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Gastrointestinal Tract Microbiology Symposium

Challenges for Microbial Digestive Ecology...
...at the Beginning of the Third Millennium

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Session I:

Microbial populations – Biodiversity

Molecular methods for bacterial community profiling in the gut. G.L. Hold, S.E. Pryde, H.J. Flint (Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK).

Our understanding of the complex bacterial communities which colonise the gastro-intestinal tract (GI tract) in mammals is still far from complete. Studies investigating GI tract pathogens are of obvious importance and the role of commensal bacteria in host nutrition, colonic health and gut development is becoming increasingly recognised. Culture-based methods are extremely time-consuming, and since they only provide information on culturable bacteria, they give a biased view of microbial diversity. Increasingly, molecular methodologies which do not rely on culturing are being exploited to examine the diversity of the gut microflora including viable but non-culturable isolates (VBNCs). Such approaches have already been used to examine the bacterial species present in complex environments including soils and hot spring microbial mats. We present data that shows the application of different molecular methodologies to the study of complex bacterial communities. Restriction Fragment Length Polymorphism (RFLP) profiling and Terminal Fluorescent labeling (TFlu-RFLP) are highlighted to look at changes in specific bacterial populations in the pig hind gut. Data highlighting the application of Denaturing Gradient Gel Electrophoresis (DGGE) and its potential use in rapid profiling of bacterial gut populations are also presented.

Development of fluorescent 16S rRNA probes for studies of rumen ecology. R.J. Forster[a], S. Koike[b], J.A. Armstrong[a], R. Teather[a], Y. Kobayashi[b] ([a] Lethbridge Research Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, T1J 4B1, Canada; [b] Laboratory of Animal Science, Faculty of Bioresources, Mie University, Mie, Japan).

Fluorescent probes targeted to specific 16S rRNA sequences have allowed the direct observation of bacteria in environmental samples, including the observation of bacteria that have not been cultured in the laboratory. There has however been a lack of such probes developed for use in the rumen, due to factors such as high background and poor signals. In this study, we used FITC and CY3 labels combined with specific filter sets to investigate probes that had been used successfully in other environments, fluorescently labeled probes previously designed for rumen 16S rRNA hybridization studies, and newly designed probes targeting rumen bacteria. Intense fluorescent signals were obtained with the general bacterial-338 probe, a general *Fibrobacter* probe and a *Bacteroides/ Prevotella* probe. A newly designed Clostridial cluster XIV probe also gave intense signals. Many of these organisms were associated with the particulate fraction of rumen contents. Cells of various morphologies fluoresced, including typical *Butyrivibrio*-like cells and cocci. A newly designed *Ruminococcus* genus probe gave adequate signals from pure cultures but weak signals from rumen particulates. A probe targeting an uncultured group of bacteria, previously identified by 16S rRNA cloning experiments to be related to cellulolytic clostridia in cluster III and IV, gave numerous signals from bacteria associated with rumen particles, but few signals from rumen fluid. Species specific probes for *R. flavefaciens* and *R. albus* and a general low G + C gram-positive probe did not work. Success or failure of the fluorescent probes could not always be predicted by location on the 16S rRNA molecule.

Characterisation and dynamics of the bacterial populations in human feces studied with 16S rRNA-based fluorescent probes. H.J.M. Harmsen, G.C. Raangs, A.C.M. Wildeboer-Veloo, R.H.J. Tonk, J.E. Degener, G.W. Welling (Dept. of Medical Microbiology, University of Groningen. Hanzeplein 1, 9713 GZ Groningen, The Netherlands).

The human gastrointestinal tract harbors a complex microbial community that can be beneficial and hazardous at the same time. It helps us with digestion and provides valuable nutrients but can also be a source of potentially pathogenic bacteria. Diet and the host surrounding this community usually maintain its balanced composition. This ecosystem is studied in great detail using culture techniques and molecular biological techniques. In our laboratory we use fluorescent in situ hybridisation (FISH) with 16S rRNA-based group-specific probes to study the composition

and dynamics of fecal flora. Studies with a first set of probes used on fecal samples of a group of volunteers showed that FISH is an accurate quantitative method (Franks et al. 1998, Appl. Environ. Microbiol., 64, 3336–3345). However, this set of probes detect only 60 to 80% of the total flora determined with the bacterial probe Bact338. In our current studies we designed new probes for coriobacteria, ruminococci and for subgroups of clostridia. These probes were validated against a large panel of reference strains. These probes were used in combination with the first set of probes, to determine the composition of the fecal flora of healthy volunteers of different age groups. This study showed that the flora of children and elderly is different from that of adults between 20 to 50 years of age. Furthermore, while the latter group has a rather similar flora composition, the flora of young infants and elderly shows large variations between individuals. Currently the probes are used in studies to modulate human flora with pre- and probiotics.

Diversity of 16S rRNA genes from anaerobic colonies reveals many novel species within the human gut. A. Suau, R. Bonnet, C. Bridonneau, V. Rochet, J. Dabard, P. Raibaud, J. Doré (Unité d'Écologie et Physiologie du Système Digestif, INRA, 78350 Jouy-en-Josas, France).

The human intestinal microflora plays a key role in nutrition and health. Its culture-independent characterisation using comparative sequencing of cloned 16S rRNA genes (rDNA) indicates the presence of numerous new molecular species. We herein report on the search for new species within the culturable fraction of the human faecal flora. The dominant culturable faecal flora of an elderly person was cultivated in an anaerobic chamber, using a BHI-hemin-yeast medium. Ninety-six colonies were selected and morphologically characterised. Seventy-three 16S rDNAs were amplified by PCR, sequenced (500 bases at the 5' end) and analysed. Thirty-eight colonies belonged to the *Bacteroides* group, 11 to the *Bifidobacterium* genus, 11 to the *Clostridium coccoides* group and the remaining 13 fell outside these main groups. These colonies were classified into 33 molecular species (at least 98% rDNA sequence similarity). Twenty species were found only once, while the three species *Bifidobacterium infantis*, *Bacteroides* sp. AR20 and *Bacteroides vulgatus*-like species, were represented by respectively 8, 9 and 12 colonies. One isolate for each of the eighteen potentially new species (no representative in the databases) were subcultured. Nine of these had a microorganism in which 16S rDNA had been detected by direct analysis, as their closest relative. Eleven colonies of interest are being further characterised. Potential biases of anaerobic culture were confirmed by in situ hybridisation on the same faecal sample, which revealed the presence of bacteria of the *Clostridium leptum* subgroup. Nevertheless, the present study shows that many culturable species of the human faecal flora have yet to be phenotypically and phylogenetically characterised.

Temporal Temperature gradient Gel Electrophoresis (TTGE) is an appropriate tool to assess dynamics of species diversity of the human fecal flora. M. Sutren[a], C. Michel[b], M.F. de la Cochetière[b], A. Bernalier[c], D. Wils[d], M.H. Saniez[d], J. Doré[a] ([a] INRA Jouy-en-Josas, France; [b] CRNH Nantes, France; [c] INRA Theix, France; [d] Société Roquette Frères, Lestrem, France).

Dynamics of species diversity within intestinal microbiota is difficult to monitor using culture based techniques. TTGE of bacterial DNA extracted from a complex flora allows for a culture independent profiling of dominant species. We assessed the relevance of this method in monitoring the dynamics of species diversity of the human faecal flora. Samples of bacterial biomass (200 mg) from in vitro continuous cultures or stools were subjected to total DNA extraction. The variable regions V6–V8 of the ribosomal DNA gene was amplified by PCR from DNA extracts. PCR products were run on the DCode system (BioRad) from 64.5 °C up (ramp rate of 0.1 °C·h^{-1}) at 60V for 16 hours and gels were read on an Image analyser. TTGE revealed that in vitro continuous cultures induced a reduction in the species diversity of the inoculated faecal flora during the first 48 hours. Different TTGE profiles, typified by one specific dominant band – presumably one species – were obtained with different substrates. In the stools from a healthy adult on a normal diet, species diversity appeared very stable day after day and over a 3 months period. A nutritional constraint lead to the detection of one single novel band that was still perceptible 3 weeks after returning to a normal diet. TTGE also allows for extraction and sequencing of the major bands from the gels obtained. We thereby showed that an in vitro culture stimulated the development of dominant species belonging to th *C. xyla-nolyticum* sub-group

within the *Clostridiun coccoides* phylogenetic group. TTGE hence proved to be a powerful tool for a culture independent monitoring of the dynamics of diversity of the human faecal flora.

Analysis of human fecal microflora by in situ hybridisation and image analysis. V. Rochet, L. Rigottier-Gois, F. Béguet, J. Doré (INRA, UEPSD, CR de Jouy-en-Josas, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France).

In situ hybridisation was developed to analyse the structure of human faecal microflora. A set of five fluorescent probes directed against the 16S rRNA of predominant groups of intestinal bacteria was used. The universal probe Eub338 was used to detect all Eubacteria. Specific probes were used to target *Bacteroides-Porphyromonas-Prevotella*, *Clostridium coccoides*, *Clostridium leptum* phylogenetic groups and the genus *Bifidobacterium*. After hybridisation, a staining with Dapi was performed to count all bacteria. Images were obtained using an Olympus BX40 microscope fitted with a Lhesa ultrasensitive camera and their analysis was performed using Optimas 6.2 software. Each step of the sample treatment was optimised: fixation, permeabilisation of cells, hybridisation and washing. The reproducibility of the method was evaluated by independently repeating the analysis on the same sample 17 times. The composition of the fecal flora of this sample was assessed very reproducibly upon multiple assays (average standard deviation inferior to 12%). The method was then used to determine the composition of the faecal microflora of 5 healthy humans. Depending on the sample, 67 to 90% of the Dapi-stained bacteria were labelled by the Eubacteria domain-probe. Cell counts expressed as average percent of Dapi-stained bacteria (range) were: 13% (7–23) *Bacteroides*, 24% (22–25) *Bifidobacterium*, 32% (29–38) *C. coccoides* and 25% (11–37) *C. leptum*. The additivity was very close to 100% (96 to 102). This method allows the detection and quantification of four phylogenetic groups which represent nearly all bacteria belonging to the human faecal flora.

Stability of individual faecal bacterial populations revealed by PCR-DGGE analysis. J.M. Simpson, V.J. McCracken, H. Rex Gaskins[a, b, c], R.I. Mackie[a, c] ([a] Departments of Animal Sciences; [b] Veterinary Pathobiology; [c] Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, IL 61801, USA).

Faecal bacterial population profiles in piglets were studied using the PCR-DGGE technique. Full sibling piglets ($n = 9$) were housed individually and fed a non-medicated weaning diet from day 21 through day 49 of the experiment. Fresh faecal samples were collected each morning between days 28–49. DNA extraction and PCR-DGGE, targeting the V3 hypervariable region of the 16S rDNA molecule, was performed as described previously (Simpson et al. 1999, J. Microbiol. Methods 36, 167–179). Gels were developed by silver staining and scanned using a GS-710 calibrated densitometer (Bio-Rad). Banding patterns were analyzed using Diversity Database^{TM} software (BioRad) and compared using the Dice similarity coefficient (D_{sc}) and the Ward algorithm. PCR-DGGE analysis of the V3 region demonstrated relatively stable banding patterns throughout the 21 day collection period. Differences were found in positions of specific bands, band intensity and number of bands present. However, for each individual animal the overall pattern was sufficiently stable to obtain a clear pattern representing temporal changes in bacterial populations within an individual animal. This was confirmed using cluster analysis based on the Ward algorithm. Overall, the data demonstrated that each animal had its own unique banding pattern and that within-animal variation was considerably less than between-animal variation. The implication of these, and other findings which demonstrate the relative stability and individuality of fecal bacterial populations suggests an unexplored role for, and the importance of, host factors.

Molecular analysis of the phylogenetic diversity of the pig gastrointestinal tract microbiota. T.D. Leser, T.K. Jensen, R.H. Lindecrona, K. Møller (Danish Veterinary Laboratory, Bülowsvej 27, 1790, Copenhagen V, Denmark).

The microbiota in the gastrointestinal (GI) tract of pigs consists of a diverse microbial community of mainly strict anaerobic bacteria, of which only a part can be cultivated using conventional microbiological techniques. To avoid the bias caused by culture dependant methods, we applied a genotypic approach to characterise the pig intestinal microflora. DNA was extracted from intestinal samples. The 16S rDNA was amplified and the products were cloned and sequenced. A comprehensive library of more than 2500 clones of 16S rDNA sequences was established. At least 270 operational taxonomic units (OTUs) were

identified. About one fifth of the OTUs had 97% or higher similarities to sequences from known bacterial species. The remaining sequences had lower similarities to any known species and are, thus, most likely representatives of yet uncharacterised species or unsequenced. Several clusters that branch deeply into the phylogenetic tree and have a low similarity to known bacterial groups were found, suggesting the presence of higher taxonomic groups that have not been cultured. The cloned sequences were affiliated with the Proteobacteria, the Planctomyces group, the Flexibacter-Cytophaga-Bacteroides group, Spirochetes, Fusobacteria, and Gram positive bacteria. Approximately 85% of the OTUs belonged to the Low G + C gram positive subdivision. The results demonstrated a so far unknown bacterial diversity in the GI tract of pigs.

Molecular analysis of anaerobic fungal populations in rumen and faeces. E. Ozkose[a, b], G.W. Griffith[b], D.R. Davies[a], M.K. Theodorou[a] ([a] Inst. Grassland and Environmental Research, Aberystwyth, SY23 3EB; [b] Inst. Biol. Sci., Univ. Wales Aberystwyth, SY23 3DA, UK).

Since their first isolation from rumen contents, anaerobic fungi have been isolated from most parts of the digestive tract, as well as from fresh and dried faeces. However, little is known about the distribution and abundance of the various anaerobic fungal taxa in gut contents and faeces. Furthermore, the effect of different isolation media on the efficiency of isolation of different species has not been studied in detail. Faecal (fresh and frozen for 30d at –20 °C) and rumen digesta (fresh only) were obtained from three fistulated cows. Fungi were isolated from serial dilutions in a basal medium supplemented with various carbon sources (either glucose, cellobiose, wheat straw, xylan or cellulose) and enumerated using the MPN technique. The abundance of thalli belonging to different fungal genera was assessed by microscopic examination. Significant differences were observed in the taxa isolated on different carbon sources. Overall the relative abundance of thalli with different morphology was 39.3% polycentric, 35.3% monocentric-rhizoidal and 25.4% with bulbous holdfasts. However, in one cow no polycentric fungi were isolated. No difference was observed in the number of fungi obtained from the rumen and faeces, though counts from frozen faeces were slightly lower. Putatively pure cultures were taken from lowest dilution MPN tubes and subcultured prior to DNA extraction. PCR-RFLP analysis of part of the rRNA gene repeat (ITS1/2 region) was used to confirm the identity of isolates and also to further elucidate diversity of the taxa present in each sample. Seven or more ribotypes were isolated from some of these samples. The results are discussed in the context of both methods of isolation and ecological differences between isolates.

Composition of the rumen microbial population fractionated by cell size. S.K. Baker[a], K.A. Munyard[a, b] ([a] CSIRO Animal Production, Floreat, 6014, Western Australia; [b] The University of Western Australia, Nedlands, 6907, Western Australia).

Microorganisms in the rumen range in cell size over 5 orders of magnitude. Strained rumen contents (25 mL) were fractionated in triplicate by centrifugal elutriation into washed fractions of 10-fold range in cell size, expressed as the diameter of a sphere of the same volume (< 1 μm, fraction SB, 1–10 μm, fraction LB, > 10 μm, fraction P) (Munyard and Baker, 1994, Anim. Prod. Aust. 20, 388). The microbial composition of the fractions is reported by these authors. The number of microorganisms in each fraction (% of total) decreased exponentially with an increase in cell size (92.5, 7.3, 0.2) ($P < 0.05$) but the microbial biomasses of the fractions, after drying at 90 °C, were similar in P and LB (55 ± 3.9 mg and 54 ± 3.9 mg) but less than in SB (170 ± 5.5 mg) ($P < 0.05$). Carbon, nitrogen and sulphur in each fraction (C, N, S, mg·100 mg^{-1} DM) were determined in a Carla Erba N1500 Analyser: samples were combusted, then reduced and concentrations of elemental C, N and S were detected by thermal conductivity. C contents in the 3 fractions (P, LB, SB respectively) were similar (51 ± 1.5, 48 ± 1.7, 54 ± 2.7) ($P > 0.05$), but N and S contents decreased progressively with a decrease in cell size ($P < 0.05$). Thus the C:N ratio increased with a decrease in cell size (10 ± 2.0, 19 ± 2.3, 50 ± 3.7) ($P < 0.05$), while the N:S ratio was similar in P and LB (6.8 ± 0.16, 7.1 ± 0.19) ($P > 0.05$) but larger than in SB (2.8 ± 0.31) ($P < 0.05$). A supply of S-containing amino acids can limit animal productivity, and diets that favour proliferation of organisms such as *Oscillospira*, *Selenomonas*, fungal zoospores and ovals may enhance the supply of N and S in the rumen microbial biomass.

A comparison of the ruminal microbial population structure in cattle browsing tagasaste (*Chamaecytisus proliferus*) in autumn and spring using 16S rRNA probes. M.Q. Thyer [a, b], N.J. Edwards[b], P.E. Vercoe[a] ([a] Dept of Animal Science, The University of Western Australia, Perth, 6009, Western Australia; [b] CSIRO Animal Production, P.O. Wembley, 6014 Western Australia).

The growth rate of cattle browsing the leguminous fodder shrub, tagasaste (*Chamaecytisus proliferus*) is highly seasonal. Despite apparently high nutritional quality of tagasaste throughout the year, cattle only maintain weight during the autumn (dry season) but grow up to 1.5kg/head/day in the spring (wet season) (Edwards et al. 1997, in Proc. XVIII Int. Grasslands Congress, Canada). This lower animal production in the autumn is associated with a lower feed intake (Edwards et al. 1997, in Proc. XVIII Int. Grasslands Congress, Canada) and it is thought that this could also be associated with poor rumen function. El Hassan and associates (El Hassan et al. 1995, in Rumen Ecology Research Planning, p. 43) demonstrated that some leguminous browse species produce anti-nutritional factors against some ruminal microorganisms, thereby depressing rumen activity. However, there have been no investigations of the rumen microbial ecology of cattle grazing tagasaste throughout the year. We have investigated the microbial population structure of the rumen in cattle relocated from annual pasture hay to tagasaste over a four week period during both the autumn and spring. This was carried out by using 16S rRNA oligonucleotide probes targeting general through to more specific groups of microorganisms. Both genomic 16SrRNA gene and rRNA templates were used in slot-blot hybridisations. Preliminary data indicate that there are differences in the structure of the microbial population between diets but not between seasons. These results will be discussed with reference to the limitations of genomic DNA and rRNA as templates for studies of microbial communities.

Use of a PCR – Single Strand Conformation Polymorphism (SSCP) method to assess the effect of diet on the microbial community of rumen. D. Macheboeuf[a], Y. Papon[a], E. Zumstein[b], B. Doreau[a], J.P. Jouany[a], J.J. Godon[b] ([a] INRA Unité de Recherche sur les Herbivores, 63122 Saint-Genès-Champanelle, France; [b] INRA Laboratoire de Biotechnologie de l'Environnement, 11100 Narbonne, France).

PCR-SSCP is a method which provides an estimate of microbial diversity and community composition without the bias imposed by culture-based approaches (Zumstein et al. 1999, Environ. Microbiol., in press). This method was used to assess the composition of microbial communities in the rumen. Rumen content samples were taken from a fistulated sheep fed on a lucerne hay diet or a 60%-barley, 40%-lucerne hay diet. Following isolation of total DNA, fragments of the bacterial 16S rDNA (V3-region) and of the protozoal 26S rDNA (D1/D2 domain) were amplified by PCR with specific primers. One of each pair of primers was 5'end labelled with a fluorescent dye. Strands were denatured and separated in non-denaturing conditions using an automated electrophoresis system. A bacteria-specific profile and a protozoa-specific profile were obtained from each sample. With the hay diet, the bacterial community was shown as a profile of 20 peaks with 13 main peaks. The protozoal community was represented by 12 peaks with 7 main peaks. Three of these peaks were determined as organisms of the genera *Polyplastron*, *Eudiplodinium* and *Entodinium*. After the diet was shifted to the high-grain level, the bacterial profile was reduced to 15 peaks (7 main) and the protozoal profile displayed 9 peaks (4 main). In this profile, 2 new peaks appeared while 5 disappeared. This study shows that the PCR-SSCP method can be used to assess the effect of dietary factors on the rumen ecosystem and that a high-grain diet induces a decrease of the microbial diversity of the dominant flora.

Remarkable archaeal diversity detected in the rumen of a cow. F. Rieu-Lesme[a], J.J. Godon[b], G. Fonty[a] ([a] Unité de Microbiologie, INRA, C.R. de Clermont-Ferrand/Theix, 63122 Saint-Genès-Champanelle, France; [b] INRA-LBE, 11100 Narbonne, France).

One major objective in microbial ecology is the understanding of biodiversity of the ecosystems. In the rumen, by using hydrogen and maintaining a low hydrogen partial pressure, methanogenic archaea play an important ecological role in fermentation processes. Methane constitutes a loss of carbon and energy for the host animal, and, in addition, is a greenhouse gas. Ruminant livestock are one of the major sources of atmospheric methane. In spite of the increasing interest for ruminal methanogenesis and lowering CH_4 production, the diversity, physiology and ecological panorama of the ruminal archaea community is not well known. Only four species have been

described so far. This limited diversity is probably merely apparent and reflects only the limits of the culture methods. Here we report a Single-strand-conformation polymorphism (SSCP) analyse of archaeal small subunit rRNA gene sequences obtained by polymerase chain reaction amplification of mixed population DNA extracted directly from the rumen contents of cows fed a grass diet. The samples were collected from the rumen just after slaughtering. Total DNA was extracted by a modification of the Marmur procedure. A fragment (approximatively 200 bp) of the bacterial 16S rRNA gene (V3 region) was amplified with one archaeal specific primer and one universal primer labelled at the 5' end. The PCR product was denaturated and separated on a non-denaturing gel. Generated SSCP profiles showed twenty distinguishable Archaeal peaks indicating as many Archaeal sequences. This study shows the potential of this approach to assess the microbial diversity of the rumen ecosystem and suggests that the Archaea community is probably more diversified than previously expected. Rumen samples taken from cows fed different diets are currently analysed with this technique.

Phylogenetic diversity of the intestinal bacterial community in the soil-feeding termite *Cubitermes orthognathus*. D. Schmitt-Wagner[a], M. Friedrich[b], A. Brune[a] ([a] Fakultät für Biologie, LS Mikrobielle Ökologie, Universität Konstanz, 78457 Konstanz, Germany; [b] MPI für terrestrische Mikrobiologie, 35043 Marburg, Germany).

The hindgut of soil-feeding termites is highly compartmentalised and characterised by pronounced axial dynamics of the intestinal pH and of microbial processes such as H_2-production, methanogenesis, and reductive acetogenesis (Schmitt-Wagner and Brune 1999, Appl. Environ. Microbiol. 65, 4490–4496; Tholen and Brune 1999, Appl. Environ. Microbiol. 65, 4497–4505). Since cultivation yielded only a small proportion of the microscopically detectable bacteria, we used a cultivation-independent molecular approach to assess hindgut microbial diversity and its axial differentiation. We prepared clone libraries of 16S rRNA gene fragments from the four major gut segments of *Cubitermes orthognathus*, using PCR amplification with a Bacteria-specific primer set. Phylogenetic analysis of >100 clones revealed that the microbial community in the anterior, highly alkaline gut segments is dominated by low-G + C-content gram-positive bacteria. In the posterior gut segments, their proportion decreased progressively, concomitant with an increase of other groups, including all groups of the Proteobacteria, the CFB group, and high-G + C-content gram-positive bacteria. The axial changes in community structure were confirmed by T-RFLP (terminal restriction fragment analysis) of PCR products of the individual hindgut compartments.

Development of the microbial community in weaning piglets. W.-Y. Zhu[a, b], B.A. Williams[a], A. Akkermans[a], S. Tamminga[a] ([a] Wageningen University, The Netherlands; [b] Nanjing Agricultural University, China).

Faecal samples from 4 piglets from the same sow were collected on Days 1, 6, 7 and 13 after weaning, and used to monitor the progress of the microbial population after the weaning process, using PCR and DGGE techniques (Zoetendal et al. 1998). On day 1 before a solid diet was offered, the four samples showed similar DGGE profiles, indicating a common microbial community. After the introduction of a solid diet, the DGGE profiles became more complex and differed between individuals. This suggests a development of a diverse bacterial community. However, as revealed by computer software analysis of DGGE profiles, the development of DGGE band complexity was relatively faster from days 1 to 7 than from days 7 to 13. This suggests a rapid development of the microbial community during the early stages after weaning. Thus, the early stages of weaning may provide an opportunity for gut microbial manipulation. It also shows that DGGE fingerprinting is a reliable approach to monitoring the development of the gut microbial community.

Dynamics of colonisation of the colon in Breast-fed infants. F. Martin[a], S.A.H. Savage[b], A.M. Parrett[b], G. Gramet[a], J. Doré[a], C. Edwards[b] (UEPSD, [a] INRA, Centre de Recherche, Jouy-en-Josas, France; [b] Department of Human Nutrition, Glasgow University, Yorkhill Hospitals, Glasgow, G38SJ, UK).

Microbial colonisation of the faecal flora in breast-fed infants was reassessed using culture-independent techniques. As indicators of bacterial communities implantation, Short Chain Fatty

Acids (SCFA) were assayed using conventional techniques. We applied quantitative Dot Blot hybridisation in order to evaluate the relative proportions of different bacterial groups. RNA was extracted from frozen faecal samples of babies at pre-, early- and late weaning. Using a set of six probes, we followed *Bacteroides, Lactobacilli*, Enterics, *Clostridium leptum* and *C. coccoides* phylogenetic groups and the genus *Bifidobacterium*. The results are expressed as the percent of bacterial rRNA. Values are given as mean ± S.D. for n subjects. The SCFA are expressed as µmol/g dry weight. Soon after birth (n = 5), the colonic flora was dominated by three groups: *bifidobacteria* (21.7% ± 6.8), *lactobacilli* (12.3% ± 3.2) and enterics (34.6% ± 0.15); the SCFA at pre-weaning were essentially, as expected, acetate (197.9) and lactate (37.4). The microflora changed as weaning took place (n = 8); *Bacteroides* (30.5% ± 9.4), *Clostridium leptum* (3.5% ± 2.7) and *coccoides* (10.6% ± 5.7) appeared in the dominant flora. In the meantime, propionate (51.7) replaced lactate (5.1). At late weaning (n = 5), *Bacteroides* and the *Clostridia* were the major groups and butyrate reached 40.5 $\mu mol \cdot g^{-1}$. These results are consistent with former data, stressing the effect of age on the dynamics of colonisation in infants.

Development of novel approaches to examine rumen microbial succession. D.R. Davies, A.E. Brooks, M.S. Dhanoa, R.J. Merry, M.K. Theodorou (Institute of Grassland and Environmental Research, Aberystwyth, SY23 3EB, UK).

Rumen microbiologists make a somewhat arbitrary distinction between liquid associated and solid associated micro-organisms. Specific groups of micro-organisms have been isolated and identified from these various niches in the rumen. This has contributed to significant advances in the understanding the rumen function. However, little emphasis has been placed on microbial succession in the rumen, particularly in relation to the colonisation and digestion of freshly ingested (living) grazed forages. Techniques are being developed to examine microbial succession and related plant enzyme mediated events (see linked paper by Kingston-Smith et al.) in freshly ingested grass boli. The grass boli are captured at the oesophageal/rumen aperture of cattle, following ingestion and chewing but before making contact with rumen contents. Initial studies have looked at microbial populations on the boli, before and after in vitro incubation (+/– rumen fluid), coupled with measurements of gas production. Anaerobic bacteria ($1 \times 10^7 \cdot g^{-1}$ fresh weight) were detected on the swallowed bolus, which is circa 1000-fold fewer than for the average rumen microbial population. Fermentation gas yield of an incubated bolus (which acted as both inoculum and substrate) was 270 mL, 68% less than one inoculated with 10% rumen fluid. Future developments will employ molecular techniques to help understand microbial colonisation and succession in the rumen with the ultimate goal of highlighting points of control in the digestion of plant biomass for improved rumen function.

A new look into the gut environment of edible Helicid snails. M. Charrier[a], A. Brune[b], G. Fonty[c], R. Roux[c], J.L. Foulon[a] ([a] UMR Ecobio 6553, Université de Rennes 1, France; [b] Fakultät für Biologie, Mikrobielle Ökologie, Universität Konstanz, Germany; [c] Lab. Microbiologie, INRA, CR de Clermont-Ferrand/Theix, Saint-Genès-Champanelle, France).

The artificial diets used in snail farming are usually derived from poultry food and are believed to be responsible for unsatisfactory results (dwarfism and sterility). In order to improve intensive rearing, more information on the gut microbial ecosystem of edible snails and their nutritional requirements is sorely needed. Using starved (at least for 3 weeks) and/or lettuce-fed individuals of *Helix aspersa*, *H. pomatia*, and *H. lucorum*, we performed: (i) a microelec-trode determination of O_2, H_2 and pH gradients along the digestive tract, (ii) counts of the total viable bacteria and methanogenic archaea and (iii) a cha-racterisation of H_2-producing bacteria. Axial O_2 measurements revealed that the gut lumen of each species was anoxic from the crop to the rectum, including the digestive gland. During starvation, H2 concentrations were around 15–75 µM, but could be 5 times higher in fed snails. The intestinal pH of starved *H. pomatia* and *H. aspersa* was between 6 and 7, but more acidic in the crop. Viable counts of anaerobic bacteria varied from 10^2 up to 10^{10} per g of tissue, depending on the gut section and the nutritional status of the snails. No CH_4 production occurred in the gut homogenates and no methano-gens were found. Two H_2-producing strains were isolated and identified as *Desulfotomaculum guttoïdeum* and *Citrobacter freundii*, based on their 16S rRNA gene sequence. These preliminary results reveal that helicid snails are possible hindgut fermenters.

Application of *Lactobacillus* and *Bifidobacterium* genus-specific PCR and DGGE to human intestinal tract ecology. R. Satokari[a], G.H.J. Heilig[a], E.E. Vaughan[a, b], E.G. Zoetendal[a, b], A.D.L. Akkermans[a], W.M. de Vos[a, b] ([a] Laboratory of Microbiology, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen; [b] Wageningen Centre for Food Sciences, PO Box 557, 6700 AN Wageningen, The Netherlands).

The introduction of molecular techniques in microbial ecology is the impetus for the renewed interest in the analysis of complex microbial communities such as the intestinal tract (Vaughan et al. 2000, Curr. Issues Intest. Microbiol. 1, 1–12). Research into the biodiversity and community behaviour over time has been especially awakened by the advent of fingerprinting techniques for complex communities. Separation of 16S rDNA PCR products generated with universal primers by denaturing gradient gel electrophoresis (DGGE) has been successfully applied to monitor the dominant human intestinal microflora (Zoetendal et al. 1998, Appl. Environ. Microbiol. 64, 3854–3859). A strategy was developed involving the use of genus specific primers to obtain PCR products for DGGE that allow the diversity and dynamics of specific groups that are present in lower numbers in the intestinal tract to be studied. We were especially interested in *Lactobacillus* and bifidobacteria that may function as probiotics and have a beneficial influence on health. By designing a *Lactobacillus*-specific primer and using genus specific *Bifidobacterium* primers, the indigenous species and the effects of probiotics on these populations in the human intestinal tract were rapidly evaluated and also monitored over time. Furthermore, by constructing clone libraries from the PCR products, *Lactobacillus* and bifidobacteria sequences were identified from different sites in the human intestinal tract and faeces. Many of these sequences shared less than 97% identity with known *Lactobacillus* and bifidobacteria in the databases.

Identification of *Bifidobacterium* sp. from piglets using differential plating, genus- and species-specific 16S rRNA-targeted probes and colony hybridisation. L.L. Mikkelsen, B.B. Jensen (Danish Institute of Agricultural Sciences, Research Center Foulum, P.O. 50, 8830 Tjele, Denmark).

A number of differential plating methods exist which seek to selectively detect and enumerate *Bifidobacterium* sp. in intestinal samples of human and animal origin. In addition, nucleic acid based methods using 16S or 23S rRNA-targeted hybridisation probes have been developed which can be adjusted to fit any taxonomic level for detection, enumeration and identification of bacteria in situ or after differential plating. The aim of this study was to combine differential plating with the use of bifidobacterial genus- and species-specific 16S rRNA-targeted oligonucleotide probes in conjunction with colony hybridisation, in order to investigate the bifidobacterial population of weanling piglets fed a prebiotic product. Samples from 2- to 4-week-old piglets were used to inoculate Beerens, Raffinose-Bifidobacterium and Modified Wilkins-Chalgren agars. Resulting colonies were randomly picked and bifidobacterial isolates were identified by demonstration of F6PPK activity and PCR using genus-specific primers. Sequencing of the 16S rDNA identified the bifidobacterial isolates as *B. boum*, *B. suis* and a possible new species. Specific 16S rRNA-targeted oligonucleotide probes were designed from the 16S rDNA sequences. Probes were alkaline phosphatase conjugated and the specificity was verified by colony hybridisation using bifidobacterial type strains, bifidobacterial isolates and non-bifidobacterial isolates from the differential plating as target organisms. The results demonstrated that the combined use of differential plating and colony hybridisation provides a powerful approach to the enumeration and identification of bifidobacteria in piglet intestinal samples.

Variability of *Butyrivibrio fibrisolvens* estimated by cellular fatty acid profiles, DNA restriction analysis and fermentation patterns. R. Marinsek Logar[a], J. Kopecny[b] M. Zorec[a] ([a] University of Ljubljana, Biotechnical Faculty, Zootechnical Dept., Groblje 3, 1230 Domzale, Slovenia; [b] Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Prague 10, Uhríneves, 104 00, Czech Republic).

Strains of *Butyrivibrio fibrisolvens* are thought to represent a significant part of rumen bacterial isolates. Presently the isolates are characterised as *B. fibrisolvens* based on certain common phenotypic characteristics despite the vast diversity and genetic unrelatedness of the strains. We analysed about 40 strains of *B. fibrisolvens* of domestic and wild ruminants to search for possibilities

of taxonomic reclassification and reliable identification. All the strains were characterised by morphology and VFA production. Their metabolic activities were tested with API 20 and showed 5 distinct groups of strains. Analysis of cellular fatty acids (FA) was done by GC following the guidelines of Microbial ID, Inc., Newark, DE, USA. The analysis confirmed the high variability of isolates and sorted them into 5 groups. C14:0, C16:0 and C18:1 were the major FAs for all isolates. DNA of selected (27) strains was isolated and 16S rDNA amplified with the aid of fD1 and rP2 primers. The distribution into groups based on 16S rDNA restriction analysis matched best with the distribution based on FA profiles (95% of similarity). The results support the possibility of using cellular FA profiles in *Butyrivibrio*-like isolates for taxonomy and identification purposes.

Flow cytometric analysis of ruminal prevotellas. G. Avgustin, L. Lipoglavsek (University of Ljubljana, Biotechnical Faculty, Zootechnical dept., Groblje 3, 1230 Domzale, Slovenia).

Flow cytometry makes the analysis of complex microbial ecosystems possible, on the level of individual microbial cells when used in combination with specific oligonucleotide probes. The speed and sensitivity of the method may surpass all other approaches given the fact that modern flow cytometers can analyse up to several thousand cells per second and usually record three or more fluoerescences and two light scatterings at the same time. Here we report one of the first attempts to use ribosomal RNA oligonucleotide probes specific for ruminal *Prevotella* species labelled with various fluorochromes for flow cytometric analysis. The cytometer we used has one laser that emits light at 488 nm only. Therefore we used the polyanionic azo dye trypan blue for discrimination between live microbial cells and dead cells as well as anorganic particles. Trypan blue has intrinsic fluorescence in the far-red region which is detected with the FL3 photomultiplier, which leaves the FL1 and FL2 photomultipliers for detection of green or orange fluorescence from the labelled oligonucleotide probes. Fluorescein rhodamine green and indo carbocyanin Cy3 proved to be the most suitable fluorochromes. We used at least three different 5' end labelled oligonucleotide probes, to increase the emitted light sufficiently for analytical purposes. Since three species-specific regions are difficult to find for most organisms we tried to increase the emitted fluorescence by using in situ PCR and in situ RT-PCR procedures rather that in situ hybridisation.

Competitive PCR and the detection and quantification of ruminal prevotellas. K. Tepsic, G. Avgustin (University of Ljubljana, Biotechnical Faculty, Zootech. Department, Groblje 3, 1230 Domzale, Slovenia).

Bacteria belonging to the genus *Prevotella* represent an important part of the microbial population in the rumen. This has been suggested by several authors and was confirmed recently by three independent molecular studies. 16S ribosomal RNA genotypes from prevotellas represent a large part of all genotypes retrieved by PCR and are the predominant type from G-bacteria. Four different species of the genus *Prevotella* were recently described, but no simple and rapid identification and quantification system for the species and for the genus is yet available. Competitive PCR allows a specific, rapid and sensitive detection and quantification of targeted organisms. A set of previously described oligonucleotide sequences, specific for *P. ruminicola*, *P. bryantii* and *P. brevis* and for genera *Bacteroides* and *Prevotella,* was used as the basis for cPCR development. Internal controls were constructed by enzymatic removal of the central part of the amplified products. cPCR primers were labelled with fluorochrome JOE and the amplicons were analysed by capillary electrophoresis. The results of the use of cPCR screenings for the presence and abundance of *Prevotella* species in cattle rumen fluid as well as in fecal samples from cattle and some monogastric herbivores are presented.

Structural organisation of the genes involved in cell division in the bacterium *Prevotella albensis*. N.R. McEwan, N.D. Walker, R.J. Wallace (Protein Metabolism Group, Rowett Research Institute, Aberdeen AB21 9SB, Scotland).

The DNA and protein sequences of components of the *P. albensis* M384 operon involved in cell division (the *fts* genes) are presented. This is the first time that genes from this operon have been described in a rumen bacterium. The structure of *P. albensis* genes and also their intergenic spacers (IGSs), in particular the IGS between *ftsQ* and *ftsA*, are discussed with regard to the

arrangement of these genes and IGSs in those species where these genes have already been identified. It is clear that there is considerable variation in the length of the IGSs between *ftsQ* and *ftsA* in a number of bacteria. In some cases there is no IGS, and the two genes overlap. The level of overlap, or size of this IGS, is discussed in terms of bacterial evolution.

Ribosomal genes from rumen *Prevotella* species – the sequence variability and impact on phylogenetic placement. M. Peterka, G. Avgustin (University of Ljubljana, Biotechnical Faculty, Zootech. Deptartment, Groblje 3, 1230 Domzale, Slovenia).

The nucleotide sequences of the small ribosomal sub-unit represent the largest biological database and the basis of the new, natural microbial taxonomic system. rRNA genes fulfil most of the demands for the ultimate molecular chronometer. However, most organisms have more than one copy of rRNA genes and the fact that these copies may differ substantially, as it was shown recently, was ignored. How these differences influence the phylogenetic positions of microorganisms, especially in the light of new molecular approaches to microbial diversity of complex ecosystems without isolation and cultivation procedures, is not yet known. The number of rRNA operons in rumen bacterial species from the genus *Prevotella* was determined. It was found that there are between four and seven rRNA operons in four different *Prevotella* species. Sequence heterogeneity between 16S rRNA gene copies was examined by direct sequencing of PCR products and sequencing of cloned ribosomal operons. In *P.ruminicola* 23^T, the analysed sequences differ up to 10%, whereas the preliminary analysis of cloned rRNA operons from *P.bryantii* B_14^T (three out of six were analysed) showed less differences. The impact of the sequence variability of 16S rRNA gene copies on phylogenetic positioning of *Prevotella* species is presented.

Phenotypic and genotypic variability in a local population of *Selenomonas ruminantium*. P. Pristas, V. Molnarova, B. Zatkovic, P. Javorsky (Institute of Animal Physiology, Slovak Academy of Sciences, 04001 Kosice, Slovak Republic).

In the last few years, a substantial diversity has been observed in many species of rumen bacteria. We have analysed the variability in 20 strains of *Selenomonas ruminantium* coming from the same local population. Tested strains showed a substantial diversity in basic biochemical properties (utilisation of sugars, growth characteristics) and in observed restriction and modification profiles. The criteria used allowed clear separation of all tested strains. In agreement with phenotypic variability, total cellular protein profile variability was observed as well. PCR DNA fingerprinting techniques based either on ERIC (Enterobacterial Repetitive Intergenic Consensus) or 16S-rDNA primers were used to cluster isolates. ERIC-PCR resulted in strain specific patterns which allowed us to define several clusters of strains. On the contrary, restriction analysis of amplified 16S-rDNA indicated close evolutionary similarity of the tested strains. All but one strain produced identical profiles using several restriction endonucleases. Genetic relatedness of strains was confirmed by sequence analysis of amplified 16S-rDNA. On the basis of the data, phenotypic variability in the *S. ruminantium* species could be attributed to intraspecific variability only.

Isolation of *Methanoculleus olentangyi* as the dominant archaea from the caecum of a donkey. F. Rieu-Lesme, G. Fonty (Unité de Microbiologie, INRA, CR de Clermont-Ferrand/Theix, 63122 Saint-Genès Champanelle, France).

In the digestive tract of herbivores the archaea methanogens play an important ecological role in the trophic chain leading to the degradation and fermentation of plant polymers. Nevertheless our knowledge of the diversity of this microbial community is still very poor. We have therefore initiated the isolation and characterisation of archaeal strains from the digestive tract of various herbivores. Here we report the isolation of *Methanoculleus olentangyi* as the dominant archaea from the caecum of a donkey. The samples were collected from the caecum and immediately serially diluted in an anaerobic mineral medium. Archaeal isolations were performed in roll-tubes on Balch medium (Balch et al. 1979, Microbiol. Rev., 43, 260–296). Cultures were incubated under H_2/CO_2 (80/20) pressurised at 200 kPa. Colonies were purified by repeated streakings and transfers under H_2/CO_2. Methane production was determined by gas chromatography. Strain Tcher 98 was the only methanogen isolated from the 10^{-8} dilution of the sample. The cells were extremely pleiomorphic and contained gas vesicles. On the basis of the full 16S rRNA sequence analysis, this isolate was

identified as *Methanoculleus olentangyi* in spite of some phenotypic differences with the type strain. This is the first report of the presence of this species in the digestive tract. Up to now this species has only been found in sediments and aquatic environments. Phenotypic characteristics of our isolates are currently under studies in order to precise their function in the rumen.

Characteristics of the cellulolytic microflora from the colon of methane-excreting individuals. C. Robert, A. Bernalier (Unité de Microbiologie, INRA de Theix, 63122 Saint-Genès-Champanelle, France).

The microflora of the human colon plays an important role, by hydrolysing and fermenting dietary fibers. However, the bacterial population involved in insoluble polysaccharides degradation is still rather unknown. The fermentation leads to the production of volatile fatty acids and gas. The hydrogen from fermentation is mainly used in situ by the hydrogenotrophic microorganisms. The human population is divided in 2 groups according to their breath CH_4 production: the methano-excretors (CH_4^+) and the non-methano-excretors (CH_4^-), correlated with the presence or the absence of archaea methanogen at a high level. Using culturable methods, we found cellulolytic bacteria in only the faeces of the CH_4^+ subjects. We have demonstrated a correlation between the size of the culturable cellulolytic microflora and the size of the methanogenic population (Bernalier et al. 1998, 8th International Symposium on Microbial Ecology, p. 104). The aim of this work was to study the diversity and the activity of 7 Gram+ cellulolytic strains (6 cocci and 1 rod) isolated from the faeces of 4 CH_4^+ subjects. According to the 16S rRNA gene sequencing, 5 strains could be closely related to the genera *Propionibacterium* (1 strain), *Enterococus* (2 strains) and *Ruminococcus* (2 strains). Two strains could not be assigned to a known genus. All the strains were able to use various sugars (fructose, mannose…) commonly found in plant cell walls, and to hydrolyse filter paper, hemicelluloses (glucomannane and xylane) and buckwheat powder. The main end-products of glucose fermentation by the *Propionibacterium* strain were propionate and acetate and those of cellulose fermentation by the *Enterococcus* and *Ruminococcus* strains were acetate, succinate and H_2. The *Ruminococcus* and *Enterococcus* strains exhibited CMCase activity and xylanase activity.

Characterisation of mucosal populations in the large gut and studies on mucinolytic bacteria in the chemostat. J.C.M. Paterson, G.T. Macfarlane, S. Macfarlane (MRC Microbiology and Gut Biology Group, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, UK).

Much of our knowledge of the human colonic microbiota derives from studies on lumenal material, but little is known of the identities and metabolic activities of bacteria inhabiting the gut mucosa. In this study, bacteria were isolated from rectal tissue and identified by FAME analysis. Over fifty different species were detected, belonging to 18 genera, but bifidobacteria and bacteroides predominated. Facultative anaerobes occurred in similar numbers to strictly anaerobic species, with viable counts ranging from 10^6 to 10^7 cm^2. Enzymological measurements showed that the isolates synthesised a variety of mucinolytic enzymes. However, none were individually capable of extensive mucin breakdown, suggesting that mucin digestion on the colonic epithelium is largely a cooperative process, in which a number of species with complementary enzyme systems, and substrate preferences, may be required. Modelling studies were used to investigate colonisation of mucin surfaces by gut microorganisms. This demonstrated that environmental factors such as carbohydrate availability were important in the ability of bifidobacteria and some bacteroides (*B. distasonis*, *B. caccae*) to form biofilms, though the establishment of other species, for example *E. coli*, *Bacteroides thetaiotaomicron*, *B. distasonis*, and various clostridia was unaffected. Measurements of mucin degrading enzymes together with chemical analysis of residual mucin oligosaccharides revealed that planktonic communities and mucin biofilm populations in the chemostats were physiologically and metabolically different, and that catabolite regulatory mechanisms played an important role in mucin digestion.

Phylogenetic relationships of butyrate-producing bacteria from the human gut. S.E. Pryde[a], A. Barcenilla[a], J. Martin[a], S.H. Duncan[a], C.S. Stewart[a], C. Henderson[b], H.J. Flint[a] ([a] Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK; [b] Robert Gordon University, Kepplestone Campus, Queens Road, Aberdeen, AB9 2PG, UK).

Butyrate is a preferred energy source for colonic epithelial cells and is thought to play an important

role in maintaining colonic health in humans. In order to investigate the diversity and stability of the butyrate-producing colonic flora, anaerobic butyrate-producing bacteria were isolated from freshly voided human fecal samples from three healthy individuals: an infant, an adult following an omnivorous diet and an adult vegetarian. A second isolation was performed on the same three individuals one year later. Of a total of 313 bacterial isolates, 74 produced more than 2mM butyrate in vitro. Butyrate-producing isolates were profiled and grouped by 16S ribosomal DNA (rDNA) PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism). The results indicate very little overlap between the predominant ribotypes of the three subjects; furthermore the flora of each individual changed significantly between the two isolations. Complete sequences of 16S rDNA genes were determined for 24 representative strains and subjected to phylogenetic analysis. 80% of the butyrate producing isolates fell within the XIVa cluster of Gram-positive bacteria as defined by Collins et al. (1994, Int. J. Syst. Bacteriol. 44, 812–826) and Willems et al. (1996, Int. J. Syst. Bacteriol. 46, 195–199), with the most abundant group (10/24 or 42%) clustering with *Eubacterium rectale*, *Eubacterium ramulus* and *Roseburia cecicola*. The majority (53%) of the butyrate producing isolates was net acetate consumers during growth suggesting that they employ the butyrylCoA/acetylCoA transferase pathway for butyrate production.

Phenotypic and physiological characteristics of the major culturable butyrate-producing bacteria isolated from human faecal samples. S.H. Duncan, A. Barcenilla, J.C. Martin, S.E. Pryde, C.S. Stewart, H.J. Flint. (Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK).

Bacterial communities in the colon ferment dietary material to volatile fatty acids (VFA) primarily acetate, propionate and butyrate (3:1:1). Butyrate is important to colonic health and is the major energy source for the colonocytes. However little information exists on the identity of the bacteria that produce butyrate in the human colon. Here we report on some phenotypic characteristics of seven isolates from two human volunteers and show they are related to genera that include *Fusobacterium*, *Coprococcus*, and *Eubacterium*. All the isolates produced excess gas and six produced hydrogen suggesting that these butyrate-producers may be closely associated with the hydrogen-utilising methanogens or acetogens. The non-hydrogen producing isolate (A2–165) was most closely related to *Fusobacterium prauznitzii* and appears to have an absolute requirement for acetate in the growth medium. Also the growth of five of the other isolates was enhanced when acetate was added to the medium. The seventh isolate (L2–50) was most closely related to *Coprococcus* sp. and growth was inhibited when acetate was present in the medium. This isolate had a requirement for VFA other than acetate, presumably required as precursors for amino acid biosynthesis. The *Coprococcus* sp. (L2–50) was also the only isolate that possessed butyrate kinase activity whilst the others possessed acetate kinase activity. A clearer understanding of the abundance and diversity of the butyrate-producing bacteria that inhabit the healthy human large intestine along with the physiological factors that influence butyrate production may be of fundamental importance to colonic health.

Effect of the diet on the caecal microflora of the growing rabbit. N. Bennegadi[a], G. Fonty[b], L. Millet[b], T. Gidenne[a], D. Licois[c] ([a] Station de Recherches Cunicoles, INRA Toulouse, 31326 Castanet-Tolosan, France; [b] Unité de Microbiologie, INRA-Theix, 63122 Saint-Genès-Champanelle, France; [c] PAP, INRA-Nouzilly, 37380 Nouzilly, France).

Plant fibres are a major component of rabbit alimentation, and digestive disorders are frequently observed when rabbits are fed low fibre diets. However it is not clear if these disorders are related to perturbations of the intestinal microflora or to physiological parameters. Therefore, our study was aimed at exploring the composition of the caecal flora of the growing rabbit in relation to the level of fibre in the diet. Nine white rabbits were fed ad libitum on a standard diet (S) (Acid Detergent Fibre = 19%) and 9 rabbits were fed a low-fibre diet (D) (ADF = 9%) from 28 to 70 days of age. The caecal microflora was analysed at days 28, 42 and 70 (3 animals/age/diet) by using dot-blot hybridisation with specific oligonucleotide probes. The composition of the diet had no significant effect on the size of the bacterial and eukaryotic communities. In contrast the size of the archaeal community was significantly lower with the D diet than with the S diet. With the two diets, the genus Bacteroides was predominant, but the size of its population was particularly higher with the D diet. With the two diets, the percentage of the 16S rRNA of the four targeted cellulolytic species (*Fibrobacter succinogenes*, *F. intestinalis*,

Ruminococcus albus and *R. flavefaciens*) represented approximately 9% of the total bacterial 16S rRNA, *F. intestinalis* and *R. flavefaciens* being the dominant species. The percentage of the 16S rRNA of R. albus tended to be higher with the D diet. No *Prevotella ruminicola*, *Escherichia coli* and anaerobic fungi were detected whatever the diet. Therefore, compared to the standard diet the targeted microbial populations were moderately affected by the low-fibre diet.

The influence of finely and coarsely ground feed fed as mash or pellets on the microbial composition in the digestive tract of broiler chickens. R.M. Engberg, B.B. Jensen (Department of Animal Nutrition and Physiology, Danish Institute of Agricultural Sciences, 8830 Tjele, Denmark).

A feeding experiment was conducted with 4 groups of broilers (240 animals per group, 8 replicate pens) receiving a wheat based diet offered as finely ground mash, coarsely ground mash, finely ground pellets, and coarsely ground pellets, respectively. During the 5th week of the experiment, 6 chickens from each pen were killed, and the contents of the gizzards, duodenum, jejunum, ileum, caeca and colon/rectum were separately collected and pooled. The pH-value was measured in all intestinal segments. Total anaerobic bacteria, coliforms, enterobacteria, lactic acid bacteria, lactobacilli, enterococci and *Clostridium perfringens* were enumerated. Generally, the influence of the form of the feed (mash vs. pellets) on the measured parameters was much greater than that of particle size of the feed. Animals fed pellets showed a higher number of enterococci and coliform bacteria in the ileum. The feeding of mash resulted in a higher number of total anaerobic bacteria, lactic acid bacteria, lactobacilli and *C. perfringens* in the lower intestine (caeca and colon/rectum). Birds receiving their diet as mash, in particular as coarsely ground mash showed a lower pH-value in the contents of the gizzard. In the contents of all other intestinal segments, the pH-value was higher than in animals fed pellets, which was surprising considering the higher number of anaerobic bacteria and lactobacilli in the mash fed animals. Other factors responding to feed structure, such as increased gastric hydrochloric acid-secretion (low gizzard pH) and increased pancreatic bicarbonate secretion (high intestinal pH) may explain these findings.

Effect of the proportion of pectin vs. starchy supplements of straw diets on rumen bacterial numbers and activity. A. Barrios Urdaneta[a], M. Fondevila[b], C. Castrillo[b] ([a] Fac. de Agronomía, Universidad del Zulia, Venezuela; [b] Depto. Producción Animal y Ciencia de los Alimentos, Universidad Zaragoza, Spain).

This work was aimed at validating in vivo previous in vitro results showing a higher bacterial fibrolytic activity over straw by adding pectin instead of starch. Four adult rumen-cannulated sheep received 400 g of ammoniated straw and 400 g of a concentrate made up with different ratios of barley and citrus pulp (3:0, M1; 2:1, M2; 1:2, M3; and 0:3, M4) daily, in a Latin Square design (4×4), made isonitrogenous with soya meal/urea. Adaptation periods to diet changes lasted 15 d. Total bacterial concentrations (10^8 mL^{-1}) were higher ($P < 0.05$) in high barley diets (22.3, 19.1, 8.3 and 3.7 for M1, M2, M3 and M4; s.e.m. = 2.96). The results were supported by the microbial synthesis estimated from allantoin excretion in urine (12.0, 11.3, 10.4 and 10.0 μmol·g^{-1} DOMI for M1, M2, M3 and M4; s.e.m. = 0.26). There was also a trend ($P < 0.10$) for higher cellulolytic counts (10^7 mL^{-1}) in M1 (11.9, 3.8, 6.8 and 1.5 in M1, M2, M3 and M4; e.s.m. = 2.17), but there were no differences in amylolytic numbers ($P > 0.10$). Total VFA concentration and proportions of acetate, propionate and butyrate were unaffected by the diet ($P > 0.05$). Total enzymatic activity against CMC, avicel or xylan was also unaffected ($P > 0.10$). Differences in total numbers were not explained by changes in rumen environmental conditions, since means weighted by time, pH and ammonia did not differ among diets. Instead, a higher rate of passage promoted by barley may have caused higher microbial synthesis efficiency. The advantages in straw fermentation observed in vitro by adding pectin instead of starch, were minimised in vivo.

Identification and distribution of bacteriocin gene homologues in the ruminal anaerobe *Butyrivibrio fibrisolvens*. M.F. Whitford, R.J. Forster, R.M. Teather. (Lethbridge Research Centre, Agriculture and Agri-Food Canada, PO Box 3000, Lethbridge, AB, T1J 4B1 Canada).

Twenty-five of fifty *Butyrivibrio fibrisolvens* isolates examined were previously shown to produce a variety of bacteriocin-like inhibitory substances (BLIS). Some of the isolates examined

were not producers but exhibited patterns of BLIS resistance similar to closely related BLIS producers. These results indicate that bacteriocin production and resistance is important in inter- and/or intra-species competition in the rumen. BLIS from two isolates (OR79 and AR10) were shown to be new Class I and Class II bacteriocins, respectively. Relatively little is known about the BLIS from the remaining *B. fibrisolvens* isolates. Using PCR and DNA sequence analysis, we identified genes homologous to the OR79 bacteriocin structural gene (*bvi79A*) in a large proportion of the other isolates, including strains that do not express the bacteriocin. The homologues generally differed in their primary sequence but the number of peptide sequence variants was limited. Genes homologous to *bvi79A* were found in members of different *B. fibrisolvens* 16S ribosomal DNA subgroups and in strains isolated from a variety of animal sources and geographical locations, suggesting an early phylogenetic origin or lateral transfer. Our results support the hypothesis that the penalty associated with bacteriocin resistance and production is often sufficient to offset its selective advantage, leading to a high incidence of variants that do not express one or both functions.

Bacteriocins of Gram-positive ruminal cocci.
M. Morovsky[a], P. Pristas[b], H. Holo[c], I.F. Nes[c], P. Javorsky[b] ([a] Department of Biochemistry, Faculty of Sciences, UPJŠ Kosice; [b] Institute of Animal Physiology, Slovak Academy of Sciences, Kosice, Slovakia; [c] Laboratory of Microbial Gene Technology, Agricultural Univerzity of Norway, N-1432 Ås, Norway).

The bacteriocin-like activity from rumen strains *E. faecium* BC25 and *S. bovis* II/1 with antibacterial activity against ruminal *S. bovis* isolates, were purified to homogeneity and characterised. Enterocin BC25, was a type II_a with significant homology to enterocin A, a bacteriocin produced by *E. faecium* CTC492/T136. DNA fragment encoding BC25 activity was isolated by PCR and completely sequenced. Antimicrobial protein isolated from the *S. bovis* II/1 was very similar to enterolysin A, isolated from *E. faecalis* LMG 2333 (Nilsen et al. 1999, submitted for publication in J. Bacteriol.) and belongs to the family III (large heat labile proteins) of bacteriocins. The oligonucleotide primers, specific for structural genes of enterocin A and enterolysin A, were used to study the distribution of sequences similar to these genes within the enterococcal and streptococcal strains isolated from the rumen by PCR. Our findings indicate a variable occurrence of these bacteriocins within the populations of Gram-positive ruminal cocci. The native ruminal bacteriocins should be used as modulators of the ruminal microbial ecosystem mainly for controlled alternation of ruminal microflora as alternatives to the ionophore antibiotics, as antimicrobial substances towards the pathogenic microorganisms colonising the digestive tract of ruminants and also for stabilisation of the cloned ruminal bacteria within the natural rumen ecosystem.

Session II:

Microbial activities and metabolism

Fibre degradation

Effect of cell wall organisation on rumen microbial degradation of fescue. G.J. Provan[a], L. Scobbie[a], A. Chesson[a], M.A. Bernard Vailhé[b], A. Cornu[b], J.M. Besle[b] ([a] Nutritional Chemistry Unit, RRI, Bucksburn, Aberdeen AB21 9SB, UK; [b] URH, INRA de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

Newly extended apical internodes of fescue were each divided into five sections, a part for analysis and a part incubated in the rumen of a canulated sheep. Lignin content increased (from 93 to 143 g·kgCW^{-1}) from the basal to the third section and then remained constant. Esterified *p*-coumaric acid (PCA) and total ferulic acid increased mostly between the basal and the second section. In contrast, the ratio of esterified FA/PCA (estFA/PCA) and non-ester linked FA (EtFA) content more closely reflected the increasing age of the tissue. Esterified hydroxycinnamic acids were rapidly hydrolysed in the rumen but EtFA to a much lesser extent. Both the extent and rate of degradation were significantly correlated with lignin and EtFA concentrations and to estFA/PCA, but the rate of degradation was better related to estFA/PCA and to EtFA than the extent of loss. The decrease in cell wall degradation (from 84% at the base to 40% at the top of the internode) could not be explained solely by the small increase in lignin content. The resistance provided by the cross-linking of the cell wall by ester and ether-linked hydroxycinnamic acids was evidently a contributing factor.

A comparison of fibrolytic and fermentative activities of the ruminal and cecal microbial ecosystems. J.P. Jouany, B. Lassalas, I. Fernandez, B. Michalet-Doreau, C. Martin (INRA, URH-DIM, Centre de Recherches de Clermont-Theix, 63122 Saint-Genès-Champanelle, France).

We compared the abilities of rumen (R) and cecal (C) digesta to degrade and ferment various cell wall carbohydrates. Four sheep fed a lucerne hay diet and fitted with R and C canulae were used. Degradation of 3 ground substrates (lucerne L, avicel A, xylan X) and fermentations were monitored for 24 h in small in vitro fermenters containing 10 mL digestive content + 20 mL of buffer + 150 mg of substrate. The specific activities of poly-saccharidases and oligo-saccharidases in digesta were 15 to 100 times higher in R than in C. However, total fermented hexoses were the same in R and C for all three substrates. The higher acetate production in C (460 mM) than in R (406 mM) was compensated for by either a decrease in methane with L, or a decrease in propionate with X. In vitro degradation of L (72 mg) was significantly lower than that of pure A (125 mg) or X (120 mg). A and X gave high atypical molar proportions of propionate (31 to 37%). Butyrate production was low for all three substrates (from 14 to 46 mM). The fibrolytic potential of the 2 ecosystems was similar when compared in vitro in the same environment, while R was much more efficient than C when digestion was measured in vivo. Such discrepancies can be explained by some cecal digesta characteristics not considered in vitro: (1) a lower supply of degradable cell walls, (2) the viscosity of C digesta which limits the exchanges between solid and liquid phases, (3) the lower retention time of solid particles. High values of propionate observed with A and X indicated that *Fibrobacter succinogenes* was probably the main fibrolytic species involved in our in vitro system.

Fibrolytic activities and cellulolytic bacterial community structure in the different rumen compartments. B. Michalet-Doreau[a], I. Fernandez[a], C. Peyron[a, b], L. Millet[b] (INRA, [a] URH-DIM, [b] Unité de Microbiologie, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

The rumen is a complex system containing liquid and solid digesta, and a very heterogeneous repartition of microorganisms between them. The objective of this study was to determine the distribution of microbial fibrolytic activities in the rumen digesta ecosystem in relation to the composition of the cellulolytic bacterial community. Rumen contents were collected 3 hours after feeding from 4 sheep fed an alfalfa hay diet. Polysaccharidase activities, expressed by the amount of reducing sugars released from xylan and avicel, and distribution of the 3 main cellulolytic bacterial species, *Fibrobacter succinogenes*, *Ruminococcus albus* and *flavefaciens* were determined in the crude (C), filtered (100 µm) (F) and filtered and washed (FW)

digesta. Cellulolytic species were detected with specific oligonucleotide probes. Specific activities of polysaccharide depolymerase enzymes (xylanase and CMCase) were higher in F than in C digesta, and in FW than in F digesta. The amount of total bacterial 16S-rRNA did not vary significantly with the nature of the samples, but the sum of the three cellulolytic bacterial species, amount and relative proportion of the total bacterial community, were higher in F than in C digesta. In all samples, the relative population size of *F. succinogenes* was much higher than those of *R. albus* and *R. flavefaciens*. The most active enzymes are secreted by the particle-associated microorganisms. The differences in the composition of microflora between solid and liquid phases suggested that bacteria are not equally distributed in the whole ruminal content. Fibrolytic species were present in a higher proportion in the solid phase.

Activity and structure of the cellulolytic bacterial community associated to the solid-adherent digesta in rumen and caecum of sheep. B. Michalet-Doreau[a], I. Fernandez[a], C. Peyron[a, b], G. Fonty[b], C. Martin[a], J.P. Jouany[a] (INRA, [a] URH-DIM; [b] Unité de Microbiologie, Centre de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

In ruminants, cell wall digestion mainly occurs in the rumen, but the caecum also plays a role in digestion, especially when digestion in the forestomachs is limited by a supplemented diet. The objective of this study was to compare the activity and structure of the solid-adherent cellulolytic bacterial community in these 2 fermentative compartments. Digesta samples were collected before feeding, and 3, 6 and 9 h after feeding from four sheep, fed alfalfa hay supplemented with barley (40/60) twice a day. Polysaccharidase activities were estimated by the amount of reducing sugars released from xylan and avicel. The populations of the 3 main cellulolytic bacteria (*Fibrobacter succinogenes, Ruminococcus albus* and *flavefaciens*) were quantified by dot-blot hybridation using specific 16S-rRNA-targeting probes. Fibrolytic enzyme activities were higher in the rumen than in the caecum, 8.95 vs. 0.38 μmol reducing sugar per mg of protein per hour for xylanase activity and 0.64 vs. 0.02 for avicelase activity before feeding. After feeding, fibrolytic activity decreased in the rumen and was not modified in the caecum. The amounts of total bacterial 16S-rRNA and of 3 cellulolytic bacterial species, were higher in the rumen than in the caecum, 696 vs. 338 $\mu g \cdot g^{-1}$ of DM and 31 vs. 13, respectively. Cellulolytic bacteria represented the same percentage of the total bacterial community (4%) in the two compartments. Therefore the lower cellulolytic activity in the caecum compared to the rumen could not be ascribed to a difference in the structure of the cellulolytic bacterial community between these 2 compartments.

Cell-wall digestibility of *Bouteloua repens* by pure and mixed cultures of rumen fungi and bacteria isolated from grazing cattle in the tropics. M.L. Arcos, T.E. Diaz (Corporación Colombiana de Investigación Agropecuaria CORPOICA, C.I. Tibaitatá, A.A. 240142, Santafé de Bogotá, Colombia).

Pure cultures of the rumen fungus *Neocallimastix frontalis* (F), mixed cultures of cellulolytic bacteria-50% *Fibrobacter succinogenes* + 50% *Ruminococcus albus* (B), as well as mixed cultures of *N. frontalis and F. succinogenes* + *R. albus* (FB) were used to test in vitro NDF digestibility of the tropical grass *Bouteloua repens*. A traditional in vitro procedure using rumen fluid (RF) as the inoculum was used as a control treatment (C). NDF digestibility of *Bouteloua repens* was higher ($P < 0.05$) for FB (67.69%) as compared with F (47.97%), B (56.76%) and C (43.61%) treatments. There were no differences ($P < 0.05$) between F and C treatments. A synergistic effect was apparent in the mixed FB culture represented by digestibility values of 19.7 percent units higher than F and 10.93 percent units higher than B. Different concentrations of fungi and bacteria in the mixed cultures also influenced the digestibility values of *Bouteloua repens.* The results suggest that using specific strains of fungi and bacteria isolated from cattle grazing low quality pastures for in vitro procedures is an alternative to estimate maximum digestibility values for tropical high cell-wall content grasses. These results also suggest a potential use for probiotics in tropical cattle feeding systems.

Cellulolytic enzyme activity relates to the growth of *Fibrobacter*, but not of *Ruminococci* populations during in vitro fermentation of different substrates with rumen fluid. S. Muetzel, E. Hoffmann, K. Becker (Univ. Of Hohenheim, Inst. 480, 70593 Stuttgart, Germany).

The fermentation of given substrates in the rumen has mostly been analysed only with respect to the formation of end products, such as SCFA, gas, ammonia, and the apparent and true digestibility of the substrate. Due to the high complexity of the rumen ecosystem comparatively little is known about the dynamic processes generating these products and the contribution of certain groups of microorganisms. The hohenheim gas test was used as a short term batch incubation system to characterise the fermentation profiles of three different substrates (barley straw, *Sesbania sesban*, *S. pachycarpa*). Population dynamics of selected groups of rumen microbes, quantified by 16S rRNA hybridisation, and enzyme activities were recorded over a 48 h incubation period, focusing on carbohydrate fermentation by cell wall degrading organisms. A very good relationship was observed between carboxymethyl cellulase activity induced with the different substrates and the *Fibrobacter* population. *Ruminococci* (*R. albus* and *R. flavefaciens*), known to use a wider range of substrates, showed variable responses to the *Sesbania* leaves. Since these are rich in xylan and pectin, additional enzyme activities such as xylanase and pectinase should be assayed to further interpret these growth kinetics. The approach will be extended further to proteolytic organisms by following protein degradation, protease activity and proteolytic populations. The results presented here demonstrate that the comparison of physiological activities and population kinetics provides a new type of information which will contribute to a better understanding of the complex process of rumen fermentation.

Isolation and evaluation of bacteria from indigenous African animals for evidence of superior cellulolytic enzymes. A.A. Odenyo[a], H. JunSang[b], P.O. Osuji[a] ([a] International Livestock Research Institute (ILRI), P.O. Box 5689, Addis Ababa, Ethiopia; [b]National Livestock Research Institute, #564, Omokchun-dong, Kwonsun-gu, Suwon, Korea).

The objective of the study was to isolate microorganisms with superior cellulolytic activities that can be used to improve digestion of poor quality feeds by domestic ruminants. Five strains of obligately anaerobic cellulolytic bacteria were isolated from indigenous African animals. These isolates were designated as KG1C.1, Kenyan giraffe; EAC.2, Ethiopian antelope; EGC.3, Ethiopian goat; EHC.4, Ethiopian horse; EDC.5, Ethiopian donkey. The isolates were tested for their ability to degrade cellulose strips (CS) and acid swollen cellulose using *Ruminococcus flavefaciens* FD-1 as a control. The results showed that EAC.2 was the fastest (hydrolysing the CS overnight) followed by EGC.1, EDC.5, and KGIC.1. *R. flavefaciens* FD-1 and EHC.4 took a similar time. The cultures were grown on cellobiose medium overnight and the supernatant and pellet were tested for CMCase and xylanase activities. Overall, more than 90% of the activity was detected from the supernatant. The freeze-dried supernatants were fractionated and the fractions were tested for CMCase and xylanase activities. Activities (CMCase and xylanase) were detected from fractions eluted between 16 and 21 min in the samples from KAC.1, EGC.3 and *R. flavefaciens* FD-1. Activities from EHC.4 and DC.5 samples were detected from fractions eluting between 16 and 21 and between 26 and 41 min. The fractions from isolate EDC.5 showed the highest activities. The fractions were separated on a polyacrylamide gel (10%) at 20 mA for 1 h. The results showed that the enzyme (s) from EDC.5 was 17 kDa. The fractions from the rest of the samples gave multiple bands. Xylanase activity was higher than CMCase in all isolates.

Isolation and characterisation of *Butyrivibrio*-like rumen bacteria posessing high xylanolytic activity. R. Marinsek Logar, M. Zorec, F.V. Nekrep (University of Ljubljana, Biotechnical Faculty, Zootechnical Dept., Groblje 3, 1230 Domzale, Slovenia).

Xylans are major components of plant materials and many rumen bacterial species possess xylanolytic enzymes. 8 xylanolytic bacterial strains were isolated from rumen contents of a black and white Friesian cow. Pure cultures were obtained using the roll-tube anaerobic technique in a modified M2 medium containing 0.6% oat spelt xylan. Two isolates (Mz5, Mz6) showed high cell bound and extracellular xylanolytic activities (1890 and 250 nM RS per mg of protein per minute respectively). Both strains were classified as *Butyrivibrio*-like bacteria on the basis of cell fatty acids, restriction analysis, VFA production and sugar utilisation. Arabinofuranosidase and β-xylosidase activities, production of bacteriocins and hydrogenation of linoleic acid to conjugated linoleic acid (antioxidant and anticancerogenic factor) were proven for both strains. Cell bound xylanolytic activity was at least 1.65 times higher than the xylanolytic activities of type strains of well known xylan-degrading

rumen bacteria (*Butyrivibrio, Fibrobacter, Ruminoccocus, Prevotella*), 10 *Butyrivibrio* sp. strains from DSMZ and 25 other *Butyrivibrio fibrisolvens* strains from different sources which were comparatively tested. SDS PAGE enzymograms revealed 4 major cell bound and 2 extracellular endoxylanases in Mz5. The extracellular xylanases were isolated and partially characterised. Because of the biotechnological potential of strain Mz5 we intend to test it as a probiotic and the isolated enzymes as feed additives in pig and poultry production.

Ability of *Epidinium ecaudatum* to digest and use some structural polysaccharides for in vitro growth. K. Wereszka[a], T. Michalowski[a], A. Kasperowicz[a], K. Rybicka[b] ([a] The Kielanowski Institute of Animal Physiology and Nutrition Polish Academy of Sciences, 05–110 Jablonna, Poland; [b] Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland).

The rumen ciliate protozoon *Epidinium ecaudatum* was isolated from the rumen of sheep and grown in vitro. Mean number of ciliates in the control cultures was about 320 cells·mL^{-1}, while only 250 and 210 cells·mL^{-1} ($P < 0.05$) were present respectively in the cultures supplemented with xylan and carboxymethylcellulose. The highest number of protozoa (440 individuals·mL^{-1}) was found in the cultures supplemented with microcrystalline cellulose ($P < 0.01$). It was found that over 80% of the ciliates were filled with cellulose particles as soon as 1 h after feeding and over 90% 11 h thereafter. Xylan was engulfed only sporadically. Enzyme preparation obtained from the ciliates incubated overnight with antibiotics (chloramphenicol, streptomycin and ampicillin, each at a concentration of 100 μg·mL^{-1}) degraded microcrystalline cellulose, carboxymethylcellulose, cellobiose and xylan. The optimum conditions for the mentioned activities were at pH values of 6.5, 5.5, 6.0 and 6.5, respectively. The degradation rates of microcrystalline cellulose and cellobiose were 0.19 and 16.2 μM glucose released·mg^{-1} protein·h^{-1}, respectively. The degradation rates of carboxymethylcellulose was 17.5 while that of xylan was 66.6 μM reducing sugars/g protein·h^{-1}, respectively. Non-denaturating polyacrylamide gel electrophoresis (PAGE) combined with CMC-ase and the xylanase zymogram detection technique revealed the presence of two protein bands active against CMC that is of a β-endoglucanase nature and a single protein band degrading xylan that is of an endoxylanase character. The end products of enzymes isolated from the gel were determined.

Activities of some fibrolytic enzymes in the rumen of defaunated and *Eudiplodinium maggii*-refaunated sheep. T. Michalowski, G. Belzecki, J. Pajak, E. Kwiatkowska (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05–110 Jablonna, Poland).

The activities of β-endoglucanase (CMC-ase) and xylanase as well as ADF and NDF contents in the rumen digesta of three ciliate-free sheep and sheep inoculated with *E. maggii* were examined. The animals were fed hay (750 g) and ground barley (130 g) at 8 a.m. and 8 p.m.. Sampling was performed before both feedings, at noon and at 4 p.m.. Enzymes were extracted from the rumen digesta and its particulate fractions and were incubated with carboxymethylcellulose (CMC) or xylan. Released reducing sugars were measured spectrophotometrically. Mean values were compared using the *t*-test. The activity of β-endoglucanase in the ciliate-free sheep fluctuated from 3.4 to 4.7 μM reducing sugars released from CMC·g^{-1} DM·min. This was about 25–59% lower than the activity in the presence of *E. maggii* ($P < 0.01$). Xylanase activity ranged from 35.8 to 66.0 μM reducing sugars released from xylan. This was lower ($P < 0.05$) in two defaunated animals; the probability level reached 10% in the third animal. The number of ciliates varied from about 6 to over 50×10^3·g^{-1} in relation to the animal, time after feeding and day of the experiment. Both the CMC-ase and the xylanase activities in the rumen digesta were positively correlated with protozoa number ($P < 0.05$). The ciliates did not affect the activity of the particle-associated CMC-ase ($P < 0.05$), suggesting that *E. maggii* did not restrict the colonisation of fibrous feed by cellulolytic bacteria (Silva et al. 1987, Br. J. Nutr. 57, 407–415). However, the activity of the particle-associated xylanase was lower in refaunated sheep ($P < 0.01$). ADF and NDF contents before feeding were not related to the presence of ciliates ($P < 0.05$).

Characterisation of a spontaneous adhesion-defective mutant of *Ruminococcus albus* 20. P. Mosoni, B. Gaillard-Martinie (Unité de Microbiologie, INRA, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

Ruminococcus albus, a predominant cellulolytic bacterium of the rumen efficiently degrades plant cell wall polysaccharides by means of several fibrolytic enzymes. The adhesion mechanisms of this species are unclear. It has been suggested that adhesion is mediated by: (1) polysaccharides of the extracellular coat (Patterson et al. 1975, J. Bacteriol. 122, 278–287); (2) glycosylated cellulose-binding proteins, with one, CbpC, which might have a fimbrial structure (Pegden et al. 1998, J. Bacteriol. 180, 5921–5927) and (3) a multicellulase complex similar to the cellulosome (Miron et al. 1998, J. Appl. Microbiol. 84, 249–254). In the present work, a spontaneous adhesion-defective mutant (D5) of *Ruminococcus albus* 20 was isolated and compared to the parent, using ultrastructural and biochemical methods in order to identify the adhesion mechanism of *R. albus* 20. TEM visualisation of cells labelled with ruthenium red showed that, when grown on cellulose, 20 had a large and homogenous glycocalyx that separated the cells from cellulose. In comparison, D5 glycocalyx was aggregated at its periphery and the cells attached loosely to cellulose. A 25 kDa glycoprotein, present in the membrane fraction and the extracellular medium of 20 cultivated on cellobiose, was not detected in the same fractions of D5. This 25 kDa glycoprotein was not a cellulose-binding protein and therefore cannot have a direct role in the adhesion of 20. It is possible that it enters into the composition of the glycocalyx and its absence might explain the altered structure of D5 glycocalyx. These results underline the implication of the extracellular glycocalyx in the adhesion of *R. albus* 20, with a possible role of a 25 kDa glycoprotein in the maintenance of the glycocalyx structure.

Zymogram analysis of polysaccharidases produced by ruminal *Prevotella* strains. H. Matsui[a], K. Ogata[a], K. Tajima[a], M. Nakamura[a], T. Nagamine[a], R.I. Aminov[b], Y. Benno[a, c] ([a] STAFF-Institute, Tsukuba, Ibaraki 305–0854, Japan; [b] Department of Animal Sciences, University of Illinois, Urbana, IL 61801, USA; [c] JCM, RIKEN, Wako, Saitama 351–0198, Japan).

Prevotella species are a genetically diverse bacterial group found in the digestive tract of ruminants, pigs, and humans. Ruminal *Prevotella* spp. are predominantly isolated from the rumen, and are considered to be involved in hemicellulose and pectin digestion. *P. bryantii* $B_1$4 has been intensively studied for glycoside hydrolases. Detailed information on polysaccharidase (PDase) are limited except for the strain. Thus, the role and significance of other *Prevotella* species in polysaccharide degradation remains obscure. We characterized carboxymethylcellulase (CMCase), xylanase and polygalacturonate (PG)-degrading enzyme activities of type strains of *P. ruminicola* JCM8958, *P. bryantii* $B_1$4, *P. albensis* M384, and *P. brevis* ATCC19188 grown on xylan, xylose, arabinose, cellobiose, and glucose as sole growth substrates. The molecular weight (MW) of each PDase was also estimated by zymograms. Zymogram analyses routinely detected the presence of high-MW (100–170 kDa) PDase in ruminal *Prevotella*. *P. ruminicola* and *P. albensis* showed CMCase induction patterns. When xylan was supplied as a sole growth substrate, xylanase activities produced by *P. bryantii* and *P. albensis* were at least 18- and 11-fold higher, respectively, than during growth on other substrates. This suggests that the regulation of the xylanases was highly specific to xylan. All strains constitutively produced PG-degrading enzymes. The corresponding activity of *P. bryantii* was more than 40-fold higher than in other strains. Characteristics of the PDase activities showed the diversity of the ruminal *Prevotella* species.

Fibre-degrading enzymes from a ruminal protozoan, *Polyplastron multivesiculatum*. E. Devillard[a, b], H.J. Flint[b], K.P. Scott[b], C.J. Newbold[b], J.-P. Jouany[a], R.J. Wallace[b], E. Forano[a] ([a] INRA C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France; [b] Rowett Research Institute, Bucksburn, Aberdeen, UK).

The fibre-degrading enzymatic equipment of ruminal protozoa was investigated using a molecular approach. A cDNA library was constructed from *Polyplastron multivesiculatum*, and screened for fibrolytic activities. In addition several clones were isolated. The inserts from XynA and XynD clones encoded two family 11 xylanases. The insert from the XynB clone encoded a protein composed of two domains, a domain homologous to thermostabilising domains and a family 10 xylanase catalytic domain. The DNA sequences of the different clones showed the characteristics of a typical protozoal genome (AT rich, highly biased codon usage…). XynA and XynB were further characterised. The protozoal origin of the two genes was demonstrated. Phylogenetic analysis on the two proteins showed that they are close to Gram-positive bacterial xylanases, suggesting horizontal transfer of these

genes. Optimal parameters of the activity of the two protozoal xylanases were determined and the role of the "stabilising" domain was investigated. This domain did not appear to be involved in the thermostability of XynB, but rather seemed to have an effect on the enzyme stability to pH variations. We also showed that two other genera of ciliates expressed genes homologous to that isolated from *P. multivesiculatum*. This work clearly shows that protozoa possess their own fibrolytic enzymes and thus play a potential active role in the degradation of plant cell wall material in the rumen.

Cellulosome organisation in *Ruminococcus flavefaciens.* M.T. Rincon[a], S.Y. Ding[b], J.C. Martin[a], V. Aurilia[d], S.I. McCrae[a], R. Lamed[b], Y. Shoham[e], E. Bayer[c], H.J. Flint[a] ([a] Rowett Research Institute, Bucksburn, Aberdeen, UK; [b] Tel Aviv University, Ramat Aviv 69978, Israel; [c] Weizmann Institute of Science, Rehovot, Israel; [d] IABBAM, Napoli, Italy; [e] Technion, Haifa, Israel).

Cellulosome complexes have been found in several cellulolytic *Clostridium* spp. where they appear to provide the key mechanism for plant cell wall breakdown. Complex formation depends on interactions between dockerin domains in fibrolytic enzymes and cohesin domains in structural, or scaffolding proteins. Despite initial uncertainty over the importance of cellulosomes in ruminal bacteria, recent evidence strongly supports cellulosome organisation in *R. flavefaciens*. Dockerin-like domains were identified from the sequences of 6 different plant cell wall degrading enzymes from *R. flavefaciens.* 17 and 2 structural components of the cellulosome complex were also identified. The proposed structural components ScaA (879 a.a.) and ScaB (1753 a.a.) contain, respectively, 3 and 7 cohesin-like domains and are encoded by adjacent genes. An isolated ScaA cohesin domain, fused to a C terminal 6His tag, was shown to bind multiple polypeptides from *R. flavefaciens* 17 culture supernatants, and also to bind to a dockerin-containing fragment of the cloned *R. flavefaciens* XynD. ScaA appeared to act as a scaffolding protein for attachment of enzyme subunits. Our results indicate that ScaA in turn binds via a C terminal dockerin to the second structural component ScaB. While the *R. flavefaciens* system parrallels that found in cellullolytic Clostridia, our results also show that the cohesin and dockerin sequences have considerable divergence. The roles of other domains present in ScaA and ScaB, and the specificity of dockerin-cohesin interactions, are under investigation.

The cellulase complex of *Ruminococcus albus.* K. Ohmiya, H. Ohara, S. Karita, T. Kimura, K. Sakka (Department of Sustainable Resource Science, Faculty of Bioresources, Mie University, Tsu, 514–8507, Japan).

Ruminants digest cellulosic materials that are too tough to be digested by human beings with high efficiency, and convert them to our essential life products such as milk, meat, leather and wool. Initiators of the conversion are cellulolytic microorganisms in the rumen. One of the organisms we were interested in, was *Ruminococcus albus* which significantly contributes to cellulose digestion in the rumen. We isolated and characterised several cellulases and xylanases from the strain *R. albus* F40 and determined the DNA sequences of their genes. A cellulase complex called "cellulosome" was found and characterized in recent studies on such enzymes from *Clostridium thermocellum.* This complex accelerates the degradation of crystalline cellulose by allowing synergistic reactions of enzymes and adsorption to cellulose. A cellulasic complex was isolated from the F40 culture broth by gel filtration column chromatography. It had a molecular size of 200 kD and contained more than 15 different proteins, some of which showed activities against CMC and oat spelt xylan after SDS-PAGE. Western blotting data indicated that many of these enzymes had a dockerin domain. In addition, immunological detection of the interaction between dockerin and cohesin in the enzyme complex suggested that some of the components reacted with cohesin-like domains in the presence of calcium ions. These results suggest that *R. albus* F40 also produces a cellulosome-like cellulase complex, which is observed on the surface of the organism by electron microscopy.

Fibre-degrading system of several strains from the genus *Fibrobacter.* C. Béra-Maillet, G. Gaudet, E. Forano (Unité de Microbiologie, INRA, C.R. Clermont-Ferrand/Theix, 63122 Saint-Genès-Champanelle, France).

Fibrobacter succinogenes, a major rumen fibrolytic bacterium, shows a high ability to solubilise different cell wall substrates thanks to a very complex fibrolytic system. Twenty-five

different glycosyl-hydrolases have been identified in *F. succinogenes*, most of them from the strain S85. This strain was originally isolated from the bovine rumen 45 years ago (Bryant and Doetsch, 1954, J. Dairy Sci. 37, 1176–1183) and has been maintained in the laboratory ever since. This may have led to genotypic changes in the organism. Furthermore, *Fibrobacter* has been shown to be a genetically diverse genus (Lin and Stahl 1995, J. Bacteriol. 177, 2543–2549). We thus compared the fibrolytic system of 8 *F. succinogenes* strains (including S85) originating from different animals, and the *F. intestinalis* type strain. Several of the *F. succinogenes* strains have been subcultured little since their isolation. The strains were first classified by DNA-DNA hybridisation and 16SrDNA sequencing. All the 10 glycosyl-hydrolase genes sequenced so far from *F. succinogenes* S85 (7 cellulase genes, one cellodextrinase, one xylanase and one lichenase gene) were found in all strains using PCR, except one gene in one strain. Cellulase and xylanase specific activity of bacterial extracts were similar from one strain to the other. All the strains also showed similar CMCase and xylanase activity profiles on zymograms. This work shows that the fibrolytic system is homogeneous within the *Fibrobacter* genus, and thus that strain S85 is well representative of the rumen *Fibrobacter* strains.

Degradation of cellulosic wastes by *F. succinogenes*. Analysis of metabolic fluxes by ^{1}H NMR and stoichiometric model. C. Creuly[a], M. Matulova[b], G. Gaudet[c], E. Forano[c], A. Pons[a], A.M. Delort[b] ([a] Laboratoire de Génie Chimique Biologique, [b] Laboratoire de Synthèse, Électrosynthèse et Étude de Systèmes à Intérêt Biologique, UMR 6504 Université Blaise Pascal – CNRS, 63177 Aubière, France; [c] Laboratoire de Microbiologie, INRA, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

With the aim of developing a biotechnological process for the degradation of lignocellulosic residues, we propose to take advantage of the very efficient enzymatic equipment of *Fibrobacter succinogenes S85*, a strictly anaerobic cellulolytic bacterium from the rumen. The development of a high-performance bioreactor is based on a general concept of "metabolic engineering": the idea is to measure metabolic fluxes in *F. succinogenes* in order to direct bacterium metabolism towards the production of biomass and enzymes of interest. In this work the metabolism of cellulose was investigated using ^{1}H NMR. In particular, the contribution of endogenous glycogen, the reversal of the succinate pathway and the utilisation of exogenous substrates (glucose, cellulose) to the synthesis of final metabolites were quantified. We used data from the biochemistry of *F. succinogenes* and NMR in parallel, in order to stipulate the structure of metabolic pathways, including 96 reactions. Validation of this model was obtained by comparison between the theoretical yields of carbon elements calculated by a computed program and the experimental yields measured during anaerobic cultures of *F. succinogenes*.

^{1}H and ^{13}C NMR study *F. succinogenes* metabolism: evidence of the reversibility of metabolic pathways. C. Matheron[a], T. Liptaj[a, c], M. Matulova[a, c] G. Gaudet[b], E. Forano[b], A.M. Delort[a] ([a] Laboratoire de Synthèse, Électrosynthèse et Étude de Systèmes à Intérêt Biologique, UMR 6504 Université Blaise Pascal – CNRS, 63177 Aubière, France; [b] Laboratoire de Microbiologie, INRA, Centre de Recherches de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France; [c] Bratislava, Slovakia).

Metabolic NMR is a powerful tool for the study of metabolic fluxes since it allows to measure isotopic enrichments and the redistribution of carbon through metabolic pathways. In this work we have used in vivo ^{13}C NMR, directly performed on resting cells incubated in the magnet, and in situ ^{1}H NMR, performed on an incubation medium, to investigate the metabolism of *Fibrobacter succinogenes*, a strictly anaerobic bacterium from the rumen. This bacterium uses cellulose, glucose and cellobiose as carbon and energy sources and produces succinate, acetate, and a little formate. A portion of metabolised carbohydrates may be stored as glycogen, which can account for as much as 70% of the dry mass of bacteria. In addition, ammonia is its only source of nitrogen.We have shown the reversibility of various metabolic pathways including: (i) the reversal of glycolysis from the triose phosphate isomerisation back to glucose-6phosphate, (ii) the reversibility of the succinate synthesis pathway, (iii) the simultaneous synthesis and degradation of glycogen in the presence of exogenous glucose (futile cycle). This methodology allowed us to quantify the modulation of metabolic fluxes in the presence or the absence of ammonia.

Nitrogen metabolism

Purification and properties of glutamate - phenylpyruvate transaminase from rumen protozoon *Entodinium caudatum*. M.R. Amin[a], R. Onodera[a], R.I. Khan[a], R.J. Wallace[b], C.J. Newbold[b] ([a] Laboratory of Animal Nutrition and Biochemistry, Miyazaki University, Japan; [b] Rowett Research Institute, Scotland, UK).

Synthesis of phenylalanine through the aminotransferase of phenylpyruvic acid (PPY) has been demonstrated in mixed rumen bacteria and protozoa and in aerobic microoganisms such as *E. coli*. The present study was aimed at purifying and investigating some characteristics of glutamate-phenylpyruvate aminotransferase (GPAase) from the rumen protozoon *Entodinium caudatum*. The enzyme was purified by ammonium sulphate precipitation (80%) and column chromatography. The reaction mixture for aminotransferase assay was composed of 0.1 M sodium phosphate buffer (pH 6.0), 0.5 mM PLP, 10 mM NH_4Cl, 15 mM L-glutamic acid, 5 mM PPY, 2.6 units GDH, 2 mM NADH and transaminase enzyme extract in a total volume of 200 µL. The reaction was initiated by the addition of an enzyme solution, incubated at 45 °C for 20 min and measured at 340 nm by a micro-plate reader. Protein concentration was measured by the method of Bradford. Molecular size of the GPAase was determined by SDS-PAGE. The GPAase from a cell free extract of *E. caudatum* was purified 74-folds by the 5 steps of column chromatography. The molecular weight of the GPAase was 65.0 kDa and it appeared to be a monomer protein. The optimum pH of GPAase was 6.0 and the enzyme was reactive over a wide range from pH 5.0 to 10.5. The maximum reaction velocity was observed at 45 °C but the activity declined markedly at higher temperatures over 55 °C. Most of the chemical agents and metal ions showed inhibition effects on GPAase activity.

Determining the origin of amino acid C skeletons and N in ruminal microorganisms. C. Atasoglu, R.J. Wallace (Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK).

Ruminal microorganisms incorporate both NH_3-N and amino acid-N during microbial protein synthesis. Labelling experiments which were aimed at determining how much microbial amino acid is formed from NH_3 and how much is formed from pre-formed amino acids have generally used either $^{15}NH_3$ or ^{14}C-amino acids as a marker. The present investigation was undertaken to trace the N and C fluxes in the same experiment. Mixed ruminal microorganisms were obtained from sheep receiving a hay/concentrate diet. They were incubated in vitro with a mixture of starch, cellobiose and xylose (2.2 $g \cdot L^{-1}$ each) in the presence of 18 mM NH_3 and 5 $g \cdot L^{-1}$ algal protein hydrolysate, in which the labels were: A] $^{15}NH_3$, B] ^{15}N algal hydrolysate, or C] ^{13}C algal hydrolysate. Label in amino acids was determined by GC-MS of tertiarybutyldimethylsilyl-derivatized 6 N HCl hydrolysate of particulate material. A comparison of ^{15}N data with total N estimates indicated that substantial turnover of microbial protein occurred during these incubations, such that net protein synthesis was only 64% of total protein synthesis in an 8-h incubation. Additionally, incorporation data from incubations with ^{13}C- and ^{15}N-labelled algal hydrolysates showed that, whereas 71% of amino acid-N was derived from algal hydrolysate, only 30% of amino acid-C was derived from algal hydrolysate. The ratio was highly variable between different amino acids. It was concluded that it is important to consider both microbial cell turnover and the impact of transamination when attempting to evaluate the origin of N and C skeletons of amino acids incorporated by ruminal microorganisms.

Properties of a lysozyme from the rumen ciliate protozoon, *Entodinium caudatum*. S.C.P. Eschenlauer[a], N.R. McEwan[a], R. Onodera[b], R.J. Wallace[a], R.E. Calza[c], C.J. Newbold[a] ([a] Rowett Research Institute, Greenburn Road, Aberdeen AB21 9SB, UK; [b] Miyazaki University, Miyazaki, Japan; [c] Washington State University, Pullman, WA. 9964-630, USA).

The breakdown of bacterial protein in the rumen leads to a nutritionally wasteful cycle of protein breakdown and re-synthesis. Engulfment and subsequent digestion by ciliate protozoa was demonstrated to be the most important cause of bacterial lysis in vitro (Wallace and McPherson 1987, Br. J. Nutr. 58, 313–323). However, despite their importance in bacterial protein turnover, little is known about the enzymes responsible for the digestion of bacteria in rumen ciliates. *E. caudatum* cells were recovered from the rumen fluid of a monofaunated sheep by filtration and sedimentation. A cDNA library was produced

and screened using an antibody raised in rabbits against partially purified lysozyme from *E. caudatum*. Among the clones isolated, one 884 nucleotides long and encoding a 240-residue protein (estimated MW 24.5 kDa) showed homology to lysozyme sequences isolated from a number of bacteriophages. The cloned activity cleaved the N-acetylmuramyl → 1,4-β-N-acetylglucosamine bond in the *M. lysodeikticus* peptidoglycan with subsequent release and destruction of muramic acid residues (0.041 vs. 0.088 mmol muramic acid/mmol glutamic acid for incubations with or without the cloned enzyme) but not glucosamine (0.110 vs. 0.115 mmol glucosamine per mmol glutamic acid, respectively) suggesting it was an N-acetylmuramidase (lysozyme). The enzyme had a pI of 5.5 and an optimal pH of 4.8.

Urease expression and activity in porcine intestinal *streptococci*. Effect of copper sulphate. O. Højberg[a], P.S. Lübeck[b], B.B. Jensen[a] ([a] Microbiology Section, Dept. Animal Nutrition and Physiology, Danish Inst. Agricultural Sciences, 8830 Tjele; [b] Microbiology Section, Dept. Ecology, The Royal Veterinary and Agricultural University, 1871 Frederiksberg C, Denmark).

The use of copper sulphate as a growth promoter for pigs has been shown to reduce the number of ureolytic bacteria (mainly *streptococci*) in the gastrointestinal tract (Varel et al. 1987, Appl. Environ. Microbiol. 53, 2009–2012). Two major species of porcine intestinal *streptococci* have been identified, namely the urease positive *Streptococcus alactolyticus* (*S. intestinalis*) and the apparent urease negative *S. hyointestinalis*. We found that out of 35 porcine streptococcal isolates analysed by universal primed PCR (UP-PCR), 17 belonged to *S. alactolyticus*, whereas 15 had band patterns common with the *S. hyointestinalis* type strain, however with an indication of a potential subgrouping within the latter species. This pattern was further supported by the finding of urease positive phenotypes among the *S. hyointestinalis* isolates. In pure cultures of *S. alactolyticus* and *S. hyointestinalis* urease was expressed and remained active in the presence of up to 1 mM copper sulphate. Urease was inactivated by 10 mM copper, however, as was the growth of urease positive as well as urease negative *streptococci*. The inhibitory concentration of copper was higher than that encountered in the GI tract of copper fed animals and seemed to affect microbial growth, independent of urease expression. Therefore mechanisms other than a direct influence of copper sulphate on urease expression and activity seem to be involved in vivo in the inhibition of ureolytic streptococci. This remains to be further studied.

Purification and characterisation of a dipeptidyl peptidase from the ruminal bacterium, *Prevotella albensis* M384. H.D. Kim, N.D. Walker, N. McKain, R.J. Wallace (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK).

Dipeptidyl peptidase (DPP) activity is the predominant type of peptidase activity involved in ruminal protein degradation. *Prevotella* is the main bacterial genus showing high DPP activity, and *Prevotella* spp. produce 4 types of DPP. This study was conducted to purify and characterise the most active of these, Ala-DPP, an enzyme which cleaves the synthetic substrate, Ala_2-*p*-nitroanilide (Ala_2-pNA) to Ala_2 and *p*-nitroaniline. *P. albensis* M384 was grown 39 °C overnight and bacterial cells were harvested by centrifugation at 27 500 × *g* for 15 min at 4 °C. The cells were washed once with 100 mM potassium phosphate buffer (pH 7.0). Bacterial soluble proteins were released by sonication and fractionated by a sequential increase of ammonium sulfate concentration. Fractions having high Ala-DPP activity were applied to several columns (Superdex 75 gel filtration, Poros 20HS cation exchange, and Poros 20HQ anion exchange column) using an FPLC system for further purification of Ala-DPP. Ala-DPP was purified 193-fold, so that only one band, M_r = 45 000, was visible with Coomassie blue staining in SDS-PAGE. A similar M_r was obtained from activity determinations in gel filtration, indicating that the enzyme was monomeric. The purified Ala-DPP hydrolysed Ala_2-pNA, LysAla-MNA, Ala4, Ala5, ValAla-AlaPhe, while it had no activity on glycine peptides or substrates for other types of DPP. Optimum pH and temperature were 7.2 and 40 °C, respectively.

Plant cell death and proteolysis in fresh white clover within the rumen. A. Kingston-Smith, F. Minchin, A. Bollard, M. Theodorou (Institute of Grassland and Environmental Research, Aberystwyth, SY23 3EB, UK).

During grazing, a significant proportion of plant cells entering the rumen are intact and able to respond to the rumen environment. The latter is anaerobic and maintained at an above air temperature, constituting a severe stress to plant metabolism. Fresh white clover leaves incubated in an in vitro simulation of the rumen environment which lacked rumen micro-organisms showed degradation of protein and nuclear DNA consistent with the onset of at least part of the senescence process. However, chlorophyll degradation was incomplete under these conditions. Plant-mediated proteolysis within fresh forage in the rumen may represent premature induction of senescence post-ingestion, prior to significant digestion of plant tissues by rumen micro-organisms. To examine regulation of plant-mediated proteolysis we used inbreeding white clover plants which do not form nitrogen fixing root nodules in the presence of N. Leaf protein content can be manipulated by altering nitrogen supply. When grown on 2.5, 5.0, 7.5 and 10.0 mM nitrate leaf protein contents were 8.3, 32.4, 37.5 and 51.9 $mg \cdot g^{-1}$fresh weight respectively. The major proteolytic activity in mature leaves was cysteine protease with an acidic pH optimum. The specific activity was highest in leaves from plants grown at the lowest nitrate supply consistent with requirements for remobilisation. During in vitro incubation in anaerobic buffer at 39 °C, degradation of plant protein proceeded rapidly in all but the lowest nitrate treatments. After 24 h protein content was equal between the treatments. Hence foliar protein content appears to influence plant protease activity and the rate of proteolysis in the rumen.

Simultaneous study of exopeptidase activities and pea peptide breakdown by rumen bacteria. A. Lambert, F. Lucas, G. Blanchart (Laboratoire de Sciences Animales, INPL-UHP-INRA, ENSAIA, 2 avenue de la forêt de Haye, 54500 Vandœuvre, France).

The incubations were conducted with mixed rumen bacteria in the presence of ^{15}N-labelled pea peptides of different sizes. Peptides were analysed on gel permeation HPLC in order to collect fractions of known molecular weight ranges. Total nitrogen and ^{15}N were analysed in each fraction by mass spectrometry. Extracellular dipeptidyl-aminopeptidase type 1 and leucine- and alanine-aminopeptidase activities were monitored throughout the incubation. The presence of exopeptidase activities from the very beginning of the incubations could explain the rapid appearance of a low enrichment in small peptides. The different evolutions of these activities showed an adaptation of activities during the incubation. DAP-1 activity per viable bacterial cell decreased continously, showing that the impact of *Prevotella ruminicola* population was less and less important. Leucine- and alanine-aminopeptidase activities both increased steadily throughout the incubation. Peak labelling in a fraction of intermediate molecular weight was reached all the more rapidly since this fraction contained peptides of a size not much lower. The delay before which no accumulation of excess ^{15}N in bacteria was observed might represent the time needed for a sufficient amount of this ^{15}N to supply the peptide fraction which can be taken up by the bacteria. Since no early increase of ^{15}N-labelling in the bacteria occurred, exopeptidase activities did not seem to be the major feature involved during the first moments of hydrolysis of high molecular weight peptides. Endopeptidases, which were not studied here, must have been active so that sufficient amounts of intermediate peptides were released and could in turn be used as substrates by exopeptidases.

Inhibitors of the dipeptidyl peptidases of *Prevotella* species. N. McKain[a], H.R. Wang[b], R.J. Wallace[a] ([a] Rowett Research Institute, Bucksburn, Aberdeen, AB219SB, Scotland; [b] Inner Mongolian Academy of Animal Science, Huhhot, Inner Mongolia, China).

In the rumen, protein is rapidly broken down, via peptides and amino acids to ammonia, much of which diffuses across the rumen wall and is lost from the fermentation. As peptides are intermediates in this process, their breakdown is a stage at which it may be possible to regulate ammonia production. Peptide breakdown in the rumen is a two-stage process where oligopeptides are broken down by dipeptidyl peptidases (DPP) and the resulting dipeptides are broken down by separate dipeptidases. The *Prevotella* spp. are active in the breakdown of peptides in the rumen and possess at least 4 DPPs (DPPI, DPPII, DPPIII and DPPIV). Several inhibitors were identified and their effect on the *Prevotella* DPPs were determined. In *Prevotella albensis* M384, the inhibitors Gly-Phe diazomethyl ketone (GPD) and Ala-Ala chloromethyl ketone (AACMK), at concentrations of 100 μM, both inhibited DPPI activity in whole cells (GPD 77%, AACMK 100%) and sonicated supernatants (GPD 90%,

AACMK 100%). In *P. bryantii* $B_1$4 there was also inhibition of DPPI with the same concentration of GPD (cells 61%; sonicated 42%). The inhibitor benserazide (DL-serine 2-(2,3,4-trihydroxybenzyl hydrazide) at 100 μM inhibited DPPIII activity in both *P. albensis* and *P. bryantii, (*M384 cells 96%, sonicated 86%; $B_1$4 cells and sonicate 87%). Diprotin A (Ile-Pro-Ile) an inhibitor of mammalian DPPIV enzyme also inhibited *Prevotella* DPPIV (M384 cells 77%, sonicated 67%; $B_1$4 cells 33%, sonicate 56%). The results show that a combination of these inhibitors will reduce peptide breakdown by *Prevotella* spp.

Effects of protozoa on nitrogen metabolism in the rumen of sheep. C.J. Newbold[a], B. Teferedegne[a], H-S Kim[a], G. Zuur[b], G.E. Lobley[a] ([a] Rowett Research Institute, Aberdeen, AB21 9SB, UK; [b] Biomathematics and Statistics, Scotland, Rowett Research Institute, AB21 9SB, UK).

Four ruminally cannulated sheep received a diet of 1 kg dried grass (as-fed basis) per day via a continuous belt feeder. Sheep were defaunated by the rumen washing technique. The same sheep were subsequently refaunated by the addition of rumen fluid from a normally faunated sheep receiving the same diet. Ruminal nitrogen kinetics were determined from a pulse dose of [^{15}N] ammonium chloride into the rumen and the measurement of ^{15}N enrichment in the ruminal ammonia pool over 40 h. The contribution of blood urea to rumen ammonia was calculated from the enrichment of ^{15}N in urine urea and rumen ammonia during a separate continuous infusion of ^{15}N labelled urea into the jugular vein. Total collection of urine was made for five days and analysed for purine derivatives to calculate microbial protein flow. Defaunation stimulated microbial protein flow from the rumen (13.3 vs. 8.9 g N·d^{-1}, SED 0.98, for defaunated and refaunated sheep respectively). The ammonia pool in the rumen decreased in defaunated animals (0.6 vs. 2.0 gN·d^{-1}, SED 0.74) as did ammonia transfer either as total flux (15.1 vs. 29.2 gN·dL^{-1}, SED 5.21) or irreversible loss (11.0 vs. 16.8 gN·dL^{-1}, SED 2.47). The flux from blood urea to rumen ammonia also declined (3.3 vs. 5.8 gN·d^{-1}, SED 1.02). As a result intraruminal N recycling was lower in defaunated animals (0.8 vs. 6.6 gN·d^{-1}, SED 3.75), both in absolute amount and as a percentage of flux through the pool (4.1 vs. 20.4%, SED 8.90).

^{15}N-ammonia incorporation by ruminal bacteria growing on different N sources. M.J. Ranilla[a], M.D. Carro[a], S. López[a], C. Valdés[a], C.J. Newbold[b], R.J. Wallace[b] ([a] Dept. Producción Animal I, Universidad de León, 24071 León, Spain; [b] Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK).

Batch cultures of mixed ruminal bacteria were used to study the effects of different N sources on ammonia incorporation by bacteria growing on two fibre (neutral-detergent fibre; 500 mg/bottle) sources (barley straw and sugar beet pulp). Eight mg of N were added to each bottle as combinations of isonitrogenous solutions of ammonia and peptides in the following ratios: 1:0 (AMO), 0.7:0.3 (PEPL) and 0.3:0.7 (PEPH). Liquid-associated (LAB) and solid-associated (SAB) bacteria were isolated by differential centrifugation and ^{15}N was used as a microbial marker. Effects of N source were analysed by orthogonal contrasts. Since there were no significant ($P < 0.05$) fibre N source interactions, means for each N source are given. The proportion of net N synthesis from ^{15}N-ammonia by LAB was decreased ($P < 0.01$) by the presence of peptides, the values for PEPH being lower ($P < 0.05$) than those for PEPL (0.92, 0.91 and 0.87 for AMO, PEPL and PEPH, respectively). In contrast, there were no differences ($P < 0.05$) due to the N source in ammonia incorporation by SAB (0.61, 0.62 and 0.60 for AMO, PEPL and PEPH, respectively). These results suggest that SAB assimilate more preformed amino acids than LAB, and indicate that increasing the peptide concentration the uptake of ammonia by LAB. In contrast, ammonia uptake by SAB is not decreased by the supply of peptides.

Effect of N source on in vitro microbial growth. M.J. Ranilla[a], M.D. Carro[a], S. López[a], C. Valdés[a], C.J. Newbold[b], R.J. Wallace[b] ([a] Dept. Producción Animal I, Universidad de León, 24071 León, Spain; [b] Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK).

The effects of different N sources on in vitro ruminal microbial growth were studied. Neutral-detergent fibre (500 mg per bottle) from barley straw (BSF) and sugar beet pulp (SBPF) were incubated in vitro with rumen fluid for 24 h. N sources provided 8 mg of N per bottle in the form of combinations of isonitrogenous solutions of ammonia and peptides in the following ratios: 1:0 (AMO), 0.7:0.3 (PEPL) and 0.3:0.7 (PEPH).

Total mixed (TB) and solid-associated (SAB) bacteria were isolated by differential centrifugation. Microbial yield was estimated by dividing the ^{15}N enrichment of total digesta (incubation residue) by the enrichment of bacterial pellets and the amount of liquid-associated bacteria (LAB) was calculated as TB minus SAB. The effects of N source were analysed by orthogonal contrasts. For both substrates, the presence of peptides increased ($P < 0.05$) microbial N synthesis compared with AMO, with PEPH supporting more growth ($P < 0.10$) than PEPL (values for BSF and SBPF: 4.2, 4.5 and 4.8, and 6.3, 6.5 and 6.8 mg N for AMO, PEPL and PEPH, respectively). However, there were no effects ($P < 0.05$) of N source on the efficiency of microbial synthesis (mean values of 24.1 and 24.3 mg of microbial N/g dry matter truly degraded for BSF and SBPF, respectively). There was also no effect ($P < 0.05$) of N source on the amount of LAB (mean values of 2.2 and 2.5 mg N for BSF and SBPF, respectively), but the presence of peptides increased ($P < 0.01$) the amount of SAB for both substrates (values for BSF and SBPF: 2.2, 2.3 and 2.6, and 3.8, 3.9 and 4.4 mg N for AMO, PEPL and PEPH, respectively). These results seem to indicate that the use of peptides stimulate microbial growth, but principally of SAB rather than LAB.

In vitro study of the interaction between two proteolytic bacteria. M. Sales, F. Lucas, G. Blanchart (INPL-INRA-UHP, Laboratoire de Sciences Animales de l'ENSAIA, 54505 Vandœuvre-lès-Nancy, France).

To characterise the effects of nutritional interdependences between bacteria on the efficiency of proteolysis in the rumen, *Streptococcus bovis* JB1 and *Prevotella albensis* were cultured in mono or cocultures in a synthetic medium containing casein or pea proteins as the only source of nitrogen. *S. bovis* grew faster on pea proteins than on casein but the final biomass was reduced. The growth of *P. albensis* was lower with the pea. The proteolytic activity of *S. bovis* was not affected by the nature of the proteins. In contrast, the activity of *P. albensis* was twice lower with the pea than with casein. Pea proteins had higher ratios of short peptides: we can suppose that both species used higher quantities of short peptides with casein than with the pea, allowing them to reach a higher biomass. *S. bovis* reached a higher μ_m with the pea: it could be attributed to a pool of peptides initially present in the pea extract. In coculture on casein, the growth was intermediate between monocultures and the proteolytic activity was not improved. The amount of residual peptides was also intermediate; the high ratio of short peptides was probably due to a lack of energy source. With the pea, the growth of the coculture was improved. The proteolytic activity was rather high but the net production of peptides was as low as with *P. albensis* alone. The ratio of short peptides was lower than in monocultures: they could have been partly used to favour bacterial growth and may have been partly deaminated so that some ammonia appeared in the medium. Two fundamental points influence bacterial growth and activity: (i) the impact of bacterial interactions on the nature of end-products of the proteolysis, (ii) the nature of the protein. Furthermore, nucleic probes could be required to specify the kinds of dependence between these two bacteria.

ABC proteins in the rumen bacterium *Prevotella albensis*. N.D. Walker, N.R. McEwan, R.J. Wallace (Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK).

The bacterium *Prevotella albensis* plays a central role in ruminal protein degradation. It is one of the few rumen microorganisms involved in the breakdown of oligopeptides to smaller peptides. Its peptidases are cytoplasmic so peptides must first be transported into the cell before they can be broken down. Thus, peptide transport is a crucial step in peptide breakdown. Peptide transport has only been studied in a few bacteria but two distinct families have been found. Most peptide transporters belong to the ATP-binding cassette (ABC) family which exists as an operon made up of five proteins, oppA, oppB, oppC, oppD and oppF. The other family is the Peptide Transport (PTR) family, which is quite distinct from the ABC-type transporter family. This study was undertaken to clone and isolate genes from *P. albensis* that encodes proteins involved with peptide transport. Degenerate primers were designed on areas of conserved homology contained within OppD, part of the oligopeptide permease operon. Amplification of genomic DNA gave two PCR products (approx. 690 bp and 450 bp) which when cloned and sequenced gave four distinct clones, one which showed significant homology to uvrA, an ABC protein, and another three which were homologous to ABC peptide transporters. The three transporter clones were used to probe a phage library and positive plaques were isolated. Chromosome walking

identified neighbouring regions that are similar to other peptide transport proteins, indicating a structure similar to the Opp operon of *E. coli*. Sub-cloning and complementation studies of peptide permease mutants will be carried out to determine functionality. This is the first report of ABC type proteins in *P. albensis*.

Others

In vivo ^{23}Na NMR quantification of Na^+ gradients in *F. succinogenes* S85. V. Schwaab[a, b], C. Matheron[a], Gaudet[b], E. Forano[b], A.M. Delort[a] ([a] Laboratoire de Synthèse, Electrosynthèse et Etude de Systèmes à Intérêt Biologique, UMR 6504 Université Blaise Pascal – CNRS, 63177 Aubière, France; [b] Unité de Microbiologie, INRA, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

Fibrobacter succinogenes is a strictly anaerobic cellulolytic bacterium from the rumen. Glucose and cellobiose, final products of cellulose degradation, are taken up and metabolised by the cells into essentially succinate, acetate and small amounts of formate. Both sugars are transported across the cytoplasmic membrane through independent constitutive transporters, which are sodium-dependent. In order to support this dependence of bacterial metabolism upon Na^+ transport, we have measured Na^+ internal concentrations in the presence of various Na^+ external concentrations by using in vivo ^{23}Na NMR. Three different shift reagents were tested: the complex $Tm(DOPT)^{5-}$ was the most efficient to separate internal and external ^{23}Na NMR signals and was not toxic to the cells as shown by in vivo ^{13}C NMR experiments. Under the range of 25 to 100 mM $[Na^+]$ex, *F. succinogenes* was able to maintain high sodium gradients ($[Na^+]ex > [Na^+]in$). We also showed that resting cells previously prepared in a buffer without sodium and re-incubated in a 75mM Na^+ buffer were enable to maintain a sodium gradient and presented a disturbed metabolism. In conclusion, these experiments show the absolute need of Na^+ gradients in *F. succinogenes* S85.

Ruminal biohydrogenation of fatty acids originating from fresh or preserved grass. M. Doreau, C. Poncet (INRA, URH, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

Dietary lipids are hydrolysed then extensively hydrogenated in the rumen by microbial enzymes. Grass is rich in linolenic acid, which is beneficial for human health (Williams, 2000, Ann. Zootech., in press). The variation in the extent of biohydrogenation due to silage or hay making was studied. A first cut of lucerne was harvested at the prebloom stage. A part was fed ad libitum as fresh grass (G) to 6 wethers. Another part was preserved as silage (with formic acid) and hay (H) that were fed to the same animals during two succesive periods. Duodenal flow was determined by the double marker technique using Ru and Cr-EDTA. Fatty acids in diets and duodenal contents were determined by gas liquid chromatography. Fatty acid amounts and composition were similar for G and S: 2.1 and 2.3% of FA in dry matter, respectively, 17.7 and 18.3% of linoleic acid, and 39.9 and 40.5% of linolenic acid in FA for diets G and S. Hay making reduced total FA in dry matter (1.0%) and the proportion of linolenic acid (14.6%) in FA, but not that of linoleic acid (17.0%). Duodenal FA flow was higher than FA intake by 39, 6 and 19% for diets G, S and H, mainly due to microbial FA synthesis. This is generally observed with diets non-supplemented with lipids (Doreau and Ferlay, 1994, Anim. Feed Sci. Technol. 45, 379–396) but inconsistent data were available with fresh grass. Linolenic acid hydrogenation, calculated as the disappearance of polyunsaturated FA, was high for the 3 diets: 90.8, 90.3 and 85.1 for diets G, S and H, whereas linoleic acid was less hydrogenated for diet G (60.6, 73.5 and 75.8% for diets G, S and H). The low extent of linoleic acid hydrogenation could be due to the incorporation of this FA by bacteria in lipidic vacuoles, avoiding the action of microbial enzymes.

Ruminal bacteria and hydration of long chain unsaturated fatty acids. B. Morvan, K.N. Joblin (Rumen Microbiology Unit, Grasslands Research Centre, AgResearch, Private Bag 11008, Palmerston North, New Zealand).

It is well established that C18 unsaturated fatty acids released from lipids in the rumen are hydrogenated by bacteria to stearic acid. However, we recently have found strains of *Enterococcus faecalis*, *Streptococcus bovis*, *Selenomonas ruminantium* and *Lactobacillus spp*. that transform unsaturated fatty acids by hydration rather than hydrogenation to yield hydroxyfatty acids. To determine whether a capacity to hydrate unsaturated fatty acids is common to all strains of

S. bovis, isolates from ovine and bovine rumen and from bovine faeces were purified and the hydrating abilities of twenty isolates together with reference strains were tested. Isolates were cultured in the presence of linoleic acid or oleic acid and transformation products were identified and monitored by thin layer chromatography and gas liquid chromatography. All isolates converted linoleic acid to only one product, 13-hydroxy-9-octadecenoic acid. None were able to hydrate oleic acid. Tests with 3 ruminal isolates in shake culture showed that growth of *S. bovis* was not strongly inhibited by linoleic acid but that hydration efficiency varied with linoleic acid concentration. At high linoleic acid concentration, hydration decreased markedly. The maximum hydroxyacid yield found was 65%. It appears that the ability to hydrate linoleic acid but not oleic acid is a feature of all strains of *S. bovis*.

The influence of heavy metals on the antioxidant enzyme activity of rumen bacteria. K. Holovská[a], V. Lenártová[a], P. Javorsky[b], K. Holovská[b] ([a] Department of Chemistry, Biochemistry and Biophysics, University of Veterinary medicine, Kosice; [b] Institute of Animal Physiology, Slovak Academy of Science, Kosice, Slovakia).

The primary antioxidant protection against reactive oxygen species is provided by the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), and glutathione reductase (GR). In our work we studied the response of the rumen facultatively anaerobic bacterium *Streptococcus bovis* 4/1 and strictly anaerobic bacterium *Selenomonas ruminantium* E-32 to environmental stress evoked by mercury and copper. Hg^{2+} increased Mn-SOD activity in both anaerobic and aerobic conditions, and significantly increased the aerobic activities of GSHPx (2.6 fold) and NADPH specific GR (1.4 fold). *S. ruminantium* showed no SOD activity and a decrease of GSHPx and GR activities after Hg^{2+} exposure. Enzyme activities were influenced in a different way with copper. Only the aerobic activity of GSHPx in *S. bovis* increased (1.66 fold). The SOD activity (anaerobic and aerobic conditions) and NADPH specific GR activity (aerobic condition) were significantly decreased. In *S. ruminantium* a significant decrease of GR activity was determined. The thiobarbituric acid reactive substances content (TBARS) was significantly increased in *S. bovis*, in the presence of Hg^{2+} (anaerobic and aerobic conditions) and 50 $\mu g \cdot mL^{-1}$ Cu^{+2} (anaerobic or areobic conditions). In *S. ruminantium*, the production of TBARS was also increased after exposure to Cu^{+2} and Hg^{2+}.

Evidence of quorum-sensing in the rumen ecosystem. D.L. Erickson[a, b], V.L. Nsereko[a], D.P. Morgavi[a], L.B. Selinger[b], L.M. Rode[a], K.A. Beauchemin[a] ([a] Agriculture & Agri-Food Canada; [b] Univ. of Lethbridge, Lethbridge AB., Canada).

We investigated the presence of acyl-homoserine lactone (AHL)- based quorum sensing systems in the rumen. Two reporter gene systems were used to assay for AHLs in rumen fluid and supernatants of pure cultures of rumen bacteria. AHLs were detected in rumen fluid from 8 animals under different dietary conditions, but were not present in the feed. Reversed phase thin layer chromatography indicated the presence of multiple signals which varied in the length of their acyl side chains, suggesting that these signals were produced by bacteria in the rumen. We tested 31 strains (15 species) of predominant rumen bacteria in a variety of liquid and solid media, in mono- and co-cultures, and at various points in the growth cycle. Surprisingly, none of the strains tested produced detectable levels of autoinducer. It is possible that the organisms we tested did not possess AHL quorum sensing mechanisms, had lost this ability through serial transfer, or that laboratory conditions were inadequate for AHL induction. We are currently continuing to screen for AHL producing organisms isolated directly from the rumen in an effort to identify the species which use quorum sensing systems. Future work should be directed towards determining which genes are being regulated and whether the resulting phenotypic changes confer a competitive advantage to the bacteria possessing this mechanism.

Session III:

Fermentations

Integrative aspects of microbial digestion

Influence of chemical composition of diet on the duodenal microbial fatty acid composition in goats. P. Bas[a], D. Sauvant[a], H. Archimède[b], M. Rigault[a], A. Rouzeau[a] ([a] Laboratoire de Nutrition et Alimentation, INRA-INAPG, 16 rue Claude Bernard, 75231 Paris Cedex 05; [b] Station de Recherches Zootechniques, INRA, BP1232, 97185 Pointe-à-Pitre Cedex, France).

A trial was performed to determine the influence of dietary parameters on lipid and fatty acid composition of microbes in the rumen. Fourteen dry goats were fed rations with 3 levels of forage (100, 70, 40% DM), 3 types of concentrate and 2 types of forage. Fatty acid composition (FA) was determined in diets and in microbial lipids (ML) taken from duodenal fluid samples. The total FA content of ML varied from 5.2 to 11.5% / DM. Two FA (C16:0 and C18:0) represented the major part of total FA (52.4 to 78.6%). The monoenic and branched chain FA percentages in ML were close and lower than 10% of FA. The percentage of roughage in the diets explained the major part of variation for the FA ML content and for most FA percentages (58.2, 78.5, 83.3, 39.2, 80.6%, for FA content, C16:0, C18:0, odd FA and branched chain FA (BCFA), respectively), but a low part for C18:1n9 and C18:2 (17.2 and 2.1%, respectively). In addition to the previous relationships, influences of diet FA composition appeared. The C16:0 percentage of diets, associated with the percentage of forage, increased the prediction of C16:0, odd FA and BCFA in ML of: 4.3, 20.3, and 6.1%, respectively. The sum of the C18 FA percentages in diets positively influenced C18:0, C18:1n9 and the sum of the other C18:1 percentages in the ML. The covariable of C18:2 percentage in diet in the The covariable of the C18:2 percentage in the diet in the model with percentage forage increased the prediction of C18:2 content in ML (/ total FA and /DM) by about 25%. FA content and composition of microbes were mainly altered by diet cell wall contents and to a lesser extent by the FA pattern of the diet.

Effect of non fermented- and fermented-liquid feeding on the intestinal microbiota of growing pigs. N. Canibe, B.B. Jensen (Danish Institute of Agricultural Sciences, Department of Animal Nutrition and Physiology, Research Centre Foulum, P.O. Box 50, 8830 Tjele, Denmark).

A total of thirty growing pigs were used to study the effect of feeding dry feed (DF), non fermented- (NFLF) and fermented- (FLF) liquid feed on the gastrointestinal ecosystem of pigs. The animals (initial weight ~25 kg) were divided into groups of 10 and each group was fed DF, NFLF or FLF. At the end of the feeding period (body weight ~100 kg), five pigs from each group were sacrificed and the gastrointestinal tracts were isolated. The liquid feed was obtained by mixing feed and water in the proportion 1:2.5. The NFLF was fed immediately after mixing the feed and water. The FLF was kept in a closed tank at 20 °C and 50% of the content was taken out and new feed introduced in the tank at each feeding (at 07.00 and 15.00). Feed conversion ratio was similar ($P > 0.05$) for all three groups varying from 2.44 for the animals fed FLF to 2.53 for those fed NFLF. The pigs fed FLF had a lower ($P < 0.05$) pH in their stomach contents (4.00) than those fed DF (4.42) or NFLF (4.61). The density of total anaerobes in the stomach and mid colon, of lactic acid bacteria in the mid colon, and coliform bacteria along the gastrointestinal tract were lower ($P < 0.05$) in the FLF group compared to the other groups. Yeast counts were higher along the gastrointestinal tract of the pigs fed FLF compared to the other two groups.

Validation of the degraded protein balance to predict efficiency of rumen N-utilisation. V. Fievez, K. De Fauw, K. Notteboom, D. Demeyer (Department of Animal Production, Ghent University, Proefhoevestraat 10, 9090 Melle, Belgium).

The effect of a negative degraded protein balance of maize silage (OEB < 0, Tamminga et al. 1994, Livest. Prod. Sci. 40, 139-155), fed at maintenance level, on the efficiency of rumen N-utilisation was studied in two Latin square experiments, using a urea supplemented ration as the balanced control. Microbial biomass production was evaluated from the renal excretion of purine derivates or blood allantoin concentrations. In the first experiment with 24 Belgian Blue suckling cows (550 ± 59 kg), urea

supplementation increased blood urea concentrations (101 ± 25 vs. 45 ± 25 mg urea-N·L^{-1}) but blood allantoin concentrations were similar (283 ± 87 vs. 285 ± 62 µmol·L^{-1}). This was confirmed in the second experiment with 4 rumen fistulated sheep (72.5 ± 8.8 kg): increased renal urea-N excretion (8.0 ± 2.3 vs. 3.19 ± 0.9 g·d^{-1}) accompanied by similar renal excretions of purine derivates (6.21 ± 1.44 vs. 6.71 ± 1.65 mmol·d^{-1}). Hence, N in maize silage does not seem to limit rumen microbial growth as balancing nitrogen and energy supply (OEB = 0) only increased nitrogen losses without increase of microbial biomass production. Such discrepancy can be due to (i) the neglect of possible urea recycling in OEB calculations (ii) the overestimation of microbial growth efficiency (122 ± 31 g microbial protein/kg FOM, calculated from purine derivate excretions in Exp. 2 vs. 150 g MP·kg^{-1} FOM in the OEB calculations) of ensiled diets because of insufficient supply of fermentable energy for microbial growth, (iii) a possible effect of slightly non-synchronised energy and nitrogen supplies, as suggested from daily patterns of rumen VFA and NH_3-N concentrations (Exp. 2) (iv) a lowered microbial growth efficiency due to increased rumen retention at a maintenance level of feeding.

Proportion of purines from bacterial origin in ruminal contents and duodenal digesta of heifers fed barley- or corn-based diets. A.N. Hristov[a], T.A. McAllister[b], G.A. Broderick[c] ([a] Department of Animal and Veterinary Sci., University of Idaho, Moscow, ID 83844, USA; [b] Agriculture and Agri-Food Canada Research Centre, Lethbridge, AB T1J 4B1, Canada; [c] U.S. Dairy Forage Research Center, Madison, WI 53706, USA).

Four heifers (566 ± 25 kg LW) fitted with ruminal and duodenal cannulae were fed two isonitrogenous diets (DM basis): 65% barley silage, 20% barley grain, 15% soybean meal (Diet 1) or 64.5% corn silage, 20% corn grain, 15.5% corn gluten meal (Diet 2) in a cross-over experiment. Intake was restricted to 90% of ad libitum. Ruminal bacterial purine N was labeled using continuous 8-day infusions of $(^{15}NH_4)_2SO_4$, providing 0.42 g ^{15}N /animal·day. Samples were taken during the last 2 days of infusion; ^{15}N-enrichment of ruminal and duodenal purine N pools were determined. ^{15}N-enrichments (atom percentage excess) of ruminal purines were (Diet 1, Diet 2, and SE, respectively): 0.1693, 0.2541 and 0.00336 (bacterial phase); 0.1433, 0.2407 and 0.00069 (ruminal solid phase, PRS); and 0.1598, 0.2454, and 0.00238 (ruminal liquid phase, PRL). ^{15}N-enrichments of duodenal purines were: 0.1248, 0.2103 and 0.00550 (duodenal solid phase, PDS) and 0.1558, 0.2405 and 0.00505 (duodenal liquid phase, PDL). Proportion of bacterial purines in PRS was higher ($P < 0.05$) for Diet 2 than for Diet 1 (0.95 vs. 0.85) but did not differ by diet for PRL (0.97 and 0.95, respectively). Proportions of ruminal bacterial purines in duodenal digesta for Diets 1 and 2 were: 0.92 and 0.95 (PLD, $P > 0.05$) and 0.74 and 0.83 (PSD, P = 0.09). The results from this experiment suggest that under these dietary conditions bacterial purines represent the largest part of purines in ruminal and duodenal digesta but a significant amount of feed purines may enter the small intestine with digesta solids.

Efficiency of rumen microbial protein synthesis on maize and grass silage based rations. L. Misciattelli, T. Hvelplund, M.R. Weisbjerg (Department of Animal Nutrition and Physiology, Danish Institute of Agricultural Sciences, 8830 Tjele, Denmark).

The rumen microbial protein synthesis was studied in an experiment with four lactating cannulated Holstein Friesian cows, arranged in a 4 × 4 Latin square design. The cows were fed grass silage or maize silage ad libitum and they were given a fixed amount of one of four different concentrates twice daily. Flow markers (Cr_2O_3 and PEG) were administered via the ruminal fistula at feeding. Samples of feed, duodenal chyme and rumen microbial isolates were obtained. Microbial protein flow in the duodenum was calculated based on both DAPA and purine flow. Data was subjected to ANOVA including effects of treatment, cow and period. When comparing orthogonal contrasts of microbial flow based on DAPA, maize silage based rations had significantly ($P < 0.05$) more microbial N (75.0 vs. 44.6 g·kg^{-1} RDOM, SEM 7.53) and microbial amino acid N (55.2 vs. 33.1 g·kg^{-1} RDOM, SEM 5.24) entering the duodenum per kg rumen digested OM (RDOM), rumen digested CHO or truly digested OM in the rumen, when compared to grass silage diets. When using purines as a marker, the same tendency ($P < 0.10$) was observed. Explanations for these differences could be rumen liquid dilution rate or rumen ammonia concentration. No significant treatment

effects ($P > 0.61$) nor contrasts ($P > 0.54$) were observed for the rumen liquid dilution rate. Furthermore differences in microbial synthesis could not be discarded by including the dilution rate as a covariant in the model. Rumen ammonia concentration did however differ significantly during parts of the day, being the highest for the maize silage based rations (average concentration 18.9 vs. 11.4 mM, SEM 1.27), but at no time were the rumen ammonia concentration on the grass silage based rations below the 5 mM that are considered limiting for rumen microbial growth.

In vitro growth and starch digestion by *Entodinium exiguum* in the presence or absence of rumen bacteria. M. Fondevila[a], B.A. Dehority[b] ([a] Dpto Producción Animal y Ciencia de Alimentos, Universidad Zaragoza, Spain; [b] Ohio Sate University, Wooster, USA).

Entodinium exiguum, isolated from sheep rumen, was cultured anaerobically with or without rumen bacteria. Its growth and amylolytic activity were studied, using corn starch as the only energy source. Basal medium M (Dehority 1998. J. Anim. Sci. 76, 1189–1196), also included (per 100 mL) 6 mL of 1% phosphate buffer, 0.1 g trypticase and 0.35 g corn starch (the starch was added before autoclaving, AUT, or just before inoculating, NOA). Where indicated, penicillin G and streptomycin sulphate were added (2000 and 130 $U \cdot mL^{-1}$). Treatments were: protozoa with antibiotics (PA), PA plus autoclaved bacteria (PAB), living bacteria (filtered through a 5 µ pore size; BAC) and protozoa plus living bacteria (PLB). Inoculum for PA and PAB was preincubated for 4 h with antibiotics. Two tubes per treatment were inoculated, incubated and sampled at 3, 6, 9, 12, 24 and 48 h. Digestion of AUT in PLB was 29.5% at 3 h, and reached 96.1% after 9 h, whereas NOA was only 23.0% at 9 h and reached 99.2% at 48 h. Starch digestion in PLB was always higher than in PA and PAB, and higher than BAC up to 24 h ($P < 0.001$). Digestion of AUT was higher in BAC than in PA and PAB from 12–48 h, but only at 48 h in NOA ($P < 0.05$). Up to 12 h (AUT) and 24 h (NOA), the sum of digestion by the bacteria (BAC) and protozoa (PA or PAB) did not equal that of PLB, suggesting some synergism. Protozoal concentrations were higher ($P < 0.01$) with AUT than with NOA at 12 h, but were just the opposite at 24 and 48 h, probably because of energy depletion in AUT. Concentrations were higher, and generation time shorter, in PLB than in PA or PAB at 24 and 48 h ($P < 0.05$). There were no differences in protozoal concentrations between PA and PAB.

Effect of some inhibitors on amylases from ruminal origin. T.F. Martinez, F.J. Moyano, M. Diaz (Departamento de Biología Aplicada, Área de Biología Animal, EPS, Universidad de Almería, Campus Universitario de La Cañada, 04120 Almería, Spain).

Despite the importance of starch in ruminant feeding, enzymatic mechanisms responsible for its degradation by rumen microbiota have been less studied than fiber or protein degrading enzymes. Some metabolic disorders are associated to the inappropriate use of highly degradable carbohydrates in feeding of ruminants, but the intimate enzymatic mechanisms involved in the digestion process are still unclear due to the rapid hydrolysis of grain starches in the rumen. In the present work, total amylase activity in ruminal extracts obtained from sheep was characterised by its sensitivity to different inhibitors. Ruminal amylases were mainly affected by chelators (ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-b-aminoethyl ether (EGTA) and 1-10-phenanthroline). Zinc sulphate was responsible for a loss of 48% in total activity, and a commercial wheat a-amylase inhibitor showed no significant effects. EDTA and EGTA resulted in a higher inhibitory effect than 1-10-phenanthroline when similar increasing concentrations were assayed, but at the maximum concentration used, the three of them were responsible for about an 80% inhibition. SDS-PAGE zymograms allowed a clear identification of different amylase active bands in ruminal extracts, showing a wide range of relative molecular masses (from 36 to more than 100 kDa). Zymograms also made the visualisation of the effect of the inhibitors on amylases from ruminal origin possible.

Influence of cereal supplementation on the viscosity of the ruminal or caecal contents of sheep. C. Martin, Y. Rochette, B. Michalet-Doreau (DIM-URH, INRA, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

In ruminants, degradation of plant cell walls occurs mainly in the rumen (R) but also in the caecum (C). The supplementation of forage diets

with cereals is known to depress ruminal fiber digestion by decreasing fibrolytic microbial activity. A limitation of microbial enzyme diffusion and an accumulation of inhibitor substances of enzyme activity associated with an increase in the viscosity of the digestive contents may also alter fiber digestion in cereal rich diets. The aim of this study was also to assess the viscosity in the R and the C in response to barley supplementation. Four sheep fitted with rumen and caecum cannula were fed twice daily a diet consisting of 100% alfalfa hay (H) or supplemented with 60% barley (HB). Ruminal and caecal contents were withdrawn on 2 consecutive days at –1 h, 2 h and 5 h after feeding. Samples were filtrated (100 µm) and 2 mL of fluid were maintained at 39 °C for measurements of viscosity with a shear-stress Carri-med CSL 100 Rheometer. Viscosities were calculated from stresses between 0.5–35 $N \cdot m^{2-1}$. Means of the three sampling times are presented. Irrespective of the diet, higher viscosities were measured in the C than in the R (3.5 times in mean for the two diets). Addition of barley in the diet increased the viscosity of the R from 3.4 to 8.4 mPas, and the viscosity of the C from 9.1 to 31 mPas. Compared to the H diet, the abundance of rapidly digested barley starch in the rumen with the HB diet involved an accelerated production of organic acids and bacterial mucopolysaccharides (slime) that might explain the increase in ruminal fluid viscosity (Cheng et al. 1998. J. Anim Sci. 76, 299–308). In conclusion, viscosity variations in digestive contents of ruminants might be involved in the plant cell wall digestion phenomena.

Does low ruminal pH affect fiber digestion and efficiency of microbial synthesis in vivo? K.A Beauchemin, L.M. Rode, W.Z. Yang (Agriculture and Agri-Food Canada, Research Centre, Box 3000, Lethbridge, Alberta, T1J 4B1, Canada).

The data from two studies (n = 16 in each) with cattle cannulated in the rumen and duodenum, were analyzed to determine whether low ruminal pH decreases fiber digestion and efficiency of microbial protein synthesis in vivo. In study 1, growing beef cattle were fed a diet containing 86% barley grain (DM basis). In study 2, dairy cows were fed a diet containing 43% barley grain. In both studies, grain processing was used to alter the availablity of starch in the rumen and cattle were fed for ad libitum intake. DM intake, ruminal NDF digestion, total tract NDF digestion, efficiency of microbial protein synthesis, mean rumen pH, and hours pH < 5.8 were: 7.0, 20.5 $kg \cdot d^{-1}$; 39.6, 49.0%; 53.4, 56.1%; 19.6, 23.7 g $N \cdot kg^{-1}$ ruminally fermented OM; 5.93, 6.01; and 9.6, 7.3 h, for beef and dairy cattle, respectively. Within study, and across studies, there were no significant relation-ships ($R^2 < 0.03$) between rumen pH (mean and hours < 5.8) and NDF digestibility (ruminal and total tract) or efficiency of microbial protein synthesis. These results contrast with in vitro studies with pure cultures that have shown activity of the predominant fibrolytic ruminal bacteria (e.g. *Fibrobacter* spp., *Ruminococcus albus*) declines ra-pidly when pH falls below 6.0. It appears that other fibrolytic organ-isms contribute significantly to ru-minal fiber digestion, thus the role of what are considered to be minor populations of rumen bacteria on fiber digestion needs to be revisited.

Fermentative pattern – Methanogenesis

Relationship between fermentation and liquid outflow rate in the rumen. D. Sauvant[a], D. Mertens[b] ([a] Laboratoire de Nutrition et Alimentation, INRA-INAPG, 16 rue Claude Bernard, 75231 Paris Cedex 05, France; [b] USDA, Madison, USA).

Liquid outflow from the rumen is mainly due to saliva production which is known to recycle buffer stabilising fermentations. A data base was extracted from the literature to study if any relationship could link ruminal liquid outflow rate (LOR) and fermentation. Based on dry matter intake (DMI) LOR was 12.92 ± 4.26 $L \cdot kg^{-1}$ DMI in 299 data issued from 75 publications on cattle. For the statistical analysis a subfile of 97 data from 40 experiments, where concentrate or NDF concentration was the experimental factor, was retained. In this data set LOR = 13.39 ± 4.35 $L \cdot kg^{-1}$ DMI. Relationships were calculated within the experiment with the GLM procedure. Rumen pH (6.22 ± 0.33) and VFA (105.1 ± 23.9) contents were significantly and non-linearly influenced by LOR/DMI (pH = 4.63 + 0.172 × LOR – 0.0037 × LOR2; n = 81, nexp = 34, R = 0.97, rsd = 0.08 and VFA = 83.4 + 5.45 × LOR – 0.25 × LOR2; n = 70, nexp = 30, R = 0.98, rsd = 6.0). All the proportions of VFAs were also altered by the LOR/DMI, as for example the acetate/propionate ratio (A/P = 3.37 ± 0.92); the equation was: A/P = 0.34 + 0.372 × LOR –0.088 × LOR2 (n = 72, nexp = 31, R = 0.95, rsd = 0.27). There was however no influence of LOR/DMI on the

concentration of NH3 and on microbial efficiency. Several dietary parameters were involved in the LOR variations. The increase of LOR/DMI was 125.6 ± 0.03 mL·%$^{-1}$ of NDF% DM increase (*n* = 97, rsd = 1.9), 67.6 ± 0.01 mL·%$^{-1}$ of concentrate%DM decrease (n = 88, rsd = 1.5) and 189.1 ± 0.06 mL·min^{1} of mastication per kg DMI (*n* = 34, rsd = 2.3). When all the data were pooled, the equations were fairly similar but their accuracy was lower. In conclusion, LOR appears as a contributive factor to fermentations in the rumen.

VFA concentration and production in defaunated and *Eudiplodinium maggii*-refaunated sheep fed hay-barley diet. G. Belzecki, E. Kwiatkowska, T. Michalowski (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jablonna, Poland).

Two male sheep were fed hay (750 g) and ground barley (130 g) at 8 a.m. and 8 p.m.. Rumen digesta DM, NDF and ADF were measured before morning and evening feedings. VFA and protozoa concentrations were estimated just before morning and evening feedings, at noon and at 4 p.m. The VFA production rate was measured at 8 a.m., at noon and 4 p.m. using "the zero time method" (Carroll and Hungate 1954, Appl. Microbiol. 2, 205-214). The number of ciliates in refaunated sheep varied from about 14 to 57 × 10^{3}·g^{-1}. VFA concentration was about 67 mM·L^{-1} before morning feeding and 108 mM·L^{-1} 4 h thereafter. It tended to be higher in ciliate free sheep. Production rate of VFA varied from about 23.8 to about 41 µM·g^{-1}rumen digesta per h. It was the highest at 4 h after feeding and was not significantly related to the presence of protozoa. The production rate of butyrate tended, however, to be higher in refaunated sheep. NDF and ADF content in the rumen varied before feeding between about 49–81 and 23–57, respectively in relation to animal and experimental day. No relation was found between the presence of ciliates and fibre content in the rumen digesta.

Changes in Gibbs free energy and stoechiometry of VFA production in rumen continuous culture. L.P. Broudiscou, D. Sauvant (Laboratoire de Nutrition et d'Alimentation INRA – INAPG, 16 rue Claude Bernard, 75231 Paris Cedex 05, France).

Using in vitro data, we tested the hypothesis of a partial control of the stoechiometry of volatile fatty acid (VFA) production in the rumen by a single thermodynamic criterion, namely the change in Gibbs free energy (G). Six dual effluent continuous cultures were fed twice a day with 22.5g·d^{-1} orchard-grass hay and 7.5 g·d^{-1} ground barley. They were run for 3 periods of 7 days. The daily outflows of acetate, propionate and butyrate ranged from 56.9 to 77.1 mmol·d^{-1}, from 15.1 to 22.2 mmol·d^{-1}, and from 10.3 to 17.8 mmol·d^{-1} respectively. These outflows were only partially predicted from the degraded amounts of NDF and readily fermentable sugars by multiple linear regression (R^2 of 0.69, 0.31 and 0.61 respectively, N = 17). Considering the real concentrations of end-products and dissolved fermentation gases in the fermenters, we calculated the changes in free energy at 311 K occurring in our experimental conditions for the main glucose fermentation pathways. The average ·G for the synthesis of acetate, propionate and butyrate from glucose equalled –408, –321 and –384 kJ·mol^{-1} respectively. The ·G for the same reactions were also calculated using the VFA concentrations predicted from the linear relationships between VFA outflows and the degradation of NDF and readily fermentable sugars. Both sets of ·G values were not significantly different (*t*-test). Thus, the variations of VFA proportions observed in the fermenters were not associated to variations in ·G.

An atypical fermentative pattern in cows fed a diet leading to sub-acidosis. M. Doreau, A. Ollier, B. Michalet-Doreau (INRA, URH, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

The occurrence of acidosis is increased by high-concentrate diets. The consequences on rumen digestion have been widely studied in experiments carried out with a low number of cannulated animals. This trial was aimed at studying acidosis on a large number of lactating dairy cows**.** Twenty-eight Holstein cows were used during the first 8 weeks of lactation (average milk yield 33 kg·d^{-1}). They received maize silage and 1 kg concentrate before calving. After calving they received a diet made up of 50% maize silage, 10% hay and 40% concentrate ad libitum given separately. Rumen liquid was sampled in the afternoon in week 4 through the rumen wall using a trocar, and blood was sampled at the caudal vein during weeks 3 and 6. Mean ruminal pH was 5.53; 27 values of 28 were comprised

between 4.9 and 6.0. Percentages of acetate, propionate and butyrate in rumen liquid were 51.4, 20.9 and 21.7% for a total acidity of 138 mM. D- and L- lactic acid concentrations were 0.210 and 0.403 $mg \cdot L^{-1}$. Blood pH and lactate were normal, at mean 7.48 and 0.642 mM. Low glycaemia (0.403 $g \cdot L^{-1}$) was observed with high concentrations of 3-hydroxybutyrate 3.55 mM) and acetone (5.65 mM), whereas non-esterified fatty acids remained low (0.116 mM). These data showed (1) a very low pH in almost all cows, (2) an atypical volatile fatty acid pattern rich in butyrate, as observed with diets rich in soluble sugars (beets), (3) the absence of typical lactic acidosis, (4) the development of subketosis, certainly related to the high flux of 3-hydroxybutyrate arising from ruminal butyrate. In this experiment, the shift of carbohydrate fermentation pattern towards butyrate could be due to modifications of the microbial ecosystem, which is not stable with high-concentrate diets.

Relationships between post-prandial pH drop and rumen soluble carbohydrate concentration. C. Maaroufi, S. Giger-Reverdin, D. Sauvant, (Laboratoire de Nutrition et Alimentation INRA de l'INA P-G, 16 rue Claude-Bernard, 75231 Paris Cedex 05, France).

An experiment was performed on 8 fistulated lactating goats to assess the link between post-prandial pH drop after a meal and the rumen juice concentration of soluble carbohydrates (SC). Four complete mixed diets were tested with two factors combined as a factorial 2 × 2 design: crude protein level (CP = 13.5 vs. 16% DM basis) and granulometry of pea seeds (Gr, coarse vs. fine) which were included at a level of 22.7% in the diet on a DM basis. Each goat received each diet. Rumen juice was removed before the morning meal, and 1, 2, and 4 hours after the onset of the meal. The 3 values obtained after the meal for each goat were pooled. The mean pH value was 5.94 ± 0.43, the mean pH drop (pHdrop) was calculated as the difference between the pH value before the morning meal and this mean pH value. It was –0.72 ± 0.38. The mean rumen concentrations were 324 ± 42 $g \cdot L^{-1}$ for SC and 206 ± 93 mg $N\text{-}NH_3 \cdot L^{-1}$ for ammonia. The mean pH drop was significantly explained by the SC concentration: pHdrop = –0.45 – 0.821 SC; (n = 31, r = 0.50, rsd = 0.34). Granulometry had no effect on this residual, and the higher dietary nitrogen level tended to limit this pH drop ($P <$ 0.10). This could be related to the higher protein breakdown and thus to buffers provided by the 16% CP diet (262 mg $N\text{-}NH_3 \cdot L^{-1}$) compared to the 13.5% CP diet (154 mg $N\text{-}NH_3 \cdot L^{-1}$). In conclusion, the postprandial drop of pH was less marked with the lowest soluble carbohydrate concentration. This observation could be of interest to better understand the mechanisms of ruminal acidosis.

Hindgut fermentation in marine herbivorous fish. D.O. Mountfort[a], K.D. Clements[b] ([a] Cawthron Institute, Nelson, New Zealand; [b] School of Biological Sciences, University of Auckland, New Zealand).

Gastrointestinal fermentation by symbiotic microorganisms facilitates plant digestion in herbivorous reptiles, birds and mammals. Gut symbionts ferment cell wall components of the diet to short-chain fatty acids (SCFA), which are then taken up and used for metabolism by the host animal. Some marine herbivorous fishes contain elevated levels of SCFA, especially acetate, in the gut. However, the contribution of fermentation to energy balance in fishes is unknown. We estimated acetate production in herbivorous silver drummer (*Kyphosus sydneyanus*), butterfish (*Odax pullus*), and marble-fish (*Aplodactylus arctidens*) collected from coastal reefs off north-eastern New Zealand. Ex vivo preparations of freshly caught fish were maintained with the respiratory and circulatory systems intact. Radiolabelled acetate was injected into ligated hindgut sections, and gut fluid wassampled at intervals for periods of up to 3 hours. Acetate production rates (see Table) were comparable with those found in the hindgut of herbivorous reptiles and mammals.

Table. Comparison of acetate production rates in herbivores.

Herbivore	Acetate production ($nmol \cdot mL^{-1} \cdot min^{-1}$)
Silver drummer (fish) hindgut	274–619
Butterfish hindgut	90–150
Marblefish hindgut	130–360
Green turtle caecum	150
Sheep rumen	383
Possum hindgut	316

These and other stoichiometric measurements including sulphate reduction and methanogenesis, indicated that over 98% of microbially-produced acetate is taken up across the gut wall of the fish. This work suggests that hindgut fermentation can function as an effective digestive strategy in ectothermic, warm temperate marine fish.

Development and efficiency of hydrogenotrophic bacteria in the rumen of methanogen-free meroxenic lambs. G. Fonty, M. Chavarot, F. Michallon, R. Roux (Unité de Microbiologie, INRA, C.R. de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France).

Reduction of ruminal methane production needs to involve a strategy whereby electron disposal via interspecies hydrogen transfer is not disrupted. The aim of this work was to study the development of non-methanogenic hydrogenotrophic bacterial communities in the rumen of methanogen-free gnotobiotically-reared lambs (meroxenic) and to evaluate their efficiency in using hydrogen in order to determine if they represent a credible alternative to methanogen during ruminal fermentative processes. Two germ-free lambs were inoculated with a 10^{-3} dilution of rumen content taken from an adult sheep. This inoculum was treated with a solution of bromo ethane sulfonic acid to kill methanogens. After weaning (week 7), the lambs were fed dehydrated lucerne hay. Two conventional lambs were used as controls. Meroxenic lambs remained in good health during the whole experiment (18 months). Compared to the conventional lambs, food consumption and ruminal fatty acid (VFA) concentration were slightly lower in the meroxenic animals. The centesimal composition of the VFA mixture was also different, the acetate: propionate ratio being lower in the meroxenics. After 6 months these animals harboured slightly larger acetogenic and fumarate-reducing bacterial communities than the conventional animals. In vitro, the efficiency of this hydrogenotrophic community for hydrogen scavenging appeared lower than that of control lambs but increased with animal age and was enhanced in the presence of fumarate. These results confirm the existence of an alternative to methanogenesis in the rumen, and suggest that the addition of fumarate in the rumen may be a tool to decrease methane emission by ruminants.

Hydrogen metabolism of human intestinal bacteria: Transfer of an in vitro model to a gnotobiotic rat model. D. Taras, L. Hartmann, M. Blaut (German Institute of Human Nutrition, Department of Gastrointestinal Microbiology, 14558 Bergholz- Rehbrücke, Germany).

The intestinal accumulation of gas is associated with various pathological and diagnostic problems. Hydrogen, which is a major component of intestinal gas, may be reoxidized by methanogenic, acetogenic, and sulfate reducing bacteria resulting in a considerable reduction of the gas volume. This is potentially beneficial to human health whereas sulfide is implicated in inflammatory bowel disease. The aim of the in vivo study was to investigate this hydrogen metabolizing community. For this reason we developed a new system for the quantitative collection and determination of hydrogen excreted by gnotobiotic rats. We determined hydrogen production before and after the association with hydrogen-producing as well as after subsequent diassociation with hydrogen-oxidizing bacteria. In addition, we compared the influence of a defined synthetic diet on hydrogen production with that of the same diet substituted with lactulose. Hydrogen excretion by rats associated with hydrogen-producing bacteria was dependent on the concentration of lactulose in the diet. Upon diassociation with various hydrogen-utilizing bacteria hydrogen excretion decreased significantly. The experiments verified the reliability and accuracy of the developed apparatus. In addition, it enabled us to collect substantial quantitative data on the effect of dietary components on hydrogen formation and on the influence of hydrogen utilizers on reducing H_2. We therefore believe that our experimental setup opens the possibility to solve the controversy over the ability of one group of hydrogen utilizing bacteria to outcompete the others for H_2 in the mammalian gut.

Biofilm forming sulphate-reducing bacteria affect butyrate metabolism in saccharolytic clostridia. E. Furrie, G.T. Macfarlane (MRC Microbiology and Gut Biology Group, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, UK).

Sulphate-reducing bacteria (SRB) belonging to the genus *Desulfovibrio* are important H_2 and lactate utilizing species in the human large bowel. However, little information is available concerning their interactions with other intestinal microorganisms. In this investigation, the effects of *Dsv. desulfuricans* isolated from a patient with ulcerative colitis were investigated in chemostats containing simplified microbiotas. Addition of the SRB to the chemostats reduced planktonic bifidobacteria and *C. perfringens* 10-fold, while numbers of *C. butyricum* increased. *Desulfovibrio desulfuricans* immediately induced wall growth in the chemostats, creating new and

distinct microenvironments in the culture vessels. High concentrations of sulphide (ca. 8 mM) occurred in the SRB chemostats, suggesting that this metabolite may have been inhibitory to some species. The introduction of *Dsv. desulfuricans* to established microbiotas resulted in profound changes in metabolism. Acetate formation was markedly increased, with concomitant reductions in propionate, butyrate and lactate. Bacteriological and fermentation product measurements demonstrated that the ecology and physiology of *Dsv. desulfuricans* was closely linked to the butyrate-forming clostridia (*C. butyricum*, *C. innocuum*). The switch from butyrate to acetate formation in saccharolytic clostridia was explicable in terms of the SRB acting as an electron sink, allowing the disposal of reducing equivalents through increased H_2 production via NADH ferredoxin oxidoreductase.

Effect of rumen protozoa on C and H distribution among the end products of fermentation. J.P. Jouany, B. Lassalas (INRA, URH-DIM, Centre de Recherches de Clermont-Theix, 63122 Saint-Genès-Champanelle, France).

Organic matter (OM) is fermented in the rumen by the anaerobic microbial ecosystem into VFAs and gases. VFAs are efficiently used by ruminants as an energy source, while gases are eructed. Energy losses through vented methane (M) can represent more than 10% of the energy intake. From a strict energy point of view, it is desirable to increase VFA production at the expense of gas production. This paper will examine the effect of rumen protozoa on this balance.

Three defaunated sheep fitted with large rumen cannulae were successively inoculated with *Isotricha* spp. and then with a conventional mixture of protozoa. Rumen digesta were sampled just before the morning meal and incubated (15 mL filtered rumen juice) in anaerobic conditions, with a Simplex Coleman buffer (25 mL) and 200 mg of a ground mixture of lucerne (61%), corn grain (26%) and grass hay (13%). Incubations lasted 24 h and were repeated three times each. Addition of protozoa to a defaunated rumen content had no significant effect on the total amount of fermented OM but it increased ($P < 0.05$) the "M/VFAs" ratio in the end products. "M/VFAs" ratios on molar basis were 0.23, 0.34, 0.33 respectively with defaunated, *Isotricha* and mixed-faunated rumen digesta. The weight of C in M was greatly decreased by defaunation (–15%) whereas defaunation did not statistically modify the amount of C in VFAs. Calculated recovery rates for defaunated, *Isotricha* and refaunated were respectively 1.07, 1.02, 0.96 for H, and 1.05, 1.01, 1.00 for C. In conclusion, as shown before for N, the presence of protozoa in rumen digesta induces a loss of available C and H in ruminants.

Effects of N fertilisation on sward composition and CH_4 production: importance of animal effects.* L. Mbanzamihigo[a], C. Da Costa Gomez[a], V. Fievez[a], F. Piattoni[a], L. Carlier[b], D. Demeyer[a] ([a] Ghent University, Department of Animal Production, Proevehoevestraat 10, 9090 Melle, Belgium; [b] Agricultural Research Center, Department of Plant Production and Ecophysiology, Burg. van Gansberghelam 109, 9820 Merelbeke, Belgium).

The effects of N fertilisation on sward composition and CH_4 production by sheep were investigated in the early summer on a mixed pasture (white clover and ray grass). Part of the pasture was fertilised with 150 kg N/ha/year, the remainder received no N. In a latin square design, in vivo CH_4 production from 4 sheep fed fresh grass ad libitum was monitored using C_2H_6 as a tracer gas during 2 periods. N fertilisation increased dry matter (DM) yield, decreased the clover proportion (35.0 to 15.9%) and increased crude protein (CP) content in DM (12.2 to 13.8%). A period effect was also observed: an increasing growth stage decreased CP content. This interaction prevented interpretation of data within a latin square design. Nevertheless, each cell of the experiment yielded CH_4 production data with paired animals on grass/clover diets containing between 37.3 and 14.3% of clover and varying in CP content between 11.9 and 15.6%. No relationship was however, observed between CH_4 production and forage CP content. This finding did not support the hypothesis that N fertilisation decreases CH_4 production because of an increase in forage CP content. In contrast, however, a clear animal effect was observed since one animal produced significantly ($P = 0.02$) less CH_4 than the three others. This difference was accompanied by a significantly ($P = 0.04$) lower effective rumen DM degradability and a lower rumen pH (5.7 ± 0.2 vs. 6.5 ± 0.2). The data clearly suggest that animal factors may be more important than diet for methanogenesis. * Project financed by the Prime Minister's Office – Federal Office for Scientific, Technical and Cultural Affairs.

In vitro comparison of dietary fats rich in medium-chain fatty acids regarding methane release from rumen fluid. F. Dohme[a], A. Machmüller[a], A. Wasserfallen[b], M. Kreuzer[a] ([a] Institute of Animal Sciences, Animal Nutrition; [b] Institute of Microbiology, ETH Zurich, ETH centre, CH-8092 Zurich, Switzerland).

Coconut oil, a fat rich in medium-chain fatty acids (MCFA, $C_{8:0}$ to $C_{16:0}$), was shown to reduce ruminal methanogenesis. Effective alternative MCFA-rich fats should be identified. In the present study, the influence of fats on methane release from rumen fluid were studied using an 8-fermenter RUSITEC-system. Six further fats with elevated contents of MCFA (palm oil, palm kernel oil, tallow, milk fat and two canola oils, genetically enriched with $C_{12:0}$) were compared to coconut oil (positive control) and rumen protected fat (negative control. The complete diets consisted of maize silage, hay and concentrate and were supplemented with 53 $g \cdot kg^{-1}$ DM of the respective fat. With protected fat, 14×10^3 mL^{-1} rumen ciliates were counted. Palm oil, tallow and milk fat inhibited ciliate counts by 26% on av., canola oil A by 64%, canola oil B (containing more $C_{18:1}$ than type A) and coconut oil by 93%. A total elimination occurred with palm kernel oil. The two last-mentioned fats also significantly decreased methanogens by 73% and 82%, respectively. Total bacteria counts were not clearly affected by the fats. The significant suppression of methane release by canola oil B (18%), coconut oil (19%) and palm kernel oil (32%) was associated with a decreased fibre degradation in these groups. Methane suppression was only found in fats with elevated contents of $C_{12:0}$ and $C_{14:0}$ and when fats were widely melted at rumen temperature.

Influence of cereal supplementation on methane production by sheep measured by the SF6 tracer method. C. Martin, Y. Rochette, M. Fabre, J.P. Jouany (Unité de Recherches sur les Herbivores, INRA, 63122 Saint-Genès-Champanelle, France).

Methane eructed by ruminants represents a loss of energy for the host animal and constitutes 15 to 20% of the global emission of methane. The daily methane production from ruminants depends on factors related to the animals (species, age, physiological state) and alimentation (intake level, nature of the diet). Four sheep (BW = 66 ± 6 kg) received 1200 g DMI·d^{-1} of a diet consisting of 100% hay (H) or supplemented with 60% barley (HB) in a 2 × 2 crossover design. Diets were fed twice daily in two equal meals at 08:00 and 20:00 h. Methane emissions from each sheep were estimated by using sulphur hexafluoride (SF_6) as the tracer gas (Johnson et al 1994, Environ. Sci. Technol. 28, 359–362). Measurements were carried out over a 12 hour period and repeated for 5 consecutive days (n = 10). Methane emissions averaged 46 ± 8 L·d^{-1} for animals fed the H diet, and 39 ± 16 L·d^{-1} for those fed the HB diet. This level of methane emissions represented a waste of 7.9% and 6.3% of the gross energy intake (GEI), or 14.6% and 9.5% of the digestible energy intake (DEI) for the H and HB diets, respectively. Comparable results were obtained from respiratory chamber measurements (Vermorel, personal communication). A larger variability among sheep was observed when animals were fed the HB diet (CV = 41%) characterised by unstable ruminal physico-chemical conditions, than when sheep were fed the H diet (CV = 17%). This individual variability in methane emissions cannot be attributed to variable intake but probably to differences between rumen microbial communities and pH conditions. The study of the origin of this variation is important in order to both understand nutritional performance and develop emission control strategies.

The impact of hexose partitioning on methane production in vitro. A.R. Moss[a], C.J. Newbold[b] ([a]ADAS Nutritional Sciences Research Unit, Alcester Road, Stratford upon Avon, Warks., CV37 9RQ, UK; [b] Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK).

Methane production represents an important sink for H_2 within the rumen. Beever (in Quantitative Aspects of Ruminant Digestion and Metabolism pp. 187–215, 1993) suggested that the partitioning of fermentable dry matter (DM) between microbial synthesis and fermentation products would alter the pattern of H_2 production and hence methanogensis. We investigated this hypothesis using 17 diets incubated in vitro with protozoa-free buffered rumen fluid. [^{15}N] ammonium chloride was used to label liquid and solid attached bacteria (SAB) and the N:^{15}N ratio in isolated SAB was used to calculate the time digestible DM, taking into account the microbial contamination of the feed residue. VFA and CH_4 production were monitored over the length of the incubation. Hexose required for the

production of VFA and the gases was calculated from the equations of Demeyer (in Rumen Microbial Metabolism and Ruminant Digestion pp. 216–237, 1991) and expressed as a proportion of truly degraded DM. The proportion of fermented material that could be accounted for in VFA and the gases (X) varied from 0.27 to 0.48. As predicted, CH_4 production (Y, mole CH_4 produced) increased as the proportion of fermented DM increased ($Y = -0.565 + 2.77X$ ($R^2 = 52.2\%$, $P < 0.05$). However this single factor could only explain 50% of the variation in methane production suggesting that other factors are also important in regulating methane production in the rumen.

Effect of a methane oxidising bacterium isolated from the gut of piglets on methane production in Rusitec. N. Nelson[a], C. Valdés[b], K. Hillman[c], N.R. McEwan[a] R.J. Wallace[a], C.J Newbold[a] ([a] Rowett Research Institute, Aberdeen, AB21 9SB, UK; [b] Universidad de León, 24071 León, Spain; [c] Scottish Agricultural College, AB21 9YA, UK).

We previously reported that a methane oxidising bacterium isolated from the gut of piglets decreased methane production when added to batch cultures of rumen fluid (Valdés et al. 1997, ITEA., 18, 157–159). The effect of this organism, which was shown to have high 16S *rDNA* sequence similarity to *Brevibacillus parabrevis*, on methane production in an artificial rumen fermentor (Rusitec) has now been investigated. *B. parabrevis* was grown aerobically in nutrient broth (100 mL) for 24 h at 37 °C then methane (10 mL) was added and the incubation continued for a further 24 h. Eight Rusitec vessels were fed 20 $g \cdot d^{-1}$ a mixed hay/concentrate diet, 4 vessels received live *B. parabrevis* (100 mL), 4 control vessels were supplemented with *B. parabrevis*, autoclaved (15PSI, 20 min) prior to addition. The experiment lasted 3 weeks, and measurements were made in the last 7 d of the experiment. There was a significant decrease in methane production in treated vessels (5.77 vs. 4.72 $mmol \cdot d^{-1}$, SED 0.785 for control and treatment vessels, respectively). This was not associated with an increased recovery of hydrogen (0.46 vs. 0.60 $mmol \cdot d^{-1}$, SED 0.40). The count of total culturable bacteria increased (4.24 vs. $5.45 \times 10^9 \cdot mL^{-1}$, SED 0.403) but there was no effect on the numbers of cellulolytic (3.5 vs. $5.2 \times 10^6 \cdot mL^{-1}$, SED 1.54) or methanogenic bacteria (3.37 vs. $2.80 \times 10^5 \cdot mL^{-1}$, SED 0.867). Thus *B. parabrevis* decreased methane production in Rusitec, and possibly as a result of its utilisation of dissolved oxygen to oxidise methane, increased numbers of anaerobic bacteria.

Methane suppressing effect of lauric acid in rumen as influenced by carbohydrate composition and calcium. C.R. Soliva, M. Kreuzer, A. Machmüller (Institute of Animal Sciences, Animal Nutrition, ETH Zurich, ETH centre, CH-8092 Zurich, Switzerland).

Among the medium-chain fatty acids, lauric acid has been identified as the most effective against rumen methanogenesis (Dohme et al. 1999, S. Afr. J. Anim. Sci. 29, 88-89). The present study investigated the interactions of lauric acid with dietary carbohydrate composition and calcium on methane suppression. These were expected from known interactions concerning other rumen fermentation parameters. A $2 \times 2 \times 2$ factorial design was applied in an 8-fermenter RUSITEC-system varying lauric acid supply, carbohydrate composition and calcium supply ($n = 4$). Concerning the dietary variation of carbohydrate composition, the calculated supply of fermentable organic matter was kept constant whereas NDF was varied by a factor of two by including diet ingredients like oat huller feed, wheat bran and straw meal instead of barley and soybean meal. The rate of organic matter degradation declined ($P < 0.001$) with lauric acid and higher dietary NDF levels but no interactions occurred. Rumen ciliates were reduced ($P < 0.001$) with lauric acid, whereas the effect of lauric acid on bacteria was influenced by the different dietary carbohydrate composition ($P < 0.05$). Methane release was suppressed with lauric acid ($P < 0.001$) and enhanced with additional calcium ($P < 0.001$). Interactions ($P < 0.05$) were found between the methane suppressing effect of lauric acid and higher NDF levels as well as with calcium supply, both diminishing the efficacy of lauric acid. These results have to be considered when applying lauric acid containing fats in vivo to suppress rumen methanogenesis.

Meta-analysis of fat supplementation influence on methane production in dairy cows. S. Giger-Reverdin, D. Sauvant, (Laboratoire de Nutrition et Alimentation INRA de l'INA P-G, 16 rue Claude Bernard, 75231 Paris Cedex 05, France).

The addition of dietary fat alters the ruminal microbial ecosystem and, in particular, the competition for metabolic hydrogen between methane and propionate production pathways. The aim of this work was to study the influence of fat supplementation (plant versus animal origin) on methane production expressed in litre per kg of feed dry matter. Since data for growing cattle and sheep were too scarce, we only considered data obtained for lactating cows (8 trials, 52 diets). The mean values (± standard deviation) were 27.2 ± 6.0 $L \cdot kg^{-1}$ for methane production (CH4), 4.76 ± 3.29%/DM for the ether extract (EE), and 28.9 ± 5.9 $g \cdot kg^{-1}$ live-weight for dry matter intake (DMI). Methane production significantly decreased when ether extract or dry matter intake increased. The variate "type of fat", which was equal to 0 or 1 for vegetable or animal origin respectively, also influenced CH4 production: CH4 = 55.5 – 0.893 DMI – 0.751 EE + 3.92 "type of fat". (RSD = 3.03, n = 52, r = 0.87.) There was no interaction between EE and type of fat. The decreasing effect of DMI and fat on methane has already been documented (Johnson and Johnson, 1995, J. Anim. Sci., 73, 2483–2492). In contrast, the difference between animal and plant fat is original. The larger depressive effect of vegetable fat could be related to the fact that vegetable fatty acids are less saturated than animal ones and are therefore able to trap more hydrogen molecules. In conclusion, methane production expressed as litre per kg feed DM is highly influenced by fat supplementation, by the degree of fatty acid saturation and the level of dry matter intake in dairy cows.

In situ determination of metabolic fluxes in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. A. Tholen, A. Brune (Fakultät für Biologie, LS Mikrobielle Ökologie, Universität Konstanz, 78457 Konstanz, Germany).

The hindguts of lower termites contain (1) large numbers of anaerobic protozoa, which are believed to ferment polysaccharides to acetate, CO_2, and H_2, and (2) high potential activities of hydrogenotrophic, CO_2-reducing acetogenic bacteria. It has been postulated that the combined metabolic activities of both groups render the digestion of lignocellulose in these termites an overall homoacetogenic process. However, microinjection of radiolabeled metabolites into intact, agarose-embedded hindguts of *Reticulitermes flavipes* showed that the in situ rates of reductive acetogenesis (3.3 nmol $termite^{-1} \cdot h^{-1}$) represent only 10% of the total carbon flux in a living termite, whereas 30% of the carbon flux proceeds via lactate. The rapid turnover of the intestinal lactate pool consolidates the previously reported presence of lactic acid bacteria and the low lactate concentrations in the hindgut fluid of *R. flavipes* (Bauer et al. 2000, Arch. Microbiol. 173, in press). The influence of the incubation atmosphere on the turnover rates and the product patterns confirmed that the influx of oxygen via the gut epithelium and its reduction in the hindgut periphery have a significant impact on carbon and electron flow within the hindgut microbial community, and add strong support to the hypothesis that the coexistence of methanogens and homoacetogens in this termite is based on the spatial arrangement of the respective populations.

Succinate accumulation in pig large intestine during the antibiotic associated diarrhea (AAD) and isolation of succinate producing bacteria. T. Tsukahara[a, b], K. Ushida[a] ([a] Laboratory of Animal Science, Kyoto Pref. Univ., 606–8522 Kyoto, Japan; [b] Kyodoken Institute 612-8073 Kyoto, Japan).

Succinate is seldom detected in the contents of the large intestine, because succinate is an intermediate metabolite which is quickly decarboxylated to propionate by the acid-utilizing bacteria in the gut microbial ecosystem. AAD often accompanies low fecal SCFA concentrations. Although, the concentration of succinate or lactate was not measured in previously published reports, we often observed high succinate content in diarrhea feces in AAD piglets. Succinate accumulation in the hindgut may be involved in AAD, because this acid stimulates water secretion from the digestive tract. In this experiment, AAD was induced by the increased doses of polymyxin B sulfate (PL; 3,000,000 $unit \cdot d^{-1}$) and enrofloxacin (ERFX; 0.6 $g \cdot d^{-1}$). Molar percentages of succinate in total organic acid in cecal and colonic digesta were 6 to 10% in PL-treated pigs, and up to 60% in ERFX-treated pigs. Succinate concentrations (mM) were 15 - 60 in both cases. In the large intestine of ERFX-treated pigs, G(–) facultative anaerobic rods phylogenetically related to *E. coli* and G(+) facultative anaerobic non- spore forming rods phylogenetically related to *Lactobacilli* were isolated as succinate producers, while only the latter were detected as succinate producers in PL-treated pigs. The contribution of these bacteria to succinate accumulation was unexpected.

Session IV:

Pathogens – Gene transfer

Dietary manipulation used to reduce the survival of *Escherichia coli* O157 in gut ecosystems. S.H. Duncan, H.J. Flint, C.S. Stewart. (Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK).

Ruminants are central to the maintenance of the pool of enterohaemorrhagic *Escherichia coli* that can result in the contamination of food and water. Human consumption of these products can result in gastrointestinal disease whilst some infections are zoonotic. Several interventions in animal husbandry practices could result in a reduction of carriage of *E. coli* O157 by ruminants, including the use of probiotics and dietary manipulation. Some dietary plant materials contain metabolites such as coumarins, and we have shown that some of these were inhibitory to the growth of *E. coli* O157 under both aerobic and anaerobic conditions. Further, the coumarin glycoside esculin was hydrolysed by a wide range of gut bacteria to the aglycone esculetin that inhibits the growth of *E. coli* O157. Batch cultures with both mixed rumen contents and mixed human faecal slurry suggested that the presence of the aglycone esculetin resulted in a marked decrease in the survival of *E. coli* O157. Survival of *E. coli* O157 under conditions simulating those in the human colon has also been investigated. A single stage fermentor was continuously fed with nutrients and inoculated with a fresh faecal inoculum from a healthy male. On subsequent inoculation with a non-verocytotoxic strain of *E. coli* O157 at approx. 10^8 cfu·mL^{-1}, the numbers decreased then stabilised at around 10^4 cfu·mL^{-1}. However the survival of *E. coli* O157 was further reduced by the addition of the coumarin esculetin and the bacterium was eventually undetectable in the system. If similar trends occurred in the ruminant gut, the use of prebiotic dietary supplements particularly in the period prior to slaughter may significantly reduce the spread of *E. coli* O157.

The effects of weak acids on *Escherichia coli* rumen and O157 H7 isolates. E.C. McWilliam Leitch, C.S. Stewart (Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, Scotland).

Weak acids at low pH have bactericidal properties. This theoretically occurs due to cytoplasmic accumulation of protons and toxic dissociated acid anions, as determined by the pH and the pKa of the acid. We examined the effect of various fermentation acids on the survival of an *E. coli* rumen isolate and an O157:H7 strain. The viability of stationary phase batch cultures in 100 mM of acid over 24 h was determined. At pH3 and 37 °C, fermentation acids caused a greater than 10^8 cfu·mL^{-1} decrease in viable cells over 24 h. Lactic acid caused the most rapid decline in cell numbers followed by *n*-butyric > *iso*-butyric > propionic > acetic acid. In contrast, HCl at pH 3 had no effect on survival. The O157:H7 strain was more susceptible to these acids than the rumen isolate. Acid susceptibility was enhanced by 5% ethanol. Incubation at 5 or 20 °C decreased acid susceptibility at pH 3. Acids at pH 5 or 7 did not affect survival at 37 °C. The greater bactericidal capacity of lactic acid is in agreement with its low pKa (3.86). However, the other acids assayed have similar pKas (4.76–4.87) but different bactericidal capacities. This suggests that current theories do not take into account all the factors that influence the bactericidal nature of weak acids.

The rumen as a reservoir for pathogenic VTEC strains. K. Boukhors[a], N. Pradel[b], J.P. Girardeau[a], Y. Bertin[a], V. Livrelli[b], M. Contrepois[a], C. Martin[a] ([a] Unité de Microbiologie, INRA, 63122 Saint-Genès-Champanelle, France; [b] Groupe de Recherche de Pathogénie Bactérienne Intestinale, Faculté de Pharmacie, 63001 Clermont-Ferrand, France).

The gastrointestinal tract of ruminants is the main reservoir for VTEC strains potentially pathogenic for humans. Although the nature of diet appears to influence the carriage of *E. coli* O157:H7, nutritional requirements of VTEC in the rumen and factors influencing survival or growth are poorly understood. Our aim was (1) to estimate the risk of zoonosis by the molecular characterisation of VTEC strains, (2) to understand the physiological basis of VTEC carriage by ruminants. We used a collection of 220 VTEC strains isolated from bovine faeces, meat, cheeses, HUS patients, in the same geographic area during a limited period of time. Characterisation of strains belonging to serotypes associated with SHU using ERIC-PCR, ribotyping and PFGE indicates that some bovine strains were genotypically identical to the HUS isolates, suggesting a clonal origin. A comparison of the in vitro growth of the same strains showed that VTEC strains

from humans and from cattle are equally adapted to growth in rumen fluid. However, physicochemical characteristics of the rumen fluid, diet and short fasting drastically influenced VTEC growth. A comparison of VTEC strains from the environment and from HUS patients in a same geographical area should allow identification of the genotypic properties of pathogenic strains. These results suggest that VTEC carriage rate and therefore food contamination depends in part on the nature of diet given to ruminants.

The effect of protozoa on the survival and flow of listeria from the rumen of sheep. J.L. Shepherd[a, b], C.J. Newbold[a], K. Hillman[b], D.R. Fenlon[b] ([a] Rowett Research Institute, Aberdeen, AB21 9SB; [b] Scottish Agricultural College, Aberdeen, AB21 9YA, UK).

Listeria monocytogenes is a potentially pathogenic bacterium, which can lead to abortion and encephalitis in ruminants. Listeria present in silage can pass through the ruminant gut, reinoculating grassland and producing a cycle of infection on the farm. In order to break this cycle it is important to understand the factors affecting the survival of listeria in the gut. Four ruminally cannulated sheep were defaunated by rumen washing. The same sheep were subsequently refaunated by the addition of rumen fluid from a normally faunated sheep. The sheep received a diet of 1 kg dried grass pellets (as-fed basis) per day via a continuous belt feeder. *Listeria innocua*, a non-pathogenic relative of *L. monocytogenes*, was added to the diet. The flow of listeria was measured at the rumen and in the faeces. Refaunated and defaunated sheep received the same amount of *L. innocua* in the diet (6.5×10^8 cfu·d^{-1}). There was a higher flow of listeria leaving the rumen of defaunated sheep (2.8 vs. 9.1×10^8 cfu·d^{-1}, SED 3.06, for refaunated and defaunated sheep respectively); however this did not influence the amount of listeria shed in the faeces (2.3 vs. 1.0×10^6 cfu·d^{-1}, SED 1.88, respectively). Thus defaunation increased the flow of listeria from the gut, presumably because in faunated animals protozoa engulfed and degraded listeria. However, while this would have increased the potential infective dose entering the lower gut, it would not influence the amount recycled onto grassland via the faeces.

***Helicobacter* infection in the bovine abomasum**. P. Gueneau, M.G. Domínguez-Bello. (Instituto Venezolano de Investigaciones Científicas, IVIC, Apartado 21827 Caracas 1020A, Venezuela).

The ethiologic role of *Helicobacter* gastric infection in human pathologies has been well established. Most *Helicobacter* gastric species have been reported in carnivorous and omnivorous mammals (humans, macaques, dogs, cats, leopards, ferrets). The purpose of this work was to determine whether ruminants could harbour gastric *Helicobacter* species in the abomasum. DNA from antrum samples taken from 30 bulls at a slaughter house were amplified by PCR using primers targeting a region of the 16S rRNA (genus specific) and *ureA* (specific for *H. pylori*) genes. Twenty of the 30 bulls (67%) were positive for *Helicobacter* sp, but none were positive for *H. pylori*. The 16S rRNA gene partial sequence (730 bp) shared 96% sequence identity with the sequence of the recently reported new species *H. bovis* (De Groote et al. 1999, Int. J. Syst. Bacteriol. 49, 1707–1715). It is remarkable that in spite of the barrier imposed by the rumen and by the gastric digestive lysozyme, ruminants are susceptible to be colonised by gastric *Helicobacter*.

Analysis of tet(W) in commensal bacteria from diverse gut habitats. C.M. Melville, K.P. Scott, D.K. Mercer, T.M. Barbosa, H.J. Flint (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK).

The widespread use of antibiotics has resulted in the rapid emergence of antibiotic resistance in both human and veterinary pathogens. However little work has been done on the incidence of resistance in the commensal gut flora of either humans or animals which may have an important role in harbouring antibiotic resistance genes with the potential for onward transfer to pathogenic bacteria. A previously undescribed tetracycline resistance gene, tet(W), was isolated by our group from the rumen anaerobe, Butyrivibrio fibrisolvens. This new ribosome protection type gene is less than 65% homologous to tet(M) and in the B. fibrisolvens strain 1.230, is carried on a 50 kb mobile chromosomal element, TnB1230, which is unrelated to known conjugative transposons. The sequence analysis of a 9 kb clone from TnB1230 reveals the presence of 5 open reading frames (orf's), one of which has

significant homology to a transfer protein of Gram positive origin. The GC content of the tet(W) gene is considerably higher than that of the flanking orf's and the Butyrivibrio host itself. We also report the isolation of a gene > 99% homologous to tet(W) from the human colonic bacteria, Fusobacterium prausnitzii and Bifidobacterium longum. In each human isolate tet(W) is present on differently sized chromosomal fragments which seem unrelated to TnB1230 from B. fibrisolvens, indicating that tet(W) is not always carried by the same mobile element. It is important now to investigate the mechanisms involved in the dissemination of tet(W), particularly since tet(W) appears to be carried on different genetic elements in different hosts. Once understood we can begin to establish the possible contribution made by tet(W) to previously unidentified tetracycline resistance in pathogenic bacteria.

Potential for onward gene transfer from genetically modified organisms in the gut. K.P. Scott, C.M. Melville, D.K. Mercer, H.J. Flint (Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK).

The increase in the use of prebiotics and probiotics with, among others, the aim of improving gut function could potentially result in the introduction of genetically modified microorganisms into the gut environment. It is important to be able to assess the persistence of both the genetically modified organism and its DNA in the gut and monitor the effect such an introduction has on the stability of the commensal flora.We have marked a range of lactic acid bacteria, important in the production of fermented foods, with the green fluorescent protein in order to track their survival under simulated gut conditions. The gfp marker was visibly detected both when plasmid encoded and when integrated as a single copy in the host chromosome. Neither marked strains of *L. lactis* nor *E. faecalis* were maintained at high numbers in the human colonic simulation although *L. lactis* did become established at a low level. No transfer of the marker gfp gene was detected to the endogenous flora using our detection method**.** The potential impact of feeding genetically modified plant material, to animals and man, is an equally important issue. Free DNA can survive for limited periods in the gastrointestinal tract and some gut bacteria are naturally transformable. One concern has been the possible introduction of new antibiotic resistance genes into the commensal flora. However we have found ampicillin resistance genes similar to those used in GM maize carrying pUC sequences in ruminal and pig isolates of *E. coli* obtained over a 10 year period from different locations, and also in pig *Pseudomonas* isolates. The significance of this finding is discussed.

Natural genetic transformation of the rumen bacterium *Streptococcus bovis* JB1. D.K. Mercer, C.M. Melville, K.P. Scott, H.J. Flint (Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK).

Little is known about gene transfer in the rumen although this knowledge is important in understanding the evolution of micro-organisms and in assessing the potential for dissemination of antibiotic resistances and of genes derived from genetically modified plant material. Previous work has concentrated on conjugative mechanisms of gene transfer, whether plasmid-mediated or via conjugative transposons, whilst the potential role of natural transformation has not been studied. Natural transformation of the ruminal bacterium *Streptococcus bovis* JB1 with plasmids carrying the green fluorescent protein marker gene was demonstrated after development of competence in normal culture medium. Transformation efficiencies were not significantly increased when heat-inactivated horse serum was added to the medium before growth. Transformation allowed the acquisition of plasmids capable of replication in *S. bovis* JB1 or integration of sequences into the chromosome via homologous recombination. No transformation was observed in the presence of undiluted autoclaved or filter-sterilised ovine rumen fluid or filter-sterilised saliva, suggesting that transformation in the ruminant digestive tract is a rare event, although transformation was observed in the presence of 1% and 0.5% filter-sterilised rumen fluid. The use of natural transformation of *S. bovis* should facilitate further molecular biological studies on this species.

Nuclease from *Prevotella bryantii* B_14^T. T. Accetto, G. Avgustin (Univ. Ljubljana, Biotechnical Faculty, Zootechnical dept., Groblje 3, 1230 Domzale, Slovenia).

Extracytoplasmic non-specific nucleases have been found and described in various bacteria and also in ruminal bacterial species like *Fibrobacter*.

Such enzymes are thought to be common, however, their function is not entirely clear. The removal of DNase activity has enabled successful transformation in some organisms implicating that such enzymes may play an important role not only in nutrition but also in the restriction of foreign DNA. Ruminal *Prevotella* species are known for their resistance to transformation with foreign DNA, and for some strains high nucleolytic activity has been described. We found that in the case of *P. bryantii* $B_1 4^T$, the cleavage of the supercoiled plasmid DNA to a linear form proceeds through an open circle intermediate which suggests a non-simultaneous cleavage of the two strands. The molecule is shortened only after it becomes linear. The cleavage is not site specific and the protein probably responsible for the activity was observed on the renatured SDS PAGE gel containing DNA at approximately 45 kDA. When measuring the nucleolytic activity of the supernatants with an adapted Kunitz assay, no evidence for quorum-sensing type of behaviour was found: the activity rose even slower than the bacterial density. No changes in the activity during growth on media containing various DNA concentrations were observed. Currently we are trying to clone the nuclease in the *E.coli* or *P.bryantii* DNase$^-$ strain.

DNA landscaping, codon indices and DNA sequence bias in cryptic plasmids from rumen bacteria. N.R. McEwan (Protein Metabolism Group, Rowett Research Institute, Aberdeen AB21 9SB, Scotland).

Cryptic plasmids may be regarded as a highly specialised form of selfish DNA, often encoding only the gene(s) necessary for their own replication and mobilisation. The size of their genome is generally at least double the size of the minimum number of nucleotides needed to encode these functions. To this effect, the sequences between the genes of cryptic plasmids provide a source of information regarding the DNA structure necessary for organisation of DNA into a form ensuring the stability of replication, transcription and mobilisation. Within a complex ecosystem such as the rumen, there is the potential for the uptake of DNA or the conjugation of plasmids between species. This work examines a number of codon indices for the genes of cryptic plasmids from different species, the DNA landscape of these genes and their intergenic spacers, and the level of nucleotide bias in both these genes and their intergenic spacers. Variation in these values within plasmids from a single species and from different species is discussed.

Session V:

Probiotics – Prebiotics – Feed additives

Effect of a live yeast supplement on microbial colonisation of the rumen of newborn lambs. F. Chaucheyras-Durand[a, b], G. Fonty[a], H. Durand[b] ([a]Unité de microbiologie, INRA, C.R. de Clermont-Ferrand/Theix, 63122 Saint-Genès-Champanelle, France; [b] Lallemand sarl, 15130 Saint-Simon, France).

Numerous attempts have been made to stimulate rumen development in pre-ruminants in order to wean them at an earlier age and to avoid digestive disorders due to feed transition. Supplementation of the diets with feed additives would therefore be a very useful tool to achieve these goals. The aim of our study was to investigate the effect of *Saccharomyces cerevisiae* CNCM I-1077 (Levucell®SC) on microbial colonisation of the rumen of newborn lambs. In this paper we report cellulolytic bacteria and protozoa establishment in the rumen of lambs (2 lambing periods), which received daily 0.2 g (4×10^9 CFU·mL^{-1}) of Levucell®SC introduced into the rumen by a stomach tube. Cellulolytic bacteria and protozoa were regularly enumerated between day 2 and day 54 after birth. In SC lambs, cellulolytic bacteria were present earlier than in controls in period 1 and their numbers were approximately 10-fold higher 8, 30 and 50 d after birth in the rumen of SC lambs compared to controls in period 2. Ciliate protozoa were established 6 days earlier in the rumen of SC lambs (day 12 in SC lambs vs. day 18 in controls), and the ciliate community tended to be more diversified in the rumen of SC lambs compared to controls. These results indicate that in the presence of yeast, ecological conditions which are required for the establishment of microbial communities typical of the adult ruminant, are created earlier. This acceleration of maturity of the ruminal ecosystem could therefore allow an earlier weaning of the animals.

Effects of a dried live yeast culture on the fibrolytic activity of the equine intestinal ecosystem. B. Medina[a], A. de Fombelle[b], E. Jacotot[b], V. Julliand[b] ([a]Alltech France; [b] Lab. nutrition des herbivores, ENESAD, 26 bd Dr Petitjean, 21000 Dijon, France).

A global approach of the activity of the equine ecosystem is required to better understand the ability of live yeast culture (LYC) on fibre digestion. Caecal ($n = 8$) and colonic ($n = 4$) contents were collected from 8 canulated horses, to determine the concentrations of the total anaerobic, lactic acid-utilising, cellulolytic bacteria, *Lactobacilli* spp and *Streptococci* spp. Caecal and colonic concentrations of ammonia, volatile fatty acids, lactate and pH were measured during the first 12 h post-feeding. The horses, allotted into pairs, were assigned to a 4×4 Latin square, in which they were fed 4 diets. The high fibre (HF) diet was based on a 54.8% alfalfa pelleted feed, whereas the high starch (HS) one contained 53.5% barley. 0 or 10 g·d^{-1} of a LYC (Yea Sacc^{1026}) were top-dressed on diets. When the HF diet was fed, the addition of LYC did not alter the cellulolytic bacteria counts. However, LYC enhanced both the acetate (C_2) concentration ($P < 0.05$) and the $[(C_2+C_4)/C_3]$ ratio ($P < 0.05$) in the large intestine. These results suggested an increase of the fibrolytic activity that was confirmed by increases of apparent digestibility of dietary nutrients and of in vitro straw degradability ($P < 0.05$), measured in parallel. When horses were fed the HS diet, LYC decreased ammonia ($P < 0.05$) and lactate concentrations ($P < 0.05$) and increased pH ($P < 0.05$) in the hindgut. These data could be explained by the stimulation of the lactic acid-utilising bacteria and the decrease of the *Streptococci spp.* shown in the colon. Thus, LYC appeared to prevent ecosystem disorders when horses were fed high starch diets. Further investigations of microbial activities and profiles are needed to establish a mode of action of LYC in the equine ecosystem.

Effect of different live yeasts (*Saccharomyces cerevisiae*) on rumen fermentation. F. Fantuz[a], F. Polidori[b], E. Salimei[c], J.C. Robert[d], P.E.V. Williams[d], J.P. Jouany[b] ([a] Fac. Med. Vet., Univ. of Camerino (MC), Italy; [b] INRA-Theix, France; [c] Dept. S.A.V.A., Univ of Molise, Italy; [d] Rhône Poulenc, France).

The aim of this study was to evaluate the effects of different live yeast preparations (*Saccharomyces cerevisiae*), provided by Aventis Animal Nutrition and coded A, B, C, D, E, on rumen fermentation. Four male Texel sheep fitted with rumen cannulae were used. During the control period the animals received a diet composed of hay (700 g), barley (400 g) and soybean meal (100 g). The animals were then adapted (14 d) to

the new diets containing yeast (3 g DM·d^{-1}). In vitro ruminal protein degradation, in sacco rumen dry matter degradation and in vivo rumen pH were determined. The results were compared with the control period using GLM and the Duncan multiple test range. Preparation C reduced ($P < 0.05$) the in vitro protein degradation (%) of soybean meal (control = 35.03 ± 1.07, C = 31.85 ± 1.05). Preparation C, D and E improved ($P < 0.05$) the in sacco ruminal degradation (%) of wheat straw at 24 h of incubation (control = 20.94 ± 2.94, C = 29.96 ± 2.64, D = 25.52 ± 4.24, E = 26.71 ± 2.56) showing no significant effects on longer incubation times. Preparation D and E, increased in vivo pH values 4 hours after the meal (control = 6.01 ± 0.04, D = 6.23 ± 0.05, E = 6.22 ± 0.06; $P < 0.05$). The results suggest that the effects of yeast culture on rumen fermentations are dependent on the specific preparation of *Saccharomyces cerevisiae* administered to the animals.

Influence of supplementary fibrolytic enzymes on fermentation of maize and grass silages by mixed ruminal microorganisms in vitro. R.J. Wallace[a], S.J.A. Wallace[a], N. McKain[a], G.F. Hartnell[b] ([a] Rowett Research Institute, Bucksburn, Aberdeen, UK; [b] Monsanto Company, Saint-Louis, MO 63167, USA).

Fibrolytic enzymes are increasingly being used as feed additives for ruminants. The aims of this project were to evaluate their effects on ruminal fermentation, and to assess whether any individual fibrolytic activity limits the rate of fibre digestion by ruminal microorganisms. Two commercial enzyme preparations only affected the digestion of maize silage and grass silage at application rates far in excess of those used commercially; furthermore, the activities added were insignificant in comparison to those already present in the feed or in ruminal microorganisms. Thus it was concluded that any benefits reported in feeding trials must result from the effects of enzymes during the pre-feeding phase, by providing a preliminary digestion which benefits ruminal microorganisms. A comparison of the effects on digestion of silages by the same two preparations at high concentrations and by 9 other commercial fibrolytic preparations with their enzyme activity indicated that the strongest correlation was with β-1,4-endoglucanase activity. There was little correlation with other polysaccharidase activities, including endo- and exo-β-1,4-xylanases, or glycosidase activities.

Effects of feeding a fungal feed enzyme preparation on the rumen microbial population. V.L. Nsereko, D.P. Morgavi, K.A. Beauchemin, L.M. Rode, A.F. Furtado, T.A. McAllister (Agriculture and Agri-Food Canada, Lethbridge, AB T1J 4B1, Canada).

Recent studies indicate that fungal enzymes improve feed utilisation in ruminants via, as of yet, an unknown mechanism. We investigated the effects of an enzyme preparation from *Trichoderma Longibrachiatum* (Monsanto Co., Saint-Louis) on the rumen microbial population. Two dairy cows fitted with rumen cannulae were each fed a diet of barley grain and maize silage supplemented with 0, 1, 2, 5 or 10 L enzyme per tonne DM. Incremental levels of this supplement stimulated numbers of total viable bacteria ($P < 0.05$) by 100, 330, 390 and 250% (quadratic effect, $P < 0.05$). Of the rumen bacteria, the most notable increases in numbers were for cellobiose utilising ($P < 0.01$), xylanolytic ($P < 0 .05$) and amylolytic ($P < 0.05$) subgroups; numbers of cellulolytic bacteria were unaffected ($P > 0.05$). Increasing concentrations of the supplement had a convex quadratic effect on protozoa numbers ($P < 0.05$), and lower protozoa numbers may partially explain the increased number of bacteria. These data suggest that exogenous enzymes enhance feed digestion at least, in part, by increasing numbers of rumen bacteria that utilise hemicelluloses and secondary products of cellulose digestion.

Effect of a *Trichoderma* feed enzyme on growth and substrate degradation by *Fibrobacter succinogenes* F85. D.P. Morgavi, V.L. Nsereko, L.M. Rode, K.A. Beauchemin, T.A. McAllister, Y. Wang (Agriculture and Agri-Food Canada, Lethbridge, AB, T1J 4B1, Canada).

Feed enzyme additives, used to improve digestion in ruminants, interact not only with the feed but also with rumen microorganisms. Some studies have shown overall increases in the rumen microbial population due to enzymes. However, it is not clear whether this effect is due directly to microbial growth stimulation or indirectly to modifying feed structure. We studied the effect of an enzyme preparation from *T. longibrachiatum* (TE) on growth of *F. succinogenes* on cellobiose, crystalline cellulose or corn silage fibre. Growth was monitored turbidimetrically. For solid substrates, fibre disappearance and fermentation parameters were also monitored. The

growth rate of *F. succinogenes* on cellobiose was not affected by TE ($P > 0.05$). The presence of TE positively affected growth in media containing cellulose but failed to increase substrate disappearance or gas production. When corn silage was used, the addition of TE increased NDF disappearance ($P < 0.05$) at 24 and 48 h (33 and 52% in controls versus 53% and 65% in TE treatments). Growth rate and gas production were also stimulated ($P < 0.05$). These results suggest that the *Trichoderma* enzyme preparation did not supply microbial nutrients or growth factors. *F. succinogenes* digests cellulose efficiently and addition of exogenous cellulases did not further increase cellulose disappearance. However, TE increased plant (corn) fibre degradation probably by providing an enzyme(s) that limited degradation.

Studies on polysaccharide degrading enzymes encoding genes. N. Özcan[a], M.S. Ekinci[b] ([a] Cukurova University Faculty of Agriculture Department of Animal Science, Adana, Turkey; [b] Kahramanaras Sutcu Imam University Faculty of Agriculture Department of Animal Science, K. Maras, Turkey).

In recent years, considerable efforts have been devoted to improving the nutritive value of animal feed stuffs through supplementation with exogenous enzymes including cellulases, hemicellulases and amylases. *Bacillus subtilis* enzymes are good sources of feed upgrading enzymes such as cellulase, xylanase, amylase and proteases. Therefore many enzymes including xylanase, CMCase, α-amylase and also bifunctional CMCase/xylanase encoding genes were isolated from *B. subtilis* RSKK246, *B. subtilis* RSKK246, RSKK245, *B. subtilis* RSKK244, and *B. subtilis* RSKK243 strains, respectively. Enzymatic assay and SDS-PAGE zymogram analyses were carried out to characterise the polypeptide pattern of each enzyme and also their stabilities under different environmental conditions. The genes were cloned into the pUB110 *E.coli*/*B. subtilis* shuttle vector, and expressed into the different *B. subtilis* strains. Expression of the genes was also observed in *B. subtilis* YB886, which has no native enzyme activity. The cell and supernatant extract obtained from that strain were used for most of the enzymatic analyses. CMCase, α-amylase and bifunctional CMCase xylanase encoding genes were also inserted into the pTRW10 *E. coli*/*Streptococus* shuttle vector to transfer these genes into the rumen bacterium, *Streptococcus bovis* and into other lactic acid bacteria including *Lactococcus lactis*, *Pediococcus acidophilus* and *Lactobacillus plantarum*. Levels of gene products in these organisms and their possible applications as silage inoculants and as probiotics for monogastric animals are under investigation.

In vitro supplementation with *Sesbania* leaves enhances the growth of ruminal bacteria and depresses eukaryotic populations. S. Muetzel, E. Hoffmann, K. Becker (Univ. Of Hohenheim, Inst. 480, 70593 Stuttgart, Germany).

Diets for ruminants in tropical regions are often based on poor roughages. Supplementation with a certain portion of fresh leaves as an additional nitrogen source can greatly enhance the productivity of these animals, but many of the trees and shrubs that can serve as additional feed sources contain antinutritional compounds. The evaluation of a supplementation strategy in an in vitro fermentation system is thus desirable prior to actual feeding experiments. The Hohenheim gas test was used as a short term batch incubation system to characterise the fermentation profiles of barley straw, *Sesbania* leaves, and different mixed ratios of these substrates. Over a 48 h period, samples were analysed for short chain fatty acid (SCFA) production, microbial biomass as estimated by total RNA, and population dynamics of some selected rumen microbes by 16S rRNA hybridisation. A clear supplementation effect was detected in total RNA with a straw: leat ratio of 6: 4. Among the cellulolytic bacteria the same effect was seen for *Ruminococcus albus* and *R. flavefaciens*, whereas the *Fibrobacter* group did not respond to the addition of *Sesbania* leaves. The eukaryotic population, including the rumen fungi, disappeared when more than 20% *Sesbania* were introduced in the fermentation. This is probably due to the saponins present in this plant, which are known to selectively destroy eukaryotic organisms. These results show that both the effects of nutrient composition and antinutritional compounds of a putative feed supplement can be simulated in vitro. The analysis of the microbial population structure allows a first insight into the modes of action of certain substances and into the regulation mechanisms of the rumen ecosystem.

Effects of essential oils on rumen fermentation. F.M. McIntosh[a], C.J. Newbold[a], R. Losa[b], P. Williams[c], R.J. Wallace[a] ([a] Rowett Research Institute, Aberdeen, AB21 9SB, UK; [b] CRINA

SA, Gland, CH-1196, Switzerland; [c] AKZO Nobel Surface Chemistry Ltd, Saint-Albans, AL1 3AW, UK).

Four mature sheep received 1 kg DM·d^{-1} of a 40:60 concentrate:forage (grass silage) mix in two equal meals. The effect of essential oils (EO; CRINA HC, AKZO Nobel Surface Chemistry Ltd, 100 mg EO·d^{-1}) was investigated in a 2×2 design with 6 week periods. EO inhibited ruminal degradation of soyabean meal N from nylon bags incubated in situ, which was reflected in a 20% (6.0 vs. 4.8%·h^{-1}, SED 0.68) decrease in the rate of degradation, equivalent to an 11% decrease in the effective degradability of soyabean dry matter (54 vs. 48% at an assumed outflow of 0.08 h^{-1}). No difference was seen in the degradation of rapeseed meal or hay in the rumen. EO had no influence on the proteolytic or peptidolytic activity of rumen fluid. However, deamination of amino acids measured in vitro decreased by 25% (204 vs. 155 nmol NH_3 produced per mg of protein per hour, SED 9.3). EO did not affect protozoal numbers in the rumen, suggesting that the effects were limited to the bacterial population. EO did not affect the flow of microbial protein from the rumen, as estimated from the excretion of purine derivatives in the urine. A commercial preparation of essential oils appeared to inhibit protein degradation in the rumen with some but not all substrates. These effects were apparently mediated via the effects of EO on the bacterial population.

Effects of flavomycin in the gastrointestinal tract of sheep. N. McKain[a], J. Edwards[a], R.J. Wallace[a], S. Edwards[b], L. Bruce[a], B.J. Bequette[a], J.C. MacRae[a] ([a] Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB; [b] Dept. of Agriculture, University of Aberdeen, 581 King Street, Aberdeen, AB24 5UA Scotland, UK).

Flavomycin is a growth promoting antibiotic used in poultry, swine and ruminant production. Its effects on some bacterial species have been determined but little is known about its effects on gut microflora. The aim of this study was to determine the effects of flavomycin on ruminal and intestinal bacteria in vitro and in vivo. Ten male, six month old, 30 kg lambs (divided into two groups) were fed a control diet of 800 g·day^{-1} pelleted dried grass for 6 weeks. For the last two weeks of this period, one group received 250 mg·day^{-1} Flavomycin·80. Samples of digesta and gut tissue were taken at slaughter. Total viable bacterial counts in the rumen increased in flavomycin-treated animals (control 1.2×10^9 g^{-1}, flavomycin 3.5×10^9 g^{-1}, $P = 0.04$): no significant changes were found in digesta samples from the rest of the tract. Proteolytic activity of digesta throughout the digestive tract, measured by ammonia production from casein, was similar in both groups, except in colonic digesta where there was a 36% decrease in flavomycin-treated animals (control 249 mg NH_3·L^{-1} and flavomycin 159 mg NH_3·L^{-1}, $P < 0.05$). Viable counts of hyper-ammonia producing bacteria and volatile fatty acid concentrations were not significantly affected by flavomycin. Gram-positive rumen bacteria were generally more sensitive to flavomycin in vitro than Gram-negative species.

Effect of NDOs on adhesion of *Salmonella* and *E. coli* in a Porcine Intestinal Organ Culture (PIOC) model. P.J. Naughton, L.L. Mikkelsen, B.B. Jensen (Department of Animal Nutrition and Physiology, DIAS, Research Centre Foulum, P.O. Box 50, 8830, Tjele, Denmark).

Non-digestible oligosaccharides (NDOs) are natural components of plants, vegetables and milk and cannot be degraded by mammalian digestive enzymes. In addition they are thought to be preferentially metabolised by beneficial intestinal microflora and their endproducts may prevent the growth of pathogenic bacteria. They may also interfere with the protein carbohydate interaction between pathogens and the host epithelial cell. The P.I.O.C. model which was adapted from an earlier method (Bäumler et al. 1996, PNAS, 93, 279–283) can test the effect of feed components such as NDOs on pathogenic bacteria. The present study consisted of 30, 4-week old piglets (10 piglets per group). Sections of the small intestines were taken aseptically from the distal ileum and mid-jejunum of the piglets which had been fed a semisynthetic control diet, a control diet supplemented with 4% Galacto-oligosaccharide (GOS) or a control diet supplemented with 4% Fructooligosaccharide (FOS), for two weeks. Jejunal organ cultures were inoculated with non-pathogenic *Escherichia coli* and ileal organ cultures were inoculated with *Salmonella typhimurium* S986 (Naughton et al. 1996, J. Appl. Bacteriol. 81, 651–656). Following incubation at 37 °C for 1 h the number of attached bacteria were quantified. In the GOS and FOS treatments, 50% of piglets showed a reduction in *E. coli* and *Salmonella* adhesion.

Effects of dietary flavonoids on the human intestinal bacteria. R. Simmering, M. Blaut (German Institute of human Nutrition, Dept. Gastrointestinal Microbiology, 14558 Bergholz-Rehbrücke, Germany).

There is a growing interest in the metabolism of dietary flavonoids owing to their pharmacological properties. It is known that flavonoids are subject to degradation by human intestinal bacteria. One of the most important flavonoid-degrading bacterial species in the human gut so far is *Eubacterium ramulus* which is able to degrade a large variety of dietary flavonoids such as quercetin and rutin. In two different human studies the influence of different dietary flavonoids on the population of *E. ramulus* was investigated using whole-cell hybridisation with a species-specific oligonucleotide probe. In addition, the effects of these substances on other phylogenetic groups were also tested. After a period of a flavonoid-free diet, the human subjects were orally given a relative large proportion of dietary flavonoids. The total fecal bacteria decreased during the flavonoid-free diet, as did the *E. ramulus*-population, in all groups. Twenty-four hours after taking the flavonoid-source, the fecal cell counts of *E. ramulus* increased dramatically. In the placebo-group no such stimulation was detected. One week after the flavonoid-free diet, the cell counts of total bacteria as the cell counts of *E. ramulus* were in the same range as before the study. These results suggest that dietary flavonoids influence human intestinal bacteria.

Isotachophoretic determination of phytate phosphorus in faeces of cattle, pigs and hens. M. Marounek[a], D. Dusková[a], V. Skrivanová[b] ([a] Institute of Animal Physiology and Genetics, Praha 10; [b] Research Institute of Animal Production, Praha 10, Czech Republic).

The direct measurement of phytic acid in samples of digesta and faeces is difficult due to the presence of colloid substances and interfering ions. In this study, a method of phytate determination by means of capillary isotachophoresis, involving phytate extraction by HCl and precipitation by $FeCl_3$ before analysis, was used. In faeces of young pigs (40–60 kg), 11.2% of total phosphorus was in the form of phytate (2618 mg of phytate P per kg DM of faeces). These pigs were fed a concentrate containing commercial phytase at 490 $U \cdot kg^{-1}$. In faeces of growing - finishing pigs (60–120 kg), 15.3% of total P was present as phytate P (2937 mg of phytate P per kg DM). In faeces of sows, 18.5% of total P was in the form of phytate P (3283 mg of phytate P per kg DM). Faeces of laying hens contained 36.2% of total P as phytate P (6676 mg of phytate P per kg DM). No phytate was found in the faeces of steers (400 kg) and dairy cows fed a high - concentrate diet. Our results indicate that (i) phytate contributes significantly to total P in faeces of monogastrics, (ii) phytase addition to a feed mixture for pigs at 490 $U \cdot kg^{-1}$ is not sufficient to eliminate phytate from faeces, (iii) on the contrary to some other reports, phytate was absent (or below the detection limit) in faeces of cattle.

Molecular study of microbial phytase and its possible application to poultry feed. M.S. Ekinci[a], M. Karaman[a], E. Efe[a], N. Özcan[b], Y. Gürbüz[a] ([a] Kahramanaras Sutcu Imam University Faculty of Agriculture Department of Animal Science, K. Maras, Turkey; [b] University of Cukurova Faculty of Agriculture Department of Animal Science, Adana, Turkey).

Enzymes that are used as animal feed supplements should be able to withstand high temperatures, which may be reached during the feed pelleting process. Enzymes should also be stable at low pH values and resistant to proteolytic activity of the digestive tract. Therefore phytase enzymes of *Aspergillus niger* and *Bacillus subtilis* were studied. Also, some rumen bacteria were screened for phytase activity. *Streptococcus bovis* and *Selenomonas ruminantium* were found to produce phytase as shown by detecting clear zones around colonies on agar plates containing sodium phytate.The *A. niger* enzyme was assayed at different temperatures and pH, and also after treatment with digestive tract enzymes including trypsine, chymotrypsine, and fresh saliva. The effects of the above treatments on enzyme activity and on polypeptide profile were determined on sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). *A. niger* enzyme has an apparent molecular mass of 60 kDa, as estimated by SDS-PAGE. Thus enzyme showed its highest activity with sodium phytate at a pH range of 3 to 5.5 and with phytic acid at a pH range 3 to 7. The enzymes were relatively stable between pH 3 and 7 and at temperatures between 37 and 55 °C. Zymogram analyses and enzyme assays showed that treatment of the enzyme with proteases and with saliva at different time intervals resulted in a loss of enzyme activity of about 40–80%. This result gives some

idea about the fate of the phytase enzyme in the digestive track of poultry after being ingested by the animal during feeding.

Effect of Potassium-Diformate (PDF) on microbial growth in the stomach and small intestine of pigs, measured in vitro. A. Knarreborg[a], N. Miquel[a], T. Granli[b], B.B. Jensen[a] ([a] Department of Animal Nutrition and Physiology, Danish Institute of Agricultural Sciences, 8830 Tjele, Denmark; [b] Norsk Hydro ASA, 3901 Porsgrunn, Norway).

In order to screen various candidates of feed additives an in vitro system was established to simulate the microbial ecology in the stomach and proximal part of the small intestines of piglets. The system was adjustable in regard to culture medium, pH, temperature and incubation period. In addition, an anoxic atmosphere was provided. The aim of the present study was to test the applicability and reproducibility of the in vitro system. Hence, three replicates of two experiments were conducted to investigate the effect of PDF on microbial growth in the stomach contents (pH 3, 4, 5) and in contents from the small intestines (pH 5, 6, 7) of pigs. Calculations of specific growth rates of coliforms and lactic acid bacteria revealed a positive relationship between growth rates and pH-level. In general, the addition of PDF decreased the specific growth rates of coliform and lactic acid bacteria. However, PDF exhibited the strongest anti-microbial properties against coliform bacteria. Furthermore, the inhibiting effect of PDF on microbial growth was most pronounced in the stomach contents. Thus, in intestinal contents the presence of PDF particularly prevented growth of coliforms, whereas a considerable killing effect on both coliforms and lactic acid bacteria were observed in the stomach contents. In conclusion, the in vitro system offers a reliable method to describe alterations in the microbiota in response to different environmental conditions and provides a valid basis for the selection of products to be tested in vivo.

A dynamic in vitro model of the gastrointestinal tract: a tool for the selection of functional micro-organisms. R. Havenaar, E. Zeijdner, M. Minekus, M. Smeets-Peeters (TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ Zeist, The Netherlands).

The selection of functional microbial strains, such as probiotics, should be based on scientific criteria which are associated with the specific health-claims. General criteria are: a long history of safe use, genetic stability, stable viability during storage and gastro-intestinal (GI) passage. The resistance of probiotic strains against the successive conditions in the GI tract, their functional properties and gene stability can be studied in dynamic models of the GI tract. The TNO gastro-Intestinal Models (TIM) are computer-controlled in vitro systems, closely simulating the conditions in the stomach and small intestines of humans (babies, adults) or animals (dogs, pigs, calves). During the passage of micro organisms through the GI tract, TIM continuously monitors and regulates: temperature, pH, gastric and intestinal mixing and transport by peristaltic movements, secretion of GI enzymes, electrolytes and bile, absorption of water and digested compounds. The influence of gastric emptying/pH, bile salt concentrations, food matrices on survival, interaction between functional/pathogenic bacteria, gene stability, and species and strain specific functions, can be studied in TIM. Various (validation) experiments of TIM with different functional, pathogenic and GM bacteria, showed that this dynamic model is a valuable tool for the selection of functional micro organisms, and the quality control of probiotic products. We give examples of studies on the fate of bacteria under various GI conditions, on the safety and functionality of probiotics, and on gene-stability studies.

Prebiotics and TNO's in vitro gastro-intestinal models. H.M.C. van Nuenen, K. Venema, M. Minekus, R. Havenaar (TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ Zeist, The Netherlands).

Many non-digestible oligosaccharides (NDOs) are considered as prebiotics, food ingredients which stimulate the growth of health promoting micro-organisms or their enzyme activities in the large intestine. In this manner NDOs contribute to general health, since a healthy microflora can improve the non-specific barrier and/or immune-defense systems in the GI tract or even participate in the prevention of cancer. The results of several in vivo studies focusing on the prebiotic effects of different oligosaccharides, are contradictory. The degree of polymerisation (DP) of the oligosaccharides investigated so far was usually low (DP 2–10). We think that the

influence of oligo/poly-saccharides with a higher DP on the intestinal microflora is worth investigating. TNO's gastro-intestinal models (TIM) are multi-compartmental, dynamic, computer-controlled models which mimic the successive kinetic events in the gastro-intestinal tract. We investigated the digestibility of a number of different oligo/poly-saccharides in TIM-1 (which simulates the stomach and small intestine). Based on these results, NDO polymers that reach the colon were selected and we studied their influence on the complex microbial ecosystems of human or animal origin in TIM-2 (which simulates the large intestine), with respect to fermentation and adaptation.

An in vitro test for prebiotics. B.A. Williams, W.-Y. Zhu, A. Akkermans, S. Tamminga (Wageningen University, The Netherlands).

Prebiotics are added to the diet, to selectively stimulate the growth of purported beneficial microbes. In vitro fermentation kinetics (cumulative gas production), was combined with DNA fingerprinting (DGGE/PCR), to detect changes in the microbial profile after fermentation of a single carbohydrate source. The modified cumulative gas production technique (Williams et al. 1995) using sugarbeet pulp (SBP) as a substrate, measured the kinetics of fermentation by the microflora of pig faeces. Samples were taken before and after the fermentation process, and a sequence analysis was performed using DGGE and PCR (Zoetendal et al. 1998). It became clear that several bands present before had disappeared after fermentation, while several had been enriched by SBP as an energy source. Cumulative gas production combined with VFA analysis indicated the type of fermentation which SBP could stimulate (likely to be fermented in the large intestine due to its slower rate of fermentation: favourable production of butyric acid). It is proposed that this combination of techniques could be used to test a range of prebiotics, and that the identification of the resulting enriched species could give an indication of which species would probably be favoured in the GIT.