *Sel. ruminantium* subsp *lactilytica* strain JDB201 and *M. elsdenii* strain JDB 301 in controlling lactic acid accumulation was then tested, and contrasted with the effect of administering 0.75 μg/ml of virginiamycin.

In a control culture of *S. bovis,* when starch was added to the medium, lactic acid concentration increased rapidly from 13 mM to 35 mM within 4 h and reached 48 mM by 8 h. When an established culture of *S. bovis* was inoculated with *Sel. ruminantium* subsp *lactilytica* strain JDB201, lactic acid increased to 60 mM within 24 hr. In contrast, when an established culture of *S. bovis* was inoculated with *M. elsdenii* strain JDB301, the lactic acid concentration was reduced by 65% to less than 20 mM. A combination of *Sel. ruminantium* subsp *lactilytica* strain JDB201 and *M. elsdenii* strain JDB301 was not as effective as *M. elsdenii* alone. In *S. bovis* cultures treated with 0.75 μg/ml of

virginiamycin alone, lactic acid accumulation was prevented for up to 12 h, but by 24 h lactate levels had increased to the level of the untreated controls. In cultures treated with *Sel. ruminantium* subsp *lactilytica* strain JDB201 plus virginiamycin or *M. elsdenii* strain JDB301 plus virginiamycin, the concentration of lactic acid in the mixed cultures was reduced by 50% and > 90% of untreated controls respectively and remained at these levels for at least 24 h.

The combination of virginiamycin and *M. elsdenii* strain JDB301 was tested for its ability to control lactic acid accumulation for up to three days in strained crude rumen fluid cultures incubated in the presence of soluble starch. Starch was added 12 h before the antibiotic/bacteria combination. At 24 h (12 h after introduction of virginiamycin and bacteria), lactic acid in treated cultures was approximately 20% of that in the control. However, by 36 h lactate had increased threefold and by 72 h was 30 mM compared with 50 mM in the controls. When the combination of virginiamycin and *M. elsdenii* strain JDB301 was introduced at 12 and again at 36 h, lactate concentrations remained below 15 mM for the duration of the experiment. Surprisingly, a combination of *Sel. ruminantium* subsp *lactilytica* strain JDB201, *M. elsdenii* strain JDB301 and virginiamycin administered together at 12 and 36 h was the most effective treatment and the concentration of lactate in the fermenter never exceeded 5 mM.

Bacterial transfer to control tannin toxicity in sheep

Feral goats thrive on a diet of Acacia (mulga) containing up to 14% by weight of condensed tannin. Domestic ruminants do not do so well. Metabolism studies were carried out on sheep inoculated with feral goat rumen fluid (FGRF) and fed a diet of mulga. Crude protein and CT levels of mulga leaf for the four measurement periods in this experiment were 142, 139, 146 and 141 g/kg and 139, 124, 120, 120 g/kg, respectively.

During the dietary adaptation stage of the experiment, N digestibility was significantly less efficient ($P < 0.05$) during period 2 than during period 1. No differences were apparent in DMI, N balance or DMD. Similarly, there were no differences between the two periods with any of the parameters measured in the domestic goats.

Following administration of FGRF, inoculated sheep had measurably higher DMI, N balance and N digestibility values in both post-inoculation periods compared with the pre-inoculation periods (Table 2). N digestibility was significantly higher during the ten days immediately following inoculation (period 3 ver. period 2). This increase was sustained in period 4 and was accompanied by significant increases in DMI and N balance compared to periods 2 and 3. Weight loss was reduced following inoculation. Dry matter digestibility did not change significantly during dietary adaptation or following FGRF inoculation. Over the test period, the untreated sheep lost approximately 40% more weight than the treated sheep. This provided inoculated sheep with a weight advantage of approximately 2.5 kg over the uninoculated sheep at the end of the experimental period.

Domestic goats inoculated with FGRF also exhibited increased N digestibility ($P < 0.05$) and DMI. N balance increased six-fold. Mean DMI, N balance and N

Table 2. *Effect of crude goat rumen fluid on sheep consuming mulga.*

Parameter	Pre-inoculation Period 1	Pre-inoculation Period 2	Post inoculation Period 3	Post inoculation Period 4	s.e.m.
DMI (g/day)	510[a]	524[a]	535[a]	611[b]	17.1
NBAL (g/day)	–0.61[a]	–0.55[a]	0.51[ab]	1.04[b]	0.4
ND (g/kg)	450[a]	417[b]	450[a]	463[a]	8.8
DMD (g/kg)	440	452	443	445	11.4
in vitro GP (µl/48 h)	nm	890[a]	nm	1483[b]	108.7
LW change (g/day)	–97[a]		–47[b]		13.9

Within rows, means with different superscripts differ significantly ($P < 0.05$).
nm—not measured.

digestibility for feral goats consuming mulga were 496 (SED = 60.8) g/day, 0.75 (SED = 0.580) g/day and 544 (SED = 62.9) g/kg respectively. For both sheep and goats, *in vitro* rumen fluid gas production increased following inoculation. This increase was significant ($P < 0.05$) for sheep.

Wool growth in mulga-fed sheep

Both inoculated groups of sheep grew more wool per unit area than the uninoculated group for three of the four periods. However, these differences were not significant. Providing a mineral supplement to the inoculated sheep produced a slight non-significant beneficial effect, reducing the rate of wool growth decline. Linear wool growth did not differ significantly between treatments.

Identification of bacteria in FGRF

Fractionation of FGRF into individual bacterial colonies on tannic acid containing plates revealed relatively few different species. One of these, subsequently named *S. caprinus*, was capable of growth in up to 3% w/v tannic acid and promotes the hydrolysis of protein-tannin complexes *in vitro*. The related species, *S. bovis* was sensitive to less than 0.5% w/v tannic acid. *S. caprinus* is now known to be widespread amongst animals browsing tannin-rich feed, but not to be normally present in domestic livestock. Another bacterium, possibly a selenomonad, expressed tannin acyl hydrolase activity and could utilise condensed tannin or tannic acid as a sole carbon source. We are currently cloning the tannin acyl hydrolase gene for possible transfer and expression in other rumen bacteria.

Discussion

Genetic engineering of rumen bacteria

Vectors for transformation and recombination in several different species of rumen bacteria have been designed and tested. A plasmid vector for gene expression in *S. bovis* has been developed, using the promoter sequence from α-amylase to drive new genes

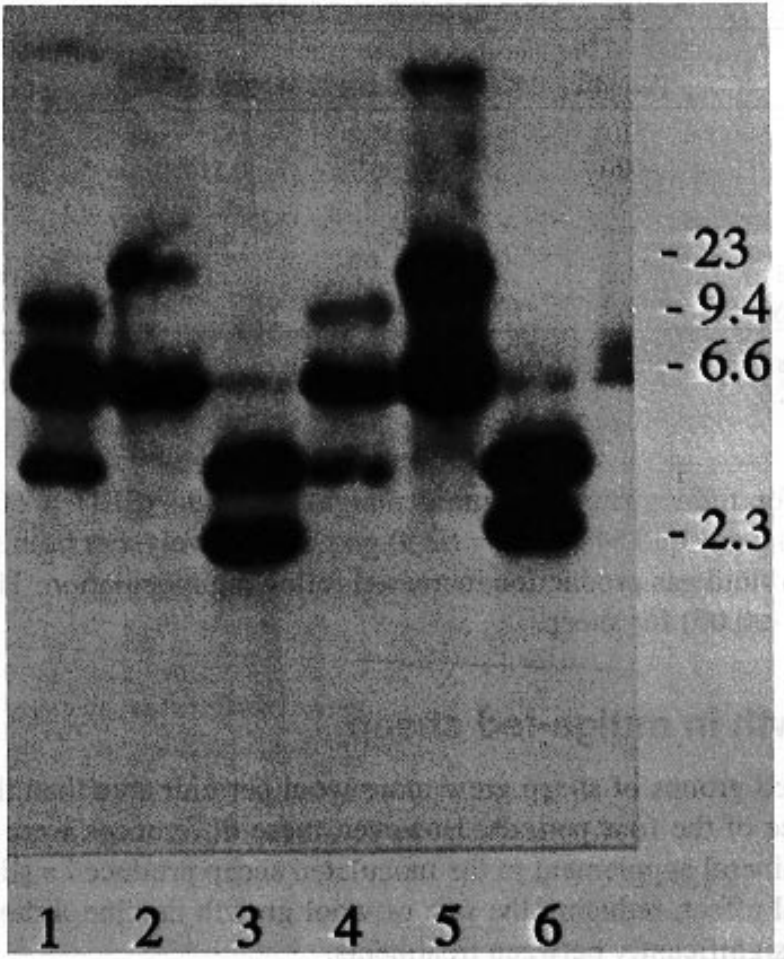

Figure 2. *Analysis of pJDB0.5 integration into* S. bovis *WI-1::Tn916. Chromosomal DNA from strains JDB-2 and JDB-3 was digested with HindIII (lanes 1 and 4), EcoRI (lanes 2 and 5), or HindIII + EcoRI (lanes 3 and 6) and analysed by agarose gel electrophoresis and Southern blot. Hybridisation was with a* ^{32}*P-dCTP labelled pMU1328 DNA.*

ligated nearby. We have also described the construction of a suicide vector that integrates in a site-specific manner into *S. bovis* WI-1::Tn*916*. Recombination into the *S. bovis* chromosome was only observed when pJDB0.5 was introduced into *S. bovis* WI-1::Tn*916*. As no erythromycin resistant transformants were obtained with *S. bovis* WI-1, it can be concluded that the plasmid is unable to replicate in this host and requires the presence of the chromosomal copy of Tn*916* to provide a site for homologous recombination. Screening is unequivocal because integration inactivates the host tet M gene, as well as permitting expression of the vector-borne antibiotic resistance marker. The patterns from the Southern blot indicate that twp copies of the plasmid were integrated into the chromosome. Since there were two copies of the transposon integrated (Brooker and Lum 1993), this could mean that each transposon sequence was interrupted by integration of the plasmid. This result is also in keeping with the loss of tetracycline resistance.

These results clearly demonstrate that expression of foreign genes in rumen bacteria can now be achieved for some species, and is likely to be achieved for others in due course. Limitations such as restriction barriers, nuclease activity and extracellular polysaccharide can be overcome. It is now possible to examine the effect of introducing and over-expressing genes such as cellulases, tannases and proteases on microbial ecology, competitiveness and function in the rumen.

Population control in the rumen

The introduction of microbial populations into the rumen can effect animal productive performance. Ruminal inoculation with lactic acid utilising bacterial species has been shown to prevent accumulation of lactic acid and thereby reduce the possibility of acidosis occurring in acutely grain-fed ruminants. The effect was sustainable for at least four days and suggests that this may provide an alternate treatment for acidosis than use of ionophores or antibiotics. The results show that at least two different bacterial species were required. This is a consequence of different metabolic directions. Under appropriate conditions, *S. ruminantium* grows rapidly but ferments glucose to produce lactic acid. *M. elsdenii* utilises lactic acid although it is slower growing. The combination of bacteria is required to maintain a reasonable growth rate and deal with lactic acid as it is synthesised.

Condensed tannins inhibit the viability and growth of many different species of microorganism (McLeod 1974). Evidence now suggests that microorganisms present in crude rumen samples from tannin-adapted animals are transferable to non-adapted ruminants to tolerate the tannins, provide microbial biomass and improve digestibility of tannin-containing forage. The results from these experiments also indicate that one organism is not sufficient and that a consortium of several different microbial species is involved *in vivo*.

Overall, these results support the view that effective manipulation of the rumen may be achieved by genetic engineering, but population control is a practical and effective alternative. This approach has certain advantages over the genetic route, including the ability to utilise the abilities of a consortium of bacteria rather than having to rely on an individual species. In the two cases above, effective control of an anti-nutritive situation was only achieved by a mixed bacterial population.

In contrast, genetic manipulation of individual species does provide opportunities for the substantial enhancement of bacterial activity and the ready transfer of the technology to other ruminants. There are advantages and disadvantages of both approaches. Notwithstanding this, manipulation of the rumen ecosystem is achievable by various routes, and will hopefully lead to increased ruminant productivity, particularly in marginal areas.

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Genetic manipulation of rumen bacteria: now a reality

K. Gregg[1], *D. Schafer*[2], *C. Cooper*[2] *and G. Allen*[2]

1. Institute of Biology, Armidale NSW 2350 Australia

2. Institute of Biotechnology, University of New England, Armidale 2351 Australia

Abstract

Techniques are now available for addition of novel genes to rumen bacteria, using plasmid vectors and high-voltage electroporation. Experiments have shown that *Butyrivibrio fibrisolvens* strain AR10 isolated from sheep can effectively colonise cattle on unimproved pastures. Strains of *B. fibrisolvens* isolated from reindeer (E14) and Canadian white-tail deer (OB156) have also been shown to colonise sheep and cattle. Bacterial populations fluctuate in number in different samples from the same animal. Tracking of four separate bacterial strains in the same two sheep has confirmed that individual strains vary in number independently of the others within any single sample. Bacterial numbers were observed during changes of diet, including a week in which the sheep were fed ground wheat straw. All four strains remained present during the 149 days of the experiment.

Strain OB156 of *B. fibrisolvens* was genetically modified by insertion of a non-transmissable plasmid containing the gene for fluoroacetate dehalogenase. The modified bacterium retained the new gene after 500 generations of non-selective *in vitro* growth. A growing population of modified bacteria was able to detoxify 10 nmol fluoroacetate per minute per mg of bacterial protein. This is calculated to be a level capable of offering some protection against the toxin for the host ruminant. Over the 149 days of the experiment, the genetically modified OB156 was detectable in most samples at levels between 10^5–10^7 cells/ml, occasionally dropping to below detectable levels (~10^3 cells/ml). There was no evidence of loss of the plasmid during major population changes, or of its transfer to other organisms. These data indicate that many of the reservations expressed about rumen bacterial genetic manipulation do not necessarily apply in practice. Expectations for the application of this technology are now considerably more confident. Equally important, the molecular techniques involved in these studies have application to a variety of ruminal ecology studies.

Introduction

Biotechnology is finding its way into many areas of agriculture and animal production through feed and crop plants, through the genetic pool of animals, and through manipu-

lation of microbes that influence the growth and development of both animals and plants. The direct genetic manipulation of microbes that inhabit the rumen has been proposed as a possible mechanism for enhancing animal production, although progress in this area has been slow. Nevertheless, the application of molecular biological techniques is beginning to contribute to our understanding of the rumen through the precise methods now available for making *in vivo* observations. Molecular techniques now allow individual micro-organisms to be tracked in the rumen, identification of organisms by genetic criteria is rapidly becoming an essential part of taxonomy, and 'tagging' bacteria with genetic markers has greatly facilitated studies of their populations *in vivo* (Gregg et al 1993).

As studies of ruminal ecology become more precise, the contribution of biotechnology methods to animal production will become continually more significant. However, in the specific area of rumen bacterial genetic manipulation, the most significant developments are very recent and have so far made little impact upon how this technology is regarded. There has been considerable reservation about the contributions that can be made by manipulating the genetics of rumen bacteria (Armstrong and Gilbert 1985; Egan et al 1992) and the basis for this reservation includes at least four areas of concern.

1. Genetic manipulation of rumen bacteria has not been demonstrated as a reproducible and reliable technique.
2. Bacteria grown in the laboratory are unlikely to retain their competitive features and are unlikely to return successfully to the rumen.
3. Genetic changes to bacteria are likely to impair their competitive fitness, particularly if the changes do not directly benefit the bacterium itself.
4. Addition of an artificially manipulated bacterium to the rumen may upset the balance of the rumen, with detrimental effects on the host animal.

In the absence of data to the contrary, these are all reasonable reservations. However, data are now being accumulated to indicate that, at least in some cases, these difficulties may have been overestimated.

Gene transfer systems for rumen bacteria

There are now several systems by which new genetic material can be introduced into rumen bacteria as plasmids (e.g. Beard et al 1995; Cocconcelli et al 1992; Thomson et al 1992). During the past ten years many different approaches have been made to the transformation of rumen bacteria, and most have shown very limited success. The technique that has proven generally useful for inserting new DNA is that of electroporation. This process uses an electrical pulse to generate openings in the cell wall, through which DNA can enter (Dower et al 1988). Combining this with plasmids originating from the bacterial species to be modified has led to at least two of the more successful processes reported (Beard et al 1995; Thomson et al 1992).

Methods that successfully achieve plasmid insertion do not necessarily provide evidence of successful genetic manipulation because there remain complications in making and transferring plasmid/gene constructs. It has been our experience that the

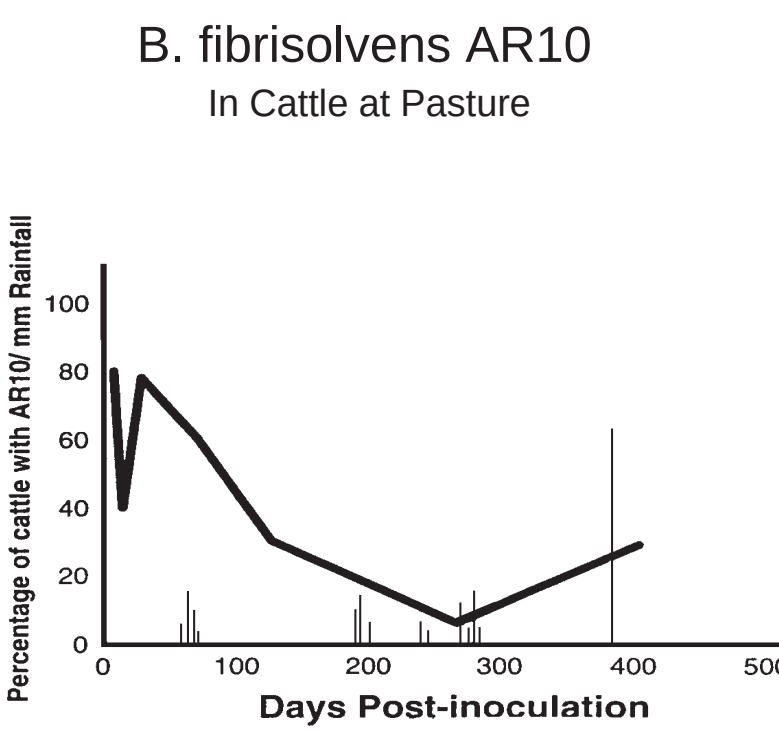


Figure 1. *The proportion of pasture-fed cattle showing the presence of* Butyrivibrio fibrisolvens *AR10 at various times after inoculation. At days 7, 14 and 168, only the 5 inoculated animals were tested. At days 28 and 70, the same 5 animals were tested together with 20 others chosen at random from the herd. At day 268, 33 animals were tested and at day 404, a total of 45 animals were tested, some of which were small calves at the beginning of the experiment, with 13 showing positive detection for AR10. The vertical lines show rainfall in millimeters.*

feasibility of adding any particular new gene to a bacterium can only be assessed empirically, even with a stable and efficient vector system. As a primary example, the usefulness and stability of the transformation system described by Beard et al (1995) for inserting genes of practical benefit into *B. fibrisolvens* is described below. Some aspects of that example demonstrate unexpected contradictions of the results predicted from basic biological and ecological principles.

Recolonisation of the rumen by laboratory-grown bacteria

The ability of *B. fibrisolvens* strain AR10 to colonise the rumen after several years storage in a laboratory has been described previously (Gregg et al 1993). Strain AR10 was isolated originally from a sheep and had been shown, through a survey of 71 animals from 14 grazing properties, to be absent from the Central Queensland region of Australia. Figure 1 shows data from an experiment in which 5 cows out of a herd of 40, on unimproved tropical pastures at one of the properties previously tested, were inoculated with strain AR10. There was rapid spread through the herd (19 out of the 24 animals tested showing easily detectable levels of AR10 after 28 days post-inoculation).

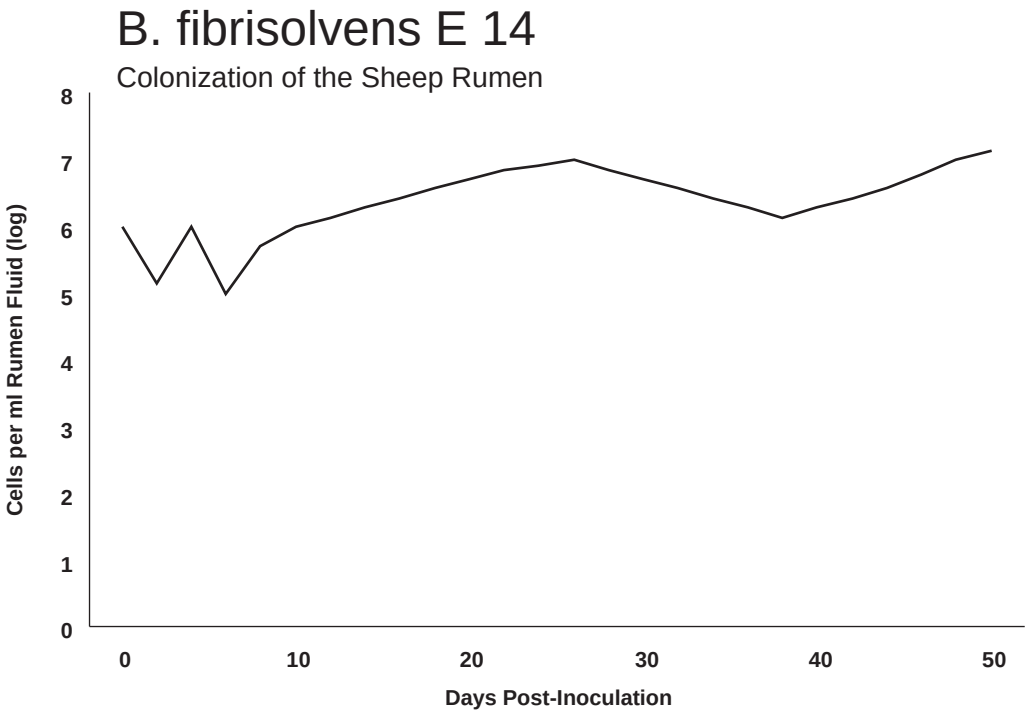


Figure 2. *Persistence of* Butyrivibrio fibrisolvens *E14 in the sheep rumen after transposon modification to impart tetracyline resistance. The addition of Tn916 required the bacterium to express a foreign gene from which it obtained no benefit* in vivo. *Modified E14 recovered from the rumen was plated on agar containing rumen-fluid medium and tetracycline, and the identity of colonies obtained was confirmed by hybridisation to chromosomal DNA of E14 at high stringency. In each animal, E14 became undetectable at various times. The line shows the mean value obtained from the four sheep.*

The conclusion from this work is that bacteria isolated and cultured in the laboratory can retain their ability to cope with rumen conditions.

A more important test for recolonisation of the rumen is to examine the ability of a bacterium to which genetic changes have been made in the laboratory to return successfully to the rumen. Strain E14 of *B. fibrisolvens* was obtained from a reindeer and provided to our laboratory by Dr C. Orpin. This strain had been 'tagged' by the addition of a tetracycline resistance gene using transposon Tn916 by Dr J. Brooker (Waite Institute, South Australia). E14 was inoculated into the rumens of four sheep in the UNE animal house and rumen samples were tested over a period of seven weeks by plating dilutions on selective plates. Colonies that grew on the plates were tested by DNA hybridisation, at high stringency, with genomic DNA from E14. The levels observed in individual animals fluctuated significantly, sometimes falling below detectable levels (10^3 cells/ml rumen fluid). On average, however, E14 was detectable in all four animals at an average density between 10^6–10^7 cells/ml (Figure 2).

The fluctuation of bacterial populations from day to day was observed in both of these experiments, but it was not possible to be certain whether it arose from genuine population changes or from sampling variability.

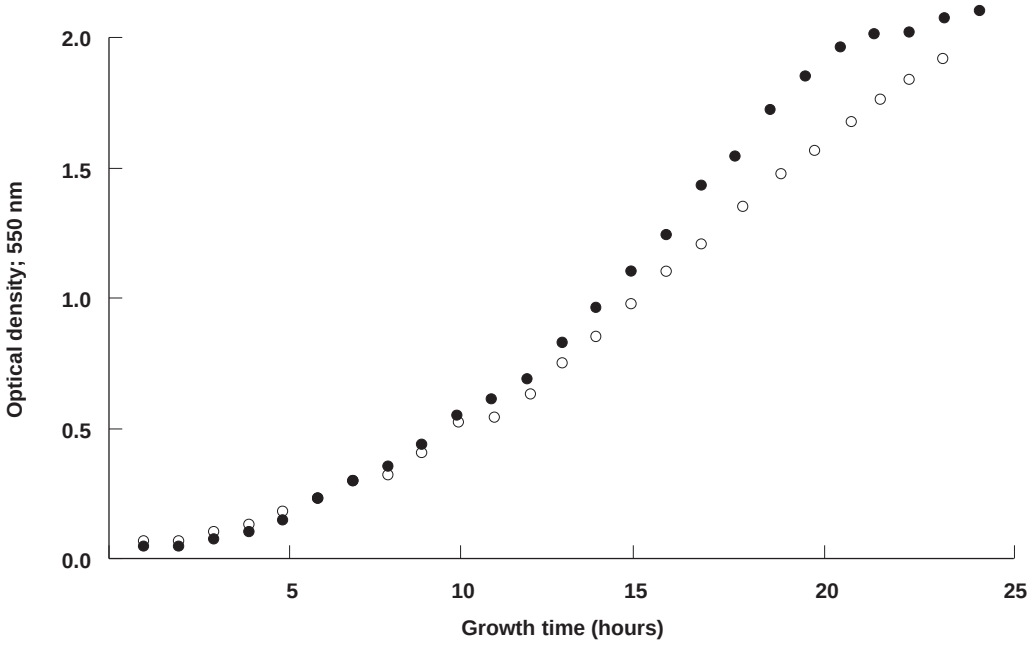


Figure 3. *Population growth in vitro of* Prevotella ruminicola *AR20 in the presence (●) and absence (o) of plasmid pBA. Plasmid-bearing bacteria were grown in the presence of clindamycin (10 mg/ml) to ensure expression of the foreign gene. Supporting plasmid replication and clindamycin resistance gene expression did not cause any detectable decrease in growth efficiency.*

Bacterial growth efficiency and the effect of an extra genetic load

Experiments described by Russell and Wilson (1988) indicated that bacteria carrying a plasmid grew approximately 30% slower than the same strain without the plasmid. It can be reasonably proposed that sustaining an additional genetic load could impede the population growth of an organism, especially under conditions in which competition for nutrients is high. However, experiments comparing the population growth of bacteria with and without a multicopy plasmid have demonstrated that harbouring a novel plasmid that has no survival benefit to the bacterium need not necessarily reduce its population growth (Gregg et al 1993; Figure 3). It is important to note, however, that this experiment was performed *in vitro* and did not address the question of direct competition for nutrients against other, non-plasmid-bearing strains.

Addition of new organisms and the ecological balance of the rumen

The concern that a modified bacterium could upset the ruminal balance, leading to other (undefined) problems, may be allayed somewhat by recent findings in rumen bacterial

taxonomy. It has been shown that phenotypically defined 'species' may actually comprise multiple, distantly related, or even unrelated, genetic groups. Such genetic diversity has been demonstrated for *B. fibrisolvens* (Hudman and Gregg 1989; Mannarelli 1988), *Prevotella ruminicola* (Hudman and Gregg 1989) and *Ruminococcus albus* (Ware et al 1989). Thus it has been known for some time that, although a 'species' such as *B. fibrisolvens* may represent 10% of the ruminal population, any individual 'strain' is more likely to represent 0.1–1.0%. This is consistent with the population levels observed for strains that have been specifically tracked in the rumen (Gregg et al 1993).

The prospect of replacing, for example, 10–20% of the rumen population with a genetically modified organism is bound to raise suggestions of major ruminal disruption. However, it is now clear that modification of one or a few rumen bacteria is not likely to effect this proportion of the total microbial population. It now appears that to alter 10% of the ruminal population will require the manipulation of many individual genetic strains. While providing a possible ameliorating factor for one aspect of this technology, this generates a perhaps equally difficult question, i.e. whether the relatively small contribution made by a single bacterial strain can provide significant biochemical effect for a modified bacterium to influence the biology of the host animal. Discussion on this point (Gregg and Ware 1990) has offered the suggestion that the effectiveness of genetic manipulation will depend to some extent upon the nature of the changes that are attempted.

Therefore, some of the reservations about rumen bacterial manipulation have been addressed and the evidence indicates that the system need not be constrained by these factors, although it remains likely that they will have relevance to a proportion of cases. The work described here addresses some of the final reservations, constituting a model system for rumen bacterial genetic manipulation.

Genetic manipulation of rumen bacteria—an example

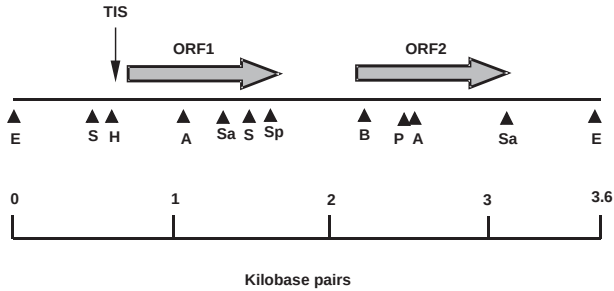
Our recent work has been directed towards practical goals for solving problems in ruminant production. In doing so, it has also addressed the questions of how stable genetic modifications to rumen bacteria may be, and how the ability of the bacterium to survive ruminal competition may be affected. These questions became very important in a project that has attempted to protect ruminants against poisoning by fluoroacetate.

Background to the problem

The compound monofluoroacetate is highly toxic to aerobic organisms, being converted metabolically to fluorocitrate which blocks the action of the enzyme aconitase (Elliot and Kalnitsky 1950). This toxin occurs naturally in at least 40 species of trees and shrubs in Australia and for many years has been known to be a problem in Africa (Marais 1943) and Central America (DeOliveira 1963). Livestock may be killed in large numbers by the toxic trees, depending to some extent upon seasonal conditions (McCosker 1989).

The problem of livestock poisoning occurs despite the existence of widespread capabilities for microbial degradation of the toxin. Many soil organisms are capable of defluorinating the compound to produce glycolate, and of using this as a carbohydrate nutrient. Perhaps because the toxin acts upon aerobic metabolism and because it has poor nutritive value under anaerobic conditions, the microbes of the rumen do not appear to have acquired the same defluorinating capability as their counterparts in the soil (Gregg and Sharpe 1991).

The soil organism *Moraxella* spp. strain B has high activity for dehalogenating fluorinated and chlorinated compounds (Kawasaki et al 1981). A gene encoding the enzyme fluoroacetate dehalogenase (haloacetate halidohydrolase, EC 3.8.1.3) has been cloned and sequenced (Kawasaki et al 1984, 1992). We repeated the cloning and sequencing after obtaining the bacterium from Prof H. Kawasaki (Osaka University) to examine the DNA regions responsible for regulation of the gene. It was shown that the dehalogenase gene was the second open reading frame (ORF2) in an operon, with the gene promoter approximately 1.4 kilobase pairs (kb) upstream (Figure 4). Removal of ORF1 and attachment of a different gene promoter demonstrated that ORF2 was sufficient for fluoroacetate detoxification.



TSI = transcription initiation site; Restriction site: A: *Ava*I; B: *Bssh*II E: *Eco*RI; H: *Hpa*I; P: *Pst*I; S: *Sal*I; Sa: *Sac*I; Sp: *Sph*I.

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Figure 4. *Diagram of the dehalogenase gene operon from* Moraxella *spp. The function of ORF1 has not been determined, but its removal does not affect dehalogenase expression.*

Experimental approach and results

The dehalogenase gene (H1) was isolated and spliced to a gene promoter from the erythromycin resistance gene (*erm*^{r}) of broad host range plasmid pAMβ1 (LeBlanc and Lee 1984) using polymerase chain reaction (Yulov and Zabara 1990). The chimaeric gene was then attached to plasmid pBH*erm* for transfer to *B. fibrisolvens* strain OB156 (Figure 5). The *erm*^{r} gene within plasmid pBH*erm* allowed transformed bacteria to be selected on culture plates containing erythromycin (Gregg et al 1994).

The genetically modified OB156 was shown to produce dehalogenase enzyme with activity of 10 nmol/min per milligramme of bacterial protein, which was calculated to be sufficient to make a significant decrease in toxicity to the host ruminant, provided the bacterium survived return to the rumen.

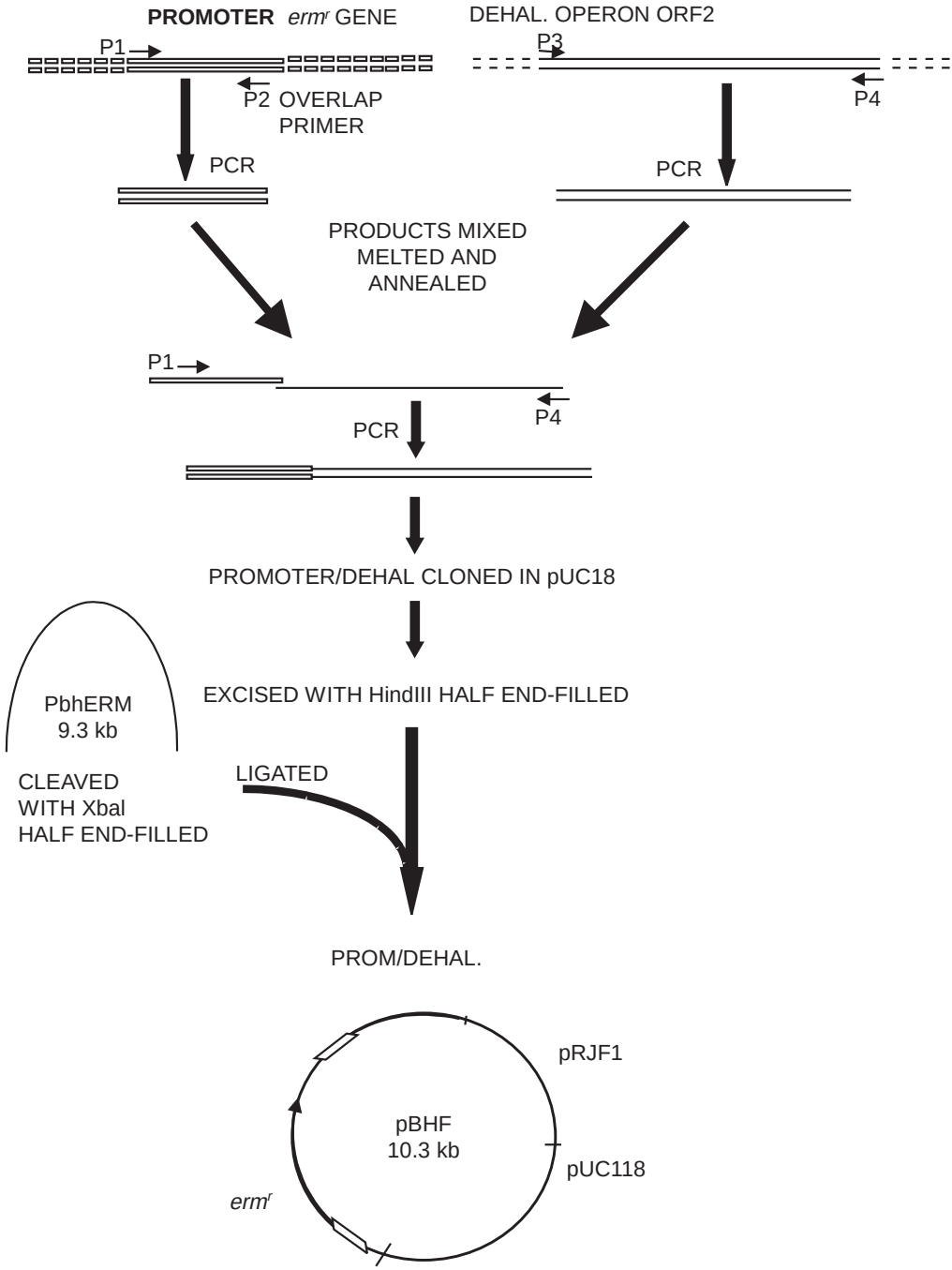

Figure 5. *Construction of the chimaeric shuttle plasmid containing the dehalogenase gene. The dehalogenase gene was joined to the promoter of the erm*^{r} *gene promoter of plasmid pAM*β*1 to ensure expression in* B. fibrisolvens.

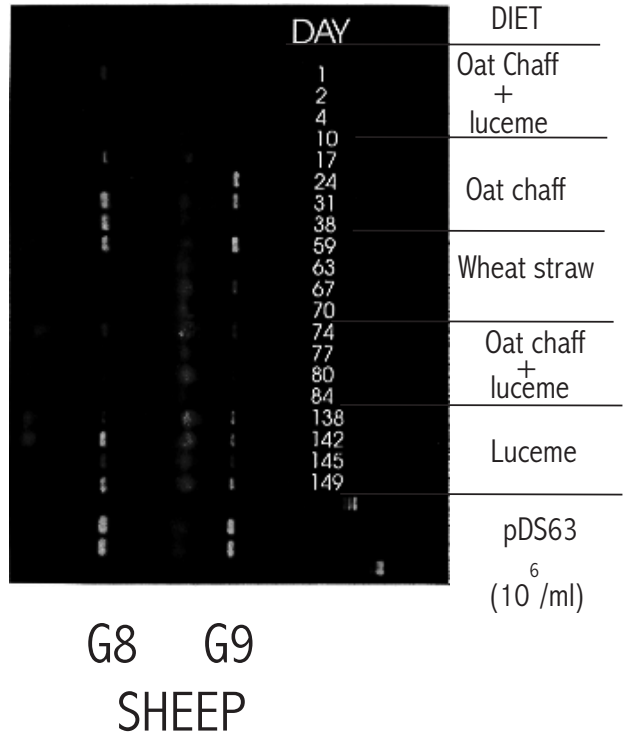

Figure 6. *Agarose gel showing PCR product from tracking* Butyrivibrio fibrisolvens *OB156. The vertical row of numbers shows the days at which samples were taken and the diet was changed as indicated on the right of the figure, to encourage competition between bacteria for nutrients.*

The stability of this plasmid-bearing organism was tested by growth for 500 generations in culture medium without the selective antibiotic. After each 100 generations, cells were plated onto duplicate selective and non-selective plates. No difference was observed between bacterial numbers on the two media and it was concluded that no loss of plasmid occurred within the cultures.

To examine the competitive ability of this organism in the rumen, it was inoculated into the rumens of two sheep, together with an unmodified strain of *B. fibrisolvens* (AR10) and two strains of *P. ruminicola* (AR20 and AR29). Over the following 149 days, the animal's diet was changed several times to encourage competition between rumen microbes and samples were removed for estimation of bacterial numbers.

Bacteria were detected by polymerase chain reaction amplification of part of their chromosome (Figure 6) and densitometer scanning gel photographs to quantitate PCR product formation (Figure 7). Plasmid within the modified strain was also tracked by PCR of plasmid sequences.

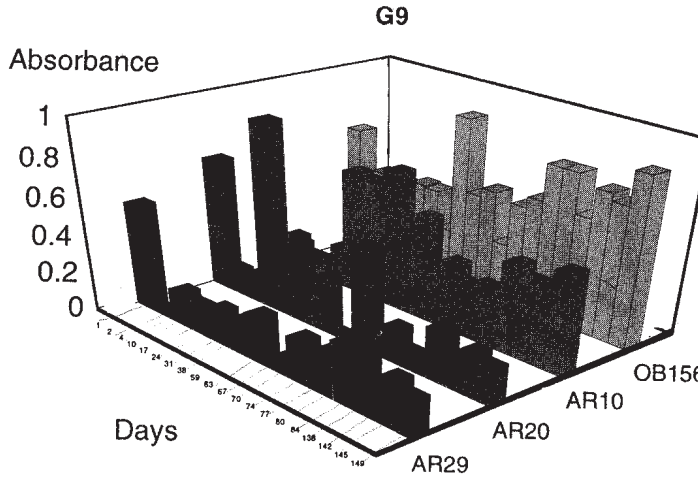

Figure 7. *Tracking genetically modified* Butyrivibrio fibrisolvens *OB156 in the sheep rumen, in parallel with an unmodified strain AR10 and two unmodified strains of* Prevotella ruminicola *(AR20 and AR29). Polymerase chain reaction products were separated by agarose-gel electrophoresis and quantitated by densitometer scanning the negative of a photograph from the ethidium bromide-stained gel.*

The results of this test showed:

1. That all four strains recolonised the rumen and remained present for the full test period. This confirmed that the ability to recolonise the rumen is common in laboratory strains and that the genetically modified bacterium was not significantly disadvantaged by its modification.
2. The levels detected for each organism fluctuated independently of the others, indicating that variation in bacterial numbers seen in previous experiments was probably genuine fluctuation, and not the result of sampling variability.
3. The plasmid detection reactions showed that levels of plasmid and host bacterium co-varied, indicating that the plasmid was not transferred to other organisms at any detectable level. This was predicted, since pBH*erm* is a non-conjugative plasmid. The results also indicate that there was no significant loss of plasmid from the OB156 population.
4. Plasmid was extracted from bacteria after they were recovered from the rumen and was shown to be structurally unchanged.

Discussion

This work proved that the genetic manipulation of rumen bacteria is feasible. Most importantly, the altered bacteria were shown to be competitive in the rumen, colonising to the same extent as three other strains that were tracked over the same period. Diet was shown to influence population levels, as expected, and the competition for nutrients under poor dietary conditions did not appear to disadvantage the modified bacterium.

Examination of the sheep during the trial period by a veterinarian confirmed that there was no change in the animals' health, apart from the predicted effects of feeding a diet of ground wheat straw for about a week.

It is now clear that rumen bacteria can be genetically modified; the modification can be stable even when carried on a plasmid; carrying plasmid does not necessarily disadvantage the bacterium energetically; laboratory-grown bacteria can recolonise the rumen; and genetically modified bacteria can do so as efficiently as their unmodified counterparts.

These observations will not necessarily apply to other genetically modified organisms that may be constructed. Each example must be tested individually before these features can be assumed. However, this work has shown that predictions based upon basic biological principles cannot substitute for testing the case empirically.

It is interesting that this modification appears stable when the dehalogenase gene confers no known advantage to the bacterium. Rationally, this represents a 'most likely to be lost' case for genetic manipulation. It appears, therefore, that the need for a modification to be useful to the modified organism is secondary in importance to the inherent stability of the plasmid or whichever other gene transfer system is used. There is currently no proven explanation for the ability of a bacterium to stably maintain a genetic feature from which it gains no direct benefit. However, we can propose that this ability stems from the fact that each bacterium maintains about 1000 genes, some of which are constitutively expressed. During the natural changes of conditions that occur within the rumen, some of these genes will inevitably be of no immediate benefit to the organism at one stage or another. If there were serious disadvantages to the bacterium in this situation, then many strains could be lost from the rumen during relatively minor changes in ruminal conditions. Our dietary change tests were for limited periods only, but indicate that the loss of bacterial strains that could be caused by such change may be more likely to occur when dietary conditions vary over much longer periods.

Biotechnology in developing countries

While the efficiency and viability of ruminant production is important to the economies of some industrialised countries, the importance for developing nations with less overseas purchasing power are more far-reaching. Where ruminants fill essential needs for food, fibre and draught power, improvements in production and maintenance efficiency contribute directly to the living standards of a high proportion of the population. Much of the nutritional improvement possible through supplementation or processing of animal feed may be difficult to implement in these situations. It is vital, therefore, that improvements in ruminant production should make maximum use of the feed sources currently or potentially available, rather than depend on adding new elements that need to be purchased.

Where a high proportion of animal feed is the residue from grain or other staple crops, digestibility is frequently the limiting factor in ruminant production. In those cases where cash crops (e.g. cocoa) leave residues with feed potential, there may be serious limitations created by the secondary compounds present in that material. There may be

considerable value, therefore, in systems that help in fibre breakdown or that detoxify anti-nutritive factors.

The capability of genetic manipulation to make rumen microbes digest plant fibres more efficiently remains to be proven. For this aim, there may be greater and more immediate gain in examining how different feed plants can be combined to supplement each other and optimise microbial growth and nutrient yield within the rumen. Microbes isolated from the fermentative organs of non-domestic animals (e.g. giraffes, camels, leaf-eating monkeys, etc.) may be directly adaptable if they are able to colonise domestic ruminants. In our laboratory we have shown that bacteria from sheep (strain AR10) will successfully colonise cattle. A strain of *B. fibrisolvens* from reindeer (strain E14) has successfully colonised both sheep and cattle and OB156 from a Canadian white-tail deer successfully colonised sheep. These three cases are the only ones we have tested to date, but all have been successful, suggesting that they are unlikely to be atypical. Attempts to transfer microbes between animal species will be facilitated by the precise molecular methods now available to track individual organisms within complex mixtures. Without precise and quantitative tracking systems, the effectiveness of any particular organism cannot be clearly demonstrated.

The potential for removing or inactivating toxins and anti-nutrients by genetic engineering of rumen bacteria has been strongly supported by the fluoroacetate case. The ease with which this approach may be applied will depend upon the complexity of reactions required for detoxification. Fluoroacetate represents the simplest system, with a single toxin inactivated by a single enzyme encoded by a single gene. The detoxification by non-oxidative means was also important for this process to be functional in the rumen. Effective removal of the goitrogen from *Leucaena leucocephala* (Jones and Megarrity 1986) by a bacterium that grows to only 10^5 cells/ml (C. Orpin, personal communication) indicates that a small number of modified strains should be sufficient to perform detoxification reactions. Modification of only a single strain might be expected to have variable success, because of the population fluctuations seen in our monitoring work. However, this may turn out, like some of the other predictions about this work, to be overly pessimistic. Trials to test this aspect of the fluoroacetate work are currently in progress.

The development of molecular mechanisms for ruminant studies, including methods for microbial strain identification, estimation of population numbers and measurement of changes, has now reached a stage where less affluent nations should be able to benefit from them. Future research will indicate which of the possible applications for genetic manipulation of rumen bacteria are likely to be successful. However, judgement of which technologies will truly benefit the developing economies remains difficult, and the subject of much conflict of opinion. If agencies assisting the developing nations are to adopt this technology, then it should preferably be for the solution of problems that are well defined.

Acknowledgements

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Working group reports, conclusions and recommendations

Terms of reference

1. Which are the potential research areas in rumen ecology?
2. Among these areas, which would be the preferred research areas for ILRI?
   a) What are the comparative advantages for ILRI in each area?
   b) Which areas require collaboration between ILRI and other institutions?
3. For the areas identified to be potential ILRI research areas, which are the resource needs?
   a) With regard to human resources.
   b) With regard to facilities.
4. a) Which are the suggested modes of collaboration within the previously identified areas?
   b) Who are the potential collaborators in each area?
5. Which are the time frames for expected output from respective research areas in ILRI?

It is important to remember the potential impact of the respective research areas within ILRI during working group discussions.

Report of Working Group 1
Potential research agenda for ILRI on rumen manipulation: potential collaborators and mode of collaboration

Chair: R.J. Wallace
Rapporteur: S. Fernández-Rivera

Day 1 (Tuesday, 14 March 1995)

The group identified the following ten research areas pertaining to rumen manipulation.

1. Fibre breakdown
2. Adhesion to fibre particles
3. Anti-nutritional factors
4. Protein breakdown
5. Microbial protein production
6. Variation in microflora/microfauna
7. Methane production
8. Defaunation
9. Feed characteristics
10. Interactions between rumen microbes

General issues

It was established that given the global mandate of the institute, the research areas to suggest for ILRI's agenda in rumen manipulation should have a broad applicability. One role of ILRI in these areas should be the development of tools robust enough to be used successfully by the national research systems of developing countries (NARS).

Fibre breakdown

It was agreed that this is an important problem that could be addressed either by improving the environmental conditions that facilitate fibre digestion, by promoting the establishment of fungi or by genetic engineering. Several felt that in the short term the manipulation of the microbial population is more likely to succeed than the high-tech approach.

Several participants felt that the data presented in the workshop on the role of fungi in fibre digestion encourage the inclusion of this group of rumen micro-organisms in the research agenda.

One topic mentioned as researchable was the process of digestion as the fibre becomes more lignified. There was a suggestion that the mechanisms involved in the improvement of fibre digestion through supplementation needs to be understood. However, there

was also the recognition that the influence of supplementation on fibre breakdown could be addressed by NARS.

There was a consensus that a main objective in this research area is the characterisation of indigenous microbes to identify superior fibrolytic strains. The approach suggested was the isolation and purification of strains. Superior strains could then be used in high-tech approaches. Some technical difficulties were identified (e.g. purity of CO_2 available in some countries, transport of microbes, etc.). However, it was considered that many of these difficulties could be overcome by involving NARS in the process of preparing the pure cultures and by using polymerase-chain reaction (PCR). Several NARS were identified as having capabilities for isolating microbes and preparing pure cultures.

Attachment to fibre particles

Participants agreed that attachment of bacteria to fibre particles is a requirement for fibre digestion and could be considered as an integrated part of the first research area. The problem of reduced fibre digestion when high levels of concentrate are used was discussed. There was a feeling that under these conditions there is no need for attachment because of preferential use of readily fermentable carbohydrates as substrate. The low pH can also affect the attachment of bacteria to fibre. It was mentioned that possibilities for manipulating microbial attachment need to be investigated. Finally it was concluded that adhesion was not a separate facet of fibre digestion and should not be addressed as a priority area.

Anti-nutritional factors (ANFs)

The group considered that the institute has some expertise in this area. There is a broad base of knowledge already available that needs to be transferred and put into practice. It was suggested that phenolics constitute the most important group of ANFs, but that there are many other types of compounds that have anti-nutritional effects. The detoxification of ANF and the breakdown of protein-tannin complexes were suggested as the main researchable problems related to ANF.

One specific area of research considered during the group discussion was the screening for the presence of ANF factors in ILRI's germplasm bank, especially in multipurpose trees (MPTs). Several participants felt that the screening should be made at early stages in the process of evaluating the feed potential of promising trees. Gas release, VFA and microbial mass production *in vitro,* as well as bioassays with small animals (e.g. the field vole, mouse deer) were mentioned as possible screening criteria. However, several participants questioned the need for a rumen microbiologist in these activities. It was unclear how a microbiologist would fit in this project. It was argued that the microbiologist should be a problem solver. This would require the identification of a specific problem caused by ANF. In addition, the problem should have broad relevance (i.e. not local or site-specific problems). Feasibility for being solved through microbiological techniques was other requirement for its inclusion in ILRI's agenda.

Several specific problems related to the utilisation of *Acacia siberiana* pods, Tagasaste, *Calliandria* and *Tephrosa* were mentioned as potential researchable issues. However, several participants felt that problems needing solution should be further and more clearly defined and should have a broader relevance. Chosen species should have a high feed potential and a wide distribution. The nature of the problem, i.e. the plant compound that causes the toxic effects, should first be identified and isolated. The causes of the anti-nutritional or toxic effects may well be at the systemic level and the solution might not be found through microbiological approaches. The possibility of a need of input from a toxicologist or a phytochemist to identify the nature of the problem was noted.

There was consensus that possibilities for transferring indigenous micro-organisms from animals that have survived and adapted under the presence of specific ANFs in their diets to domesticated animals should be addressed. The input from the microbiologist would be the isolation and culture of the microbes that detoxify the targeted ANF.

Day 2 (Wednesday, 15 March 1995)

General issues

1. The feasibility for isolating and preparing pure cultures in NARS was revisited. There was concern that the technical skills required are relatively high. Therefore it might be difficult to find effective collaborating NARS in this area. Several participants felt that in general, with some basic training and the provision of some key materials not readily available in some countries, this should not be a major problem.
2. A framework for identifying research priorities was proposed. The framework was based on the nature of the constraints to livestock production, i.e. nutritional and non-nutritional factors. The latter (economic, social, political, disease, genetic factors) would influence the appropriateness of each potential nutritional solution proposed in a given farming system. The nutritional constraints were arranged in a matrix with the limiting factors on one axis and possible solutions on the other. It was suggested that rumen microbiologists had a potential contribution in the identification of which areas of the matrix could be focused on.
3. It was considered that the proposed activities on rumen ecology should be viewed as part of ILRI's feed utilisation programme. The group should focus on how expertise in rumen microbiology will contribute and enhance the relevance of the other research activities in this programme. It was suggested that a great deal of specificity or focus was needed to make reasonable progress in five years. A specific area that was suggested was the possibility for identifying natural defaunating agents. Others replied that such a topic would be the subject of a PhD thesis, rather than the core of a research programme. It was suggested that the feasibility of transferring defaunating technology either commercially or through trees grown on-farm should be considered. It might be possible that even if defaunating agents exist, the indigenous protozoans have developed a resistance to these agents.

4. As the discussion on ANFs continued, the need for identifying the relative importance of ANFs (i.e. the farming systems, regions, seasons where they act as limiting factors), as well as the chemical nature and mechanisms of action was indicated. On the question of how much progress ILRI had made on these aspects, there was a response that there was some information on their effects, but the chemical characterisation would require the input of a phytochemist.
5. Among the ten research areas listed during the first group discussion, the priority areas for ILRI's research agenda were identified. It was considered that several areas (e.g. methanogenesis, protein supply) actually derive from fibre breakdown; others (e.g. defaunation) could be addressed through collaboration with other institutes, but would not be broad enough to be the core of the programme; others (e.g. feed characteristics) are part of other activities in feed evaluation or fibre breakdown. There was consensus that the two most important areas are fibre breakdown and ANFs. The identification of microorganisms that have superior fibrolytic activity or capabilities to degrade ANFs was identified as the major contributions of rumen ecology to ILRI's research work.

Fibre digestion: needs, objectives and outputs

The feasibility of measuring the ability to degrade fibre in pure cultures was discussed. It was mentioned that the isolated microbes could be characterised on the basis of substrate utilisation, particularly in terms of relative rates of fibre digestion. Several participants argued that this is already known while others suggested that the substrate used for screening should be made up of locally available and important fibrous feeds. This would ensure relevance of comparisons among strains. It was also suggested that fungi should be included in the characterisation of indigenous microbes. Although the collection of micro-organisms was important it was not seen as the end or objective of the project.

The possibility for storing 'ecosystems', i.e. mixed rumen populations, was also discussed. Although technically it appears possible (transport of rumen fluid, use of glycerol + soluble carbohydrates, dilution series, etc.), some participants questioned the value of this approach.

Several members of the group mentioned the possibility that in a reasonable period of time (three years) the programme could: a) assess the fibrolytic activity of few species, b) assess how well these species are fitted to digest specific substrates and locally available feeds, and c) describe the indigenous microbial populations in some of the mandate areas. It was argued that the programme should be problem-oriented and that more work to identify the constraints to fibre digestion, similar to the experiments conducted in Niger, should be made. In reply, it was noted that such work identifies the problem, not the solution. There is the possibility that the constraints are nutrients and that the problem might not require microbiological techniques to solve. There is a need to establish if the constraints are nutrient deficiencies. If this is the case, interventions to overcome these constraints should be tested along with measures of how the fibrolytic bacteria respond to such interventions.

The influence of synchrony in the availability of nutrients to microbes on fibre digestion was suggested as a research issue. Several participants thought that this is not an activity for a microbiologist and noted that it was not clear how this work would serve the feed utilisation programme.

General objective

There was consensus that the general objective of the research activities on fibre breakdown is 'the use of rumen microbiological techniques for alleviating constraints to fibre digestion breakdown in ILRI mandate areas'.

Aims

It is expected that in a period of five years the research activities on fibre breakdown will result in:

- Characterisation of fibrolytic activity of microbial populations.
- Selection of isolates with enhanced fibrolytic activity towards relevant fibrous feeds.
- Assessment of cross inoculation and pure culture inoculation for improved fibre breakdown.

Outputs

In a period of five years, the research on fibre breakdown should produce the following outputs:

- Inoculants for enhanced fibre utilisation
- Improved feed utilisation
- Improved skills and techniques
- Collection of potential bio-resources

ANFs and MPTs

The need for microbiological work on MPTs was discussed at length. ILRI would not have a comparative advantage from a microbiological point of view. The comparative advantage of the institute is the access to the materials and the agronomic knowledge base. Since more progress appears to be taking place in Latin America, it was suggested that ILRI should focus on Africa and Asia and learn about the on-going work in Latin America.

It was suggested that the most important group of ANFs is constituted by phenolics and that there is need for knowledge on how the microbiological system in the rumen responds to these compounds. Some group members thought that too much work is already being done on phenolics at the expense of investigating the role of other important ANFs.

Mechanisms of action of ANFs (i.e. at systemic or microbiological level) and the needs for toxicological work were revisited. Several participants thought that this might

not be a microbiological problem. The need for a sharp definition of the problem to be solved and the MPT species to be worked on was mentioned again. It was suggested that trees to be used in ANF work should rank highly in relevance (broadly distributed), feed potential (consumption by livestock, concentration of nutrients), yield potential and severity (economic importance) of the problem caused. The specific problems observed in Debre Zeit with Tagasaste, *Tephrosa* and *Calliandria* were rediscussed. It was argued that some of the problems with MPTs are related to feed characterisation and not rumen ecology. Some participants thought that it might be easier to make an impact at farm level, through interventions including MPTs, than through enhancing fibre digestion.

The group was reminded that work on mimosine required a lot of manpower and it was unlikely that, with limited resources, ILRI could make a substantial impact in this area in the short term.

After long discussions the group agreed that rumen manipulation work should focus on fibre breakdown, but that the rumen microbiologist should collaborate with and support the work on MPTs as the opportunities arise. Two potential areas that were suggested as worth looking into were the characterisation of microbes in animals that have evolved when forages high in ANFs are consumed and the possibilities for cross inoculation to domesticated animals.

Resources needed for rumen manipulation work

The group advised that the rumen microbiologist would need back-up in three important areas.

1. Analytical chemistry
2. Nutrition
3. Molecular biology

The group was informed about ILRI's present analytical capabilities in Addis Ababa. It was considered that the present facilities would be sufficient for only the most common analytical needs. Some additional equipment would have to be purchased (see below), but more advanced analytical needs demanding cutting-edge technology and expertise would be contracted to advanced institutions and/or consultants.

The group was also informed that ILRI's facilities for molecular biology in Nairobi are state-of-the-art but are heavily used by other programmes. There is no expertise in rumen microbes. If the rumen ecology work were based in Addis Ababa, the needs for back-up in molecular biology could be met either in institutions already working in rumen microbes or in Nairobi.

The group considered that at least a senior scientist and a post-doc with two full time well qualified technicians are needed to cover the proposed rumen microbiology work.

Given that the work on rumen ecology would be part of the feed utilisation programme, it was considered that it would be best placed in Ethiopia, where most of the work on nutrition research is taking place. The laboratory should be placed where cannulated animals are readily accessible.

The following equipment was considered necessary for the programme on rumen manipulation: anaerobic cabinet and gas mixers, high-speed refrigerated centrifuge, incubators, shaking water bottles, spectrophotometer (VIS/UV) and thermal block, GC (FID and TZ), four-place balances, microscope and camera, liquid scintillation counter, furnace and ovens, autoclave, colony counter, and automatic pipettes. Subsequent discussion indicated that a micro-plate reader would be extremely useful in any programme for the screening of ANFs.

Collaboration

The group agreed that collaboration with NARS and advanced institutions would be critical for the success of the programme. No specific institutions for collaboration were proposed. The group felt that this decision should be taken at a later stage and would depend, at least in part, on the appointee(s).

Report of Working Group 2 Improved efficiency of use of feed resources by tropical livestock

Chair: R.I. Mackie
Rapporteur: N. Murphy

The group identified the following seven constraints to feed resource utilisation by tropical livestock.

1. Shortage of feed resources
2. Seasonal fluctuations
3. Feed quality
4. Toxic compounds
5. Applicability of methodologies
6. Environmental and health aspects
7. Low input/access

Opportunities

In considering the constraints, opportunities which could arise through a focused research effort in feed utilisation and rumen microbiology were discussed and the following seven identified.

1. Increase conversion efficiency
2. Enhance degradation
3. Detoxification
4. Provision and evaluation
5. Biodiversity
6. Ameliorate stress
7. Technology transfer

Potential research areas in rumen microbiology and ecology

The agreed seven opportunities were discussed in turn with the focus on researchable areas, available information, constraints and potential outputs. The group did not place major emphasis on budgetary or personnel constraints that might exist in the institute during this discussion, as information on this was lacking.

1. Conversion efficiency
    - particle reduction
    - improved growth efficiency (C, N, minerals)
    - engineer protein quality
    - intrarumenal N recycling/defaunation
    - reduce methanogenesis/increase acetogenesis

2. Enhance degradation
   - enzymatic enhancement (fibre, protein)
   - alleviate nutritional limitations
   - microbial interactions
3. Detoxification
   - identification and characterization (plant and bugs)
   - biochemical pathways
   - relative contribution (bugs versus host)
   - strategies for intervention
4. Evaluation
   - chemical, microbial and animal
   - seasonal and environmental variation
   - develop appropriate methodology
5. Microbial diversity
   - numbers and types
   - choice of animal model
   - adaptation (bugs versus animal)
6. Ameliorate stress
   - nutrition/immune interaction
   - heat generation by fermentation
   - biocontrol of parasites
7. Technology transfer is a requirement
   - consultation
   - collaboration
   - Preferred research area
   - Focus on forage legumes which include multi purpose trees (MPTs).

Hypothesis

increased fibre degradation
⇓
better nutrient supply
⇓
enhanced biotransformation of ANFs
⇓
improved livestock efficiency

Why ILRI?

Advantages

- germplasm resources (ILRI, CIAT, ICRAF)
- research experience (characterisation)
- strong nutrition programme and facilities

- location (plant and animal)
- access/biodiversity
- global relevance
- global application (research)

Challenges

- scale (selection)
- target

Resource needs

Human

- two teams
- one rumen microbiologist/ecologist
- one rumen plant biochemist
- six to eight associates (allocation variable
- integrated with other expertise

Facilities

- space (three laboratories)
  - microbiology
  - molecular
  - biochemistry

It was agreed that there would be extensive interaction between both research groups and that, therefore, it would be preferable for them to be located at the same place. Further discussion on this was deferred until the two working groups met to discuss the final recommendations.

Modes of collaboration

- science to drive collaboration
- collaborative grants
- international network
- contract research
- PhD students and post-docs
- review meetings
- potential collaborators unspecified

Time frame

- scope of work with five-year milestone
- mid-term review

Conclusions and recommendations

The International Livestock Research Institute (ILRI) inherits considerable research experience on the development of livestock feeds in Africa and their utilisation by tropical livestock. The institute is in the process of formulating a defined programme on feed utilisation, the overall goal of which is the improved efficiency of use of feed resources by tropical ruminants. The broad programme objectives are to (i) evaluate digestibility and nutritional quality of traditional feedstuffs through animal responses; (ii) determine how feed utilisation can be enhanced; and (iii) define adaptation mechanisms of tropical ruminants to low quality feeds and other stresses (work, heat, water, etc.).

Feedstuffs consumed by ruminants is exposed to the fermentative activity in the rumen prior to gastric and intestinal digestion. The fermentation yields characteristic products which provide nutrients for metabolism by the host animal. The quality and quantity of these products are dependent on the types and activities of the microbes in the rumen. This, in turn, has major impacts on nutrient output and productive performance of ruminants.

It was therefore considered that manipulation of rumenal function, particularly by influencing the microbiological activity of the rumen, may have a significant impact on feed utilisation. Research in this area therefore might contribute directly to the second objective of the feed utilisation programme.

The Rumen Ecology Research Planning Workshop was therefore convened with the following objectives:

- to identify and prioritise areas of rumen ecology research which are promising for their potential impact on improving the nutritional status of tropical ruminants;
- to develop, if appropriate, a rumen ecology research programme for ILRI based on relevance to developing countries and ILRI's comparative advantage vis-a-vis other institutions; and
- to identify potential collaborators in advanced research institutes and define mode(s) of collaboration.

Potential research areas in rumen microbiology and ecology

The workshop participants collectively identified the following research areas:

1. Improvement of conversion efficiency by:
   - better microbial adhesion to fibre particles
   - reduction in particle size
   - improved microbial growth efficiency (C, N, minerals)
   - capture of limiting nutrients (NH_3, BCVFA)
   - engineering of protein quality
   - intrarumenal N recycling/defaunation
   - reduced methanogenesis/increased acetogenesis
2. Enhancement of degradation of feeds by:
   - enzymatic enhancement of fibre and protein degradation

- alleviation of nutritional limitations
- optimisation of microbial interactions

3. Detoxification of toxic and anti-nutritional compounds in feeds by:
   - identification and characterisation of toxins and their effects (plant and microbial)
   - identification of their biochemical pathways
   - understanding the relative effects of toxins/anti-nutritional factors (ANFs) on rumen microflora and the host animal
   - determination of strategies for intervention
4. Evaluation of toxins/ANFs by:
   - development of appropriate methodologies for their determination
   - chemical, microbial and animal assays
   - examination of seasonal and environmental variation
5. Determination of microbial diversity by:
   - determination of numbers, types and inter-strain variation
   - choice of animal model
   - adaptation (microbial flora vs animal host)
6. Amelioration of stress by:
   - improving nutrition/immune interactions
   - reduction of heat generation by fermentation
   - biocontrol of parasites
7. Technology transfer is a requirement through
   - consultation
   - collaboration
   - technologies appropriate to end-users

Within these research areas the participants evaluated the requirements of ILRI and ILRI's comparative advantage in undertaking research in any of the areas. The workshop determined that any development of capacity should be through enhancement of the existing feed utilisation programme; on the basis that:

- the most promising strategy is the exploitation of supplementary plant materials (this research is in progress and represents a comparative advantage of ILRI);
- but the presence of ANFs in these potential supplementary plant feeds represents a major constraint.
- nutritional evaluation programmes, largely for fodder trees, are currently based on chemical analyses, degradation studies and feeding trials.

The workshop therefore developed two recommendations, the final wording of which was determined in plenary session.

Recommendation 1

Within the existing programme, develop additional screening procedures at ILRI incorporating microbiological, chemical and animal assessments of these supplementary plant materials relevant to their effects on ruminant productivity.

The expected outcome will be:

1. Improved methods of assessment of the potential utility of fodder trees and shrubs (FTS) for enhancing the quality of low quality diets.

2. More effective use of resources by concentrating on those FTS that do not have significant ANFs.
3. Development of research strategies to overcome effects of ANFs for these accessions that are otherwise of high merit.
4. Identification and selection of superior plant accessions as supplementary feeds.

Recommendation 2

Establish capacity in rumen microbiology/ecology at ILRI to capitalise on institutional and international programmes aimed at increasing the utilisation of low quality feeds through improved rumen function with emphasis on FTS to supply limiting nutrients.

The expected outcome will be:

1. Assessment and exploitation (as appropriate) of indigenous or engineered microbes, or changed population balance, on rumenal efficiency and the adaptation of tropical livestock.
2. More complete characterisation of selected FTS and combination with available feeds to optimise rumen function (especially with respect to detoxification, fibre digestion and defaunation) and subsequent animal productivity.
3. Provision of more comprehensive and quicker attainment of the goals of the feed utilisation programme.
4. Methodologies and materials for transfer to national programmes for improved animal productivity on locally available feedstuffs.

Resource needs

The participants collectively agreed that the following human and material resources would be required to carry out the recommendations when integrated with ILRI's existing resources.

1. Human—Two distinct but related efforts would require:
   - one rumen microbiologist/ecologist
   - one plant biochemist
   - six to eight associates (at the postdoctoral and postgraduate levels)
   - integration with other technical expertise and linkage to collaborating institutions
2. Facilities
   - three discrete laboratory areas for:
     - plant biochemistry (with appropriate chromatographic and analytical facilities)
     - microbiology of anaerobic organisms
     - molecular biology[1]
   - isolation facilities (e.g. for transfer/adaptation experiments involving rumenal microbes)

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1 It should be noted that the collective view was that ILRI, at this stage, would require molecular biology facilities for DNA probe preparation, and microbial typing and quantification by PCR methods. Genetic engineering of rumen microflora is not presently anticipated.

- animal facilities including cannulation capacity
- experimental surgery

3. Arrangement—Juxtaposition of the two research efforts (in phytochemistry and rumen ecology) is clearly desirable. Workshop participants were of the view that some disaggregation of component parts may be feasible but did not seek to make a recommendation as to how this would be managed by the institute.
4. Modes of collaboration—An ILRI programme of scientific excellence developing applicable research findings will attract collaborators. This could be supported through the pursuit of collaborative grants, exchange of postdoctoral and PhD students and, potentially, contract research. This interaction, the current workshop and scientific review meetings would help establish the ILRI programme within the worldwide network of laboratories active in the field of rumen ecology. Because collaborations will be established after programme development and in response to changing research needs, the workshop did not see the need to establish a list of potential collaborators, although the workshop participants and their associated institutes provide a research pool from which ILRI might draw.
5. Actions and time scale—Two senior appointments be made as soon as practicable (start 1996). Development of chemical, microbiological and animal/tissue for screening ANFs in plant materials on a large scale will be a priority (variable but within one to two years).

   Early work will require establishment of additional methods of identifying and quantifying important rumen microbes, including fungi (some assays are available, others to be developed by associate scientific support or collaboration within two years). Concurrently, a search can be made for suitable rumen microbial populations from other mammals able to tolerate FTS known to contain toxins or ANFs. These capacities will allow ILRI to interact with advanced research institutes (ARIs) conducting complementary and supporting research on the rumen ecosystem.

   A strategy of research for improving the utilisation of selected FTS through the above could be expected between year 2 and 3 of the research (when a further small-scale, peer review of the sub-programmes is suggested) with impact from selected improvements estimated within five years of establishment of the programme in ILRI.

Appendix: list of participants

Workshop participants.

List of participants

Anindo, D.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Brooker, J.D.
Department of Animal Science
University of Adelaide
Glen Osmond, 5064 Australia
Fax: (618) 303 7114

Cobos, M.A.
Programa de Ganaderia
Colegio de Postgraduados
Montecillo, México 56230
Fax: 52 595 45 265

Cook, R.M.
Michigan State University
East Lansing
Michigan 48824, USA
Fax: (517) 353 1699

Dolan, T.T.
International Livestock Research Institute
P.O. Box 30709
Nairobi
Kenya
Fax: (254-2) 63 14 99

Fernández-Rivera, S.
International Livestock Research Institute
c/o ICRISAT Niger
P.O. Box 12404
Niamey
Niger
Fax: (227) 73 43 29

Fitzhugh, H.A.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Gardiner, P.
International Livestock Research Institute
P.O. Box 30709
Nairobi
Kenya
Fax: (254-2) 63 14 99

Gordon, G.R.
Division of Animal Production
Commonwealth Scientific and Industrial Research Organisation (CSIRO)
Prospect, Sydney, New South Wales 2148
Australia
Fax: (61-2) 840 2940

Gregg, K.
Institute of Biotechnology
Armidale, NSW
2351 Australia
Fax: 67 72 8235

van Gylswyk, N.O.
Department of Animal Nutrition and Management
Swedish University of Agricultural Sciences
Kungsängen Research Centre
S-753 23 Uppsala, Sweden
Fax: 018 67 29 95

Hanson, J.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Johnsson, I.D.
Meat Research Corporation
P.O. Box 498
Sydney South NSW 2000
Australia
Fax: 02 380 0699

Kaitho, R.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Kope...ný, J.
Institute of Animal Physiology
and Genetics
Czech Academy of Sciences
Prague 10, Uhlínves, 104 00
Czech Republic
Fax: (42-2) 759 182

Kudo, H.
Department of Animal Physiology
National Institute of Animal Industry
Ministry of Agriculture, Forestry
and Fisheries
Inashikigun, Ibaraki
305 Japan
Fax: 0298 38 8606

Lahlou-Kassi, A.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Mackie, R.I.
Department of Animal Sciences
and Division of Nutritional Sciences
University of Illinois at Urbana-
Champaign
Urbana, Illinois 61801
USA
Fax: 217 333 8804

Mohamed-Saleem, M.A.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Mpairuse, D.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Murphy, N.
International Livestock Research Institute
P.O. Box 30709
Nairobi
Kenya
Fax: (254-2) 63 14 99

Nsahlai, I.V.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Odenyo, A.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92, (251-1) 33 87 55

Ørskov, E.R.
Rowett Research Institute
Bucksburn, Aberdeen
Scotland, AB2 9SB
Fax: 44 224 716 687

Osuji, P.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92, (251-1) 33 87 55

Topps, J.H.
Department of Animal Science
University of Zimbabwe
P.O. Box MP167
Mount Pleasant, Harare
Zimbabwe
Fax: 263 4333 407

Umunna, V.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Zerbini, E.
International Livestock Research Institute
P.O. Box 5689
Addis Ababa
Ethiopia
Fax: (251-1) 61 18 92

Wallace, J.
Rowett Research Institute
Bucksburn, Aberdeen
Scotland AB2 9SB
Fax: 44 224 716 687