4 Discussion

DL1-DL6 Plant collection

The objective of this project was to investigate the potential use of plant extracts to solve welfare and environmental problems associated with ruminant livestock production. A major in vitro screening of 500 plants and plant extracts by the consortium was very useful with respect to the generation of information on these plants. The scale of the work carried out would only have been possible with the joint cooperation of all the members of the consortium.

The four academic partners were contracted under WP1 to collect and annotate 100 samples of plant material, and to provide the material to the coordinator. The commercial partners were to provide 50 natural plant-derived specimens and to provide the material to the coordinator (WP2). The coordinator was then to collate descriptions (WP3).

The partners undertook the task in slightly different ways. Partner 1 (RRI) enlisted the help of Professor Ian Alexander of the University of Aberdeen, head of the botany department and curator of the Aberdeen Botanic Garden. Professor Alexander compiled his collection from plants growing in the Aberdeen area, mainly in the botanic garden, on the basis that they either had known secondary metabolites of potential usefulness, or they had traditional uses that indicated antimicrobial activity, or both. The fresh samples were stored in plastic bags, and frozen at –20°C. They were freeze-died, ground to 1mm, and stored in the dark at room temperature.

With Partner 2 (UH), the samples comprised herbaceous plants of known medicinal uses, spices, or weeds as well as tree foliage. Some cultivated oil or dye plants were included, the gross biomass of which has not been used in animal nutrition so far. The plants were collected from the Botanical Garden of the University of Hohenheim, from Rieger-Hofmann GmbH, a commercial supplier of seeds for wildflowers and grasses, the research and demonstration farm "Flachshof",and from home gardens or from the wild, where sufficient amounts were known to grow from personal observations over several years. The plants were cut, stored in plastic bags, transported to the lab as soon as possible, and frozen at –20°C. They were freeze-died, ground to 1mm, and stored in glass jars. The species list with the required descriptions was delivered by e-mail Sep. 25, 2002.

Partner 3 (UL) collected wild plants collected from uplands and mountain areas situated in the surroundings of the city of Leon (up to 100 km from the city). The plant part sampled was different for each species, sometimes collecting the whole plant, and sometimes a specific part (leaves, stems, etc.). Collection was carried out under the supervision of botanists, whose advise was required for the plant identification and the decision about which part was to be collected. Sampling was carried out by hand or with scissors, samples were collected in plastic bags and taken immediately to the lab, where they were frozen within 2-6 h post-harvesting. Then samples were freeze-dried, and milled in a hammer mill (Cullati) using a 1-mm screen. Ground samples are stored in plastic containers placed a cool and dark place. Small portions of each sample were distributed to each partner.

Partner 4 (UR), with the exception of five seaweeds, collected all plant material from within a 35-km of radius of Reading. Some of these, however, were not indigenous to this immediate area but were purchased from local wholesalers. The seaweeds were obtained from the Severn Estuary (Clevedon, Avon) and the North Sea (Dunbar, East Lothian). Detailed information regarding the immediate site from

which each sample was obtained was not collected due to the assumption that, while environment plays an enormous role in defining the composition of the test material, differences between species, plant components and physiological growth stage are likely to have had a greater effect on the outcomes observed. Equally it is likely that, if environment is considered a descriptive factor, then year-to-year variation will also have to be included. No difficulty was encountered in obtaining the samples although in some cases their availability was highly seasonal (e.g. tree fruits). Unless stated the majority of the material comprised aerial vegetative components and all samples were pre-dried and rotor milled to a pass a 1.0 mm diameter screen. Identification was confirmed using standard texts (e.g. Clapham *et al*., 1973) and through consultation with University of Reading botanists. The rationale used for the selection of these plants was either a known "pharmacological" activity (via a literature search) or that they represented a group of readily accessible plants or co-products which were currently not being utilised e.g. heath and aquatic plants, waste from glasshouse crops and bulb production.

Partner 5 (Alltech) made a collection of 50 plant materials based on commercial availability and traditional uses. Partner 6 (Crina) selected, described and provided 50 samples of essential oil compounds.

DL7 General effects of 500 plants on fermentation

The *in vitro* fermentation screen utilised at Reading had the advantage of being able to identify interactions (positive and negative) between the basal feedstuff and the test materials. Where only one level was used, as the degradability characteristics of the test substrate were unknown, an alteration in any of the parameters examined could not automatically be assumed to be a direct substrate effect. For instance an increase in fermentation gas release could result from either a more fermentable material being included or an enhancement of basal substrate degradation by the supplement. An additional advantage of using a dynamic system (Hohenheim and Reading) is that kinetic effects can also be identified. Where supplementation reduced degradation rate although end-point values remained unaffected, offering such materials to ruminants tends to depress intake - an effect which could not have been predicted from end-point assays. Equally changes to the pattern of fermentation can be identified, for instance, where degradation of a particular component e.g. fibre, had been influenced.

Three partners (León, Hohenheim and Reading) evaluated general effects of the samples on fermentation by rumen microorganisms. Concern was raised at the initial meetings (Rowett / León) over variation between the *in vitro* systems and data analyses used. While it was felt important not to change protocols that had the confidence of the partners, common data had to be generated so that comparisons could be made. A compromise 24 h incubation period was agreed, with measurements to include total gas, methane, dry matter and NDF degradation and VFA analysis as appropriate. The degree of replication was determined according to the normal practice of each laboratory, with partners free to analyse further times and parameters as they saw fit. At Hohenheim, the Menke syringe technique was replaced with the RPT, following coordinator discussions (October 2002). With these modifications it was considered that these systems were sufficiently similar to detect treatment effects, such that further standardisation was unnecessary. Management of the donor animals, their feed and the basal substrate differed between sites, in part representing geographical differences. Variation between the systems and methodologies (as detailed in the patent application) are given in Table 4.1. The higher quantity of

inoculum used by León will have tended to increase the initial rate of degradation; however end-point values are unlikely to have been altered and will be comparable with those of the other sites. Using two inclusion levels, the studies at Reading were able to differentiate and quantify true treatment responses i.e., an enhancement of fermentation resulting from an interaction rather than a direct (supplementary) effect due to the inclusion of a more degradable substrate. End-point degradation was estimated differently at each site. Both León and Reading recovered fermentation residues by filtration through sintered crucibles which were then dried at 100°C (24 h). In contrast Hohenheim transferred flask contents into polyester bags which were then sealed and treated with neutral detergent solution (60 mins) prior to drying at 105°C overnight. In all cases DM disappearance loss was considered to represent the extent of degradation.

León estimated NDF degradation by assaying NDF residue content, while Reading ashed fermentation residues (500°C, overnight) and calculated organic matter degradation by difference. Fermentation efficiency, calculated as DMD (mg) / cumulative gas release (ml) provided an indirect estimate of relative proportion of VFA and microbial protein produced, with high values indicating lower fermentation losses.

Table 4.1 Variation in the *in vitro* systems applied to identify effects of samples on the pattern of fermentation by rumen microorganisms.

	Laboratory		
	León	Hohenheim	Reading
Methodology	RPT modified	RPT[a]	RPT
Substrate type	Alfalfa hay: grass hay: barley (5:4:1)	Grass silage	Maize silage
Quantity incubated (mg)	500	750	1000
Inclusion level (mg g^{-1})[b]	100	100	100, 400
Inoculum : buffer ratio	1:4	1:9	1:9
Incubation volume (ml)	50	75	100
Gas measurement	End-point (24 h)	2, 4, 6, 8, 12, 16 and 24 h	2, 4, 6, 8, 10, 12, 16, 18, 21 and 24 h
Technique	Pressure / syringe	Pressure	Pressure

[a] Mauricio *et al*. (1999) [b]Level of test material inclusion in the basal feed (substrate)

The combined general fermentation effects database, the result of a substantial effort by many researchers over a prolonged period of time, is a highly valuable and unique information resource. However relatively little direct use of the information has been made (within Rumen-Up) as its primary function was decision support, especially with respect to selection of substrates for further development within the project. For instance a decrease in gas release following the inclusion of a particular substrate is of interest with respect to reducing methane production, however if this is associated with a significant inhibition of degradation then the substrate would have been discarded. In part a reflection of the quantity of data generated, but relatively few original results are included in the database, e.g. end-point cumulative gas values rather than gas release kinetics. The information is summarised, with the majority of effects expressed relative to their respective controls. Thus absolute values are not shown but rather the relative impact observed following the inclusion of specific substrate on a particular parameter is provided. Although this could be considered

limiting it has the advantage of allowing the data to be interrogated using simple comparisons or rankings, so rapidly refining the number of substrates to be selected for further, more detailed, evaluation. The entire base has however been made available for the follow-up EU FP6 project REPLACE.

A number of useful developments was achieved. Generally, during product development, researchers actively look for methods to prevent or reduce the use of test animals. Where the use of test animals cannot be avoided, we try to reduce the number of test animals and to refine the experimental design to achieve this. In this sense, the development within this project of a set of *in vitro* test procedures to search for selected target features in materials is to be seen as an important step in such a direction. We expect that the refinement and optimization of the presently developed approaches will lead to a substantial decrease in the number of animals needed to develop new products.

Moreover those approaches could contribute to the development of *in-vitro* models to replace animal testing for licensing/registering purposes; further, studies would profit by a reduced use of animals e.g. to test for efficacy, safety and quality as needed in the production of nutritional, biological, and pharmaceutical products for animals.

The databank of information generated on the 500 plants and plant extracts will serve as a useful source for the potential development of products in the future. In addition, it currently serves as a starting point for the FP 6 project Replace.

DL8 Effects on microbial protein yields

This one of the disappointing aspects of the project. UHOH had a method, involving the measurement of RNA concentration at different times during fermentation, which proved to be unreliable. The peaks in RNA concentration appeared at times which were not predictable, so no reliable estimate of microbial protein yield was obtained by RNA analysis. Instead, the influence of plant samples on microbial protein synthesis can be inferred by taking together (in the general effects on fermentation, Annex B) the substrate fermented and the fermentation product concentrations. A carbon balance would enable and estimate of microbial yield. However, this analysis did not give the partners sufficient confidence to proceed to animal trials with any of the samples identified by these criteria. The information will, however, be useful for anyone who wishes to examine the potential of any of the plants in the collection in the future.

DL9 Antiproteolytic and antiprotozoal properties

Antiproteolytic. Two methods were employed in the initial screening of 500 samples. The 14-C-casein method used by the RRI detects lower activity values in the supernatant that are mainly due to the precipitation of protein and not to a retarded proteolysis, especially since the method is based on the degradation of casein and a short incubation time. When testing different protein substrates in the studies that led to UHOH's final proteolysis test system, we found that casein was degraded within the first two hours and degradation was not affected by monensin (data not shown, see intermediate report), therefore we concluded that casein is unsuitable as a substrate for our purposes. Variability was found between concessions of *K. arvensis*

Different accessions of *K. arvensis* had different efficacy in inhibiting proteolysis. The variability between accessions might be due to different sampling

regions and soil and climate conditions, or to the different stages in development of the plants, such as pre-flowering or seed production.

The addition of *Knautia arvensis* in continuous culture in two independent experiments resulted in significant changes in the fatty acid metabolism; a retardation of protein degradation as seen from the RPT system, was not observed. The addition of *Knautia arvensis* in continuous culture in two independent experiments resulted in significant changes in the fatty acid metabolism; a retardation of protein degradation as seen from the RPT system, was not observed.

Experiments with continuous fermenters indicated that the effects of *K. arvensis* declined with time. However, many other adaptive changes occurred with time, as evidenced by molecular population analysis (see below). This points towards an adaptation to fermenter conditions that masks, at least with respect to protein degradation, a potential adaptation to the plant additive.

Antiprotozoal. Overall, a substantial number of samples exhibited antiprotozoal activity. The best of these were analysed further; however, inevitably this selection meant that other samples, with possibly great potential, were left behind. Two samples were investigated in most detail, namely daisy (*Bellis perennis*) and willow gentian (*Gentiana ascledipea*). *B. perennis* was the more potent, but still required 2-3 g of plant per L of ruminal fluid for effective inhibition. Neither appeared, from 24-h adaptation measurements, to have problems in activity being lost rapidly. While this may appear to be rather a short adaptation period, RRI's experience indicates that the samples more vulnerable to adaptive detoxification would be detected by the method. Different accessions of *B. perennis* were effective, adding confidence to its use. In contrast, different samples of *Lonicera* were variable in their effectiveness.

DL10 Effects of plants on bloat and lactic acid

"Acidosis" takes many forms, from the slight, often chronic, depression of rumen pH resulting in reduced fibre degradation and lower intakes – the so-called sub-acute ruminal acidosis or SARA - to the severe pH decrease, favouring lactic acid producing bacteria and possible death of the animal. For the latter to occur *in vitro* unless the VFA produced from substrate fermentation are removed, they tend to buffer the medium pH at about 4.8 (their dissociation constant). This prevents pH declining further and allowing *Lactobacilli*, and similar lactic acid producing species to dominate. This probably explains why greater reductions in pH or higher concentrations of lactic acid were not observed.

Ranking the substrates, according to the extent to which 24 h pH was depressed, identified 67 in which pH declined faster than that of the positive controls. Although this tends to suggest that the other substrates showed a positive effect (maintained a higher pH), to a degree this may have occurred due to supplementation reducing total degradability. Relative to the positive controls 289 substrates showed a similar or reduced level of end-point acidity. To simplify selection a positive effect of on acidosis was identified as reduced rate of pH decline to 24 h, a higher end-point pH and a lowered lactic acid concentration. When ranked the most promising candidate plants (as proposed at the Hohenheim meeting, April 2003) were:

H058	*Symphytum officinale*	UR131	*Helianthus annuus*
E047	*Pentaglotis sempervirens*	H015	*Echium vulgare*
H014	*Amorcaria rusticana*	E151	*Potentilla reptens*

R054 *Mentha arvensis*

However it was considered that while a reasonable *in vitro* attempt had been made to mimic conditions under which bloat occurred at no time were "classic" bloat observations recorded. For example, even the rumen contents of the cattle which supplied the inoculum, did not exhibit symptoms normally associated with bloat e.g., excessive gas / froth or exocellular polysaccharide accumulation. Gas production kinetics were well defined however neither the viscosity nor foam measurements obtained were sufficiently descriptive. An alternative technique to estimate the influence of exocellular polysaccharide on foam production and persistency was recently identified, in which following incubation, a known quantity of finely dispersed carbon dioxide is pumped into the fermentation medium and the resulting foam examined in terms of height and persistency over time.

Bloated animals present a considerable pressure within the rumen, suggesting that during a bloat incidence the reticulo-oesophageal orifice is either physically blocked or a compound produced during the fermentation of bloatagenic substrates blocks signals from stretch receptors in the rumen wall and in some way hinders eructation. The cyanogenic glycosides present in white clover, which cause smooth muscle paralysis, have been implicated in this respect. Releasing this pressure, similar to shaking then opening a bottle of carbonated water, creates the foam that is generally associated with bloat, however it may be more appropriate to considered this an outcome rather than cause. Thus if bloat results from a constriction of the sphincter that controls eructation, itself a consequence of an interaction between the parasympathetic and sympathetic groups of the autonomic nervous system, this cannot be identified using such simple *in vitro* models. In hind sight, while it has been possible to generate some bloat symptoms (e.g., gas kinetics, viscosity and foam production) these are of little use as assays where bloat is a physiological effect.

It was therefore concluded that due to the difficulty of testing such products *in vivo* and to the lack of response identified, it was decided that no further bloat work would be conducted.

DL11 Collated effects and selection of phase 3 plants

The following extracts from the meeting held in Clermont-Ferrand in June 2004 provide discussion of the processes and considerations that led to the selection of the most promiising samples:

"Final choice of antiprotozoal samples (RN)
An assay was run on 7 samples to look at dose response. Linear regressions were used on the results, and it was found that daisy (*Bellis perennis*) and gentian (*Gentiana asclepiadea*) gave the best results and were selected for the persistency study. This was monitored for 24 h after which there was found to be no adaptation. The chemical nature was analysed, both plant activities were not affected by heating up to 100°C (in closed containers) or autoclaving. Extraction was carried out. The plants were homogenised, centrifuged and separated. *B. perennis* was found to have high amounts of saponins. Soxhlet solvent extraction was conducted and it was found the antiprotozoal activity increased in more polar solvents. These effects were only noted in the supernatant after homogenisation therefore the active compound is intracellular. The new collections of *B. perennis* and *G. asclepiadea* were tested and the results were similar, suggesting a true effect. NS suggested that if *B. perennis* was selected then it should be run through the UHOH antiproteolysis assay.

Final choice of antimethane samples (SL)
This investigation was conducted in 3 stages – plant selection, dose response and persistency. In UHOH in 2003 5 plants were selected by their reducing methane production by 15%. Two were

omitted from that list and were included in this investigation – Quercus (unsure of species) and *Rheum nobile*. All 7 plants were re-tested for the same parameters as before in 3 different runs each with 3 replicates. Three plants were selected – Quercus, *R. nobile* and *Carduus pycnocephalus*. Dose response was conducted at 5, 10, 15 and 20%, and no conclusive results shown. The maximum reduction in methane was shown in *R. nobile* and *C. pycnocephalus* at the 10% level. There were no dose titration effects with Quercus and it was shown that the new collection of Quercus didn't have a methane reducing effect! Persistency studies were conducted in RUSITEC, where the new collections of the 3 samples were included as 7.5% total diet, and after 12 d there was a consistent reduced methane production with both *R. nobile* and *C. pycnocephalus* . This was repeated using a different basal diet (concentrate based) but at roughly the same additive inclusion level (8%). There was still a reduction in methane production with *R. nobile* and *C. pycnocephalus* but it wasn't statistically significant.

Points discussed: –

The difference between the two Quercus samples was discussed. RJW said the two collections were at different times of the year, but EH suggested that a pattern can't be deduced from a limited number of samples. It was concluded that the anti-methane effect observed with *R. nobile* and *C. pycnocephalus* is more pronounced with a forage-based diet, which is where methane production is more of a problem.

Final choice of antiproteolytic samples (EH)
A standardized dot blot test was developed, using monensin as a positive control. The substrate for the assay had to be chosen according to solubility. It was decided to use a soyabean/BSA mix, and monensin at a concentration of 3μM (to minimise VFA profile disturbance). The 21 antiproteolytic samples from phase 2 were screened, and were shortlisted relative to the control (monensin). RJW mentioned that the SDS-Page technique depends on the size of the molecule present, and that if a protein molecule decreases in size eventually it won't appear on the dot blot. It was unsure what the minimum size a protein has to be to appear.

NS then discussed the extraction procedure on the selected sample, *K. arvensis*. Very little of the original sample remained so a new batch was collected, and when tested for antiproteolytic effect it was noted that the original batch was more effective (and 2 collected later in the year in Germany weren't effective). *Knautia arthopurpurea* had less effect than *K. arvenis.* SL mentioned that the botanists in Spain said the plant could be *K. arvensensis* rather than *K. arvensis*."

Further discussion included the following observations:

SAMPLES WITH POTENTIAL FOR CONTROLLING CILIATE PROTOZOA

Thirty-eight samples produced a decrease of >50% in protozoal activity when added at a concentration of 5 g/l. Of these, the following were selected for further development.

Lonicera j*aponica* (A025, A026, A027)
Three samples contained *L. japonica*, which is Japanese honeysuckle. A026, an extract from the flower, abolished protozoal activity but had no other effects. A025, which is a seeds preparation, also depressed protozoal activity, but only by 60%; again, there were no other effects on fermentation. A sample containing a mixture of *Magnolia officinalis* and *L. japonica* was almost as potent as A026, causing 95% loss of protozoal activity while having little influence on other parameters. The related species, *Lonicera periclymenum*, had only a very small effect on protozoal activity.

These samples were provided by Alltech. A quick internet search revealed that several companies sell Japanese honeysuckle extracts. Commercial availability therefore would be the preferred option for future development. A number of commercial samples will be purchased and examined for their efficacy.

Japanese honeysuckle is edible and medicinal. High in calcium, magnesium, and potassium, the leaves can be parboiled and eaten as a vegetable. The edible buds and flowers are used to make a syrup or puddings. The entire plant has been used as

an alternative medicine for thousands of years in Asia. Honeysuckle is particularly good for upper respiratory tract infections. The stems and flowers are used together in infusion or decoction. The stems are used internally in the treatment of acute rheumatoid arthritis, mumps and hepatitis. Honeysuckle is antibacterial, anti-inflammatory, antispasmodic, diuretic, febrifuge, and is also used to reduce blood pressure. Experimentally, the flower extracts have been shown to lower blood cholesterol levels and are antibacterial, antiviral and tuberculostatic. Externally, the flowers are applied as a medicinal wash to skin inflammations, infectious rashes and sores. Leaves and flowers are traditionally used to treat chicken pox.
The active constituents include calcium, elaidic acid, inositol, linoleic acid, lonicerin, luteolin, magnesium, myristic acid, potassium, tannin and zinc. Triterpenoid saponins have been found in the aerial parts. Polyphenolic compounds isolated from *L. japonica* inhibit human platelet activation and provide protection from cellular injury, and thus help maintain human vascular homeostasis.

It can be concluded that *L. japonica* is a traditional medicine with many useful properties. There are no contra-indications regarding toxicity. The compound(s) which might be responsible for the suppression of protozoa are probably triterpenoid saponins.

Gentiana asclepidea (R038)
This sample comprised leaf and stem of the low-growing shrub, willow gentian. Addition of *G. asclepidea* abolished protozoal activity by 92%, while having minimal effects on other fermentation parameters, except a possible slight increase in microbial protein production, a positive effect that would result from suppression of protozoal activity.

This sample was taken from the botanic garden at the University of Aberdeen, where there are several plants. If *G. asclepidea* were to be required for a trial, it would be necessary to cultivate the plant, and it would be necessary to establish the plant during 2003 for harvesting during 2004.

Gentiana is a large genus of herbaceous plants belonging to the natural order Gentianaceae. The genus comprises about 300 species, most of them perennial plants with tufted growth, growing in hilly or mountainous districts, chiefly in the northern hemisphere, some of the blue-flowered species ascending to a height of 16,000 ft. in the Himalaya Mountains. All the species of the genus are remarkable for possessing an intense but pure bitter taste and tonic properties. About forty species are used in medicine in different parts of the world. By far the most important of the species used in medicine is *G. lutea*, a large plant 3 or 4 ft. high, growing in open grassy places on the Alps, Apennines and Pyrenees, as well as on some of the mountainous ranges of France and Germany, extending as far east as Bosnia and the Danubian principalities. Its use in medicine is very ancient, tracing back to the Greeks. The root is used as a means of dilating wounds, and is generally the part used in medicine. It has a pure bitter taste and faint distinctive odour. The bitter principle, known as gentianin, is a glucoside, soluble in water and alcohol. It can be decomposed into glucose and gentiopicrin by the action of dilute mineral acids. It is not precipitated by tannin or subacetate of lead. A solution of caustic potash or soda forms with gentianin a yellow solution, and the tincture of the root to which either of these alkalis has been added loses its bitterness in a few days. Gentian. root also contains gentianic acid which is inert and tasteless. It forms pale yellow silky crystals, very slightly soluble in water or ether, but soluble in hot strong alcohol and in aqueous alkaline solutions. This substance is also called gentianin, gentisin and gentisic acid. The root also contains 12 to 15% of an uncrystallizable sugar called gentianose, of which fact advantage has

long been taken in Switzerland and Bavaria for the production of a bitter cordial spirit called Enzianbranntwein. The use of this spirit, especially in Switzerland, has sometimes been followed by poisonous symptoms, which have been doubtfully attributed to inherent narcotic properties possessed by some species of gentian, the roots of which may have been indiscriminately collected with it; but it is quite possible that it may be due to the contamination of the root with that of Veratrum album, a poisonous plant growing at the same altitude, and having leaves extremely similar in appearance and size to those of *G. lutea*.

Gentian is one of the most efficient of the class of substances which act upon the stomach so as to invigorate digestion and thereby increase the general nutrition, without exerting any direct influence upon any other portion of the body than the alimentary canal. Having a pleasant taste and being nonastringent (owing to the absence of tannic acid), it is the most widely used of all bitter tonics. The British Pharmacopoeia contains an aqueous extract (dose, 2-8 grains), a compound infusion with orange and lemon peel (dose, 3/4-I ounce), and a compound tincture with orange peel and cardamoms (dose 4-I drachm). It is used in dyspepsia, chlorosis, anaemia and various other diseases, in which the tone of the stomach and alimentary canal is deficient, and is sometimes added to purgative medicines to increase and improve their action. In veterinary medicine it is also used as a tonic, and enters into a well-known compound called dia pente as a chief ingredient. Other uses include as a tonic as antipyretic (reduce fever), anti-spasmodic, depurative (promotes elimination via natural channels of the body), antihypertensive, anti-TB, anti-diarrhoeal, anthelmintic and anti-inflammatory.

It can be concluded that *Gentiana* in general may have many useful properties. There are no contra-indications regarding toxicity. The compound(s) which might be responsible for the suppression of protozoa are unclear, although gentianin is an obvious candidate.

Eugenia caryophyllata (A016)

This was a commercial sample of cloves, which is the dried embryo seed of the *E. caryophyllata* tree. Ninety-eight % inhibition of protozoal activity occurred with A016, with the only other change being also beneficial, namely an increase in microbial protein production of 10-20%. Interestingly, the main essential oil from cloves, eugenol (C023), had no influence on ciliate protozoa at the concentration used (100 ppm).

The sample was provided by Alltech. It is available commercially, therefore would not present a problem for future trials.

Cloves is a widely used medicine in Asian herbalism. It has been considered an aphrodisiac in China since the 3rd century B.C. It also promotes digestion. The Swahili used a bark decoction as a remedy for diarrhoea. Egyptians used cloves to strengthen the respiratory system. Eugenia is widely used in Paraguayan folk medicine for treating gout. Clove oil is best known as a local anaesthetic for toothache. Eugenol, its principal constituent, is used synthetically in the dental industry for numbing the gums. Compounds in clove oil have shown strong activity against bacteria associated with plaque and gum disease. This stimulating oil is widely used in mouthwash and gargle.

If eugenol is not the factor responsible for the antiprotozoal effect, it is important to identify if it is another compound in the oil, or a component unrelated to the essential oils. Thus, experiments will have to be carried out with clove oil as well as the clove extract (A016).

Cloves therefore have many traditional uses. The only contraindication is skin irritation. Bulk commercial quantities would be the route of choice for *in vivo* exploitation.

Bellis perennis (R017)

The common daisy, *B. perennis*, was collected from domestic pasture. The dried plant, comprising mainly leaves, inhibited protozoal activity by 93% at 5 mg/ml. A small (6.5%) decrease in methane formation was observed.

B. perennis was contributed by the Rowett. It was collected from the wild. While collection of greater quantities would be possible, it would be tedious, and cultivation would be preferable.

A description of its properties, safety and use in human and veterinary medicine is given in Annex A. *B. perennis* extracts are used as an expectorant or astringent. Daisy is said to be useful for coughs and catarrh, and has a reputation of value in arthritis and rheumatism as well as in liver and kidney problems. Due to its astringency it is also useful for diarrhoea. In animals, it is used for skin complaints, or as an anti-inflammatory or anti-spamodic agent. There are no reports of toxicity in man or animals. Daisy may be used freely and safely, according to herbalists.

Daisy has a complex array of secondary compounds, including triterpenoid saponins, flavonoids and phenolic acids, as well as essential oils and tannins. Once again, it seems that the triterpenoid saponins might be the active principle.

On the grounds that the *B. perennis* sample was effective, with no safety or toxicity problems observed or anticipated, it may be a good sample for development. It would be easy to re-collect small quantities. For larger quantities, cultivation would be required. A number of chemicals may be responsible for the observed benefit, most likely triterpenoid saponins.

Olea europaea (A016)

The sample was dried olive leaves. It decreased protozoal activity by 88%. Other parameters were affected little. Minor improvements were seen in fermentation efficiency and microbial biomas production (León).

The sample was submitted by Alltech. Olive by-products are not only readily available, they present a disposal problem in many areas. The cost would be very low.

Olive leaves have been examined as a feedstuff, as explained in a document taken from FAO. The FAO document can be found at http://www.fao.org/DOCREP/003/X6545E/X6545E03.htm. Leaves and branches are traditionally used fresh in many countries and can be a substantial fodder resource. However, there is no indication that olive leaves have been examined for their effects as feed additives. There seems no question that they will be safe for animal feeding. Olive tree leaves distributed fresh during the winter season are usually consumed willingly by animals without any problem of adaptation, even over a long period. In terms of traditional uses, the ancient Egyptians may be been the first to put the olive leaf to practical use. They regarded it as a symbol of heavenly power, and in keeping with that belief, they extracted its oil and used it to mummify their kings. Later cultures found the leaf was better utilized for the living than the dead. Over the ages, there is documentation that it was a popular folk remedy for combating fevers. Decades later, scientists isolated a bitter substance from the leaf and named it oleuropein. It was found to be one ingredient in a compound produced by the olive tree that makes it particularly robust and resistant against insect and bacterial damage.

From a technical angle, oleuropein is an iridoid, a structural class of chemical compounds found in plants. It is present in olive oil, throughout the olive tree, and is, in fact, the bitter material that is eliminated from the olives when they are cured. Oleuropein lowers blood pressure in animals. It could also increases blood flow in the coronary arteries, relieve arrhythmias, and prevent intestinal muscle spasms. The active ingredient in oleuropein is a substance he called elenolic acid. It was found to have a powerful anti-bacterial effect. Microchemical tests have indicated the presence of alkaloids, saponins and tannins, but not of cardiac or cyanogenic glycosides. The presence of the flavonoids apigenin, luteolin, chrysoeriol and their derivatives is also established. The quinoline group alkaloids cinchonine and cinchonidine have been isolated from leaves of Algerian, Moroccan, French and Italian collections of *Olea europaea.* Also present are various common triterpenes e.g. β-amyrin, oleanolic acid and unusual monoterpenes e.g. the secoiridoids oleuropein and oleacein. Thus, there are several compounds that could be responsible for the effects observed on rumen ciliate protozoa.
On the basis of safety and availability, *O. europaea* must be an excellent candidate for development. The chemical component must be isdenitified, and some investigation about methods of preparation and preservation of samples must be carried out.

Symphytum officinale (H058)
The sample was of all over ground plant. This sample gave an 87% inhibition of protozoal activity. It also appeared to benefit acidosis. There was an indication from León data that digestion might be slowed; as no similar indication was obtained at Hohenheim or Reading, it was concluded that *S. officinale* might be unsuitable for higher-fibre diets, but could be useful otherwise. Increases in fermentation efficiency were also obtained with *S. officinale.*
The most common name for this plant is Comfrey. It was collected by Hohenheim. The plant is abundant in the wild and trial quantities could readily be collected from the area around Aberdeen.
Comfrey has been used as a herbal remedy or medicine. It is generally suggested that Comfrey (either species) is for external use only and that children and pregnant or nursing women should avoid this plant completely. Large doses taken internally for extended (three or more months) may cause HVOD (hepatic veno-occlusive disease (narrowing of blood vessels in the liver thus reducing liver's effectiveness)) and/or liver cancer. It should be noted that Comfrey has never been identified as the cause of any case of liver cancer in humans (though there are some reported cases of liver and bladder tumors being caused by Comfrey in animals (no species of comfrey noted)) and only two cases of HVOD have been blamed on Comfrey, despite all the Comfrey that is consumed every day and has been many years. There are therefore contra-indications that Comfrey, when consumed for a prolonged period, might be toxic. Comfrey contains a number of pyrrolizidine alkaloids, which may be the agents responsible for the antiprotozoal effect. However, they also may be the cause of the medical contraindications. Tannins are also present.
In conclusion, *S. officinale* benefits fermentation, although with some concern for its use with high-fibre diets. The plant could be collected easily for further experimentation. It has been used in traditional medicine, but toxic effects have been noted. We should be cautious about this plant.

β-*Myrcene* (C037)

Myrcene had a much less pronounced effect on protozoal activity than the other samples which were selected, causing a 32% decrease in protozoal activity. Its only other significant effect was a 10% decrease in methane formation. As the dose of myrcene could be increased with ease, it was decide to include this compound in the next phase.
β-Myrcene was supplied by Crina. It is a component of essential oils of many plant species. It is found in fennel (*Foeniculum vulgare*), which was not antiprotozoal when tested alone. □-Myrcene has been in public use since the 1950s. The greatest use of myrcene is as an intermediate in the commercial production of terpene alcohols: geraniol, nerol, and linalool, which serve as intermediates for the production of large-volume aroma and flavor chemicals. It is also used in large quantities in the manufacture of specialty aroma compounds (myrcenol and its derivatives). It is found in verbena oil, galbanum oil, and Formosan and West Indian lemongrass oil, and is the major constituent of hop and bay oils which are used in the manufacture of alcoholic beverages. Depending on variety, □-myrcene generally accounts for 40 to 70% of the total oil content but is readily oxidized and lost after harvest. □-Myrcene and essential oils containing this monoterpene have been widely used as scenting agents in cosmetics, soaps, detergents, and as flavoring additives in food and beverages. β-Myrcene is a peripheral analgesic substance and the active ingredient in lemongrass (*Cymbopogon citratus*) tea. This potion is widely used in Brazilian folk medicine to treat gastrointenstinal disturbances and as a sedative and antipyretic.
The chemical name of β-myrcene is 2-methyl-6-methylene-2,7-octadiene. It is an oil at room temperature.
Myrcene is therefore likely to be safe and is readily available. Whether it will prove to be sufficiently potent will be investigated in the next phase.

SAMPLES WITH POTENTIAL FOR CONTROLLING PROTEOLYSIS

The screening revealed that 45 samples gave an apparent decrease in the rate of proteolysis as determined by the rate of breakdown of ^{14}C-casein added to ruminal fluid. Some of the experimental runs were suspect and are being repeated. At the present time, the following positive hits are intended to be investigated further.

Potentilla anserina (H068)
Four species of *Potentilla* are included in the collection. Three of these, samples H041, H068 and R100, from species *P. aurea*, *P. anserina* and *P. palustris*, gave significant inhibition of proteolysis. The fourth, *P. reptens* (E151), needs to be repeated. The results also indicated that *P. anserina* benefited microbial biomass production.

P. anserina was collected by Hohenheim. Other names include Cinquefoil, Crampweed, Goose Tansy, Goosegrass, Moor Grass, Silverweed, Trailing Tansy, Wild Agrimony. *Potentialla* is a low, perennial, flowering plant, common in Europe and America. It is available commercially, but probably only in small quantities. Collection of bulk quantities would be difficult, though do-able.
P. anserina is known as a traditional medicine, as a remedy for diarrhoea, premenstrual syndrome and sore throat. In the past, Potentilla was considered a treatment for lockjaw, jaundice (liver disease) and menstrual cramps, and was used to remove freckles and prevent scarring from smallpox. Today, it is included in various tea mixtures for treating sexual disorders, nervous agitation, and nausea. Its effectiveness for such uses remains unproven. No toxicity is reported

Potentilla species in general contain high concentrations of polyphenols and polyprenols, including tannins. It is likely to be these polymers which cause the anti-proteolytic activity. It would be interesting if it were the polyprenols, as opposed to polyphenols, which were effective.

In conclusion, *Potentilla* spp., of which *P. anserina* is an example, are probably safe materials to use as inhibitors of proteolysis. Their collection for bulk trials might be difficult. Identification of the active principle may enable better species to be identified.

Gleditsia japonica (H086)

This sample decreased proteolysis by 40%. Microbial biomass production tended to be increased. No detrimental effects were noted.

G. japonica was collected by Hohenheim. It was a sample of leaves and young shoots, taken from this deciduous Japanese decorative tree. It would be difficult to gather sufficient material for bulk trials, and probably too long a period of cultivation to produce enough material for study.

The chemical constituents of *G. japonica* include saponins, but although tannins and other phenolics are probably present little information appears to be available.

Given the difficulty of collection, *G. japonica* may not be strongly recommended for further study in Rumen-up.

Anthoxanthum odoratum (R098)

Sweet vernal grass gave a 39% decrease in proteolysis, accompanied by a 33% decrease in protozoal activity. No negative effects were observed.

The sample was provided by the Rowett. It is abundant on acid grassland, although it would have to be established by seed one season before collection for bulk trials.

A. odoratum, as its name implies, gives off a pleasant aroma. It is sold by herbalists, but its proven application is unclear. The aroma is due to coumarin. The problem with this plant is that, when it forms a high proportion of the diet in cattle, coumarin is converted to dicoumarin. Dicoumarin is toxic and can lead to death (Runciman et al., Austr. Vet. J. 80, 28, 2002). Surprisingly, coumarin itself (C018) did not have any effect at 100 ppm.

The sample may therefore give problems in several respects. At this stage, it is important to find out next what the effective dose is, and whether the inhibition is sustained.

Fagopyrum esculentum (H096)

Buckwheat caused a 30% inhibition of proteolysis in the initial screen, which was not statistically significant and which therefore needs repetition. However, 38% inhibition of protozoal activity, together with increases in microbial biomass, suggested that this sample could be beneficial in more than one way.

F. esculentum, alternatively known as *Polygonum fagopyrum*, is an erect, annual herb with hollow stems; leaves alternate, simple, arrow-shaped; flowers small, white, fragrant, in terminal or axillary clusters; fruit a 3-sided nutlet. It was provided by Hohenheim. It is cultivated in Southern Europe as a food crop, so ample quantities would be available for trials. The part of the plant processed was the upper part, in late bloom.

F. esculentum is considered, as a whole plant, to be poisonous. It causes photosensitisation in sensitive individuals. On the other hand, flour made from its dried fruits is edible. According to Chinese traditional medicine, buckwheat is good

for inflammation, bleeding, and the young leaves are good for the eyes and ears. Even though most people eat the grain, the young leaves are very nutritious. They are high in vitamin B_1 and many minerals. Traditionally, buckwheat was used for the treatment of circulatory problems, lowering blood pressure.
The toxic principle which causes photosensitisation is fagopyrin. Buckwheat seeds also contain complex tannins, condensed tannins, flavonoids and hydrolysable tannins. The tannins content probably explains the antiproteolytic activity. Rutin, a flavonoid, is reputed to be advantageous to circulation and blood pressure.
Overall, so long as the dose to be used is relatively small and sufficient to avoid photosensitisation, buckwheat may be a useful material. It will be plentiful and easy to obtain.

Methyl salicylate (C036)
Methyl salicylate produced a 34% decrease in proteolytic activity. It had a slight tendency to decrease gas production and digestibility. As methyl salicylate was used at the very low concentration of 100 ppm, it was decided that higher concentrations would be tested.
Methyl salicylate, also known as Oil of Wintergreen, is a local anaesthetic agent and disinfectant commonly used in toothpaste, mouthwashes, and perfumes. It is available as a chemical from the lubricants industry.
Traditionally, Oil of Wintergreen is used in rubs, mouthwashes etc. If ingested, it can cause poisoning when used in too great doses. Methyl salicylate is the most toxic form of the salicylates. Doses of less than 1 teaspoonful have been lethal in small children.
Methyl salicylate is the active ingredient of a new insecticide to be used in packaging materials which has been approved by the Environmental Protection Agency. It is a safer alternative to traditional insecticides and fumigants because it is used in the packaging itself and does not come into contact with the food product or other material in the container, the agency said.
The value of methyl salicylate as a proteolysis-controlling feed additive therefore will depend on its effective dose and persistence. It would be readily available for trials.

DL12 Persistence and chemical nature of antiprotozoal agents

Neither *B. perennis* nor *G. acsledipea* appeared, from 24-h adaptation measurements, to have problems in activity being lost rapidly. While this may appear to be rather a short adaptation period, RRI's experience indicates that the samples more vulnerable to adaptive detoxification would be detected by the method.

Both *B. perennis* and *G. ascledipea* contained saponins, which have been shown by others, as described in Chapter 1, to have antiprotozal activity. Chemical analysis indicated that they were triterpenoid saponins. However, the pattern of banding in TLC indicated that the saponins actually present in both samples were very different. The haemolytic activity of the two main extracts was compared with that of authentic saponins. It emerged that the haemolytic activity of *B. perennis* resided in the methanolic extract, and that the methanolic extract had similar potency to authentic *Quillaja* saponins (QS). In contrast, *G. asclepiadea* (R038) had no discernible haemolytic activity. Thus, while *B. perennis* was selected for its more immediate availability, it could be that *G. ascledipea* would be more promising for development, because of a lower toxicity. [Having said that, no toxicity was observed with *B. perennis* in sheep, see below].

DL13 Characterisation, proteolysis inhibitors

The RPT incubations with methanol extracts of *Knautia arvensis* provided by a company confirmed the results of a retarded proteolysis obtained with methanol extracts produced in UHOH lab. However, further characterization of the active component was not possible due to small recoveries and consequently insufficient amounts of extracts available to insert them in our test system and demonstrate their antiproteolytic activity.

Protein degradation parameters were investigated further for the 4 concentrates designed for the antiproteolysis trial. The soluble protein released from the pellets after 1 h correlated with the crude protein content of the feeds, but even for the higher protein content it was well below the standard substrate used in the *in vitro* assays. Degradation proceeded at similar rates, but due to the lower starting point soluble protein concentrations came already close to the background level (0.1 mg/ml) after 6 and 9 h for the low and high CP feeds, respectively. P2 and P4, which contained the antiproteolytic additive, ranged slightly but not significantly above the values of the corresponding controls. This, however, was backed up by significantly reduced release of iso-SCFA in the presence of the additive up to ca. 10 h of fermentation. At the end of the fermentation period, this difference was no longer detectable. Ammonium concentration, on the other hand, showed a tendency to lower concentration in the presence of the additive.

Thus, altogether, there were indications of the described effects of *K. arvensis* to reoccur on the background of the concentrate mix, but they were not as clear as in the standard test system. Further optimization of the concentrate composition with respect to these properties would have been advantageous.

DL14 Persistence and chemical nature of anti-methane agents

The aims of this component of the project were to determine the effects of the agents on fibre digestion, end-product formation, microbial growth and on whether adaptaion occurred in the microbial community, rendering the agents only transiently effective.

A first selection of candidates as possible antimethane plants was decided at month 18 based on the effects on methane production (selecting plants causing a decrease greater than 15% as compared with the control cultures) and on digestibility, gas production and total VFA production (confirming the lack on inhibiting side-effects on these variables) measured in vitro in the screening trials carried out under WP4.

Using this information, the suggested candidates were:

- *Salix caprea* (R69)
- *Carduus pycnocephalus* (E96)
- *Populus tremula* (R61)
- *Prunus avium* (R63)
- *Paeonia alba* (radix) (A40)
- *Quercus robur* (R65)
- *Rheum nobile* (R66)

Further in vitro incubations were carried out to investigate consistency of the response observed in the screening trial because the high variability observed (only 3 replicates

for plant had been used in the screening) and to check if there could be any "Run effect" (plants had been tested in different incubation runs)

Incubations with the addition of any of the seven selected plants were repeated following the methodology used in WP4, but:

- Three - four incubation runs were carried out, with 3 replicates for each plant in each run, hence at least nine observations were available for each plant
- The seven plants were tested in three incubation runs to minimize possible "incubation run effects"

In this trial, Q. pedunculata and R. nobile caused the greatest and most consistent decrease in methane production.

None of the plants selected showed depressing effects on other parameters (digestibility, total gas production, total VFA production) of the rumen fermentation. Thus, the plants *Carduus pycnocephalus*, *Quercus robur* (*Q. pedunculata*) and *Rheum nobile* were selected for further studies.

Dose response studies

A preliminary single assay was carried out in which the seven plants were added at rates of 8 and 16%, but results were not conclusive.

Then a dose response trial with *in vitro* batch cultures was performed, in which the three plants (*Carduus pycnocephalus*, *Quercus robur* and *Rheum nobile*) were added at 4 doses (5, 10, 15 and 20%) using a diet with 50% alfalfa hay, 40% grass hay and 10% barley. There were 3 incubation runs with 3 replicates for each experimental treatment (plant and dose) in each run, giving a total of 9 observations per treatment.

These results can be summarised as follows:

- Regardless of the dose used (up to a level of inclusion of 20%), there were no consistent effects on ruminal fermentation parameters such as digestibility, total gas or VFA production
- The addition of the plants had some effect on methane production, but dose response effects could not be demonstrated; the relationship between methane decrease and dose did not follow any particular pattern.

The lack of a significant dose response could be due to confounding effects when adding the plant that may provide an active "anti-methane" principle but may also supply a variable amount of fermentable substrate directly related to the dose incorporated. To minimise this effect two control cultures were used (with either 400 or 500 mg diet, but even so the possible dose effects could not be detected and identified.

Persistency studies

According to the work plan, two experiments were conducted to test longer term effects of the addition of the three plants selected (*Carduus pycnocephalus*, *Quercus robur* and *Rheum nobile*), on methane production when either a forage- (Experiment 1) or a concentrate-diet (Experiment 2) were incubated in an artificial rumen system (semi-continuous fermenter), simulating ruminal fermentation for a few weeks.

The first experiment consisted of 2 trials, using in each eight vessels set up as described above. Thus, measurements were taken on sixteen experimental units (two trials 8 +8 = 16 fermentation vessels) assigned randomly to one of the four

experimental treatments (two vessels per treatment in each trial, giving a total of for observations per treatment): control (no plant), Carduus, Quercus and Rheum. The feed used in these trials was a forage based diet (20% barley grain + 10% straw + 35% grass hay + 35% alfalfa hay), of which 16 g were provided daily to each vessel. The dose of additive was 1.2 g / d (7.5% of the diet, 1500 ppm as concentration of the vessel contents). The additive was ground finely, and weigh out in a small polyester bag, pore size 25 μm, which was soaked in 40 mL of the vessel supernatant and then placed in the food container beside the bags containing food. The bag containing the additive was replaced daily.

The three plants caused a decrease in methane production, although in the case of *Q. robur* this decrease was small and did not reach statistical significance. The difference was statistically significant for *C. pycnocephanus* and *R. nobile*, that caused decreases in methane production of 16% and 22%, respectively, compared to the control vessels.

The decrease in methane production observed with *C. pycnocephalus* and *R. nobile* was steady during the 12 days of the measurement period, indicating that this effect was persistent during the experiment. This was confirmed in the statistical analysis, as the interaction between plant additive and time (day of measurement) was not significant ($P > 0.05$).

The addition of any of the selected plants did not affect significantly apparent DM digestibility, VFA production or VFA molar proportion.

It was concluded:

- *Carduus* and *Rheum* caused a significant decrease in methane production without affecting other ruminal fermentation parameters
- The effect was persistent over the time the Rusitec experiment lasted
- *Quercus* had no significant effect
- Although the plants did not affect negatively the rumen fermentation, the decrease in methane production was not accompanied by changes in the fermentation end-products that could be beneficial to the animal.

The second experiment consisted of only one trials using eight fermentation vessels assigned randomly to one of the three experimental treatments two vessels as control treatment (no plant), three vessels receiving *Carduus*, and the other three receiving *Rheum*.

The feed used in these trials was a concentrate based diet (66% of a commercial concentrate containing barley, maize, soya bean meal, molasses and corrector and 33 % of a mixture of straw, grass hay and alfalfa hay), of which 15 g were provided daily to each vessel. The dose of additive was 1.2 g / d (8% of the diet, 1500 ppm as concentration of the vessel contents). The additive was ground finely, and placed in a small polyester bag, pore size 25 μm, which was soaked in 40 mL of the vessel supernatant and then placed in the food container beside the bags containing food. The bag containing the additive was replaced daily.

Methane was also decreased with the addition of Carduus and Rheum to the concentrate diet, although to a lesser extent than with a forage diet, and did not reach the level of statistical significance. Compared with the control, the decrease in methane production was 5% with *Carduus* and 15% with *Rheum*. There was not a time-effect, so the differences between control and treatment vessels were similar over the Rusitec experiment. The addition of Carduus or Rheum did not have any significant effect on other fermentation parameters in the Rusitec.

Thus, *Carduus* and *Rheum* affected methane production in the RUSITEC to lesser extent when a concentrate diet was used, and the effects did not reach statistical significance. The effects of the addition of *Carduus* or *Rheum* on methane production observed with a forage diet were not detected when a concentrate diet was used.

Chemical nature of anti-methane agents

In the meeting held in Dublin in March 2004, it was agreed that ULE would determine chemical fractions by proximate analyses and also tannins by conventional procedures in all the nine plants taken to the last stage of Rumen-Up as the most effective as antiprotozoal (*Bellis perennis* L., *Gentiana asclepiadea* L.), antiproteolysis (*Knautia arvensis* L., *Peltiphyllum peltatum* (Torr. ex Benth.) Engl.), antiacidosis (*Lactuca sativa* L., *Urtica dioica* L.) or antimethane (*Rheum nobile* Hook. f. & Thoms., *Carduus pycnocephalus* L., *Quercus robur* L.) agents.

Peltiphyllum, *Rheum* and *Quercus* can be considered plants with high concentrations of tannins. *Knautia* seemed to have a high content of tannins using the Folin-Ciocalteau procedure of Makkar, whereas with the acid butanol the concentrations detected in this substrate were negligible.

To follow with the study of the chemical nature of antimethane agents, ULE tested extracts of *Carduus pycnocephalus* L. and *Quercus robur* L. provided by RRI (partner 1) after extraction with different organic non-polar solvents. These assays showed no antimethanogenic effect, indicating that either the active ingredient had been diluted out or destroyed during the extraction procedures.

DL15 Persistence and chemical nature of anti-bloat and anti-lactic acidosis agents

While the inclusion of *U. dioica* and *L. sativa*, as plant material, in previous studies had produced a significant dose related treatment effect with respect to the maintenance of a higher fermentation medium pH, no such effects were identified in this study. The solvents selected differed in their polarities and therefore likely to extract a range of molecules, especially lipid-soluble or lipid-associated molecules. However the lack of effect suggests that the active component is either found in an alternative fraction e.g. water soluble or that a problem with preparation of the extracts occurred. The small quantity of material available may also have caused problems as might the inability of the extract to solubilise in the fermentation medium and so remain “inactive”.

DL16 Palatability and toxicity determination

Acceptability – short term. The first three plants tested (*L. sativa, K. arvensis* and *B. perennis*) produced very different intake patterns. Although little interest was shown in any of the plants in the first three day period, intake of *L. sativa* improved over the second period suggesting an increasing degree of acceptance. However that of *K. arvensis* was minimal throughout, while intake of *B. perennis* decreased, indicative of poor acceptability and even a possible aversion, respectively. In contrast the second study where *U. doicia*, *P. pelatum* and *C. pycnocephalus* were offered, resulted in much higher intakes even in the initial three day period. Although the sheep offered *U. doicia* tended to consume the leaves first then the stem the entire allocation was rapidly consumed. A similar pattern was identified for *C. pycnocephalus*, while that of *P. pelatum* increased although the material was not totally consumed. These results

suggest that when offered alone, intakes of *K. arvensis* and *B. perennis* will be limited.

Acceptability – long term. Combining the test material with hay produced a slightly different intake pattern to that observed with the short-term studies. With *L. sativa, K. arvensis* and *B. perennis* total intakes gradually increased over the first four or five days to about 1100 g daily with *L. sativa and B. perennis* , but only to 800 g for the sheep offered *K. arvensis*. While when *U. doicia* and *C. pycnocephalus* were offered, intakes of both (despite their defensive mechanisms still being active) were high and in some cases totally consumed. In contrast *P. pelatum* intake declined over the ten day feeding study suggesting an aversion to the material, even in a mixed diet. It is therefore concluded that lettuce, daisy, thistles and nettles were all readily consumed as part of a mixed diet, however neither *K. arvensis* nor *P. pelatum* (especially at higher levels) were acceptable to sheep offered hay.

Tolerance. The ability of sheep to tolerate the test substrates was examined by comparing the concentrations of various blood metabolites and enzymes at the start and end of each phase. These sheep were all aged, and the possibility that they had some pre-existing liver fibrosis or other tissue damage from previous infections was high. When analysing the results, it was observed that many values were outside the normal range expected. Therefore to analyse the results, group means were taken for each metabolite/enzyme, and the population mean and standard error calculated. Any adverse effect imposed on the physiology of the sheep by the test material was assumed if the activity or concentration of that metabolite in the second sample (day 21 or 22) differed from that of the population mean for that set of samples.

With the exception of the *B. perennis* group no marked differences in blood metabolites or enzyme levels were found as a result of including any of the plant materials in the diet. Although the enzyme activities were highly variable within groups, one individual offered *B. perennis* showed an increase in all enzymes assayed over the trial period. Overall the activities of GLDH and AST measured for this group were 50 % higher than the population mean; 109 and 219 U/l, compared to the population means of 65.2 and 157.4 U/l for GLDH and AST, respectively.

From this tolerance study it can be observed that there are no marked adverse reactions to any plant of blood metabolites or serum liver enzymes examined. A possible exception could be the slight increase in two enzymes found with the sheep offered *B. perennis*. However it cannot be concluded that the consumption of daisies by sheep causes some slight hepatic or other damage, as the sheep allocated to this group may have had some pre-existing condition. In addition the short-term nature of this study, levels offered and poor replication have to be considered. Live weight decreased in all sheep over the experimental period and is not considered to be treatment related.

DL17 Antibacterial effects, population analysis

The DGGE banding patterns from samples of the antiproteolytic trial (E073) revealed a strong influence of the *in vitro* conditions on community structure. Further, the influence of the feeding level masked the effect of the plant addition. Thus, no clear influence of *Knautia arvensis* on investigated microbial groups was obtained with the samples chosen. DNA samples of fermenter experiment F4 or the currently performed

combined fermenter / *in vivo* trial with cannulated sheep using P1 and P2 would probably yield better results. However, these samples had not been not available in time for molecular analysis within the project.

The banding patterns from samples of the antiprotozoal trial (R017) showed an influence of the plant addition on all bacterial groups studied, but not on protozoa. Although protozoa were the organisms targeted by the addition of *Bellis perennis*, there was no reduction in the number of bands or any obvious influence of the diet on bandings patterns Sequencing of selected excised bands would be of great interest.

On all levels studied an influence of time was visible in the appearance/disappearance of single bands. The banding patterns from samples of the antiacidotic trial (A049) revealed an influence of the "acidotic" diet or time of feeding of the concentrate diet on the eubacterial and protozoal community structure; on more specific levels also indications of differences between control and nettles diet were obtained. As with the pure-culture studies, Gram-positive bacteria were generally more resistant to plant samples than Gram-negatives, althouth the proteolysis inhibitors had effects on the Gram-negative *Bacteroides*-like organisms.

DL18 Antibacterial effects, culture analysis

The pure-culture analysis illustrated a number of general features of interest. In general, the cellulolytic bacteria were more vulnerable to growth inhibition than other species.

Of the antiproteolytic plants, *G. asclepiadea* was harmless to all species, whereas *B. perennis* slowed growth of a few species, notably the cellulolytic *F. succinogenes* and the pectinolytic *L. multipara*; thus, once again, the *Gentiana* might be considered a potentially safer (in terms of fibre digestion) antiprotozoal candidate than *B. perennis*.

P. peltatum, one of the most potent antiproteolytic plants, slowed the growth of several species, being particularly toxic to *R. flavefaciens.* The protein-metabolising *Prevotella* spp. Were sensitive to *P. peltatum*, which is consistent with the high tannins content of *P. peltatum* reacting with the enzymes of *Prevotella* and interfering with the processes of protein, peptide and amino acid analysis. The other antiproteolytic sample, *K. arvensis*, was generally much less toxic.

Q. robur (also high in tannins) inhibited the different bacterial species in a manner similar to *P. peltatum*, but the other antimethanogenic sample, *C. pycnicephalus*, was generally non-toxic, except for *F. succinogenes.* It would be useful to know why the *Carduus* was effective. However, because the antimethanogenic activity declined both with time and throughout extraction, the active component was not identified.

Perhaps surprisingly, the antiacidosis samples had no influence on the growth of lactate- producing bacteria, but both inhibited the growth of *F. succinogenes* S85 and *L. sativa* inhibited *R. flavefaciens* Fd1 (Fig. 3.7.4).

In summary, cellulolytic bacteria were most vulnerable to the added plants. The tannins-containing plants, especially *P. peltatum* and *Quercus robur*, also affected other species, notably some *Prevotella.*

DL19 Product quality assessment

Knautia arvensis

In *in vitro* tests, *Knautia arvensis* was shown to decrease the proteolytic activity of rumen microbes such that at an inclusion level of 18% concentrations of soluble substrate protein after 12 h were ca. 60% higher than in the corresponding control. At the same time iso-SCFA were ca. 20% lower. This was an efficacy of ca. 50 % of that of 3 μM monensin in the same test system. General fermentation (gas, SCFA) was slightly enhanced in the presence of *K. arvensis*. The plant was therefore chosen for a feeding trial to test its efficacy *in vivo*.

Under the given experimental conditions the protein sparing effects of *K. arvensis* observed in vitro were not reflected in a corresponding positive response in the feeding trial. A few parameters did indicate a potential impact of the additive on the protein metabolism of lambs, but the effects were not consistent, neither within this experiment, nor in comparison to the production trial performed in Leon.

At UL, the initial weight of the lambs was 15 kg, and all of them were slaughtered when their weight approached 25 kg. The inclusion of *Knautia* in the concentrate did not affect lamb growth rate, nor was there any significant effect of the plant additive on wool growth. Feed digestibility was unaffected by the plant additive. Although protein digestibility was 6% lower and N balance 23% lower with the *Knautia* concentrate compared with the control group, differences did not reach the level of statistical significance ($P > 0.10$). There were no significant differences between experimental treatments in the carcass weight, kill-out or subjective indicators (conformation and fat scores or fat consistency) of carcass quality, or in the percentage of the commercial cuts obtained of the lambs used in this experiment. There was a significant difference ($P < 0.05$) between the control and the *Knautia* groups in the protein content of the carcass, which was 8% lower in the carcass of the lambs receiving the plant additive. As a consequence, the carcass fat content tended ($P < 0.10$) to be higher in the lambs of this latter group. This result was unexpected, although could be associated with the lower protein digestibility and N balance observed with the *Knautia* diet. In agreement with the results observed for the chemical composition of the carcass, the protein content of the muscle longissimus dorsi was 5% higher ($P < 0.05$) in the lambs of the control group than in those receiving the plant additive. No significant differences between experimental groups were observed in the other chemical components, or in other meat characteristics, such as pH, water holding capacity or cooking losses. As for the sensorial quality, no differences between experimental groups were detected in the "instrumental" meat attributes (colour and texture or hardness). The results of the sensorial assays were: 11 correct judgements (out of 24 tasters) in the triangular test, and 12 (out of 24) tasters showing a preference for the chops from lambs of the *Knautia* group. With these data, the conclusion is that there is no significant ($P > 0.05$) discrimination or preference between meats of animals from both experimental groups. In comparison with the control group, the inclusion of *Knautia* in the concentrate did not have any significant effect on the ruminal mucosa or on the fermentation parameters measured in the rumen contents of the lambs collected post-mortem.

Bellis perennis

According to the *in vitro* screening, *B. perennis* had been identified as a top candidate plant for antiprotozoal effects. Its effects were shown to be dosage dependent and, based on a 24-h in vitro test, predicted to be persistent. The plant material was readily palatable to sheep. Based on these results the plant was chosen for a feeding trial with

sheep, in order to prove both the effects on rumen function and the persistency *in vivo*.

The UHOH trial with *B. perennis* indicated that, although a partial defaunation could be detected based on the rumen fluid samples, this difference was not manifested in animal performance. All balance data collected were equal for treatment and control group. While the diet was formulated to have a protein content that would give a response to increased protein flow, the increase in protein flow may not have been large enough to detect in a sufficiently large response to be detected on this scale. Over the entire course of the UHOH experiment, the sheep showed a steady weight gain of (on average) 122 g/d for the control and 148 g/d for the treatment group, although the difference between the diets was not statistically significant. It is worth noting that the effects of monensin, which is used universally in North America for economic reasons, has a response of only 7%, which would probably not have been detected here. Thus, the absence of response should not lead to the immediate dismissal of *B. perennis* as an antiprotozoal feed additive. There was an obvious trend for higher counts when the control diet was fed, and lower counts in the presence of daisy, even after the diets were switched over. This, however, was often obscured by the high standard deviations. With respect to general fermentation patterns, there was a slight decrease in average total SCFA concentration (101.8 vs. 94.9 mM) and a slight increase in iso-SCFA (1.36 vs. 1.50) in response to the addition of daisy ($p<0.05$ and $p<0.01$). C2/C3 ratio and NH4 concentration were not affected. A slightly lower average concentration of soluble protein was observed in the presence of daisy (0.366 vs. 0.396), but as only a subset of samples (212 data points) was analysed for this parameter the difference was not significant. All fermentation parameters were highly dependent on the individual sheep.

Methanogenic archea are reported to live in close association with rumen protozoa, so that partial defaunation might also influence methane production by the community. Based on the respiration data in the UHOH trial, the CH_4/CO_2 ratio was calculated. There were no differences between control and treatment group.

At UL, both concentrate feeds had similar energy and protein contents in the lamb trial. The protein content was relatively low (12%). Sugar beet pulp was included as a source of soluble fibre and both concentrates included bicarbonate to buffer possible drops of pH in the rumen associated with the fermentation of non-structural carbohydrates of cereals. With these ingredients it was intended to favour the presence and growth of ruminal protozoa. Under these circumstances, if the plant additive has any defaunating effect suppressing some of the protozoal population, it could be expected that a greater amount of protein would reach the small intestine resulting in an increase of the protein supply to the animal. The hypothesis is that the final result would be some improvement in the growing lamb performance. The initial weight of the lambs was 15 kg, and all of them were slaughtered when their weight approached 25 kg. The inclusion of *Bellis* in the concentrate did not affect lamb growth rate. There was not any significant effect of the plant additive on wool growth. The daily intake of concentrate and straw, and feed conversion rate were similar when lambs received the concentrate containing *Bellis* compared with those receiving the control concentrate. Feed digestibility and N balance were unaffected by the plant additive. There were no significant ($P > 0.05$) differences between experimental treatments in the carcass weight, kill-out or subjective indicators (conformation and fat scores or fat consistency) of carcass quality or in the percentage of the commercial cuts obtained of the lambs used in this experiment. Feeding the lambs with the concentrate containing *Bellis* did not have any significant effect on the

chemical composition of the carcass. In agreement with the results observed for the chemical composition of the carcass, no significant differences between experimental groups were observed in the meat chemical components determined in the muscle longissimus dorsi. Other meat characteristics, such as pH, water holding capacity or cooking losses were not affected by the inclusion of *Bellis* (5%) in the concentrate. As for the sensorial quality, no differences between experimental groups were detected in the "instrumental" meat attributes (colour and texture or hardness). The results of the sensorial assays were: 12 correct judgements (out of 24 tasters) in the triangular test, and 13 (out of 24) tasters showing a preference for the chops from lambs of the *Bellis* group. With these data, the conclusion is that there is no significant ($P > 0.05$) discrimination or preference between meats of animals from both experimental groups. In comparison with the control group, the inclusion of *Bellis* in the concentrate did not have any significant effect on the ruminal mucosa or on the fermentation parameters measured in the rumen contents of the lambs collected post-mortem.

Urtica dioica

In the initial screening, *Urtica dioica* decreased the concentration of H^+ ions by 23% during 24h fermentation in the *in vitro* RPT system at an inclusion rate of 10% using ground wheat as a substrate. This *in vivo* trial aimed to investigate the possible effect of a 5% inclusion of *Urtica dioica* under an acidotic feeding regime on the pH of sheep.

The results from the continuous pH measurements *in vivo* at UHOH indicate that the diet N5A (inclusion of 5% nettles) induced higher concentrations of H^+ ions in the rumen compared to the control feed N0A. These results do not support, but are contrary to the results obtained in the screening and persistence studies in batch *in vitro* systems (RPT, RUSITEC), in which nettles reduced acidity. However, in these *in vitro* experiments either 100% ground wheat or a mixture of 30% wheat and 70% maize silage served as substrates, whereas in the *in vivo* trial the diet was based on various grains (16% wheat, 44% barley, 12% maize). It cannot be excluded that the efficacy of the plant additive is highly depending on the composition of the diet. The experimental diets should have been tested with the *in vitro* system used for the screening to exclude this possibility before starting the *in vivo* trials.

Lactate concentrations were generally low except of temporarily high concentrations in single samples taken shortly after feeding. However, from these concentrations it can be concluded, that the chosen feeding regime did not lead to an accumulation of lactate, which can occasionally reach 100 mM under acidotic feeding conditions. In accordance pH values did not correlate with lactate concentration but with the concentration of short chain fatty acids or total acid load (sum of lactate and short chain fatty acids).

At León, both concentrate feeds had similar energy and protein contents. The protein content was relatively high (14%). The concentrates had a high percentage of cereals, including wheat, and no fibre sources or no buffers (bicarbonate) were included in the concentrate. Thus, the aim was to have a very acidogenic feed, with a fast fermentation of starch and low buffering capacity that may cause marked drops in ruminal pH. Under these circumstances, if the plant additive had any buffering effect alleviating the effects of a low pH in the rumen, it could be expected that a higher and more stable feed intake, and also an enhancement of animal welfare (preventing the effects of acidosis). The hypothesis is that the final result would be some improvement in the growing lamb performance.

The initial weight of the lambs was 15 kg, and all of them were slaughtered when their weight approached 25 kg. The daily weight gain was 9% higher in the lambs fed the *Urtica* concentrate, although the difference with the average daily gain observed in the lambs of the control group was not statistically significant ($P > 0.05$; Table 3.3.2). There was not any significant effect of the plant additive on wool growth. The daily intake of concentrate was 9% higher when lambs received the concentrate containing *Urtica* compared with those receiving the control concentrate (Table 3.3.3), and this difference tended ($P < 0.10$) to be significant. The average intake of straw was unaffected. As both growth rate and intake were increased to the same extent, the average feed conversion rate was the same in both experimental groups. Feed digestibility and N balance were unaffected by the plant additive (Table 3.3.4). It is noteworthy that fibre digestibility was 14% higher with the concentrate containing *Urtica*, although teh difference was not significant ($P > 0.05$). Although the weight at slaughter was the same in both groups, the carcass weight was 4% greater ($P < 0.05$) for the lambs receiving the plant additive. There were no significant ($P > 0.05$) differences between experimental treatments in the kill-out or subjective indicators (conformation and fat scores or fat consistency) of carcass quality, or in the percentage of the commercial cuts obtained of the lambs used in this experiment. Feeding the lambs with the concentrate containing *Urtica* did not have any significant effect on the chemical composition of the carcass. In agreement with the results observed for the chemical composition of the carcass, no significant differences between experimental groups were observed in the meat chemical components determined in the muscle longissimus dorsi. Other meat characteristics, such as pH, water holding capacity or cooking losses were not affected by the inclusion of *Urtica* (5%) in the concentrate. As for the sensorial quality, no differences between experimental groups were detected in the "instrumental" meat attributes (colour and texture or hardness). The results of the sensorial assays were: 12 correct judgements (out of 24 tasters) in the triangular test, and 10 (out of 24) tasters showing a preference for the chops from lambs of the *Urtica* group. With these data, the conclusion is that there is no significant ($P > 0.05$) discrimination or preference between meats of animals from both experimental groups. In comparison with the control group, the inclusion of *Urtica* in the concentrate had a significant effect on number of papillae per cm^2 the ruminal mucosa). Under ruminal acidosis, the number of papillae tends to decrease as the mucosa is damaged as a consequence of low pH values in the rumen digesta. There was not a significant effect of the administration of *Urtica* on blood parameters considered indicators of the acid-base status of the animals, such as pH or bicarbonate concentration. Most of the fermentation parameters measured in the rumen contents of the lambs were unaffected by the administration of *Urtica* in the concentrate. However, there was a significant ($P < 0.05$) difference between both experimental groups in the concentration of lactate in the rumen contents. This acid is responsible for the low pH of the rumen digesta in situations of acidosis, and was decreased when animals received the concentrate containing the plant additive

DL20 Feasibility, technical, economic evaluation

General:

The interaction between the industrial and the academic partners was and is of great value and has already produced further co-operations within (REPLACE) and outside

(Contract Research) the RUMEN-UP consortium.

In vitro screening and databank:
The objective of this project was to investigate the potential use of plant extracts to solve welfare and environmental problems associated with ruminant livestock production. A major in vitro screening of 500 plants and plant extracts by the consortium was very useful with respect to the generation of information on these plants. The scale of the work carried out, would only have been possible with the joint cooperation of all the members of the consortium.
A number of useful developments were achieved. Generally, during product development, researchers actively look for methods to prevent or reduce the use of test animals. Where the use of test animals cannot be avoided, we try to reduce the number of test animals and to refine the experimental design to achieve this. In this sense, the development within this project of a set of *in vitro* test procedures to search for selected target features in materials is to be seen as an important step in such a direction. We expect that the refinement and optimization of the presently developed approaches will lead to a substantial decrease in the number of animals needed to develop new products.
Moreover those approaches could contribute to the development of *in-vitro* models to replace animal testing for licensing/registering purposes; further, studies would profit by a reduced use of animals e.g. to test for efficacy, safety and quality as needed in the production of nutritional, biological, and pharmaceutical products for animals.
The databank of information generated on the 500 plants and plant extracts will serve as a useful source for the potential development of products in the future. In addition, it currently serves as a starting point for the FP 6 project Replace.

Selection of candidate materials:
The main selection criteria for materials entering final test stages were exclusively of scientific nature. We suggest that, in other similar projects, additional technical and practical parameters of such materials, such as supply, drying, transport, long term availability and potential to cultivate, be taken into consideration at early stages of the selection procedure.

Animal trials:
The somewhat disappointing results of the animal trials emphasize the fact that attempts to correlate *in vitro* data with their *in vivo* counterparts are by far not simple processes. The *in vivo* trials of the present project provided many positive indications; since most of those results were not statistically significant, it was – retrospectively considered - extremely optimistic to hope that such limited *in vivo* trials would provide a definitive proof of the target effect(s). Industrial experience shows that often a number of trials are required before conditions such as dose rate, animal condition, and other factors become sufficiently well known to allow to determine and to demonstrate significant positive effects. For example, known highly successful products such as ionophores and essential oils would very likely share the same kind of success in a similar project as those in the trials carried out in the present project.

Knowledge protection:
The protection of the knowledge, raised from the project, via a patent filing is important for potential future implementation of commercial products. The real value of this patent can be evaluated at the time when products will be launched on the

market.

Possible industrial implementations:
As described above, due to the lack of clearly positive answers of the selected materials from the animal trials, a direct implementation of project results into a commercial product is not foreseeable in the near future; however, the commercial partners do not exclude it for the more distant future. Further trials have to be performed before a decision can be taken to finally develop a product.

Overall conclusions and considerations:
Strengths of this project:
The main strength of this project is the quantity of data generated about a diverse collection of plant extracts. This data will be useful in the future as the requirement for natural alternatives to chemical additives in the animal feed industry will become more important.
The opportunity for the range of scientists from different institutes to co-operation in this project has been important. This co-operation has led to various collaborations between the partners and between the industrial partners and the academic institutes. Furthermore, it has led to the subsequent participation in a Framework Programme 6 project (Replace).
Weaknesses of this project:
There was a lack of foresight as to the risks involved with the collection process for the 500 plants, such as seasonability of some plants and the difficulty obtaining large quantities of the plants
Many of the plants showed positive results during *in vitro* screening however, when the *in vivo* trials were carried out, results were not as positive. Perhaps a preliminary dose response trial should have been incorporated in to the project.

DL21 Project evaluation and recommendations

All partners agreed that the project had been successful, using criteria that all project deliverables had been delivered and milestones passed. Additionally, the exploitation would benefit all partners. Further important outcomes of the project were the strong academic links formed between the partners and the experience gained, particularly with respect to being able to anticipate problems of seasonality and availability of plant materials for anlaysis, particularly on a field-trial scale.

The partners concurred with the conclusions made by the commercial partners on commercial and feasibility aspects (Annex C).

The development of the work into new areas of application, using the common resource of the plant collection, was agreed and implemented via the REPLACE project in FP6.

The partners were in agreement that the collection, its attendant descriptions and general effects on ruminal fermentation formed a body of information that they had not anticipated would be so rich in potential yet which, for most of the samples, seems certain to be underexploitated. The partners and the Commission should look for ways of making certain the this value is maximised once the confidentiality period has passed.