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Application of Biotechnology to Improve Post-Ingestion Forage Quality in the Rumen

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Abstract: Several methods are currently employed to manipulate rumen fermentation to enhance post-ingestion nutritive value of fibrous forages through use of biotechnology including inoculants of native and recombinant rumen microorganisms, natural adaptation and microbial feed enzymes. In this essay, progress and problems related to manipulation of rumen ecosystem through inoculation of natural and genetically modified rumen microorganisms are discussed. Also advancements in exogenous fibrolytic enzymes to improve digestibility of fibrous diets is briefly reviewed.

Key words: Fibrous forages, ruminal bacteria, fibrolytic enzymes

Introduction

The major feed resources for ruminants particularly in arid and semi-arid areas are fibrous residues from cereal crops and pasture or cut grasses from wastelands. Usually both sources of ruminant diets are low in nitrogen (N) and digestibility (Leng, 1991). The nutritive value of the feed consumed and the efficiency with which rumen microorganisms convert dietary carbohydrate to end product of fermentation i.e., Volatile Fatty Acids (VFA), determines the nutrient supply to the ruminant animal (Weimer, 1998). Therefore, maximizing efficiency of breakdown and digestion of plant cell walls in the rumen can have a marked effect on animal productivity (Hoover and Stokes, 1991; McSweeney *et al.*, 1994).

Several methods are currently employed to manipulate rumen fermentation to enhance post-ingestion nutritive value of fibrous forages through use of biotechnology including inoculants of native and recombinant rumen microorganisms, natural adaptation and microbial feed enzymes (Flint and Scott, 2000). In this essay, progress and problems related to manipulation of rumen ecosystem through inoculation of natural and genetically modified rumen microorganisms will be discussed. Advancements in the use of exogenous microbial enzymes for increasing digestibility of fibrous diets will also be taken into account.

Natural ruminal microorganisms: The rumen is a very diverse and complex microbial ecosystem (Hespell *et al.*, 1997) and there is mounting evidence for genotypic and phenotypic diversity of the major functional groups of ruminal microorganisms in different ruminant species and geographical regions. Based on other ecosystems it is likely that less than 10-20% of the rumen microbial population on roughage/forage based diets is culturable and those strains currently held in culture collections represent only a

minor fraction of the existing diversity (McSweeney *et al.*, 1999).

Grazing ruminants often rely on highly fibrous diets as source of energy, but in many cases greater than 50% of the dietary fibre passes through the digestive tract in an undegraded form (Cherney *et al.*, 1991). In this context *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes* are regarded as the primary fibre degrading bacteria in the rumen (Krause *et al.*, 1999c; Weimer *et al.*, 1999).

The genetic diversity and phenotype of *Ruminococcus* have received little attention and very few investigations have been carried out in a systematic manner, but for other fibrolytic bacteria, a number of studies is available (Amann *et al.*, 1992; Lin and Stahl, 1995). DNA-DNA hybridization studies have shown that *Butyrivibrio* can be divided into at least five groups, suggestive of five different species (Mannarelli *et al.*, 1990). Comparatively a more recent 16S rDNA analysis has confirmed the DNA-DNA hybridization results of *B. fibrisolvens* and show that this bacterium is polyphyletic (Froster *et al.*, 1996). Furthermore, all strains for *B. fibrisolvens* so far sequenced fall within cluster XIV of the genus *Clostridium* (Collins *et al.*, 1994). Krause *et al.* (1999a) have undertaken a study to assess the genotype of 26 *Ruminococcus* strains isolated from cattle and sheep and their ability to digest plant cell walls. Only three of these strains had the same genotype and extent of fibre digestion of a medium quality grass varied from 0-61%. The genotype was based on ribosomal RNA sequence analysis. It is believed that if this phenotypic diversity is represented within an individual rumen, then increasing the number of highly fibrolytic *Ruminococci* could potentially improve the rates of cell wall digestion in the rumen. In another study, Krause *et al.* (1999b) collected the highly fibrolytic *Ruminococcus* strains and evaluated them for their ability to colonize the rumen and enhance fibre digestion. Tracking systems based on strain

specific 16S rDNA sequences indicate that inoculated *Ruminococcus* strains did not persist for longer than 3 weeks before reaching undetectable levels. The resolution of this issue is fundamental to understanding the true contribution that individual strains make to fibre degradation and to the population of species and genera in the rumen and overall fibre digestion.

Compared with bacteria, the role of the fungi in rumen physiology is less well understood. Five genera of anaerobic chytridomycetous fungi have been isolated from the rumen: *Anaeromyces*, *Caecomyces*, *Neocallomastix*, *Orpinomyces* and *Piromyces* (Ho and Barr, 1995) and are considered to be involved in fibre degradation (Hespell *et al.*, 1997). Cellulases and xylanases are the enzymes produced by these fungi and are the most active fibrolytic enzymes (Gilbert *et al.*, 1992; Trinci *et al.*, 1994). Remarkably high activity xylanases have been isolated from *Neocallimastix patriciarum* and from *Orpinomyces joyonii* (Gilbert *et al.*, 1992; Li *et al.*, 1996).

Genetically modified ruminal microorganisms: With regard to fibre degradation in the rumen, much effort has been expended in developing genetically modified bacteria that would have superior fibre-degrading abilities. The construction of genetically modified bacteria has proceeded under the assumption that the rumen microbiota does not produce the correct mixture of enzymes to maximize plant cell degradation. It is well established that the principal fibrolytic bacteria of rumen are *Ruminococcus* and *Fibrobacter*, but it is thought that they do not produce exocellulases that are active against crystalline cellulose, so that adding this activity would make them more potent.

Ruminal bacterial species such as *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* are found widely in ruminant animals on varied diets and are found in significant numbers regardless of the ruminal environment. These species, therefore, are logical choices to introduce new or enhanced genetic material into the rumen (Selinger *et al.*, 1996).

Over 100 different genes encoding enzymes for fibre digestion have been identified and cloned from ruminal bacteria such as *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. At least 30 genes from ruminal fungi have been isolated that encode cellulase, xylanases, mannanases and endoglucanases. Almost 50% of the fibrolytic genes cloned have been sequenced (Bowman and Sowell, 2003). These genes are of particular interest due to their powerful fibrolytic activity and ability to break down very resistant cell wall polymers.

Two plasmid vectors developed for use in other Gram-positive bacteria have been introduced into four different *Ruminococcus albus* strains by electroporation (Cocconcelli *et al.*, 1992) and an efficiency of 3×10^5

transformants/ μ g was achieved with one of the plasmids pSC22. A low frequency of transfer of the broad host range plasmid pAM β 1 was also achieved into *R. albus* by conjugation from *Bacillus thuringiensis* BT351 (Aminov *et al.*, 1994). Despite these encouraging developments there have been no reports of introduction of vectors into *R. flavefaciens* strains and no studies on gene inactivation or the expression of genes introduced into *Ruminococcus*. Similarly there are no reports of indigenous plasmids or of successful attempts at gene transfer into this important cellulolytic species (Flint and Scott, 2000).

In addition, cellulase and xylanase genes from ruminal protozoa have been cloned and most of the fibrolytic genes cloned have also been sequenced (Selinger *et al.*, 1996). Protein bioengineering has been used to increase the catalytic activity and substrate diversity of fibrolytic enzymes from ruminant microbes. This has resulted in enzymes which with up to 10 times higher specific activity, changed pH and temperature optima and increased substrate binding activity than the enzymes from which they originated (Selinger *et al.*, 1996).

Streptococcus bovis is found to be tolerant to O₂ and depressed rumen pH, unlike most cellulolytic bacterial species in the rumen. In addition, there are convenient gene transfer methods available for this species that make it a candidate as a host for the expression of genes from other organisms (Bowman and Sowell, 2003). Ekinici *et al.* (2002) were able to use a β (1,3-1,4)-glucanase promoter found in *S. bovis* to express a cellulase gene from the anaerobic rumen fungus *Neocallimastix patriciarum* that is found in very low levels in the rumen and is important for the degradation of crystalline cellulose. The resulting enzyme product was active against a wide variety of cellulosic substrates. The advantages of using fungal enzymes are their stability to low pH and their high activity level.

Xue *et al.* (1997) were successful in introducing a xylanase gene from the anaerobic ruminal fungus *Neocallimastix patriciarum* into *Butyrivibrio fibrisolvens* and achieving secretion of the enzyme. More recently, Krause *et al.* (2001) constructed a recombinant *Butyrivibrio fibrisolvens* that expressed a xylanase enzyme from the ruminal fungus *Neocallimastix patriciarum*. The recombinant *Butyrivibrio fibrisolvens* did have an increased ability to digest fibre, but it did not persist in the rumen for more than 22 days. Hence, the biggest problem is the ability to introduce and maintain the new strain in the mixed rumen population and survival of new strains is not well understood (Wallace, 1994).

Reintroduction of natural and genetically modified microbes to the rumen: The ecology of the introduced strains has been overlooked to a large degree and the success of this technology may ultimately depend on a better understanding of factors that determine

establishment in a complex microbial ecosystem, survival and population density (McSweeney *et al.*, 1999). Introduction of a defined genotype into a complex microbial ecosystem is probably more difficult than originally expected (Stewart *et al.*, 1988). Expression of modified *Neocallimastix patriciarum* xylanase in *Butyrivibrio fibrisolvens* H17c (xynA and pUMSX) resulted in more fibre digestion but could not effectively compete with native highly fibrolytic bacteria under the *in vivo* environmental condition of the rumen (Krause *et al.*, 2001). Kobayshi and Yamamoto (2002) investigated the factors that limit maintenance of recombinant bacterium (*Butyrivibrio fibrisolvens*) expressing a foreign xylanase gene (*B. fibrisolvens* NO₄) and found that depression in survivability of the recombinant was mainly due to heat-sensitive antibacterial factors associating with microbial cells in the rumen. Conversely, Ziemer *et al.* (2002) found that when a natural bacterium *Butyrivibrio thetaiotaomicron* was added to a rumen model, it increased the fibre digestion.

Matin *et al.* (2001) found that the fibrolytic activity of *Fibrococcus mucinogens*, *Ruminococcus albus* and *Ruminococcus flavefaciens* was depressed with cereal supplementation and there was no modification of the balance of the three cellulolytic bacterial species examined.

Krause *et al.* (2003) found that re-establishment of either natural or recombinant ruminal bacteria in the rumen is both variable and unpredictable and there are even reports of the disappearance of the inoculant from the rumen after very short time period (Wallace and Walker, 1993; Miyagi *et al.*, 1995). Furthermore, the probability of colonizing the rumen with a bacterium not originating from the rumen is low (Wallace, 1994; Cotta *et al.*, 1997). Therefore, the ability of reintroduced bacterium to survive in the rumen is determined by many factors, which are (McSweeney *et al.*, 1999);

- 1) Competition with indigenous microbes for substrates that are used for growth
- 2) Growth at a rate faster than the dilution rate of the rumen
- 3) Adaptation to and tolerance of the chemical and physical environment in the rumen
- 4) Capability of withstanding engulfment by protozoa and,
- 5) Resistance to inhibition by bacteriocins and infection by bacteriophage

Therefore, systemic research is needed to further explore these factors that determine what additional conditions are required to ensure survival of introduced ruminal microorganism strains. Model systems such as the gnotobiotic animal may be very useful to progress this work, as this approach has demonstrated that adherent cellulolytic bacteria are more difficult to establish in the rumen than non-structural carbohydrate

fermenting microorganisms (Durand and Fonty, 2001).

Use of exogenous fibrolytic enzymes: Enzyme preparations for ruminants are evaluated primarily on the basis of their capacity to degrade plant cell walls and are of bacterial (mostly *Bacillus* spp.) and fungal (mostly *Trichoderma longibrachiatum*, *Aspergillus niger*, *Aspergillus oryzae*) origin (Pendleton, 2000). It is relevant to mention that most of the fibrolytic enzymes used as feed additives in ruminant diets were originally developed as silage additives (Feng *et al.*, 1996). Differences in the relative proportions and activities of individual enzymes have an impact on the efficiency of cell wall degradation. Even within a single microbial species, the types and activity of enzymes produced can vary widely depending on the strain selected and the growth substrate and culture conditions employed for enzyme production (Gashe, 1992; Lee *et al.*, 1998). Rode *et al.* (1999) carried out a study on efficacy of an exogenous dose of fibrolytic enzymes (mainly cellulase and xylanase) in cows and found that total digestibility of nutrients was dramatically increased by enzymes. Morgavi *et al.* (2000) found a synergistic effect between ruminal and exogenous fibrolytic enzymes, which increased the hydrolytic potential within the rumen environment. They concluded that synergism is likely a significant mechanism by which enzyme additives improve feed digestion.

For increasing fibrolytic activity and increasing post-ingestion quality of forages, Kaiser (1999) Wallace (2001) and Wang *et al.* (2001, 2004) found that by applying the fibrolytic enzymes onto feed prior to feeding was more effective than dosing directly into rumen. Contrary to these results, there are some studies which indicate that the use of exogenous microbial feed enzymes did not result in significant increase in the digestion of fibrous diets in ruminants (Hristov *et al.*, 2000; Bowman *et al.*, 2003). Finally, there is comparatively more evidence that suggest an improvement in fiber digestion within the rumen on addition of fibrolytic enzymes either direct into rumen or pre-treating the feed with the enzymes.

Conclusions: The use of biotechnology to improve post-ingestion quality of fibrous forages is on the verge of delivering practical benefits to ruminant production system. The microbial flora of the rumen can be successfully manipulated if such manipulations are consistent with the principles of microbial ecology. Also adding exogenous fibrolytic enzymes to ruminants can potentially improve cell wall digestion and the efficiency of feed utilization.

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