Effect of Nutrient Supply on Ruminal Microbial Populations.

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The Rumen Microbial Ecosystem

Many mammals are herbivores, and most herbivorous mammals live on a diet that makes cellulose digestion essential. The ruminants, which include some of our most important domestic meat- and milk-producing animals - cattle, sheep and goats - have a specialized digestive tract which is highly adapted and has evolved to digest highly fibrous plant materials which non-ruminants are unable to utilize. In ruminants, the stomach consists of several compartments; the abomasum, which is the true digestive stomach is preceded by the reticulum, rumen and omasum. The rumen serves as a large vat in which the food, mixed with saliva, undergoes extensive fermentation by the resident microbial population. The products of fermentation, mainly volatile fatty acids and microbial protein then become available to the host. Volatile fatty acids can account for up to 80% of the host animal's requirements and microbial protein leaving the rumen may account from between 50 and 90% of the protein, depending on diet, entering the small intestine. Ruminants are one of the few animals in which microbial derived protein is nutritionally available to the host. The rumen microbial population contains a highly diverse and complex mixture of bacteria, protozoa, fungi, archaea, viruses (phage) and mycoplasmas. The roles of the bacteria, protozoa and anaerobic fungi in dietary material breakdown, have been extensively studied and are particularly well defined for the bacteria. In comparison, little is known about phage and mycoplasmas which are thought not to aid in feed breakdown but are believed to have a more opportunistic parasitic role living off the bacterial population. Phage however can lyse their bacterial host, thus contributing to microbial recycling and due to their large numbers (10^9 particles/ml; Klieve and Swain, 1993) they can have a significant impact upon the dynamics of the bacterial population and consequently upon ruminal fermentation.

Conditions in the rumen are strictly anaerobic, although small trace amounts of oxygen may be found, particularly in close proximity to the rumen wall and in ruminal gas. Temperature is maintained at 38 to 42°C which enables optimum growth of the bacteria and if animals are fed a balanced ration of forage and grain the pH lies between 5.8 and 6.4 which allows the growth of many different types of bacteria.

Bacteria are present in large numbers, with estimations in excess of 10^{10} cells/ml and they are the main contributors to ruminal fermentation. More than 200 different species have been isolated and many more unculturables identified through the sequencing of clone libraries derived from rumen contents. Different bacteria have different roles in dietary breakdown and are generally categorized into different functional groups by the role they play. The main classes are fibrolytic, amolytic, or proteolytic bacteria, which preferentially digest structual carbohydrates, non-structural carbohydrates, and protein, respectively. Other hydrolytic, fermentative and hydrogenotrophic bacteria are present. No single species of bacterium can display all of the enzymatic properties required to degrade all dietary constituents although many can utilize more than one substrate. In so doing they produce many different products which can

in turn be utilized as a growth substrate by another organism. This has led to interspecies dependence and interaction in the rumen with each microorganism carrying out a specific role and filling an ecological niche. Interactions between dietary particles, other microorganisms and even the host animal are therefore important factors which may affect the composition of the population. Diet in particular can have a significant effect upon the diversity and population sizes of different groups of bacteria and can affect their relative proportions. Bacterial numbers tend to be higher on high grain diets than on high forage diets, although this may be a simple reflection on ease of enumeration as bacteria attached to feed particles can be more difficult to count than bacteria associated with the liquid phase.

Due to their size protozoa can comprise almost 50% of the rumen microbial population and are found at concentrations of 10^4 - 10^6 cells/ml (Williams, 1986). They mostly have a predatory role, feeding off bacteria thus contributing to nitrogen recycling, although some are able to digest starch and plant particles. The most important rumen protozoa are anaerobic ciliates and these are classified according to their morphological traits. Two families of ciliate protozoa are found in the rumen. The holotrichs, Isotrichidae, have cilia over their entire body surface and the entodiniomorphs, Orphyroscolecidae, have regions of cilia concentrated in tufts. At least 25 different genera have been identified. These organisms live in a symbiotic relationship with the methanogenic archaea, the methanogens profiting from hydrogen produced by the protozoa and the protozoa profiting by hydrogen removal. Ciliate protozoa, unlike the bacteria, are not essential for ruminal fermentation, but their activity may be considered beneficial or detrimental to nutrition depending on the animal's nutritional status (Williams and Coleman, 1988). Protozoa are generally more sensitive to dietary changes than the bacterial population and populations tend to alter more with time and between animals than do bacterial populations. Generally speaking, ciliate species are less diverse in browsing ruminants. This is thought to be due to these animals feeding on more fibrous foods.

The rumen is one of the few environments where strictly anaerobic fungi are found. Although not as numerous or as diverse as the bacteria or protozoa, under certain dietary conditions, particularly highly fibrous diets, they may account for 8% of the microbial population and zoospore numbers in the ruminal fluid phase have been estimated at 10^3- 10^5 cells/ml (Orpin and Joblin, 1988). These organisms have high hemicellulase and cellulose activity and are thought to play an important role in plant cell wall weakening. It is thought that they may act as initial colonizers of lignocellulose and increase the rate of cellulose digestion by the bacteria, thus they may be more important than is suggested by their low concentrations in the liquid phase of ruminal contents.

Starch utilizing microorganisms

A large number of ruminal bacteria, protozoa and fungi are able to use starch. The principal amylolytic bacteria, and numerically predominant, are *R. amylophilus*, *Prevotella* sp., *S. bovis*, *Succinimonas amylolytica*, many strains of *Selenomonas ruminantium*, *Butyrivibrio fibrisolvens*, *Eubacterium ruminantium* and *Clostridium* spp. (Cotta, 1988; Kotarski et al, 1992). Some of these bacteria possess both endo and exo enzymes which break the α-4 and α-6 bonds of amylose and amylopectin. However, not all of these bacteria are equipped with a complete array of digestive enzymes for starch breakdown and the maximal digestion of starch to monosaccharides

requires synergistic interaction among several bacterial species. Cotta (1992) demonstrated that coculture of *S. bovis*, *B. fibrisolvens* or *Prevotella ruminicola*, with *S. ruminantium* led to high growth rates and complete digestion of starch. Experiments with gnotobiotic lambs fed starchy diets in which a defined bacterial flora was established and normal growth and ruminal function was achieved, led to the suggestion that it is probable that the major bacteria involved in starch digestion have been identified (Hobson et al, 1981). These experiments also demonstrated that amylolytic protozoa and fungi are not essential elements for ruminal starch utilization.

Ruminal protozoa play a role in engulfing and ingesting starch particles which may also have bacteria attached to their surface. This engulfment process is believed to limit access to the starch by the rapidly fermenting amylolytic bacteria and slows its degradation and the consequent lowering of ruminal pH. The rate of starch uptake varies greatly with species, with *Entodinium* spp. engulfing starch grains very rapidly. Nearly all of the larger entodiniomorph protozoa are amylolytic. Starch is broken down to maltose and then glucose and either used as an energy source or stored in the ectoplasm. The rate of starch uptake and breakdown is governed by the concentration of starch or amylopectin inside the protozoa.

Fibre degrading microorganisms

Fibre breakdown in the rumen is catalysed by a complex community of fibrolytic microorganisms. Fibrolytic bacteria tend to degrade the more readily digestible fibrous structures and rely on help from the ruminal fungi to weaken the plant cell wall.

The major fibrolytic bacteria include the Gram negative organism *Fibrobacter succinogenes* and two species of Gram positive bacteria, *Ruminococcus albus* and Ruminococcus flavefaciens. Fibrolytic activity and growth of these organisms are severely affected by low pH. *In vitro* incubations have shown that below pH 6 the growth and enzymatic activity of these major fibrolytic bacteria is inhibited. This is of particular note when animals are fed high concentrate diets where ruminal pH is regularly below pH 6. Unlike most ruminal bacteria which can ferment carbohydrates and are capable of using numerous monosaccharides and disaccharides as growth substrates, *F. succinogenes* and the ruminococci are nearly restricted to cellulose and its hydrolytic products as growth substrates.

F. succinogenes possesses a complex battery of fibrolytic enzymes and is one of the few microorganisms isolated from the rumen which is capable of digesting crystalline cellulose. Endocellulase, endoxylanase and licheninase activity from several glycosyl hydrolase families have all been identified in this organism. This organism can interact synergistically with non-cellulolytic bacteria during forage digestion. Co-culture of *F. succinogenes* with the hemicellulolytic bacterium *Prevotella ruminicola* resulted in a 2-fold increase in the breakdown of orchard grass (Osborne and Dehority, 1989). *Ruminococcus albus* and *Ruminococcus flavefacie*ns also possess a large number of glycosyl hydrolases involved in the breakdown of cellulose and hemicellulose, several which have been isolated, purified or cloned. Both *R. albus* and *R. flavefaciens* have high xylan degrading activity. At least seven different endoglucanases have been identified in R. albus as well as a β-glucosidase. The cellulose system of *R. flavefaciens* is composed of several endoglucanases, an exoglucanase and a cellodextrinase.

Other important bacteria involved in fibre breakdown include the *Butyrivibrio* and *Prevotella* spp. Although cellulolytic strains of *Butyrivibrio* have been isolated from the rumen, this trait is generally lost upon cultivation under laboratory conditions. It does however retain its ability to

rapidly utilize xylans and an abundance of xylanase genes have been identified. This group of organisms is thought to be one of the most metabolically versatile ruminal bacteria and can use simple sugars, starches, pectic polysaccharides and other non-cellulolytic polymers for growth. It is also one of the main organisms involved in ruminal biohydrogenation and the metabolism of linoleic acid (LA). *Prevotella* spp. are numerically predominant under several different dietary regimes. They can exclusively degrade the non-cellulose components of plant cell walls and possesses several xylanases and is an important contributor to xylan degradation in the rumen. These organisms are also of particular interest in the breakdown of dietary protein and peptides. *Lacnospira multiparus* is the most major pectinolytic bacterium and posseses both an endo-acting pectate lyase and an exo-acting polygalacturanase digalacturonohydrolase which cleaves polygalacturonate to galacturonate residues.

The fungi have an important role in fibre digestion because they are able to penetrate both the cuticle and cell wall of lignified tissue, suggesting the presence of cutinase activity (Akin, 1986). When incubated with barley straw, increased degradation was observed by the fungi when compared with fibrolytic bacteria (Joblin et al, 1989). Filamentous growth by the fungi aids its ability to penetrate plant tissue. These organisms have a broad range of highly active fibrolytic enzymes and are the only rumen microorganisms with exocellulase activity. They have the capacity to attack all carbohydrate components of the cell wall and can slowly solubilize lignin. However, they are relatively slow growing and their ability to persist in the rumen is limited by their growth rates which are much lower than the rumen dilution rate. The best characterized ruminal fungi are the *Neocallimastix* spp. which are highly efficient at degrading crystalline cellulose. Fibrolytic activity and growth of this organism may be enhanced by co-culture with hydrogen-utilizing methanogens, but may be repressed by *Ruminococcus* spp. This antagonistic effect only affects the cellulases of the fungi but not its growth, therefore only cellulolytic activity is affected and appears to be due to the production of extracellular proteins which bind either to the cellulose substrate or the fungal cellulase. Numbers of anaerobic fungi tend to be highest on highly fibrous diets and diminish with increasing concentrate (Gordon, 1984).

Approximately 25 – 33% of fibre breakdown in the rumen is protozoal. Defaunation, or removal of the protozoa, results in a decrease in fibre breakdown (Bonhomme, 1990; Ushida et al, 1990; Yang and Varga, 1993). All of the rumen entodiniomorphid protozoa, except for *Entodinium* spp. possess cellulase activity, with highest activity in *Eudoplodinium maggi*. Xylanases are also present and a broad range of glycosidase activities are observed.

Thus fibre breakdown is carried out by a complex consortium of different microorganisms, key members of which can be significantly influenced by changes in diet or by interactions with other microorganisms.

Effect of diet on certain microbial populations

Ruminants evolved to consume fresh forage diets, particularly those of poor quality. However, because of intensive production systems and the need to increase meat and milk production, ruminants are instead fed diets composed of high quality forages and grains to meet their high demands for nutrients. This may have a significant effect upon the composition of the microbial ecosystem. High concentrations of dietary starch and low concentrations of effective fibre are factors which may lead to a reduction in ruminal pH and a greater predisposition to digestive disorders, like acidosis.

Acute Lactic Acidosis

The sequence of events and the role the ruminal microbial population plays in the onset of acute acidosis have been well described (Nocek, 1997). A sudden increase in rapidly fermentable carbohydrates from feeding grain can eventually result in accumulation of large amounts of lactic acid in the rumen and a sharp decline in ruminal and, subsequently, blood pH. Initially, the addition of rapidly fermentable carbohydrates results in an increase in the total bacterial population which in turn leads to an increase in fermentation and VFA production which decreases ruminal pH and motility. One consequence of a decrease in motility is a decrease in rumination and less production of saliva leading to a reduction in the buffering capacity of the rumen (Crichlow and Chaplin, 1985). Numbers of *Streptococcus bovis* increase due to the increase in availability of growth substrate and as this organism has a high specific growth rate it rapidly out-competes other members of the bacterial population (Russell and Hino, 1985). This causes the balance of the normal ruminal microflora to be disrupted and the population shifts from one where gram negative and gram positive organisms are in balance to one in which gram positive lactate producers (*S. bovis* and *Lactobacillus* sp.) predominates. Ruminal pH decreases further, and in this more acidic environment the normal gut microflora is affected; fibrolytic bacteria are inhibited and lactate utilizing organisms (*Megasphaera elsdenii*, *Selenomonads*) are unable to cope with the level of lactate production and lactate accumulates. This reduces the pH even further and when the pH drops below 5.5, no fibrolytic and relatively few saccharolytic bacteria survive, including important VFA producers like the *Prevotella* sp.. The population of lactate utilisers also decreases, resulting in even more lactate accumulation until conditions are too extreme even for *S. bovis* (pH <5) and its growth is inhibited. Then the lactobacilli are able to take over and continue to produce lactic acid as the pH drops. This downward acidoic spiral continues until a state of acute acidosis is reached, the consequences of which may even result in death of the animal.

Normal concentrations of lactate in the rumen are less than 5 mM, however under conditions of acute acidosis they may exceed 100 mM, and the relative proportions of D(-) and L(+)lactate changes as the pH decreases. Below pH 5, the D(-) isomer may make up 50% of the lactate present and as this isomer is less readily metabolized than the L-isomer its accumulation increases and the host has difficulty in clearing it from it’s system. Low pH results in the death and lysis of the normal microflora and leads to the release of endo and exotoxins which can result in the formation of histamine and activation of metallomatrixproteases which can be involved in the destruction of the hoof laminae and in the onset of laminitis, the effects of which will not be seen until several weeks after the first initial grain insult.

Acute acidosis can be regarded as the extreme end of the spectrum, with relatively very few extreme cases actually occurring. More frequently, cases of subacute ruminal acidosis will be observed in dairy herds.

Subacute Ruminal Acidosis (SARA)

Subacute Ruminal Acidosis (SARA) can be characterized by a rumen pH between 5 and 5.5, where the total VFA concentration has been increased and the relative proportions of acetic acid, propionic acid and butyric acid has been shifted towards the production of propionic acid and butyric acid and where accumulated levels of lactic acid in the rumen fluid does not exceed 5 to 10 mM (Hibbard et al, 1995). Whereas acute acidosis has been defined as occurring when ruminal pH is below 5 and where lactate accumulation can occur to levels far higher than 40 mM

(Owens et al, 1998). The composition of the microbial population is also markedly different between the two states of acidosis. In acute acidosis, no protozoa are present, and the flora is dominated by Gram positive bacteria, the majority of which are Lactobacilli. Whereas in the animal suffering SARA, there is still a large proportion of Gram negative bacteria present in the population, although the numbers of Gram positive bacteria are increasing. Numbers of bacteria may also increase in response to increased available substrate, but the lactate utilisers are able to keep pace with the production of lactate and it does not accumulate to the levels observed in cases of acute acidosis. *S. bovis* and lactobacilli numbers may be elevated, but have not proliferated in the same manner observed in acute cases. Protozoal numbers are also decreased, or may even be absent, as a result of the decrease in pH (Goad et al, 1998), and a shift may be observed in the composition of the protozoal population. Species belonging to the genera *Charonina*, *Ostracodinium*, *Diplodinium*, *Metadinium*, *Polyplastron* and *Ophryscolex* are sensitive to low pH, whereas *Isotricha* and *Entodinium* are more resistant (Nagaraja and Towne, 1990).

Although the results are not as severe in SARA animals as those observed in animals suffering acute acidosis, they do take their toll on animal health and productivity, with erratic appetite leading to variable feed intake, body weight loss, diarrhea and lameness being taken as indicators of cases of SARA. Increased incidences in liver abscesses can also be noted. Effects on the milk composition with a depression of milk fat percentage may also be observed in some individuals.

Milk fat depression

The terms low milk fat syndrome or milk fat depression (MFD) are frequently used to describe a situation where there is a considerable depression in milk fat, largely due to effects of feeding a diet high in concentrate and low in fibre or structured fibre. Feeding of processed roughage or supplementation of unsaturated fatty acids may also lead to incidences of MFD, primarily due to the effect that unsaturated fatty acids have on the main group of organisms which carry out biohydrogenation the *Butyrivibrio* spp..

In MFD changes are observed in the ruminal fermentation pattern, acetate is generally decreased while propionate levels are increased (Van Beukelen et al, 1985; Murphy et al, 2000; Khorasani and Kennelly, 2001). In some instances butyrate levels rise (Van Beukelen et al, 1985; Murphy et al, 2000), although in others it decreases in line with the acetate (Storry et al, 1974, Kennelly et al, 1999). In all of these experiments induction of MFD was associated with a drop in ruminal pH. MFD is also now known to be primarily caused by the accumulation in the rumen of long chain trans fatty acids, particularly the trans-10, cis-12 isomer of conjugated linoleic acid (CLA). The t10,c12 CLA is particularly potent as an inhibitor of de novo milk fat synthesis (Moore et. al. 2004).

Only a few organisms have been isolated from the rumen that are able to form t10,c12-CLA from linoleic acid (LA). This isomer may in turn be further reduced to form trans10-vaccenic acid which previously has been taken as an indicator of MFD occurrence. The main route of LA metabolism leads to the formation of cis9, trans11-CLA, by an isomerisation reaction, which is further reduced to trans-11 vaccenic acid. t11-VA may either leave the rumen and be converted back to c9,t11 CLA by the action of delta9-desaturase in the mammary tissue or may be further reduced in the rumen by a small, select group of ruminal bacteria to form stearic acid C18:0. The *Butyrivibrio* spp. are the main group of organisms involved in this biohydrogenation reaction and

carry it out as a detoxification process to saturate the double bond and in so doing reduce the toxicity of these long chain FA. A trace amount of t10,c12 CLA is also formed by the *Butyrivibrio*, but the majority of LA is converted to vaccenic acid. The final step in the biohydrogenation process is the formation of stearic acid by a small sub-population of the *Butyrivibrio* which are extremely sensitive to the effects of PUFA and whose growth is eliminated by the addition of fish oil to the diet. Decreased pH can have a major impact on the main ruminal biohydrogenating population as the *Butyrivibrio* spp. are sensitive to changes in pH, thus it would be expected on high grain diets that ruminal biohydrogenation would be affected. These organisms are also adversely affected by the addition of ionophores and PUFAs.

To date, only a few bacteria have been isolated from the rumen which are able to form t10,c12 CLA. Several strains of Propionibacteria spp. and a strain of Lactobacillus have been found which convert LA to this isomer (R.J. Wallace, personal communication). Previously, *Propionibacterium* isolates from other habitats have also been shown to form *trans*-10,*cis*-12-CLA (Verhulst *et al.*, 1987; Jiang *et al.* 1998). Kim et al (2002) isolated a strain of *M. elsdenii* which formed t10,c12 CLA, however subsequent attempts to isolate other strains of *M. elsdenii* capable of performing the same reaction, have failed. Further examination of the original culture of Kim et al has revealed that it was contaminated with Propionibacteria. Re-isolation of the two composite organisms showed that the isomerisation activity was actually associated with the contaminating Propionibacterium. High grain starchy diets which normally induce MFD are diets would be expected to potentially increase the populations of both Propionibacteria and Lactobacilli. This may explain the increase in t10,c12-CLA and partially account for the increase in propionate associated with MFD-inducing diets.

Another possible route in the formation of t10,c12 CLA may lie in the conversion of hydroxy acids formed in the rumen by *S. bovis*, *E. faecium* and several lactic acid bacteria, (Hudson et al, 1995; 1996; 1998; 2000); all organisms which would proliferate on a high grain ration. To date however, no organism has been identified yet which may carry out these dehydration reactions. Further work is necessary to elucidate all of the possible routes of LA conversion before the key microorganisms may be identified.

Stabilising Ruminal pH

Central to all of these disturbances in either digestive function or in the normal composition of milk is the effect that high concentrate: low forage diets have on the ruminal microbial population and the resulting decrease in ruminal pH. Maintaining a good balance between effective fibre and grain is a key factor in obtaining a stable ruminal pH and stable ruminal population.

If the animal is adapted gradually to high concentrate rations from a high forage diet, the drastic shift in microbial population observed in cases of acute acidosis can be avoided leading to a more stable ruminal population. Chemical buffers like bicarbonate or bentonite may be added to the feed ration as a means of stabilizing ruminal pH. Other dietary additives which have a positive effect upon ruminal pH include antimicrobial feed additives, like ionophores, or the addition of live lactic acid fermenting bacteria, or dicarboxylic acids to promote the growth of lactate-utilizing organisms, supplemental fat and DFMs. The addition of yeast DFMs have been shown to aid in the stabilization of ruminal pH through several modes of action. Yeast DFMs may stimulate the growth of both fibre degraders and lactate utilizing organisms. They may also reduce populations of *S. bovis* by competing for growth nutrients.

To conclude, diet can have a significant effect upon the composition and stability of the ruminal microbial population and subsequently on ruminal fermentation. As ruminal fermentation is an essential component of nutrient input for the animal, any disturbance or disruption of microbial activity may have an effect on the health and productivity of the host animal. Therefore it is important to achieve a better understanding of all of the factors which affect rumen function and the ruminal microbial ecosystem so that we can achieve a positive effect on animal health and productivity.

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