Microbial fermentation in the rumen gives cattle and sheep the ability to utilise dietary forage that they would otherwise be unable to be digest. However, as a consequence of this anaerobic fermentation, ruminants are significant contributors to the production of greenhouse gases (methane and nitrous oxide) and environmental pollutants (ammonia). Globally, livestock agriculture produces nearly a quarter of the total anthropogenic emissions of methane. The development of mitigation strategies to address these inherent problems in livestock farming is urgently needed and requires an improved understanding of the way that rumen microbes use the nutrients contained in ingested plant material. The following two articles explore the way that current research into rumen function is directed towards addressing these issues.

**Exploiting the dynamic nature of plant-microbe interactions in the rumen**

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Rumen fermentation is an anaerobic process known to involve diverse populations of bacteria, fungi, protozoa and methanogens. By considering these micro-organisms as nodes on a "food web", with the end products generated by one microbial species being subsequently utilised by other species, different nutrient flow pathways are possible depending on the competition established by the microbial diversity in the ecosystem (Fig. 3.3). As such interactions could substantially influence the composition of fermentation end products, there is an immediate need for a deeper understanding of the ecological relationships between the members of these rumen communities.

**Microbial biofilms**

In nature, bacteria, fungi and protozoa commonly exist as biofilms, whereby attached microbes envelope themselves in extracellular polymeric substances (EPS), often termed 'slime'. We have found that the rumen is no exception, regularly observing microbial biofilm communities on forages in this environment. Surrounding themselves in such a 'slimy' matrix could offer the rumen bacterial species protection against predation by the protozoa, as well as the ability to concentrate the microbial enzymes required to break down plant materials to obtain the required nutrients.

Recent studies at Gogerddan have used molecular approaches to show that the first step in rumen microbial colonisation of fresh perennial ryegrass (*Lolium perenne*) takes place very rapidly, occurring within minutes (Figure. 3.2). Rumen bacteria and protozoa both rapidly attach to the forage, with the attached protozoal population sizes stabilising more quickly than attached...
bacterial populations. In contrast, colonisation by anaerobic fungi is delayed by several minutes and does not stabilise within the same time-frame. Following attachment, EPS production ensues, reaching a maximum after 2 hours of incubation, at which point the biofilm communities are clearly visible by scanning electron microscopy (Figure 3.3). Molecular profiling techniques have also revealed that changes in the diversity of attached bacteria occur after forage has been in the rumen for 2 hours, indicating that some microbes detach whilst other, secondary colonisers, start to attach to the forage. If biofilm microbes do not detach to colonise other plant materials, they will then pass to the lower intestinal tract, together with the partially degraded forage particles to which they are attached.

Based on these findings, biofilm formation and collapse in the rumen can be summarised into four stages: attachment, EPS production, EPS collapse along with detachment of some primary colonisers with influx of secondary colonisers, and lastly detachment or passage to the lower intestinal tract (Figure 3.4).

The plant factor

Forage composition has been shown to have a major effect on microbial colonisation. For example, stems and leaves of perennial ryegrass (*Lolium perenne*) differ in their chemical content, with stem material being lower in nitrogen and total lipids but higher in water soluble-carbohydrates and lignins compared with leaves. Following incubation of separated stems and leaves with a rumen microbial inoculum, molecular profiling techniques have revealed that the bacterial species colonising these two types of material are quite distinct, with just 40% similarity detected during an 8 hour period (Figure 3.5).
As well as chemical variability between plant parts and cultivars, fresh forage has been found to undergo subtle changes in composition as a direct consequence of ingestion. Plants have no means of escaping adverse environmental conditions and, instead, rely on physical and metabolic mechanisms to enable them to withstand environmental stresses. Some of these adverse conditions are also encountered in the rumen which, in addition to containing micro-organisms, is an anaerobic, pH neutral environment, maintained at 39°C. For example, anaerobic defences are more usually associated with flooding, while heat stress reactions are normally encountered during the summer when leaf temperature can exceed 45°C. Plants are also continually attacked by a range of airborne microbial plant pathogens, leading to a well-characterised ‘arms race’ between the genomes of plant and pathogen.

Current estimates suggest that, during grazing, approximately 50% of the plant cells entering the rumen are intact and viable. Therefore, it is of little surprise to discover that plants instigate some of these survival strategies upon exposure to the rumen environment. Such metabolic defence strategies are important because the reactions involved could affect nutrient availability and so influence the progression of colonisation.

An adequate supply of protein and soluble carbohydrates is essential for microbial growth. However, within the plant cell these nutrients are contained within sub-cellular organelles, so they may not be readily available to colonising micro-organisms. Sucrose is stored within the plant cells in vacuoles but heat stress in the rumen causes membrane damage, thereby permitting sucrose and larger chain carbohydrates (fructans) in fresh forage to be released at a linear rate. Soluble carbohydrates are strong chemotactic signals for microbes, but the rate of sucrose release from fresh forage is likely to be limiting for microbial growth so would result in competition within the microbial ecosystem. Likewise, the majority of the protein in plant cells is contained in the chloroplast. Data indicate that conditions in the rumen are sufficient to induce autolysis, where the plant proteins are broken down by the plant cell’s own proteases. Too much protein breakdown in the rumen not only restricts the amount of protein available to the microbial ecosystem, but an abundant supply of protein breakdown products is known to increase the potential for excessive ammonia generation. This inefficient utilisation of plant protein is directly related to undesirable levels of nitrogen excretion and forms a major driver for our research.

Successful pathogen attack on leaves results in localised or extensive browning as bacterial or fungal growth is fuelled by the nutrients contained in the leaf. In contrast, if the leaf successfully resists the infection, the only visible symptoms may be localised lesions (Figure 3.6). In this latter case, the plant has induced a specific programme of localised cell death in the cells surrounding the invading micro-organism to act as a barrier. This cell death involves breakdown of DNA and production of characteristic defence proteins, such as PR1. Similar characteristic symptoms of induced cell death have been observed in forage white clover in response to the conditions in the rumen.

Figure 3.5: Cluster analysis of population profiles showing that the rumen bacterial populations colonising fresh perennial ryegrass leaf and stem material are distinct.

Figure 3.6: Wild fire disease on tobacco caused by *Pseudomonas syringae* pathovar tabaci bacteria. (Courtesy of Luis Mur).
Plants and microbes interacting

Because the genetic background of the plant is a major determinant of its stress tolerance, the plant-derived nutrients presented to rumen micro-organisms will alter according to both the time post-ingestion and the plant variety/genome concerned (Figure 3.7). This means that competition pressures between colonising rumen microbes are unlikely to be static in the grazing animal. Through a combination of skills in plant biology, microbiology and molecular biology, as well as access to the diverse plant genetic resources available at IBERS, the impact of plant genome on successional microbial colonisation in the rumen can be assessed.

Elucidation of the role of the plant genome as a determinant of nutrient release to rumen micro-organisms can therefore enable the development of plant-based solutions to the environmental problems associated with livestock and greenhouse gases. Furthermore, consumers are placing increasing emphasis on safety, traceability and health benefits of food. Understanding the nutrient balances required to select for those specific pathways through the rumen microbial network which result in desirable products could, therefore, deliver forage crops improved for both enhanced sustainability (lower emissions) and product quality.

Yeast culture

The use of yeast in ruminant diets has a long history. Reports from the 1920s indicate brewer’s yeast having been successfully used as a protein source in ruminant diets for many years. The application of low levels of yeast (<1% of dietary dry matter) to cattle diets first received attention in the 1950s, with reports that the inclusion of 50 g/day of an active yeast increased milk yield by 1.1 kg/day. Other results from this period were more variable, however, with little or no increase in productivity being reported. It is only in the last two decades that a considerable literature has appeared concerning the use of yeast culture to improve the health and productivity of ruminant livestock.

Several products based on Saccharomyces cerevisiae, also widely used in the brewing and baking industry (Figure 3.8), are commercially available. In all cases, the products contain live cells plus a growth medium, although there are considerable differences in the number of live cells and the nature of the growth medium between products. We have noted that not all strains of the yeast are capable of stimulating rumen digestion. The differences were not related to the number of viable yeast cells in the preparations but may be linked to differences in metabolic activity.

When available results from the literature are considered, milk yield is seen to increase by an average of 4.5% and liveweight gain in
growing adult cattle by 7.5% in response to yeast addition at rates ranging from 0.5 g to 20 g of live yeast per day. These responses are both diet- and animal-dependent, with greater responses reported in early lactation and in animals fed high concentrate diets. There is general agreement that production responses are the result of the action of the yeast within the rumen. An increase in the number of total culturable bacteria that can be recovered from the rumen would appear to be one of the most consistently reported responses to yeast addition. While these increases in culturable bacteria might not often reach statistical significance, studies in which yeast culture fails to stimulate bacterial numbers are rare. There is further general agreement that this increased bacterial count seems to be central to the action of the yeast (Figure 3.9), driving an increased rate of fibre degradation within the rumen and an increased flow of microbial protein from the rumen, both of which lead to increased animal productivity. However, it is not clear if this is due to a general stimulation of all bacteria in the rumen or a selective effect in which the yeast stimulates only certain microbes.

Molecular microbiology

Traditional studies on rumen microbiology have relied on our ability to culture and characterise the constituent microorganisms. While significant progress has been made using these techniques over the years, it has now been recognised that only a relatively small proportion of the microbes within the rumen are recovered by such techniques, leaving us ignorant about the roles and activities of the vast majority of this microbial ecosystem. Modern molecular techniques based on amplification of ribosomal genes have allowed both quantitative and qualitative studies on wider microbial populations in the rumen to be carried out. Ribosomal genes are present in all cells; they have highly conserved regions that allow the use of universal primers to amplify all the microbial ribosomal DNA in a sample (typically 16S rRNA for studying bacteria (Figure 3.10) and 18S rRNA for studying the fungi and protozoa), and variable regions that allow us to distinguish between different species.

Yeast in the rumen

Recently we have used 16S rRNA to characterise changes in the rumen bacterial population of cattle fed supplemental live yeast. Three cannulated lactating cows (Holstein/Friesian) received a daily ration (24 kg/day) of corn silage and concentrates. The effect of yeast (BIOSAF SC 47 at 0.5 or 5 g/day) was compared to a control (no additive) in a Latin square design with 24-day periods. Samples from the liquid, solid and solid plus liquid phase of the rumen were then collected for DNA extraction.

Variation in the bacterial community between treatments was assessed by using terminal Restriction Fragment Length Polymorphism (tRFLP) based on the 16S rRNA gene. In brief, we amplified the gene from previously extracted DNA using universal primers that recognise all known bacterial gene sequences, then cut the resulting DNA products with a range of restriction enzymes which operate on particular nucleotide sequences. The DNA fragments produced were then separated on a capillary DNA sequencer to give a trace similar to that shown in Figure 3.11, where each peak represents a unique bacterial grouping. Having produced these bacterial profiles for each experimental treatment, we made comparisons to see which samples had the greatest degree of similarity. As can be seen in Figure 3.12A, there was a clear separation between samples taken from the liquid phase of the rumen and those taken from the solids, confirming the observations of our colleagues in the companion article that a unique microbial population is attached to different types of feed material in the rumen.
It was also obvious that a unique and different bacterial population had developed in the rumen of the animals receiving either 0.5 or 5 g/day yeast (Figure 3.12B).

We have tried to characterise further what these differences might be. Recent advances in massively-parallel pyrosequencing (‘sequencing by synthesis’ in which the sequencing of a DNA template is achieved by synthesising the complementary strand along it, one base pair at a time, and detecting which base was actually added at each step based on chemiluminescence) have opened up the possibility of sequencing a segment of the 16S rRNA genes from all the bacteria within a sample.

In a pilot experiment using a mix of all the fractions from the experiment described above, pyrosequencing (using a Genome Sequencer FLX system; 454 Life Sciences™) generated 5516 sequences of 250 nucleotide average length read from the V3 hyper-variable region of the 16S rRNA gene. Sequence identification, followed by assignment to bacterial phylogenies based on 16S rRNA sequences available in online databases, found that Firmicutes accounted for 50 - 60% of the recovered sequences (depending on the treatment), Bacteroidetes (34-40%),...
Proteobacteria (1.2-2%), Actinobacteria (0.4-1.2%) and Fibrobacteres (0.6-1.5%), together with eight minor phyla (<0.5%). The relative occurrence of Bacteroidetes and Proteobacteria decreased in yeast-fed animals, whilst Firmicutes, Fibrobacteres and Actinobacteria increased. When bacteria were classified in functional groupings based on known metabolic activity, a significant decrease in the taxa representing starch-consuming bacteria (Ruminobacter) and proteolytic bacteria (Prevotella) contrasted with an increase in the taxa representing fibrolytic bacteria (Fibrobacter, Ruminococcus Eubacterium) and lactic acid-utilising bacteria (Megasphaera and Selenomonas) in yeast-fed animals (Figure 3.13). IBERs scientists are currently undertaking a more complete study comparing the effects of yeast and sodium bicarbonate in the rumen in which we will generate some 500,000 16S rRNA sequences.

In conclusion, the use of modern molecular techniques has shown that yeast not only stimulates bacterial activity in the rumen but also changes the composition of the bacterial population. We are now using the techniques described to investigate the use of yeast, other probiotics and plant extracts to manipulate the rumen microbial population in order to reduce the emission of methane and nitric oxide from cattle and sheep.

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