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Session I:

Genetic diversity of gut bacteria

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O-1* **Novel groups of culturable bacteria are numerically abundant in the porcine gastrointestinal tract.** B.B. Jensen, L.L. Mikkelsen, O. Hojberg (Danish Institute of Agricultural Sciences, P.O. Box 50, 8830 Tjele, Denmark)

Despite fast elucidation of bacterial diversity by 16S rDNA cloning and sequencing, the functional characteristics of bacteria cannot be extrapolated from these data alone. Sequence databases, however, reveal the gaps in our culture collections and expedite the quest for important, yet uncultured groups of bacteria. In the present study ~2000 colonies have been isolated from porcine gastrointestinal tract lumen content and classified into so far ~120 species/operational taxonomic units (otus) on the basis of morphology, cytology, fermentation patterns and 16S rDNA sequences. Despite high diversity, 75% of the isolates were covered by eighteen otus (> 1% of isolates each) representing twelve genera. This could be due to selectivity of culturing, but is actually in good accordance with a comprehensive 16S rDNA clone library (Appl. Environ. Microbiol. 68 (2002) 673–690), where 23 of 375 defined otus (> 1% each) made up 52% of 4270 clones. Seven of our eighteen dominant otus showed less than 97% similarity to 16S rDNA sequences of identified species, however most isolates matched to porcine bacterial clone sequences. Clearly, some of the dominant clone library otus were not among our isolates. On the other hand, one of our dominant species (4% of colon isolates) belonging to the *Sporomusa* subgroup showed less than 97% similarity to any database sequence. The fermentation patterns revealed the latter as well as other unknown species as major producers of butyrate, lactate and formate. These metabolites are considered crucial factors in preventing pathogen invasion and development of colon cancer. To our knowledge, the present work is one of the most comprehensive studies on the culturable commensal microbiota of monogastrics and indicates that major groups of so far unidentified bacteria potentially involved in stabilising a healthy gut can actually be cultured and characterised.

* O- oral presentation, P- poster, IL- invited lecture

O-2 **Development of a microarray for structural analysis of pig gastrointestinal bacterial communities.** T.D. Leser, M. Boye, J. Z. Amenuvor, T.K. Jensen, K. Møller (Danish Veterinary Institute, Dept. of Bacteriology, Bülowsvej 27, 1790, Copenhagen V, Denmark)

375 phylotypes were identified previously from a 16S rDNA clone library from the gastrointestinal (GI) tract of pigs. These data provide an inventory of the phylotypes that can be found in the GI tract of Danish pigs, however the distribution and abundance of individual phylotypes in different compartments of the GI tract and among different animals cannot be deduced from this type of analysis. To furnish this kind of information a microarray of specific probes for those detected phylotypes is being developed. The probes are 17- to 20-mer DNA oligonucleotides targeting specific sequences of the 16S rRNA. The probes are spotted onto glass slides. Whenever possible, at least two probes are designed for each phylotype. For optimizing the procedure, a prototype array of 36 probes targeting 17 individual phylotypes was designed. These phylotypes represented various phylogenetic groups. Universal probes for the *Bacteria* and also non-sense probes were included in the array. To test the microarray, the lumen and the mucosa was sampled in the ileum and the top spiral colon of two pigs. RNA was extracted immediately from the fresh samples, labeled with CY3, and hybridized to the microarray. After scanning of the microarrays the fluorescent signals were normalized to the universal probe, EUB338, and the hybridization patterns compared between samples. The results show that the microarray can detect differences in bacterial community structure in the individual compartments of the GI tract and between animals. Some phylotypes were confined to specific niches in the GI tract, while others were globally distributed.

O-3 **Chicken gut bacteria: ecological studies and beyond.** J. Gong (Food Research Program, Agriculture & Agri-Food Canada, Guelph, Ontario, N1G 5C9, Canada)

Studies of gut bacteria are a prerequisite for the development of probiotics and their most effective use. The gut bacteria in adult broiler chickens were investigated by molecular analyses

of 16S rRNA genes, including DNA sequence, T-RFLP, and DGGE techniques. Bacteria in the cecal mucosa were highly diverse and predominantly Gram-positive with low % G + C. *Fusobacterium prausnitzii* and butyrate-producing bacteria comprised the largest groups among 116 cloned 16S rDNA sequences. Twenty five percent of the clones had less than 95% homology to database sequences. Many cloned sequences were related to those of uncultured bacteria identified in human feces or the bovine rumen. Bacteria in the ileal mucosa were also mainly Gram-positive with low % G + C. More than 70% of cloned 16S rDNA sequences (total of 51 clones) were lactobacilli and *Enterococcus cecorum*. Only two sequences had less than 95% homology to existing database sequences. Lactobacilli, *Enterococcus cecorum*, and butyrate-producing bacteria were the three major groups detected in the mucosa of both ilea and ceca. T-RFLP analysis revealed differences between bacterial populations present in the mucosa and lumen of the two gut regions. It also demonstrated a less diverse bacterial population in the ileum than in the cecum. The gut bacteria from free-range and in-house reared chickens were analysed using PCR-DGGE. Differences among the bacterial populations were also identified. The dissimilarity of these bacterial populations as well as between cecal/ileal bacteria is highlighted by this study. The potential of our findings for the development of novel probiotic bacteria is being explored.

O-4 **The effect of condensed tannins from *Acacia angustissima* on bacterial diversity and metabolic activity in the rat gastrointestinal tract.** A.H. Smith[a,b], R.I. Mackie[a] ([a] Department of Animal Sciences, University of Illinois, 1207 W. Gregory Drive, Urbana, IL 61801, USA; [b] Animal Nutrition and Animal Products Institute, Irene, South Africa)

Tannin-tolerant gastrointestinal bacteria may contribute to herbivores' ability to utilize tannin-containing diets. Using rats as a model and condensed tannins extracted from leaves of a potential fodder legume *Acacia angustissima* the effect of dietary tannins on the gastrointestinal bacteria was monitored. Tannin-tolerant bacteria in the feces of rats on the control diet comprised 1.6% (SE 4.3) of the total culturable bacteria. After three weeks on a low *A. angustissima* tannin diet (0.7%) the proportion of tannin-tolerant bacteria increased significantly ($P = 0.0242$) to 18.1% (SE 5.9) and increased to 32.3% (SE 3.7) ($P < 0.0001$) on a high tannin diet (2%). Within three days of returning to the control diet tannin-tolerant proportions returned to pre-exposure levels. Bacterial metabolic fingerprints of fecal samples revealed that functional activities of culturable bacteria were affected by the presence of tannins. A permanent shift in bacterial populations was indicated as metabolic fingerprints did not return to pre-exposure patterns. This was confirmed by molecular fingerprints of the fecal bacteria by Denaturing Gradient Gel Electrophoresis (DGGE) as the observed shift in bacterial populations due to tannins in the diet did not return to pre-exposure patterns over four weeks of sampling. There was less bacterial diversity in the groups fed tannin-containing diets based on the number of bands in the DGGE gels and by sequence analysis of predominant bands. Isolated tannin-tolerant bacteria belonged to the *Bacteroides* group or the Enterobacteriaceae and this corresponded with the bacterial sequences which predominated in animals on tannin diets determined using DGGE analysis.

O-5 **Development of oligonucleotide probes to detect and quantify the numbers of potential butyrate-producing bacteria present within the human faecal flora**. A. Schwiertz[a], G.L. Hold[b], M. Blaut[a], S.H. Duncan[b], H.J. Flint[b] ([a] DIfE, Potsdam, Germany; [b] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

16S rRNA-targeted oligonucleotide probes were designed for the detection of selected butyrate-producing bacteria isolated from human faeces. The targeted bacteria clustered within two bacterial groups, *Clostridium* clusters IV and XIVa, identified previously as comprising a significant proportion of the total microbial diversity within the human gastrointestinal tract. The specificity of the probes was tested against a range of common intestinal bacteria before being applied to faecal samples obtained from 10 healthy volunteers. The results showed that the *Roseburia intestinalis*-cluster, and the *F. prausnitzii*-group, accounted for up to 10.9% of the total bacteria flora. Species-specific probes were also designed for other butyrate-producing species, but these were shown to comprise only a minor part of the

total microflora. Taking into consideration that both the *Roseburia intestinalis*-cluster and the *F. prausnitzii*-group mainly consist of butyrogenic bacteria, the results indicate that significant portions of the human faecal microbiota are butyrogenic. Since, in addition, *Roseburia* spp. and *F. prausnitzii* were previously shown to produce large amounts of butyrate in vitro, relative to other isolates, these species are likely to make a significant contribution to butyrate formation in the large intestine.

P-1 **Comparison of DNA extraction and purification procedures for luminal samples from the swine gastrointestinal tract.** K.L. Anderson (United States Department of Agriculture, National Swine Research and Information Center, Ames, IA 50011, USA)

The swine gastrointestinal tract contains a large number of bacterial species that are not detected by standard cultivation procedures. Thus, non-culture methods must be employed. One such method, the polymerase chain reaction (PCR), enables the amplification and specific identification of small quantities of DNA from samples of digesta. This permits PCR to not only provide a means of detecting the presence of uncultured species of bacteria, but also of estimating their population size and distribution. However, the results obtained from PCR amplification are directly affected by the efficiency of extracting genomic DNA from the sample. This extraction efficiency can be affected by various factors, including incomplete cell lysis, DNA sorption to particulate material, and degradation or damage of the DNA. A comparative analysis of various extraction methods was achieved by obtaining luminal samples from both the cecum and colon of three pigs. Total DNA was extracted from these samples using 19 different DNA extraction methods. These methods consisted of contrasting physical, chemical, and enzymatic protocols. Of these 19 methods, four recovered a significantly greater ($P < 0.05$) quantity of total DNA from both cecum and colon samples than the other methods. In addition, using a universal primer for PCR amplification of eubacterial 16S rDNA, no PCR inhibitory agents were detected in DNA obtained by any of the four extraction methods. The results of this study provide a guideline for determining the most appropriate DNA extraction methods for luminal contents from the swine.

P-2 **Examination of changes in the gut microflora using DGGE and T-RFLP.** N. Bernbom, P. Saadbye, B. Nørrung (Institute for Food Safety and Nutrition, The Danish Veterinary and Food Administration, Copenhagen, Denmark)

Several investigations have shown that the microflora of the gastrointestinal (GI) affects the well being of humans. Different diets and the use of antibiotics and probiotics may affect the composition of the microflora in the intestinal tract. The use of bacteriocin-producing microorganisms as biopreservation for foods has become very popular especially in order to inhibit the growth of pathogenic microorganisms such as *Listeria monocytogenes*. Nothing is known, however, about the possibility that bacteriocin-producing lactic acid bacteria interfere with the normal microflora of the gastrointestinal tract. The purpose of this study was to examine the effect of bacteriocins and bacteriocin producing strains on the normal and pathogenic microflora of the GI tract. The human intestinal microflora is a very diverse ecosystem, which is estimated to harbour more than 400–500 different microbial species. Until now the primary methods to investigate the gut microflora have been culturing studies, but it has been shown that only a fraction of the microflora is actually culturable. Therefore new methods have been/are being developed, where the culturing step is being eliminated. To investigate the intestinal microflora, and later possible changes caused by ingestion of bacteriocin producing microorganisms, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) were chosen. These are both non-culturable methods to view complex microbial ecosystems. In both methods DNA is amplified by PCR followed by a separation in different electrophoresis systems. The DNA extraction and purification from faecal samples of rats have been performed using a mechanical method, a Bead beater, followed by purification steps using a kit (QIAamp). Results from DGGE and T-RFLP on rat faecal samples are presented.

P-3 **Influence of dietary changes on human colonic bacteria in an anaerobic fermentor system.** S.H. Duncan[a], K.P. Scott[a], A.G. Ramsay[a], C.S. Stewart[a], H.J.M. Harmsen[b], G.W.

Welling[b], H.J. Flint[a] ([a] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK; [b] Department Medical Microbiology, University of Groningen, Groningen, The Netherlands)

Bowel disorders are a major cause of health problems in North America and Northern Europe. Dietary intervention can help to prevent or alleviate disease, but the influence of dietary changes on the colonic microflora and their metabolism is poorly understood. Here the predominant bacterial groups that inhabit the large intestine were monitored in response to dietary shifts within anaerobic fermentors using fluorescent in situ hybridisation (FISH) probes. The duplicate fermentor systems gave remarkably reproducible results. FISH analysis indicated that some groups of bacteria e.g. *Fusobacterium prausnitzii* survived poorly in the fermentors when compared to their abundance in faecal samples, while *Bacteroides* spp. came to predominate. Feeding inulin to the fermentors resulted in a marked increase in the abundance of some groups of bacteria including the *Ruminococci* and *Eubacterium cylindroides* groups. The fate of three marked bacterial strains belonging to the cluster XIVa *Clostridium* subphylum, namely *Roseburia intestinalis* L1-82, *Roseburia* sp. A2-183 and *Eubacterium* sp. A2-194 was also monitored following inoculation into the fermentors. The three introduced strains exhibited markedly different behaviour in the complex fermentor ecosystem although all three strains were able to survive in the presence of the faecal microflora. Inulin enhanced the growth of *Eubacterium* sp. A2-194. This suggests that inulin which escapes digestion in the small intestine is likely to promote a variety of colonic bacteria.

P-4 **Use of odd-chain fatty acid profiles to study microbial colonisation of boli from freshly ingested herbages.** E.J. Kim, J.K.S. Tweed, R.J. Dewhurst, R.J. Merry, D.R. Davies, M.K. Theodorou (Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion, SY23 3EB, UK)

The objective of this study was to use fatty acid profiling to examine microbial colonisation of boli (masticated herbage) from contrasting fresh forages (grass or white clover). The boli were collected following hand-feeding of forage to cows after rumen emptying. Approximately 3 kg of rumen boli were collected from each animal and 150g portions were weighed immediately into pre-weighed dacron bags and incubated in the rumen for 0, 1, 2, 4, 8 and 24 hours. After removal from the rumen, bags were gently rinsed in a sink of cold water, freeze-dried and weighed for dry matter disappearance. Fatty acid methyl esters were extracted and determined by GC using tricosanoic acid as an internal standard. Two odd-chain fatty acids (*iso* C15:0 and *iso* C17:0) were not detected in grass or white clover from pasture, but are found in washed bacterial preparations from the rumen, indicating that they could be used as microbial markers. The concentration of both these fatty acids in harvested boli of both forages increased with incubation time. The relationship between the concentration of *iso* C15:0 ($mg \cdot kg^{-1}$ DM) and DM disappearance ($g \cdot g^{-1}$ DM) was described by linear regression: for the grass: $Y = 317.3X - 104.5$ ($r^2 = 86.9$, s.e. 19.8, $P < 0.01$); and for white clover: $Y = 96.1X - 13.2$ ($r^2 = 66.4$, s.e. 18.0, $P < 0.01$), respectively. The results clearly demonstrate rapid and continued microbial colonisation of both grass and white clover. However, the diversity of the developing microbial communities in residues will remain unclear until molecular analysis is completed.

P-5 **Bacterial translocation in weaning piglets analysed by 16S rDNA based approaches.** S.R. Konstantinov[a], T.D. Leser[c], W.M. Akkermans-van Vliet[a], B.A. Williams[b], W.M. de Vos[a], A.D.L. Akkermans[a] ([a] Wageningen University, Laboratory of Microbiology, 6703 CT Wageningen, The Netherlands; [b] Wageningen Institute of Animal Sciences, Wageningen University, The Netherlands; [c] Danish Veterinary Institute, 1700 Copenhagen V, Denmark)

Translocation is a process of active or passive passage of bacteria across the epithelial barrier from the intestinal lumen to extra intestinal sites such as the mesenteric lymph nodes and other organs of the host. The present study was initiated to assess bacterial translocation from the gastrointestinal tract into mesenteric lymph nodes of weaning piglets. Bacteria were detected and identified based on PCR amplification of their 16S rDNA and molecular profiling techniques

such as terminal-restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE). DGGE and T-RFLP fingerprints of the bacterial communities in the mesenteric lymph nodes (MLN), jejunum, ileum, caecum and colon of six weaning piglets were screened for amplicons common in all samples. T-RFLP analysis of MLN revealed that the number of terminal restriction fragments (TRs) differed among the piglets, and only a few TRs were found in MLN and GI tract of all piglets. In comparison to T-RFLP, DGGE analysis detected approximately two times lower numbers of 16S rDNA amplicons of the MLN and GI tract samples. These results demonstrate high bacterial diversity in the MLN and reveal that specific parts of the microbiota in the gastrointestinal tract can be translocated from the intestine of weaning piglets.

P-6 **Taxonomy and role of *Butyrivibrio* and *Pseudobutyrivibrio* species in microbial ecosystem from the digestive tract of ruminants**. J. Kopečný[a], R. Marinšek-Logar[b] ([a] Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Prague 10, Uhříněves, Czech Republic; [b] University of Ljubljana, Biotechnical Faculty, Domžale, Slovenia)

Butyrivibria are a major component of the ruminal microflora and have been isolated from the gastrointestinal tracts of various animals. They contribute to fiber digestion through degradation of plant hemicellulose, cellulose, starch and protein. Currently are recognized two butyrivibrio species: *Butyrivibrio fibrisolvens* and *Butyrivibrio crossotus*. Genetically closely related species are *Pseudobutyrivibrio ruminis* and *Clostridium proteoclasticum*. We have tested 62 "butyrivibrio" isolates from the rumen of the cow and sheep. There were estimated fermentation products, morphology, substrate utilization, enzyme production, composition of cellular fatty acids, RFLP of 16S DNA, DNA-DNA hybridization and 16S rDNA sequences. Obtained data enabled us to describe two new species: *Butyrivibrio hungatei* and *Pseudobutyrivibrio xylanovorans*, which were the most common species in our set of isolates. All bacteria tested were clustered into two genetically related groups: Butyrivibrio group included *Butyrivibrio fibrisolvens*, *Clostridium proteoclasticum* and *Butyrivibrio hungatei* isolates. Pseudobutyrivibrio group consisted of species *Pseudobutyrivibrio ruminis*, *Butyrivibrio crossotus* and *Pseudobutyrivibrio xylanovorans*. Pseudobutyrivibrio group included another cluster of bacteria isolated mainly from the rumen fluid of deer and sheep, which is not taxonomically defined. Both Pseudobutyrivibrio and Butyrivibrio group can be distinguished with specific PCR primers. *Pseudobutyrivibrio xylanovorans* isolates showed the highest ability to degrade plant polysaccharides. Beside that, they were producing, together with isolates of *Clostridium proteoclasticum*, the highest proteinase activity. All other species tested were utilizing metabolic intermediates of fiber degradation only. (The project – 524/99/0602 – was supported by The Czech Grant Agency).

P-7 **Molecular ecology of *Oscillospira guillermondii* – a large, morphologically conspicuous, but uncultured rumen bacterium.** R.I. Mackie[a], R.I. Aminov[a], W. Hu[a], M.A. Olsen[a,b], A.V. Klieve[c], Y. Kamagata[d] ([a] Department of Animal Sciences, University of Illinois, Urbana, IL 61801, USA; [b] Department of Arctic Biology and Institute of Medical Biology, University of Tromso, 9037, Tromso, Norway; [c] Queensland Beef Industry Institute, Queensland Department of Primary Industries, Moorooka, Australia; [d] National Institute of BioScience and Human Technology, Agency of Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan)

Detection and identification of microbial populations are the most basic prerequisites for microbial ecology studies. Although *Oscillospira* have been observed microscopically, based on their large size and conspicuous morphology, for over 90 years, they remain uncultured and their phylogeny unknown. Based on PCR-retrieved 16S rDNA sequences of *Oscillospira* spp., we have developed PCR, PCR-DGGE and FISH techniques for cultivation-independent monitoring of *Oscillospira* spp. to estimate the occurrence of this bacterium in different ruminant species, during diet shifts in cattle, and to evaluate the level of its genetic diversity. *Oscillospira*-specific sequences were detected in a broad range of geographically distant ruminant species: North American domestic cattle, sheep from Australia and Japan, and Norwegian reindeer. Phylogenetic analysis of the sequences obtained enabled

us to define three operational taxonomic units (OTUs) within the *Oscillospira* assemblage. Consistent with this genetic diversity, we observed other, atypical morphotypes of *Oscillospira*, detected with the use of the *Oscillospira*-specific FISH probe. Despite the visual disappearance of typical *Oscillospira* morphotypes during the switch from green pasture to indoor housing, its presence was still detected by *Oscillospira*-specific PCR. Together with their detection in geographically distant ruminant species fed a range of diets, these observations suggest the ubiqitous presence of *Oscillospira* species in various rumen ecosystems with the numbers, and types (possibly species) responding to diet and geographic location.

P-8 **Development of a competitive PCR for detection and enumeration of *Butyrivibrio* and *Pseudobutyrivibrio* strains in the rumen ecosystem.** J. Mrázek[a], J. Kopečný[a], G. Avguštin[b] ([a] Institute of Animal Physiology and Genetics, Prague 10, Czech Republic; [b] University of Ljubljana, Domžale, Slovenia)

Motile and butyrate-producing anaerobic bacteria in the rumen are represented mainly by *Butyrivibrio* and *Pseudobutyrivibrio* strains. They play an important role in the degradation of proteins as well as structural and storage plant polysaccharides. Recent studies based on 16S rDNA sequential analysis showed that those species are clustered into six distinct groups. The aim of this project was to develop a competitive PCR, which could easily distinguish *Butyrivibrio* and *Pseudobutyrivibrio* strains and quantify them in environmental samples. Twenty strains of butyrivibria were divided into two main groups on the basis of 16S rDNA sequences comparison. The primers specific for both groups were designed and tested on 50 strains of butyrivibria and pseudobutyrivibria. The highest specificity for butyrivibria was found in case of 71f primer (5- CGG AGA ATT TAC GCT GAT GAA G –3), and for pseudobutyrivibria was the most suitable primer F2 (5- AAT TTT CTA CGA TCC CTT CGG GG –3). The internal controls for cPCR systems for both groups were prepared using a "double primer method". Standard curves were constructed using a known number of cells from a pure culture of butyrivibria (counted by flow cytometer) and a serial dilution of internal control. The Agilent bioanalyzer 2100 was used for the analysis of cPCR products. The standard curves can be used for enumeration of the number of cells of butyrivibria in the range from 4.1×10^7 to 1×10^6 cells per mL and for pseudobutyrivibria in the range from 6.1×10^8 to 6.1×10^5 cells per mL of rumen fluid.

P-9 **An optimised RNA extraction protocol for rumen samples.** S. Muetzel, K. Becker (University of Hohenheim, Inst. for Animal Production in the Tropics and Subtropics, Dept. for Animal Nutrition and Aquaculture, 70593 Stuttgart, Germany)

For the analysis of complex microbial ecosystems quantitative recovery of nucleic acids is a prerequisite. Contamination of the extracted nucleic acids with carbohydrates, protein or polyphenols and the lysis of recalcitrant organisms are the two main problems during nucleic acid extraction. RNA recovery from rumen fluid samples was compared using two RNA extraction methods. The method that yielded higher group specific RNA concentrations was further modified to allow extraction of RNA in the presence of tannins. Furthermore the lysis protocol based on bead mill disruption was optimised in order to recover a maximum of RNA from recalcitrant and fragile organisms from rumen fluid samples. Quantification of the RNA was done by densitometry from polyacrylamide and agarose gels in the study of RNA-tannin interactions and by membrane hybridisation with group specific RNA probes targeting total rRNA, Bacteria, Archaea, Eukarya, *Ruminococcus flavefaciens* and the genus *Fibrobacter* for the lysis procedure study. The RNA extraction procedure presented is a rapid, inexpensive method with good reproducibility, yielding high amounts of RNA suitable for 16S rRNA targeted membrane hybridisation.

P-10 **Culture independent molecular analysis of the elderly faecal microflora reveals an extreme complexity.** K. Saunier[a], K. Tuohy[b], M. Sutren[a], A. Cresci[c], J. Doré[a] ([a] Institut National de la Recherche Agronomique, UEPSD, 78350 Jouy-en-Josas, France; [b] Unit of Food

Microbiological Studies, University of Reading, Reading RG6 6BZ, UK; [c] Dipartimento di Scienze Morfologiche e Biochimiche Comparate, Università degli Studi di Camerino, 62032 Camerino, Italy)

Understanding age-related alterations in the gastrointestinal tract is important in the design of preventive nutrition strategies. The elderly fraction of the population is currently rising in Western societies, and yet specificities of its gut microbiota, involved in health, remain largely unknown. The present study was conducted to evaluate the bacterial diversity of the dominant faecal microbiota of elderly. We used comparative sequencing of 1260 cloned 16S rDNA from faecal DNA of 9 healthy elderly persons (6 women- and 3 men, aged 69–87, mean 78). By BLAST, we identified 21 RDP-defined phylogenetic groups. Among them, the 6 groups commonly found as dominant in adults: *C.leptum* (38.8% of cloned sequences on average; prevalence 9/9), *C.coccoides* (25.7%; 9/9), *Bacteroides* (18.1%; 9/9), *Enteric* (2.4%; 7/9), *Lactobacilli* (1.7%; 5/9) and *Bifidobacterium* (1.4%; 7/9) constituted an important part of the elderly microbiota (88.1%). We further observed other dominant groups: *Sporomusa* (1.6%; 7/9), *Acholeplasma-Anaeroplasma* (1.3%; 9/9), and *Atopobium* (0.9%; 7/9) as well as an important part of unaffiliated bacteria (5.9%). Other remaining groups were less represented with a weaker prevalence. Finally, the number of novel sequences (< 2.5% identity with GENBANK sequences) was 60% on average. These results reveal an extremely high complexity of the elderly microbiota. Hence, to better appreciate the composition and dynamics of the elderly gut microbiota, new probes should be defined for hybridisation-based analysis.

P-11 **Physiological affiliations of *Dorea longicatena* gen. nov., sp. nov. in gastrointestinal ecology.** D. Taras[a], M. Blaut[b] ([a] Free University of Berlin, Institute for Animal Nutrition, 14195 Berlin, Germany; [b] German Institute of Human Nutrition, Department of Gastrointestinal Microbiology, 14558 Bergholz-Rehbrücke, Germany)

In recent years the investigation of the bacterial diversity in the gastrointestinal tract using molecular tools has attracted major interest and led to sequencing and phylogenetic classification of several novel species. But due to difficulties in isolation and cultivation of these organisms the biochemical characterization has often remained undone, which hinders the complete understanding of their physiological function in the human gut. We isolated two strains of a Gram-positive, strictly anoxic, non-sporeforming, rod-shaped bacterium from human faeces. Based on phenotypic and phylogenetic considerations we proposed that the hitherto unknown rod-shaped bacterium together with *Eubacterium ormicigenerans* (6% sequence divergence) be classified in a new genus *Dorea*, as *Dorea longicatena* gen. nov., sp. nov. and *Dorea formicigenerans* comb. nov., respectively. Experiments with a specific 16S rRNA directed oligonucleotide indicated that *D. longicatena* is present in all human volunteers studied so far at cell counts of approximately 10^9 per gram of dry weight faeces. On average *D. longicatena* represented 0.96% of the total faecal flora and, at least for some individuals studied, is a member of the numerically dominant gut microflora. The major products of hexose fermentation are ethanol, acetate, formate and hydrogen. In mixed cultures with *C. coccoides*, which requires formate and hydrogen while growing on CO_2, an interspecies hydrogen and formate transfer was demonstrated, which led to a metabolic shift in *D. longicatena*. The conclusion is tempting that this mutually beneficial interaction between *D. longicatena* and *C. coccoides* as well as with other equally common acetogens growing on H_2/CO_2 occurs also in the human gut.

P-12 **Isolation and characterization of ammonia-hyperproducing bacteria from stored swine manure.** T.R. Whitehead, M.A. Cotta (USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL 61604, USA)

Storage of swine manure is associated with the microbiological production of a variety of odorous compounds, including ammonia, organic acids and alcohols, and sulfides. These compounds can contribute to health problems for swine facility workers and animals, as well as odors that affect local human populations. Previous research in our laboratories has demonstrated that the microbial populations in swine manure stored in deep pits are composed primarily of low % G + C, Gram-positive anaerobic

bacteria. Examinations of predominant isolates from this ecosystem found that little ammonia was produced by these bacteria. Therefore, a selective medium containing 1% tryptone/1% casamino acids (and no carbohydrates) was used to isolate bacteria capable of growth on these compounds. Isolates were obtained from stored manure that were capable of growth on the selective medium and produced large amounts of ammonia, ranging from 20 mM to 80 mM, from 1% tryptone/1% casamino acids. These levels are similar to those found with ammonia-hyperproducing bacteria isolated from the rumen of cattle. Identification of the bacterial isolates by 16S rDNA gene sequencing indicated that the majority were low % G + C, Gram-positive bacteria. Several of the isolates were presumptively identified as *Clostridium* or *Peptostreptococcus* species, while others could not be identified. A number of isolates were capable of growth on individual and combinations of amino acids, and produced high levels of ammonia. Growth of all of the Gram-positive isolates was inhibited by addition of the ionophore monensin. These results suggest that production of ammonia during storage of manure may be due to the presence of ammonia-hyperproducing bacteria, and that ammonia production may be reduced by the addition of monensin to the manure.

Session II:

Manipulation of microbial activities

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O-6 **The bacterial community in the ileum of broiler chickens at various ages is influenced by dietary fat source and subtherapeutic levels of antibiotics.** A. Knarreborg[a], M.A. Simon[b], R. Engberg[a], B.B. Jensen[a], G.W. Tannock[b] ([a] Research Centre Foulum, 8830 Tjele, Denmark; [b] Department of Microbiology, University of Otago, Dunedin, New Zealand)

The ecosystem in the small intestine of young broilers can easily be disturbed and can be manipulated by diet composition and subtherapeutic levels of antibiotics. In particular, the carbohydrate fraction of the diet has been intensively studied, whereas little is known about the role of dietary fat in relation to avian gut ecology. The effect of dietary fat source (soy oil or a mixture of lard and tallow) and antibiotic supplementation (a combination of avilamycin; 10 mg·kg·feed^{-1} and salinomycin; 40 mg·kg·feed^{-1}) on the bacterial community in the ileum of broiler chickens at different ages (7-, 14-, 21-, 35 days) was studied using PCR-denaturing gradient gel electrophoresis (DGGE) analysis in combination with bacteriological culture. The DGGE profiles of the total bacterial community (HDA-primers) and DGGE profiles using primers specific for lactic acid bacteria (Lac-primers) and *Clostridium perfringens* (Cpa-primers) showed that the composition of the microflora was age-dependent and influenced by fat source and antibiotic treatment. An increased detection of streptococci and *Clostridium perfringens* with age of the chickens was demonstrated. Lactobacilli predominated in the ileum of chickens and the composition of the *Lactobacillus* population was altered in response to age and diet. *Lactobacillus salivarius* and *Clostridium perfringens* were the bacterial groups most affected by the diets. Further, different strains of *Clostridium perfringens* type A were detected in relation to age and diet. The results of bacteriological culture and DGGE confirmed and complemented each other, hence, detailed microbial ecological information was obtained.

O-7 **Effect of a live yeast culture on cellulolytic activities of liquid and solid associated microorganisms in the equine hindgut ecosystem.** B. Medina[a], E. Jacotot[b], G. Bertin[a], V. Julliand[b] ([a] Alltech France, 95190 Goussainville, France; [b] ENESAD, 21079 Dijon, France)

Depending on the forage to grain ratio in equine diets, the addition of *Saccharomyces cerevisiae* (SC) is correlated with different effects on the apparent fiber digestibility. The aim of our study was to investigate the effect of SC (Yea Sacc®1026) on intestinal fibrolytic activities and bacterial communities in relation to the composition of the diet. Four mature geldings were fed a high fiber (HF) or a high starch (HS) diet daily, supplemented with 0 or 10 g (5×10^9 CFU·g^{-1}) of SC. Bacterial communities and live yeast cells were enumerated from cecal and right ventral colonic contents, collected 4 hours after feeding. Polysaccharidase and glycosidase activities were determined on liquid associated microbial (LAM) and solid adherent microbial (SAM) populations by measuring the release of reducing sugars from polysaccharide purified substrates or the release of *p*-nitrophenol from glucoside specific substrates, respectively. The counts of cellulolytics in the hindgut were not changed with SC. Polysaccharidase activities were significant in LAM compared to SAM. We observed no diet or SC effect on the LAM activities. SAM glycosidase activities decreased with the HS diet compared to the HF. SAM specific activity of CMCase, β-D-cellobiosidase, β-D-glucosidase, α-L-arabinosidase and β-D-xylosidase increased with SC in the hindgut. Quantitatively the stimulating effect was higher on cellulolytic than on hemicellulolytic activities. The specific activity of β-D-cellobiosidase and β-D-xylosidase was higher in the cecum than in the colon with YS. This was correlated with a higher count of SC in the cecum (4×10^6 CFU·mL^{-1}) than in the colon (5×10^4 CFU·mL^{-1}) of horses supplemented with SC.

O-8 **Natural immune responses in sheep to methanogenic archaea.** P.E. Holloway[a,b], S.K. Baker[a] ([a] CSIRO Livestock Industries, Floreat Park Research Laboratory, Private bag 5, Wembley, 6913, Western Australia; [b] Current address: University of Tasmania, Sandy Bay, 7004, Australia)

In foetal ruminants and immediately post-partum, immunoglobulins (IgG) which bind to individual rumen eubacteria are undetectable, but titres of these IgG increase as functional rumen eubacterial populations develop until as mature ruminants there are detectable (but low) titres of

plasma IgG binding to individual eubacteria that are found normally in the rumen. Methanogenic archaea also are found normally in the rumen. Methanogenic archaea are antigenetically distinct from eubacteria, and in this study titres (mL^{-1}) of plasma IgG binding to individual species and strains of methanogenic archaea were determined using an enzyme-linked immunosorbent assay. Titres of plasma IgG were expressed as titres greater than those found in foetal lamb plasma, which contains no detectable IgG to methanogens. Plasma samples from ten two-year-old Merino wether sheep were used. Detectable titres of IgG (between 10^1 and 10^4 mL^{-1}) were found in plasma of all of the sheep in this study, binding to *Methanobrevibacter smithii* strain PS, *Methanobrevibacter ruminantium* strain M-1, *Methanobrevibacter sp.* strain ZA-10, *Methanobrevibacter arboriphilicus* strain DH-1, *Methanobacterium formicicum* strain MF, *Methanosarcina barkeri* strain MS, *Methanomicrobium mobile* strain BP, *Methanosphaera stadtmanae* strain MCB3, *Methanoculleus bourgensis* strain MS2, *Methanococcus vannielii* strain SB, *Methanospirillum hungatei* strain JF-1, and *Methanofollis (Methanogenium) liminatans* strain BM1. Many of the methanogens that were used in this study have not been identified as rumen methanogens. This indicates the ubiquity of methanogens in the environment and/or a commonality of some surface antigenic components amongst the methanogenic archaea.

O-9 **Bacterial flagellin, but not LPS, is required for the induction of human beta-defensin 2 in human intestinal and gastric epithelial cells.** D.A. O'Neil, S. Conway, G. Grant, K.E. Garden, E.T. Logan, D. Wilson, D. Kelly (Division of Gut Microbiology and Immunology, The Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

In the present study, we address the role of two key membrane components of enteropathogenic bacteria, namely LPS and flagellin, in the induction of enteric human beta-defensin (HBD)2. HBD2, an endogenous antimicrobial peptide of the gastrointestinal tract, is a central component of innate host defence and, by its chemotactic activities, acts as a biological bridge between the innate and adaptive phase of the immune response. We demonstrate a low level expression of human Toll-like receptor (TLR)4 and TLR5 in Caco-2 human intestinal and AGS gastric epithelial cells, also the TLR-associated adapter proteins, MyD88 and Tirap and the LPS receptor, CD14. Parallel to the rapid induction of HBD2 expression in Caco-2 and AGS cells, levels of TLR4, TLR5, MyD88 and Tirap mRNA were downregulated in response to challenge with *Salmonella enteritidis*, suggesting the involvement of either, or even both, of these receptors in the HBD2-mediated response to *Salmonella* challenge. In order to assess the influence of LPS and flagellin, the proposed ligands for TLR4/CD14 and TLR5 respectively, on HBD2 induction, we challenged Caco-2 and AGS cells with a panel of wild type (Fla+), aflagellate (Fla–) and flagellin-mutant (Fla-m) *S. enteritidis*. HBD2 induction was only observed in cells challenged with Fla+ and Fla-m bacteria, but not Fla– strains. Stimulation with ultrapure Salmonella-LPS also failed to induce HBD2 expression in Caco-2 and AGS and Gram-positive Listeria strains induced HBD2 in these cells to the same extent as Salmonella. This points to flagellin, not LPS, as the key factor in regulating enteric HBD2 expression. Further, bacterial-cell contact, not invasion, is the governing factor in HBD2 induction, as live and immobilised Fla+ *S. enteriditis* induced HBD2 to the same extent. Finally, while flagellin governs HBD2 induction, it does not affect the microbicidal activity of HBD2 as Fla+ and Fla– *S. enteritidis* were shown to be equally susceptible to HBD2-mediated killing.

O-10 **Bifidobacteria upregulate Toll-like receptor 3 mRNA levels in epithelial cells.** E. Furrie, S. Macfarlane, G.T. Macfarlane (MRC Microbiology and Gut Biology Group, University of Dundee, Dundee, UK)

Toll-like receptors (TLR) play a key role in recognition of bacteria and yeast in the innate immune response. TLR comprise a group of at least ten receptors that have different expression patterns in immune and non-immune cells. They are responsible for binding of bacterial surface antigens, and subsequent signalling to the cell that results in expression of inflammatory mediators. Their ligands are currently unknown, but bacterial products such as LPS, peptidoglycan and lipoteichoic acids may be involved. Detection of mRNA for TLR 1,3,4,5,7,9 and 10 can be observed on both HT29 and Caco-2 colonic

epithelial cell lines. We have examined the effects of mucosal bacterial isolates on the synthesis of TLR mRNA using real time PCR. RNA was standardised by cell number and beta-actin/GADPH levels of expression, and the products were cloned and sequenced to check specificity. TLR-1 mRNA in HT29 and Caco-2 cells was unaffected by Gram negative or Gram positive bacteria. TLR-3 mRNA was unchanged when untreated cells were compared to those treated with Gram negative species such as *E. coli* or *Gemminger formicilis*. In contrast, *Bifidobacterium adolescentis* and *Bifidobacterium angulatum* strains upregulated TLR-3 mRNA synthesis compared to the controls, as did *Enterococcus faecalis* and *Peptostreptococcus anaerobius*. However, individual isolates exhibited great variablity in their ability to induce expression of TLR-3. TLR-3 expression is upregulated in colonic epithelial cells when they encounter the surface of Gram-positive bacteria, which may be important in epithelial cell bacterial interactions on the mucosal surface.

P-13 **Caecal microflora of growing-rabbits affected by a non specific enteropathy**. N. Bennegadi[a], G. Fonty[b], L. Millet[b], T. Gidenne[a], D. Licois[c] ([a] INRA, CR de Toulouse, 31326 Castanet-Tolosan, France; [b] INRA, CR de Clermont-Fd/Theix, 63122 Saint-Genès-Champanelle, France; [c] INRA, CR de Tours, 37380 Nouzilly, France)

Enteropathology, which mainly occurs in young rabbits after weaning, is the major problem encountered in rabbit breeding. Diarrhoea is the most common clinical symptom of the sick animals. These enteropathies which increase morbidity and mortality rates can be caused by a specific pathogenic agent but often no clear pathogenic origin is detected. The ingestion of a low-fibre diet increases the incidence of these non specific enteropathies. In the present study, we compared the balance of some microbial populations in the caecum of rabbits presenting diarrhoeic symptoms to that observed in healthy animals. One group of rabbits was fed with a standard ("S") diet (Acid Detergent Fibre: 19%) and the second received a fibre-deficient diet ("D") (ADF: 9%). Detection and quantification of microbial populations were performed by dot-blot hybridization with oligonucleotidic probes. The sanitary risk (mortality + morbidity rates) was significantly higher ($P < 0.05$) with the "D" diet than with the "S" diet (59% versus 41%). The enteropathies appeared mainly between day 43 and d 56. Compared to healthy rabbits, the microbial community of the fresh caecal content of diarrhoeic animals was characterized by a marked decrease of archaeal community and *Ruminococcus* sp. population sizes, a strong increase of eucaryal community and *Fibrobacter* sp. population sizes. The size of the population of *Bacteroides – Porphyromonas- Prevotella* group was the same in healthy and sick rabbits. No *Escherichia coli* and anaerobic fungi were detected. These results show that the caecal microflora of growing rabbits suffering of a non specific enteropathy is greatly altered.

P-14 **Feed structure and processing and addition of formic acid to the feed influences the microbial ecology of the gastrointestinal tract of 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, Denmark)

In the search for alternatives to antibiotic growth promoters, coarse feed or feed added formic acid have been reported to decrease enterobacteria, such as Salmonella and coliform bacteria, in the gastrointestinal tract (GIT) of pigs. A study was performed to investigate the effect of feed structure and addition of formic acid to the feed on the microbial ecology of the GIT of pigs at various times post-feeding. The experimental diets were: fine, heated and pelleted (Fine); fine, heated and pelleted added 1.8% formic acid (Fine + 1.8%); coarse, non-heated and non-pelleted (Coarse). Addition of 1.8% formic acid to the diet resulted in lower counts of lactic acid bacteria, enterobacteria and yeasts along the GIT of the pigs, whereas the effect on the counts of total anaerobes in the caecum and colon was smaller. Feeding diet 'Coarse' stimulated the growth of lactic acid bacteria in the stomach and the small intestine, tended to reduce the counts of enterobacteria along the GIT and tended to increase the counts of yeasts compared to diet 'Fine'. The concentration of formic acid was highest in the stomach and the small intestine of the pigs fed diet 'Fine + 1.8%', whereas the concentration of lactic acid was highest in the stomach and proximal small intestine of the pigs fed

diet 'Coarse'. Microbial activity (ATP) and in vitro production of organic acids in digesta from various sites of the GIT were also measured. In conclusion, changes in feed structure or processing, and addition of formic acid to the feed affect the microbial ecology of the GIT of pigs.

P-15 **Survival of microorganisms after exposure to organic acids.** N. Canibe, B.B. Jensen (Danish Institute of Agricultural Sciences, Department of Animal Nutrition and Physiology, Research Centre Foulum, P.O. Box 50, 8830, Denmark)

The new situation in pig farming, devoid of antibiotic growth promoters, has lead to the search of alternative feeding strategies. Addition of organic acids to the feed or water is among the alternatives to antibiotic growth promoters. However, adaptation of microorganisms of the gastrointestinal tract to acidic environments has become a concern. An initial experiment was designed to study whether addition of organic acids to piglet diets increases the acid resistance of microorganisms of the gastrointestinal tract. We fed weaners (six per diet) four diets: a standard weaner diet with no added organic acids, the same diet added 1% K-diformate, 1% benzoic acid, or 1% sorbic acid. The animals received the diets during six weeks, after which they were slaughtered. Digesta from the distal third of the small intestine was collected, and incubated at 38 °C during one hour at pH 3 without addition of acids, at pH 4 without and with addition of 20 mM formic acid, benzoic acid or sorbic acid, and at pH 5 without and with addition of 20 or 40 mM of the same acids. The initial levels of microorganisms in the ileal digesta were different for the four diets, which reflects the antimicrobial effect of the various acids. At pH 3, the survival of lactic acid bacteria and enterobacteria was 1–11%. pH 4 resulted in survival of 27–58% of lactic acid bacteria and of 38–160% of enterobacteria, and pH 5 of 87–138% lactic acid bacteria and 210–271% enterobacteria. Addition of acids decreased survival of the microorganisms. The data did not point to higher survival of the microorganisms collected from animals fed diets added 1% organic acids compared to those fed diet with no added acids.

P-16 **Functional cell model of non-tumorigenic intestinal epithelial cells IPEC–J2 to study probiotic-pathogen-gut epithelium interactions.** A. Cencič[a], M. Jakobsen[b] ([a] University of Maribor, Faculty of Agriculture, Vrbanska c. 30, 2000 Maribor, Slovenia; [b] Royal Veterinary and Agricultural University, Dept. of Dairy and Food science, Rolighedsvej 30, 1958 Frederiksberg C, Denmark)

The Caco-2 cell line (human colon adenocarcinoma) has been widely used in studies of probiotic-pathogen-gut epithelium interactions. Although it has been shown that Caco-2 cells exhibit some phenotypic features similar to those of normal intestinal epithelial cells, they have mainly tumorigenic phenotypes distinguished from the normal gut epithelia by modified surface glycoconjugates and physiological responses. Therefore the purpose of our work was to use established normal pig intestinal epithelial cells (obtained from Prof. A. Blikslager, North Carolina University, USA) that were growing on microporous inserts, where epithelial cells develop characteristic epithelial morphology and a functional polarity, as is the case in vivo. A comparison between primary pig intestinal cells and Caco-2 was made. Like primary porcine intestinal epithelial cells, IPEC-J2 cells express alkaline phosphatase in a polarised manner, towards the apical compartment. Positive staining in Schiff-periodic acid assay indicated apical expression of mucins. As the main target for microorganisms to attach are glycoconjugates located at the apical surface of the gut epithelial cells, a surface glycoconjugate analysis was made. Results obtained using selected lectins showed a similar pattern as in the primary porcine enterocytes. The probiotic bacterium *Lactobacillus reuteri*, originally isolated from pig intestine, was used in attachment assays and showed surface binding of approximate 20%. Moreover, upon treatment with porcine interferon gamma (PoIFN-γ), an inflammatory cytokine, and a significant apical expression of MHC class II antigens was observed, indicating an immunomodulatory response of this intestinal epithelial cell line.

P-17 ***Butyrivibrio* sp. strain Mz5 – a possible probiotic candidate for monogastric animals.** T. Cepeljnik, M. Zorec, F.V. Nekrep, R. Marinšek-Logar (Zootechnical Department, BF, University of Ljubljana, Groblje 3, SI – 1230 Domžale, Slovenia)

The beginning of the third millennium sets new challenges in animal science. Growth promoters are being replaced by new biologically active substances. Among these are enzymes and organic acids which can mediate effects of probiotic bacteria as is the case with some lactic acid bacteria, and perhaps other less-studied bacterial groups. We have isolated many butyrate producing bacteria from the rumen of Holstein-Friesian cow on the xylan containing medium. The highest xylanolytic activity was detected in isolate Mz5 (three endoxylanases of 58, 51 and 26.7 kDa were partially isolated and characterized). Several independent studies have revealed its classification in the genus *Butyrivibrio* where species delineation is being intensively reestimated. This isolate has some exquisite properties which favour its application as a probiotic. We revealed its relative low sensitivity to oxygen; very good growth and xylan hydrolysis was measured even at redox potential above –100 mV. The advantages of its effective and pH stable xylanolytic system can be used in xylan digestion that results in xylooligosaccharides that promote growth of other beneficial microflora. Among other products of its metabolism are the most notable high levels of butyrate, which is preferential energetic source for the gut cells and conjugated linoleic acid that acts as antioxidative, antimutagenic and anticarcinogenic substance. Bacteriocin production resulted in the growth inhibition of some rumen bacteria and is a future target of our research, as are adhesion and anticarcinogenic effects upon Caco-2 cells. The results are very promising and favour the use of the strain Mz5 as a probiotic for pigs and rabbits.

P-18 **Identification and characterisation of bacteria in the gut flora of neonates.** G. Cooke, M. Costello, J. Behan (Institute of Technology, School of Science, Tallaght, Dublin 24, Ireland)

The occurrence of late haemorrhagic disease, particularly in breast fed neonates, has been associated with low levels of vitamin K. Vitamin K, an essential component of the blood clotting mechanism, is provided in the diet (phylloquinones) or produced by intestinal bacteria (menaquinones). Preventative treatment for late haemorrhagic disease has involved the administration of vitamin K either intramuscularly or orally using various regimes. Specific bacteria within the gut flora contribute significantly to individual vitamin K requirements. The neonate initially has a sterile gut. This project looks at the establishment of the various bacteria of the gut flora at three time points in the 0–6 week range differentiating between breast fed and bottle fed babies. Microbiological methods have been used to specifically enumerate *Lactobacillus* sp., *Bifidobacteria* sp., *Enterococci* sp., *Staphylococci* sp., *Bacteroides* sp., *Clostridia* sp. and coliforms present in the gut. Further studies will look at the production of vitamin K in selected bacteria with the aim of ascertaining the contribution of isolated vitamin K producing bacteria of the gut to the overall vitamin K concentration in the intestine.

P-19 **The influence of *Lentinus edodes* preparations on bacteriological and morphological aspects of the small intestine in piglets.** J.A. Decuypere, C.J. Van Nevel, N. Dierick, K. Molly (Department of Animal Production, Ghent University, Melle, Belgium and Seghersnutrition Sciences, Drongen, Belgium)

β-1,3–1,6 glycans (Lentinan) present in *L. edodes* mushrooms are non-digestible oligosaccharides with immunostimulatory properties against bacteria. Their effect on bacteriological and morphological aspects in the digestive tract of piglets was investigated. Four groups of 5 weaned piglets (28 d) were fed: a control diet C (diet 1) and C supplemented with resp. 50 ppm of Avilamycin (diet 2), 0.1% of *L. edodes* extract (min. 25% polysaccharides; diet 3) and 5% of dried *L. edodes* mycelium (diet 4). After 11–12 days, the animals were euthanized and viable counts of groups of bacteria (total bacteria, *E. coli*, streptococci, lactobacilli) were done on luminal contents (stomach, proximal and distal jejunum) and mucosa (jejunum only). The following observations were done on jejunal tissue samples: villus height and crypt depth, number of goblet cells and intra-epithelial lymphocytes (IEL), and mitotic index in the crypts and apoptotic index (villi). Decreases of viable counts (0.5–1.8 $\log_{10}$ CFU·g^{-1}; $P < 0.1$) of all bacterial groups were observed in luminal contents and mucosal samples only with diet 4. Feeding diets 3 and 4 had no effect on crypt depth, but increased villus height in proximal and distal jejunum, but those were only statistically

significant with diet 4 (10–15% increase). Both diets decreased the number of IEL in mucosal samples, e.g. in distal part from 36 cells/100 enterocytes with diet 1 to 23 with diets 3 and 4. Compared to diet 1, apoptotic index was lower when supplemented diets were fed, while mitotic index was only slightly changed from 62% to 58% mitotic cells per crypt with diet 4. The influence of *L. edodes* on several parameters indicated a lower turnover rate of the intestinal epithelium probably due to a lower bacterial load. The effect of *L. edodes* mycelium on the intestinal flora was probably due to the presence of antimicrobial compounds, e.g. lenthionine, rather than to an immunostimulating activity of β-glycans with increased release of IgA onto the mucosa surface, as also supported by the fact that addition of Lentinan to incubations with ileal contents lowered bacterial counts.

P-20 **Medium chain fatty acids as a nutraceutical alternative for in-feed antibiotics in piglet nutrition.** N. Dierick[a], J.A. Decuypere[a], K. Molly[b], E. Van Beek[c], E. Vanderbeke[d] ([a] University of Ghent, Faculty of Agricultural and Applied Biological Sciences, Department of Animal Production, Melle, Belgium; [b] Vitamex N.V., Drongen, Belgium; [c] Kemin Europa N.V., Herentals, Belgium; [d] Aveve N.V., Merksem, Belgium)

In order to prevent diarrhoea or poor performance, antimicrobial feed additives are usually applied in weaner and grower diets. In recent years, public concerns about development of cross resistance in human pathogens and residues in animal products and the environment, have caused in the EU a pressure to search for consumer friendly alternatives. A concept was developed based on studies with medium chain fatty acid containing triacylglycerols (MCTAGs; C6:0–C12:0) and selected lipases for in situ generation of free medium chain fatty acids (MCFAs) in the stomach and proximal gut of piglets as a potential noncaloric alternative for in-feed antimicrobial agents. The concept was tested out in vitro and validated in vivo, with gastric cannulated piglets and under field conditions, including effects on zootechnical performances. The addition of MCFAs containing oils (coconut, butter or particular MCTAGs oils, *Cuphea* seeds; 25–50 $g \cdot kg^{-1}$) to piglet diets in combination with microbial lipases (1 $g \cdot kg^{-1}$; lipase activity: 6500–9500 $U \cdot g^{-1}$) results in a physiological environment in the stomach and foregut which regulates and stabilizes the flora. A minimal concentration of 0.025 M free MCFAs in the intestinal contents was necessary for obtaining a significant (> 10 fold) suppression of the luminal flora (total anaerobic count, lactobacilli, *E. coli)*. It is striking that the controlled release of MCFAs in the lumen parallels with the degree of suppression of the bacterial load and with the extent of improvement of the mucosal health status and growth performance of the piglets, which make the concept a valuable alternative for in-feed nutritional antibiotics.

P-21 **Intestinal bacteriological changes in piglets after weaning onto a diet based on wheat**. C. Favier, J.P. Lallès, B. Sève (INRA-UMR Veau et Porc, 65, rue de St-Brieuc, 35042 Rennes, France)

The EU ban on in-feed antibiotics has raised the question of how to evaluate antibiotic alternatives for stabilising the gut flora in young farm animals. In the present work the consequence of a high incorporation rate of wheat in weaning diets for piglets on the bacterial community was evaluated by plate counting. According to the literature, wheat may increase the frequency of digestive disorders and diarrhoea. Wheat was therefore chosen as a non-pathogenic model of gut disorder induction. The piglets were weaned at 21 days of age and then starved for two days in order to reproduce the transient post-weaning anorexia. Then they were stomach tube-fed for 13 days to reduce individual variations for feed intake. A reference diet and a simplified diet enriched with wheat were provided. Piglets were slaughtered at day 0, 8 and 15 post-weaning and digesta collected. In the caecum, the ratio of lactobacilli:anaerobes increased and the ratio of enterobacteria:aerobes decreased from day 0 to day 15. By contrast, in the jejunum, changes in the bacterial ratios were variable. By day 15, the ratios enterococci/anaerobes were lower but the ratios of enterobacteria:anaerobes were higher at both sites for the diet containing wheat. The higher proportion of enterococci in the reference diet observed by day 15 may explain the decrease in pH observed in the caecum of the animals fed with this diet. Indeed, the enterococci belong to the group of lactic acid bacteria. The high proportion of enterobacteria found in the jejunum

and in the caecum relative to the total anaerobic population in the wheat-fed piglets suggests that wheat could favour intestinal disorders.

P-22 **Infiltrating polymorphonuclear leukocytes in ulcerative colitis have an enhanced response to members of the normal microbiota due to enhanced antibody response towards cell surface structures of these bacterial species.** E. Furrie, S. Macfarlane, G.T. Macfarlane (MRC Microbiology and Gut Biology Group, University of Dundee, Dundee, UK)

Members of the commensal microbiota live in close proximity to the host immune system without eliciting an inflammatory immune response. Polymorphonuclear leukocytes (PMN) or neutrophils are the first line of defence against bacteria. They migrate to sites of invasion and phagocytose foreign microorganisms. This process can be greatly enhanced by opsonisation by specific antibodies. Ulcerative colitis (UC) is a disease where this tolerance to commensal organisms is lost, resulting in a state of chronic inflammation in which the mucosa of patients is infiltrated with PMN and plasma B cells secreting antibody. In these studies the ability of 15 different bacterial species isolated from the rectal mucosa of UC patients to induce respiratory bursts in PMN was investigated. The bacteria were opsonised using serum from UC patients and healthy controls to determine whether the environment created in the inflammed mucosa of UC contributes towards prolongation of the disease. It was found that untreated organisms elicit a respiratory burst in PMN, with bifidobacteria inducing the largest respiratory bursts. After opsonisation with serum, it was observed that bacteria coated in antibodies from UC patients demonstrated an enhancement of the PMN respiratory burst of over 100%. The greatest stimulation was seen with *Bacteroides fragilis, Bacteroides vulgatus, Escherichia coli, Enterococcus faecalis* and *Peptostreptococcus anaerobius*. Furthermore, respiratory bursts towards opsonised bacteria were significantly faster and higher in magnitude. These data provide evidence that the antibody response in individuals suffering from UC is directed towards the surface of normal commensal organisms, and indicate that this would facilitate progression and maintenance of a chronic inflammatory state.

P-23 **Factors affecting intestinal flora construction in infant rat.** R. Inoue, K. Ushida (Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan)

We investigated the factors affecting the intestinal flora construction in pups. Three pregnant Wistar rats bred in barrier units were purchased. They were housed under non-barrier circumstances in our animal facility and fed on a standard pelleted diet. The littermates were not separated until weaning (day 20). After weaning, pups from one dam were offered the same diet as their mother. Pups from the second dam were divided in two groups at day 20. One group received the same-pelleted diet and the other received fructooligosaccharide (FOS)-supplemented diet. Three pups within a litter of third dam were orally inoculated *Bifidobacterium animalis* at day 18. Faecal bacteria were analyzed by ARDRA and TGGE for all animals at days indicated below. An additional experiment was done with one SPF SD pregnant rat to evaluate the influence of microbial circumstances on the adult. At day 18, the flora constitution of pups was simple and primarily consisted of *E. coli*. At day 40, it became complex and anaerobic bacteria constituted it in large part. The bacterial OTUs detected (approx. 30) in dams were all detected in their pups. However, 25 to 30 OTUs were newly detected in pups. The SPF rat had a far simpler intestinal flora and the number of OTU was not increased during the experiment. The commercially available conventional rats have simple intestinal flora and their flora constitution, once established, might not be influenced by the microbial circumstances. Pups received bacteria from the environment during day 18 to 22. Dietary FOS influenced strongly the development of intestinal flora during this period. *B. animalis* transmitted to the littermates after inoculation, but two pups were not colonized. Involvement of the immune response will be discussed.

P-24 **Development of probiotics for canine and feline nutrition.** R. Knorr, F. Praplan, V. Rousseau, C. Cavadini (Nestlé Research Center, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland)

The successful use of probiotics in human nutrition and in farm animal feeding promises similar

benefits for canine and feline nutrition and makes the application of lactic acid bacteria in pet food a quite attractive concept. We describe adequate in vitro methods for the development of probiotics for use in dog food, especially the development of a model system simulating canine small intestinal conditions. About one hundred lactobacilli strains were isolated from dog and cat faeces, purified, physiologically characterised, and screened to meet the following requirements: (1) resistance to canine gastric conditions, (2) resistance to canine small intestinal conditions, (3) physiological activity under small intestinal conditions with and without addition of pet food, and (4) bactericidal activity against pathogens implicated in canine enteric infections. 18 strains exhibited bactericidal activity. They comprised the following species according to their fermentation patterns: the heterofermentative *L. fermentum/reuteri* and *L. salivarius* groups, and the homofermentative *L. acidophilus/crispatus* group. Out of these strains, the 13 that were able to ferment starch, the main carbohydrate provided with the pet food, showed the highest antimicrobial activities. These strains were all typed *L. acidophilus* and could be divided into three distinct groups regarding their ribotype patterns. The first two groups (7 strains) were antimicrobially active due to production of high amounts of lactic acid (> 90 mM) and a concomitant decrease of the pH down to 4.5. The last ribogroup seemed to employ a different active principle, effective even at neutral pH values. Studies investigating this antimicrobial agent are ongoing. These screening results enable us to select promising probiotics for evaluation of efficacy in clinical studies.

P-25 **Receptors recognized by bifidobacteria on intestinal epithelial cells.** M. Kostrzynska, J. Dixon, D. Lepp (Agriculture and Agri-Food Canada, Food Research Program, Guelph, Ontario, Canada)

Bifidobacteria have potential beneficial effects on the host that are generally related to inhibition of pathogens, maintenance and restoration of normal intestinal flora and increased immune response. We investigated adherence properties of *Bifidobacterium infantis* R0033 and identified receptors recognized by this bacterium on human intestinal epithelial cells. Various soluble carbohydrates and glycolipids were tested as potential inhibitors of *B. infantis* R0033 adhesion to Caco-2 cells. Fucosylated compounds such as fucosyllactose and Lewis b tetrasaccharide (histo-blood group determinant) significantly inhibited adhesion of *B. infantis* R0033 to intestinal epithelial cells. Bacterial adherence was also mediated by asialoganglioside-GM1 and by acidic glycosphingolipids - sulfatides. These results suggest that fucosylated Lewis b antigen, sulfatides and asialo-GM1 serve as adhesion receptors for *B. infantis* R0033.

P-26 **Comparison of the effects of adding antibiotic or probiotic as growth promotor in ration on broiler performance.** M. Modirsanei, S.M.M. Kiaei, M. Farkhoy (Department of Animal and Poultry Health and Nutrition, Faculty of Veterinary Medicine, University of Tehran, P.O. Box: 14155-6453 Tehran, Iran.)

An experiment was conducted in order to compare the effects of adding an antibiotic (Virginiamycin = VM) or a probiotic (PR = a mixed culture of *Bacillus subtilis* CH 201 and *Bacillus licheniformis* CH 200) into diets on body weight (BW), feed intake (FI), and feed conversion ratio (FCR) of broiler chicks. Five hundred forty day-old male Ross broiler chicks were randomly divided into three dietary treatments. Each treatment contained six replicate floor pens of 30. One treatment considered was as the control and received a diet without any antibiotic, growth promotor and coccidiostat. For feeding the chicks in two other treatments, 1 $g \cdot kg^{-1}$ PR or 0.1 $g \cdot kg^{-1}$ VM were added into diets, respectively. Feed and water were provided ad-libitum throughout the experimental period. Results showed that at the end of the trial, adding VM into diet increased BW significantly ($P < 0.01$), while supplementing the diet with PR had no significant effect on BW. There was no significant difference between BW of chickens fed diets supplemented with VM or PR. When compared with chicks fed diets containing PR, adding VM into diet caused a significant increase in FI ($P < 0.05$). Supplementation diets with VM or PR improved FCR, significantly ($P < 0.01$), in comparison with control, but no significant differences were observed between these two dietary treatments.

P-27 **The effect of feed form on the microflora at the terminal ileum of the young pig.** C.A. Moran[a], P.H. Brooks[b] ([a] Alltech Inc. Nicholasville, KY 40356, USA; [b] Seale-Hayne Faculty, University of Plymouth, Newton Abbot, Devon TQ12 6NQ, UK)

Until recently, the industry managed digestive problems in the young pig by the use of prophylactic antibiotics. The objective of the study reported here was to examine the effect of feed form on the microflora of the young pig's gut. Twenty-four piglets, weaned at 23 ± 2 days of age were randomly allocated (eight per treatment) to one of three dietary treatments, non-fermented liquid feed (NFLF), fermented liquid feed (FLF) or dry feed (DF). A further eight piglets were suckled by their dam (S). All feeds were fed ad libitum for 14 days. Gastrointestinal tracts were removed under terminal anaesthesia. Samples of the terminal ileum were taken for determination of SCFA concentration (HPLC), pH and enumeration of lactobacilli and coliform populations (plate count techniques). No coliform bacteria (< 3.0 $\log_{10}$ CFU·mL^{-1}) were detected in pigs fed FLF compared with 8.5, 8.1, and 6.0 $\log_{10}$ CFU·mL^{-1} digesta in DF, NFLF and S pigs respectively. Significant ($P < 0.01$) differences in the concentrations of acetic, formic and propionic acids were found in pigs fed FLF and NFLF compared to DF and S piglets. The lactobacilli populations were significantly affected by dietary treatment with the lowest in the dry fed piglets and the highest in those fed a fermented diet ($P < 0.001$). There were significantly higher ($P < 0.01$) concentrations of acetic, formic and propionic acids found at the terminal ileum of the pigs fed FLF and NFLF compared to dry and suckled pigs. The short-chain fatty acid profiles reflect changes in microbial populations in the terminal ileum due to dietary treatment. A combination of reduced pathogen load, acidified feed, high lactic acid bacteria numbers and the short chain fatty acid concentrations resulted in a significant reduction in coliforms at the terminal ileum. This has implications in terms of piglet health and dietary prevention of enteric diseases.

P-28 **Functional foods as a tool to modulate the gastrointestinal ecology and reduce antimicrobials used in broiler chickens diets.** G. Nava, N. Ledesma, B. Hargis, A. Donoghue, G. Tellez (Departamento de Producción Animal: Aves, UNAM-Mexico DF 04510, Mexico)

The effect of functional food (FF) supplemented with *Aspergillus* meal (AM) prebiotic in broilers was investigated. Two experimental sorghum-soybean diets, control-diet and FF with 0.2% of AM, were administrated from day 1 to 20 of age. Mucosa morphology, crop and cecal pH, short chain fatty acids (SCFA), protein and energy (P-E) in ileal digest, mineral content in tibia (MCT), gastrointestinal transit time (GITT), body weight (BW), BW uniformity (BWU), gut aerobic and anaerobic microflora, and *Salmonella enteritidis* (SE) organ invasion were evaluated. Increases in the GITT, total CFU of aerobic, anaerobic (*Lactobacillus*) microflora and a reduction on SE organ invasion were observed in the group fed with FF compared with control-diet ($P < 0.05$). No differences were observed on total CFU of *Clostridium* between both groups. FF increased the villi height in the ileum at 20 days. Although there were no differences on crop pH at any time of evaluation, an increase in cecal pH in FF group was observed at 20 days. No significant changes in crop SCFA were observed between groups at any time. Interestingly, at 20 days, a reduction of cecal SCFA concentrations was observed in the FF group. This effect and the alkalinity of cecal pH support the concept that absorption of SCFA may be concentration-dependent. Chicks fed with FF showed the best P-E utilization in the ileum and an increase in MTB. FF supplementation did not affect BW, but improved BWU. The results of the present study describe the possible physiological pathways by which FF could be a good tool to modulate the gastrointestinal ecology and reduce antimicrobials used in poultry diets.

P-29 **Transition of probiotic bacteria, *Lactobacillus casei* strain Shirota, in gastrointestinal tract of pigs.** Y. Ohashi, R. Inoue, K. Ushida (Laboratory of Animal Science, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan)

It is widely recognized that probiotics made of lactic acid bacteria have a beneficial health effect. They are more effective for the host if more viable probiotic bacteria reach their site of action. Therefore, resistance to gastric and bile acids is important. The survival of probiotic strain has

been evaluated by their recovery from faeces. However, the transition of viable cells in gastrointestinal tract is still unclear. In this study, we investigated the passage of a probiotic strain in the gastrointestinal tract of pigs that received a commercially available fermented milk which contained *Lactobacillus casei* strain Shirota (LCS). Three female pigs fistulated at the caecum were fed 130 mL fermented milk, which contained over 10^{10} (CFU) LCS, with daily meal for 8 days. On the 8th day, pigs received two transit markers with a morning meal. $YbCl_3$ and Co-EDTA were used to estimate the transit of solid and liquid components of the digesta. Caecal digesta were sampled through fistulae every 2 h for 24 h. The viable cell number of LCS and the concentrations of transit markers in each digesta sample were determined. The viable cell numbers (log CFU·g^{-1}) of LCS ranged from 3.56 to 6.58. It was suggested that all LCS did not reach the caecum in pigs. LCS number reached the maximum level 6h after dosing. Four doses every 6h may be required to keep the maximum LCS level at caecum. The viable cell number of LCS was significantly correlated with the relative concentration (Sum of the marker concentration for 12 samples = 100) of Co ($r = 0.794$, $P < 0.01$) and Yb ($r = 0.458$, $P < 0.01$). These results indicate that LCS moved with the liquid component.

P-30 **Modulation of piglet gut microflora post-weaning.** J.A. Pickard, C.E.R. Dodd, J. Wiseman (School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK)

Post-weaning growth check in piglets is currently a major problem that is characterised by low voluntary food intake, poor growth performance, expression of enteric diseases and mortality. Investigations into interactions between pre- and post-weaning nutrition and gut physiology, and their relevance to performance and health, are of paramount importance in solving these problems. Piglets fed an immunostimulant supplemented diet immediately post-weaning displayed a significant reduction in the number of coliforms present in small intestinal digesta ($P = 0.033$). Additionally, coliforms decreased linearly throughout the experimental period (14 days), on both the control and supplemented diets ($P = 0.002$); there was a significant day × diet interaction ($P = 0.067$ (L)). There was no significant effect of diet on digesta lactobacilli or bifidobacteria counts ($P = 0.785$; $P = 0.111$ respectively). However, there was a significant time effect, with lactobacilli ($P = 0.008$ (Q)) and bifidobacteria increasing ($P = 0.006$) throughout the experimental period. A similar pattern was displayed in faecal samples, as lactobacilli and bifidobacteria counts increased over time ($P < 0.001$ (Q)). There was however a significant dietary effect ($P < 0.001$) with animals fed the immunostimulant supplemented diet shedding higher numbers of lactobacilli than control animals. There was a trend towards a reduction in coliform numbers ($P = 0.079$) for animals fed the treatment diet, and a trend towards a general linear reduction in coliforms over time ($P = 0.055$ (L)). Subsequently, an immunostimulant supplemented diet was fed for 14 days pre-weaning in addition to 14 days post-weaning; both coliform and *E. coli* numbers decreased with time ($P < 0.007$ (L); $P = 0.007$ (L) respectively), whilst bifidobacteria increased ($P = 0.012$). The lactobacilli to coliform ratio decreased over time ($P = 0.005$) although there was no effect of diet or time on lactobacilli counts ($P = 0.238$). Beneficial modulation of gut microflora is therefore possible through dietary manipulation.

P-31 **Microbial changes in liquid piglet feed formulations.** C. Plumed, M. Pagès, A. von Wright (Institute of Applied Biotechnology, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland)

Liquid piglet feed formulations, in farm conditions and laboratory experiments, were studied in order to detect developments in the microbial populations during the three-month feeding period. In addition, the feed was inoculated with an isolated *Lactobacillus* to check whether these bacteria could control the feed fermentation process. Total aerobic bacteria, proteolytic, amylolytic, lipolytic bacteria, bacillus, lactic acid bacteria, yeast and moulds were checked using selective plating. Two substrates, barley and maize, were used for the fermentation process. The results demonstrated a tendency to elevated bacterial counts towards the end of the feeding period, except for the amylolytic bacteria and bacilli that were only occasionally detected. Yeast and moulds remained at low concentration. Microbial counts were higher with barley than with maize substrates before the 24 hours

incubation. At 24 hours, the counts were similar. The fermentation with the inoculated lactobacillus demonstrated that the strain could establish itself and become dominant in the fermentation conditions. In conclusion, the changes in the microbial composition of the liquid piglet feed reflect the formation of a certain microbiological balance in the feeding system. Barley-based and maize-based feed were capable of spontaneous fermentation and they are good substrates for lactic acid bacteria.

P-32 **Immunisation of mice with *Lactobacillus plantarum* NCIMB8826 expressing the antigen UreB of *Helicobacter pylori*: evaluation of stability of the recombinant strain and its immunogenicity for vaccine development.** Y. Roussel, M. Grammatikaki, A. Beugnet, M. Wilks, S. Tabaqchali (Queen Mary University of London, UK)

This study describes the construction of *Lactobacillus plantarum* NCIMB8826 (Lp 8826) expressing the *Helicobacter pylori* antigen UreB, its survival in the mouse gut and its efficacy to protect mice against a Helicobacter infection. The gene encoding the urease subunit B (*ureB*) of *Helicobacter pylori* was cloned in the intracellular expression vector pNX. Expression of *ureB* in Lp 8826 pNXureB was analysed by Western blot where cell lysates revealed a reactive band of 66 kDa corresponding to the predicted molecular weight of UreB. The amount of UreB produced was estimated to 10 µg per 10^9 CFU. When assayed in vitro, pNXureB was found to be segregationally stable in Lp 8826 after 50 generations in medium without antibiotic pressure but showed a reduced expression of UreB. The ability of the recombinant strain to colonise the gut of C57BL/6N mice was assessed by quantitative culture of faeces obtained after oral feeding. Lp 8826 pNXureB and Lp 8826 pNX could be recovered up to 48h after the cessation of feeding, indicating that they had a limited colonising ability in mice. For immunisation experiments, mice were orally administered live cells or cell lysates of Lp 8826 pNXureB, either with or without the mucosal adjuvant cholera toxin. Low or no immune responses (serum IgG and IgA) were detected in all mice although some of them were found to be protected against a *Helicobacter pylori* challenge.

P-33 **Development of probiotic strains for pet food.** R. Simmering, J. Benyacoub, R. Knorr, T. von-der-Weid, C. Cavadini, F. Rochat (Nestlé Research Center, P.O. Box 44, 1000 Lausanne 26, Switzerland)

The intestinal micro-flora have a major impact on gastrointestinal function and therefore on the health of the host. Probiotics are defined as "live microbial feed supplements, which beneficially effect the host animal by improving its intestinal microbial balance". The majority of probiotic products are designed for humans or cattle. However, it is reasonable to assume that similar beneficial effects can be conferred by probiotic use in pets. To investigate the potential health benefits of probiotics in pets, over 50 different strains of lactic acid bacteria were isolated from cats and dogs. These were identified and characterised for relevant technological and physiological properties. From the results of this initial screening, six strains were selected and their potential to inhibit the growth of pet enteropathogens was investigated in co-cultivation experiments in an in vitro model simulating small intestinal conditions. One potential probiotic strain, Lb2628, showing promising results in vitro was used in a series of in vivo studies in gnotobiotic mice. The anti-pathogenic effects against *Salmonella enterica* Serovar *Typhi* and *Clostridium perfringens* together with the immune-modulating effects of this particular strain were investigated. In these experiments the probiotic strain was able to decrease the translocation of *S. enterica* into mouse tissue. In addition, Lb2628 modulated and stimulated immune responses to *S. enterica* and *C. perfringens*, respectively, as suggested by the antibody isotypes measured in blood and faecal samples. These data were supported by in vitro assessments of cytokine profiles in canine leukocytes. Further in vivo studies investigating the probiotic potential of this bacterial strain are underway.

P-34 **Effects of an oregano-based feed additive on the porcine intestinal microflora in vitro.** E. Veligratli[a,b], K. Hillman[a], S. Stefopoulou[a], V.R. Fowler[b] ([a] SAC, Craibstone, Aberdeen AB21 9YA, UK; [b] University of Aberdeen AB24 5UA, UK)

Orego-Stim®, an oregano-based feed additive, has previously been shown to improve the health

and growth of pigs. It was postulated that this additive may function, at least in part, through a direct influence on the microflora of the porcine intestine. The effect of this additive on the intestinal microflora was examined using two simultaneous in vitro simulations of the porcine colon. These were inoculated with fresh porcine faeces and allowed to stabilise for four days. Subsequently, one of the vessels was dosed daily with 20 mg of Orego-Stim®. Bacteriological enumeration was performed after four days dosing. Bacterial species enumerated were total coliforms, *Escheri-chia coli*, total aerobes, total anaerobes, *Lactobacillus* spp., *Clostridium* spp. and *Bacteroides* spp. Control and test vessels were alternated on replication. The additive reduced the numbers of almost all the bacterial groups examined, with the exception of total anaerobes and *Bacteroides* spp., although only *E. coli* was reduced to a statistically significant level. The data obtained in this study indicate that Orego-Stim® has little effect on strictly anaerobic bacterial species, but can influence the growth of aerotolerant and facultative bacteria within the porcine intestinal microflora. As the most numerous group (total anaerobic bacteria) were unaffected by the additive, there is no support in this data for the popular idea that improved growth results from an overall reduction in the intestinal microflora. However, the suppression of growth of *E. coli* may be important in the promotion of animal health, as this is indicative that the formulation may also be effective against enteropathogenic *E. coli* and related coliform pathogens.

Session III:

Pathogens and antibiotic resistance transfer

Reprod. Nutr. Dev. 42 (2002) S27–S40

IL-1 **Transfer of antibiotic resistance genes between gut bacteria – Are gut bacteria reservoirs for resistance genes?** A.A. Salyers (Department of Microbiology, University of Illinois, Urbana, IL, USA)

The notion that human intestinal bacteria might serve as reservoirs for antibiotic resistance genes has been discussed for many years, but only recently has it been possible to refine and test this hypothesis. More specifically, the hypothesis is that bacteria that pass transiently through the human intestine may donate genes to or acquire genes from bacteria that permanently colonize the intestine. Our work has focused on the genus *Bacteroides*, which is one of the numerically predominant genera of bacteria in the human colon. In the laboratory, *Bacteroides* donors transfer DNA by conjugation to *Bacteroides* recipients. They can also transfer DNA to members of other genera such as *Escherichia coli* and *Prevotella ruminicola*. Yet, even under optimized laboratory conditions, the frequencies of transfer are relatively low. This observation raised the question of whether such transfers were likely to occur in the colonic environment. We have now shown that such transfers appear to be more common than expected. Genes such as *tetQ* and *ermF* are found in many different *Bacteroides* species, which are genetically distant from each other. Moreover, these genes are found on conjugative elements, primarily conjugative transposons. Evidence for transfer of resistance genes between *Bacteroides* species and Gram-positive bacteria comes from finding genes like *ermB* and *ermG*, genes that have previously been found primarily in the Gram-positive bacteria, in *Bacteroides* species isolated from the colon. Recently, we have found that both *ermB* and *ermG* are carried on conjugative transposons. Although it is not yet possible to determine the direction in which these transfers occurred or whether transfer occurred in one or many steps, our results strongly support the hypothesis that conjugal transfer of antibiotic resistance genes occurs between diverse types of bacteria, including those which are permanent residents of the colon and those which are only transiently present in the colon.

O-11 **Evolutionary and ecological implications of antibiotic resistance.** R.I. Aminov[a,e], J.C. Chee-Sanford[b], N. Garrigues[c], I.J. Krapac[d], R.I. Mackie[a] ([a] Department of Animal Sciences and [c] Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign, USA; [b] USDA-ARS, Urbana, IL 61801, USA; [d] Illinois State Geological Survey, Champaign, IL 61820, USA; [e] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

The emergence and rapid dissemination of antibiotic resistance is a major problem in medicine and agriculture. Traditionally, this area has been confined to studies of antibiotic resistances among the pathogenic microbiota. However, broader evolutionary and ecological studies are needed to understand the phenomena of the origin and dissemination of antibiotic resistance. Phylogenetic reconstruction of the evolutionary history of the *tet* and *erm* genes suggests the scenario of early branching and extensive diversification of these genes well before the modern "antibiotic era". Also, phylogenetic reconstruction does not support the model of antibiotic resistance gene exchange between the antibiotic-producing and non-producing bacteria. While the functional role of these proteins in antibiotic-producing bacteria is evident, their presence and function in bacteria of other ecological niches that have no or limited contact with the antibiotic-producing representatives of soil microbiota is more challenging to explain. Our ecological studies demonstrated the occurrence of *tet* genes in the environment as a direct impact of antibiotic usage in agriculture. At the same time, our study of the environment and gut microbiota of the Galapagos archipelago, which is minimally affected by human activities, implies that despite the apparent absence of antibiotics, the environment and microbiota of local animals carry a substantial pool of antibiotic resistance determinants. Together, these observations indicate that besides the clear-cut case of antibiotic selective pressure, there are other, as yet unidentified, factors selecting and maintaining antibiotic resistance genes in bacterial populations of the gut and environment.

O-12 **Identification of novel ribosome protection type tetracycline resistance genes *tet*(W) and *tet*(32), amongst predominant gut commensal anaerobes.** K.P. Scott[a], C.M. Melville[a], P. Mullany[b], H.J. Flint[a] ([a] Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, UK; [b] Eastman Dental Institute, University College London, London, UK)

Despite the rapid emergence of antibiotic resistance in both human and veterinary pathogens little work has been done on the incidence of resistance in the commensal gut flora. Two previously undescribed tetracycline resistance genes of the ribosome protection type, *tet*(W) and most recently *tet*(32), were isolated by our group. *tet*(W) was isolated from the rumen anaerobe *Butyrivibrio fibrisolvens* and *tet*(32) from a human-gut Gram-positive anaerobe *Clostridium* spp, strain K10. *tet*(W) shares less than 65% amino acid identity with *tet(*M) and in the *B. fibrisolvens* strain 1.230 is carried on a 50 kb mobile chromosomal element, Tn*B1230*. *tet*(W) appears to be plasmid-borne in some hosts. Screening for the presence of *tet*(W) in diverse ecosystems suggests that it may be as abundant as *tet*(M) in nature. *tet*(32) shares 76% amino acid sequence identity with *tet(*M) and only 68% identity with *tet*(W). *tet*(32) is also carried by a highly mobile chromosomal element and is transmissible to both *Eubacterium* spp. and *B. fibrisolvens* in vitro. This novel ribosome protection type gene expresses a higher level of Tc^R than other ribosome protection proteins and is abundant in the ovine rumen, human and porcine gut. The potential exists that these novel tetracycline resistance genes, carried by conjugative transposons, may account for a significant proportion of unknown resistance amongst clinical pathogenic bacteria.

O-13 **Molecular characterisation of Shiga-toxin producing *Escherichia coli* isolated from cattle, food and patients in Central France.** V. Livrelli[a,b], N. Pradel[b], Y. Bertin[a], K. Boukhors[a], N. Banchet[b], C. Martin[a] ([a] Laboratoire de Microbiologie, Centre de Recherche INRA Clermont-Fd-Theix, France; [b] Pathogénie Bactérienne Intestinale, Faculté de Pharmacie, 28, place Henri-Dunant, 63001 Clermont-Ferrand, France)

Shiga-toxin producing *Escherichia coli* (STEC) have been associated with food borne diseases, ranging from uncomplicated diarrhoea to hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS). While most outbreaks are associated with *E. coli* O157:H7, mainly in North America and Japan, non-O157:H7 serotypes are frequently isolated from sporadic cases in Continental Europe. Healthy cattle appear to be the main reservoir of STEC strains, which are transmitted to humans through foods or water contaminated by faecal material. We established a collection of 220 STEC strains isolated from bovine faeces (n = 186), food samples (n = 24) and stools from asymptomatic children (n = 10). To identify specific properties of pathogenic strains, the collection was characterized with respect to a number of putative virulence factors, and compared to 15 STEC isolated from patients suffering from HC/HUS in the same geographical area. The presence of genes encoding virulence factors such as Shiga-toxins (stx_1, stx_2 and variants), Intimin (*eae*) and other adhesins (*efa1*, *saa*), Enterohemolysin (*ehx*A), Serine-protease (*esp*P), Catalase-peroxidase (*kat*P) was checked by PCR and/or hybridization with specific DNA probes. A high level of cytotoxicity towards Vero cells, and the presence of the stx_2, *eae* and *kat*P genes were clearly associated with pathogenic strains. A detailed analysis of STEC strains belonging to serogroups O91 and OX3 was performed using ribotyping and PFGE. It revealed a great diversity among non-O157:H7 STEC strains isolated in France and underlined the risk of zoonosis.

O-14 **A role for lipopolysaccharide (LPS) but not intimin in *Escherichia coli* O157:H7 persistence in a specific pathogen free (SPF) chick model.** A. Best[a,b], R.M. La Ragione[a], C.D. O'Connor[b], M.J. Woodward[a] ([a] Department of Bacterial Diseases, Veterinary Laboratories Agency Addlestone, Surrey, KT15 3NB, UK; [b] Department of Molecular Biology and Biochemistry, University of Southampton, Bassestt Crescent East, Southampton, SO16 7BX, UK)

Escherichia coli O157:H7 is a major cause of food-borne disease in humans. Cattle are cited as a significant reservoir, but the mechanisms of O157 persistence in this and other animals have not been determined. We have developed a surrogate chick infection model to study the role of surface antigens of *E. coli* O157:H7 in persistence. Groups of day-old specific pathogen free chicks were dosed orally with shiga-toxin negative *E. coli* O157:H7 strain NCTC 12900 or LPS (DM2) or intimin (DM3) derived knockout mutants either singly or pair-wise (competitive index). At post mortem, significant numbers of NCTC 12900 and DM3 were recovered from the caeca on days 1, 2 and 5 post dosing in both types

of inoculum group. DM2 was detected but at lower numbers. Weekly cloacal swabbing of double inoculum groups revealed that NCTC12900 and DM3 both persisted for 112 days, whereas DM2 only persisted for 49 days. In single inoculum groups DM3 persisted for 211 days, NCTC 12900 persisted for 168 days and DM2 persisted for 140 days. Contamination of egg shells by NCTC 12900 (6%) and DM3 (14%) was noted. DM2 versus DM3 in the same model has demonstrated that the LPS mutant was out competed by the intimin mutant. The data demonstrated that LPS, but not intimin, is required for persistence in the day-old chick. We are currently investigating other knockout mutants and lateral and environmental transfer of wild type and mutant.

O-15 **Interactions of probiotic bacteria and *E. coli* in the intestinal tract of young animals.** A. Bomba, R. Nemcová, S. Gancarčíková, D. Mudroňová, P. Pecúch, R. Herich (Research Institute of Veterinary Medicine, Hlinkova 1/A, 040 01 Košice, Slovak Republic)

Despite a lot of research effort, the mode of action of probiotics has not yet been fully explained. The inhibitory action of probiotics against pathogens may be mediated by competition for receptors on the gut mucosa, competition for nutrients, the production of antibacterial substances, and the stimulation of immunity. In the young, enterotoxigenic *Escherichia coli* appear to be the most frequent diarrhoea-causing agents. Employing lactobacilli in the form of probiotics seems to be a very efficacious method of preventing and treating diseases caused by pathogenic microorganisms in young animals. Our studies in gnotobiotic lambs showed that the competition for adhesion receptors on the intestinal mucosa did not play an important role in the mechanism of inhibiting enterotoxigenic *E. coli*. More likely seemed to be a metabolite–mediated inhibition. The results of our experiments on gnotobiotic pigs suggest that the significantly increased levels of organic acids produced by lactobacilli in the mucosal film of the jejunum and the ileum, in comparison to the luminal contents, may present an efficient barrier inhibiting the adherence of digestive tract pathogens to the intestinal mucosa. Our group confirmed that maltodextrin KMS X-70 and fructooligosaccharides potentiated the inhibitory effect of *Lactobacillus casei* against the colonisation of intestinal tract by *E. coli*. The administration of polyunsaturated fatty acids positively affected the adhesion of lactobacilli in the digestive tract. The elucidation of mechanisms of the synergistic inhibitory effect of probiotic bacteria and some components of natural origin may enable the development of more effective probiotic preparations, or “potentiated probiotics”.

P-35 **Restriction endonucleases from ruminal strains of the genus *Prevotella*.** T. Accetto, G. Avguštin (Zootechnical Department, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia)

Restriction seems to hinder transformation of *Prevotella* strains with DNA of heterologous origin. In addition, some of these organisms possess extracytoplasmic non-specific nucleases that may also degrade plasmid DNA and prevent its establishment in the cell. Strains with little or incapacitated non-specific nucleases and well defined restriction-modification systems are therefore required for successful transformation. They could permit development of faster and simpler ways of gene introduction and facilitate genetic research of ruminal prevotellas. In cell free extracts of *P. ruminicola* 23 and *P. bryantii* TC1-1, strains with low non-specific nucleolytic activity, site specific endonucleolytic activity was observed. Upon partial purification of this activity on heparin-sepharose and tests on bacteriophage lambda and other well characterized substrates it became apparent that the activity of the strain 23 was identical to the activity of enzyme *Hae*III. The activity from TC1-1 corresponded to that of *Sau*3AI. The site of cleavage was determined by analysing radioactively labelled primer extension products of pUC19 treated with endonucleases from TC1-1 and 23. They proved to be true isoschizomers of *Sau*3AI and *Hae*III. PCR product fragments obtained with endonuclease from TC1-1 were successfully ligated to *Bam*HI cleaved vector demonstrating functional interchangeability with *Sau*3AI. We propose the names *Pru*2I and *Pbr*TI for endonucleases from *P. ruminicola* 23 and *P. bryantii* TC1-1 respectively. Plasmid and bacteriophage lambda DNA was protected from cleavage by *Pru*2I and *Pbr*TI by incubating it

with cell free extracts of *P. ruminicola* 23 or *P. bryantii* TC1-1. DNA was also successfully protected from the action of *Pru*2I by the commercially available *Hae*III methylase. The effect of protection on the electroporation frequency of strain TC1-1 with plasmid pRH3 is being investigated.

P-36 **Enteric viruses as quality markers for natural drinking water sources of dairy cattle.** P.M. Becker, S.J.W.H. Oude Elferink, G.A.L. Meijer (ID-Lelystad, ID TNO Animal Nutrition, P.O. Box 65, 8200 AB Lelystad, The Netherlands)

Pathogenic agents in natural drinking water supplies – even if present in low numbers – can form a serious threat to dairy cows, because of their high daily water intake. An indicator for the sanitary quality of natural waters would hence be helpful. Because faecal viruses were detected in water free of faecal indicator bacteria, the adequacy of bacterial indicators for water quality assessment is questionable. In this study, possible viral indicators of pathogens for dairy cattle in surface water were to be specified. Cattle pathogenic viruses that are transmissible by waters are diverse, comprising RNA and DNA viruses with both single and double stranded genomes. Most of the bovine enteric viruses do not apparently cross species. Viruses with interspecies transmission belong to the parvoviruses, the rotaviruses, and the pestiviruses. It is neither possible to specify a single representative marker virus for these enteric viruses, nor for any other pathogenic organism. In case of an acute circulation of an infectious pathogen, only the pathogen itself can indicate the specific hazard. However, some bacteriophages, which infect enteric bacteria, are promising as general markers of a faecal contamination. Male-specific F-RNA coliphages or *Bacteroides fragilis* phages do not multiply out of their host macro-organism, they survive as well as, or even better than, enteric viruses in the environment, and they are not pathogenic to macro-organisms. To identify the source of a contamination, the human or animal specificity of F-RNA-phage serogroups as well as the macro-organism specificity of *Bacteroides fragilis* host strains could be taken advantage of.

P-37 **Subtyping of virulence factors in Shiga toxin-producing *Escherichia coli* (STEC) isolated in France from healthy cattle and food products.** Y. Bertin[a], K. Boukhors[a], V. Livrelli[a,b], N. Pradel[b], C. Martin[a] ([a] INRA, Centre de Clermont-Ferrand-Theix, France; [b] Faculté de Pharmacie, Clermont-Ferrand, France)

Enterohemorrhagic *Escherichia coli* (EHEC) are important food-borne pathogens causing bloody diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. Although *E. coli* O157:H7 is the main causative agent of hemolytic uremic syndrome (HUS) due to EHEC outbreaks, more than 100 other serotypes have been associated with sporadic cases of human disease. Ruminants are thought to be the principal reservoir for Shiga toxin-producing *E. coli* (STEC) potentially pathogenic for humans. We have screened a collection of 220 STEC strains isolated in central France from healthy cattle and cattle-derived food products for the presence and subtypes of genes encoding the main EHEC virulence factors: the Shiga toxins (Stxs) that are responsible for the principal manifestations of HUS, and the locus of enterocyte effacement (LEE) which encodes genes involved in effacement of intestinal epithelial cell microvilli and in intimate adherence between bacteria and the epithelial cell membrane. We found a great diversity in the combination of *stx* genes, with a high number of strains harbouring two or more *stx* subtypes. The *stx2* and *stx2vh-b* genes were prevalent and correlated with a high toxicity on Vero cells. A new *stx2* variant was characterised. A small number of strains harboured the LEE. Subtyping of the variable espA, espB, *tir*, and *eae* genes showed a variety of pathotypes, including pathotypes frequently found in EHEC isolated from HUS. In conclusion, it appeared that the majority of STEC strains isolated from cattle and food products in central France did not seem dangerous for humans. However, a small number of them presented a high virulence potential and could be a source of zoonosis.

P-38 **Role of the Shiga toxin in the ecology of *Escherichia coli* O157:H7.** N.A. Cornick, H.W. Moon (Iowa State University, Ames, IA 50011, USA)

E. coli O157:H7 and other Shiga toxin-producing *E. coli* (STEC) are an important cause of

food-borne illness in humans. Ruminants are considered to be the major reservoir of these bacteria although they are occasionally isolated from other animal species. While much has been learned about the pathogenesis of STEC, their propensity to colonize the ruminant gastrointestinal tract has not been adequately explained. Shiga toxins (Stx) are encoded by lamboid-like prophages inserted within the bacterial chromosome. We hypothesized that Stx and/or the Stx-converting phage confers an ecological advantage to *E. coli* O157:H7 to become established in the ruminant gastrointestinal tract. To test this hypothesis young adult sheep were inoculated with 10^{10} CFU of either wild type *E. coli* O157:H7 (Stx2 pos), an isogenic Stx2 deletion mutant or a naturally occurring Stx-negative strain of *E. coli* O157:H7. At 2 days, 2 weeks, 1 month and 2 months post-inoculation there were no significant differences in either the magnitude or duration of fecal shedding of the Stx-negative strains compared to the wild type *E. coli* O157:H7. We are currently conducting experiments to determine whether the absence of the entire Stx phage genome influences the magnitude and/or duration of *E. coli* O157:H7 shedding by sheep.

P-39 **Use of green fluorescent protein as visual marker**. M.S. Ekinci[a], K.P. Scott[b] ([a] Kahramanamras Sutcu Imam University, Faculty of Agriculture, Department of Animal Science, Kahramanmaras, Turkey; [b] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

The development of sensitive methods for observing individual bacterial cells in a population in experimental models and natural environments is of great importance for studying the systems. The gene encoding the green fluorescent protein (gfp) from the jellyfish *Aequorea victoria* has recently become an important visual marker of gene expression in eukaryotic and prokaryotic organisms such as mammals, fish, insects, plants, yeasts and broad variety of bacteria. The main advantage is that protein maturation and expression of green fluorescence do not require the addition of any substrate except oxygen. A red-shift mutant gfp gene was previously used in cloning to construct pKPSPsgfp in *E.coli/ Streptococcus* shuttle vector pTRW10 (Scott et al., FEMS Microbiol. Ecol 26, 219–230 (1998)). In this work, the construct pKPSPsgfp (8.1 kb) containing the *gfp* was transferred into *Streptococcus thermophilus* by electroporation. Survival of genetically modified *S. thermophilus* in the natural environment in competition with other lactic acid bacteria was investigated using the *gfp* gene as a visual marker. Milk was inoculated with recombinant *S. thermophilus* expressing GFP and with a mixed population of yoghurt inoculants. The survival of recombinant organisms in the mixed yoghurt population was estimated after overnight incubation by serial dilution of samples on agar plate, and screening for green fluorescence.

P-40 **Screening of lactic acid bacteria from ruminant gastrointestinal tract for inhibitory activity against *Escherichia coli***. J.M. Gough, L.L. Conlan, D.O. Krause, W.J.M. Smith, C.S. McSweeney (CSIRO Livestock Industries, Indooroopilly, Qld. 4068, Australia)

The number of new enterohemorrhagic *Escherichia coli* (EHEC) serotypes implicated in food-borne illness has increased in recent years. Ruminants are considered a principal natural reservoir of EHEC, and pathogens are purportedly transmitted by cattle faeces. Reduction of *E. coli* numbers in the gastrointestinal tract (G.I.T.) by autochthonous lactic acid ruminal bacteria (LAB) able to produce inhibitory compounds may be possible. LAB isolated from the G.I.T. of cattle, were initially screened for inhibitory end-products using a deferred-antagonism plating assay against *E. coli* (serotypes 0157:H7, 026, 0111). Isolates demonstrating zones of growth inhibition were further evaluated with a micro-gel well diffusion assay. To accomplish this, inhibitory compounds were concentrated by ultrafiltration and dialysis of the supernatant and subsequently tested against *E. coli* serotypes by means of a microtitre plate assay. This assay has proved economical for large scale screening assays, is reliable and reproducible, and inhibition can be quantified using a microtitre plate reader. Results suggest that a number of the tested isolates produce compounds toxic to *E. coli* growth. These compounds may provide a useful tool for future biological control of pathogenic *E. coli* populations in the ruminant G.I.T.

P-41 **Antimicrobial properties of dietary potassium diformate in pigs.** T. Granli[a], M. Øverland[a], K.C. Kjeldsen[a], Z. Mroz[b] ([a] Hydro Formates, P.O. Box 2516, 3907 Porsgrunn, Norway; [b] IDTNO, ID-Lelystadt, P.O. Box 65, 8200 AB Lelystad, The Netherlands)

The diminishing use of antibiotic growth promoters (AGP) in animal production creates a need for alternatives. In-feed organic acids have proven to be promising candidates. Potassium diformate (KDF) is a crystalline compound based on formic acid. It is the active substance in Formi® which is approved in EU (July 2, 2001, EC No. 1334/2001) as the first non-antibiotic growth promoter for pigs. On average, KDF increases growth rate of piglets by 11% and improves feed efficiency by 6%, which is in line with what is commonly achieved with the use of AGP. An important mode of action is assumed to be the antimicrobial effect of the compound in the gastro-intestinal tract, which primarily depends on: pH, concentration of formate and action time, but which also varies with type of microorganism. We have investigated these parameters in vivo using duodenal-jejunal cannulations and the slaughter technique. Our results indicate that the antimicrobial effect of KDF is restricted to the stomach and first part of jejunum where the substance reduces pH and a high concentration of formate is still present. Nevertheless, the result of the antimicrobial effect can be observed throughout the gastro-intestinal tract and expressed in three ways: (1) a general reduction of microbial activity; leaving more nutrients for the animal and less harmful bacterial metabolites; (2) a special effect against opportunistic pathogens such as *E. coli*; (3) an improved bacterial composition of lactic acid bacteria vs. enterobacteria and lactic acid producers vs. lactic acid consumers.

P-42 **Salmonellosis in the rat: a model for gastroenteritis-type infection?** G. Grant[a], M. Duncan[a], J.M.C. Robertson[a], P.J. Naughton[b,c] ([a] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK; [b] The Robert Gordon University, Aberdeen AB10 1FR, UK; [c] University of Ulster, Coleraine, Co. Londonderry BT52 1SA, UK)

Salmonella typhi and *S. paratyphi* elicit typhoid-type and often lethal infections. There is no direct animal model for these disorders. However, *S. typhimurium* causes a severe systemic and ultimately fatal infection in susceptible mouse strains. This is widely used as a model for typhoid-like infection. The disease elicited by *S. typhimurium* or *S. enteritidis* in susceptible mice is nonetheless atypical for these serovars. They generally cause a self-limiting gastroenteritis in humans and most domesticated animals. *S. typhimurium*-infected calves display an enteritis-type disorder. However, from a practical and cost standpoint, there are significant limitations to their routine use as a model of the disease. Alternatives are needed. Male rats were given a single oral dose of *S. typhimurium* or *S. enteritidis* (10^9 CFU). Both serovars colonised and persisted in the gastrointestinal tract. They invaded primarily via the ileum, translocated to the mesenteric lymph nodes and spread in moderate numbers to the liver and spleen. However, no proliferation in the liver and spleen or development of bacteraemia was evident. In fact, the numbers of *S. enteritidis* in systemic tissues declined rapidly between 6 and 12 days post-infection whilst the levels of *S. typhimurium* remained constant. Infiltration by inflammatory cells and fluid accumulation in the gut was evident by 3 days and disruption of the villi and crypt cell hyperplasia thereafter. Faecal water and dry matter outputs were greatly increased but scouring (transient) was evident only with a limited number of rats (< 5%). The rats continued to grow throughout the study, albeit more slowly than controls. *S. typhimurium* had more marked effects than *S. enteritidis*. Salmonellosis in the rat, although not identical, has many features that are similar to the gastroenteritis-type infection observed in humans. It can therefore be useful in study of this disorder and of the effects of dietary, environmental and bacterial factors on pathogenicity.

P-43 **Interactions between pathogenic and probiotic bacteria in the porcine intestinal microflora via quorum-sensing mechanisms.** K. Hillman (SAC, Craibstone, Aberdeen AB21 9YA, UK)

The antibacterial activity of many probiotic *Lactobacillus* cultures was observed to be lost on repeated subculture. This was originally thought

to be caused by the selection of non-inhibitory subspecies by the process of subculture. The addition of non-growing *Escherichia coli* to these subcultures was found to result in the retention of the inhibitory capabilities of the lactobacilli, and the same result could be obtained with a cell-free filtrate of *E. coli* culture. Surprisingly, it was found that certain inactive cultures could be reactivated by growing them in the presence of *E. coli* cells or cell free filtrate. This led to two conclusions: (a) The inhibitory capability of these cultures had not been lost on subculture, merely inactivated. This was therefore not a case of selection of a subspecies, but of inactivation of a gene or genes. (b) The lactobacilli were capable of detecting the presence of *E. coli* and reacted by producing an antimicrobial. This detection did not require the presence of active *E. coli* cells. Therefore, the lactobacilli had reacted to something produced by the coliform, by activating bacteriocin-producing genes: in effect, an inter-species quorum-sensing mechanism. A similar effect was observed when *E. coli* was replaced with *Salmonella* spp. A nalidixic-acid-resistant *Salmonella poona* was used to examine these findings in the presence of the entire intestinal population in vitro. After 3 days dosing with a cell-free filtrate of *S. poona* culture, an inoculum of active *S. poona* was removed from the dosed in vitro system significantly faster than from a concurrent control system.

P-44 **Post mortem evolution of the concentration of *Clostridium perfringens* in the bovine digestive tract.** V. Julliand[a], C. Philippeau[a], C. Reibel[a], S. Goncalves[b], M.R. Popoff[c] ([a] Enesad, BP 87999, 21079 Dijon, France; [b] Chambre d'Agriculture de Côte d'Or, 21000 Dijon, France; [c] Institut Pasteur, 75724 Paris, France)

Enterotoxaemia represents a major cause of sudden death amongst beef cattle. Commensal bacteria, mainly *Clostridium perfringens* and *sordelli*, are implicated. A concentration between 10^6 and 10^7 CFU *Cl. Perfringens*·mL^{-1} of intestinal content (*Popoff, 1989; Manteca et al., 2001*) is considered to characterise a bovine enterotoxaemia. This microbial aetiology is difficult to assert because pathogenic bacteria develop in natural post-mortem conditions. The aim of our experimentation was to determine the post mortem evolution of the concentration of *Cl. perfringens* in the digestive tract of healthy bovines. Three calves and three young bulls of the Charolais breed were sacrified and the content of their cecum, jejunum, rumen and abomasum was collected every 3 hours for 24 hours. The concentration of *Cl. perfringens* was measured after an immediate inoculation in VL media (*Sebald and Petit, 1997*) supplemented with sheep blood on Petri plates, under anaerobic conditions. No *Cl. perfringens* was counted in the ruminal content. The threshold of 10^7 CFU *Cl. Perfringens*·mL^{-1} of jejunal content was reached after 3 hours post-mortem and got up to a maximum of 10^8. In the cecum of 2 animals, the concentration of *Cl. perfringens* reached 10^7 CFU per mL of content after 9 hours and got up to 10^7. The concentration of *Cl. perfringens* in the abomasum was very low and never exceeded 10^5 CFU·mL^{-1} of contents. Our results showed that the natural post-mortem development of *Cl. perfringens* call into question the threshold of 10^7 CFU *Cl. perfringens*·mL^{-1} of jejunal contents. More data are needed, especially for animals suspected to be dead with enterotoxaemia, to determine the microbial etiologic diagnosis of this disease.

P-45 **The use of Lactobacilli as a competitive exclusion agent for control of bacterial pathogens in poultry.** R.M. La Ragione[b], A. Narbad[b], N. Horn[b], H. Evans[b], M.J. Gasson[b], M.J. Woodward[a] ([a] Department of Bacterial Diseases, Veterinary Laboratories Agency, Addlestone, Surrey KT15 3NB, UK; [b] Institute of Food Research, Food Safety Science Division, Norwich Research Park, Colney, Norwich NR4 7UA, UK)

Poultry are a major reservoir of bacterial pathogens including *E. coli*, *Salmonella* and *Clostridia*. In recent years the removal of growth promoters from animal feed has lead to an increase in disease in food producing animals. As a consequence alternative intervention strategies are urgently required. Competitive exclusion (CE) provides such a strategy, where use is made of the natural gut micro-flora to reduce colonisation and persistence. Lactic acid bacteria (LAB) have been shown to be an essential component of CE agents. This project is aimed at the isolation and in vitro and in vivo characterisation of LAB of chicken gut origin, which have effective CE properties. A library of Lactobacilli strains were characterised in vitro using a number of in vitro adhesion assays. One isolate, strain

Lactobacillus gasseri, FI9785 was selected for further analysis in vivo. Specific pathogen free (SPF) chicks were orally inoculated with Lactobacilli FI9785 prior to challenge with one of the following organisms, *E. coli* O78:K80, *Salmonella enteritidis* and *Clostridium perfringens*. These studies concluded that a single oral inoculum of 1×10^9 CFU Lactobacilli was sufficient to suppress colonisation and persistence of *E. coli* and Clostridia in the SPF chick model. However, only marginal reductions in colonisation were observed for Salmonella. Further studies have focused on the localisation and mechanism of the protective effect conferred by the Lactobacilli.

P-46 ***Escherichia coli* O157 and non-O157 isolates are more susceptible to L-lactate than to D-lactate.** E.C. Mc William Leitch, C.S. Stewart (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

Infections caused by *Escherichia coli* O157, though infrequent, are associated with a high level of morbidity and mortality. As a result public confidence in food safety has been reduced. Organic acids have been traditionally used in the animal feed, food and pharmaceutical industries to control pathogens. One of the most effective acids against *E. coli* O157 is lactate. Investigations into the antimicrobial effect of lactate have previously focused on the commercially available D,L-lactate mixture. We decided to compare the relative contribution of the stereoisomers to the antimicrobial effect of lactate on various *E. coli* O157 and non-O157 isolates. Stationary phase cells (10^9 CFU·mL^{-1}) were treated with L-lactate or D-lactate such that the final pH was 3.8 and incubated at 37 °C. The viability was determined by standard methods. The proton motive force was determined by the centrifugation method following incubation of cultures in D- or L-lactate. L-lactate was much more bactericidal than D-lactate over a range of concentrations for an *E. coli* O157 strain. The efficacy of both isomers was dose-dependent. A similar effect was observed across a wide range of *E. coli* O157 and non-O157 isolates suggesting this effect is prevalent amongst *E. coli* strains. However, there was no difference between D-lactate and L-lactate-treated cells regarding the transmembrane pH gradient, intracellular pH or membrane potential. The viability results may have implications for the use of lactate as an antimicrobial agent and lactic acid bacteria as probiotics. As components of the proton motive force did not differ between D- and L-lactate-treated cells, it is unlikely that these factors are involved in the antimicrobial action of L-lactate. This study also highlights the potential use of the isomers of lactate as tools to elucidate the mechanism of action of lactate.

P-47 **The role of protozoa in the survival of *E. coli*, Verocytotoxin encoding bacteriophage and their VTEC lysogens in the rumen.** F.M. McIntosh, K.N. Stanley, C.S. Stewart, C.J. Newbold (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

The survival of *E. coli*, and in particular Verocytotoxin (VT) producing *E. coli* (VTEC), in the ruminant gut is an area of public health concern not only in terms of transfer to the human food chain but also in maintaining a reservoir of infection within the farm. Breakdown of bacteria in rumen fluid was determined from the release of acid soluble [^{14}C] from [^{14}C]-leucine labelled bacteria in the presence of an excess of unlabelled leucine. All *E. coli* stains tested were broken down more rapidly than commensal rumen bacteria by rumen protozoa. Since VT genes are encoded on a lysogenic bacteriophage we have investigated the release of a detoxified insertion mutant VT bacteriophage (*vt*2a $\phi 24_B$::kan) from lysogenic *E. coli* and survival of released VT bacteriophage incubated in washed protozoal fractions and cell sonicates. In washed suspensions of *Entodinium caudatum*, lysogenic *E. coli* *vt*2a $\phi 24_B$::kan were broken down faster than the naïve strain not carrying the bacteriophage. This relationship was not however observed with mixed large entodiniomophid protozoa. Phage release from lysogenic *E. coli* F38 ϕ24B::kan was stimulated when incubated with a sonicated extract from a mixed protozoal preparation but not in the presence of intact protozoa. There was a steady decline in VT bacteriophage titre in experimentally inoculated rumen fluid over a 50 h period, the rate of this decline being slightly higher in the presence of protozoa. In conclusion different patterns of *E. coli* degradation were observed among rumen protozoa populations. Although *E. coli* carrying the VT2 bacteriophage were broken down faster by *Entodinium caudatum*, the overall effect of protozoal grazing on survival of VTEC lysogens compared to non VTEC and on VT bacteriophage concentrations in the rumen is likely to be negligible.

P-48 **A novel conjugative transposon, TnB *1230*, is responsible for the transmissible tetracycline resistance between strains of *Butyrivibrio fibrisolvens*.** C.M. Melville[a], P. Mullany[b], H.J. Flint[a], K.P. Scott[a] ([a] Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, UK; [b] Eastman Dental Institute, University College London, London, UK)

The world-wide use of antibiotics in both human and veterinary therapeutics and as prophylactics and growth promoters, has resulted in rapid emergence and spread of antibiotic resistance. Whilst studies so far have concentrated on resistance in pathogens, commensal bacteria are fast becoming recognised as important reservoirs for the transfer of these genes. Recently we identified a novel tetracycline resistance gene, *tet*(W) amongst important rumen, porcine and human gut commensal bacteria. The remarkable sequence conservation between different copies of *tet*(W) found in environmental and phylogenetically distinct bacteria implies rapid transfer of this gene in nature. Previous work showed that *tet*(W) could be transferred in vitro between strains of the rumen anaerobe *Butyrivibrio fibrisolvens* and that resistance transfer was accompanied by transfer of a 50–60 kb chromosomal fragment. Apart from this, however, very little is known about the transfer of *tet*(W) in *B. fibrisolvens* or in other Gram-positive anaerobes known to harbor *tet*(W). This work provides the first information on a genetic element involved in transfer of *tet*(W), and the first identification of a conjugative transposon from a rumen anaerobe. Analysis of the regions flanking *tet*(W) in *B. fibrisolvens* reveals two direct repeat structures that represent possible IS elements and a series of open reading frames whose closest similarity is with a conjugative transposon from *Enterococcus faecalis*. The identification of novel tetracycline resistance genes amongst some of the most predominant gut anaerobes highlights the importance of commensal bacteria as reservoirs for the onward spread of antibiotic resistance.

P-49 **A bioreactor system to study the survival of *Salmonella* in pig gut content.** P.J. Naughton[a,b], B.B. Jensen[b] ([a] Northern Ireland Centre for Food and Health, School of Biomedical Sciences, University of Ulster (at Coleraine) BT52 1SA, UK; [b] Microbiology Unit, Dept. of Animal Nutrition and Physiology, Foulum Research Centre, P.O. Box 50, 8830, Tjele, Denmark)

The removal of antibiotics from pig feed and the resultant need to find alternatives in order to reduce the incidence of pathogenic bacteria in the pig gastrointestinal tract has led to the need for appropriate in vitro systems to test such alternatives. The aim of this study was to develop an in vitro method to model the effects of different diets and feed additives on the incidence of *Salmonella* and to test how different organic acids at different pH effected the survival/growth of *Salmonella* in stomach content. Six bioreactors with an operating volume of 800 mL were used with pooled digesta from the stomach of slaughter pigs. The slurry used in the bioreactors consisted of content diluted 1:1 with anaerobic salt medium. The pH was maintained using a pH controller. Each bioreactor was inoculated with 10^9 *Salmonella enterica* var Typhimurium DT12. Samples were taken at 0, 1, 2, 3, 4 and 6 hours. We examined various organic acids and lactic acid for their effects on *Salmonella* survival in stomach content at pH 4, 5 and 6. Lactic acid and benzoic acid were found to be the most effective in reducing *Salmonella* growth. This in vitro system provides a useful and relatively inexpensive tool to screen how various feeding conditions and feed additives effect the incidence of *Salmonella* in the pigs and hence aids in the selection of products to be tested in vivo or to explain the results of products tested in vivo.

P-50 **Mutations in a bacterial host affecting CAT-mediated chloramphenicol resistance mechanism.** J. Potrykus, G. Węgrzyn (Department of Molecular Biology, University of Gdańsk, Kładki 24, 80-822 Gdańsk, Poland)

Antibiotic resistance of bacterial pathogens poses a serious health problem, rendering the commonly used antibacterial agents ineffective. Evaluating the impact of specific genetic traits in the bacterial host on the expression of resistance mechanisms is a complicated issue, potentially useful in designing new drugs. Studies on a model non-pathogenic gut commensal, *Escherichia coli*, might contribute to our understanding of its pathogenic counterparts. In our laboratory we are investigating a mutant

E. coli strain CM2555, which is sensitive to chloramphenicol despite the presence and expression of *cat*, the resistance gene. Quite paradoxically, the gene product, CAT (chloramphenicol acetyltransferase), retains its biological and biochemical activity in this strain. Our recent studies show that the mutant carries a mutation in *acrA*, a gene encoding a component of TolC-AcrAB efflux system, which in this genetic setting contributes to the chloramphenicol-sensitivity phenotype. Deletion of the *acrAB* locus caused chloramphenicol sensitivity of some other, but not all, *cat*-expressing *E. coli* strains tested. At least one additional mutation seems to be responsible for the observed antibiotic sensitivity. Our preliminary results imply a defect in the synthesis of Acetyl Coenzyme A. A decline in Acetyl-CoA level was observed in the *cat*-carrying mutant upon the addition of chloramphenicol. This effect was reversed when the growth medium was supplemented with sodium acetate, an alternative source for Acetyl-CoA production, leading to stable Acetyl-CoA levels in the presence of chloramphenicol. This was also observed for a control strain with an *acrAB* deletion, carrying *cat* on a multicopy plasmid. We are currently working on mapping the specific mutation responsible for the phenotype of CM2555 strain.

P-51 **DNA modification in rumen bacteria – occurrence of Dam and Dcm modification.** P. Pristaš[a], M. Neuzilova[b], P. Javorský[a] ([a] Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovakia; [b] Department of Biochemistry, Faculty of Natural Sciences, Pavol Josef Safarik University, Košice, Slovakia)

While DNA methylation is the main mechanism of gene expression control in eukaryotes, the only documented case of prokaryotic methylation involved in the regulation of cellular processes is Dam methylation. Screening of large numbers of bacteria has revealed the presence of Dam methylation in cyanobacteria as well as in the group of related families of *Enterobacteriaceae* (which includes *E. coli*), *Parvobacteriacea*e and *Vibrionaceae*. The presence of N6-methyladenine and C5-methylcytosine at GATC (Dam) and CCA/ TGG (Dcm) sequences in DNAs of 45 strains belonging to 11 prevailing species of rumen bacteria was investigated using sensitive methylation discriminating isoschizomeric restriction enzyme analysis. While all but one strain was deficient in Dcm modification, methylation at GATC sequences was detected in 18 out of 42 tested strains, predominantly in bacterial species belonging to the *Sporomusa* subbranch of low % G + C Gram-positive bacteria (*Seleno-monas ruminantium*, *Megasphaera elsdenii*, *Mitsuokella multiacidus*). Using *E. coli* dam specific primers no sequences similar to *Eco-dam* gene were found in any Dam positive strain, indicating that the appearance of Dam modification in *Sporomusa* could be due to the presence of evolutionary new Dam encoding gene.

P-52 ***Salmonella enterica*** **serovar Enteritidis: involvement of flagella and fimbriae in infection of the rat.** J.M.C. Robertson[a], N.H. McKenzie[a], M. Duncan[a], E. Allen-Vercoe[b], M.J. Woodward[b], H.J. Flint[a], G. Grant[a] ([a] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK; [b] Veterinary Laboratories Agency (Weybridge), Addlestone, Surrey KT15 3NB, UK)

S. enterica serovar Enteritidis (*S. enteritidis*) continues to be a major source of infection in humans and domesticated animals. In the majority of cases, it causes self-limiting gastro-enteritis. However, it can occasionally elicit a chronic systemic infection that may lead to death. Alternatively, it may establish an asymptomatic carrier state. The nature and severity of the infection is linked to expression of virulence factors, such as adhesins, flagella, toxins and other cell-modulating compounds by the pathogen. The roles of five fimbriae ([SEF14, SEF17, SEF21, pef, lpf] and flagella in pathogenicity have been investigated using a rat model of salmonellosis. Insertionally inactivated mutants of *S. enteritidis* unable to express SEF14, SEF17, SEF21, pef and lpf (*fim–/fla+*) colonised the gut and spread systemically to the mesenteric lymph nodes, liver and spleen as effectively as wild-type parent (*fim+/fla+*) strains. In contrast, aflagellate (*fim+/fla–*) strains were less able than *fim+/fla+* to persist in upper gastrointestinal tract, the inflammatory responses they elicited in the gut were less severe and spread to the liver and spleen was transiently reduced. Despite this, single (*fim+/fla–*) and multiple (*fim–/fla–*) deletion mutants still caused a significant and persistent infection, albeit of lower severity than that elicited by *fim+/fla+* strains. In the short-term, *fim+/fla–* mutants

appeared slightly more able than fim–/fla– strains to remain in the upper gastrointestinal tract. Functional flagella were thus important for induction and persistence of salmonellosis in the rat. In contrast, SEF14, SEF17, SEF21, pef and lpf did not appear to be a prerequisite for development of infection, although they may have had transient roles. Deletion of flagella and SEF14, SEF17, SEF21, pef and lpf (*fim–/fla–*) significantly reduced but did not abolish pathogenicity. Other virulence factors therefore compensated for the loss of these components.

P-53 **The effect of "propolis-ginseng" extracts in anti-*Helicobacter pylori* therapy.** A.I. Sidorov[a], E.M. Sulman[a], L.E. Smirnova[b], V.F. Vinogradov[b] ([a] Tver State Technical University, 22, A. Nikitin str., Tver, 170026, Russia; [b] Tver State Medical Academy, Sovetskaya str., 4, Tver, 170642, Russia)

The purpose of the present study was to evaluate the effect of "propolis:ginseng" extracts in anti-*Helicobacter pylori* therapy. Acid suppressant therapies are known to aid *H. pylori* eradication but the reduction of acid secretion leads to the enteric flora increasing and probably reduces the resistance to occasional infections. Here, two groups of patients were tested in anti-*H. pylori* therapy. The main group took the extracts of propolis and ginseng along with the therapy. Ethanolic extracts of "propolis:ginseng" were prepared via ultrasonic extraction in the weight ratios of 3:7, 5:5 and 7:3 using 30%, 50%, 70% ethanolic solutions. Propolis and ginseng samples were collected in Tver region (Russia). The extracts were analyzed by IR- and UV-spectroscopy to identify the total flavonoids and ginsenosides content. Acceleration of *H. pylori* eradication and normalization of T-cell immunity by 30% (comparing to the control group) were found for the main group patients. In vitro experiments showed that the propolis-ginseng extracts are able to modulate the heterofermentation processes.

P-54 **Antibacterial effect of fatty acids on *Escherichia coli.*** V. Skřivanová, M. Marounek (Research Institute of Animal Production and Institute of Animal Physiology and Genetics, Uhříněves, Prague 104 00, Czech Republic)

Antibacterial activity of C_2–C_{18} fatty acids was tested in three strains of *Escherichia coli*. Bacteria were grown under a CO_2 atmosphere in a medium containing glucose (5 g·L^{-1}), bactopeptone and yeast extract. Acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic and oleic acid were added at 0, 0.1, 0.2, 0.3, 0.5, 1, 2, 3 and 5 mg·mL^{-1}, together with an equivalent amount of NaOH. Cultures were cultivated at 39 °C for 24 h, then the pH was measured and glucose determined enzymatically. The residual glucose concentration was plotted against concentration of a fatty acid. The IC_{50} was the fatty acid concentration in which only 50% of the initial glucose was utilized. In control cultures and those containing C_2–C_6 fatty acids the optical density at 640 nm was measured. In control cultures the pH fell from 6.70 ± 0.09 to 5.73 ± 1.57 during incubation. *E. coli* strains proved sensitive to medium-chain fatty acids. Utilization of glucose was inhibited by caprylic (C_8) and capric (C_{10}) acid. In cultures of the strain ATCC 25922, CCM 3954 and CCM 4225 the IC_{50} values were 0.90, 0.45 and 0.28 mg of caprylic acid per mL, respectively. Corresponding IC_{50} values in cultures with capric acid were 2.70, 2.03 and 1.25 mg·mL^{-1}. Other acids did not influence glucose utilization. Caproic acid (C_6) decreased optical density of cultures in a dose-dependent manner, by 44– 57% at 5 mg·mL^{-1}. Effect of valeric acid (C_5) was less pronounced. No effect of C_2–C_4 fatty acids was observed. (This study was supported by grant 523/02/0460 of the Grant Agency of the Czech Republic).

P-55 **Transfer and survival of verocytotoxin encoding bacteriophage in the ruminant gut.** K.N. Stanley[a], C. James[b], H. Allison[b], J. Saunders[b], A. McCarthy[b], H.J. Flint[a], C.S. Stewart[a] ([a] Gut Microbiology and Immunology, Rowett Research Institute, Aberdeen AB21 9SB, UK; [b] Environmental and Molecular Microbiology Group, School of Life Sciences, University of Liverpool, Liverpool, UK)

The main reservoir of Verocytotoxin (VT) producing *Escherichia coli* (VTEC) is ruminant animals reared for meat. The potential for transmission of *vt* genes to *E. coli* or other enteric bacterial species via the lambdoid bacteriophage is of considerable public health concern. These bacteriophage could contribute to the survival

and dissemination of *vt* genes in the gut microflora of animals. A recombinant VT2 bacteriophage has been created by interruption of the vt_2A subunit gene through insertion of a kanamycin resistance cassette (*aph3*). This bacteriophage has been used to study the survival of free bacteriophage and transfer of *vt* genes between wild type *E. coli* in rumen fluid, faecal batch culture and rumen and colon simulating continuous culture fermentors. We have produced evidence that this bacteriophage can infect and lysogenise *E. coli* strains under the conditions found in the ruminant gut. Bacteriophage survival in a mixed flora background was determined using a plaque assay and putative lysogens were selected on kanamycin agar. Lysogens were confirmed by colony hybridisation and DNA amplification of *vt2a*::*aph3* followed by induction of the lytic cycle with norfloxacin to release phage particles. The data demonstrate that the ruminant gut is a potential site of *vt* gene transfer via bacteriophage.

P-56 **Use of fermentable fibre in diets for weaning piglets.** F. Tagliapietra, M. Bonsembiante, S. Schiavon, L. Bailoni (Department of Animal Science, University of Padua, Agripolis, Legnaro, Italy)

The inclusion of fibrous ingredients to manipulate the gut microflora in early weaning piglets must be evaluated taking into account the effects on feed intake, growth and health state. Two antibiotic free diets, a control and a diet (SBP) containing 120 $g \cdot kg^{-1}$ of unmolassed sugar-beet-pulp, were fed to 668 piglets 3 to 10 weeks old, divided in two groups. Live weight and feed consumption were measured every 2 weeks. The number and weight of dead and removed animals were recorded. During the first 4 experimental weeks faecal samples were collected from 32 subjects to be analysed for proximate composition, end products of fermentation and microbial count. Bioptic samples of rectum mucosa were collected and analysed for myeloperoxidase content and DNA adducts. No signs of diarrhoea were observed. SBP exhibited a significant (χ^2, $P < 0{,}01$) reduction of piglets dead and removed (13% and 5%, respectively) as respect to the control. Meningitis due to *Streptococcus spp.* was the major cause of mortality. Small differences of live weight (26.9 and 25.6 kg, $P < 0.01$) and feed conversion rate (1.45 and 1.47) were observed at the end of the trial for the control and SBP groups, respectively. SBP increased significantly ($P < 0.01$) the faecal concentration of acetic and propionic acid (11.9 to 16.4, and 6.0 to 7.1 $\mu g \cdot g^{-1}$ dm, respectively). A significant reduction ($P < 0.01$) of *Escherichia coli* (8.0 and 7.1 $\log_{10}$) and *Staphylococcus ssp.* (8.1 and 6.7 $\log_{10}$) were also observed in the SBP faeces during the first two weeks from weaning. No differences were detected for the analytical measurements performed on tissues. SBP can be conveniently used to improve the health state and maintain growth performance of weaned piglets.

P-57 **Identification of tetracycline resistance determinants in *Salmonella* within swine production facilities in Illinois.** B. Teferedegne, D.A. Barber, R.E. Isaacson, R.M. Weigel, B.A. White (University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA)

The distribution of tetracycline resistance (tet^r) efflux genes amongst 206 *Salmonella* isolates collected from ten swine farms was determined. *Salmonella* were isolated from fecal samples from swine, cats, mice, feed, homogenized insect body parts, floor samples, boot scrapings and bird fecal droppings. The presence of tetracycline resistance genes encoding the efflux phenotype was assessed by the polymerase chain reaction using degenerate primers for various genotypes. There were different patterns of distribution of tet^r amongst and within these farms. The overall prevalence of tet^r in all the farms was 79%. The TetC determinant was identified in 70% of the isolates, whereas the TetA and TetB determinants were identified to a lesser extent (35% and TetB 4%, respectfully). More than 20% of the isolates that tested positive for one class of determinant also harbored another class of determinant (TetA and TetC, 20% and TetB and TetC, 3.4%). No PCR amplicon was detected for any of the other tet^r determinants. The prevalence of isolates which were phenotypically tetracycline resistance negative, but genotypically tetracycline resistance positive was 8.7%. The diversity of these different classes of tet^r genes from different sources was analyzed using denaturing gradient gel electrophoresis (DGGE). DGGE analysis did not detect any diversity

within these classes of genes which might suggest that the same tet^r genes were circulating within and between farms. DNA sequence analysis of these amplicons is needed to confirm this hypothesis. These isolates were also screened for phenotypic resistance to a number of antibiotics. There was no correlation between resistance to multiple antibiotics and the tet^r genotype.

P-58 **Distant interactions regulating bacterial growth.** M.V. Trushin, I.G. Syomina, V.M. Chernov (Kazan Institute of Biochemistry and Biophysics, 420111 Kazan, P.O. Box 30, Russia)

We have investigated distant interactions of bacterial cells mediated by physical fields. Previously it was shown that distant interaction plays a key role in regulation of: (i) cell division; (ii) adaptation of microorganisms to stress conditions; (iii) adhesive capacities of cells. Our experiments were performed with *Escherichia coli*. Growth media was LB. Experiments were designed using device "flask-in-flask". Growth were monitored using light scattering rate at $\lambda = 600$ nm (optical density - OD) measured with the use Specord M40 Spectrophotometer. Data have been processed with statistical program STATISTICA for Windows (release 5.0) using tests of Wilcoxon and sign. All the experiments were performed in duplicates and were repeated no less than 4 times. It was founded that cells with initial OD 0.2–1.2 (signal transmitters) decreased specific speed of logarithmic growth and the harvest of signal recipient cells. In the same time the duration of lag-phase depended on initial OD of signal transmitter cells. The influence of signal transmitters with initial OD 0.2–0.4 resulted in reduction of lag-phase in recipient cells. In other cases the effect was reversed. We assume that these effects are attributed to the emission of electromagnetic signals in the visible region.

P-59 **Colonization and transformation capacity of *Acinetobacter calcoaceticus* and *Bacillus subtilis* in the intestinal tract of gnotobiotic rats.** A. Wilcks, B.L. Jacobsen (Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration, Moerkhoej Bygade 19, 2860 Soeborg, Denmark)

The naturally competent bacteria *Acinetobacter calcoaceticus* and *Bacillus subtilis* can be isolated from different sources, including the environment and food. Both bacteria are able to take up DNA by transformation in aquatic and terrestrial environments, and for *B. subtilis* transformation in different foodstuffs has also been reported. The objectives of this study were to ascertain whether *Acinetobacter* and *B. subtilis* are able to colonise the gastrointestinal tract of gnotobiotic rats, and take up DNA by transformation. *Acinetobacter* was unable to colonise the germ-free rats. Even though the rats were dosed several times, no living bacteria could be detected in faeces 24 h after dosing. DNA from the bacteria could be detected by PCR in the faeces samples. *B. subtilis* colonised the rats poorly; although the animals were dosed each day with 10^9 CFU·mL^{-1} of bacteria, the concentration in faeces only reached about 10^5 CFU·g^{-1} faeces. The animals were fed with plasmid DNA for three weeks, but no transformants could be detected in faeces. At the end of the experiment the animals were sacrificed, and intestinal samples were taken out and analysed for the presence of transformants and plasmid DNA. Again no transformants were found, but DNA could be detected in all parts of the gastrointestinal tract. Some of the samples were able to transform competent *Escherichia coli* cells by electroporation, indicating that the plasmid DNA was intact after passage of the gastrointestinal tract.

P-60 **Suppression of enterotoxigenic *E. coli* population in piglet intestines by a *Bacillus* probiotic.** L. Zhao[a], L. Sun[a], G. Song[b], G. Wei[a] ([a] Shanghai Jiao Tong University, Shanghai, China; [b] Shanxi University, Taiyuan, China)

A probiotic based on *Bacillus subtilis* YK-1R provided similar if not higher efficacy to antibiotics in controlling bacterial diarrhea among nursing piglets. Structural shifts of intestinal microflora were systematically monitored in two groups of day 1 old diarrheal piglets receiving antibiotic vs. probiotic treatment. After treatment with antibiotics for 4 days, piglets stopped diarrhea but developed into constipation after one week. In the probiotic treated group, diarrhea stopped after 24–48 hrs and remained healthy thereafter. At the time of weaning, the body weight of piglets in the probiotic treated group

was ca 8% higher than the antibiotic control. The enterotoxigenic *E. coli* population in the diarrheal intestines treated with this probiotic decreased to a level non-detectable by Southern blotting with the enterotoxin Stb gene probe 2–3 days earlier than the antibiotic control. The Shannon's index based on ERIC-PCR fingerprints with total fecal DNA as templates indicated that the probiotic group achieved a more diversified and balanced microbial community structure in the intestine during the treatment. One 1.2 kb size, disease-associated and one 2.7 kb health- associated signature band were identified in the ERIC-PCR profiles for fecal population structures. Cloning and sequencing of DNA fragments revealed a predominant clone 100% homologous with *E. coli* genome from the former, and a clone with no homology with all sequences in GenBank from the latter. Specific primers designed to amplify the *E. coli* clone and the unknown health-associated bacterium were used successfully as biomarkers for a microbiologically balanced intestine. This therapeutic *Bacillus* probiotic has the potential to be an alternative to antibiotics in therapy and prevention of bacterial diarrhea among piglets.

Session IV:

Fermentation (1) – substrate breakdown

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O-16 **Cellulosome organisation in *Ruminococcus flavefaciens* 17 involves multiple dockerin-binding specificities.** S.I. McCrae[a], M.T. Rincon[a], S.-Y. Ding[c,d], R. Lamed[d], J.C. Martin[a], V. Aurilia[b], V. Shoham[e], E.A. Bayer[c], H.J. Flint[a] ([a] Microbial Genetics Group, Rowett Research Institute, Aberdeen AB21 9SB, UK; [b] IABBAM Napoli, Italy; [c] Weizmann Institute, Rehovot, Israel; [d] University of Tel Aviv, Israel; [e] Technion, Haifa, Israel)

Cellulosome complexes have now been described from a range of Gram-positive cellulolytic bacteria. These complexes are characterised by interactions between dockerin sequences in enzyme subunits and cohesin domains present in structural proteins found on the cell surface. Recent work has demonstrated that the rumen bacterium *Ruminococcus flavefaciens* 17 produces at least two structural proteins, designated ScaA and ScaB, that carry multiple cohesin domains (Ding et al., J. Bacteriol. 183 (2001) 1945-1953). The seven cohesins of ScaB were found to interact specifically with a dockerin present at the C-terminus of ScaA, rather than with enzyme subunits, and ScaB is therefore considered as a putative cell wall anchoring protein to which ScaA molecules attach. We have now shown that the *R. flavefaciens* enzymes EndB, XynB and XynD, whose dockerin sequences resemble each other, all interact specifically with cohesins from the 130 kDa ScaA protein. On the other hand the enzymes XynE and CesA, whose dockerins show some divergence from the other enzymes in their sequences (Aurilia et al., Microbiology 146 (2000) 1391-1397), failed to bind to ScaA, and instead recognise other *R. flavefaciens* proteins. Thus, also including the specific interaction between the ScaA dockerin and ScaB cohesins, three distinct dockerin-cohesin binding specificities have been identified to date in *R. flavefaciens* 17. This provides clear evidence that different groups of enzyme from a single cellulolytic species can differ in their dockerin-binding specificities, and implies that more than one type of plant cell wall-degrading enzyme complex may be elaborated by *R. flavefaciens.*

O-17 **Adhesion to cellulose of the Gram-positive rumen bacterium *Ruminococcus albus* involves type IV pili.** H. Rakotoarivonina, G. Jubelin, B. Gaillard-Martinie, E. Forano, P. Mosoni (Unité de Microbiologie, INRA, Centre de Recherches de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France)

Ruminococcus albus is a predominant cellulolytic bacterium from the rumen. Adhesion to cellulose, which is an important step in the cellulolytic process, appears to involve several mechanisms in this species. This study aimed at characterising a cell surface 25 kDa glycoprotein (GP25) that was previously shown to be underproduced by a spontaneous adhesion-defective mutant D5 of *R. albus* 20. An antiserum against wild-type strain 20 adsorbed with the mutant (Anti-Adh serum) blocked adhesion of *R. albus* 20 and reacted mainly with GP25 in bacterial and extracellular protein fractions of strain 20. The N-terminal sequence of purified GP25 was identical to that of CbpC, a 21 kDa cellulose-binding protein (CBP) of *R. albus* 8. The gene encoding GP25 was isolated and sequenced. It encoded a protein of 165 amino acids and 16.9 kDa that showed 73% identity with CbpC and presented homologies with type IV pilins of Gram-negative pathogenic bacteria. Negative-staining electron microscopy revealed fine and flexible pili surrounding *R. albus* 20 cells while mutant cells were not piliated. Immunoelectron microscopy showed that the anti-Adh serum, probing mainly GP25, completely decorated the pili surrounding *R. albus* 20, thereby showing that GP25 was a major pilus subunit. This study shows for the first time the presence of pili at the surface of *R. albus* and identifies GP25 as their major protein subunit. Though GP25 was not identified as a CBP, isolated pili were shown to bind cellulose. In conclusion, these pili, which belong to the family of type IV pili, mediate adhesion of *R. albus* 20 to cellulose.

O-18 **Proteomics-based analysis of *Ruminococcus albus* 8 adhesion-defective mutants.** E. Devillard, D. Goodheart, M. Morrison (The Ohio State University, Columbus, OH, USA)

We report here the use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), mass-spectrometry (MS) methods and genome sequence data, to examine mutant strains of *Ruminoccus albus* 8 that are defective in their adhesion to cellulose. These mutant strains have been cultured with both cellobiose and cellulose,

and their cell surface-associated proteins were extracted with N-Lauroyl Sarcosine. When cultured with cellobiose, all the mutant strains were found to lack two proteins (PpaA and PpaB), which in separate studies, have been shown to increase in the wild-type strain when either ruminal fluid, or phenylacetic and phenylpropionic acids (PAA/PPA), are added to the media. Cellulose degradation and growth of these mutant strains are very similar to that observed when the wild-type strain is cultured in the absence of ruminal fluid or PAA/PPA, that is, much less than what is observed under permissive conditions. We have compiled N-terminus and peptide sequence, and peptide mass fingerprints for both PpaA and PpaB, by Edman degradation and MALDI-TOF analysis, respectively. The peptide sequences have been used to query the unfinished sequence for the genome of *R. albus* strain 8, available from The Institute for Genomic Research. All putative open reading frames identified by these BLAST searches show a high degree of sequence similarity with multi-domain cellulases, although type I dockerin sequences are absent from most of these ORF's. However, none of the presumed "full-length" ORF's identified in these searches possessed a peptide mass fingerprint that closely matches those obtained for PpaA and PpaB by MALDI-TOF analysis. Currently, these discrepancies may be explained by either incomplete sequence data (some ORF's remain truncated) and (or), a high degree of post-translational modification of PpaA and PpaB, presumably by glycosylation.

O-19 **The *Fibrobacter succinogenes* strain S85 genome sequencing project.** K. Nelson[a,b], R. Aminov[a], C. Forsberg[a], R.I. Mackie[a], J.B. Russell[a], B.A. White[a], D.B. Wilson[a], S. Mulligan[b], K. Tran[b], H. Carty[b], H. Khouri[b], W. Nelson[b], S. Daugherty[b], C. Fraser[b], M. Morrison[a]. ([a] The North American Consortium for Genomics of Fibrolytic Ruminal Bacteria, and [b] The Institute for Genomic Research, Rockville, MD, USA)

The North American Consortium for Genomics of Fibrolytic Ruminal Bacteria is a USDA-supported initiative established in 2000, and our overarching objective is to generate and exploit genomics-based information to better understand the genetics, physiology and ecology of predominant fiber-degrading rumen bacteria. We report here the progress to date with the *Fibrobacter succinogenes* S85 genome project. The random sequencing phase of the project was completed in January 2002, from 75 461 sequences with an average edited length of 641 bp. There were 266 assemblies, for a total of 3.916 Mbp, approximately 0.5 Mbp larger than the genome size predicted from physical maps. Of the initial 179 sequencing gaps identified, only 18 now remain to be closed by multiplex PCR and final editing. The sequence data has been searchable since July 2001, at www.tigr.org. From the preliminary gene list, we have identified 24 genes encoding endoglucanases and cellodextrinases: thirteen belong to family 5; seven in family 9; one in family 45, and three more like CelF, which possess multiple catalytic domains. Another 23 genes have been identified as encoding xylanases and related enzymes, and there are additional glycosyl hydrolase genes as well, belonging to families 2, 3, 8, 10, and 13. Many of these genes had not been previously identified in clone libraries propagated in *E. coli*. Furthermore, we have yet to identify any exo-acting cellulase (from family 6 or 48), nor is there a processive endoglucanase apparent in the genome sequence data available. Both type I and type II secretion systems are present, in addition to Pil-T1 homologs that coordinate twitching motility in other gram-negative eubacteria. Whole genome annotation by the Consortium should begin in late March or early April.

O-20 **Identification of functional gene subsets from the cellulolytic organism *Ruminococcus flavefaciens* using a comparative genomics approach.** D.A. Antonopoulos, B.A. White (University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA and the North American Consortium for Genomics of Fibrolytic Ruminal Bacteria)

Subtractive hybridization has been utilized to compare the genomes of two closely related, highly cellulolytic strains of *Ruminococcus flavefaciens* that differ at the 16S rDNA level by 2%. PCR-based subtractive hybridization amplifies unique regions of a genome, relative to another, based on modification of restriction enzyme sites. Conservation of specific restriction enzyme

sites in two genomes effectively "subtracts" the intervening region between restriction sites from further analysis. The PCR-based subtractive hybridization was highly efficient at recovering sequences unique to strain FD-1 and not JM1 (based on a 288-clone sampling size). Examples of the unique sequences retrieved included regions with similarity to a non-ribosomal peptide synthetase (*Streptomyces avermitilis*), and a *Sal*I restriction endonuclease (*Streptomyces albus*). Bacteriocin-mediated competition has been demonstrated in *Ruminococcus*, and thus a non-ribosomal peptide synthetase would be consistent with production of bacteriocin-like products by this organism. Additionally, the presence of a *Sal*I restriction endonuclease complements previously demonstrated *Sal*I isoschizomer activity (*Rfl*FII) isolated from this strain. These sequences exhibit very weak similarity with those described in the current *Ruminococcus albus* 8 genome sequence dataset (preliminary sequence data was obtained from the TIGR website; funded by the USDA via the NACGFRB). Retrieved sequences with significant levels of similarity to those in the *R. albus* 8 dataset include a putative carbon monoxide dehydrogenase and nitrogenase (78% and 60% conservation at the nucleotide level respectively). The sequence information gained here further reiterates the competitive nature between strains in the ruminal ecosystem and provides a functional basis for strain differentiation.

P-61 **Characterization of a family 11 carbohydrate-binding module from CelJ: importance of the CBM to cellulose hydrolysis.** T. Arai, S. Karita, T. Kimura, K. Sakka, K. Ohmiya (Faculty of Bioresources, Mie University, Tsu 514-8507, Japan)

CelJ, a 178-kDa major component of the *Clostridium thermocellum* cellulosome, is a modular enzyme. It is composed of an N-terminal signal peptide and six modules in the following order: a family 11 carbohydrate-binding module (CBM), an immunoglobulin-like module (Ig), a family 9 catalytic module (CM9), a family 44 catalytic module (CM44), a dockerin module, and an X module of unknown function. Truncated derivatives of CelJ were constructed: CBM-CM9 consisting of CBM and CM9, Ig-CM9 consisting of Ig and CM9, the polypeptide CM9P of CM9 only, CBML consisting of CBM and Ig, and CBMS consisting of the CBM only. CBM-CM9 showed strong activity toward carboxymethylcellulose (CMC), barley β-glucan and lichenan, hydrolysis of cellooligosaccharides, and low activity toward Avicel, acid-swollen cellulose, and xylan. Analysis of CMC hydrolysis suggested that CBM-CM9 is a semiprocessive enzyme with both endo- and exoglucanase activities. By contrast, Ig-CM9 and CM9P devoid of the CBM showed negligible activity toward CMC, i.e. about 1/1000 of the activity of CBM-CM9, suggesting that the CBM participates in the catalytic function of the enzyme. Affinity of the CBM for insoluble polysaccharides was measured. The CBM bound Avicel, ball-milled cellulose, acid-swollen cellulose, lichenan, agarose, and chitin. The capacity of CBML and CBMS to bind to a series of different soluble polysaccharides was qualitatively evaluated by affinity native gel electrophoresis. The migration of the CBM was significantly retarded by inclusion of hydroxyethyl-cellulose, methyl-cellulose, lichenan, and barley β-glucan. The affinity of CBML and CBMS for cellooligosaccharides was analyzed by isothermal titration calorimetry. The K_a (binding constant) values were determined to be 2.7×10^5, 3.9×10^4, 2.8×10^3, respectively.

P-62 **In vitro fermentability of carbohydrates by faecal microflora of pigs at weaning.** M.W. Bosch, B.A. Williams, L.W. Bos, M.W.A. Verstegen (Animal Nutrition Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen, The Netherlands)

Prebiotics are considered to be a possible alternative for antibiotic growth promotors for weaner piglets. Prebiotics lead to a shift in the microbial population in the GIT which will be beneficial for host health. To formulate a diet with potentially suitable prebiotic properties, a range of carbohydrates was tested for fermentability using the in vitro cumulative gas production technique. This technique is used to measure fermentation kinetics (total gas production and rate of gas production) as well as fermentation end-products (VFA, NH_3). Contents from the second half of the colon of six unweaned pigs (no creep feed or antibiotics) was mixed and used as an inoculum to assess the fermentability of

45 feedstuffs. Rapidly fermentable carbohydrates are more likely to be fermented, earlier in the digestive tract (ileum), while substrates with a slower rate of fermentation are more likely to be fermented later in the tract (hindgut). The maximum rate of gas production, and the time at which this rate occurred were 11.5 $mL \cdot h^{-1}$ and 13.6 h for sugarbeet pulp, 8.2 $mL \cdot h^{-1}$ and 27.9 h for wheat starch, 21.6 $mL \cdot h^{-1}$ and 9.3 h for lactulose and 19.8 $mL \cdot h^{-1}$ and 8.7 h for inulin. Based on these data, in combination with values for other end-products, these four ingredients were added to a diet for an in vivo experiment with weaner pigs. It was hypothesized that this combination would stimulate fermentation along the whole tract.

P-63 **Concurrent maltodextrin and cellodextrin synthesis by *Fibrobacter succinogenes* S85 as identified by 2D NMR.** A.-M. Delort[a], M. Matulova[a], R. Nouaille[a,b], G. Gaudet[b], E. Forano[b] ([a] Laboratoire de Synthèse et Étude de Systèmes à Intérêt Biologique, UMR 6504 Université Blaise Pascal - CNRS, 63177 Aubière cedex, France; [b] Unité de Microbiologie, INRA, Centre de Recherches de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France)

Fibrobacter succinogenes S85, one of the main cellulolytic rumen bacteria, degrades cellulose into glucose and cellobiose which are further metabolised to succinate, acetate and a small amount of formate. It is also able to store large amounts of glycogen. In this work, 1D and 2D NMR experiments were used to analyse the synthesis of various metabolites by resting cells of *Fibrobacter succinogenes* S85 when incubated with [1-^{13}C]glucose, both in the extracellular and in the cell media. Besides the expected glycogen, succinate, acetate, glucose-1-*P* and glucose-6-*P*, the presence of maltodextrins and cellodextrins was detected. Maltodextrins, whose synthesis is demonstrated here for the first time, were excreted into the external medium. They were found to have linear structures with a maximum degree of polymerisation (DP) of about 6–7 units. Cellodextrins were located in the cells (cytoplasm and /or periplasm), their DP was up to 4. Both labelled ([1-^{13}C] and [6-^{13}C]) and unlabelled maltodextrins and cellodextrins were detected showing the contribution of carbohydrate cycling in *F. succinogenes*, including the reversal of glycolysis and the futile cycle of glycogen. Mechanisms of oligosaccharide synthesis are discussed. (Matulova M., et al., Eur. J. Biochem. 268 (2001) 3907–3915; Bibollet X., et al., J. Biotechnol. 77 (2000) 37–47; Matheron C., et al., Appl. Environ. Microbiol. 65 (1999) 1941–1948; Matheron C., et al., Appl. Environ. Microbiol. 64 (1998) 74–81; Matheron C., et al., Biodegradation 9 (1998) 451–461).

P-64 **Degradation of cellulose and ^{13}C-enriched lignocellulosic fibres by *Fibrobacter succinogenes* S85. A liquid and solid state NMR study.** A.-M. Delort[a], R. Nouaille[a,b], M. Matulova[a], E. Forano[b] ([a] Laboratoire de Synthèse et Étude de Systèmes à Intérêt Biologique, UMR 6504 Université Blaise Pascal – CNRS, 63177 Aubière cedex, France; [b] Unité de Microbiologie, INRA, Centre de Recherches de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France)

In the rumen, *Fibrobacter succinogenes* S85 becomes predominant among the cellulolytic bacteria when ruminants are fed with a poor diet, i.e. one which is highly lignified. The enzymatic equipment of *F. succinogenes*. Firstly, this bacterium degrades cellulose via a very efficient cellulolytic system. Cellulose is depolymerized at the bacterial surface by different cellulases and the released cellodextrins are hydrolysed to glucose and cellobiose in the periplasm. Secondly, it produces ferulic acid and acetylxylan esterase, and arabinofuranosidase that are necessary to cleave the ester bonds linking hemicelluloses to lignin, or to debranch xylans. Finally, several different xylanases and an α-glucuronidase complete the cellulolytic system. Although these fibrolytic enzymes have been identified, little is known about their concurrent mode of action on solid substrates. For instance, is amorphous cellulose degraded faster than crystalline cellulose? Which chemical linkages are cleaved first, and therefore which saccharides are released first in the medium? To answer to these questions, we have used solid state NMR, ^{13}C-CP-MAS (cross polarization magic angle spinning) to monitor the action of fibrolytic enzymes on cellulose (Avicel) and lignocellulosic fibres (more specifically ^{13}C-enriched wheat straw) during growth of *F. succinogenes* S85 on these substrates.

This technique allows analysis in situ of the chemical components of these fibres and also quantification of the crystalline/amorphous cellulose ratio. ^{1}H and ^{13}C liquid state NMR gave complementary information about the sugars released the culture medium.

P-65 **Genetic transformation and expression in mutant bacteria.** M.S. Ekinci, M. Buyukcelik, A. Kamalak, Y. Gurbuz, E. Ozkose (Kahramanmaras Sutcu Imam University Faculty of Agriculture Department of Animal Science, Kahramanmaras, Turkey)

Since the importance of microbial enzymes in improving digestion of plant cell wall polysaccharides was first recognised, considerable effort has been devoted to isolating, overproducing and understanding the mode of action of these enzymes. A number of genes encoding plant cell wall polysaccharides have been isolated from both Gram-positive and Gram-negative rumen bacteria and from anaerobic fungi. Such genes have been studied mostly in *E. coli*, where expression is often anomalous because of internal translational start sites and where extensive proteolysis breakdown occurs. Internal translational start-points and proteolysis may complicate interpretation of cloned gene products from rumen microorganisms expressed in *E. coli* that recognise promoters different from those used in the original host organisms. Therefore, it is of interest to examine expression of cloned genes in Gram-positive species more closely related to ruminal species. Protease-negative strains of *Bacillus subtilis* and *Streptococcus thermophilus* were obtained by ethidium bromide mutation. Glucanase and xylanase encoding genes were isolated from the rumen microorganism *S. bovis* and from *B. subtilis* RSKK246 and introduced into both mutant and native *B. subtilis* and *S. thermophilus*. Expression levels of the enzymes from mutant and native strains of *B. subtilis* and *S. thermophilus* were determined and compared. Results showed increases of approximately 40% in β-glucanase and 30% in xylanase activity in mutant *S. thermophilus* and of approximately 30% in β-glucanase and 35% in xylanase activity in mutant *B. subtilis*, compared with both native recombinant organisms.

P-66 **Effects of a methanogen on the lignocellulolytic ability of gut anaerobic fungi from different herbivores.** K.N. Joblin, G.E. Naylor (Grasslands Research Centre, AgResearch, Palmerston North, New Zealand)

The degradation of purified cellulose or xylan by the gut anaerobic fungi, potent degraders of plant cell walls, is strongly increased when fungi are co-cultured with methanogens. Perhaps unexpectedly, this increase in degradation often is minimal or not observed when lignocellulosic tissues are the substrates. To obtain further information, 30 purified anaerobic fungi isolated from 7 herbivores were cultured on large stem fragments of lucerne (*Medicago sativa*) hay both in the presence or absence of *Methanobrevibacter smithii* and the extent and rate of degradation were measured. In most cases, growth in methanogenic co-culture had no significant effect on stem degradation despite establishment of functional syntrophic co-cultures. For some fungi however, growth in methanogenic co-culture significantly increased the extent of degradation, the rate of degradation or both. For instance, for 3 of 17 *Piromyces* isolates tested, growth in the presence of *M. smithii* strongly increased the rate but not the extent of degradation. *Piromyces* spp. isolated from seaweed-eating feral cattle on a sub-Antarctic island showed an unusual pattern of stem degradation but degradation did not increase in the presence of the methanogen. In the case of isolates belonging to *Caecomyces*, a genus regarded as ineffective against refractory lignocellulosic tissue, degradation by 2 of 4 isolates increased markedly in the presence of the methanogen to reach levels similar to those of the best *Neocallimastix* and *Piromyces* spp. This effect has not been observed previously. Interactions between anaerobic fungi and methanogens and the consequences for plant cell-wall degradation may be more complex than believed previously.

P-67 **Ability of the rumen bacterium *Treponema* strain kT to utilize fructose and fructose polymers for growth.** A. Kasperowicz, T. Michałowski (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences. 05-110 Jabłonna, Poland)

Growth of the rumen bacterium *Treponema* strain kT in medium containing fructose, sucrose, the Timothy grass fructan and inulin as the sole sourcse of carbon was studied. The strain examined originated from our own collection. Of the carbohydrates used, the Timothy grass fructan and fructose were the best in supporting bacterial growth when measured by the optical density of the cultures. The best growth was accompanied by the highest utilization of both substrates. The cultured bacteria were also able to develop on glucose. Examination of the bacterial cell extracts showed the presence of enzymes degrading sucrose, inulin and the Timothy grass fructan with the rate of 1.9, 2.9 and 39.2 μmol·mg^{-1} protein·h^{-1}, respectively whereas the pH optimum for degradation of the substrates was 6.0, 6.2, and 6.4. Separation of the cell extract at 100 000 g demonstrated the presence of fructanolytic activity in both the sediment and supernatant fractions. Native polyacrylamide gel electrophoresis combined with zymograms revealed the presence of single fructanolytic enzyme in each fraction. The enzyme present in sediment degraded only the Timothy grass fructan to oligosaccharides as the end products. The enzyme from the supernatant fraction was able to degrade the Timothy grass fructan, inulin and sucrose to monosaccharides. Bacteria growing on glucose were able to produce the fructanolytic activities. This suggests that the enzymes were of a constitutive character.

P-68 **Competitive PCR-aided monitoring of cellulolytic bacterial species attaching to orchardgrass hay incubated in the rumen.** S. Koike, Y. Kobayashi, K. Tanaka (Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan)

Close association of ruminal cellulolytic bacteria with plant material occurs while cellulose-rich plant cell wall material is being degraded, suggesting that bacterial attachment is an important step of fiber breakdown in the rumen. Therefore, monitoring of fiber-associated bacteria is a key to understanding mechanisms of fiber digestion by ruminants. In the present study, we evaluated attachment of representative ruminal cellulolytic bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* to plant fiber by competitive PCR. Stems of orchardgrass hay in the nylon bag were incubated in the rumen of sheep for the period from 5 min to 96 hr. Total DNA was extracted from incubated stem and employed for competitive PCR assays to quantitate *F. succinogenes*, *R. flavefaciens* and *R. albus*. After 5 min incubation, *F. succinogenes* and two ruminococcal species were attached to the stem at levels of 10^5 and 10^4 g^{-1} dry matter (DM) of the stem, respectively. These attached cell numbers for all three species increased 10 fold after 10 min incubation. Although slight differences were observed among the three species, the subsequent rate of increase of cell number was almost the same (× 1.5–2.5 h^{-1}) up to 6 hr incubation. Thereafter, attached cell numbers of the three species gradually increased and reached the maximum at 24 h (10^9 g^{-1} DM for *F. succinogenes* and 10^7 g^{-1} DM for *R. flavefaciens*) or 48 h (10^6 g^{-1} DM for *R. albus*). After 96 hr incubation, associated population sizes for all three species had decreased to 20% of each maximum level. These results suggest that attachment of the representative ruminal cellulolytic bacteria is primarily achieved within 10 min; their growth may progress up to 48 h and then their detachment occurs with aging of stem in the rumen.

P-69 **Activity of pectin-degrading enzymes in rabbit caecal strains of *Bifidobacterium pseudolongum*, and in rumen bacterium *Streptococcus bovis*.** M. Marounek, L. Slováková, D. Dušková (Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Uhříněves, Prague 104 00, Czech Republic)

In strains P2 and P6 of *Bifidobacterium pseudolongum* grown on pectin the following activities, expressed in nmols of substrate split or product released per min per mg of protein, were found : intracellular pectinase (P) – 3.96 ± 0.26, 3.74 ± 0.22; extracellular P – 83.0 ± 16.0, 87.4 ± 16.5 ; exopectate lyase (EL) – 0, 0 (both intra- and extracellular); intracellular 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPGA) – 610 ± 212, 919 ± 274. In cells of *Streptococcus bovis* X4 grown on pectin plus glucose activities of pectin-degrading enzymes were as follows : intracellular P – 3.45 ± 0.26; extracellular P – 11.55 ± 0.30; intracellular EL – 0; extracellular EL – 102.4 ± 22.1; intracellular KDPGA – 0. It can be concluded that strains P2 and P6 of *B. pseudolongum*

have enzymes necessary for complete degradation of pectin. Exopectate lyase, the principal enzyme degrading the pectin macromolecule in rumen bacteria was absent in both bifidobacteria. *S. bovis* X4 possessed both exopectate lyase and pectinase activity. No growth, however, of *S. bovis* X4 on pectin was observed. The lack of growth of *S. bovis* X4 on pectin is probably caused by the lack of KDPGA activity in this bacterium. (This study was supported by grant 524/99/0480 of the Grant Agency of the Czech Republic).

P-70 **Expression of cellulase genes from anaerobic bacteria in *Clostridium paraputrificum* and *Streptococcus bovis*.** K. Ohmiya, K. Morimoto, H. Taguchi, M. Kawase, T. Kikuta, T. Kimura, K. Sakka (Applied Microbiology, Faculty of Bioresources, Mie University, Kamihama-cho 1515, Tsu 514-8507 Japan)

Huge amounts of cellulosic biomass are synthesized from solar energy by fixing CO_2 on the earth. However, the biomass is so resistant to hydrolysis by microorganisms and/or cellulolytic enzymes within a short period that the majority of the bulk is utilized with difficulty. For enhancing solubilization of cellulosic biomass and its conversion to feeds and fuels, we have been studying the improvement or addition of cellulolytic ability to anaerobic bacteria, such as *Clostridium paraputrificum* and *Streptococcus bovis*. *C. paraputrificum* is our isolate which grows on chitin and *N*-acetylglucosamine and produces significant amount of hydrogen gas. Hydrogen productivity of the bacterium was increased up to 1.8-fold by integrating and expressing hydrogenase gene of the host organisms by electroporation with a vector pJIR751 which was kindly provided from Rood (Plasmid, 27 (1992) 207–219). As the host organism grows negligibly on cellulose, the endoglucanase I gene (*egI*) from *Ruminococcus albus* and xylanase A genes (*xynA*) from *Clostridium stercorarium* and *Clostridium thermocellum* were successfully expressed in *C. paraputrificum* using the vector when the promotor of a hydrogenase gene from the host organism was placed upstream from the target genes. *S. bovis* is a starch-utilizing rumen anaerobe, from which a plasmid was isolated and modified as a shuttle vector between *S. bovis* and *E. coli* by Nakamura et al. (Current Microbiology 43 (2001) 11–16). Since the vector was also kindly provided to us, we could express *egI* in *S. bovis* by electroporation using a promotor from a-amylase of the host organism.

P-71 **Pectinases in the rumen bacteria *Ruminococcus flavefaciens* 17.** G. Palmieri[a], D. Di Liberto[a], L. Ferrara[a], H.J. Flint[b], V. Aurilia[a] ([a] C.N.R.-I.A.B.B.A.M., Italian National Research Council, Via Argine, 1085-Napoli, Italy; [b] Rowett Research Institute, Aberdeen, UK)

The rumen bacterium *Ruminococcus flavefaciens* plays an important role in the degradation of plant cell wall polysaccharides. Pectin is a valuable component for feed of ruminants and although there are some studies on the metabolism of pectin in mixed and pure cultures of rumen pectinolytic bacteria, there is little information on molecular properties of pectinase enzymes involved in the degradation process. We report here preliminary results on enzymes that act on the pectin polymer, in particular we have evidence of pectate lyases (PL) and pectin methyl esterases (PME). The bacterium was grown anaerobically on media containing cellobiose and 30% or 67% esterified citrus pectin. In all culture conditions examined substantial levels of extracellular PME activity were detected but in the presence of high-methylated pectins a tenfold increase of PME activity was observed. Furthermore, PME enzymes produced in the different cultures were examined by activity staining of pectin esterase (PE) following SDS-PAGE. Results revealed the presence of PE isoforms which are different between the substrates used for the growth. The most abundant PE enzyme of about 35 kDa, produced in the cellobiose medium has been partially purified. As far as pectate lyase is concerned, the activity was detected by dot-spot experiments using the supernatant culture of the bacteria grown both on cellobiose and pectin. A genomic library was constructed in *Escherichia coli* and screened. One positive clone exhibiting pectate lyase activity was directly detected by an in situ plate assay. The plasmid isolated from the clone contained a 1.7 Kb genomic fragment from *Ruminococcus* and sequence analysis is in progress. Further investigations on the enzyme characterisation and gene cloning are in progress in order to improve our knowledge on this system.

P-72 **Extracellular α-amylases from human colonic strains of *Butyrivibrio fibrisolvens* and related Gram-positive anaerobes.** A.G. Ramsay, K.P. Scott, H.J. Flint (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

Resistant starch makes up a large proportion of the dietary substrate reaching the human colon, where it is fermented by human colonic bacteria to produce short-chain fatty acids (SCFA) as major end-products. Resistant starch is a potential prebiotic, but knowledge at a molecular level of the enzymes involved in starch degradation by colonic commensal bacteria is limited to the gram negative *Bacteroides* spp. Recent studies have shown that low % G + C Gram-positive bacteria are among the most abundant components of the adult faecal flora. Amylases of several new isolates belonging to the Clostridial cluster XIVa were visualised using zymogram analysis and found to be of high molecular weight. An amylase gene from the human colonic strain *B. fibrisolvens* 16.4 was sequenced following polymerase chain reaction (PCR) amplification with degenerate oligonucleotides and chromosome walking. The putative amylase consists of an open reading frame of 4038 bp encoding a protein of 1346 amino acids with a calculated M_r of 146 177. The catalytic domain shows high sequence homology with a previously characterised α-amylase from a ruminal *B. fibrisolvens* strain and shares the conserved active centres in the N-terminal half of the protein. The highly charged C-terminus contains two repeat regions of unknown function that also show some homology to the ruminal amylase. Functional studies should help elucidate the function of this novel C-terminus and provide insights in how extracellular enzymes are organised in this group of bacteria.

P-73 **A new family of cellulose binding modules from *Ruminococcus flavefaciens* 17**. M.T. Rincon, S.I. McCrae, J.C. Martin, J. Chiquette, K.P. Scott, H.J. Flint (Microbial Genetics Group, Rowett Research Institute, Aberdeen AB21 9SB, UK)

The molecular mechanisms by which *R. flavefaciens* cells and enzymes attach to cellulosic substrates remain unclear. Plant cell wall degrading enzymes appear to be organised into cellulosome-like multienzyme complexes in this species. However the scaffoldin-type protein ScaA that has been identified as binding enzyme subunits in *R. flavefaciens* 17 does not contain an identifiable cellulose-binding module (CBM). On the other hand, we recently identified a region in the cellulosome-located enzyme EndB that acts as a CBM, but is not closely related to other published protein sequences (Rincon et al., Appl. Environ. Microbiol. 67 (2001) 4426–4431). Purified 6His-tagged fragments of the complete EndB enzyme, as well as the isolated CBM, were shown to bind to crystalline cellulose. Since this novel module is also the first CBM to be reported from this species, we decided to determine whether it was present in other *R. flavefaciens* proteins. Polyclonal antibodies were raised against the purified CBM fragment, and these were shown to recognise several *R. flavefaciens* proteins ranging in size from 50 to almost 300 kDa. Meanwhile a PCR product corresponding to the EndB CBM coding region was used as a hybridisation probe to detect clones from a *R. flavefaciens* library that have partially homologous inserts. We can conclude that several proteins in addition to EndB are likely to carry closely related CBMs, and that the EndB CBM represents a new family of carbohydrate binding module capable of attachment to crystalline cellulose. The relative contributions to substrate attachment of CBMs associated with enzyme subunits, structural proteins or pili-like structures have yet to be established in this species.

P-74 **Western immunoblot analysis of *Ruminococcus albus* and *Fibrobacter* spp. for proteins antigenically similar to scaffoldin modules of *R. flavefaciens*.** M.T. Rincon[a], J. Jacobovitch[b], J. Miron[b], R. Lamed[c], E.A. Bayer[d], M. Morrison[a] ([a] The Ohio State University, Columbus, OH, USA; [b] The Volcani Research Institute, Bet Dagan, Israel; [c] Tel Aviv University, Israel; [d] The Weizmann Institute, Rehovot, Israel)

Ruminococcus albus and *R. flavefaciens* both produce cellulosomes, but their complexity, in terms of the number and forms of integrative and catalytic proteins, is undefined. Additionally, *R. albus* produces a pilus-like structure (CbpC) that appears to coordinate a non-cellulosomal mechanism of adhesion to cellulose. Western

immunoblotting was employed to examine additional *Ruminococcus* and *Fibrobacter* spp. for proteins containing epitopes recognized by antibodies raised against the CbpC protein from *R. albus* strain 8, as well as modules present in the ScaA and ScaB proteins from *R. flavefaciens* strain 17. The anti-CbpC antiserum crossreacted with a polypeptide of ~30 kDa from R. albus SY3, as well as several other strains of *R. albus*. Interestingly, the anti-CbpC antiserum also crossreacted with several *F. intestinalis* proteins, suggesting that the bacterium possesses protein(s) structurally analogous to the *R. albus* CbpC protein. Western immunoblots with antisera, raised against either the X-domain or cohesin modules from the *R. flavefaciens* ScaA and ScaB proteins, showed that both *R. albus* strains 8 and SY3 possess epitopically similar proteins to *R. flavefaciens*. Notably, a protein of 200 kDa in *R. albus* SY3 crossreacted strongly with the anti-ScaA antiserum. All three antisera crossreacted, albeit weakly, with high-molecular-weight proteins from *R. albus* 8, and unlike strain SY3, the strongest interaction occurred with two polypeptides of less than 20 kDa, and with the anti-ScaB-X-domain antiserum. Such crossreactivity suggests the presence of ScaA and ScaB homologs in *R. albus*, but perhaps, not exclusively as scaffoldin modules.

P-75 **Real-time RT-PCR quantification of cellulase and xylanase genes expression in *Fibrobacter succinogenes*, a major ruminal fibrolytic bacterium.** F. Suau, C. Béra-Maillet, E. Forano (Unité de Microbiologie, Institut National de la Recherche Agronomique, CR de Clermont-Fd/Theix, 63122 St-Genès-Champanelle, France)

Fibrobacter succinogenes is particularly efficient in degrading various forms of cellulose and shows a high ability to solubilise different plant cell wall polysaccharides. Studies of its fibrolytic equipment have shown it to be extremely complex, comprising more than 25 glycoside hydrolases. However, little is known about the regulation of *F. succinogenes* fibrolytic gene expression and the importance of these enzymes in the total cellulolytic activity. The mRNAs from all glycoside hydrolase genes sequenced so far were first quantified using real-time RT-PCR (LightCycler) in cells grown in vitro on glucose, cellobiose or filter paper cellulose, confirming our previous results showing that all genes are transcribed in growing cells. Optimal efficacity of the fragment amplifications was obtained with a high sensitivity of detection, resulting in the quantification of the gene transcripts even in cells growing on soluble sugars. The mRNAs were then quantified in rumen content of two gnotoxenic lambs with a controlled ruminal flora containing only *F. succinogenes* S85 strain as a cellulolytic microorganism, and in the rumen of a conventional lamb.

P-76 **A pepD-like peptidase from *Prevotella albensis*.** N.D. Walker, N.R. McEwan, R.J. Wallace (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

Peptide breakdown in the rumen is a biphasic process where oligopeptides are broken down by dipeptidyl peptidases (DPPs), and the resulting di- and tripeptides are broken down to free amino acids by separate di- and tripeptidases. The *Prevotella* spp. produce four different DPPs and separate tri- and dipeptidase activities and thus play a significant role in peptide breakdown. Studies were carried out in which a peptidase-deficient strain of *E. coli* CM107 was complemented with genomic DNA from one of the type strains of this group of organisms, *Prevotella albensis* M384, in order to investigate the properties of *P. albensis* peptidases. Competent cells of CM107 were transformed with a pBlueScript plasmid genomic library from *P. albensis*. Recombinants were screened for peptidase activity using an overlay technique. One positive peptidase clone was obtained. Characterisation of the clone revealed that it was a metallopeptidase that had broad specificity, cleaving a single amino acid from the N-terminus. The clone had a preference for dipeptides, although some tri- and oligopeptidase activity was observed, and required a free N-terminus, indicating it was an exopeptidase. Sequence analysis revealed that the clone shared significant identity with PepD, a broad specificity dipeptidase found in several different prokaryotes. A putative metal-binding region was identified. Within this region, significant identity with other members of the same peptidase family, PepT, (a tripeptidase) and PepA, (an oligopeptidase), was observed. The theoretical M_r weight of PepD was 53000 and

gel filtration indicated it exists as a dimer in its native state. This is the first report of the successful complementation of a peptidase-deficient strain of *E. coli* with DNA from a ruminal bacterium.

P-77 **Sequence analysis and cloning of the *dppIV* gene from *Prevotella albensis* in *Escherichia coli.*** N.D. Walker, N.R. McEwan, R.J. Wallace (Rowett Research Institute, Aberdeen AB21 9SB, UK)

Prevotella spp. are important ruminal bacteria involved in protein breakdown in the rumen. They possess at least four different dipeptidyl peptidases, (DPP-I, DPP-II, DPP-III and DPP-IV). DPP-IV was cloned from *Prevotella albensis* M384 and expressed in *Escherichia coli.* DPP-IV is one of the most highly conserved DPPs and is found in several different organisms. Degenerate primers were designed based on conserved regions of the amino acid sequence of previously described DPP-IV genes and used to amplify genomic DNA from *P. albensis* to obtain part of the *dppIV* gene. The sequences of the flanking regions of the gene were determined by genomic sequencing. Following sequencing of the entire gene plus 500 bp flanking sequence either side, the gene plus flanking sequence was amplified by PCR and the product was cloned into *E. coli*. The biochemical properties of the cloned enzyme were similar to those of *P. albensis* and other DPP-IV enzymes in terms of substrate and inhibitor specificity, optimum temperature and pH. Sequence analysis revealed that the enzyme contained a conserved motif that is associated with the S9 class of prolyl oligopeptidases, and that the gene that encoded this protein appears not to be part of a contiguous operon. Construction of a phylogenetic tree demonstrated that the DPP-IV of *P. albensis* clusters with others found in bacteria of the *Cytophaga/ Flexibacter/Bacteroidaceae* (CFB) phylum, and these are more closely related to eukaryotic DPP-IV than the DPP-IV-like enzyme (pepX) of the lactic acid bacteria. This is the first report of the successful cloning of an oligopeptidase from a ruminal bacterium.

Session V:

Fermentation (2) – anaerobic metabolism

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O-21 **Identification of new derivatives of sinigrin and glucotropaeolin produced by the human digestive microflora, using ^{1}H NMR spectroscopy analysis of in vitro incubations**. A.-M. Delort[a], B. Combourieu[a], L. Elfoul[b], S. Rabot[b] ([a] Laboratoire Synthèse et Étude de Systèmes à Intérêt Biologique, UMR 6504 CNRS-Université Blaise Pascal, 63177 Aubière Cedex, France; [b] Unité d'Écologie et de Physiologie du Système Digestif, INRA, 78352 Jouy-en-Josas Cedex, France)

1D and 2D ^{1}H NMR spectroscopy were used to study the biotransformation of two dietary glucosinolates, sinigrin and glucotropaeolin, by the human digestive microflora in vitro. The molecular structures of the new metabolites were identified and the modulation of carbon metabolism was studied. Unambiguously, and for the first time, it was shown that sinigrin and glucotropaeolin were transformed quantitatively into allylamine and benzylamine, respectively. The comparison of the kinetics of transformation of sinigrin and glucotropaeolin with and without glucose clearly showed that the presence of glucose did not modify either the nature of the metabolites or the rate of transformation of the glucosinolates (complete degradation within 30 h). The main end-products of the glucose moiety of glucosinolates were characteristic of anaerobic carbon metabolism in the digestive tract (acetate, lactate, ethanol, propionate, formate and butyrate) and similar to those released from free glucose. This work represents the first application of ^{1}H NMR spectroscopy to the study of xenobiotic metabolism by the human digestive microflora, demonstrating allyl- and benzylamine production from glucosinolates. Whether these amines are produced in vivo from dietary glucosinolates remains to be established. This would reduce the availability of other glucosinolate metabolites, notably cancer protective isothiocyanates. (Combourieu B., et al., Drug Metab. Dispos. 29 (2001) 1440–1445).

O-22 **Butyrate production from lactate by human colonic microflora.** C. Bourriaud[a], S. Akoka[b], S. Goupry[c], R. Robins[b], C. Cherbut[a], C. Michel[a] ([a] UFDNH - INRA, B.P. 71627, 44316 Nantes cedex 03, France; [b] LAIEM, Université de Nantes, B.P. 92208, 2 rue de la Houssinière, 44322 Nantes cedex 03, France; [c] ENV, UNA, Route de Gachet BP 40706, 44307 Nantes cedex 03, France)

Butyrate produced by human colonic microflora has a potentially beneficial effect on intestinal health. However, its bacterial origin is only partially known. Non-digestible carbohydrates, which favour butyrate production, generally also stimulate the growth of lactate-producing bacteria (prebiotic effect) thus leading to raised intracolonic concentrations of lactate. We hypothesise that, in appropriate conditions of pH, butyrate might be produced by the metabolism of lactate by the lactate-using bacteria. Consequently, we have investigated whether the intestinal flora metabolises lactate into butyrate and whether pH influences this conversion. DL-Lactate conversion into butyrate was demonstrated at pH 6.5 using continuous and batch cultures of human faeces, in which lactate supplementation (30 mM) led to 18 mM butyrate (c. 30% of total short chain fatty acids) as compared to 7 mM (c. 16%) for the basal medium. This conversion was significantly affected when the pH was held at 5.8 (22 mM or 38%) compared to the basal medium (9 mM or 22%). L-Lactate alone led to similar results (19 mM or 32%), indicating that the conversion does not depend on the form of lactate present. Conversely, butyrate concentration varied according to the origin of the flora (10, 22 and 24 mM using 3 different donors), which represented 23 to 43% consumption of the initial lactate, as shown by GC-MS with [3-C^{13}]lactate. Studies using NMR have been initiated to determine the metabolic link between lactate and butyrate in these fermentations. Despite this demonstrated utilisation of lactate, no change was observed in the total number of lactate-using bacteria in continuous culture. However, cultures of these bacteria will probably enable the isolation and the identification of potential new butyrate-producing bacteria.

O-23 **The growth promoting mode of action of flavomycin in ruminants.** J.E. Edwards, R.J. Wallace, N.R. McEwan (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

The growth promoting phosphoglycolipid antibiotic, flavomycin, has a different and narrower ruminal antibacterial spectrum compared to the well-characterised ionophore antibiotics. Previous in vitro work showed that *Fusobacterium necrophorum* and some hyper-ammonia producing (HAP) bacteria are sensitive to

pharmacological concentrations of flavomycin. This communication describes an experiment which looks at the effect that the antibiotic has on these bacterial populations in vivo. Four adult cannulated sheep, on a hay-concentrate diet received 250 mg Flavomycin^{80} per day for a period of 2 weeks. Rumen digesta was collected at 0 and 2 weeks; rumen VFA and NH_3 concentrations and microflora were assessed. The concentration of ruminal NH_3 decreased by 14% ($P < 0.001$) and a significant ten-fold drop in the viable counts of HAP bacteria was observed. Viable counts of *F. necrophorum*, the main aetiological agent of liver abscess, also decreased significantly in response to dietary flavomycin. Ruminal pH and VFA analysis showed no responses that would contribute to a growth promotion mechanism. The decrease in ruminal NH_3, through direct action on the HAP bacterial population by flavomycin, may result in more efficient utilisation of feed. Suppression of ruminal *F. necrophorum* by dietary flavomycin would be considered beneficial, as ruminal wall attachment and invasion is likely to be minimised. A corresponding mechanism may occur in non-ruminant species that also show a beneficial response to dietary flavomycin.

O-24 **Effect of tropical forage legumes and the addition of *Sapindus saponaria* on rumen fermentation and methane release in vitro.** H.D. Hess[a], L.M. Monsalve[b], C.E. Lascano[b], J.E. Carulla[c], T.E. Díaz[d], M. Kreuzer[a] ([a] Institute of Animal Sciences, ETH-Zurich, ETH-Zentrum/LFW, 8092 Zurich, Switzerland; [b] CIAT, Cali, Colombia; [c] National University, Bogotá, Colombia; [d] Corpoica, Bogotá, Colombia)

Ruminants of low productivity held in the tropics and subtropics contribute significantly to global methane emissions. Feeding strategies to improve animal productivity include the dietary supplementation with forage legumes and saponin-containing feeds. The effect of such strategies on methane release are unknown. In a Rumen-Simulation Technique (Rusitec) experiment a basal diet of *Brachiaria dictyoneura* was tested either without legume or with 33% (dry matter basis) of one tropical forage legume of contrasting quality each, i.e., *Calliandra calothyrsus* (low quality), *Cratylia argentea* (medium) and *Arachis pintoi* (high). All diets were evaluated with and without the addition of *Sapindus saponaria* fruits. *A. pintoi* and *C. argentea* increased bacteria counts and *A. pintoi* increased protozoa counts. Both legumes enhanced organic matter and fibre degradation. *C. calothyrsus* had no effect on microbial counts and reduced organic matter and fibre degradation. Methane release was increased by *C. argentea* and *A. pintoi* to almost 3- and 4-fold levels, whereas *C. calothyrsus* reduced methane release by 50%. *S. saponaria* reduced methanogenesis by 11% on average. Interactions between addition of legumes and *S. saponaria* were mostly insignificant, except in protozoa counts (reduced with *S. saponaria* only in the diet containing *A. pintoi*). Our results suggest that methanogenesis can be reduced with *S. saponaria* in unimproved and improved forage-based tropical diets and that tannins present in *C. calothyrsus* could be associated with methane reduction. The higher methane amount with high-quality legumes probably will be counterbalanced by a correspondingly higher animal productivity.

O-25 ***Megasphaera elsdenii* JCM1772^T regulates hyper lactate production in the rat large intestine.** K. Ushida[a], K. Hashizume[a], T. Tsukahara[a,b], K. Yamada[b], K. Koyama[c] ([a] Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan; [b] KYODOKEN Institute, Shimoitabashi, Kyoto 612-8073, Japan; [c] Fujisawa Pharmaceutical, Tokodai, Tsukuba 300-2698, Japan)

Hyper lactate production in the large intestine is often related to disorders of the intestine such as inflammatory bowel diseases, dyspepsia and antibiotic associated diarrhoea. Lactate is an intermediate product in the colonic fermentation, which is converted to SCFA under normal conditions. *Megasphaera elsdenii* is able to convert lactate to butyrate, a physiologically important SCFA for colonic epithelial cells. In this experiment, we introduced *M. elsdenii* orally into rats to control experimentally induced high lactic fermentations. Fifteen SD male rats (6 wks old, SPF, Japan Slc) were used. The rats received a semi-purified diet supplemented with 10% fructo-oligosaccharide. Faeces were collected from the rectum at 17:00 and immediately analysed for organic acids by HPLC. Rats with faecal lactate concentrations higher than 30 mmol·kg^{-1} were divided into two groups of which one (5 rats) was orally dosed *M. elsdenii*

JCM1772^T (1.4×10^{13} CFU) at 10:00 for 3 days. The remainder (7 rats) were treated with saline. After 3 days rats were slaughtered under the general anaesthesia and ceacal contents were collected. Faecal lactate started to decrease 7 h after the first dose of *M. elsdenii* (37.3 vs. 61.5 mmol·kg^{-1}) and significantly decreased after 2 doses. The decrease in faecal lactate was reflected in an increase in butyrate concentration. The dry matter content of faeces was also higher in *M. elsdenii* treated rats. Using competitive PCR, 7×10^6 *M. elsdenii* CFU·g^{-1} were recovered from the caecum of treated rats.

P-78 **Effect of antibiotics or acidifiers in feed on rabbit caecal population and metabolism given maize or acorn/sorghum diet.** L. Abecia[a], M. Fondevila[a], J. Balcells[a], A. Belenguer[a], M. Decaux[b] ([a] Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza, M. Servet 177, 50013 Zaragoza, Spain; [b] Cargill Animal Nutrition, Sant Joan 193, Barcelona, Spain)

A basal diet with no additives (control) or with different levels of chlortetracycline (CT; 0.2, 0.4 and 0.8 g·kg^{-1}), bacitracin (BC; 0.05, 0.1 and 0.2 g·kg^{-1}) and fumaric acid (FA; 5 and 10 g·kg^{-1}) were given to growing New Zealand male rabbits (8 animals/diet). Digestibility trials were carried out with urine collection for determination of purine derivatives excretion. Four animals per treatment were then slaughtered and caecal metabolic parameters measured. There was no consistent trend for the levels of included additives in any parameter studied. Average neutral detergent fibre digestibility was higher with BC (27.9%) than control (23.9%; $P < 0.10$), CT (24.2%; $P < 0.05$) and FA (23.4%; $P < 0.01$). There was no treatment effect over caecal content weight, pH or ammonia concentration, although the former tended ($P < 0.10$) to be higher with CT than BC (105 vs. 90 g). Urinary excretion of purine derivatives also increased ($P < 0.05$) with BC compared to CT or control (1.53, 1.22 and 1.09 mmol·d^{-1}), indicating a higher microbial caecal population. However, this contrasted with total bacterial counts, that were higher with CT than FA, BC and control (54.5 vs. 21.5, 19.2 and 20.3×10^8 g^{-1}, respectively; $P < 0.05$). Average cellulolytic counts (10^5 g^{-1}) were higher with FA (64.3) than CT (4.8; $P < 0.05$) and BC (7.69; $P < 0.10$), but differences were mostly caused by the higher level of FA. Despite the high variability, amylolytic concentration (10^8 g^{-1}) was higher with CT (31.2) than BC (9.7; $P < 0.05$) and control (6.8; $P < 0.10$).

P-79 **Effects of the intake of condensed tannins on the fermentative activity of sheep rumen liquid.** H. Ammar, S. López, M.L. Tejido, J.S. González, M.J. Ranilla (Department of Producción Animal, Universidad de León, 24071 León, Spain)

Some ruminants (feral goats) can tolerate much higher dietary levels of tannins than other animals, possibly owing to the presence of rumen microbial populations that are resistant to these compounds. Tannin-tolerant bacteria have been isolated from the gastrointestinal tracts of animals consuming high tannin diets. The numbers of tannin-tolerant bacteria can be increased by feeding the animals with tannin containing plants, and therefore can be considered an adaptive response of the microbial population. The aim of this work was to study the medium-term effects of the intake of quebracho condensed tannins on fermentative activity in the rumen of sheep. Eight Merino rumen-cannulated sheep fed chopped alfalfa hay were used. Four sheep were given alfalfa hay treated with 50 g quebracho·kg^{-1} DM for 60 days (group Q), whereas the other animals were always given untreated alfalfa hay and used as the control group (C). Differences in the fermentative activity were examined in vitro in batch cultures inoculated with rumen fluid obtained on day 60 from both groups of sheep. In vitro dry matter digestibility (IVD) and gas production kinetics were determined for the foliage of indigenous shrub species *(Erica australis, Cistus laurifolius, Quercus pyrenaica, Cytisus scoparius and Rosa canina).* In most cases IVD was higher when rumen fluid from Q sheep was used. For the same substrate, the inoculation with rumen fluid from Q sheep resulted in a greater extent of DM degradation, a higher gas production at 24 h of incubation and faster fractional gas production rates. The extent of this effect was smaller when material with lower tannin contents was incubated (*C. scoparius* leaves). In conclusion, rumen fluid from sheep fed a diet supplemented with condensed tannins showed a higher fermentative activity, giving

rise to greater in vitro degradability of browse plant foliage. This could be due to the appearance and proliferation of tannin-tolerant bacterial species or to the induction of changes in the existing bacteria to enhance their tolerance to these phenolic compounds.

P-80 **A deletion approach to assess the amino requirements for optimum fermentation by mixed micro-organisms from the sheep rumen.** C. Atasoglu, A.Y. Guliye, R.J. Wallace (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

Two main approaches have been taken previously to identify key amino acids for rumen fermentation. The first involved supplementation with single or groups of amino acids, which failed to identify key amino acids. The other used labelling methods which identified proline, glycine, valine and threonine as amino acids whose biosynthesis was most sensitive to repression by added amino acids. Supplementation with these amino acids, however, failed to replicate the stimulatory effect of a mixture of amino acids. Here we report the results of a third approach, in which individual amino acids were deleted from a complete amino acids mixture and their effects of the deletion on fermentation were determined. Rumen samples were taken before feeding from four rumen-fistulated adult sheep, strained through linen cloth, and diluted in buffer (1:2). Gas, VFA and microbial yields from a mixture of soluble starch, cellobiose and xylose were measured, in the presence of NH_3 alone, a mixture of all 20 amino acids (0.25 $g \cdot L^{-1}$ each), or mixtures with single amino acids omitted. The complete amino acids mixture increased fermentation rate by 10.3% 6 h after feeding. The deletion of phenylalanine and leucine decreased fermentation rate most, while deleting eight other amino acids individually also decreased ($P < 0.05$) the initial fermentation rate. Removal of leucine caused a 28% decrease ($P = 0.03$) in the stimulation of growth yield but no other deletion affected yield significantly ($P > 0.05$). Thus, the deletion approach confirmed that no single amino acid limits ruminal fermentation, and identified phenylalanine, leucine and serine as key amino acids limiting fermentation rate and/or efficiency with soluble, rapidly degraded materials as energy substrates.

P-81 **Influence of fermentable carbohydrates on fermentation in piglet GIT at weaning.** A. Awati, B.A. Williams, M. Bosch, M.W.A. Verstegen (Animal Nutrition Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen, The Netherlands)

With increasing concern about antibiotic resistance in bacteria, the search for an alternative to the use of antibiotic growth promotors is of great interest, although the exact mechanisms of their action are still unclear. Sudden weaning results in serious stress for young animals, which can predispose them to a variety of health problems ranging from diarrhoea to sudden death. By addition of fermentable carbohydrates to the diet, our aim was to alter the pattern of GIT fermentation in the piglet, and thereby improve piglet health at weaning. An in vivo study was conducted, to test two diets: one containing added fermentable carbohydrates (FER), and a control (CON) – neither contained antibiotics nor added copper. Thirty-six piglets from 4 litters were weaned at 4 weeks of age (no creep feeding), and introduced to one of the two diets. Piglets were slaughtered on 1st, 4th and 10th day of each period. Digesta samples were collected from the first and second halves of the small intestine, caecum and colon. The dry matter, VFA profile, ammonia, lactic and formic acids were analyzed at all locations for all animals. Feed intake and growth were also measured per animal, and feed conversion ratio calculated. This study was carried out in triplicate. The FER diet led to a shift in VFA production, particularly in the ratio between the straight-chain versus branched-chain VFA. The FER diet also led to less ammonia present in the digesta compared with the CON diet. There was no significant difference in production performance between dietary groups.

P-82 **The effect of dietary fermentable carbohydrates on microbial activity within piglet faeces, studied in vitro.** A. Awati, B.A. Williams, M. Bosch, M.W.A. Verstegen (Animal Nutrition Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen, The Netherlands)

Prebiotics are substances added to diets, which will lead to shifts in GIT microbial activity which will be favourable to host health. However, most studies reported seem to focus on the counts of certain specific microbial species, without

actually considering microbial activity of the whole population. The in vitro cumulative gas production technique can be used to assess microbial activity of a complex population, in relation to fermentation of a particular energy source. An in vivo study was conducted, to test two diets: one containing added fermentable carbohydrates – including sugarbeet pulp (SBP) and wheat starch (WST) (FER), and a control (CON) – neither contained antibiotics nor added copper. Twenty-four piglets selected from 12 litters (2/litter) were weaned at 4 weeks of age (no creep feeding), and introduced to one of two diets. After 9 days on the diets, faecal samples were collected from selected animals, and tested for their activity in terms of gas production kinetics, and end-products such as VFA, ammonia, and DM disappearance of the two test substrates SBP and WST. Faeces from piglets fed the FER diet had a more rapid fermentation and a tendency to more total gas for both substrates, compared with the faeces from piglets fed the control diet This suggests an adaptation of the GIT microflora to components present within the digesta (prebiotics). It was concluded that the addition of fermentable carbohydrates to the diet, can lead to a change in the GIT microbial activity.

P-83 **Effect of tannins and other secondary plant products on microbial populations and gut function.** J.D. Brooker[a], S. Rakhmani[a], L. O'Donovan[a], D.O. Krause[b] ([a] Animal Science Department, Adelaide University, Roseworthy Campus, South Australia 5371, Australia; [b] CSIRO Livestock Industries, Long Pocket Laboratory, Indooroopilly, Brisbane 4068, Australia)

Forage tannins reduce nutritive value through reduced palatability and inhibitory effects on intestinal functions. This includes effects on microbial populations as well as structure/function interactions in the gut. Cellulolytic bacteria appear to be inhibited to a greater extent than proteolytic organisms or ruminal fungi, possibly due to inhibition of attachment mechanisms. The effect of tannins and essential oils on pathogen colonisation in the GI tract is currently being investigated. HPLC and LC-MS analysis of plant tannins demonstrates complex patterns that differ between species, and even between accessions of the same species; polymeric proanthocyanidins correlate with protein binding activity and dry matter digestibility by ruminants. Tannin-degrading microorganisms occur widely in nature and some animals harbour tannin-degrading microorganisms, possibly as an adaptive response to diet. *Streptococcus caprinus (gallolyticus)* decarboxylates gallic acid to pyrogallol and is resistant in vitro to at least 7% w/v tannic acid and 4% w/v Acacia condensed tannin. Initiation of logarithmic growth of *S. caprinus* is tannin concentration-dependent. Extracellular polysaccharide (EPS) is also produced in response to tannins, although EPS by itself is not protective against tannins. *Selenomonas ruminantium* K2 synthesises a tannin-inducible tannin acylhydrolase (tannase) which is active against simple and complex gallo-tannins. Tannins also inhibit nutrient digestion and uptake in the abomasum and small intestine of ruminants. Alkaline phosphatase and aminopeptidase-N activities are reduced and intestinal microvilli appear fragile. The protein-complexing action of tannins may have both beneficial and harmful effects on gastrointestinal function and microbial interactions in the gut.

P-84 **Effect of *Saccharomyces cerevisiae* CNCM I-1077 on protein and peptide degrading activities of some rumen bacteria grown in vitro.** F. Chaucheyras-Durand[a,b], G. Fonty[b], S. Masséglia[a,b] ([a] Lallemand Animal Nutrition, 42 av. du Général de Croutte, BP 1021, 31023 Toulouse cedex 1, France; [b] Unité de Microbiologie, INRA CR Clermont-Ferrand/Theix, 63122 Saint-Genès-Champanelle, France)

In ruminants, the utilization of dietary nitrogen is often inefficient because most of the proteins ingested by the animal are rapidly degraded by rumen microorganisms (mainly bacteria and protozoa) which convert these proteins into peptides, amino acids and ammonia. Although a part of ammonia is re-utilised as a nitrogen source by several bacterial species, and a part is recycled by the animal, an important fraction of the ammonia produced in the rumen is excreted and represents a nitrogen loss for the animal and an environmental pollutant. We investigated the potential of the additive *Saccharomyces cerevisiae* CNCM I-1077, on protein and peptide degrading activities of proteolytic rumen bacteria, *Prevotella albensis* M384, *Streptococcus bovis* DSM 20480 and *Butyrivibrio fibrisolvens* DSM 1374 grown in vitro. Alive or heat-killed yeast cells were added to bacterial cultures at two different

concentrations in a complex casein-glucose medium. After a 24 hour-incubation at 39 °C under O_2-free CO_2, proteinase and peptidase activities were determined in the absence or in the presence of yeasts. Proteinase activities were also detected after SDS-PAGE in gelatin-copolymerized gels. Results showed that live *S. cerevisiae* I-1077 limited proteinase activity. Bacterial peptidase activities were also reduced; the mechanism implicated seemed to be different to that for proteinase activity, since live as well as dead yeast cells had the same effect. The factors responsible for such effects are currently under investigation.

P-85 **Potential health benefit of H_2-consuming acetogenic bacteria from the human colon: new nutritional strategy targeting digestive troubles associated with gas production.** C. Del'Homme[a], A. Yazourh[b], S. Rabot[b], A. Bernalier-Donadille[a] ([a] Unité de Microbiologie, INRA Clermont-Ferrand/Theix, France; [b] Digestar, Biopôle Clermont Ferrand-Limagne, France)

In humans, the potential health benefit of fermentation of dietary fibres in the colon is now well recognized. However, gas production associated with this fermentative process can be responsible for digestive troubles that often lead individuals to eliminate dietary fibre from their diet. Hydrogen, one of the main fermentative gases, is partly excreted but the main pathway of H_2 disposal remains its utilization by H_2-consuming microorganisms. Among the three hydrogenotrophic mechanisms existing in the human colon, reductive acetogenesis is of particular interest since acetate formed from CO_2 reduction by H_2, is non-gaseous and an energy source for eukaryotic cells. The objective of our work was to investigate the ability of *Ruminococcus hydrogenotrophicus*, an H_2-utilizing acetogen isolated in our lab, to re-utilize H_2 produced during fermentation of dietary fibre. In vitro, *R. hydrogenotrophicus*, could efficiently re-utilize H_2 produced from cellulose fermentation by cellulolytic isolates. This H_2-transfer induced a metabolic shift via acetate in some cellulolytic strains, which was associated with an enhancement of cellulolysis. In vivo, the daily ingestion of *R. hydrogenotrophicus* by human-flora associated rats led to an increase in the faecal acetogenic population and induced an important decrease in total gas excreted (H_2 and $H_2 + CH_4$) by animals. In conclusion, *R. hydrogenotrophicus* showed great ability to utilize H_2 produced during the fermentative process. This acetogen was also able to survive through the upper digestive tract and remained active in the colon. Therefore, *R. hydrogenotrophicus* appears as a potential probiotic that could be used to decrease colonic gas production and alleviate digestive troubles associated with dietary fibre fermentation.

P-86 **Efficacy of siesta grazing to improve rumen microbial growth.** V. Fievez[a], C. Dragomir[b], B. Vlaeminck[a], D. Demeyer[a] ([a] Department of Animal Production, Ghent University, Belgium; [b] Department of Animal Nutrition, I.B.N.A.-Balotesti, Romania)

Common dietary strategies may induce periods of severe rumen N/energy imbalance. Siesta grazing – allowing limited grazing with an intermediate period indoors (e.g. 1 p.m. to 7 p.m.) – intended to improve rumen N utilisation through a more even distribution of high and low protein feeds. During two consecutive 2-week-periods, traditional (T) and siesta (S) grazing were compared based on feed, milk and spot urine samples, taken from 30 dairy cows on a private farm. The main components (on DM basis) of stable diets were maize silage (55%), sugar beet pulp (14%), grain distiller silage (12%) and a protein mixture (9%). Crude protein contents (CPC) of stable diets were 121 ± 8 (T) and 140 ± 12 (S) $g \cdot kg^{-1}$ DM, whereas those for fresh grass were 211 ± 26 (T) and 188 ± 15 (S) $g \cdot kg^{-1}$ DM. Milk production did not differ between the two grazing strategies [26.1 ± 6.6 (T) vs. 26.2 ± 7.2 (S) $kg \cdot cow^{-1} \cdot day^{-1}$, $n = 120$]. The slight decrease ($P < 0.05$) in allantoin/ creatinin ratios in spot urine samples during siesta grazing [3.2 ± 0.8 (T, $n = 81$) vs. 2.9 ± 0.8 (S, $n = 53$) $mmol \cdot mmol^{-1}$] suggests no improvement of rumen microbial growth, probably because the CPC of the stable diets did not cause a severe N limitation for rumen microbial growth or the slow energy release from maize silage. Higher ($P < 0.01$) milk urea concentrations [253 ± 49 (T) vs. 302 ± 8 (S) $mg \cdot L^{-1}$, $n = 120$] even suggest a reduced efficiency of rumen N utilisation, but also might indicate an increased ingestion of fresh grass during siesta grazing, as reported in recent Dutch research. The research

has been supported by a grant from the Belgian Federal Office for Scientific, Technical and Cultural Affairs and the Flemish Administration for Agriculture and Horticulture.

P-87 **Microbial caecal fermentative activity in Iberian or Landrace pigs given maize or acorn/sorghum diets.** M. Fondevila[a], J. Morales[b], J.F. Pérez[b], J. Gasa[b] ([a] Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza, M. Servet 177, 50013 Zaragoza, Spain; [b] Departament de Ciència Animal y dels Aliments, Universitat Autónoma de Barcelona, 08193 Bellaterra, Spain)

The caecal contents of Iberian (IB) pigs, a fat breed produced in extensive conditions, and Landrace (LD) pigs given maize (MZ) or acorn/sorghum (AS) based diets from 88 to 133 kg were obtained after slaughter and studied in a 2×2 factorial design (6 animals per treatment). Microbial concentration, estimated as mmol purine bases$\cdot g^{-1}$ DM, was lower in IB than LD (26.3 vs. 42.0; $P < 0.001$). Guanine:adenine ratio (1.48 vs. 1.32; $P < 0.01$) might suggest differences in the type of population. Although not statistically significant, total enzymatic activities against polysaccharides in IB were 34 to 49% larger than LD. Besides, bacterial activities (per unit of purine bases) against CMC and starch were higher in IB than LD (11.0 vs. 6.4 and 26.8 vs. 11.9; $P < 0.01$). There were no differences in total volatile fatty acid concentration, but propionate and butyrate proportions were higher and lower, respectively, in IB (27.5 vs. 22.9 and 9.9 vs. 12.2; $P < 0.05$). The fast transit time in IB may tend to negate the benefit from the higher bacterial enzymatic activity, and thus minimise differences between breeds. Regarding diets, the resistance of acorn starch gives a larger starch arrival to the caecum, promoting a more butyric fermentation (9.1 vs. 13.1 with MZ and AS; $P < 0.001$). A trend for a lower bacterial activity with AS ($P = 0.06$) may be associated with its tannin content.

P-88 **The effect of oligosaccharides and resistant starch in cat and dog rations on faecal NH_3 and H_2S emission.** M. Hesta, G.P.J. Janssens, J. Debraekeleer, R. De Wilde (Laboratory of Animal Nutrition, Ghent University, Belgium)

Prebiotics are commonly used in commercial dog and cat foods. The aim of the present experiment was to evaluate the addition of fructo- (FOS), isomalto-oligosaccharides (IMO) and resistant starch (RS) to a commercial diet on faecal odour components (ammonia and H_2S) in dogs and cats. Fifteen cats were supplemented with 0 or 3% FOS, 3% IMO or 3% RS in 2 periods. The faeces and urine were collected during a 5-day collection period. Food and water intake as well as faecal and urine production and faecal consistency were noted daily but did not differ significantly. All faeces were collected daily and stored at –18 °C. The faeces were defrosted and stored in air closed erlenmeyers. The faeces were incubated for 48 hours at room temperature. H_2S and NH_3 concentrations were measured in the headspace but did not differ significantly between the treatments. The same protocol was used in dogs in a 2×4 Latin square design. Fresh faecal samples were collected and stored at –18 °C. The same method for the measurement of faecal odour was used but an anaerobic environment was simulated by flushing with N_2. The faeces were incubated for 48 hours at 38 °C. This was done to simulate the colonic environment, and to maximise the production of faecal odour. No significant differences were noted for water intake, urine and faecal production and faecal pH. Fecal consistency was normal in all supplemented groups although there was a trend for slightly higher faecal moisture content in the FOS group ($P = 0.065$). In contrast with the experiment in cats, the ammonium concentrations were below detection limit (< 0.2 ppm) in all faecal samples. There were no significant differences between the 4 supplemented groups concerning H_2S concentration.

P-89 **Methane release from feeds characterised by different carbohydrates as measured with Rusitec.** I.K. Hindrichsen[a], H.-R. Wettstein[a], A. Machmüller[a], C.R. Soliva[a], J. Madsen[b], M. Kreuzer[a] ([a] Institute of Animal Sciences, ETH-Zurich, ETH-Zentrum/LFW, 8092 Zurich, Switzerland; [b] Royal Veterinary and Agricultural University, 1870 Copenhagen, Denmark)

Although dietary carbohydrates make a particular contribution to methanogenesis in ruminants, a wide range of carbohydrates has not yet been examined in detail with respect to their methane production potential. Therefore, this study investigated effects of feeds characterised by

different carbohydrates (lignified and non-lignified cellulose, pectin, hemicellulose, galactomannan, inulin, saccharose and starch) on rumen fermentation, particularly on methane release. Eight isoenergetic and isonitrogenous diets with forage (maize silage, grass silage, hay) and concentrate in a ratio of 1:1 were examined with Rusitec. The concentrates differed by the inclusion of either oat hulls (50%), soybean hulls (70%), apple pulp (54%), beet pulp (61%), guar gum (51%), Jerusalem artichoke (68%), beet molasses (18%) or wheat (46%). Bacteria counts were significantly higher with the guar gum and the Jerusalem artichoke diets than with the apple pulp and oat hull diets, while effects on protozoa counts were not significant. NDF degradation was highest with guar gum, but total VFA concentration was significantly lower compared to Jerusalem artichoke. Methane release ($mmol \cdot day^{-1}$) increased in the order of inclusion of oat hull (5.5), wheat (7.5), guar gum (7.5), soybean hull (7.9), beet pulp (8.4), apple pulp (8.5), Jerusalem artichoke (9.7) and molasses (10.3), with methane from molasses treatment being significantly higher than with oat hulls, wheat and guar gum. Accordingly, compared to wheat, some feeds with easily-fermentable carbohydrates (guar gum) do not enhance methane production while others (molasses, Jerusalem artichoke) do so. Therefore equations available for the calculation of methane release seem to be oversimplified when only cellulose and hemicellulose contents are considered.

P-90 **An extract of *Moringa oleifera* seeds modulates protein fermentation by rumen microbes in vitro.** E.M. Hoffmann, S. Muetzel, K. Becker (University of Hohenheim, Inst. for Animal Production in the Tropics and Subtropics, Dept. for Animal Nutrition and Aquaculture, 70593 Stuttgart, Germany)

Feeding ruminants in intensive production systems, especially for dairy production, requires the supply of a very high level of energy and protein. Under such conditions various strategies have been applied to delay ruminal protein fermentation and supply bypass protein to the lower digestive tract. They were usually based on chemical treatments or antibiotics, and lately have received considerable criticism. A natural compound extracted from the seeds of the pantropical tree *Moringa oleifera* may have a similar potential to increase the efficiency of protein utilization in high yielding cattle, without the negative side effects. The efficacy of the new substance, which is neither a tannin nor a saponin, was clearly demonstrated in an in vitro system. The *Moringa* extract modulated ruminal fermentation in a selective manner. It strongly delayed protein degradation by rumen microbes, and at the same time only moderately inhibited the fermentation of carbohydrates. The inhibition of protein fermentation was specific in the sense that a model protein (BSA), the major protein of fresh forage (Rubisco), and a number of soy proteins were affected, but casein was not. Also, the extent of inhibition depended on the underlying carbohydrate source. All of the observed effects were dosage dependent. Three varieties of *Moringa* of different geographic origin were tested and the active principle was present in all of them. Current work tries to identify the active compound and isolate it from the crude extract.

P-91 **Are ruminal bacteria protected against environmental stress by plant antioxidants?** K. Holovská[a], V. Lenártová[b], K. Holovská[b], P. Pristaš[a], P. Javorský[a] ([a] Institute of Animal Physiology, Slovak Academy of Sciences, 04001 Košice, Slovakia; [b] Department of Chemistry, Biochemistry and Biophysics, University of Veterinary medicine, 041 81 Košice, Slovakia)

Ruminants can be exposed to the toxic concentrations of different environmental pollutants, including heavy metals, by consumption of contaminated feed and water. These toxic substances can be inhibitory to both the fermentative activity and growth of the microbes, thereby changing the physiological steady-state of rumen fermentation. The consumed xenobiotics also contribute to enhancing of free oxyradicals content, which could generate the oxidative stress of ruminal microbes. The cells are usually protected against the environmental stress by activation of own antioxidant enzymes as a superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) and glutathione reductase GR), which exterminate the toxic free radicals. In our previous experiments we have detected a significant increase of SOD activity in the ruminal strain *S. bovis* 4/1 with different concentrations of

mercury. In the present study we have documented the positive effect of nonenzyme antioxidant substances (alpha tocopherol, beta carotene, seleno-L-methionine) on the elimination of environmental stress, evoked by mercury, in ruminal bacterium *S. bovis* 4/1. We have observed a significant decrease in the activity of SOD of *S. bovis* 4/1 in the presence of mercury (II) chloride (5 $\mu g \cdot mL^{-1}$) and of potential plant antioxidants, except melatonin, under anaerobic conditions in in vitro culture. The GSHPx activity of *S. bovis* 4/1 was not significantly changed under the same cultivation conditions and a significant decrease of GSHPx activity was found only in the presence of beta carotene. The role of defence mechanisms in rumen bacteria against antioxidants of plant origin requires further more complete studies.

P-92 **Effect of dietary supplementation of Japanese horseradish on methane production, rumen fermentation and digestibility in steers.** H. Itabashi[a], N. Mohammed[a,b], N. Ajisaka[b], Z.A. Lila[a], Koji Hara[b], K. Mikuni[b], Kozo Hara[b], S. Kanda[b] ([a] Tokyo University of Agriculture and Technology, Fuchu-shi, Tokyo 183-8509, Japan; [b] Bioresearch Corporation of Yokohama, 1-1-1 Fukuura, Kanazawa-ku, Yokohama 230-0004, Japan)

Ruminal methane emissions have received attention in vitro due to their contribution to global warming. Many studies have been made to reduce methane production, however practical methods have not been established. In this paper, we evaluated the effect of horseradish-cyclodextrin (HR-CD) on ruminal methane, volatile fatty acids and microbial populations in steers. Four Holstein steers were fed hay plus concentrate diet (control diet), and then were fed a control diet plus HR-CD (2% of DM). Both periods lasted for 17 days. Methane production was determined by head-cage chamber. Supplementation of HR-CD inhibited methane production by 21%, increased ruminal propionate by 36%, and decreased acetate and ammonia. Ruminal protozoa were unchanged. Digestibility of DM and NDF were increased by HR-CD. The postprandial blood glucose was increased and urea-N was decreased indicating improved gluconeogenesis and nitrogen utilization. Total viable count was slightly increased and methanogenic bacteria were decreased, while cellulolytic and sulphate reducing bacteria remained unchanged by HR-CD. These results suggest that supplementation of HR-CD could reduce methane production and improve energy utilization.

P-93 **In vitro study of isomerisation and biohydrogenation of polyunsaturated fatty acids (PUFAs) in the rumen.** J.-P. Jouany, B. Lassalas (INRA-URH, Centre de Clermont-Theix, 63122 St-Genès-Champanelle, France)

Conjugated linoleic acids (CLAs), which are synthesised from PUFAs in the rumen, have attractive biological properties. These substances undergo further hydrogenation giving trans-monounsaturated acids (MUFAs), which are used as precursors of CLAs in the mammary gland, and found as they are in edible animal products with negative consequences for consumers. We followed, in vitro, the kinetics of PUFA conversion in the rumen from the polyunsaturated to the saturated state. Linseed oil was used as PUFA source and represented 10% of the substrate [40% hay + 60% barley] in the incubators. Thirty-three percent of C18:3n-3 and 43% of C18:2n-6 disappeared during the first 5 h; only 1 and 7% remained at time 24 h. Total CLAs increased regularly in time and reached a maximum at 24 h (2.2% Total FAs). C9-t11, cis-cis, t11-t13 and trans-trans represented 55, 18, 1 and 1% of total CLAs respectively. Among MUFAs, *t*-vaccenic acid increased at the same rate as CLAs while C18:1n-9 decreased slightly with incubation time. Owing to hydrogenation of MUFAs, the proportion of C18:0 in total FAs rose in time. CLA synthesis was a rather slow process when large amounts of linolenic acid were supplied in the incubators. The highest concentration was reached at 24 h. Trans-vaccenic acid and stearic acid increased also with time, confirming the sequencing process of biohydrogenation: PUFAs, CLAs, MUFAs, SFAs. The proportion of the other SFAs, from C12:0 to C17:0, was not significantly changed during incubation. A similar course was noted in the metabolism of PUFAs when hay was used as substrate, but the rate of biohydrogenation was slower. (The authors are indebted to the European Community for financial support through the HEALTHY BEEF project).

P-94 **Gas pressure inside a rumen in vitro system stimulates the use of hydrogen.** J.-P. Jouany, B. Lassalas (INRA-URH, Centre de Clermont-Theix, 63122 St-Genès-Champanelle, France)

Among the multitude of devices for in vitro culture of rumen microbial ecosystem , some allow gases produced during fermentation to accumulate inside the incubators, while the gas pressure remains at a low level in other systems. We compared the end products of fermentation and estimated the recovery rate of metabolic hydrogen in these two extreme conditions. (S+) incubators where gases were continuously collected in syringes fitted on the rubber cap of the bottle, and (S–) incubators in which gases accumulated for 6 or 24 h, were supplied with either a lucerne hay (H) or a barley/lucerne hay (BH) substrate. Animals used as donors were fed the same diets. Methane production was significantly ($P < 0.0001$) stimulated, and the amount of hydrogen in the gas mixture was significantly ($P < 0.0001$) decreased by gas pressure in (S–), with both substrates (H) and (BH). More acetate ($P < 0.01$) and propionate ($P < 0.05$) were produced in (S–). As a consequence, more theoretical hexoses were fermented ($P < 0.01$) and pH was lowered ($P < 0.0001$) in (S–). The 2H recovery calculated as $100 \times [(2H \text{ accepted})/(2H \text{ released})]$, was markedly improved ($P < 0.0001$) by gas accumulation in (S–): 81.9 ± 7.6 in (S+) vs. 103.7 ± 6.6 in (S–). These results are in line with the increase of the calculated 2H recovery with incubation time (Demeyer, unpublished data), and it could be argued that hydrogen is more efficiently metabolised by rumen microbes when the gas phase located above the liquid phase is maintained under pressure in the incubators, thus increasing gas solubilisation. When gaseous hydrogen is collected in a syringe, or a bag, immediately after being produced during fermentation, metabolic hydrogen could be less available for reaction with electron acceptors. (The authors thank Pr D.I. Demeyer and Dr Veerle Fievez (Gent University, Belgium) for their helpful contribution in the discussion of these results).

P-95 **The effect of sample processing on gas production kinetics of four different maize silages.** A. Kamalak, M.S. Ekinci, Y. Gurbuz, M. Karaman, E. Ozkose (Kahramanmaras Sutcu Imam University Faculty of Agriculture Department of Animal Science, Kahramanmaras, Turkey)

The production of gas during fermentation has been used as an indirect measure of substrate degradability, most commonly referred to as the Menke gas production technique. The aim of this work was to study the effect of sample processing on gas production kinetics. Four maize silages were ground to pass either a 1 or a 6 mm screen. Incubation were carried out for 2, 4, 7, 17, 24 and 48 hour. The gas production data were fitted to the equation $y = A - BQ^{t}Z^{\sqrt{t}}$. When maize silage samples were ground to pass through a 1 mm screen there was no significant ($P > 0.05$) difference between maize silages in terms of gas production and estimated parameters whereas there was a significant difference ($P < 0.001$) between silages in term of gas production and estimated parameters when silage samples were ground to pass a 6 mm screen. It was concluded that sample processing has a significant effect on the interpretation of results obtained from in vitro gas production measurements.

P-96 **The antioxidant enzyme activity of *S. bovis*.** V. Lenártová[a], K. Holovská[a], K. Holovská[b], P. Javorský[a] ([a] University of Veterinary Medicine, Košice, Slovakia; [b] Institute of Animal Physiology, Slovak Academy of Science, Košice, Slovakia)

The first line of defence against reactive oxygen species (ROS) are the enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSHPx). We investigated the presence of these enzymes in the Gram-positive obligately anaerobic bacteria *S. bovis 4/1* isolated from the rumen of sheep. Electrophoretic studies confirmed a single band Mn-SOD that was not inhibited by cyanide or hydrogen peroxide. Paraquat increases oxidative stress directly by generating oxygen radicals. Therefore we tested whether paraquat resulted in an alteration of SOD and GSHPx activities under both aerobic and anaerobic growth conditions. Only 0.5 mM paraquat evoked a significant increase of GSHPx activity and 1 mM paraquat a significant increase of SOD activity as well, under aerobic conditions. All oxygen reactions generating ROS proceed through heavy metal catalysis. In the present study we have tested the influence of different

concentrations (5 μg and 50 μg·mL^{-1} respectively) of mercury, copper and chromium on SOD and GSHPx activities under both aerobic and anaerobic growth conditions. A significant increase of SOD activity was observed only in the presence of mercury. Copper and chromium significantly inhibited SOD activity. GSHPx activity, was significantly increased in the presence of mercury and copper (5 μg·mL^{-1}) under the aerobic growth conditions. The fact that different SOD and GSHPx induction evoked by mercury, copper and chromium supports the idea that there is no general mechanism of bacterial heavy metal resistance and that enzymatic detoxification differs with the toxic heavy metal ion.

P-97 **Effect of essential oils on ammonia production by rumen microbes.** N.R. McEwan[a], R.C. Graham[a], R.J. Wallace[a], R. Losa[b], P. Williams[c], C.J. Newbold[a] ([a] Rowett Research Institute, Aberdeen AB21 9SB, UK; [b] CRINA S.A., 15, Chemin de la Combe, Gland, 1196, Switzerland; [c] AKZO NOBEL Surface Chemistry Ltd., D2 The Courtyard, Alban Park, Hatfield Road, St Albans AL4 OLA, UK)

Essential oils are the volatile oils obtained from plants or from parts thereof by, for example, steam distillation and/or water distillation. These components are responsible for the characteristic aroma of spices. Recently, it was shown that a commercial blend of essential oil compounds, essential oils, decreased rumen ammonia concentrations in sheep. The aim of this study was to investigate further the effects of essential oils on ammonia production in rumen fluid. Four rumen-cannulated sheep received a diet of grass silage and concentrate (60:40). Treatments consisted of a high or low protein concentrate plus or minus essential oils (CRINA RUMINANTS, CRINA S.A. Switzerland, fed to supply 110 mg per sheep per day). Treatments were compared in a 4 × 4 Latin square with 6 week periods. Numbers of hyper ammonia producing bacteria (HAP) in the rumen were determined by a most probable number technique based on the ability to grow with trypticase as the sole energy source. Diversity within the HAP was visualised based on single strand conformational polymorphism (SCCP) using a fragment of the 16S *rDNA* gene amplified with a primer recognising an area found in all bacterial small sub-unit genes (around position 400) together with a primer corresponding to the sense strand of an area around position 200 of the gene in a number of members of the *Bacillus* /*Clostridium* cluster. On the low, but not high, protein concentrate essential oils decreased both the number and diversity of "hyper-ammonia-producing" bacteria in the rumen. This was associated with a decrease in the rate of ammonia production from amino acids. In conclusion essential oils inhibited ammonia production from amino acids in a diet dependent manner. These effects were apparently mediated via the effects of essential oils on HAP bacteria in the rumen.

P-98 **Effect of essential oils on protein digestion in the rumen.** N.R. McEwan[a], R.C. Graham[a], R.J. Wallace[a], R. Losa[b], P. Williams[c], C.J. Newbold[a] ([a] Rowett Research Institute, Aberdeen, AB21 9SB, UK; [b] CRINA S.A., 15, Chemin de la Combe, Gland, 1196, Switzerland; [c] AKZO NOBEL Surface Chemistry Ltd., D2 The Courtyard, Alban Park, Hatfield Road, St Albans AL4 OLA, UK)

Microbial fermentation in the rumen allows ruminants to utilise sources of energy not available to other animals. However, fermentation can also be wasteful if the rate of protein degradation exceeds the rate at which liberated ammonia nitrogen can be incorporated into microbial protein. The objective of this study was to investigate the effect of a dietary additive based on essential oil compounds, which is claimed to reduce the rate of protein breakdown in the rumen. Four rumen-cannulated sheep received a diet of grass silage and concentrate (60:40). Treatments consisted of a high or low protein concentrate plus or minus essential oils (fed to supply 110 mg CRINA RUMINANTS, CRINA S.A. Switzerland per sheep per day). Treatments were compared in a 4 × 4 Latin square with 6 week periods. The in vitro degradability of peas, fishmeal, soyabean meal, sunflower and rapeseed meal in rumen fluid withdrawn from the sheep was determined using the inhibitor in vitro method described by Broderick. The same substrates were incubated in nylon bags in the rumen for 4 h prior to microbes bound to substrates in the bags being extracted using lysozyme and carbon tetrachloride and proteolytic activity being measured. Essential oils decreased the ruminal

degradation of peas and rapeseed meal (measured both in vitro and in vivo), but not other protein sources. The decrease in degradability of peas and rape meal was associated with a decrease in the attachment and colonisation of these feeds by proteolytic microbes. It is suggested that essential oils decreased microbial attachment to substrates in the rumen reducing the rate of protein degradation in the rumen.

P-99 **Influence of dipeptidyl peptidase inhibitors on peptidase activity, growth and ammonia production by ruminal microorganisms**. N. McKain[a], H.R. Wang[b], R.J. Wallace[a] ([a] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK; [b] Inner Mongolian Academy of Animal Science, Huhhot, Inner Mongolia, China)

The dipeptidyl peptidases (DPPs) of *Prevotella* spp. are important in catabolic peptide metabolism in the rumen. Potential inhibitors of DPPs were investigated for their effects on the growth and catabolic activity of pure and mixed cultures of ruminal microorganisms, in order to assess their possible usefulness as protein-sparing feed additives. Four inhibitors, Gly-Phe diazomethyl ketone (GPD), Ala-Ala chloromethyl ketone (AAC), benserazide (DL-serine 2-(2,3,4-trihydroxybenzyl)-hydrazide) and diprotin A (Ile-Pro-Ile) were used in this study. These compounds were found previously to inhibit DPP activities of *Prevotella* spp. GPD and AAC were inhibitory to DPP-I, benserazide inhibited DPP-II-like activity, and diprotin A inhibited DPP-1V. The only effect of the inhibitors on growth of 18 species of ruminal bacteria in a complete ruminal fluid-containing medium (M2) was a 26% decrease by benserazide with *Megasphaera elsdenii*. In contrast, growth of *P. albensis* M384, which has an auxotrophic requirement for methionine, was inhibited by GPD in Pittman and Bryant defined medium (PR) when methionine was supplied in Trypticase or the peptide Ala_3Met but not when methionine was supplied as the free amino acid. Benserazide was inhibitory in all three PR media, while diprotin A and AAC had no effect. The inhibitors had no significant effect on protozoa as determined by their influence on the rate of breakdown of radiolabelled *Selenomonas ruminantium* in vitro. Ammonia production from casein by mixed ruminal microorganisms was inhibited significantly ($P < 0.05$) by AAC (29% inhibition) and benserazide (33%). These results suggest that DPP inhibitors could improve nitrogen retention by ruminants and have the potential to be developed as growth-promoting feed additives.

P-100 **Effect of gliotoxin, an *Aspergillus fumigatus* mycotoxin, on rumen microbial fermentation in vitro**. D.P. Morgavi, H. Boudra, D. Graviou, J.-P. Jouany, B. Michalet-Doreau (INRA, Centre Clermont-Theix, Unité de Recherche sur les Herbivores, 63122 Saint-Genès-Champanelle, France)

The mycotoxins present in poorly preserved silage and hay have not received as much attention as those usually found in cereals. However, they may affect ruminant production and health as well as contaminate animal products destined for the food industry. *Aspergillus fumigatus*, one of the predominant species isolated from molded preserved forages can produce several toxic metabolites, including gliotoxin. Gliotoxin, which has antimicrobial and immunosuppressive properties, plays an important role in the development of aspergillosis. It has also been linked to a case of feed intoxication in camels. Up to date there is no report describing the effect of gliotoxin on rumen microbial fermentation. A completely randomized study was carried out to investigate the effects of gliotoxin on rumen fermentation in vitro. Ethanol solutions of gliotoxin (0.25 mL) were applied to 0.5 g alfalfa hay contained in vials and the ethanol allowed to evaporate at room temperature. Fermentations were done using six vials per treatment and were replicated in time. Each vial contained 40 ml of rumen buffer and 10 ml rumen fluid and they were incubated for up to 24 h at 39 °C. Rumen fluid was obtained from three forage-fed wethers, prior to feeding. Final concentrations of gliotoxin were 0.1, 0.05, 0.01, 0.001, and 0 $\mu g \cdot mL^{-1}$. Gas production, volatile fatty acids, and dry matter digestion were used to monitor microbial activity. Gliotoxin did not negatively affect ($P > 0.05$) microbial fermentation of alfalfa hay in vitro under the conditions studied. The fate of this toxic fungal metabolite in the rumen and animal tissues after ingestion remains to be investigated.

P-101 **The effect of tyrosine supplementation on the production of *p*-cresol by monocultures of the gut microflora during in vitro batch fermentations.** P. Neysens[a], B. Degeest[a,b], W. Vansieleghem[a], B. Pot[a,b], L. De Vuyst[a] ([a] Research Group of Industrial Microbiology, Fermentation Technology and Downstream Processing (IMDO), Department of Applied Biological Sciences, Vrije Universiteit Brussel, 1050 Brussels, Belgium; [b] Yakult Belgium N.V., 1070 Brussels, Belgium)

The intestinal microflora plays an important role in the metabolism of both carbohydrates and proteins. In the colon carbohydrates are mainly converted into short chain fatty acids. Proteins result by putrefaction in the formation of potentially toxic compounds such as ammonia, amines, mercaptans, and phenol derivatives. All these metabolites play an important role in the pathophysiology of the colon. The quality and quantity of these metabolites derived from bacterial metabolism are dependent on the characteristics of the gut flora, the transit time through the colon, and the availability of essential nutrients. For instance, the toxic molecule *p*-cresol is formed as the end-product of tyrosine metabolism by some intestinal anaerobic bacteria. It binds strongly to plasma proteins and hence it is retained in the blood stream when the kidneys fail. For instance, during in vitro batch fermentations it was shown that *Clostridium perfringens* LMG 11264 was capable of forming *p*-cresol when tyrosine was added to the growth medium. With increasing concentrations of tyrosine lower viable cell counts were obtained but higher conversion rates of tyrosine were observed. After addition of tyrosine to an active growing bacterial population a higher amount of *p*-cresol was produced. Other members of the gut microflora that are often excessively present in the colon of uremic patients such as *Escherichia coli* (LMG 2092) and *Bacteroides fragilis* (LMG 10263) did not form *p*-cresol from tyrosine. Finally, it was shown that *Lactobacillus casei* YIT 9092 (a probiotic strain), *Enterococcus faecalis* LMG 7937 (a common lactic acid bacterium member of the gut), and *Bifidobacterium longum* LMG 10497 (a health supporting species of the gut microflora) did not experience any effect upon tyrosine supplementation. The probiotic strain did not show any uptake of the toxic molecule either, whereas the *E. faecalis* strain converted tyrosine into a metabolite that could not be identified up to now. Hence, the advantageous effect of probiotic lactobacilli will exist in the increase of biomass at the cost of undesirable gut bacteria and at the same time in favour of removal of toxic compounds via the feces.

P-102 **Propionate uptake by rumen microorganisms.** P. Nozière, S. Gachon, C. Martin, M. Doreau (URH, INRA, Theix, 63122, France)

Ruminal infusions of volatile fatty acids (VFA) are widely used to investigate their production rate and metabolism during absorption. We assessed if short- and long-term ruminal infusion of propionate may induce uptake of this VFA into rumen microbes. Four ruminally cannulated sheep were fed 1000 g hay in 8 meals·d^{-1}. Treatments consisted of no infusion (C), ruminal infusion of propionate (86 g·d^{-1}) for 1 (P1) and 7 d (P7), and of salts for 7 d (S7), in a 4×4 Latin square design. The infusion of propionate increased its ruminal molar proportion from 19 (C, S7) to 32% (P1, P7). Ruminal pH, osmolality, amount of protozoa, and protozoal genera remained similar among treatments. Rumen contents (100 mL liquid + 100 g solid) were incubated at 39 °C in anaerobic flasks containing 200 mL buffer, 0.88 g $(NH_4)_2SO_4$, 17 g ground hay, and 0.45 µCi [2-^{14}C]propionate. After 6 and 16 h, clarified fermenter fluid (CFF), liquid-associated protozoa (LAP), and liquid-associated bacteria (LAB) taken as representative of total bacteria were separated by fractional centrifugations. Microbial pellets were washed with saline before ^{14}C determination. In flasks, pH, osmolality, gas, VFA production, protozoal counting and ^{14}C repartition were similar among treatments. Between 6 h and 16 h, the amount of ^{14}C decreased in CFF and increased in LAB and LAP. Amount of estimated microbial DM was 7.2 g/flask. Amounts of ^{14}C incorporated into microbial fractions averaged 3.5 and 6.5% of total amount of ^{14}C after 6 h and 16 h incubation, respectively. This indicates that uptake of propionate by ruminal protozoa and bacteria exists, is quantitatively low, and does not increase when rumen microbes are submitted to propionate infusion.

P-103 **Rumen microorganisms are not almighty in protein nutrition of ruminants.** R. Onodera, S. Wadud, M.M. Or-Rashid (Laboratory of Animal Nutrition and Biochemistry, Miyazaki University, Japan)

Amino acid nutrition of ruminants has been assumed to depend only on rumen microbes growing with urea as a sole source of nitrogen. In this sense nutritionists have considered that rumen microbes were fundamentally almighty in protein nutrition of the host animals. However, in a series of our experiments to examine the abilities of rumen microbes to synthesize amino acids, which had been regarded as essential for rats, it has been revealed that histidine was not synthesized de novo at all by mixed rumen bacteria and protozoa, which were collected from the rumen contents of goats reared with haycube and concentrates. Similarly, threonine was also shown not to be synthesized de novo by mixed rumen protozoa. On the basis of our own observations concerning the nutritive value of rumen microbes and contents, histidine turned out to be the first limiting amino acid of rumen microbes and hence rumen contents when evaluated by chemical score using beef protein as a reference protein. On the other hand, some reports have indicated that methionine, lysine and threonine were limiting in this order in rumen microbial protein when compared with requirements of the ruminants tested. Histidine, however, was not included in the limiting amino acids. These contradictory facts hinted that ruminants might have an ability to synthesize histidine by themselves. Experiments carried out with crude enzymes of cattle organs such as liver, kidney, muscle and so on have revealed a relatively high activity of histidinol dehydrogenase, which works at the last step in de novo histidine synthetic pathway. From these facts, rumen microbes of this sort can be considered not almighty in supplying amino acids to the host.

P-104 **Effects of malate on in vitro rumen fermentation of cereal grains.** M.J. Ranilla[a], M.D. Carro[a], J. Mesià[b] ([a] Dept. Producción Animal I, Universidad de León, 24071 León, Spain; [b] División NATURE. NOREL & NATURE, S.A. Calle Industria, 1. 08185 Llicà de Vall, Spain)

The effects of malate (Rumalato®; Norel & Nature, S.A., Spain) on rumen fermentation of corn and barley grains were investigated in vitro using batch cultures. Ruminal fluid was collected from 4 rumen-cannulated sheep fed hay ad libitum and supplemented with 400 g of concentrate daily. Samples were incubated with ruminal fluid for 17 h at 39 °C. Rumalato® was added to the incubation bottles to achieve final malate concentrations of 0, 4, 7 and 10 mM. For both substrates, volatile fatty acid (VFA) production increased ($P < 0.001$) linearly with increasing concentrations of Rumalato®. Final pH also increased ($P < 0.001$) linearly from 6.14 and 6.27 for corn, and 6.17 and 6.29 for barley, for 0 and 10 mM malate concentrations, respectively. Propionate production increased linearly ($P < 0.001$) from 1.38 to 1.62 mmol for corn (values for 0 and 10 mM malate, respectively) and from 1.40 to 1.67 mmol for barley. Whereas increasing amounts of the additive produced a linear increase ($P < 0.05$) of acetate production for corn grains, no changes ($P > 0.05$) in acetate production were detected for barley grains. The acetate:propionate ratio decreased linearly ($P < 0.001$) from 1.49 to 1.36 and from 1.53 to 1.33 for corn and barley grains, respectively. The addition of Rumalato® decreased linearly the concentration of L-lactate for corn ($P < 0.01$; from 203 to 103 mg·L^{-1}) and barley ($P < 0.05$; from 179 to 126 mg·L^{-1}) grains. Malate supplementation tended ($P < 0.10$) to decrease the production of methane for barley, but no effect ($P > 0.05$) was observed for corn. In conclusion, Rumalato® stimulated the in vitro rumen fermentation of corn and barley grains by increasing concentrations of propionate and total VFA and decreasing L-lactate concentrations, leading to an increase of the final pH.

P-105 **Distribution of liquid- and solid-associated bacterial populations in the Rusitec system: effect of forage to concentrate ratio in the diet.** M.J. Ranilla, M.D. Carro, M.L. Tejido (Dept. Producción Animal I, Universidad de León, 24071 León, Spain)

The proportion of bacteria associated with particulate matter is important in meeting the nutrient requirements of ruminants. The relative proportion of solid-associated (SAB) and liquid-associated (LAB) bacteria in the rumen can be affected by dietary factors such as forage to concentrate ratio. The effects of two different

forage to concentrate diets on the distribution of SAB and LAB bacterial populations were studied in an artificial rumen (Rusitec). Diets consisted of chopped alfalfa hay and concentrate (cracked barley grain, cracked corn grain and soybean meal, 0.39:0.44:0.17) in the proportions of 0.8:0.2 (C20 diet) and 0.2:0.8 (C80 diet). SAB and LAB were isolated by differential centrifugation and ^{15}N was used as a microbial marker. Daily microbial yield was similar ($P > 0.05$) for both diets (114 vs. 116 mg microbial N for the C20 and C80 diets, respectively), but the amount of SAB was higher ($P < 0.05$) with the C20 diet (77 mg $N \cdot d^{-1}$) than with the C80 (63 mg $N \cdot d^{-1}$) diet. As a result, the proportion of SAB was higher ($P < 0.001$) with the C20 diet (0.67 vs. 0.54 for C20 and C80), with a consistently lower LAB proportion ($P < 0.001$) for the C20 (0.33) than for the C80 (0.46) diet. The high ratio of forage: concentrate (C20) decreased ($P < 0.01$) ^{15}N enrichment of both the LAB and the SAB and increased ($P < 0.01$) the N content in SAB. The results seem to indicate that the forage to concentrate ratio can alter bacterial colonisation of rumen particles and the chemical composition and the relative proportion of LAB and SAB.

P-106 **The nutritional importance of ruminal bacterial extracellular polysaccharides**. L.M. Rode, V.L. Nsereko, K.A. Beauchemin, D.P. Morgavi (Agriculture and Agri-Food Canada, Research Centre, Lethbridge T1J 4B1, Canada)

Extracellular polysaccharides (EPS) are produced by several bacterial species in their natural environment. While it is known that some rumen bacteria possess EPS, the nutritional and energetic implications of bacterial EPS production in the rumen have been virtually ignored. Cultured bacteria produce less EPS, and their carbohydrate fraction is more digestible than that from mixed bacteria obtained from the rumen. If this difference can be ascribed to the presence of EPS, increasing its digestion has the potential to enhance the dietary energy available to ruminants. To test this hypothesis EPS obtained from rumen and duodenal contents of cattle fed different diets was quantified and characterized in terms of sugar composition and degradability by polysaccharidases. The material obtained had a monosaccharide profile different from that of feed components present in the diets with a relative abundance of galactose, rhamnose, and fucose, while xylose was practically absent. EPS was not digested by the mammalian polysaccharidases amylase and amyloglucosidase. Mixtures of fungal enzymes, able to digest plant fibre, were at least 10 times less effective at degrading EPS than at degrading cellulose. Total concentrations of EPS collected from the rumen were 30, 45, and 160 g/100 L fluid for forage, dairy, and concentrate-fed animals ($n = 3$), respectively. In duodenal samples of dairy cows ($n = 6$), EPS concentrations ranged from 2.5 to 4.5% DM. EPS from mixed ruminal bacteria were resistant to degradation by mammalian and exogenous enzymes. However, quantities of EPS present in rumen and duodenum of ruminants were not large enough to sustain the hypothesis that utilization of this material would significantly increase the dietary energy available to the animal.

P-107 **Effect of ammonia rumen content on proteolysis in ewes, as a consequence of a change in the diet.** M. Sales-Duval, G. Blanchart (ENSAIA, Laboratoire de Sciences Animales, Vandoeuvre-les-Nancy, France)

In order to study the effect of a small difference in starch and nitrogen availability on proteolysis, two different diets were successively supplied to four ewes fitted with rumen fistulae. They differed in the ratio of fermentable nitrogen (FN) over fermentable energy estimated by the fermentable organic matter content (FOM) with 144 g FN per kg FOM for diet I and 126 g FN per FOM for diet II. Samples of rumen juice were taken at 0, 0.5, 1.5, 2.5, 4 and 6 hours after access to the meal in each animal. The rumen content was fractionated into liquid phase and small particles (< 0.2 mm). pH and VFA's did not significantly change between the two diets ($P > 0.05$) but the lactate content was three times higher with diet II. The disappearance rate of soluble proteins was 2.5 times higher with diet II. At the same time, the total proteolytic activities of the particulate and the liquid compartments were significantly stimulated with diet II ($P < 0.05$) while no change was measured with the diet I during this time. This fact could help in explaining the higher disappearance of dissolved proteins with diet II. This was accompanied by a

significantly lower concentration of ammonia (from –28 to –43%). Moreover, in the liquid phase exopeptidase activities increased more with the diet II, especially leucine aminopeptidase and dipeptidyl peptidase I. No significant difference was observed at the particulate level. We then suspected a positive effect of ammonia when it is liberated at low concentrations in the rumen, as shown with diet II, but this hypothesis cannot be distinguished from the increase of the ratio of FOM in this diet: this probably favoured the development of a distinct flora as the increase of the lactate concentration and of the leucine aminopeptidase activity suggests.

P-108 **Influence of pattern of energy supply on the degradability of grass and microbial numbers in the rumen simulating fermentor Rusitec**. M.L. Tejido[a], A.Y. Guliye[b], S. López[a], R.J. Wallace[b], C.J. Newbold[b] ([a] Universidad de León, 24071 León, Spain; [b] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

Recently there has been increased interest in matching or synchronizing the supply of energy and nitrogen in the rumen in order to improve microbial growth. However we found no effect on microbial synthesis of changing the pattern of pre-formed amino acid supply in a rumen-simulating fermentor fed a diet of NDF. Here we investigate the effect of changing the energy supply in a fermentor fed grass. Twelve vessels were supplied at the same time every day with 20 g of frozen grass (circa 4 g DM). Treatments were allocated at random to 3 vessels each and were: grass fed once daily unsupplemented (G), grass supplemented with maltose as continuous infusion (GMI), grass with maltose infused over the 6 h immediately after feeding (GMA) and grass with maltose infused over the 6 h immediately prior to feeding (GMP). Maltose concentrations were adjusted to add 1 g per vessel per day to supplemented vessels. The experiment lasted 21 days. As might be expected VFA production rates were higher in maltose-supplemented vessels. Ammonia outputs from the fermentors were low (reflecting the low level of DM input) but were reduced by maltose addition (11.3, 1.4, 3.4, 3.7 SED 1.14 $mg \cdot d^{-1}$ for G, GMI, GMA and GMP vessels respectively). Treatments had no effect on the degradability of the grass in the fermentors and while total bacterial numbers were higher in maltose-supplemented vessels there were no significant treatment effects (3.63, 4.5, 4.4 and 5.9 SED 0.98×10^8 mL^{-1} respectively). In conclusion while maltose addition stimulated ammonia uptake and may have stimulated bacterial growth the pattern of maltose supply did not seem to be important.

P-109 **Effect of supplementing a high fibre basal diet with two N sources (ammonia and soyabean protein) on microbial growth in the rumen simulation fermenter Rusitec.** M.L. Tejido, M.J. Ranilla, S. López, R. Bodas, M.D. Carro (Dept. Producción Animal I, Universidad de León, 24071 León, Spain)

Many studies have demonstrated benefits to feeding pre-formed amino acids in terms of increased rumen microbial growth. In most of these studies a sample of liquid-associated bacteria (LAB) was considered to be representative of the total population leaving the rumen. The aim of this study was to investigate the effect of two N sources (ammonia and isolated soyabean protein) on microbial growth of both LAB and solid-associated bacteria (SAB) in the rumen simulation fermenter Rusitec. Each vessel was fed daily 16 g of an almost N-free basal diet containing 700 $mg \cdot g^{-1}$ of neutral-detergent fibre extracted from grass hay and 300 $mg \cdot g^{-1}$ of starch. Nitrogen (300 $mg \cdot d^{-1}$) was supplied by the continuous infusion of solutions containing just ammonia (4 vessels) or a mixture (0.5:0.5) of ammonia and soyabean protein (4 vessels). ^{15}N was used as a microbial marker. Two weeks after the beginning of the trial, samples of digesta and effluents were collected and pellets of SAB and LAB were isolated for the study of microbial growth. In comparison with ammonia as the only N-source, the supplementation with soyabean protein resulted in a greater ($P < 0.001$) microbial growth of both SAB (32.5 vs. 25.9 mg microbial $N \cdot d^{-1}$) and LAB (55.2 vs. 45.9 mg microbial $N \cdot d^{-1}$). Apparent disappearance of organic matter (OM) was greater ($P < 0.05$) for the soyabean treatment (458 vs. 436 $g \cdot kg^{-1}$). Microbial efficiency, expressed as g microbial $N \cdot kg^{-1}$ OM apparent disappearance, was increased ($P < 0.01$) by supplementation with protein (12.0 vs. 10.3). The results of this study would indicate that N forms other than ammonia are required for maximum growth of both SAB and LAB in the rumen.

P-110 **Effect of crude fibre content in grass silage on milk odd-chain fatty acids.** B. Vlaeminck[a], M.H. Bruinenberg[b], V. Fievez[a], K. Raes[a], D. Demeyer[a] ([a] Department of Animal Production, Ghent University, Belgium; [b] ID TNO Animal Nutrition, Lelystad, The Netherlands)

The lipids of rumen fibrolytic and amylolytic bacteria are distinguished by their odd-chain fatty acid (OCFA) pattern. As bacterial fatty acids are partly deposited in milk lipids, milk OCFA content might be a useful tool in the characterisation of the rumen bacterial population. Four Holstein cows were used in a 4 x 4 Latin square to study the effect of grass silage, differing in botanical composition, on milk OCFA. Experimental diets consisted of 15 kg dry matter (DM) either from 100% intensive ryegrass silage (IRGS) (I), 80% IRGS + 20% meadow bird grass silage (MBGS) (II), 40% IRGS + 60% MBGS (III) or 40% IRGS + 60% herbage rich grass silage (IV), supplemented with a protein-rich concentrate (4.5 kg DM, NEL 7.4 $MJ \cdot kg^{-1}$). After a two week adaptation period, milk samples were collected during four consecutive milkings and analysed for fatty acid methyl esters (FAME) (until present, only 1 sample per cow for each diet, $n = 16$). An increase in crude fibre content of the diet (259 ± 18, 266 ± 16, 278 ± 14 and 290 ± 9 $g \cdot kg^{-1}$ DM for respectively diet I, II, III and IV) was accompanied by a decrease in the proportion of C15:0 ($r_{pearson} = -0.769$, $P < 0.001$) and an increase in that of C17:1 ($r_{pearson} = 0.665$, $P < 0.01$). This finding might be related to lower and higher levels, respectively, of these fatty acids in the bacteria present in the solid phase of the rumen. (B. Vlaeminck was supported by a grant from the Flemish Institute for the Promotion of Scientific-Technological Research).

P-111 **Effects of daidzein on in vitro fermentation of micro-organisms from the goat rumen.** W.-Y. Zhu[a], S.-Y. Mao[a], Q.-J.Wang[a], W. Yao[a], Q. Liu[a], M.K. Theodorou[b] ([a] College of Animal Science and Technology, Nanjing Agricultural University, China; [b] Institute of Grassland and Environmental Research, Aberystwyth SY23 3EB, UK)

Daidzein is an isoflavonoid compound with estrogenic activity, naturally present in legumes. The estrogenic activity of these compounds can become more effective after rumen metabolism and may result in higher feed conversion efficiency in ruminants. In this work we investigated the effect of daidzein (a synthesized product) on the fermentation profiles and ammonia production of microorganisms from the rumen of goats. Strained rumen fluid was incubated with ground rice straw ($7\ g \cdot L^{-1}$) and soybean concentrates ($3\ g \cdot L^{-1}$) for 24h in a habitat-simulating medium containing daidzein (5–200 $mg \cdot L^{-1}$). Ammonia and volatile fatty acids (VFA) were determined after 24 h incubation. Results showed that there were no significant differences between treatment and control incubations, except for incubations involving 10 $mg \cdot L^{-1}$ of daidzein where ammonia concentrations were 13% lower than that in the control ($P < 0.05$) after 24 h of incubation. VFA concentrations (moles) were also unaffected by daidzein treatment. However, in terms of molar proportions, incubations with 5 or 10 $mg \cdot L^{-1}$ of daidzein caused a significant ($P < 0.05$) reduction in acetate and a significant ($P < 0.05$) increase in propionate relative to the control. No significant difference was observed in the concentration of microbial cell protein between treatments and control. In more detailed investigations with 10 $mg \cdot L^{-1}$ of daidzein, changes in the profile of VFA molar proportions were evident after one hour of incubation, whereas differences in ammonia concentration became apparent after five h of incubation. The results suggest that daidzein with concentration around 5 to 10 $mg \cdot L^{-1}$ could alter VFA profiles in the rumen propionate and away from acetate.

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IL-2 **Rumen ciliate protozoa.** B.A. Dehority (Ohio State University, Wooster, OH 44691, USA)

Recent studies with in vitro monocultures have provided a new look at some physiological and morphological characteristics of rumen protozoa. Generation times (GT) for *Entodinium caudatum* and *Epidinium caudatum* measured by growth from a small inoculum were 23 and 26 h, respectively. However, when estimated by transferring at various time intervals, GT for these two species were 13 and 12.5 h, which helps explain how they can maintain their numbers in vivo. The GT for *Entodinium exiguum* was 13.5 h. Longer GT were observed for the higher ophryoscolecids. Since feeding of high concentrate diets generally results in an *Entodinium*-only fauna, it has been suggested that the *Entodinium* are more pH tolerant than the other genera of ciliates. Using in vitro cultures grown and transferred in poorly buffered medium to simulate rumen fluctuations in pH, *Entodinium caudatum*, *E. exiguum*, *Epidinium caudatum* and *Ophryoscolex purkynjei* all had a minimum pH value between 5.3 and 5.4. No adaptation to low pH was observed in *Epidinium* cultures after recovery of one or two viable cells remaining in pH 5.4 medium. Unexpectedly, and differing from the other species, *Ophryoscolex* concentrations were highest between pH 5.6 and 5.8. Numerous reports suggest that rumen protozoa require live bacteria for growth in vitro. Using *Entodinium caudatum* and *E. exiguum* cultures, without bacteria, plus dead bacteria or plus live bacteria, no differences in concentration were observed up through 48 h. However, higher concentrations were present with live bacteria at 72 and 96 hr for *E. exiguum* and *E. caudatum*, respectively. Starting with clone cultures of *Epidinium*, possessing different numbers of caudal spines, caudal spination was followed after dividing the culture and two transfers. Caudal spination was quite variable, but surprisingly, the five-spined form (*parvicaudatum*) appeared to predominate.

O-26 **Characterisation of XYNB, a modular xylanase from the ruminal protozoan *Polyplastron multivesiculatum*.** E. Devillard[a], C. Béra-Maillet[b], H.J. Flint[a], K.P. Scott[a], C.J. Newbold[a], R.J. Wallace[a], J.-P. Jouany[b], E. Forano[b] ([a] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK; [b] Unité de Microbiologie, Institut National de la Recherche Agronomique, CR de Clermont-Fd/Theix, 63122 St-Genès-Champanelle, France)

Contribution of the ruminal protozoa in the degradation of plant cell wall polysaccharides ingested by the host-animal was recently confirmed by the isolation of several glycosyl-hydrolase genes from *Polyplastron multivesiculatum*. Characterisation of the *xynB* gene and of its product are presented here. XynB is the first protozoan glycosyl hydrolase to be completely characterised and is a modular enzyme comprising a signal peptide, and a family 22 carbohydrate binding module (CBM) preceding a family 10 catalytic domain. The CBM22 was shown to be a true carbohydrate binding module and no evidence was found that it affected enzyme thermostability or pH stability. Unlike other modules from the same family, the XynB CBM22 had no affinity for xylans or mixed linkage glucans, but is the first example of a module of this family that binds to Sigmacell cellulose. The optimal temperature and pH for activity of XynB were respectively 39 °C and 7.0, these values being close to those of the ruminal ecosystem. The phylogenetic relationships between the XynB CBM22 or catalytic domain and related sequences from ruminal microorganisms were analysed.

O-27 **Cryopreservation of rumen ciliate protozoa; creation of a European bank of cryopreserved ciliates.** E. Nsabimana[a], S. Kišidayová[b], D. Macheboeuf[a], J.-P. Jouany[a] ([a] INRA-URH, Centre de Clermont-Theix, 63122 St-Genès-Champanelle, France; [b] Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice, Slovakia)

Rumen ciliates make a significant contribution to the digestive microbial ecosystem and to digestion in ruminants. They also play a detoxifying role, thus protecting the animals against plant and mould toxins. The study of individual species is difficult because it has proved impossible to maintain them in axenic culture. The feasibility of storing rumen ciliates as viable frozen stocks was therefore considered. Then, frozen cells can be transferred to other European laboratories for study. First, we prepared defaunated sheep which were inoculated with a few isolated cells belonging to the same species of rumen ciliates so as

to obtain mono-faunated sheep. Ciliates were concentrated from one litre of rumen content sampled from mono-faunated sheep by sedimentation to eliminate contamination by feed particles. The two-step freezing method was applied under the following conditions: ciliates were suspended in rumen juice with DMSO as cryoprotectant at various concentrations: 4, 5, 6, 10; equilibration time and temperature were 5 minutes and 25 °C respectively. After 45 minutes at the holding temperature set at –30 °C, cells were immersed in liquid nitrogen. Samples were taken at the end of the holding phase to test the viability of ciliates after thawing in rumen juice. This technique was applied to the following species: *Entodinium caudatum, Polyplastron multivesiculatum, Epidinium ecaudatum caudatum, Isotricha prostoma, Dasytricha ruminantium, Eudiplodinium maggii*. The survival rate measured after two weeks of cryopreservation, ranged from 58% to 100% and depended on the species of ciliates. (The authors are indebted to the European Community for financial support through the ERCULE project).

O-28 **Diversity of rumen ciliates revealed by 18S ribosomal DNA analysis.** Seung Yeo Moon-van der Staay[a], G.W.M. van der Staay[a], P. Javorský[b], J.-P. Jouany[c], T. Michałowski[d], E. Nsabimana[c], D. Macheboeuf[c], S. Kišidayová[b], Z. Váradyová[b], N.R. McEwan[e], C.J. Newbold[e], J.H.P. Hackstein[a] ([a] Department of Evolutionary Microbiology, University of Nijmegen, Toernooiveld 1, 6524ED Nijmegen, The Netherlands; [b] Institute of Animal Physiology, Slovakia; [c] INRA, France; [d] Kielanowski Institute of Animal Physiology and Nutrition, Poland; [e] Rowett Research Institute, Buckburn, Aberdeen AB21 9SB, UK)

The rumen hosts an extremely numerous and complex microbiota. Besides astronomical numbers of bacteria, the rumen contains up to 10^{11} ciliates. Traditionally, these ciliates have been identified on the basis of morphological characters. However, this approach appears to have its limitations. Here, we describe a molecular approach to study the biodiversity of rumen protozoa at the molecular level. Clone libraries of 18S rDNA genes from ciliates were established after PCR amplification of DNA of total rumen contents with suitable primers. The diversity of rumen protozoa was analysed by sequencing randomly selected clones. About 400 partial ciliate 18S rDNAs have been sequenced that had been obtained from the rumen contents of goat, sheep and cow. They were compared to sequences from isolated pure cultures obtained from GenBank and within the current project. The phylogenetic analysis suggests that the molecular biodiversity of rumen ciliates exceeds the diversity determined by "classical" means by far. (This project was supported by the EU infrastructure grant QLRI-CT-2000-01455: www.ercule.com).

O-29 **Understanding the molecular ecology of the rumen fungi – a DGGE approach.** M.J. Nicholson[a,b], E.J. Kim[a], M.K. Theodorou[a], J.-L. Brookman[a] ([a] Dept. Animal Science and Microbiology, Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, UK; [b] School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK)

The rumen ecosystem is a complex, open ecosystem harbouring a mixed population of anaerobic bacteria, fungi and protozoa. Feed boli entering the rumen are colonised by micro-organisms and this leads to the digestion of plant tissue which ultimately serve to 'feed' the ruminant host. Although there is a large body of evidence on the individual species inhabiting the rumen and their respective degradative capabilities, characterisation of the relationship between plant and microbe, particularly during the initial stages of colonisation of the feed bolus is less well understood. Molecular methods for the description and characterisation of bacterial populations within complex ecosystems such as the rumen have been described. A variety of techniques including denaturing gradient gel electrophoresis (DGGE) have been used successfully. We have developed a DGGE-based system to characterise the anaerobic fungal populations in the rumen ecosystem. Initial development and optimisation has used axenic cultures and anaerobic fungi found in ruminant faeces. The faecal samples were collected from farmed cattle and sheep in Australia and native African ruminants. Comparisons have been made between dominant population members between and within groups. In general, within-sample diversity was similar but with different representation from the anaerobic fungal genera between samples.

The optimised DGGE protocols are now being used on feed boli recovered from the rumen of animals fed on either grass or clover. A progress report on this programme suggesting the pattern of early colonisation events will be presented.

O-30 **Novel group of archaea recovered from 16S rRNA clone libraries prepared from ovine rumen contents**. A.-D.G. Wright, A.J. Williams, S.K. Baker (CSIRO Livestock Industries, Private Bag 5, Wembley, WA 6913, Australia)

Molecular diversity of rumen archaea was investigated using 16S rDNA clone libraries prepared from the rumen contents of six merino sheep grazing pasture. Clones were constructed using methanogen-specific primers designed in our laboratory to PCR amplify the 16S rRNA gene from rumen contents from each sheep. A total of 326 clones containing almost the complete 16S rRNA gene sequence (1300 bp) were generated from six clone libraries. *Hae*III-Riboprint analysis was used to presumptively identify the methanogen clones against our methanogen riboprint database. Those with novel *Hae*III-riboprint patterns were completely sequenced and a sequence similarity search was performed against our database of new methanogen sequences and against the GenBank® database. Our libraries yielded 16S gene sequences which were similar to cultivated methanogens belonging to the Methanobacteriales. In addition, there were two dissimilar sequences that had very little sequence similarity to any cultivated methanogen and appeared to represent a novel group of rumen archaeal sequences which are atypical for this system. The two clones, CSIRO2.96 and CSIRO9.5, were encountered eight times from two of the six animals and are only distantly related to each other (d = 89.4%). The two novel clones were distantly related ($d \approx 91.1\%$) as the sister groups to a clade consisting of an unidentified group of archaea from swine waste, unidentified archaea from a wine aerobic digester in France, and a group of unidentified archaea recently discovered from a 16S clone library prepared from ovine rumen contents in Japan. These sister groups form a distinct branch whose genetic distance from other methanogens is greater than the genetic distance between the orders Methanobacteriales and Methanomicrobiales. This suggests the erection of a new order of methanogens once cultivars are isolated to validate this proposal. Furthermore, in the absence of cultivated isolates the biology and ecology of this novel group in the rumen remains unclear.

P-112 **Methanogens in kangaroos.** S.K. Baker[a], T. Schoep[a,b], N.J. Edwards[a,c], A.-D.G. Wright[a] ([a] CSIRO Livestock Industries, Floreat Park Research Laboratory, Floreat, 6014, Western Australia; Currently: [b] Murdoch University, Murdoch, 6150, Western Australia; [c] SARDI, Naracoorte, 5271, South Australia)

Evidence of methane (CH_4) production by kangaroos is conflicting. Kangaroos are herbivores. Dense microbial populations in the forestomach ferment dietary polymers, yielding volatile fatty acids in proportions similar to those in the ruminant forestomach. To determine if there are methanogens in *Macropus rufus* (red kangaroo) and *M. fuliginosus* (western grey kangaroo), CH_4 production was determined after incubation of forestomach contents in broth media. Forestomach contents from two *M. rufus* and two *M. fuliginosus* were incubated (8% inoculum, 39 °C, under N_2) in media comprising sterile, strained forestomach contents from either sheep or kangaroo, and Na-acetate, yeast-extract, $(NH_4)_2SO_4$, and cysteine-S (0.125, 0.2, 0.875, 0.375% (w/v) respectively). Forestomach contents from the kangaroos were incubated also (10% inoculum, 39 °C) in media commonly used to culture methanogens, comprising sterile, clarified forestomach fluid from either sheep or kangaroo (10% (v/v)), trypticase, yeast-extract, Na-acetate, Na-formate, NH_4Cl, cysteine-S (0.2, 0.2, 0.25, 0.25, 0.1, 0.375% (w/v) respectively), macro- and trace-minerals, and vitamins. The culture broths contained either cephalothin (6.7 $\mu g \cdot mL^{-1}$) plus clindamycin (1.7 $\mu g \cdot mL^{-1}$) (to retard eubacterial growth) and were incubated either under H_2/CO_2 (80:20) or N_2/CO_2 (80:20), or they contained bromoethane-sulphonate (BES, 50 mM) (to retard methanogen growth) and were incubated under H_2/CO_2 (80:20). BES inhibited CH_4 production by microbial populations from the kangaroo forestomach. CH_4 production was greater when media were prepared with forestomach contents from a kangaroo than from a sheep, suggesting that the former provided a better balance of nutrients or specific nutrients. Strong CH_4 production with incubation under N_2/CO_2

suggested that methanogens in the kangaroo forestomach use sources of hydrogen other than di-hydrogen to reduce CO_2 to CH_4.

P-113 **The role of the ciliate *Eudiplodinium maggii* in starch digestion in the rumen.** G. Bełżecki, T. Michałowski (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland)

Three Polish Merino sheep were used to determine the fate of dietary starch in the rumen in relation to microfauna composition with particular respect to the presence of the ciliate species *Eudiplodinium maggii*. The animals were fed every 12 h with hay (750 g) and ground barley (130 g). The sheep were either ciliate free or faunated with only *Eudiplodinium maggii* or *Eudiplodinium maggii* and *Entodinium caudatum*. The number of *Eudiplodinium maggii* was 15.9–38.5 $\times 10^3$ g^{-1} when this species was alone in the rumen and was reduced by about 28–68% when *Entodinium caudatum* was added. The proportion of cells filled with starch grains increased from about 9–31% before feeding to 85–94% at 4 h after feeding of sheep. *Entodinia* reduced starch engulfment by *Eudiplodinium maggii* by about 16%. Amylolytic activity in rumen digesta of defaunated sheep was 13.4–18.0 μmol glucose released from starch·gDM^{-1}·min^{-1}. It increased after *Eudiplodinium maggii* was added. Amylolytic activity of *Eudiplodinium maggii* contributed about 12–76% of the total activity in the rumen, in relation to animal, time after feeding and presence of *Entodinium caudatum*. The specific amylolytic activity of *Eudiplodinium maggii* was 29.3 ± 9.22 pmol reducing sugars released from starch/ciliate cell·min^{-1}. The amount of starch detected at 12 h after feeding was 10.5, 13.1 and 1.6 g/whole rumen in defaunated and refaunated with only *Eudiplodinium maggii* and *Eudiplodinium maggii* and *Entodinium caudatum*, respectively. These values represent, however, both the undigested dietary starch and storage polysaccharides in the ciliates.

P-114 **Ruminal fermentation characteristics and protozoal population in induced latent acidosis.** L. Brossard[a,b], C. Martin[a], F. Durand-Chaucheyras[b], B. Michalet-Doreau[a] ([a] Institut National de Recherche Agronomique, Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France; [b] Lallemand Animal Nutrition, 42 avenue Général de Croutte, BP1021, 31023 Toulouse Cedex 1, France)

During acute acidosis in ruminants, the ruminal microbial ecosystem and fermentative pathways are largely modified towards an accumulation of propionate and lactate resulting in a very low ruminal pH. Although more frequent, latent acidosis is less known and more difficult to describe because of the instability and the fluctuation of the ruminal microbial ecosystem. The objective of this work was to study the ruminal fermentative changes during an experimentally induced latent acidosis. Four sheep fitted with ruminal cannula were fed twice daily 90% ad libitum. After a 100% hay diet (H) and a 5 days transition period, animals received a 40% hay + 60% wheat diet (W). Compared to H diet, ruminal mean pH decreased (6.48 vs. 5.97) and the area under pH 6 increased considerably (2.78 vs. 276 min × unit pH) with the W diet. Ruminal fermentations of wheat lead to an unusual VFA pattern for such an acidotic diet: low in acetate (68 vs. 62%) and propionate (20 vs. 18%) but high in butyrate (7 vs. 13 %). Lactate concentration increased with the W diet (0.94 vs. 2 mM) but stayed at low levels. Total protozoal counts increased by 2.5 (140 vs. 359 $\times 10^3$ mL^{-1}) with the W diet. The latent acidosis status was not lactic but characterized by a VFA profile rich in butyrate and by a large protozoal population. This suggests the major role for protozoa in determining of fermentative pathways during latent acidosis. A more complete microbial study will go deeper in the explanation of these pathways.

P-115 **Viability of *Entodinium caudatum* cultures, estimated by fluorescence microscopy.** G. de la Fuente[a], M. Fondevila[a], J.A. Cebrián[b] ([a] Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza, M. Servet 177, 50013 Zaragoza, Spain; [b] Departamento de Bioquímica y Biología Molecular y Celular, Universidad de Zaragoza, M. Servet 177, 50013 Zaragoza, Spain)

The viability (% of non-damaged cells) of an in vitro culture of the rumen protozoa *Entodinium caudatum*, was studied using fluorescence microscopy after incubation in different media.

The staining procedure was adapted from the two-step propidium iodide-fluorescein diacetate method, and is based on the different colour of cells depending on their membrane integrity, since the nucleus of damaged cells is selectively stained. Aliquots (4 mL) of *E. caudatum* culture were grown in triplicate tubes at different pH for 3 h, using 0.5 M acetic acid to adjust pH. After incubation, tubes were centrifuged (1000 × g, 5 min), the supernatant was used for pH measurement and the residual 0.5 mL were sampled for fluorescence staining. Proportions of viable protozoa were 92.1 ± 1.53, 79.4 ± 2.6, 63.3 ± 5.9 and 10.4 ± 6.0% at average pH of 6.6, 6.2, 5.6 and 5.0, respectively. Viability decreased linearly ($P < 0.001$), but a quadratic effect was also manifested ($P = 0.002$). The same technique was also applied to study the effect of refrigerating or freezing cultures. Fluorescence showed that the proportion of viable protozoa can be maintained over 70% after 30 min at 5 °C. Some viable cells were still observed after 30 min at –20 °C, but their proportion is overestimated by the high number of broken cells with this treatment. The fluorescence technique is more accurate and less time-depending that microscopic measurement of motility, and can be a rapid tool for evaluating cell damage in cultures of rumen protozoa.

P-116 **Why cannot some species of protozoa grow in the rumen?** J.-P. Jouany[a], E. Nsabimana[a], S. Kišidayová[b], T. Michałowski[c], D. Macheboeuf[a] ([a] INRA, Research Centre of Clermont-Theix, 63122 Ceyrat, France; [b] Slovak Academy of Science, Soltésovej 4–6, 040 01 Košice, Slovakia; [c] Institute of Animal Physiology and Nutrition, 05-110 Jabłonna, Poland)

Pure species of rumen protozoa sometimes need to be isolated in large quantities for certain studies. Given the difficulty maintaining them in in vitro large scale cultures, growing them in the defaunated rumen has been proposed. However, some species of protozoa may fail to grow when inoculated into a defaunated rumen. In France, *Polyplastron multivesiculatum* failed to maintain in two defaunated sheep initially harbouring a B-type fauna, after being inoculated several times. Another unsuccessful attempt was made in a sheep already contaminated with *Epidinium ecaudatum caudatum*. In Slovakia, *Ophryoscolex caudatus trichoronatus* was inoculated three times in two sheep, but no cells could be detected. *P. multivesiculatum* was inoculated in one of the two previous sheep and subsisted at a low level (40 to 10 cells·mL^{-1}) for 7 months. The other sheep received *Entodinium caudatum*, which maintain at a high level (4×10^5 mL^{-1}) for 3 years. In Poland, *Diploplastron affine* inoculated in a defaunated sheep appeared at a low concentration eight days later, but then disappeared definitively. Two hypotheses are advanced to account for these results. (1) Ruminants produce specific antibodies against some protozoa. This could explain why some animals in a herd fed similar diets harbour different species of protozoa. (2) Protozoa are unable to adapt to the rumen environment when in vitro or in situ cultures have been initiated from only a few cells of the same species and grown for long periods. In Poland, *D. affine* took well after being grown in vitro for three weeks, but not when cultured for 11 months. (The authors are indebted to the European Community for financial support through the ERCULE project).

P-117 **Electromigration of rumen ciliate protozoa.** S. Kišidayová, Z. Váradyová (Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic)

The ability of rumen protozoa to move in a unidirectional electrical field from the anode to the cathode was tested in large-volume electromigration equipment; the aim was to concentrate the protozoa and clean them of impurities. As an electromigration system, a cylindrical separating funnel of 150 mL volume was used. The filling compartment served as the starting part, and the mouth of the funnel behind the tap as the collecting part, with a 4 mL volume. 150 mL of fresh rumen fluid with a mixture of ciliates or *Entodinium caudatum* in vitro culture were filled into the starter part of funnel. The collecting part was filled with 4 mL culture medium. During electromigration in the rumen fluid and at a voltage of 10 V (I = 0.8 mA) Isotricha *(Isotricha prostoma, Isotricha intestinalis)* were the first to swim towards the cathode; one minute later they were followed by *Dasytricha ruminantium.* Entodiniomorphous ciliates (small Entodinium as well as large species) displayed minimum movement towards the cathode. The yield of electromigration of *Entodinium caudatum* from the in vitro culture ranged within 2–6%

when using 75–100 V (10 mA) and 60 V (5 mA) voltage, respectively. At 10 V (0.8 mA) Entodinium cells did not move towards the cathode. On the basis of these experiments it can be presumed that the behavior of trichostomatids (dasytrichae and isotrichae) in the tested electromigration equipment differs from that of the entodiniomorphous rumen ciliates. Concentration and cleaning of entodiniomorphous ciliates using electromigration in the tested equipment proved to be ineffective.

P-118 **Codon bias in the rumen ciliates.** N.R. McEwan[a], F.M. McIntosh[a], J.-P. Jouany[b], E. Nsabimana[b], D. Macheboeuf[b], C.J. Newbold[a] ([a] Rowett Research Institute, Aberdeen AB21 9SB, UK; [b] INRA, Research Centre of Clermont-Theix, 63122 Ceyrat, France).

We have already reported that the rumen ciliates have a very unusual codon utilisation pattern. However this work was reported primarily on cDNA sequences derived from a single species of protozoan – *Entodinium caudatum* – supplemented by one or two partial sequences from other rumen protozoa. As part of the EU-funded program ERCULE we have now obtained additional sequence information from the rumen ciliates *Epidinium ecaudatum caudatum*, *Polyplastron multivesiculatum*, *Isotricha prostoma* and *Eudiplodinium maggii*. These sequences confirm our original observation regarding the unusual codon usage pattern, whereby there is a relatively small sub-set of potential codons used on a regular basis, and that there appears to be a strong molecular bias exerted towards their use. In particular there is a strong bias toward AAA, CAA and GAA, rather than AAG, CAG and GAG to encode lysine, glutamine and glutamic acid respectively. In addition, there is a strong bias in favour of AGA as the codon of choice for arginine. Since these organisms are assumed to be monophyletic, and a similar codon usage pattern is not observed in other organisms present in the rumen (both fungal and bacterial), it appears that the original progenitor of the rumen ciliates probably made use of this strong codon bias, rather than it being something which has been acquired following these microbes becoming rumen dwellers. (This project was supported by EU infrastructure grant QLRI-CT-2000-01455: www.ercule.com).

P-119 **Transfer RNA molecules in the rumen ciliate *Entodinium caudatum.*** N.R. McEwan, L.A. McLeod, C.J. Newbold (Rowett Research Institute, Aberdeen AB21 9SB, UK)

In many ciliated protozoa the codons TAA and TAG, which are used as stop codons in the 'universal' genetic code, are used to encode glutamine residues. Furthermore, they do not use the codons CAA and CAG, which encode glutamine in the 'universal' genetic code. From earlier work we have demonstrated that in many of the cDNA clones isolated from *E. caudatum* TAA is the only potential stop codon present in the correct reading frame. The aim of this work was to augment the earlier circumstantial evidence regarding the proposed anti-codon region of tRNA molecules, in particular those encoding glutamine residues. Total genomic DNA was extracted from *E. caudatum* and genes encoding tRNA molecules were amplified using the degenerate primers TAGYBYAGYGGTT and GGTTCRADTCC. Successful amplification was shown by electrophoresis and PCR products were cloned in TOPO® vectors (Invitrogen). Clones carrying a plasmid with an insert were selected and prepared for DNA sequencing. DNA sequences were analysed using the tRNAscan-SE Search Server via the Internet (www.genetics.wustl.edu/eddy/tRNAscan-SE). Two different clones were found to possess genes encoding tRNA molecules. One clone contained a cluster of three tRNA genes; $tRNA^{TAG}$ (an anti-codon for leucine), $tRNA^{GCA}$ (an anti-codon for cysteine) and $tRNA^{GAA}$ (an anti-codon for phenylalanine). The other clone contained the gene $tRNA^{TTG}$ (an anti-codon for glutamine). These four genes would recognise the codons CTA, TGC, TTC and CAA respectively. It is the presence of the fourth of these genes that is of particular interest as this demonstrates that the rumen ciliate *E. caudatum*, unlike many other ciliates, encodes a gene which allows it to encode glutamine residues using the 'universal' genetic code.

P-120 **Effect of dietary fibre and calcium content on methane inhibition by myristic acid in sheep.** A. Machmüller, C.R. Soliva, M. Kreuzer (Institute of Animal Sciences, ETH-Zurich, ETH-Zentrum/LFW, 8092 Zurich, Switzerland)

Interactions within the rumen will largely determine whether or not strategies to suppress methanogenesis in ruminants will be successful or not. In the present study interactions between the methane-suppressing medium-chain fatty acid myristic acid and the fibre and calcium content of the diet were investigated. Six castrated male sheep (live weight, 41 ± 2 kg) were subjected to a 6 × 6 Latin square arrangement. Pure myristic acid (0 and 5%) was supplemented to two basal diet types differing in forage to concentrate ratio (1:1.5 and 1:0.5). Levels of dietary calcium were 0.4 and 0.9% (the latter only in combination with the myristic-acid supplemented diets). The experiment comprised six subsequent 24d measurement periods consisting of 14d of adaptation, 8d of complete collection of faeces and urine and 2d of quantitative methane measurement in open-circuit respiratory chambers. Rumen fluid samples were taken at the end of each measurement period. The study showed that the extent of the inhibitory effect of myristic acid on methane emission will depend on basal diet type and on the level of dietary calcium. Supplementing 5% myristic acid to the basal diet with a high concentrate proportion significantly suppressed methane emission of the sheep by 58% and 47%, supplying 0.4% and 0.9% of dietary calcium, respectively. With the basal diet including a high forage proportion, the extent of the methane-suppressing effect of 5% myristic acid was lower (22%) and became insignificant when the level of dietary calcium was increased. Total methanogen counts in rumen fluid were significantly depressed with myristic acid supplementation. The composition of the methanogen population appeared to be unaffected by treatments.

P-121 **The effect of microfauna composition on the fibrolytic enzyme activities, bacterial mass and fibre disappearance from the rumen of sheep**. T. Michałowski, G. Bełżecki, E. Kwiatkowska, J.J. Pająk (The Kielanowski Institute of Animal Physiology and Nutrition Polish Academy of Sciences, 05-110 Jabłonna, Poland)

Three growing Polish Merino sheep, fed hay and ground barley, were defaunated and then faunated with only *Eudiplodinium maggii,* with *Eudiplodinium maggii* plus *Entodinium caudatum* or with the both ophryoscolecids and *Dasytricha ruminantium*. The establishment of *Entodinium caudatum* was followed by a decrease in the number of *Eudiplodinium maggii* already existing in the rumen by about 28–68%. Appearance of *Dasytricha ruminantium* did not markedly restrict the populations of either ophryoscolecids. The establishment of the ophryoscolecid ciliate population in the rumen of sheep resulted in an increase in both the CMC-ase and xylanase activities ($P < 0.01$) while the development of a population of *Dasytricha ruminantium* was followed by a decrease in the activity of xylanase. A positive relationship was found between the fibrolytic activities in rumen digesta and *Eudiplodinium maggii* numbers ($P < 0.05$), irrespective of presence or absence of other species of ciliates in the rumen. Total bacterial mass in the rumen was measured by DAPA concentration. The presence of *Eudiplodinium maggii* did not negatively effect the bacterial flora. Appearance of *Entodinium caudatum* and *Dasytricha ruminantium* in the rumen was accompanied by a decrease in bacterial mass in two of the three examined sheep. The increase in the fibrolytic enzyme activities resulting from the establishment of protozoa populations was accompanied neither by an increase in DM nor in ADF or NDF disappearance from the rumen. A decrease in disappearance rate of all three examined substrates was observed following the establishment of *Dasytricha ruminantium* in all three animals. (This project is supported by The Polish State Committee for Scientific Research, Grant No. 5P6E03615).

P-122 **Some factors affecting growth of the rumen ciliate *Epidinium caudatum f. fasciculus* in vitro.** R. Miltko, A. Kasperowicz, K. Wereszka, T. Michałowski (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland)

The rumen ciliate *Epidinium caudatum f. fasciculus* was isolated from the rumen fluid of a cow in three different media the “caudatum” and “simplex” salt solutions (Coleman et al., J. Gen. Microbiol. 75 (1972) 509–521) and the salt solution of Hungate (Hungate, Biol. Bull. 83 (1942) 303–319). The ciliates survived only in the first. However, they were able to survive in both the initially unaccepted media after adaptation to the in vitro conditions. Three experiments were

performed to study the growth of ciliates. Ciliates were maintained for 28 days in all three culture media saturated with CO_2. However, they disappeared within 8–12 days from "Hungate" and "simplex" medium, if a mixture of N_2 (95%) and CO_2 (5%) was used. Protozoa were fed with food (0.25 mg·mL^{-1}·d^{-1}) consisting hay (60%), wheat gluten (16%) and barley flour (24%). Ciliate number varied between 110 and 150 individuals·mL^{-1}. The same food was given to protozoa at between 0.125 and 0.75 mg·mL^{-1}. The number of ciliates changed from about 50 to over 500 cells·mL^{-1} in relation to food dose and culture medium composition. Survival of ciliates in relation to the food composition was examined in experiments using of Hungate solution. Protozoa received hay (0.3 mg·mL^{-1}·d^{-1}), hay supplemented with wheat gluten (0.08 mg·mL^{-1}·d^{-1}) or with gluten and either starch or cellulose (0.12 mg·mL^{-1}·d^{-1}) and a mixture of cellulose and starch (0.12 mg·mL^{-1}·d^{-1}). Ciliates did not survive when only hay was added. The highest numbers of ciliates (up to 200 cells·mL^{-1}) was found in incubations supplemented with starch. Further experiments are being performed to study the effect of soluble carbohydrates on survival of ciliates in vitro. (This project was supported by EU infrastructure grant QLRI-CT-2000-01455. www.ercule.com).

P-123 **Identification of rumen protozoa by PCR-RFLP.** C.J. Newbold[a], B.A. Dehority[b], J. Sylvester[b], J. Firkins[b], M. Morrison[b], Z. Yu[b], G. van der Staay[c], J.H.P. Hackstein[c], P. Pristaš[d], S. Kišidayová[d], J.-P. Jouany[e], T. Michałowski[f], N.R. McEwan[a] ([a] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK; [b] Ohio State University, USA; [c] University of Nijmegen, The Netherlands; [d] Institute of Animal Physiology, Slovakia; [e] INRA, France; [f] Kielanowski Institute of Animal Physiology and Nutrition, Poland)

Identification of protozoal genera has traditionally been based on microscopic identification. With appropriate training these methods are fast and accurate, however they are not appropriate for samples where cells may be partially digested such as duodenal digesta. Based on 18S rDNA sequences from 19 rumen ciliates a primer set was designed to amplify a 1650 bp product. Based on available sequences we predicted that the restriction enzymes TscI (cut ACGT') should give a unique fragments of 280, 500 and 850 base pairs with small Entodinium, while DraI (TTT'AAA) would give unique products at 1000 & 660 with all holotrichs, and MniI (CCTC-nnnnnnn') should give products of 1020 and 600 with large entodinomorphs. DNA was extracted from cultures of *Epidinium*, *Entodinium rectangulatum*, mixed small *Entodinium* and a mixed protozoal preparation prepared from rumen fluid. The 18S rDNA was amplified and digested using the primers and restriction enzymes above. In all cases the fragments produced were as predicted. It appears that it may be possible to use PCR-RFLP to determine the composition of the protozoal populations. In addition we were able to recover PCR products from duodenal samples and to quantifiably recover products from duodenal samples spiked with washed protozoa suggesting that it might be possible for PCR-RFLP methods to quantify the flow of different protozoal groups at the duodenum. (CJN acknowledges the support of OECD Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems. This project was supported by EU infrastructure grant QLRI-CT-2000-01455: www.ercule.com).

P-124 **Saponin fractions in *Sapindus rarak*: effects on rumen microbes.** R.W.S. Ningrat[a], P.C. Garnsworthy[a], C.J. Newbold[b] ([a] Division of Agricultural Science, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Leicestershire LE12 5RD, UK; [b] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

Elimination of ciliate protozoa from the rumen stimulates microbial protein production. Recently, there has been increased interest in plant secondary metabolites, e.g. saponins, for use as possible defaunating agents. Raw and extracted pericarps of *Sapindus rarak* (Sapindaceae) were screened for their effects on rumen protozoa and bacteria. The pericarps of *S. rarak* were extracted using a C_{18} solid-phase column with an aqueous methanol mobile phase. Fractions were collected and monitored by TLC. The active compounds were tested in vitro by visual assessment of protozoal viability and by measuring the degradation of ^{14}C labelled bacteria by rumen protozoa. The influence of methanol extracts of *S. rarak* on growth of pure cultures of rumen bacteria was also examined. The saponin fractions of *S. rarak*

exhibited antiprotozoal activity, as confirmed by visual assessment and by measurement of the breakdown of ^{14}C-leucine-labeled *Prevotella bryantii* in rumen fluid incubated in vitro. No evidence was found of differential susceptibility between different protozoal genera. Inclusion of a methanol extract of *S. rarak* in the growth medium of the pure cultures of rumen bacteria inhibited slightly the growth of cellulolytic bacteria. In conclusion, extracts prepared from *S. rarak* may be useful as defaunating agents, but more studies are needed to investigate the effect of the extracts on ruminal fibre digestion.

P-125 **Molecular ecology of anaerobic fungi.** E. Ozkose[a], D.R. Davies[b], M.S. Ekinci[a], M.K. Theodorou[b], G.W. Griffith[c] ([a] KSU, Agriculture Faculty, 46100 Kahramanmaras, Turkey; [b] IGER Aberystwyth SY23 3EB, UK; [c] Inst. Biol. Sci., University of Wales Aberystwyth, SY23 3BY, UK)

Anaerobic fungi are important components of the rumen ecosystem. Whilst there is information about total fungal numbers for different host animals on various diets quantitative information about the relative abundance of the different taxa is sparse. Physiological information about the various fungi suggest that they may occupy slightly different ecological niches, although it is not clear how many species may coexist in a single host animal, nor whether the various species differ in their patterns of substrate colonisation. With the aim of addressing these questions, we have conducted detailed analysis of fungal populations from rumen digesta and faeces of silage-fed cows. Using the MPN technique with various carbon sources as an enrichment source, quite different fungal taxa were isolated. For instance, *Caecomyces* spp. were more abundant on cellobiose. In the course of this work we also isolated and characterised a new genus of anaerobic fungus *Cyllamyces aberensis*. Difficulties in the reliable morphological identification of these fungi led us to explore the use of PCR-RFLP of the ITS region of the rRNA locus as a tool for identifying fungi isolated from MPN tubes. Sequence analysis of these regions was used to validate restriction enzyme patterns. Ambiguous RFLP patterns were found to be attributable to between-repeat polymorphisms within the ITS region of single isolates. ITS2 sequences from a single isolate diverged by 5% or more and there was more variability between repeats than between quite unrelated isolates. The possible origin of these divergent repeats and the implications of these results for the utility of the ITS region for molecular ecology of anaerobic fungi are discussed.

P-126 **Methanogen growth on formate in batch culture.** S.M. Rea, S.K. Baker (CSIRO Livestock Industries, Floreat Park, WA 6913, Australia)

The free energy available for methanogenesis is greater for H_2/CO_2 than for formate (–130 and –119 kJ·mol^{-1} CH_4, respectively). Furthermore, methanogen growth on formate is often slower than on H_2/CO_2 and cell yields are generally reduced, due to decreasing formate concentrations and pH. The aim of this study was to determine if increasing concentrations of formate would increase cell yields in batch cultures. Mesophilic methanogen strains isolated from diverse environments were chosen. *Methanobacterium formicicum* strain MF, *Methanoculleus bourgensis* MS2, *Methanococcus vannielii* SB, *Methanobrevibacter smithii* strain PS, *Mbr. ruminantium* M1 and 2 *Mbr*. strains (ZA-10 & KM1H5-1P) were grown in batch cultures at 39 °C in a bicarbonate/phosphate buffered medium (Balch 1 + NH_4Cl) with Na-formate under N_2:CO_2 (80:20) as the sole substrate. The initial concentration of formate in the medium varied in 0.07 M increments from 0.15 M to 0.44 M. Growth (OD_{660nm}) was maximized if the formate concentration in the medium was 0.29 M for strains PS, MS2 and SB, although formate was completely utilized by strain PS in all cultures (0.15 M–0.44 M formate) and the pH range never exceeded ~1 unit (7.06–8.12). Optimal growth occurred in a formate concentration of 0.22 M for strains KM1H5-1P and MF; the former utilizing all substrate. Strains M1 and ZA-10 were inhibited by increased initial formate concentration (> 0.15 M) in the medium, suggesting that the increasing salt concentrations were inhibitory or that growth cannot be maximised on formate in a closed system. The pH (~7–8) was maintained within 1 unit of the optimal pH for all the strains by the strongly buffered medium indicating that at least in small volumes, pH does not need to be controlled externally.

P-127 **Rapid identification of rumen protozoa by restriction analysis of amplified 18S rRNA gene.** M. Regensbogenova[a], P. Pristaš[a], S. Kišidayová[a], T. Michałowski[b], P. Javorský[a], S.Y. Moon-van der Staay[c], G.W.M. van der Staay[c], J.H.P. Hackstein[c], C.J. Newbold[d] ([a] Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovakia; [b] Kielanowski Institute of Animal Physiology and Nutrition, Jabłonna, Poland; [c] Catholic University of Nijmegen, Nijmegen, The Netherlands; [d] Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

Rumen protozoa represents a substantial part of the rumen microbial population and some 200 species have already been described from this environment. However, due to fastidious growth requirements and their complex morphology our understanding of the role of protozoa in rumen fermentation is still limited. Rapid methods have been developed for molecular identification of rumen protozoa without the need for cultivation. Total DNA was isolated from single picked protozoal cells by the Chelex method and used as a target for PCR amplification using primers directed to the ribosomal RNA operon. Amplified DNA was then subjected to cleavage by tetranucleotide sequence recognizing restriction endonucleases and specific DNA fingerprints were obtained after agarose gel electrophoresis. The highest discriminatory power was observed for *Hae*III and *Msp*I endonuclease, which could separate the subfamily *Diplodinineae* from the other species. The method was found to be able clearly discriminate between all strain studied (18 strains belonging to 12 species). No differences have been observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species, indicating limited intra-species variability of studied protozoa. The method described here provides a rapid and convenient way for identification and diversity studies of rumen protozoa. (This project was supported by EU infrastructure grant QLRI-CT-2000-01455: www.ercule.com).

P-128 **Isolation and identification of rumen anaerobic fungi from sheep in Iran.** M. Rezaeian, A. Khosravi (Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran)

The anaerobic fungi have been isolated from very diverse herbivorous animals in different parts of the world. They are now known as one of the significant groups of rumen microorganisms.

A serum bottle technique was used to isolate anaerobic fungi from the rumen of fistulated Shal sheep. Infested barley straw, after 24 hours of bag incubation in the rumen, were used to inoculate medium C with chloramphenicol solution. Fungal isolates were identified based on their morphological characteristics after 48 hours of culture incubation at 39 °C using light microscope. Uniflagellate and polyflagellate zoospores were observed with 1 or 2–16 flagella respectively. Zoospores were variable in shape (globose to irregular) and size. Ovoidal, ellipsoidal and very elongated sporangia were also observed in examined samples. Sporangia were highly variable in size attached to their rhizoid systems with or without sporangiophor. Rhizoidal systems of the isolates also differed in their size and branching types. These observations indicated that various genera of anaerobic fungi could inhabit the rumen of Iranian sheep in which the most predominant genera were *Neocallimastix* and *Piromyces*. Further structural and nutritional examinations are needed to identify the various genera and species of the isolates precisely and to determine the potential activity of these microorganisms in comparison to the isolates from other countries.

P-129 **Distribution of the anaerobic fungi along the digestive tract of sheep.** M. Rezaeian[a], G.W. Beakes[b], D.S. Parker[b] ([a] Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [b] Faculty of agriculture, University of Newcastle Upon Tyne, NE1 7RU, UK)

An experiment was designed to investigate the presence of the anaerobic fungi and their survival within the digestive tract and to assess the chitin component of the digesta as a marker of fungal biomass. Three sheep were habituated to a diet consisting of 1.6 kg chopped and pelleted lucerne. The sheep were killed by injection of Euthatal 30 h after feeding. The component weights of each part were measured. Triplicate samples of digesta content were then collected from each part of the digestive tract for dry matter determination, fungal isolation, SEM study

and chitin assay. Anaerobic fungi were isolated from the abomasum, small and large intestine, caecum and faeces of sheep whilst attempts to isolate them from the ruminal and omasal digesta were unsuccessful. However, SEM examination of the samples confirmed the presence of fungi in these organs. SEM examination of the particles obtained from the large intestine and caecal samples showed circular surface pitting sporangia which possibly represent putative survival structures. No rhizoidal structures were identified from any of the samples taken from the digestive tract. Estimation of the fungal biomass in the whole rumen digesta by the use of chitin assay indicated that they may account for up to 20% of the total microbial biomass. The results support the view that anaerobic fungi produce survival structures in the lower part of the digestive tract. However, the factors responsible for the induction of these resistant zoosporangia have yet to be identified.

P-130 **Medium-chain fatty acids and their interaction in suppressing ruminal methanogens and methanogenesis in vitro.** C.R. Soliva[a], I.K. Hindrichsen[a], L. Meile[b], M. Kreuzer[a], A. Machmüller[a] ([a] Institute of Animal Sciences and [b] Institute of Food Science, Laboratory of Food Microbiology, ETH-Zurich, ETH-Zentrum, 8092 Zurich, Switzerland)

Dietary fats rich in medium-chain fatty acids (MCFA) have the potential to suppress ruminal methanogenesis. Three in vitro experiments using the Hohenheim gas test apparatus were carried out to investigate the effects of single MCFA directly on ruminal methanogens and methane release. Therefore, incubations were performed without feed but with sufficient supply of H_2 and CO_2 to maintain methanogenesis. In experiment 1, increasing levels of lauric ($C_{12:0}$), myristic ($C_{14:0}$) and, as a control, stearic ($C_{18:0}$) acid were added to rumen fluid. To examine whether MCFA act in a synergetic way, in experiment 2 increasing levels of $C_{12:0}$ were supplied at four different levels of $C_{14:0}$. In experiment 3, keeping the entire MCFA supply constant, different proportions of $C_{12:0}$ and $C_{14:0}$ were used to evaluate the most effective combination in suppressing methanogenesis. Provided as single MCFA, only $C_{12:0}$ suppressed methanogenesis but both, $C_{12:0}$ and $C_{14:0}$, decreased counts of methanogens. When adding both MCFA together, synergetic effects in suppressing methanogenesis and methanogens occurred. $C_{14:0}$ added to $C_{12:0}$ increased the efficacy of $C_{12:0}$ against methanogenesis (experiment 2) and mixtures of $C_{12:0}$ and $C_{14:0}$ in proportions of 1:0.1 to 1:1.5 showed the same effects on methanogenesis as the same amount of $C_{12:0}$ provided alone (experiment 3). Higher proportions of $C_{14:0}$ had decreasingly lower effects. The present results illustrate the advantage of using mixtures of $C_{12:0}$ and $C_{14:0}$ to suppress ruminal methanogenesis. This was verified in a Rusitec experiment by supplying MCFA mixtures to complete ruminant diets.

P-131 **Effects of combinations of 3-butenoic acid and two inhibitors of methanogenesis on in vitro ruminal fermentation.** E.M. Ungerfeld, S.R. Rust, R. Burnett (Michigan State University, East Lansing, MI 48824, USA)

We hypothesized that the use of 3-butenoic acid as an electron sink could relieve the constraints on fermentation caused by the methanogenesis inhibitors lumazine and propynoic acid. In two experiments with 24-h batch ruminal cultures, lumazine (0, 0.6 and 1.2 mM, Exp. 1), and propynoic acid (0, 2 and 4 mM, Exp. 2), were each incubated in Wheaton bottles (n = 4) with 3-butenoic acid (0 or 4 mM). Ground lucerne hay was the substrate. In Exp. 1, lumazine decreased ($P < 0.01$) methanogenesis by 15 and 24% at 0 and 4 mM 3-butenoic acid, respectively (linear interaction $P = 0.03$). In Exp. 2, propynoic acid linearly decreased ($P < 0.01$) methanogenesis by 68%. 3-Butenoic acid decreased methanogenesis by 30% at 2 mM propynoic acid, but did not affect it at 0 or 4 mM proynoic acid (quadratic interaction $P < 0.01$). Lumazine did not affect the substrate fermentation (estimated through a mass balance), while propynoic acid decreased it ($P < 0.01$) by 13.1 percentage units. However, 3-butenoic acid compensated ($P = 0.01$) for the decrease in the substrate fermentation caused by propynoic acid. 3-Butenoic acid also stimulated ($P < 0.01$) the substrate fermentation in Exp. 1 by 9 percentage units. Lumazine did not affect the acetate to propionate ratio at 0 mM 3-butenoic acid, and increased ($P < 0.01$) it at 4 mM 3-butenoic acid. Propynoic acid decreased ($P < 0.01$) the acetate to propionate ratio. 3-Butenoic acid increased ($P < 0.01$) the acetate

to propionate ratio in Exp. 1, but not in Exp. 2. Both methanogenesis inhibitors, and 3-butenoic acid, increased ($P < 0.01$) butyrate molar percentage. The combinations of either inhibitor with 3-butenoic acid decreased methanogenesis while maintaining or improving the substrate fermentation.

P-132 **The fibrolytic activity of the rumen ciliate *Diploplastron affine***. K. Wereszka, T. Michałowski, A. Kasperowicz (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland)

The rumen ciliate *Diploplastron affine* was isolated from the rumen fluid of a cow and cultured in vitro as a clone. The standard feed given to protozoa consisted of powdered hay (60%), wheat gluten (16%), barley flour (12%) and microcrystalline cellulose (12%). It was found that protozoa were able to grow in a medium composed of ($g \cdot L^{-1}$) potassium phosphate (11.3), sodium chloride (0.65), sodium acetate (0.75), magnesium sulfate (0.045) and calcium chloride (0.045) when saturated with CO_2. The ciliates survived for 30 days in the culture buffer supplemented with only powdered straw but the concentration of ciliates did not exceed 400 cells$\cdot mL^{-1}$. Protozoal numbers increased by about 70% when straw was replaced by powdered hay. These findings show that fibrous food given to protozoa satisfied their nutritional requirements. On the other hand neither oat spelt xylan nor crystalline cellulose increased population density. Barley starch was a stimulating factor, however the most dense population was found when a mixture of starch and microcrystalline cellulose was added to the diet composed of hay and wheat gluten. Crude enzyme preparations obtained from the broken cells of ciliates were able to digest crystalline cellulose, carboxymethylcellulose, cellobiose and xylan (0.06 ± 0.02, 20.6 ± 3.23, 8.7 ± 0.12 and 117.8 ± 3.14 μmol equivalents of glucose or xylose$\cdot mg^{-1}$ protein$\cdot h^{-1}$, respectively). The highest rate of cellulose, cellobiose and xylan digestion was observed at pH 6.0 and with CMC at pH 5.0. Native electrophoresis combined with zymograms revealed the presence of CMC-ase and xylanase enzymes in protozoal protein. End products of the carbohydrate hydrolysis were identified by TLC technique. (This project is supported by EU infrastructure grant QLRI-CT-2000-01455: www.ercule.com).

P-133 **Molecular characterisation of bacterial communities in the guts of marine herbivorous fishes.** I. Pasch[a], S. Turner[a], K. Clements[a], D. Mountfort[b] ([a] School of Biological Sciences, University of Auckland, New Zealand; [b] Cawthron Institute, Nelson, New Zealand)

It is well established that terrestrial vertebrate herbivores are nutritionally dependent on the fermentation products of their gastrointestinal microflora. However, the significance of microbial fermentation processes in the nutritional ecology of herbivorous fishes is still unclear. Redox potentials indicative of anaerobic conditions and elevated levels of short chain fatty acids (SCFAs) in the posterior intestines of marine herbivorous fishes support the notion that the hindgut is a site of active bacterial fermentation. However, evidence supporting a microbially-based digestive process has lacked a clear characterisation of the gastrointestinal microflora of these animals. Here we describe the application of a molecular strategy, Amplified Ribosomal DNA Restriction Analysis (ARDRA) and comparative sequence analysis, to characterise the posterior gut flora of three marine herbivores, *Kyphosus sydneyanus*, *Odax pullus* and *Aplodactylus arctidens*. This analysis indicates that members of the genus *Clostridium* dominate the posterior regions of the gut in these fishes. While the Clostridia are metabolically diverse, the genus is generally characterised by spore formation, strict anaerobiosis and fermentation of carbohydrates to SCFAs. This study thus provides evidence for the abundance of fermentative bacteria in the intestine of marine herbivorous fishes and supports recent work on fermentation rates in these animals.

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Gut flora and health – new perspectives

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IL-3 **Bacterial biofilms in the large intestine.** G.T. Macfarlane (University of Dundee, MRC Microbiology and Gut Biology Group, Ninewells Hospital Medical School, Dundee DD1 9SY, UK)

In the human large bowel, bacterial biofilms exist on the mucosal surface, in the mucus layer, and on the surfaces of digestive residues in the gut lumen. Scanning electron microscopy and fluorescent in situ hybridisation (FISH) experiments using group specific 16S rRNA oligonucleotide probes showed that mucosal bacteria occurred in microcolonies, especially on the rectal epithelium, as well as in diffusely spreading communities between the crypts. Live/dead staining indicated that the bacterial microcolonies contained a mixture of living and dead organisms. Culturing studies on bacterial populations colonising the rectal epithelium showed that bacteroides and bifidobacteria predominated in healthy individuals. However, these investigations also demonstrated that a wide range of bacteria were present on the rectal mucosa, with 72 species being identified (belonging to 18 different genera) on the basis of automated cellular fatty acid analysis. Comparison of healthy subjects with ulcerative colitis (UC) patients indicated that they had higher numbers of mucosal bifidobacteria and lower numbers of enterobacteria and Gram positive cocci than the UC patients.

Microbiological analysis of strongly adherent bacterial populations on food particles indicated that they were broadly similar in composition to non-adherent communities, and that clostridia were not present to a significant degree. However, FISH studies showed that large numbers of these organisms could be present in lumenal biofilms. Adherent bacteria differed enzymically with respect to glycosidase and polysaccharidase expression, and were metabolically distinct from their non-adherent counterparts. This was evidenced by measurements of specific rates of SCFA production and the types of fatty acids that were formed. Bacteria in the biofilms generally produced more acetate, while non-adherent populations formed proportionately more butyrate.

IL-4 **Transformation of flavonoids by human intestinal bacteria.** M. Blaut, A. Braune, H. Schneider, R. Simmering, L. Schoefer, C. Herles (Department of Gastrointestinal Microbiology, German Institute of Human Nutrition Potsdam-Rehbruecke, DifE, Germany)

Fruit, vegetables and cereals contain a wealth of secondary plant metabolites which have been implicated in the promotion of health. To understand the mechanism of their action it is necessary to gain more information on their fate in the body following their ingestion. A certain proportion of ingested secondary plant constituents may escape absorption in the small intestine and therefore may undergo transformation by intestinal micro-organisms. To study the transformation of secondary plant metabolites by bacteria, *Eubacterium ramulus* was isolated from human faeces and incubated with selected flavonoids. *E. ramulus* is a strictly anaerobic bacterium which was found to be present in the gastrointestinal tract of most individuals. It cleaves the ring system of several flavonols and flavones giving rise to the corresponding hydroxyphenylacetic and hydroxyphenylpropionic acids, respectively, acetate and butyrate. Degradation pathways were proposed based on the intermediates detected by high performance liquid chromatography (HPLC) and HPLC coupled with mass spectrometry (LC-MS) and the detection of enzymes that catalyse reactions such as taxifolin isomerization, phloretin hydrolysis and phloroglucinol reduction. The dearomatising phloroglucinol reductase, presumably part of all flavonoid degradation pathways, was purified and characterised. In addition, the gene encoding the phloretin hydrolase was cloned from a *E. ramulus* gene library taking advantage of a newly developed fluorescence test for activity screening. Moreover, a new intermediate was discovered and identified by MS and ^{1}H- und ^{13}C-NMR-analysis as alphitonin. To investigate the degradation potential of *E. ramulus* under in vivo conditions, germfree rats were associated with *E. ramulus*. Following the intragastric application of quercetin-3-glucoside, urine and faeces of gnotobiotic rats were analysed for degradation products originating from quercetin-3-glucoside. In faeces of rats monoassociated with *E. ramulus*, 3,4-dihydroxyphenylacetic acid was found, indicating that this organism was able to cleave quercetin under in vivo conditions. To investigate in which way the dietary flavonoid content affects the concentration of *E. ramulus* in the human intestinal tract, twelve human subjects consumed a flavonoid-free diet for one week and subsequently a flavonoid-rich diet. Fecal samples from both phases of the study were analysed by in situ hybridisation for total bacterial counts and counts of *E. ramulus*. Total cell counts and the cell counts of *E. ramulus* decreased significantly

during the flavonoid-free period, while there was an up to tenfold increase in the *E. ramulus* counts during the flavonoid-rich period indicating that dietary secondary plant metabolites may have an influence on the intestinal microbiota.

IL-5 **Human gut flora and its modulation using functional foods.** A. McCartney (Food Microbial Sciences Unit, School of Food Biosciences, University of Reading, UK)

The human colonic microflora is a large and complex bacterial community, which has been shown to interact intrinsically with host health. The acquistion of this bacterial ecosystem begins at birth and a number of factors impact its composition, including diet, age, health, stress and environment. Metchnikoff's correlation between diet and longevity (in the early 1900s) led to the advent of functional food science. Initially the emphasis concentrated on fermented milk products containing lactic acid bacteria (LAB). However, as interest in the health benefits of such foods and LAB in general grew, so dawned the era of *probiotics*. That is 'live microbial feed supplements which aid in host health'. Today such food products are not only produced and marketed as health promoting but also for prophylactic and/or therapeutic purposes. The health benefits attributed to LAB include colonisation resistance, anti-carcinogenic activity, immune stimulation, re-establishing a balanced microflora, cholesterol lowering activity, vitamin synthesis, alleviation of lactose intolerance. More recent developments in functional food science have targeted enhancing indigenous LAB populations, as opposed to oral administration of exogenous strains. This largely revolves around the *prebiotic* concept. Furthermore, the application of probiotics for particular endpoints (that is specific strains for specific functions) and the potential of prebiotics (selective enhancement, possibly even to species or sub-species levels) has spawned a further category of functional foods; *synbiotics*, the combination of probiotic(s) and prebiotic(s). Dietary intervention studies (using functional foods) have clearly demonstrated the ability to modify the gut flora. Increasingly, clinical studies are being performed to demonstrate the applications of functional foods in vivo, with specific interest in target populations such as individuals with a compromised microbiota.

IL-6 **Development of the gut flora in the infant: implications for health.** C.A. Edwards (Department of Human Nutrition, University of Glasgow, Yorkhill Hospitals, Glasgow G3 8SJ, UK)

The colonisation of the infant gut is a complex process that is very little understood. The critical periods are thought to be in the days after birth and during weaning. The microflora of the breast fed infant is quite different from that of the infant fed formula milk. In the first few weeks of life, the gut of the exclusively breast fed infant is dominated by lactic acid bacteria, particularly bifidobacteria and lactobacilli, in contrast to the formula fed infant who has more *Enterobacteriaciae* and *Bacteroides* spp. Breastfeeding is associated with a lower risk of diarrhoea than formula feeding and this may be due in part to the difference in the flora. The factors determining this difference in microflora are not certain but probably relate to several properties of human milk including the presence of non-digestible oligosaccharides, nucleotides and lactoferrin. The microflora of these two infant groups also has a different metabolic profile. Breast fed infants have more acetic and lactic acid in faeces with a lower pH than formula fed infants who have a short chain fatty acid profile dominated by acetic and propionic acid with a small amount of butyric acid. Other metabolic products associated with the adult microflora are also at higher concentrations in the formula fed infant before weaning. Although many of the metabolic differences between the microflora of breast fed and formula fed infants persist into the early stages of weaning it appears that flora of the breast fed infant diversifies earlier than this. The fermentation capacity of the flora of breast fed infants develops slowly especially for complex carbohydrates. That of formula fed infants develops more rapidly. The gradual development of the flora and its metabolic activities in infancy may provide an opportunity to manipulate the colonisation process in a way that has health benefits in infancy and later.

Concluding Lecture

IL-7 **Anaerobic metabolism in the gut.** C.S. Stewart (Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK)

In pre-gastric fermentors such as ruminants, anaerobic rumen microbes enable the host animal to access energy and nitrogen sources in feed plants that would otherwise be unavailable. Similar microbes are also vital to the health of post-gastric fermentors such as humans, but the impact of their activity may be somewhat different. The anaerobic bacterium *Oxalobacter formigenes* is found in many animals, protecting herbivores from oxalate poisoning. In humans, microbial colonic oxalate degradation provides protection against hyperoxaluria and the development of kidney stones. Some people may lose *O. formigenes* as a result of antibiotic or drug therapy, but tests on the use of this bacterium as a probiotic have proved promising. Butyrate producing bacteria are also very widespread in animal species; in the human colon interest is focussed on the role of butyrate as an energy source for colonocytes and as a signal molecule in the host cell development cycle. Progress is being made in identifying key butyrogenic bacteria including *Faecalibacterium, Roseburia, Eubacterium Anaerostipes* and *Coprococcus* species and in elucidating the different pathways of butyrate production. Resistant starch (RS) consumption correlates most closely with butyrate formation in the GI tract; understanding the effects of different types of RS on the colonic microbes, particularly in relation to butyrogenensis, is of obvious importance. Apart from establishing links between diet and health, the study of transformations by GI tract anaerobes can lead to unexpected discoveries. For example, coumarin aglycones released by hydrolysis of some common glycosides are bactericidal to some human pathogens. The wide range of metabolic transformations performed by GI tract anaerobes is likely to provide substantial opportunities for biotechnological exploitation in the future.

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