2 Material and methods

2.1 Sample collection and processing

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.

2.2 Gas production, fermentation properties

2.2.1 Hohenheim

General fermentation parameters. General fermentation parameters of the 500 Rumen-up plant samples were determined in short term batch incubations using the Reading Pressure Technique (RPT) in principle as described by Mauricio et al (1999). Incubations were conducted in pre-warmed 125 ml serum flasks sealed with rubber stoppers. Rumen fluid was collected manually prior to morning feeding from three cannulated non-lactating Holstein cows receiving a grass silage hay mix at maintenance fed in two equal portions at 8.00 h and 16.00 h. Rumen fluid was filtered through a 100 µm nylon net and diluted 1:10 with pre-warmed reduced buffer medium (Table 2.2.1.1). All steps were carried out at 39°C under CO_2 to maintain anaerobic conditions. Substrates were incubated at a ratio of 10 mg/ml medium, i.e. 750 mg of 1 mm ground grass silage was incubated with 75 ml of buffered rumen fluid containing 7·5 ml of filtered rumen fluid (10% v/v rumen fluid). Total running time was 24 h. Gas volume or the pressure was recorded at 2, 4, 6, 8, 10, 12, 16, and 24 h of incubation and samples were taken after 6, 8, 10, and 24 h. Six flasks containing only buffered medium and rumen fluid inoculum served as blanks to correct for gas release and fermentation residues resulting from the inoculum. Six flasks containing buffer-inoculum mixture and grass silage only served as controls. Test plants were added at an inclusion level of 10% replacing the corresponding amount of grass silage. The effects of each plant were tested in three independent runs with rumen fluid of three different donor animals in four parallel flasks, two of which served for repeated sampling and two for gas reading and determination of digestibility after 24 h.

Table 2.2.2.1 Composition of buffer medium, reducing solution and micromineral solution used for RPT incubation.

RPT buffer

	Components	final conc.
	$NH_4 (CO_3)_2$	13·5 mM
	$Na (CO_3)_2$	86·5 mM
	Na_2HPO_3	5·5 mM
	KH_2PO_3	9·5 mM
	$Mg SO_4 7xH_2O$	0·5 mM
	Microminerals	0·020 %
1 %	Reszurine	0·001 %
	dH_2O	
	Reducing solution	6 %
	Rumen fluid	10 %

Reducing solution

	Components	final conc.
	Cystein HCl	0·118 M
1 M	NaOH	0·040 %
	Na_2S	0·026 N
	total volume	make up to with dH2O

0.5x Microminerals

Components	final conc.
$CaCl 2xH_2O$	0·45 M
$MnCl 4xH_2O$	0·25 M
$CoCl 6xH_2O$	0·02 M
$Fe (Cl)_3 6xH_2O$	0·15 M
total volume	make up to with dH2O

Analyses. Gas pressure was recorded in the serum bottles of the RPT system and converted into volume using an experimentally determined calibration curve. After each measurement the gas was released from the serum bottles.
In vitro true digestibility was determined by transferring the incubation content to preweighed polyester bags (Ankom 51 µm pore size). The bags were heat-sealed, cooked in neutral detergent solution for 1 hour, dried at 105 °C over night and weighted for determination of digestibility.
SCFA were determined by gas chromatography using a stainless steel column packed with GP 10% SP 1000 1% H_3PO_4, Chromosob W AW (Suppelco Inc. Bellafonte, PA) (Holtershinken et al. 1997). An aliquot of 900 µl samples was centrifuged (30,000 g, 10 min, 4°C) and to 0·9 ml of the incubation supernatant 0·1 ml formic acid containing the internal standard (1% Methylbutryric acid) was added. Proteins were precipitated over night at 4°C. Samples were centrifuged (30,000 ***g***, 10 min, 4°C) and the supernatant was collected into appropriate GC-vials for analysis.
RNA was extracted according to a modified phenol chloroform protocol from samples taken at the maximal gas production rate. 600 µl pH 5·1 phenol, 270 µl pH 5·1 buffer, 30 µl SDS (20% w/v) and 1·0 g of zirconium beads were added to the samples (300 µl). Cells were lysed by beating the samples for 2 x 2 minutes in the bead-beater (50 Hz) interrupted by a 10 min incubation at 60°C. Samples were cooled on ice for 10 min and 300 µl chloroform were added. After shaking vigorously and another 10 min at RT samples were centrifuged (10,000g, 5 min, 4°C) to separate aqueous and organic phases. Aqueous phase was removed quantitatively and transferred to a vial containing 300 µl NH_4-acetate (7·5M) and 900 µl isopropanol. Samples were incubated over night at -20°C and nucleic acids were precipitated by centrifugation (16,000g, 10 min, 4°C). Supernatant was discarded and the samples were washed once in 80% ethanol. Nucleic acids were loaded on agarose gels and quantified densitometrically after staining with ethidium bromide against a calibration curve (25 –300 ng/µl).

The RNA concentration (µg/ml) in the sample taken at the point of maximum gas production rate was used as indicator for microbial biomass. The fermentation efficiency was calculated according to (Blümmel 1997) as PF = mg truly digested substrate/ml gas produced (both at 24 h and per g substrate incubated).

2.2.2 Reading

A series of thirteen consecutive fermentations, one per week, was undertaken using the Reading Pressure Technique (RPT, Maurico *et al*. 1999), to identify potential effects from including these test materials on the fermentation and degradation characteristics of a basal forage (maize silage). All materials (508) were examined in triplicate and at two inclusion levels (100 and 400 mg g^{-1} DM substrate), with the exception of the "Crina" "oils" (10 and 40 mg g^{-1} inclusion levels). The maize silage was pre-dried and milled to pass a 2-mm screen. A total of 1.0 g blended substrate (silage plus substrate) was placed in each fermentation flask. Ninety ml buffer solution was then added, the flasks sealed and stored overnight at room temperature. Prior to inoculation flask contents were raised to 39°C. The inoculum was prepared from rumen fluid (hand-squeezed contents) obtained prior to feeding (07.00 h) from two lactating dairy cows offered a maize/grass silage:concentrate (60:40) ration *ad libitum*. The same cows were used for each sampling throughout this series of studies. The fluid was filtered through two layers of muslin and held under CO_2 at 39 °C until use. Ten ml prepared fluid was added to each flask and these incubated at 39 °C for the duration of the study. All feeds were examined in triplicate. Six negative controls

(buffered medium plus rumen fluid only) were included in each run to correct gas values and degradation residues for direct rumen fluid effects. In addition six flasks of unsupplemented maize silage (1.0 g flask^{-1}) were included in each run. Results within studies were expressed relative to the positive control values.

Headspace gas pressure readings were obtained 2, 4, 6, 8, 10, 12, 14, 16, 18, 21 and 24 h post-inoculation. Following the last measurement, 3.0 ml fermentation medium was sampled from each flask and bulked by test material / inclusion level and stored frozen (-20 °C) until later analysis for VFA composition (if required) using a Varian 3600 GC. Fermentation residues were recovered by filtering flask contents through tared 60 ml Gooch crucibles (porosity 1, 100 to 160 μm) under light vacuum. The residues were then dried (100 °C for 24 h), weighed, ashed (500 °C overnight) and reweighed. The quantity of dry matter (DM) and organic matter (OM) determined were used to assess iDMD and iOMD (*in vitro* DM and OM degradation, respectively). The extent (ml gas g^{-1} OM incubated) and rate of fermentation gas release (ml h^{-1}) were generated using a previously derived quadratic function to convert pressure to volume. Feed fermentation efficiency (FE) was estimated as iDMD (g kg^{-1})/cumulative gas (ml) release at 24 h post-inoculation. Statistical differences between treatment means and the positive control (maize silage alone) were assessed using Students T-test and significance declared at P>0.05). To identify possible beneficial plant materials for Phase II, all the following selection criteria were applied:

1. gas production should be decreased by 5%
2. iDMD should be increased by 3%, and
3. FE should be improved by 10%.

Substrates which fulfilled all three conditions were identified as beneficial to the rumen environment with respect to feedstuff degradation. Rankings were compiled of plants or plant products that both positively and negatively impacted on rumen fermentation and degradation characteristics.

2.2.3 León

This study was conducted with 500 samples, 100 collected from our group, 300 supplied by the other 3 academic partners and another 100 supplied by the commercial partners. Procedures for the trial were discussed and agreed in the meeting held in Leon in April 2002, and are described in detail elsewhere (López et al., 2005).

Rumen cannulated sheep fed on 1.2 kg of a diet consisting of 750 g/kg alfalfa hay and 250 g/kg barley (free access to vitamin/mineral supplements and water) were used as donors for rumen fluid. Rumen fluid was collected just before the morning meal.

The substrate used for the batch cultures was the same diet fed to the animals (750 g/kg alfalfa hay and 250 g/kg barley), milled in a hammer mill with a 1-mm screen. The amount of substrate used was 500 mg, and inclusion rate of the test plant was 10% (ca. 50 mg). Both were weighed into serum 120 mL bottles, where 50 ml of buffered rumen fluid (containing 10 ml of filtered rumen fluid and 40 ml of the medium described by Goering & Van Soest, 1970) were dispensed anaerobically. The bottles were sealed and incubated at 39ºC. After 24 h of incubation, the ensuing measurements were recorded:

- Total gas production (ml), using a pressure transducer and collecting the gas in a calibrated syringe following the procedure described by Theodorou et al. (1994).
- pH in the incubation medium.

- Volatile fatty acids (VFA) concentration in the incubation medium, by GC (VFA was also measured at inoculation time to calculate VFA production after 24 h).
- Dry-matter (DM) incubation residue, by filtering the bottle contents in sintered crucibles and weighing the residue after drying. Thereafter, the neutral detergent fibre (NDF) content of the residue was determined to calculate the amount of undegraded NDF. From this, DM and NDF disappearance were estimated. To calculate NDF disappearance, NDF content of the 450 plants used in the study was determined by the method of Van Soest et al. (1991).
- Indirect measurements: fermentation efficiency (digestible DM per ml gas produced) and microbial biomass (using digestible DM and total gas and VFA production).

Three replicates per plant were incubated, and blanks (no substrate no plant) and controls (500 mg substrate without any plant, and 550 mg substrate without any plant) were used.
Results were expressed relative to the control values. Statistical differences between treatment means and control (no plant added) were assessed using Student *t*-test declaring significance at $P < 0.05$.

2.3 Antiprotozoal assay

The initial screening of plant samples was carried out based on their ability to inhibit the breakdown of ^{14}C-leucine-labelled *Selenomonas ruminantium* as described by Wallace and McPherson (1987) and Newbold *et al.* (1997).

2.3.1 Preparation of labelled bacteria

S. ruminantium Z108 was maintained on modified Hobson's medium no. 2 (Hobson,1969). The medium contained 20% autoclaved and clarified rumen fluid that was obtained from a cow at the Rowett Research Institute. It was labelled by growing overnight at 39 ^{o}C in Wallace and McPherson medium (1987) containing [^{14}C]-leucine as the sole N source. Bacteria were harvested by centrifugation (3000 ***g***, 15 min) and resuspended in 7 ml of anaerobic Coleman's solution D containing 5 mM unlabelled L-leucine to prevent re-incorporation of released [^{14}C]leucine.

2.3.2 Bacteriolytic activity of protozoa

Ruminal fluid was obtained 2 h after feeding from three sheep and strained through two layers of muslin. A portion of strained ruminal fluid (4.5 ml) was added to a Hungate tube (Bellco Glass Inc., Vineland NJ. USA) containing 0 or 5 g/l ground (1 mm) sample final concentration, or 100 ppm for essential oils (dissolved in ethanol). This mixture was pre-incubated for 1 h at 39 ^{o}C before adding 0.5 ml of [^{14}C]leucine labelled *S. ruminantium*. Unlabelled L-leucine was included in all incubations at a final concentration of 5 mM to prevent re-incorporation of released [^{14}C]leucine. The incubation continued under CO_2 in shaking water bath (Grant Instrument Ltd, Cambridge, 80 strokes/min) at 39 ^{o}C. Samples (0.5 ml) were removed at 0 h and at 1 h intervals up to 3 h into microcentrifuge tubes containing 0.125 ml trichloroacetic acid (25%, w/v) which were centrifuged (11000 ***g***, 5 min) and samples of the supernatant fluid were counted by liquid-scintillation spectrometry (Packard 1900 CA, Berkshire, UK). The degradation of [^{14}C]leucine labelled *S. ruminantium* at each incubation time

was calculated from the acid-soluble radioactive label and expressed as a percentage of the total dpm present in labelled bacterial suspension. The rate of degradation per hour was calculated as difference from the linear portion of the degradation curve (normally 0-3 h). Six incubations were performed for each test plant, using rumen fluid from 3 sheep in duplicates. The rate of bacterial breakdown in the control (in the absence of test plants) is expressed as 100%. Given values indicate residual activity, such that e.g. 20% indicates an inhibition of protozoal activity by 80%.

2.4 Antiproteolytic activity

2.4.1 Adaptation of in vitro incubation system RPT to test proteolysis

The RPT-incubation system described above for the general screening was modified to serve as standardized test system for ruminal proteolysis. Various protein substrates as well as various concentrations and combinations thereof were tested to establish well reproducible protein degradation kinetics. Monensin was introduced in the system as external standard, and its optimum concentration in the RPT was determined. The typical effects described for monensin in literature were observed *in vitro*. Effects of antiproteolytic plant additives could thus be evaluated against the efficacy of monensin, allowing corrections for differences between inocula. The key features of the final test system for proteolysis are as follows:

Substrate consisted of 450 mg maize silage, 225 mg barley grain, 150 mg soy protein (Sigma), and 10 mg BSA (fractionV, Sigma) in 75 ml incubation volume. Test plants replaced the silage to the desired inclusion level. Controls and blanks were as described before. Monensin served as an additional control, applied in 11·25 µl ethanol to a final concentration of 3 µM. Incubation was conducted for 10 to 12 h with hourly gas reading and 5 sampling times per run. Samples were analysed for SCFA concentration and composition, ammonium, soluble protein and insoluble protein concentration, and protein patterns.

The system was used to screen a shortlist of 21 candidate plants (18% inclusion rate) and select the final samples included in the patent. It was further applied to test the efficacy of different accessions of the top candidate, *Knautia arvensis*, and the fractions obtained by different solvent extractions. Dosage studies with entire plant material and with the methanol extract were done with this system. In order to identify effects due to tannins in the plant additive, parallel incubations were performed with and without 450 mg polyethylenglycol (PEG).

2.4.2 Analyses

Additional analytical methods were required to study protein degradation by the rumen microbes.

Protein concentration was quantified by dot blot (Hoffmann et al. 2002). Samples were centrifuged (30000 g, 10 min, 4°C), and soluble protein was directly determined from the supernatant. The corresponding pellets were solubilized in denaturing buffer (Laemmli 1970) to determine insoluble protein, which comprises both, microbial protein and precipitated substrate protein. Individual substrate protein bands were traced, either in the supernatant or in the pellet, after separation by PAGE according to Laemmli (1970) and Neuhoff et al. (1985).

Ammonium was determined by the phenol-hypochlorite method, a photometric assay according to Koroleff (1976).

2.4.3 ^{14}C-labelling method

Samples of ruminal fluid were removed 2 h after morning feeding and strained through 2 layers of muslin cloth before use in experimental measurements. Ruminal degradation of proteins was investigated using casein (Sigma Chemical Co, Poole, UK) reductively methylated with [^{14}C] formaldehyde (Wallace, 1983). 0 or 5 g/l ground (1 mm) sample final concentration was added to the incubation mixture, or 100 ppm for essential oils (dissolved in ethanol . The assay contained 1.0 ml strained ruminal fluid to which was added 2.0 ml anaerobic 50 mM potassium phosphate buffer, pH 7.0, containing 4 mg ^{14}C-labelled casein ml^{-1}. After 60 min incubation at 39 ^{0}C, the reaction was stopped by the addition of 1 ml 25% trichloroacetic acid. Samples were chilled at 4 ^{0}C and then centrifuged at 13,000 ***g*** for 5 min. Incubations were carried out in duplicate. Acid-soluble ^{14}C in the supernatant fluid was measured by liquid-scintillation spectrometry. The activities of different sheep were analysed by ANOVA using Genstat 6 software.

2.4.4 Dosage and persistency studies Accessions of Knautia arvensis.
The materials tested included the original Rumen up sample derived from Spain in June 2001 (E073), a sample collected in Bühl, Germany, in September 2003 (E073-Bühl), and two commercial samples, K. arvensis from Rieger-Hofmann, harvested post-blooming in September 2003 (E073-Rieger), and Herba scabiosae from Galke GmbH, Germany, for which regional origin and time of harvest are unknown. (E073-Galke). A second batch was ordered from the latter supplier for the in vivo trials (E073-GalkeMS).

Dosage. Dosage studies were performed in the RPT system with inclusion levels of 12, 18 and 24% of the entire plant material E073 and using the 95% methanol extract of E073 with 3·6, 7·2, 10·8, 14·4 and 18·0% inclusion levels, corresponding to final concentrations of 0·4, 0·8, 1·2, 1·6, 2·0 mg/ml.

Persistency studies in the continuous fermenter. A continuous fermenter system was used to test the efficacy of *K. arvensis* or its methanol extract in long-term incubations and evaluate the persistence of the effect. The system is described in detail in Mützel et al (2005). Briefly, the fermenter unit consists of six fermentation vessels running simultaneously. Automated feeders dispense substrate in two feeding periods per day, simulating the usual feeding regime of animals. Different velocities of stirring are possible to mimic rumen contraction. Incubations are run at 39°C with a buffer inflow rate of one fermenter volume every 24 hours (1Vol/d). Overflow is collected over a 24 h period in a 4°C water bath and sampled for chemical analyses and digestibility.

Fermentation is monitored by continuous pH and temperature measurements. A daily sample or samples during daily courses are withdrawn from each vessel to determine SCFA concentration and composition, and ammonia concentration. Additional parameters, such as protein concentration and pattern, enzyme activities, and microbial community composition were determined according to the analytical protocols detailed elsewhere.

Two experimental approaches were used to study persistency and adaptation:
1. Addition of BSA. A concentrated stock solution of BSA was added to the fermenter vessels shortly before morning feeding to a final concentration of 0·5 mg BSA/ml. Samples were taken at regular times, e.g. 0, 2, 4, 6 h after addition. Proteolysis was then monitored by dot blot (total protein) and PAGE (BSA). Different kinetics in treatments as compared to controls indicated efficacy.

2. RPT incubations with fermenter fluid. An aliquot of the fermenter content was diluted 1:4 and used to inoculate a short-term incubation (RPT), under the standardized conditions described above. Nine bottles were inoculated from each fermenter, three of which contained the control substrate, another three contained substrate plus additive, and the third set substrate plus monensin. If the inoculum originates from a control fermenter, a typical response should be observed in the RPT, i.e. protein degradation should be delayed in the presence of the additive as compared to the control bottles. If, on the other hand, the inoculum originates from a fermenter fed with the additive, a diminished or absent response will indicate an adaptation of the microbial community. Again, monensin served as standard.

Two runs were performed to test the effects of addition of *K. arvensis*. The substrate incubated was the same mix of maize silage, barley grain and soy protein as used in the RPT test system (56%, 27% and 18% of total substrate, respectively; BSA was omitted). In the first fermenter run three different feed levels were applied, with 12, 20 and 28 g per fermenter and day, fed in 2 periods over 1, 2 and 3 h, respectively, and referred to as low, medium and high feed level. *K. arvensis* was added to one of the two parallels of each level at a ratio of 20% of the total substrate, replacing the silage, from fifth day of the experiment onwards. Daily samples were withdrawn at 14.00 h. Protein degradation was monitored by BSA addition on days 5, 6, 11 and 15, and by a final RPT incubation on day 20. The second fermenter run used a medium feed level of 18 g fed in two periods of 1 h. Two vessels served as controls, two received the entire plant material as additive (20% of total substrate, as before), and two a methanol extract from *K. arvensis* (2 mg/d, equivalent to 20% plant material), the latter applied in a single dose during morning feeding. Daily samples were taken at 14.00 h, protein degradation was monitored by BSA addition on days 2, 7 and 14.

2.5 Acidosis and bloat screening

2.5.1 Acidosis

All Rumen-Up samples were evaluated using an *in vitro* screen in which the maintenance of fermentation pH and suppression of lactic acid production were considered indicators of their potential to offset acidotic effects. Eight consecutive 48-h fermentation series were conducted, with each substrate examined in triplicate. Ten negative controls (buffered rumen fluid) and ten positive controls (buffered rumen fluid plus milled wheat) were included in each series.

Milled wheat (2 mm) was used as the basal substrate and the test materials included at 100 mg g^{-1} DM (or 10 mg g^{-1} DM for the Crina samples). Each 125 ml flask contained 1.0 g substrate and 90 ml buffer solution, prepared at 0.5 standard concentration to allow for the buffering capacity of the incubation medium to be exceeded, pH to decline and the growth of *Lactobacillus* spp. and other lactic acid-producing bacteria, encouraged. Ten ml prepared rumen fluid, obtained from two fistulated cows offered an early lactation ration with a high level of coarse milled wheat, was added to each flask. Fluid pH measurements were made one hour post-inoculation (start pH) then at 24 and 48 h, by inserting a pH electrode directly into each flask. Immediately following the last measurement approximately 3.0 ml fluid were taken from each flask, bulked by sample and stored frozen at –20 °C until analysed for L(+) lactic acid by a method, based on that proposed by Gutmann and Wahlefield (1974). Readings were made using a UV-1201 Shimadzu spectrophotometer at 340nm.

Following the Hohenheim meeting, it was considered that due to insufficient time between trial completion and selection, the initial data should be re-assessed (as detailed below). From that 100 samples were selected for further examination.

Using data from the first set of acidity results, samples with acidity values (calculated on the basis of hydrogen ion concentration) <0.8 controls were selected. Of these any showing a decreased in iDMD (24 h values @ 10% inclusion) or increase in gas release relative to the controls were rejected. The 131 remaining 105 substrates were then ranked in order of fermentation efficiency and the top 100 selected for re-examined. In addition twenty samples considered acidogenic, were included as positive controls. Lactic acid values from the first study were not used in this selection due to the marginal levels generated.

The *in vitro* screen used was similar to the initial (i.e. 50% buffer solution, 48 h incubation period and rumen fluid from a similar source) except that the quantity of milled wheat incubated was increased to 2.0 g. Incubations were again conducted in triplicate, with the basal pH values taken 60 min post-inoculation to allow the flasks to equilibrate, then at 24 and 48 h. Samples of the incubation medium were taken at 48h, bulked and stored frozen (-20 °C) prior to analysis for D and L-lactic acids using the same methodology as in the initial screen.

2.5.2 Bloat

Two models were examined in an effort to estimate whether the Rumen-Up samples showed any potential to ameliorate the range of parameters associated with the condition known as bloat or ruminal typmany. In this condition ruminants are unable to release fermentation gases due either to its excessive rate of production or to a thickening of the ruminal fluid (microbial mucilage) resulting in the production of foam-like consistency.

In the first model, the rate and extent of fermentation gas release from the degradation of red clover (*Trifolium pratense*) hay was examined together with an assessment of incubation medium viscosity at 24 h (fermentation end-point). These fermentations were conducted in triplicate, following the standard RPT methodology. As previously, 1.0 g blended substrate was incubated per flask with the test materials included at 100 and 10 mg g^{-1} DM. Viscosity was measured using a LVT Dial Viscometer (Brookfield Engineering, Ma., USA), the flask contents having first been initially filtered through a 2-mm metal sieve. In the second model foam height and strength were determined, by incubating 800 mg blended substrate in 200 mm pyrex test tubes (21 mm internal diameter). To this was added 10 ml buffer solution and two hours later (16.00 h), 30 ml prepared rumen fluid. The tubes were then closed with Parafilm in which a small hole was made to allow fermentation gas release and the tubes incubated at 39 °C. Appropriate controls were included. Foam height was measured after 8 and 16 h incubation, with the tubes shaken after the first measurement to disperse any accumulated foam. After the second measurement the tubes were secured in a custom-made rack. Foam strength was assessed by placing a weighted plunger (80 g, diameter 20 mm with a compressive force circa 0.25 g mm^{-2}) on to the surface of the foam and recording the vertical distance travelled after 15 seconds. For both models rumen fluid was obtained from two lactating dairy cows offered a diet containing 30% red clover hay, together with grass silage, hay and concentrate, *ad libitum*. For the first model fluid was taken prior to the morning feeding (07.00 h), while samples were obtained at 15.30 for the second. As previously, results are presented relative to those obtained from the controls (red clover only) within each fermentation series. Five and eight consecutive fermentation series were required to complete these assessments.

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

2.5.3.1 Persistence of effect

The ability of plant materials to maintain an elevated fermentation pH were examined by incubating the samples over a 48 h period using the Reading Pressure Technique (RPT, Mauricio *et al*., 1999), the inoculum for which was derived from either rumen fluid or following prolonged perturbation in the Rumen Simulation Technique (RUSITEC, Czerkawski and Breckenridge, 1977). Two substrates were examined – *Lactuca sativa* (lettuce) and *Urtica dioica* (stinging nettles). Both had been identified via the *in vitro* general fermentation and the acidosis screens. Both materials were pre-dried and ground (rotor-milled) to pass a 2 mm screen.

The study was designed to answer a number of questions, including:

1. whether the response to the materials was dose related
2. whether the effects could be repeated over time, and
3. the extent to which persistency maintained following long term exposure of the microflora to the material.

This was achieved using the following methodology. RPT fermentation flasks containing 1.0 g DM, comprising ground wheat (2 mm screen), supplemented with 0, 10, 20, 50 and 100 mg g^{-1} DM of the additive, were inoculated with 10 ml prepared rumen fluid, obtained pre-feeding (07.00h) from two cows offered a high concentrate / early lactation diet). Ten flasks per treatment were used together with negative controls (buffer plus rumen fluid). The standard RPT buffer was prepared using a 0.5 dilution and 90 ml dispensed into each flask. Gas release profiles were determined from five flasks over the 48 h incubation period, while the remaining flasks had permanent needles fitted to allow continuous gas release. Headspace gas pressure was assessed at 2, 4, 6, 8, 10, 12, 15, 19, 24, 30, 36 and 48 h – the measurements being taken prior to that for pH. Initial rumen fluid pH values were estimated as was flask pH at time zero (30 minutes post-inoculation) and after 24 and 48 h. The study was conducted three times, twice with rumen fluid at an interval of seven days, with the same cattle sampled on both occasions and on the second occasion also with an inoculum obtained from a controlled [RUSITEC] fermentation.

A standard RUSITEC protocol was applied to seven identical vessels to create and maintain a short-term [seven day] incubation, the fluid from which was used to provide an inoculum to evaluate persistency of effect. As such three control vessels and two vessels for each of the two treatments [*Lactuca* and *Urtica*] were used to ensure sufficient fluid for inoculation. The following fermentation parameters were applied:

Rumen fluid / solids: obtained concurrently with the material used in the first RPT study.
Infusion rate: ca 650 ml day^{-1}
Feeds: rolled wheat (RW), maize silage (MS)
Feeding level: 12 g increasing to 15g DM daily after three days
Treatments (in feed)
- controls: 0.3 RW / 0.7 MS
- treated: 0.27 RW / 0.63 MS / 0.1 nettles or lettuce

Gas: measured / discarded
Effluent: measured / discarded
pH: at servicing and at 16.00h daily

Duration: 7 days

The short-term incubation period was used to minimise divergence between vessels with similar treatments and to reduce the possible occurrence of experimental errors. In addition it was conjectured that if persistence was observable after seven days it was likely to be maintained. While the RUSITEC fermentations were not specifically designed to demonstrate treatment effects, but rather to produce inoculum for a further RPT study, observations were made with respect to gas and effluent volume and fermentation vessel pH. The vessels were inoculated with 500 ml rumen fluid (obtained simultaneously from the same cows as that material for the first of the two RPT studies), 200 ml buffer and 100 ml distilled water, together with 70 g rumen solids (hand-squeezed) retained with in a feed bag, replaced after 24 h (first service) with the test feed. After an initial three days for fermentation to equilibrate, followed by a seven-day incubation period, vessel fluid, including that from bag washings, was harvested and bulked by treatment.

RPT flasks were inoculated with either 20, 30, 40 or 50 ml bulked fluid to which 0.5 buffer and distilled water had been added such that the final liquid volume in all flasks was 100 ml (Table 2.5.3.1.1). Fluid from the control vessels was used to inoculate flasks containing 1.0 g DM wheat or wheat supplemented (100 mg g^{-1} DM) with lettuce or nettles, while the lettuce and nettle perturbed RUSITEC fluid was used to inoculate flasks containing wheat and either wheat + lettuce or wheat + nettles, respectively (**Table 2.5.3.1.2**). Negative controls (no substrate) were inoculated with fluid from all three treatments. At time zero (30 minutes post-inoculation) and at 24 and 48 h, pH was measured in all flasks. Headspace gas-pressure data were assessed as previously detailed with the measurement being taken prior to that for pH. All treatments were replicated four times with two flasks used to assess gas release kinetics.

Table 2.5.3.1.1 RUSITEC vessel fluid inclusion level

	Volumes (ml $flask^{-1}$)			
RUSITEC fluid	20	30	40	50
0.5 Buffer	60	40	20	0
Distilled water	20	30	40	50

Table 2.5.3.1.2 RUSITEC vessel fluid inoculations

RUSITEC vessels	Positive	Negative	Lettuce	Nettles
Controls	x	x	x	x
Lettuce	x	x	x	
Nettles	x	x		x

Direct treatment effects [additive / inclusion level] with respect to acidity were identified by examining the changes in hydrogen ion concentration both over time and relative to that of the controls. These values, derived from pH measurements, have been found to be a much more reliable measure than the use of pH values. In addition

the comparison of results obtained with control RUSITEC fluid and those obtained with the nettle or lettuce modified fluids using both control [wheat alone] or wheat plus either additive was used to identify persistency effects. This study was conducted simultaneously with the second RPT study using rumen fluid.

2.5.3.2 Chemical nature, solvent fractionation studies

Three solvent extracted fractions of *Urtica dioica* and *Lactuca sativa* were evaluated *in vitro* in an attempt to identify which contained the active component(s) considered responsible for the maintenance of the observed higher fermentation medium pH. From the available literature Lactucarium and chicoric acid are possible active compounds in *Lactuca*, while terpenes and formic acid are both present in *Urtica*. The fractions, prepared at the Rowett Research Institute, were first re-dissolved in the appropriate solvent [hexane, dichloromethane or methanol], then diluted, such that quantities equivalent to 20, 50 and 100 mg DM original material g^{-1} substrate were added to 70 ml fermentation flasks and the solvent evaporated under nitrogen. Four replicates were used per inclusion level, with 450 mg milled wheat and 40.5 ml 50% buffer solution added to each flask. These were stored overnight and the temperature raised to 39°C prior to inoculation with 4.5 ml prepared rumen fluid [obtained from two high producing dairy cows]. Rumen fluid pH and that of the flasks after 30 min [baseline], 24 and 48 h incubation were measured by inserting a pH probe into each flask. The values were examined as acidity following conversion to hydrogen ion concentration [H^+].

2.6 Antimethane activity

2.6.1 Initial screening

This study was conducted with 500 samples, 100 collected from our group, 300 supplied by the other 3 academic partners and another 100 supplied by the commercial partners. The study was carried out using batch cultures of mixed ruminal microorganisms and 24-h incubations of a diet consisting of 750 g/kg alfalfa hay and 250 g/kg barley, following the same procedures described in section 2.2.3. The diet to be used had been discussed and agreed in the meeting held in Leon in April 2002. It was a forage based diet, so that a high methane production per unit of degraded substrate was expected. Therefore, any inhibiting effect of the plant additive would be more noticeable. After 24 h of incubation, and once total gas production was measured, a representative sample of the gas produced was taken for subsequent analysis by gas chromatography. Sampling was conducted using special gas-tight glass syringes fitted with a valve allowing for sample locking and storage within the syringe. A sample was taken directly from the headspace, after inserting the needle through the septum of the stopper, taking a sample in the syringe and closing the valve. Finally, the needle of the syringe was inserted into the injection port of the GC, the valve opened and the sample injected. The GC Parameter Settings (configuration) in our laboratory were:

Instrument: Shimadzu GC-14B

Column: 2.3 meters length x 2.1 mm internal diameter stainless steel column packed with 60/80 mesh Carboxen 1000 stationary phase

Injection volume: 300 to 500 µl of gas injected directly into the GC using the special gas-tight syringe.

Carrier gas: Helium, flow rate (100 kPa), Constant flow mode

Detector: FID. Temperature 200ºC, Synthetic Air flow rate (50 kPa), H_2 flow rate (50 kPa)
Injector: Temperature 200ºC
Column-oven: 170ºC (isothermal)
In this screening, three replicates per plant were incubated, and blanks (no substrate no plant) and controls (500 mg substrate without any plant, and 550 mg substrate without any plant) were used. Results were expressed relative to the control values ($Effect\ (\%) = \frac{CH_4 additive}{CH_4 control} \times 100$), and statistical differences between treatment means and control (no plant added) were assessed using Student *t*-test declaring significance at $P < 0.05$.

2.6.2 Further selection
Based on the results obtained in the initial screening, and after evaluating the information available on the most promising plants, seven plant species were selected for 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 "batch effect" (plants had been tested in different incubation runs). The seven plants used in this assay were:
Carduus pycnocephalus (E-096)
Paeoniae alba radix (A-40)
Populus tremula (R-61)
Prunus avium (R-63)
Quercus robur (R-65)
Rheum nobile (R-66)
Salix caprea (R-69)
Batch cultures of mixed ruminal microorganisms using the same diet and methodology described in sections 2.2.3 and 2.6.1 were used, with the exception that:

- 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".

This design allowed for a more robust assessment of the effects of each plant additive relative to control values.

2.6.3 Dose response studies
The methodology (inoculum, substrate, medium, etc.) used in this trial is the same described in sections 2.2.3 and 2.6.1. In this case, the only difference is that selected plants were added at different inclusion levels in order to establish the dose response curve.
A preliminary single assay was carried out in which the seven plants used in setion 2.6.2 were added at rates of 8 and 16%, but results were not conclusive.
After assessment of results, the selected candidates for further assays were:
Carduus pycnocephalus (E-096)
Quercus robur (R-65)
Rheum nobile (R-66)
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

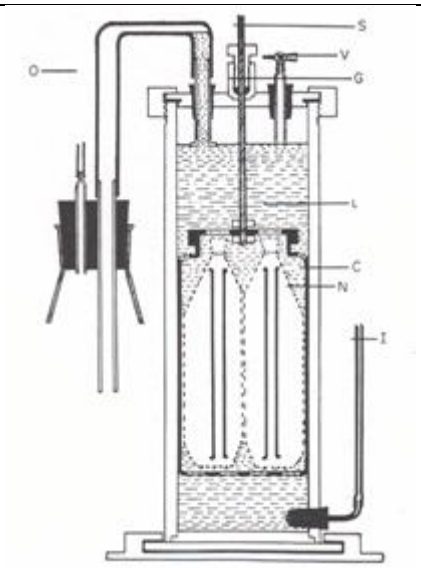
at 4 doses (5, 10, 15 and 20%) using a diet with 50% alfalfa hay, 40% grass hay and 10% barley. There were 3 incubation runs with 3 replicates for each experimental treatment (plant and dose) in each run, giving a total of 9 observations per treatment.
The experimental design followed for each plant additive is shown in the ensuing Table.

Experimental design of the dose response assays

Dose	Diet (mg)	Aditive (mg)	Total (mg)
5 %	400	20	420
10 %	400	45	445
15 %	400	70	470
20 %	400	100	500
0	400	0	400
(Control)	500	0	500

2.6.4 Persistency studies

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 rumen-simulation technique (Rusitec) was used as described by Czerkawski & Breckenridge (1977) and Newbold et al. (2005). The nominal volume in each reaction vessel was 850 mL and the dilution rate was set at 0.8 per day, the infused liquid being artificial saliva at pH 8.4. Inocula for the fermentation vessels were obtained from a pooled sample (liquid and particulate rumen contents) from three rumen-cannulated sheep fed 1.4 kg/d of alfalfa hay. On the first day of the experiment, 300 mL of strained rumen fluid and 300 mL of artificial saliva were transferred to each reaction vessel. Solid rumen contents (80 g) were weighed into a nylon bag which was placed inside the food container in each vessel together with a bag of food. The food was provided in nylon bags (pore size 50 □m) which were gently agitated in the liquid phase. Two bags were present at any time and one bag was replaced each day to give a 48 h incubation. The bags that were removed from the vessels were placed in plastic bags, and their contents washed and squeezed with 40 mL of artificial saliva. This was done twice for each bag, and the combined washings were poured back into the reaction vessels. Fermentation vessels were flushed with anaerobic grade CO_2 before filling, after filling, and then every day during feeding (when the nylon bags with the food were changed). The duration of each trial was 19 days (7 days adaptation + 12 days measurement). Gas samples were taken over days 8 to 19 and analysed for methane by gas liquid chromatography, as described in section 2.6.1.
Fermentation products were determined on samples taken from the liquid overflow. Fermentation acids (acetate, propionate and butyrate) were detected by gas chromatography and ammonia was measured by colorimetry using the phenol-hypochlorite method. The volume of the liquid overflow together with the concentration of fermentation products was used to calculate their daily output. pH was measured in samples of fermentation fluid withdrawn from around the nylon bags at the time of feeding. The digestibility of the diet was estimated from the dry matter remaining in the bags after 48-h incubation. Dry matter in feed samples and in incubation residues was determined by drying at 105° C for 48 h. Microbial protein was determined using N15 as microbial marker as described by Gomez et al. (2005).



I = infusion of saliva; N = nylon bag containing food; C = food container; L = liquid supernatant; S = stirrer; O = effluent ourput

Fig. 2.6.1 Schematic representation and portrait of a fermentation vessel of the Rumen SImutalion TEChnique (RUSITEC)

Experiment 1

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 weighed out in a small polyester bag, pore size 25 □m, 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.

Experiment 2

The second experiment consisted of only one trial 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 □m, 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.

2.6.5 Assessment of antimethane activity of solvent extracts

In these in vitro assays, the antimethane activity of solvent extracted fractions of *Carduus pycnocephalus* (CP) and *Quercus robur* (QR) was examined, trying to identify which fraction contained the active component(s) considered responsible for the effects observed when the plant material was used as additive in the incubations. Fractions were obtained at RRI after extraction with different organic non-polar solvents: n- Hexane (Hex), Dichloromethane (DCM) and Methanol (MeOH). The methods used for in vitro incubations and methane analysis have been described previously in sections 2.2.3 and 2.6.1, respectively. The substrate used in this case was 50% alfalfa hay + 40% grass hay + 10% barley.

Based on recovery data obtained at RRI, extracts were re-dissolved in corresponding solvent. Then, 50 μL of this solution were added to each culture. The amount of extract added was calculated to be equivalent to the amount that would be extracted from 50 mg of each plant. Calculated amounts of each extract added were:

	μg
CP-Hex	1.19
CP-DCM	0.80
CP-MeOH	6.72
QR-Hex	1.65
QR-DCM	1.33
QR-MeOH	3.95

Two in vitro assays were performed. In the first one the different extracts of the two plants were used at a single level of addition following the ensuing experimental design:

Experimental treatments

50 μL extract CP-Hex

50 μL extract CP-DCM

50 μL extract CP-MeOH

50 μL extract QR-Hex

50 μL extract QR-DCM

50 μL extract QR-MeOH

Control, 500 mg substrate, NO extract

Blank, no substrate no extract

In the second assay, the extracts showing the best results in the first assays were used at different levels of addition, according to an experimental design with the following experimental treatments:

Experimental treatments

50 µL extract CP-Hex

100 µL extract CP-Hex

200 µL extract CP-Hex

50 µL extract CP-DCM

100 µL extract CP-DCM

200 µL extract CP-DCM

100 µL extract CP-MeOH

Control, 500 mg substrate, NO extract

Blank, no substrate no extract

Four replicates per treatment were included to assess statistical significance of the effect relative to control cultures.

2.7 Acceptability/toxicity

Using a range of *in vitro* methodologies, twenty-three candidates from over 500 plant samples and extracts, with either anti-acidosis, -protozoa, -methanogenesis or anti-proteolytic activity were identified for potential patenting. From this six plant material were selected for examination in field trials. However as only the most suitable of these, within each mode of action, will be tested the following protocols were applied to evaluate these materials for their acceptability and tolerance by ruminants. Two studies, each with three feeds, were conducted and can be considered as comprising an "acceptability" and a "tolerance" phase with a total duration of 28 days. The six plant materials and their respective main identified activities are:

Bellis perennis (R017) – common daisy (anti-protozoal) [1]
Lactuca sativa (UR013) – lettuce (anti-acidosis) [2]
Knautia arvensis (E073) - field scabious (anti-proteolytic) [3]
Urtica dioicia (A049) – stinging nettle (anti-acidosis) [2]
Peltiphyllum pelatum (R114) – indian rhubarb (anti-proteolytic) [1]
Carduus pycnocephalus (E096) - slender or Italian thistle (anti-methane) [4]

The superscript refers to the collaborator who sourced these materials:

[1] Rowett Research Institute, Aberdeen, UK
[2] University of Reading, Reading, UK
[3] University of Hohenheim, Stuttgart, Germany
[4] University of León, León, Spain.

The plants were offered in a pre-dried, coarse chopped state. The experimental animals (barren mule ewes) were sourced from the CEDAR flock and were housed at the University's Animal Production Research Unit in individual pens. For the duration of the study they were offered grass hay at the maintenance level of feeding, with water and a salt lick freely available. They were individually penned and bedded on sawdust. Although it would have be preferable to conduct the work as a single study, due to availability of test substrates and animals, two studies were conducted using the same nine sheep. The studies were conducted with full compliance to any UK Home Office regulations with respect to good husbandry, welfare and ethics.

2.7.1 Acceptability study

This study was conducted using groups of three sheep, naïve to the plant material being examined. The sheep were weight blocked and allocated to groups such that total live weights were similar. Treatments were then randomly assigned to each group. Following an introductory period when the sheep were acclimatised to the housing, routine and basal diet (good quality grass hay) offered at maintenance, the test feed was offered. This involved removing the basal feed for a period of one hour at the same time (11.00h) each day for three consecutive days. In its place the sheep were offered a fixed quantity (50 g ± 1 g) of the test substrate. Eating behaviour was monitored including time taken for the feed to be consumed, any apparent aversion or appetite stimulation and feeding frequency. At the end of 60 minutes any residues were removed and weighed and the basal feed returned. This procedure was repeated after four days (five days for the first phase), such that intake behaviour was assessed on days 1, 2 and 3 and 8, 9 and 10 (9, 10 and 11 for first phase) days of the study. The pattern of consumption over the two periods was evaluated and an assessment made as to whether the feed was unpalatable (not eaten), poorly or well tolerated (small or medium proportion consumed) or readily consumed (all feed eaten rapidly). A further assessment of long-term "acceptability" was made in the second stage of the study - the "tolerance phase" - where sheep were offered hay plus the "additive" at maintenance as a mixed ration.

2.7.2 Tolerance study

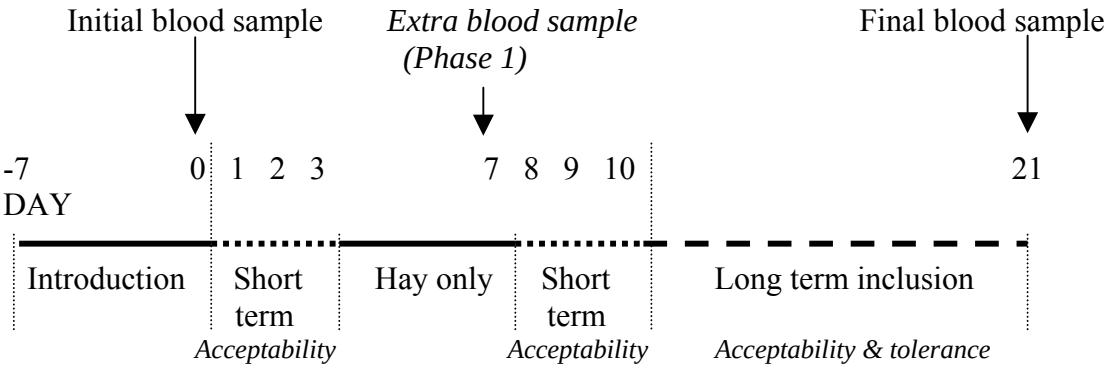
The second phase of the study provided an opportunity to examine whether the consumed "additive" was tolerated by the host animal as assessed by changes in blood biochemistry parameters and to examine long-term acceptability. It was not the intention of this study to identify whether these materials are toxic *per se*, but more that they produce no apparent adverse effects at inclusion levels likely to be required to produce the desired effect. The relative short-term nature of the study, together with the small number of animals was imposed in part by substrate availability and by a degree of prior knowledge of the biochemical activity of the samples which suggested no adverse effect, but in the main from an ethical / welfare point of view to minimise the number of animals used. The animals were offered hay at maintenance plus additive included at the 10 % level as a mixed ration. The ration was offered in two equal quantities at 08.00 and 16.00 h. Intake was measured daily over a ten-day period. Rejection of feed or the relative level of intake compared with pre-inclusion levels provided evidence of palatability effects under "mixed diet" conditions. Blood samples were taken from each animal at the start of the study (day 0), day 7 of the first phase and at the end of the continuous inclusion period by venupuncture of the jugular vein, using appropriate (size and treated) vacuutainers, by appropriately trained and HO licensed personnel. The samples were sent to Compton Paddocks Laboratories, (Newbury, Berkshire, UK) for analysis for the following metabolites and liver enzymes:

total protein, albumin, urea, glucose and β-hydroxy butyrate
GGT – gamma-glutamyl transferase
GLDH – glutamate dehydrogenase
AST – aspartate aminotransferase
ALT – alanine aminotransferase

The choice of these metabolites and enzymes was made after consultation with the local veterinary surgeon. The metabolites indicated whether the sheep protein and

energy requirements were satisfactorily supplied. GGT is widely used in diagnosing hepatic disorders in domestic animals as its activity is relatively high within the liver. GLDH is highly concentrated in the ovine liver also. AST is not liver specific but elevated serum activity indicates myocardial, skeletal diseases. Elevated serum ALT indicates hepatic necrosis. The experimental animals were then returned to the flock. The results from these analyses were discussed with two veterinary surgeons (Chris Trower (Larkmead Veterinary Group, Cholsey) and Maurice Allen (Compton Paddocks Laboratories, Newbury).

Trial Timetable:



Day 1 – 3 maintenance hay + 50 g sample offered for 1 h
Day 4 –7 maintenance hay only (2 meals/day)
Day 8-10 maintenance hay + 50 g sample offered for 1 h
Day 11-20 maintenance hay plus 100 g sample mixed (2 meals/day)

2.8 Antimicrobial effects

The antimicrobial activity of the samples was determined in two ways. The sensitivity of major bacterial species was determined by cultural means. Changes in community structure were investigated by retrospective molecular ecological analysis based on denaturing gradient gel electrophoresis (DGGE) of cloned 16S rDNA.

2.8.1 Culture methods

The influence of the selected plants on rumen microbial populations was examined using pure cultures of ruminal bacteria from the Rowett Research Institute culture collection.

The selected plant materials (35 mg) were gamma-irradiated at 25 kGy by Isotron Plc. in Bellco anaerobic culture tubes in order to sterilise them at low temperature for use with pure cultures. The growth medium was the liquid form of medium 2 of Hobson (1969). Seven ml were added aseptically to the Bellco tubes to give a final concentration of 5 mg/ml, then the tubes were inoculate 5% v/v with fresh culture, then incubated at 39 °C for 24 h. After 24 h incubation, the suspensions were filtered using filter paper (Whatman #1541, diameter 11cm, pore size 20-25 μm). The absorbance was read at 650 nm.

2.8.2 Molecular techniques for microbial community analysis

2.8.2.1 DNA extraction and PCR conditions

Aliquots of 300 µl sample were extracted using bead beating, hot phenol-chloroform and RNAse digestion. The primers Gm5f (Muyzer et al. 1995) and Gm1r (Muyzer et al. 1993) were used to amplify eubacterial 16S rDNA fragments, which were separated by denaturing gradient gel electrophoresis (DGGE). More specific primers for the amplification of 18S rRNA gene fragments of ciliates, and of 16S rDNA fragments of Gram positive bacteria and members of the Bacteroidetes were used to unravel shifts in specific microbial groups of interest. Primers, PCR conditions and specific settings for DGGE applied are given in Table 2.8.2.1. A 40 bp GC-rich nucleotide sequence (GC-clamp) was attached to the 5´-end of one of the primers to stabilize migration of the DNA fragments in DGGE (Muyzer et al. 1993). PCR amplifications were performed as follows: 1 µl of extracted DNA (1-10 ng), 10 pmol of each of the appropriate primers, 12·5 nmol of each deoxyribonucleoside triphosphate, 5 µl of 10x RedTaq^{TM} PCR buffer (Sigma, Deisenhofen, Germany), 5 µl bovine serum albumin (final concentration 3 mg ml^{-1}), and 0·5 units RedTaq^{TM} DNA polymerase (Sigma, Deisenhofen, Germany) were adjusted to a final volume of 50 µl with PCR-grade water. The amplicons were examined on 1·4% agarose gels stained with ethidium bromide (1 µg ml^{-1}) (Sambrook et al. 1989).

Table 2.8.2.1 Primers used in this study and their specificity, specific PCR and DGGE conditions applied, and references

Primer	Specificity	PCR	DGGE	Reference
Gm5f-clamp / Gm1R	Eubacteria	3 min 95°C; 2x (1 min 95°C; 1 min 65→56°C; 2 min 72°C); 16x (1 min 95°C; 1 min 55°C; 2 min 72°C); 7 min 72°C	40-70% denaturing gradient	Muyzer et al. 1995 Muyzer et al. 1993
LGCa / 685r-clamp	Gram +	2 min 95°C; 2x (1 min 95°C; 1 min 62→58°C; 2 min 72°C); 27x (1 min 95°C; 1 min 57°C; 2 min 72°C); 7 min 72°C	40-70% denaturing gradient	Meier et al. 1999 Johnson 1994
cf286f / cf719r-clamp	Bacteroidetes	3 min 95°C; 35x (1 min 95°C; 1 min 55°C; 2 min 72°C); 7 min 72°C	30-70% denaturing gradient	Weller et al. 2000
ciliF / ciliR-clamp	Ciliates	3 min 94°C; 37x (1 min 94°C; 1 min 60°C; 2 min 72°C); 8 min 72°C	30-60% denaturing gradient	Regensbogenova et al. 2004

2.8.2.2 DGGE and cluster analysis

DGGE gels of 0.75 mm thickness and 8% (wt/vol) polyacrylamide concentration were run with the CBS system (C.B.S. Scientific Company Inc., CA, USA) in 1x TAE for 17 h at a constant voltage of 100 V and stained with SYBRGold (Molecular Probes) according to the manufacturer's instruction. The denaturing gradient of the gels varied according to the primer pair used as shown in table 2. Bands of interest were excised and stored in 100 µl PCR-grade water at -20°C until further processing. Banding patterns of gels were analysed using pearson correlation as similarity coefficient and curve based UPGMA (unpaired group method of analysis) to generate the dendrogram (GelCompar, Applied Maths). Background subtraction was applied

depending on the signal-to-noise ratio of the corresponding gel and band position optimization was adjusted according to the calculations of the optimal position tolerance level performed by the software.

2.9 Solvent extraction, chemical analysis

2.9.1 Solvent extraction

Rowett extraction. Two types of extraction procedure were carried out to extract the active compound(s). In Method 1, samples were extracted in a soxhlet extraction apparatus (Fig. 2.9.1). Complete extraction of natural products requires a series of solvents varying in polarity. A succession of solvents (150 ml each) was used to extract 5 g of sample, beginning with petroleum ether, chloroform, and ethyl acetate, then more polar solvents, butanol and methanol, and finally water for the removal of the most polar components. The solvents were evaporated in a rotary evaporator at 40 C.

Fig. 2.9.1 Soxhlet extraction apparatus

In Method 2, samples were extracted successively with 250 ml of hexane, then dichloromethane (DCM), then methanol.

Hohenheim extractions and incubation of fractions.

Chloroform. Chloroform will remove very long chained aliphatic alcohols and cuticular waxes, which are likely to interfere with the biological activity of secondary compounds. Therefore part of the plant material was pre-treated with a chloroform rinse. Ten grams of freeze-dried material was ground to 1 mm, placed on a folded filter (Whatman no.1) in a glass funnel, and was slowly rinsed with 200 ml chloroform for 2 min. The residue was air-dried at 40-50°C and ground to a fine powder for further extractions.

Petrolether. Two to 3 g dry matter of either chloroform-extracted or untreated plant powder were extracted in a Soxhlett-apparatus according to common protocols at a

temperature of 90°C. Subsequently petrolether was evaporated from the extract and recovered as "oil" fraction. The residue was air-dried at 40-50°C.

Methanol. Two to 3 g dry matter of untreated, chloroform-extracted, or petrolether-extracted plant powder were suspended in methanol (ca. 0·2 g/ml), stirred and sonicated for 20 min at RT. The suspension was then centrifuged (10 min, 8000 g, RT), the supernatant collected, and the pellet resuspended in another equal volume of methanol. Extraction was repeated three times, and the supernatants were pooled. Methanol was removed from the extract with a rotary evaporator and both, extract and residue were air-dried at 40-50°C. 95% aqueous methanol was used in the first extractions, 20 to 100% methanol were tested in later experiments.

Water. Two to 3 g dry matter of untreated plant powder or methanol residue were suspended in nanopure H_2O (ca. 0·2 g/ml), stirred and sonicated for 20 min on ice. The suspension was then centrifuged (10 min, 8000 g, 4°C), the supernatant collected, and the pellet resuspended in another equal volume of H_2O. Extraction was repeated three times, the supernatants were pooled, and both, extract and residue, were freeze-dried.

Acetone. Concentrated methanol extracts were re-dissolved in actone.

The efficacy of all extracts and residues as well as extracts obtained from various extractions by the RRI was tested in the standardized RPT-system, using inclusion levels equivalent to the original plant material. Dosage studies were performed with the methanol extract.

2.9.2 Chromatography

Petroleum ether, chloroform and ethyl acetate extracts were resuspended in their corresponding solvents and subjected to thin-layer chromatography (TLC) on silica gel using three different solvent systems:

1. Chloroform
2. Petroleum ether : ethyl acetate = 75 : 25
3. Toluene : ethyl acetate = 80 : 20

Colour development was observed in visible and UV light.

Butanol, methanol and water fractions were analysed using a further three solvent systems:

1. Toluene : chloroform : acetone = 40:25:35 (phenolics)
2. Chloroform : methanol : water = 65 : 35 : 10 (saponins)
3. Chloroform : acetone : diethylamine = 80 : 20 (alkaloids)

Detection was carried out under UV light (short and long wavelength), followed by spraying using different reagents for different specific target compounds. 4-Hydrobenzaldehyde-sulphuric acid was used as a spray reagent for sapogenins following the method of Stevens (1964). The plate was heated for 3-4 min at 105 °C, yielding yellow to pink spots consistent with the presence of sapogenins. Another plate was sprayed with zinc chloride, which detects steroidal sapogenins.

Sapogenesis. Another preliminary indication of the saponins content in plant material was obtained by the generation of foam. Here, 5 g of sample were extracted in 100 ml 80% methanol. The mixture was incubated at 37 °C in a shaking water bath overnight. The suspension was centrifuged (4000 ***g*** for 10 min). The supernatant and residue were freeze dried. 9 ml of water were added to 1 ml of the methanol fraction. One ml of this solution was vortex-mixed in a small test tube for 30 s. After 15 min, the height of foam in the tube was measured.

2.9.2 Chemical characterization

Crude nutrient analysis. Crude nutrient contents and gross energy were determined according to the methods of the Association of Official Analytical Chemists (1990). Fibre fractions were quantified by the method of Goehring and van Soest (1970). True protein concentration of ground plant material was determined by the previously described dot blot method using various dry matter/Laemmli buffer ratios (2·5, 5, 10, 20 and 50 mg/ml).

Tannin analysis. Freeze-dried plant samples were ground to a fine powder and tannins were extracted in aqueous methanol (500 ml/l) as described in Makkar et al. (1988). Total phenols and total tannins were determined by the ferric chloride assay using the entire extract, or after tannin-protein precipitation with BSA, respectively, as described by Makkar et al. (1988). Protein determination in the tannin-protein pellets was carried out as described in Hoffmann et al. (2002).

GC/MS analysis. Extracted solutions of herbs and dried herbs were analyzed using a Varian Saturn 2000 equipped with a MS detector. A VF-5ms column (30 m x 0.25mmID) coated with 5% phenyl and 95% dimethylpolysiloxane[1] and (Df=0.25) . Oven temperature is programmed from 40°C for 1 min to 140°C at the rate of 4°C/min, then to 220°C at the rate of 10°C/min; injector temperature was 260°C; helium was used as carrier gas at a constant flow of 1 ml/min.; splitless mode. The GC/MS interface line and the ion trap temperatures were 260°C and 190°C, respectively.

The mass spectrometer was scanned from 50-400 amu at 0.5 seconds/scan in the EI mode (ionization voltage of 70 eV).

SPME conditions

Between 1.5 and 4.0 [g] of dried herb were introduced into a 20 ml SPME vial for CPAL autosampler. The fiber used is coated with 75 μm Carboxen™/Polydimethylsiloxane (CAR/PDMS)[2]. The incubation was performed with three different temperatures firstly at 40°C, then at 60°C and 80°C in order to ensure that all the volatile components are released by herb matrix.

The CPAL autosampler was programmed as follows:

CYCLE	SPME
SYRINGE	Fiber
Pre Inc Time	0:20:00
Incubat Temp	40°C, 60°C or 80°C
Agi Speed	250 rpm
Agi On Time	0
Agi Off Time	0
Vial Penetr	22.0 mm
Extract Time	0:20:00
Desorb to	Front

[1] Similar phases: DB-5, HP-5, CP-sil 8 CB, etc.
[2] According to SUPELCO1 Carboxen-PDMS seems to be the fiber for our application

Inj Penetr	44.0 mm
Desorb Time	0:15:00
Fiber Bakeout	0:00:00

Knautia Arvensis: *Preparation of liquid samples for GC-MS injections*
About 2 g of dried herb are extracted with acetone (100 ml, Soxhlet method) for 3 hours. The extraction is carried out two times (2927A: 2.004 g; 2927B: 2.005 g). An extraction with methanol (100 ml) of about 3 g of dried herb (Soxhlet method) is also carried out for 3 h (2927C: 3.035 g).
The three extracts from Hohenheim University, Extract G9 (9 mg), Extract G9A (10 mg) and Extract 11 (7 mg) were diluted in 1 ml MeOH for GC-MS analysis.

***HS-SPME of dried* Knautia arvensis**
2.003 g of dried sample (ID 2927) were analyzed by HS-SPME (as before).
Dried herb from ID 2928 to ID 2937 samples
Sample preparation for GC-MS injections
A known amount of dried herb (see table below) is extracted with methanol (100 ml) according to Soxhlet method for 3 h.
The amounts of herbs in [g] for HS-SPME analyses are reported in the table below.

ID number	Herb	Soxhlet	SPME
2928	***1.1.1.2Rheum nobile***	1.799	1.000
2929	***1.1.1.3Carduus pychnocephalus***	1.996	1.998
2930	***1.1.1.4Lonicera japonica***	1.778	2.002
2931	*Peltiphyllum peltatum*	2.473	0.999
2932	*Gentiana asclepiadia*	2.258	1.003
2933	*Gentiana lutea (root)*	2.577	2.005
2934	*Bellis perennis (leaves)*	3.035	2.003
2935	*Bellis perennis (flower)*	2.285	1.998
2936	*Lonicera japonica (leaves)*	3.692	2.001
2937	*Locinera japonica (flower)*	3.099	2.005

Haemolytic activity. The haemolytic activity of the plant extracts was evaluated by determining the released haemoglobin of 2% suspensions of fresh sheep erythrocytes at 540 nm. Sheep red blood cells were centrifuged and washed three times with PBS pH 7.4, from which stock solutions were made. A volume of 2 ml of the cell suspension was mixed with 2 ml diluent containing sample solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 g ml^{-1} in PBS. The mixture was incubated at 39 °C for 30 min, then centrifuge tubes at 2500 rpm for 10 min. 200 µl of supernatant was transferred in triplicate to a microtitre plate, and absorbance was read at 540 nm. Zero percent and 100% hemolysis were determined in PBS and water, respectively. The haemolysis percentage was calculated using the following equation
% haemolysis = (A_{540} in soln – A_{540} in PBS) / (A_{540} in water – A_{540} in PBS) x 100.

2.9.3 Chemical characterization - León studies
In the meeting held in Dublin in March 2004, it was agreed that UL 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.

Dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (EE, method ID 920.30) and crude protein (CP, method ID 984.13) contents were determined following the methods of AOAC (1999). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined with the ANKOM fibre analyser (Ammar et al., 1999) using the reagents described by Van Soest et al. (1991). Non-structural carbohydrates (**NSC**) were estimated by difference as OM – EE – CP – NDF.

For the measurement of phenolics and tannins, freeze-dried ground samples (200 mg) were weighed out into glass tubes and pigments were removed by a double extraction with 10 ml of diethyl ether containing 1% glacial acetic acid, sonicating the tubes for 5 min at room temperature, followed by centrifugation at 3000 *g* for 10 min at 20ºC. The supernatant was decanted and the solid residue remaining in the tubes was dried at 50ºC for 2 h. Phenolic compounds were extracted from the solid residue following the procedure described by Makkar (2003), by adding 15 ml of 70% aqueous acetone to each tube and gassing the headspace with N_2, followed by a gentle stirring for 15 min, sonication for 20 min at 4ºC and finally centrifugation at about 3000 *g* for 10 min at 4ºC. The supernatant was maintained at 4ºC and used as the 'original extract'. Total phenols (TP) were determined according to the method of Julkunen-Tiitto (1985), using Folin-Ciocalteau solution (1 N) and aqueous solution of Na_2CO_3 (20%) as reagents, and tannic acid in aqueous solution (0.1 mg/ml distilled water) as standard. Absorbance was measured against a blank at 725 nm using a Kontron Spectrophotometer (Uvikon 940). Tannins were estimated indirectly (Makkar, 2003) after adsorption on and precipitation with insoluble polyvinylpyrrolidone (PVP). This synthetic polymer was added to the "original extract" (110 mg PVP per ml "original extract"), mixing thoroughly and centrifuging (3000 *g*, 10 min, 4ºC) to precipitate tannins. Total phenols were measured in the supernatant (non-precipitable phenols, NPP) as described above, and extractable tannins (ET) were calculated by difference, as follows: ET = TP – NPP. Extractable condensed tannins were measured using either the vanillin assay (CTv) of Broadhurst and Jones (1978) or the butanol-HCl assay (CTb) reported by Porter et al. (1986) with the modifications of Makkar (2003). In the first method (CTv), vanillin (40 g / L methanol) and concentrated HCl were used as reagents, and a solution of catechin (3.5 mg/ml aqueous acetone 70%) was used as standard. Absorbance was read against a blank at 500 nm using a Kontron Spectrophotometer (Uvikon 940). For CTb, butanol/HCl (95:5 v/v) and ferric ammonium sulphate (2 g in 100 ml HCl 2N) were used as reagents, and a solution of purified quebracho tannin (1 mg/ml aqueous acetone 70%) was used as standard. Absorbance was measured against a blank at 550 nm in a Biokinetics ELISA-microplates reader (Cultek TL 340). The butanol-HCl technique was used to quantify the bound condensed tannins (BCT) in the solid residue remained after extraction; thus, the same reagents and standard used previously for CTb were used in this assay, following the procedures of Pérez-Maldonado & Norton (1996). Total condensed tannins (TCT) were calculated as TCT = CTb + BCT. Concentration of phenolics and tannins was expressed in g/kg DM, standard equivalent.

2.10 *In vivo* trials

Two sets of *in vivo* trials were carried out to determine the effectiveness of selected samples under conditions similar to those which obtain in livestock production.

2.10.1 *In vivo* trials, Hohenheim

All animals were kept according to the animal welfare regulations under the supervision of the animal welfare representative of the University of Hohenheim and cared for by professional animal keepers and the veterinarians of the University of Hohenheim.

2.10.1.1 *In vivo* trial on antiproteolytic effect

Twenty weaned male Merino lambs from the University's Agricultural Station were kept in a group stable with ad lib access to water and weighed in regular intervals until they entered the experiment at a bodyweight of 26 ± 2 kg. Four experimental concentrates (P1-P4) were prepared, containing different levels of crude protein (18 and 14%), each with and without 12% *K. arvensis* (i.e. 10% in the final diet including some hay). A grass meal substituted the additive in the controls (Tables 2.10.1.1 and 2.10.1.2). In five consecutive weeks groups of four animals each were selected and each of the animals was assigned to a diet. The sheep were transferred to single boxes and were fed a ration of 800 g concentrate and 300 g hay, shared in two equal meals per day. They were pre-fed for one week with the control diets (P1, P3), to adapt them to the respective protein levels, without exposure to the additive. If feed intake was satisfactory, they were given another week of adaptation to the actual experimental diets, i.e. two animals continued to receive the controls, the other two the concentrates containing the additive (P2, P4). Then all four sheep were put on metabolic cages for a five-day quantitative collection of faeces and urine. In the final week each animal was kept in a respiration chamber for two consecutive days where O_2, CO_2 and methane were recorded. The data collected in the balance and respiration periods allowed calculating C-balance, N-balance, energy balance and efficiencies. Five such runs were completed, each shifted by a one-week interval. A sixth repetition with two spare animals was added in the end, as there was one incidence of a sheep refusing its feed in the respiratory chamber.

Table 2.10.1.1 Ingredients of concentrate pellets RU-P1 to P4 used in the antiproteolysis trial.

(%)	P1	P2	P3	P4
Barley	25	25	55	55
Peas	25	25	14	14
Beans	31	31	13	13
Grass hay	12	0	12	0
E073	0	12	0	12
Mineral mix	2	2	2	2
Oil	2	2	2	2
Lime	1	1	1	1
Molasse	1	1	1	1
sum	100	100	100	100

Table 2.10.1.2 Dry matter (DM), crude ash (CA), crude nutrients (CP= crude protein, CL = crude lipid, CF = crude fibre), gross energy (GE) and fibre fractions (NDF = neutral detergent fibre, ADF = acid detergent fibre) of concentrate feeds.

Feed	DM	CA	CP	CL	GE	NDF	ADF
	% FM	% DM	% DM	% DM	% DM	% DM	% DM
N5 A	93.3	6.95	18.2	2.0	nd	15.2	6.4
N0 A	93.0	6.71	17.4	2.0	nd	16.2	7.4
D5 Z	93.7	7.57	15.9	2.0	nd	17.7	9.0
D0 Z	93.6	7.43	15.5	2.0	nd	20.0	9.4
RU-P1	89.6	6.79	19.6	4.6	18.3	18.3	9.8
RU-P2	89.6	6.11	19.5	3.2	18.5	16.8	9.5
RU-P3	89.8	6.39	15.8	3.6	14.9	20.5	9.7
RU-P4	89.7	5.86	15.3	3.6	14.0	20.0	9.7

2.10.1.2 *In vivo* trial on antiprotozoal effect

Eight rumen fistulated and castrated male Merino lambs with an average body weight of 43 kg received a ration of 1100 g of concentrate and 400 g of hay, shared in two equal meals per day. For an initial, two-week pre-feeding period all eight sheep received the control concentrate D0Z. After that four animals were designated to the control group, and four to the treatment group receiving concentrate with a 5% inclusion of *Bellis perennis* (D5Z). The composition of the feeds is detailed in tables II and III. The animals received these diets for sixty days during which the rumen content was sampled in regular intervals using a manual pump, which was pushed through the feed mat to withdraw fluid from the liquid phase in the ventral sac of the rumen. Two daily courses with four time points each were sampled during the pre-feeding period. Based on these results 14.00 h was chosen as best time for routine sampling. A five-day balance study followed by a two-day respiration measurement was carried out in the middle of this period. Finally the diets were switched over, and samples at narrow intervals drawn around the change of diets. Samples of rumen fluid were analysed for protozoal counts, soluble protein, ammonium and SCFA concentration and composition. Selected samples were analysed for shifts in microbial community structure by 16 and 18Sr RNA gene fragment banding patterns using DGGE and cluster analysis thereof.

2.10.1.3 *In vivo* trial on antiacidotic effect

In the first feeding period, six rumen cannulated castrated male Merino lambs (body weight 49·1 to 56·3 kg) received hay *ad libitum* at 8.00 h and 16.00 h for three weeks. In a following crossover feeding experiment with two feeding periods of five weeks each, the animals were fed a high starch diet consisting of 900 g concentrate and 400 g hay per day given in two equal portions at 8.00 h and 16.00 h. At a time three lambs were fed the control concentrate NOA and three sheep were fed concentrate N5A in which grass hay was replaced by the plant additive *Urtica dioica* (A049) at an inclusion level of 5%. The detailed composition and crude nutrient analyses of the concentrate feeds are given in Tables 2.10.1.2 and 2.10.1.3. Sampling by a manual pump was done as described on a daily basis except for weekends. After an adaptation period of two weeks a daily course was sampled followed by balance studies over five days and continuous pH measurements over three days.

Table 2.10.1.3 Composition in % of the concentrates used in the antiprotozoal and antiacidosis experiments.

	Defaunating (Bellis)		Antiacidosis (Urtica)	
	Control D0Z	Treatment D5Z	Control N0A	Treatment N5A
Wheat	-	-	15.8	15.8
Barley	40.4	40.4	44.4	44.4
Maize	22.7	22.7	11.8	11.8
Soybean (44)	9.9	9.9	14.8	14.8
Sugar beet pulp	11.8	11.8	-	-
Sugar Molasses	3.9	3.9	3.9	3.9
Bicarbonate	2	2	-	-
Carbonate	1.5	1.5	1.5	1.5
Phosphate	0.2	0.2	0.2	0.2
Salt	0.7	0.7	0.7	0.7
Other (pelleting agents)	1.0	1.0	1.0	1.0
Vitamines/ Minerals	1.0	1.0	1.0	1.0
Grass	4.9	-	4.9	-
Plant	-	4.9	-	4.9
Total	100	100	100	100

2.10.1.4 Additional analytical methods

Balance and respiration measurement. Lambs were kept in metabolic crates on five consecutive days under the respective feeding regime and had *ad lib* access to water. Faecal bags were fixed to the animals and emptied twice a day. Total faeces were collected and kept at 4°C. Total fresh weight was determined and subsamples were taken for determination of DM, GE, C and N content. Urine was collected in a tray below the crates leading into a flask filled with 200 ml 12% H_2SO_4. Weight and volume of the samples were determined daily and subsamples pooled for analysis of C and N content.

*Carbon and N content of feed and faece*s Carbon content of samples from all *in vivo* experiments was determined by elementary analysis using the 'Elementaranalysator Vario MAX CN' (Elementar Analysen-systeme GmbH, Hanau, Germany) according to the manufacturer's specifications. The same procedure was used for Nitrogen analysis in the Daisy and Urtica trials. In the Knautia trial Nitrogen analysis of feed, faeces, and urine was done by Kjeldahl destillation (AOAC 1990).

Respiration measurements applied the principle of indirect calorimetry in an open circuit (McLean and Tobin, 1987). They were performed in gas tight respirations chambers maintained at 18°C and 65% relative humidity for 2 x 24 h under the same feeding conditions as in the metabolic crates. Gas was analysed for CH_4 and CO_2 by URAS 10E (Hartmann & Braun, Frankfurt), and for O_2 by Magnos 16 Advance Optima (ABB Analytical, Hartmann & Braun, Frankfurt).

Protozoa. Aliquots of 0·5 ml rumen samples were fixed and stained with the same amount of methylgreen-formalin and counted microscopically using a Fuchs-Rosenthal Haemocytometer.

Measurement of pH. Rumen samples were kept on 39°C and measured immediately by a pH probe. Continuous measurement of pH was performed with a pH probe (single-rod measuring cell with gel amplifier, Hanna instruments) permanently inserted through the fistula directly into the in the ventral sac of the rumen. Values of a 15 minutes interval were averaged and recorded by a computer unit.

Lactate. Lactate concentration was determined enzymatically according to Gutmann & Wahlefeld (1974), adapted to microplate dimensions. Rumen fluid samples were deproteinized over night with TCA (final conc. 7·5%) at 4°C. The precipitate was removed by centrifugation (10 min, 30000 g, 4°C), and the supernatant was neutralized with 2M $KHCO_3$, mixed thoroughly, and centrifuged again. The final supernatant was used in the assay. LDH from rabbit muscle (Sigma L-2500) was diluted 1/10 with ddH_2O immediately before use. NAD (2 mg/ml) was dissolved in hydrazine-glycine buffer (pH 9) immediately prior to use. On a 96 well microplate, each cavity was loaded with 50 µl sample or standard, 250 µl of NAD-solution, and 5 µl enzyme solution or water. Four parallels were pipetted per sample, two of which received the enzyme, the other two received H_2O. Absorption at 340 nm was monitored for 1 h with 5 min reading intervals in a TECAN Sunrise Microplate reader set to 37°C. The end point of the reaction was reached after 30 min and the difference in absorbance between the enzyme and water containing parallels was calculated and converted to lactate concentration using a calibration curve from 20 – 400 µM lactate. At least two independent runs were performed for each sample.

2.10.1.5 Statistics

Influence of various factors and interactions thereof were tested by general linear models (SAS V9.1).

2.10.2 *In vivo* trials, León

2.10.2.1 Animal and diets

Each study was conducted using weaned Assaf lambs. The 24 animals were randomly divided into two groups: one group was used as control (*Control* group; n = 12) and fed with barley straw and concentrate ad libitum. The other group received ad libitum barley straw and the same concentrate in which grass meal was substituted with the plant additive used in each case (*Additive* group; n = 12). Both concentrates were formulated to have the same energy and protein contents.

Common experimental design

Two experimental groups

	Control	Additive
FEEDING	Barley straw *ad libitum*	
Concentrate *ad libitum*	Ingredients: - Cereals - Protein supplements - Oils - Molasses - Min + Vit	Ingredients: - Cereals - Protein supplements - Oils - Molasses - Min + Vit
	Grass	Plant additive

At the beginning of the experiment, animals were housed individually in 1.5 m x 1.5 m pens and drinking water was always available. Previously, they had remained with their mothers and had been given free access to a commercial starter concentrate and alfalfa hay until the commencement of the trial (when live weight was approx. 15 kg). Lambs were treated with sodium selenite and α-tocoferol acetate (Vitasel; Lab. Ovejero, Spain) immediately after birth to avoid pale muscle disease, and later on

with Miloxan (Merial Lab., Spain) to prevent enterotoxaemia and with albendazol 2.5% (Ganadexil®; Industrial Veterinaria, Spain) to control parasites.

The experiment was carried out in accordance with European Council Directive 86/609/EEC of 24 November 1986 for the protection of animals used for experimental and other scientific purposes.

2.10.2.2 Experimental procedures

Control of feed intake, live body weight and wool growth. The forage (barley straw) and the concentrate were offered separately once a day. The amount of feed offered was adjusted daily on the basis of the previous day intake, allowing refusals of 15-20%. Orts were withdrawn, weighed and dried daily to determine voluntary DM intake throughout the experiment. Samples of the feeds offered and rejected were collected weekly and analysed individually.

All lambs were weighed once a week before the morning feeding until they reached 23 kg and then every 3-4 days up to 25 kg of live weight (LW). At the beginning of the experimental period in each lamb a 15 x 5 cm^2 area was sheared in either shoulder or leg of the right side. On the day of slaughter, the same areas were sheared and wool samples were placed into a preweighed nylon bag, dried in a forced-draft oven at 60 ºC and then weighed to determine wool growth (g dry matter/cm^2).

Digestibility and N balance. At 20 kg of LW, four lambs for each experimental group were randomly selected to provide digestibility and nitrogen balance data. Animals were placed in metabolism crates for 9 days. Total collection of faeces and urine were conducted daily for the last 7 days. Urine was collected in buckets containing H_2SO_4 as preservative to keep pH below 5. Faeces and urine were subsampled daily (5% of total output), composited over the period and stored at –20 ºC until analysed as described below. Apparent digestibilities of dry matter (DMD), organic matter (OMD), crude protein (CPD) and neutral detergent fibre (NDFD) of diets were calculated. N retention was calculated by difference between N intake and N excreted in faeces and urine.

Slaughter. Animals were slaughtered when upon reaching the intended weight, considered a common market weight for this type of animals (approx. 25 kg). Just before slaughtering, lambs were weighed and then slaughtered by exsanguination from the jugular vein, eviscerated and skinned. The whole body of each lamb was dissected into carcass, which included thymus, testicles, kidney and perirenal-retroperitoneal fat, and the "non-carcass" component, weighing both fractions. Each carcass was weighed twice: immediately after slaughter (hot carcass weight) and then again after 24 h of chilling at 4ºC (cold carcass weight). Chilling losses were calculated as the difference between hot and cold carcass weights, expressed as proportion of the initial weight. Dressing percentage or kill-out (%) was calculated as the cold carcass weight expressed as percentage of slaughter weight.

Rumen measurements. Total digestive tract and reticulum-rumen were weighed before and after emptying. Rumen fluid was strained through two layers of cheesecloth, pH was immediately measured and samples were collected for subsequent ammonia-N (10 ml rumen fluid acidified with 10 ml 0.2 N HCl), VFA (0.8 ml rumen fluid added to 0.5 ml of deproteinising solution containing 20% metaphosporic acid and 0.4% crotonic acid, w/v) and lactate analyses. Samples were stored at –30 ºC until analysis.

Samples of rumen wall were taken for anathomo-pathological examination. Sections of tissue (approx. 8-10 cm^2) were excised from the posterior dorsal and anterior ventral areas of the rumen and weighed. An outline of each section was

drawn on transparent acetates and the area of each section determined using a plannimeter (ΔT Areameter, MK2). Samples were stored in 10% formalin until counting the number of papillae per cm^2.

Blood biochemistry. In order to examine signs of toxicity, blood samples from each lamb were collected before slaughtering by jugular venepuncture using heparinized vacutainers (Venoject®, Belgium). Immediately after collection, samples were centrifuged at 1900 x g for 30 min at 4 ºC and plasma samples were taken for subsequent biochemical analysis. Samples were stored at –30 ºC until analysis. Further blood samples were collected from animals used in Experiment 3 to detect clinical signs of metabolic acidosis. These samples were analysed immediately after collection in a Stat Profile phOx Plus blood analyzer (Nova Biomedical, USA) to determine pH, CO_2 pressure (pCO_2), bicarbonate concentration (HCO_3^-) and packed cell volume.

Carcass measurements Each carcass was subjectively assessed for conformation, fatness and fat consistency using a scoring method applied to this type of light carcasses (Colomer-Rocher et al., 1988). Linear measurements described by Palsson (1939) and Boccard et al. (1958) were taken on the whole and left half carcass to determine carcass morphology. These measurements were carcass external and internal length, pelvic limb length and buttock girth and perimeter. As described by Colomer-Rocher et al. (1988), left half carcass was cut into seven commercial joints: ribs (rack-loin), leg, shoulder, neck, breast, flap and tail, that may be grouped into three categories: extra (ribs and leg), first (shoulder) and second (neck, breast, flap and tail). All components of left half carcass, except the loin, were minced together, mixed and homogenised in a commercial blender. Samples of each carcass were taken and frozen for chemical analysis.

Meat quality measurements Meat quality was evaluated using the *Longissimus dorsi* muscle of the left side of the carcass. Then, pH was measured at the 6^{th} rib site using a Metrohm® pH meter, equipped with a penetrating electrode. CIE-L* (lightness), a* (redness) and b* (yellowness) colour parameters were determined at the same site using a Minolta CN2002 spectrophotometer. An outline of the section of the muscle L. dorsi at 6^{th} (craneal side) and 13^{th} (caudal side) rib was taken on a transparent acetate, and then the muscle area determined using a plannimeter (ΔT Areameter, MK2).

Then, the muscle was weighed and cut into two parts (thoracis and lumborum). After removal of the epimysium, the Longissimus lumborun was minced, frozen a –20 ºC, freeze dried and stored at 4ºC until chemical analysis. A subsample of L. thoracis was taken to determine the water holding capacity, and the remaining portion was stored at –20º C until cooking losses and instrumental hardness measurements. Water holding capacity was determined using the filter paper press method described by Grau and Hamm (1953), modified by Sierra (1973). Cooking losses were calculated after cooking meat samples in a 75 ºC water bath for 15 minutes. Cooked muscle cores with a cross section of 10 mm^2 and 20 mm long were cut parallel to the muscle fibres and shear force measured using a Warner-Bratzler device mounted on an Texture analyzer (QTS, CNS, Farnell).

Sensorial studies The rib cut from the right side carcass was packed under vacuum and stored at –30 ºC until required for sensory analysis. Cuts were thawed for 24 h at 4 ºC before cooking. Then, each cut was chopped off into 15-mm slices and cooked on an electric grill. Once grilled, the chops were offered to the members of the tasting panel for sensory evaluation. The panel included 24 non trained tasters. Two tests were performed to evaluate the effect of the plant additive on meat sensorial quality:

- A triangular test (ISO 4120), in which each taster is offered three chops, two from a lamb of one of the experimental groups, and the other from a lamb of the other experimental group. The taster has to identify which chop is different from the other two. The statistical significance of the discrimination between control and additive rib chops is assessed from the number of correct judgements, taking into account that there is a 1/3 probability of a positive judgement by chance without a real discrimination (Roessler et al., 1978).
- A paired preference test (ISO 5495), in which each taster is offered two chops, from lambs of different experimental groups and will say which one prefers and why. A significant preference occurs if a certain number of tasters show a clear preference for the chops of the lambs of a given experimental group (Roessler et al., 1978).

2.10.2.3 Chemical analysis

Feeds offered and faeces were dried in an oven at 50ºC to constant weight and then analysed for Kjeldahl N and ash by AOAC (1999) procedures and for neutral-detergent fibre according to Van Soest et al. (1991). Urine samples were analysed for Kjeldahl N. Freeze-dried carcass and meat samples were analysed for crude protein (N × 6.25), fat and ash, following AOAC (1999) procedures. Chemical composition of carcass was corrected taken into account the chilling losses.