Project workplan for RUMEN-UP

a) Introduction

The aim of the project is to discover and characterize new materials of plant origin which may be used to decrease environmental and animal welfare problems associated with ruminant livestock production. The workplan consists of three phases.

- The first phase is the selection of suitable plant specimens.
- The second phase involves the use of *in vitro* techniques with digesta withdrawn from cattle and sheep on typical European diets: the composition and volumes of gases formed, the small molecular weight fermentation products and the microbial protein formed will be measured; in individual laboratories, the effects on ciliate protozoa, protein breakdown, lactic acidosis and bloat will be determined. These experiments will identify specimens which may be useful in achieving the objectives.
- The third phase focuses on the specimens with regard to their effects on the specific target problem, in terms of microbiological responses and the chemical nature of the active component.

b) Project planning and time table

Phase 1

Assembling the specimens will be partly the responsibility of the two industrial partners, each of whom have collections of candidate materials suitable for testing, and the academic partners, who will visit national botanical collections, research institutes and university departments in order to obtain best advice on other specimens which might be included. The conclusion of this phase will be a meeting, together with a small team of international botanical consultants with expertise in this field, to agree which samples should be tested, at what concentration, and in which diets.

Phase 2

The test systems involve the incubation of the specimens with rumen fluid *in vitro* in order to determine their effects on rumen fermentation. Techniques for measuring the production of fermentation gases, microbial protein, ammonia and volatile fatty acids are well established (25) and can be automated to deal with large numbers of samples. Each of the academic partners will undertake this routine screening, using diets typical of different production systems in Europe. This screening will pick up methane inhibitors and will help eliminate materials which are generally toxic.

Antiprotozoal effects are better detected separately. The test system involves the labelling of rumen bacteria with ^{14}C-leucine and following the release of radioactivity in the presence of 5 mM unlabelled leucine (26). Bloat can be simulated *in vitro* by the use of appropriate substrates and viscosity measurements (27).

Project time table

Task	Task description	Month No.											
		0 - 3	4 - 6	7 - 9	10-12	13-15	16-18	19-21	22-24	25-27	28-30	31-33	34-36
WP1	Collection and description of 400 samples												
WP2	Collection and description of 100 samples												
WP3	Collation of descriptions; collection of samples												
WP4	General effects on ruminal fermentation,												
	fibre digestion and methane												
WP5	Effects on microbial protein synthesis												
WP6	Effects on proteolysis and ruminal protozoa												
WP7	Effects on lactic acid production and bloat												
WP8	Collation of data with descriptions; selection												
	of phase 3 materials												
WP9	Persistence and chemical nature of												
	antiprotozoal agents												
WP10	Characterisation of inhibitors of proteolysis												
WP11	Persistence and chemical nature of												
	anti-methane agents												
WP12	Persistence and chemical nature of												
	anti-bloat and anti-lactic acidosis agents												
WP13	Palatability and toxicity determination												
WP14	Antibacterial effects, population analysis												
WP15	Antibacterial effects, culture analysis												
WP16	Product quality assessment												
WP17	Feasibility, technical, economic evaluation												
WP18	Project evaluation and recommendations												
Milestones and deliverables													
Report	Collated description of 500 samples												
Report	Collated effects and selection of phase 3												
Report	Persistence and chemical nature of antiprotozoal agents												
	Characterisation, proteolysis inhibitors												
	Persistence and chemical nature of anti-methane agents												
	Persistence and chemical nature of anti-bloat and anti-lactic acidosis agents												
Report	Palatability and toxicity determination												
Report	Antibacterial effects, population analysis												
	Antibacterial effects, culture analysis												
Report	Economic and technical feasibility												
	Project evaluation and recommendations												

Additional objectives/milestones following completion of project

- Intellectual Property, patent protection
- Distribution of results via company literature to 5000 clients
- Description of project at designated session, international biotechnology symposium, 2004
- Opening of consortium website, with results and conclusions, to all

Lactic acid is produced in response to readily available starch. The *in vitro* screen will involve the measurement of D,L-lactic acid concentrations in response to the addition of soluble starch in the fermentations.

The participants and consultants will meet again, once the results of the *in vitro* incubations are known. It will then be possible to assess, for each specimen, whether it possesses any potentially useful properties and whether these useful properties would be negated by other observations. For example, the partner assessing antiprotozoal effects might have found 10 specimens which decrease bacterial protein breakdown by protozoa. It may transpire that 7 of these have been observed by other partners to have detrimental effects on fibre digestion. The 3 which had no detrimental effects would therefore take priority over the others – unless there were agronomic or commercial considerations which made it desirable to try to fractionate the material in order to eliminate ill-effects without conpromising useful ones. Each of the decisions will be therefore be made by considering several factors in addition to the observed beneficial effects.

Phase 3

This phase will focus on the individual skills of the different groups:

1. Specimens which inhibit methane formation will be tested for dose response and for longer-term effectiveness *in vitro*. Rapid adaptation of methanogens to become resistant to antimethanogenic chemicals has been a recurring feature of research in ruminal methane formation. Microbiological studies will determine whether the effective specimens inhibit methanogenic archaea directly or if other bacterial species are affected.

2. Those materials which inhibit ciliate protozoa will also be tested for the development of adaptive resistance. Previous experience suggests that effective materials will vary in their persistence (28). Adaptation may be picked up by pre-incubating the specimens with rumen fluid for 24 h before exposing them to protozoa; if bacterial detoxification is going to occur, it is our experience that this pre-incubation will detect it. Otherwise, it will be necessary to feed sheep the plant materials and to take daily samples of ruminal fluid for protozoal counts.

Continuous *in vitro* methods are less suited to detecting effects on protozoa.

3. Specimens which affect ammonia formation will be numerous. They may inhibit the desired reactions, but other functions will give false positives. Many of the false positives will result from the effects of the material on overall fermentation (29); these should be eliminated by comparing with rates of gas production and fibre breakdown. Some will result from the presence of carbohydrates, the fermentation of which will cause ammonia to be assimilated by the microbes as they grow; they will be picked out by analysis of the sugar content of all of the positives. The specimens which remain will be tested for their effects on the individual proteinase, peptidase and amino acid deaminase activities of mixed rumen fluid. Their effects on the main species of peptidolytic and ammonia-producing bacteria will be determined in pure culture.

4. Any materials which affect lactic acidosis will be tested for their effects on the causative organisms, namely *Lactobacillus* spp. and *Streptococcus bovis* (23). *In vitro* defined mixed cultures will be set up, similar to those which were done previously to demonstrate the effectiveness of ionophores (30). It should thereby be possible to assess the antimicrobial role of the plant materials.

5. With anti-bloat materials, adaptation is expected to be less of a problem, because there would be no selective advantage for bloat-forming bacteria to adapt in order to resist the anti-bloat agent.

With all of the plant materials which are found to have potentially beneficial effects, further chemical analysis will be carried out. This will vary according to the plant material and the application. Solvent extraction may remove tannins or saponins (31, 32). Polymers such as polyethylene glycol titrate out the effects of tannins (33). Molecular weight characterization by MALDI (partner 1) will help give an initial impression of the chemical nature of the compound. More precise chemical characterization may be done by the companies, who carry out many analyses of this type, or sub-contract the work to others. Covalent modification of materials may be considered, based on chemical advice, in order to enhance the effectiveness of the new materials.

Most of the effects will be caused by modulation of the microbial population. This will be assessed by traditional means using culture collections at the research centres. It will also be assessed by modern 16S rDNA probe-based methods, in order to ensure that the results include analysis of isolates which may not be culturable.

Palatability is a limitation to the use of many plant materials. An unpleasant taste is in itself a defence mechanism for the plant. It would be hoped that the quantities of plant materials to be used would be small, and palatability would therefore not present a problem. However, two of the research centres will carry out preliminary studies, in which palatability and veterinary measurements will be made of blood and urinary metabolites, to highlight as soon as possible any of the materials which may have problems with acceptibility or toxicity to animals.

The two commercial partners will, during the last six months of the project, each commission a field trial to test what they consider to be the most promising of

the extracts. The trials will assess the efficacy of the manipulation and its persistence. Attention will also be paid to the quality of the product. Modern shear-force measurements will be made to quantify meat tenderness, and "sniff"-gas chromatography will be used to quantify changes in flavour components. Safety is recognised to be paramount, but safety assessment is a major task that would be undertaken only when efficacy had been established and product quality evaluated.

Conclusion

The project will end with a meeting in which the main conclusions of the project will be agreed and recommendations for implementation of the findings drawn up. The structure of the Final Report will be decided, and the Technical Implementation Plan (TIP) agreed and formulated.

Outcomes

The results will be disseminated in the usual way in peer-reviewed international scientific journals.

In addition, a session of an international biotechnology symposium in 2004 will be devoted to the work; a review book and CD will accompany this symposium.

The results will be conveyed to the farming and feed manufacturing industry by means of regular mail shots which both companies use to communicate with clients and potential clients. At least 5,000 potential end-users will be circulated in this way. Presentations will also be made to the European Association for Animal Production and to national farmers' organisations.

Provision is made in the finances to obtain preliminary protection of the Intellectual Property, with a view to full international patent protection being sought later by interested partners. The Technical Implementation Plan, to be drawn up at the last meeting, will be key to future development.

The web pages created for the project will be available for consultation by scientists and non-scientists alike, interested in the science and the social and agricultural implications of the results. Locally, national newspapers will be targetted as a mechanism for drawing attention to the results. It is anticipated that an Accompanying Measures proposal will be made in the next Call for Proposals in order to fund the information dissemination programme that will be necessary if the information is going to reach all of the target end-users.

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