Influence of Oxygen on Ensiling

R.E. Muck

Introduction

Crops are commonly ensiled without proper sealing. Concrete stave tower silos are rarely sealed with plastic after filling, and as a consequence a substantial spoiled layer may develop at the top of the silo prior to the start of emptying. Similarly, bunker silos are frequently not covered. A situation that small farms face is that of having only one silo per crop. Under such circumstances the farmer may feed out of the silo immediately before an adequate fermentation can take place.

The objective of this study was to look at what happens in the first four weeks of ensiling of both alfalfa and corn when the top surface is left open to the air. The goal was to help answer two questions: how far down must one go to find a normally fermented silage and how long must one wait to get a stable silage.

Methods

Alfalfa and whole-plant corn were harvested with normal field equipment. Four cuttings of alfalfa were ensiled at dry matter (DM) contents of 29, 30, 49 and 41%. One trial was performed with corn at 35% DM. The crops were hand-packed into 15 cm dia. x 60 cm long PVC pipe silos. The wet weights ensiled per silo varied with each trial: 7800, 7800, 6600, 6600, 6500 for the four alfalfa trials and one corn trial, respectively. Thus wet densities ranged from 613 kg/m^3 for the corn silage to 736 kg/m^3 for the wettest alfalfa silages. The tops of all silos were kept open to air. The walls were covered with approximately 9 cm fiberglass insulation, and thermocouples placed at 5, 20 and 35 cm from the open face. All silos were stored inside at room temperatures.

Two silos were destructively sampled at 1, 2, 5 (or 3), 7, 14 and 28 d. Samples were taken at 5, 20, 35 and 50 cm from the open face for oxygen content of the silo gas, pH, DM content, fermentation products, nitrogen fractions, and various microbial groups.

Results

In all five trials, oxygen levels at 5 cm below the open face were generally above 10% v/v throughout the 28 d of storage. At the lower levels, oxygen levels were usually below 1% except toward the end of ensiling when oxygen concentrations at 20 cm rose to 1 to 5%. These results indicate that respiration, both plant and microbial, near the open face used up the oxygen entering the silage. By the end of the 28 d period, apparently much of the rapidly degradable compounds in the silage near the face must have been exhausted, allowing oxygen to penetrate further and causing spoilage deeper within the silos. This is also evident from the pH and fermentation data.

The pHs for the wettest and driest alfalfa trials and the corn trial are shown in Figures 1 to 3, respectively. In spite of the high oxygen levels at 5 cm, there was evidence of fermentation early in the storage period in the two wettest alfalfa trials and in the corn trial (Fig. 1, 3). However this forage rapidly spoiled as noted by the high pHs at later times. In the alfalfa, the primary spoilage microorganisms in this layer were bacilli and/or acetic acid bacteria. Yeasts and molds were not a significant factor over 28 d. In corn, acetic acid bacteria and yeasts dominated early spoilage in the upper layer. Later bacilli and mold counts rose to significant levels in the corn silage.

At 20 cm and deeper from the surface, fermentation proceeded in an apparently normal fashion in all trials, most likely due to the low oxygen levels. The time to reach a minimum pH in alfalfa was between 7 to 14 d across the four trials. In contrast, the corn fermented rapidly (less than 5 d) to a stable, low pH. By day 28, the pH at 20 cm was beginning to rise in some of the

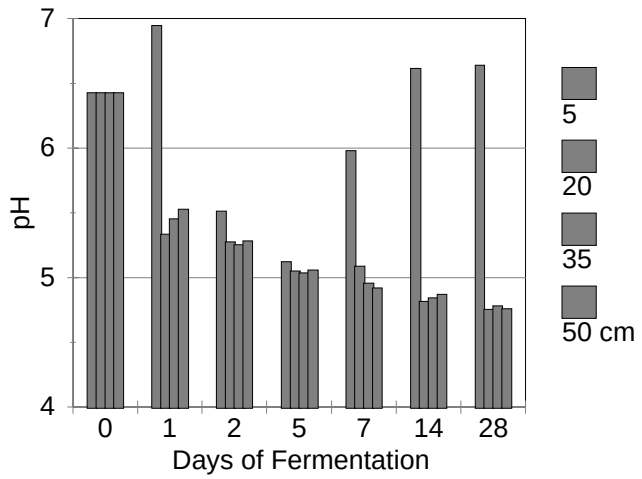

Figure 1. Change in pH in 29% DM alfalfa after ensiling with respect to time and depth from the open face.

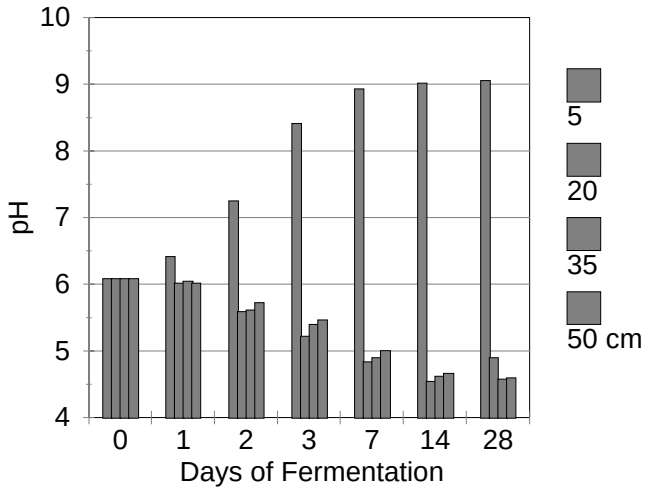

Figure 2. Change in pH in 49% DM alfalfa after ensiling with respect to time and depth from the open face.

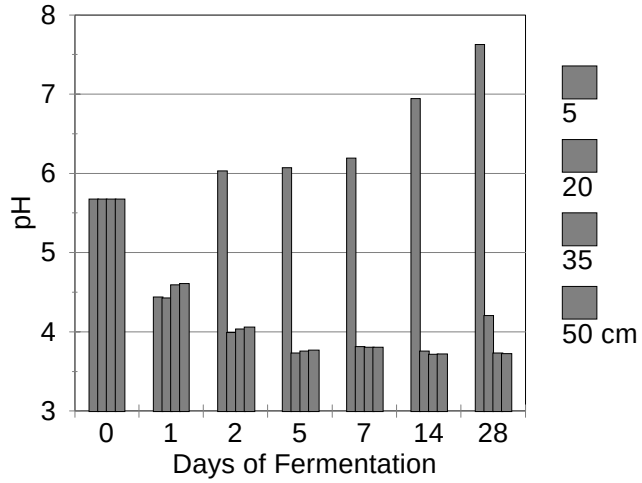

Figure 3. Change in pH in 35% DM corn after ensiling with respect to time and depth from the open face.

trials (e.g., Fig. 2 and 3) and fermentation products were declining, indicating the activity of spoilage microorganisms. At 28 d at the 20 cm level, bacilli were the predominant spoilage group in alfalfa whereas all major spoilage groups were at substantial levels in corn.

Additional time beyond that for active fermentation is usually necessary for populations of aerobic spoilage microorganisms to be reduced. This is desirable to make a silage which is not susceptible to heating and spoilage during feeding. At the two locations farthest from the face of the corn silage, yeast and acetic acid bacteria were still at high levels ($>10^6$ per g silage) after fermentation was complete (day 5) and did not drop below 10^5/g until the sampling at days 14 and 28, respectively. At day 14 in alfalfa, bacilli were above 10^5/g in two cases and acetic acid bacteria were above that level in the other two. Yeasts and molds were at low levels in all the alfalfa silages after one week.

Conclusions

At moderate silage densities and DM contents between 30 and 50%, a normal fermentation occurred consistently at 20 cm below a face open to air at temperatures of 20 to 25°C. However, there was substantial spoilage at 5 cm, and in most trials spoilage was becoming evident at 20 cm by the end of the 28-day trial. In both alfalfa and corn, substantial levels of one group of spoilage microorganisms were still present after 14 days ensiling at the most anaerobic levels in the silos. These results suggest that a one month ensiling period prior to feeding is beneficial in reducing aerobic spoilage microorganisms.

Acid Ionization Constant of Ammonia in Silage

R.E. Muck and J. Cellerino

Introduction

Nitrogen losses during ensiling are approximately half the dry matter (DM) losses. Under typical U.S. conditions, there are two mechanisms of loss: nitrate reduction to nitrogen and nitrogen oxide gases and ammonia volatilization. The former mechanism may be important in heavily fertilized or drought-stressed corn and grasses where the crop may have elevated levels of nitrate. However, ammonia volatilization is likely to be the most common mechanism in silages.

The rate of ammonia volatilization in a silo will be determined by the level of air exchange and the pH of the silage. The pH is a factor because volatilization is related to the concentration of unionized ammonia in silage, which is a function of the acid ionization constant (pK) and pH. The pK is simply the pH at which the ionized and unionized concentrations of ammonia are equal.

The objective of the study was to determine the pK of alfalfa and corn silages as a function of temperature.

Methods

Samples of six alfalfa silages and three corn silages were collected from a variety of bunker, tower and bag silos. The DM contents ranged from 22 to 55%. Silage (4 g) was diluted with distilled water to 200 g in a blender jar and macerated for 30 sec. The diluted silage was tranferred to a beaker and placed on a magnetic stir plate. The pH was reduced with HCl to pH 3 or less to remove carbonate. Then the silage was titrated with 0.1 N NaOH to pH 11.5 over approximately 20 min using a peristaltic pump and temperature-compensated pH meter. Temperature and pH were recorded by computer at 15 sec intervals. The pK was determined graphically from the titration curve. Titrations for each silage were performed with and without the addition of NH_4Cl at room temperature, in a hot room (39°C) and in a cold room (5°C). The NH_4Cl improved the accuracy of the pK determination, particularly of the corn silages which had low ammonia contents.

Results

The pKs for ammonia could not be determined in unamended corn silage and some of the alfalfa silages because the buffering of other constituents masked the buffering range of ammonia. However, NH_4Cl addition made it possible to consistently determine the pK for ammonia. The pKs from the titrations of the NH_4Cl-amended alfalfa and corn silages are shown in Fig. 1. There were no significant differences in the results between corn and alfalfa. There was greater variability in the alfalfa results due to difficulties in rapidly mixing the titrant with the more fibrous alfalfa slurries. At lower temperatures, the pKs were similar to those for ammonia in water. At higher temperatures, pKs in the silages were higher than those for water.

Conclusions

Based on work in animal manures, we would anticipate that the pKs in undiluted silages would be raised slightly relative to the values that we obtained here. Nevertheless, these results will be useful in helping to estimate both the loss of nitrogen from silage as well as air exchange that occurs in silos under field conditions.

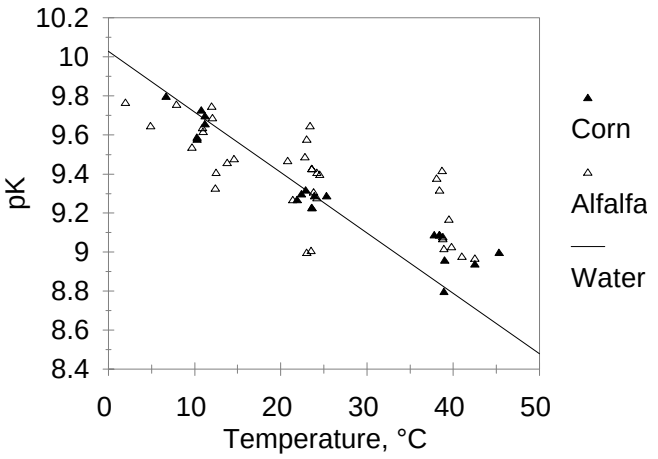

Figure 1. The pK of ammonia in diluted alfalfa and corn silages as compared with that of ammonia in water.

Phytase From Transgenic Alfalfa

R.G. Koegel, S. Austin-Phillips, E.T. Bingham, R.J. Straub and M.E. Cook

Introduction

Buildup of phosphorus in the environment and the resulting degradation of water resources is of mounting concern. Much of this buildup is traceable to human activities. Important among these is livestock production. Monogastric animals, such as poultry and swine which can solubilize only a small fraction of the phosphorus in their grain-based rations, while excreting the remainder, have come under increased scrutiny. Supplementation of inorganic phosphorus into rations to meet animal nutritional requirements exacerbates the problem.

Much of the phosphorus in grain is in the form of insoluble phytates. Researchers have shown that supplementing poultry and swine rations with the enzyme phytase can lead to solubilization of the phosphorus, thus eliminating the need for phosphorus supplementation and concurrently reducing the level of phosphorus in the excrement to approximately one-half of that normally experienced.

Because of relatively higher population and animal densities in western Europe, concern over phosphorus buildup has preceded that in the U.S. Accordingly, certain areas, like the Netherlands, have mandated limits on animal numbers and/or required the use of phytase in animal rations.

The enzyme phytase derived from *Aspergillus niger* has, to date, generally been produced in fermentation vats using genetically engineered microorganisms. It has been estimated that the cost of phytase supplementation with this material would be about three times the cost of conventional supplementation with dicalcium phosphate.

As an approach to reducing the cost of phytase production, a multi-disciplinary ARS-UW team at Madison, Wisconsin has produced transgenic alfalfa with the capability of expressing phytase. This phytase can be recovered from juice extracted from the herbage. Other constituents of the juice including xanthophyll, used to pigment egg yolks and broiler skin, high levels of dietary protein, and various vitamins and minerals add to its value in rations. The use of whole alfalfa herbage, however, would not be desirable due to its high fiber content. Since phytase would potentially be needed in great quantities, but not in very pure or concentrated form, it is believed that the economic advantage of production in "plant bioreactors" such as alfalfa would be great. The advantage in capital costs is particularly great. Ideally, the cost of phytase supplementation should be competitive with the traditional dicalcium phosphate supplement, with the environmental benefits as an added incentive.

Methods

Sixteen phytase-producing alfalfa transformants were originally created. Early bioassays indicated that these transformants produced phytase at a range of levels. These transformants were vegetatively propagated in the greenhouse during the winter and spring of 1997 and approximately 7500 plants were set out into the field in mid-May in both replicated research plots as well as larger "production plots." The plant densities in the two plot types were 9680 and 13936 per acre, respectively. The plots were harvested on August 1 and September 15. Concurrent with this work, transformants were crossed with elite production strains to produce plants which will be repeatedly back-crossed until seed is produced which will yield approximately 95% phytase producers which have desirable persistence and production characteristics.

Results

The transformants generally did well under field conditions. The August 1 harvest was affected by rather severe insect damage. Insects were subsequently controlled by spraying.

Phytase concentrations of the best six transformants ranged from approximately 0.85% to 1.8% of soluble protein and from approximately 21,000 to 36,500

phytase activity units per liter of juice expressed. Suggested activity units per ton of feed are 400,000 and 1,200,000 for poultry and swine, respectively. Therefore, the ranges of juice product required per ton of feed would be from 11-19 liters for poultry and 33-57 liters for swine. The per acre-year anticipated yields and values are shown in Table 1 for five high-yielding transformants. A value of $1.50 per ton of feed is arbitrarily assigned. This is approximately half of the current cost of supplementation with dicalcium-phosphate.

Conclusion

Alfalfa plants vegetatively propagated from the original transformants performed well in the field producing phytase at economic levels. Field work will continue in 1998 with plants produced from seed.

Table 1. Quantity and value of phytase produced in five alfalfa transformants.

Plant No.	Activity units/ Liter juice	Activity units/acre (1 cutting)	Activity units/ acre-year (20 ton fresh wt)	Tons poultry feed/acre-yr @ .4 x 10^6 units/ton	Value/acre-yr in poultry feed @$1.50/ton	Tons swine feed/acre-yr @ 1.2 x 10^6 units/ton	Value/acre-yr in swine feed @ $1.50/ton
360	36,500	37.7 x 10^6	282 x 10^6	705	$1056	235	$353
410	30,000	24.2 x 10^6	264 x 10^6	660	990	220	330
472	29,000	32.4 x 10^6	261 x 10^6	653	978	218	327
411	23,000	30.6 x 10^6	197 x 10^6	493	740	164	246
420	21,000	24.0 x 10^6	161 x 10^6	403	605	134	201

Concentration of Phytase in Alfalfa Juice by Ultrafiltration

C. O'Donnell, R.G. Koegel and R.J. Straub

Introduction

Using transgenic alfalfa to produce enzymes such as phytase has many attractions including the potential to produce large quantities of enzyme at a lower cost than more typical microbiological fermentations (Austin and Bingham 1996). Phytase has an application as a monogastric feed supplement, to improve the animal's utilization of organic phosphate in the diet and therefore to reduce the environmental impact of the manure.

Product recovery from alfalfa typically involves extraction of juice from the fiber and subsequent processing of the juice to obtain the desired product. This can be a difficult downstream processing problem due to the complex composition of the extracted juice. The problem may be somewhat simplified in the case of phytase because the protein in the juice may be incorporated into the ration thus reducing the purification needs. Concentration of extracted juice in the field would also reduce transport costs to a processing plant. The aim of this study was to evaluate the potential of dynamic ultrafiltration to concentrate alfalfa juice containing phytase.

Materials and Methods

Ultrafiltration was carried out in a pilot apparatus (VSEP) (New Logic International, Emeryville, CA) fitted with a 10,000 molecular weight cutoff (MWC) regenerated cellulose membrane. The unit consisted of an annular membrane (area of 0.0465 m^2) that was vibrated by a torsion spring at 60 Hz creating high shear forces at the juice-membrane interface, thereby increasing mass transfer rates over traditional cross flow apparatus. The apparatus was operated with an amplitude of vibration of approximately 25.4 mm at the periphery, and a transmembrane pressure of 100 psi. Filtration was carried out without pretreatment and at temperatures between 15 and 35°C. Initial sample volume was approximately 40 l and retentate was recycled to achieve concentration.

Results and Discussion

Phytase activity. Phytase activity increased in the retentate fraction throughout the experiment but activity expressed per g DM was reduced after about 6h (Fig. 1). This may have been due to long processing times or loss of activity due to stress from mechanical shear with entrained air or from dehydration. A final DM concentration of 20 to 25% was achieved when the whole juice was processed.

There was some phytase activity in the permeate after 4h but this disappeared at later times (Fig. 1). The appearance of activity in the permeate indicates that phytase, which is typically reported to have a subunit MW of 40 to 60 kD, is not totally rejected by a membrane with a nominal MWC of 10 kD. It is often found that the nominal membrane MWC is not closely related to its rejection of a particular molecule, thus creating the requirement for extensive testing of membranes to determine particular process performance. The disappearance of activity from the permeate at later times may be due to fouling, resulting in a decrease in the effective MWC of the membrane

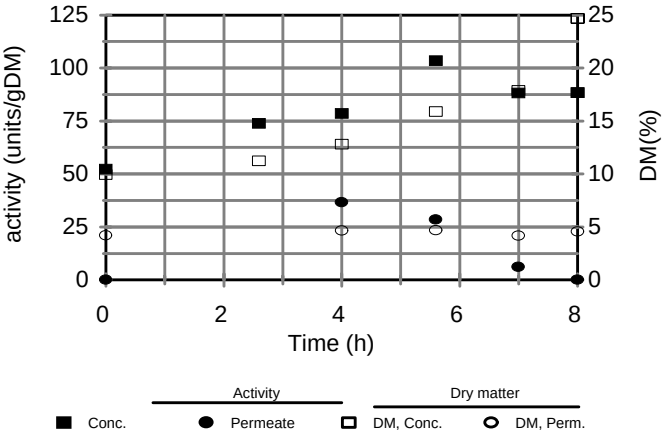

Figure 1. Phytase activity and juice DM during concentration of alfalfa juice by ultrafiltration through a 10 kD regenerated cellulose membrane. An activity of one unit is release of m-mole inorganic phosphorus per minute from excess phytate at 37°C.

and subsequent dilution of the phytase in the permeate fraction. The loss of activity over time is also likely to be a contributing factor.

Processing rates. The ability of a processing unit to concentrate the juice at a rate compatible with expected harvesting rates is a performance criterion of any proposed field apparatus. To satisfy typical field conditions, it was calculated that a permeation rate of 600 ml/m^{2}min was the minimum acceptable. This is the rate that would allow the juice collected over a 10 h harvesting period to be concentrated from 12 to 20% DM in a 20 h period. From the graph of permeation rate (Fig. 2), it appears that this minimum standard could be reached with the full scale VSEP unit (27.9 m^2 membrane area) although linear scaleability was assumed, which may not be correct.

The fouling index (FI) in Figure 2 was calculated as the flux of water before membrane use, divided by the measured permeate flux. The membrane had a lower initial water flux, and a much lower FI, than other tested membranes with greater nominal pore sizes. The ideal situation would be a high water flux and a low FI to produce high processing rates for the alfalfa juice.

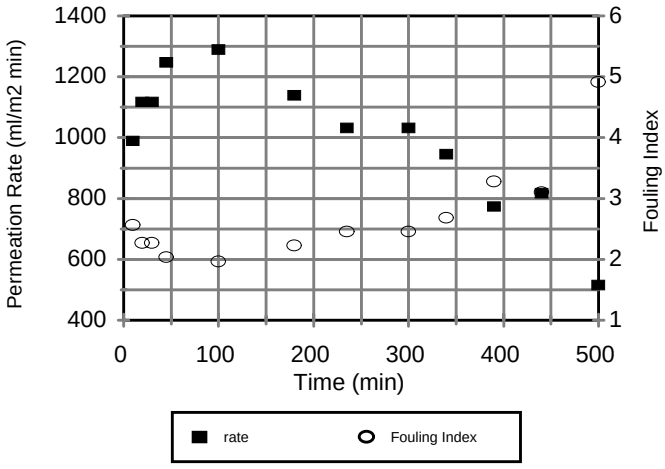

Figure 2. Permeation rate of alfalfa juice and fouling index on 10 kD regenerated cellulose ultrafiltration membrane at a transmembrane pressure of 100 psi.

There was some evidence that the permeation rate was affected by temperature which affects the viscosity of the feed. The concentration of the juice also affects viscosity and there appeared to be a limit of around 20% DM in the retentate stream for satisfactory permeation rates.

Conclusions

The performance of the VSEP was superior to previous attempts to concentrate whole alfalfa juice by ultrafiltration (Ostrowski-Meissner 1983). There appears to be considerable potential to use dynamic filtration to concentrate alfalfa juice in the field, although there are still many issues to be resolved. The phytase was concentrated in the retentate fraction which indicates that, at present, the only processing option is concentration of the juice. Attempts to fractionate the larger chloroplastic protein from the smaller soluble protein fractions, including the phytase, using larger membrane pore sizes were not successful because fouling reduced the effective MWC of the membranes. It is important to identify and characterize the performance of suitable membranes and operating conditions for processing alfalfa juice.

There are also several pretreatment options to remove the chloroplastic protein and allow better UF performance. These include temperature and pH precipitation which are well established in alfalfa juice processing.

References

Austin S., and E.T. Bingham. 1996. The potential use or transgenic alfalfa as a bioreactor for the production of industrial enzymes. In: McKersie, B.D. and Brown, D.C.W., eds., CAB International, Biotechnology and the Improvement of Forage Legumes. Pg. 409-424.

Ostrowski-Meissner, H.T. 1983. Protein concentrates from pasture haylage and their fractionation into feed and food-grade products. In: Telek, L. and Graham, H.D., eds., Leaf Protein Concentrates. Pg. 437-465.

Liquid Hot Water (LHW) Pretreatment of Alfalfa Fiber Destined for Ethanol Production

R.G. Koegel, H.K. Sreenath and R.J. Straub

Introduction

Alfalfa "fiber" referred to here is the result of wet fractionation of fresh alfalfa herbage into a juice fraction and a fiber fraction. While there are a number of other uses for this fiber fraction (Lu et al. 1979; Rowell et al. 1997), this study deals with its conversion to ethanol or organic acids by saccharification and fermentation.

It is frequently stated that the greatest single cost in producing ethanol from ligno-cellulosics is for the enzymes used to hydrolyze the fiber to fermentable sugars. It is therefore conventional wisdom that a chemical/thermal pretreatment to partially hydrolyze the fiber is necessary to improve processing economics. A number of such pretreatments have been proposed and studied. Many involve acids or bases used with elevated temperatures. Others involve softening with steam or liquid ammonia followed by an abrupt pressure drop to atmospheric. The pretreatment chosen for this study is referred to as liquid hot water, LHW, or "Aquasolv" (Mok and Antal 1992; VanWalsum et al. 1996). It consists of treating the feedstock with water at 220°C, pressurized to hold it in the liquid state, for two minutes. Advantages claimed for this process include: (1) no chemicals required which can ultimately lead to costs and waste products; (2) almost total hydrolysis of hemicellulose; and (3) partial hydrolysis of lignin. Enzymes are still needed for reducing the oligomers (short chains of sugars) in the pretreatment extract to monomers (single molecules) required by the fermentation organisms. Since hydrolysis of hemicellulose leads mainly to five-carbon sugars and hydrolysis of cellulose to six-carbon sugars, the fermentation organism(s) must be capable of fermenting both types of sugars. This dual capability is not frequently found but has been achieved in genetically engineered organisms.

Methods

The alfalfa "fiber" used in this research was the result of wet fractionation in which freshly cut alfalfa herbage was macerated using a rotary impact device and was immediately dejuiced by means of a screw press. The resulting fiber contained 70-75% of the dry matter of the original herbage. Its average composition (see Fig. 1) was cellulose, 33%; hemicellulose, 18%; lignin, 8%; protein, 11%; ash, 9%; and solubles, 22% (by difference). This material was air dried and stored for future use.

The pretreatment consisted of flowing preheated water at 220°C through 30 g of sample for two minutes. The water was held at sufficient pressure to insure that it remained in the liquid phase. Fig. 3 is a schematic of the apparatus. After the treatment chamber, the water with hydrolyzed dry matter was throttled through a valve to atmospheric pressure. This caused approximately one-third of the liquid to vaporize. After some initial treatments, a water-cooled condenser was added to allow retention of the vapor fraction for analysis. After the treatments, the fibrous residue was removed, oven-dried and stored for further analysis or treatment. The liquid "extract" was weighed and sampled to determine dry matter content. It was then stored under refrigeration for further use. Fiber and LHW products were saccharified using commercially available cellulases and pectinases. Fermentation was carried out using the genetically engineered yeast *Candida shehatae*, capable of fermenting both pentoses and hexose.

Results

The division of the fiber dry matter brought about by the LHW treatment is shown in Fig. 4. Almost 60% of the dry matter was extracted leaving around 40% of the original dry matter as fibrous residue. Only about 45% of the original dry matter was in the extract, however, leaving about 14% of the dry matter unaccounted for. It was initially assumed that this was the result of dry matter being volatilized. However, in later experiments to be reported elsewhere where 0.07% sulfuric acid was added to the treatment water

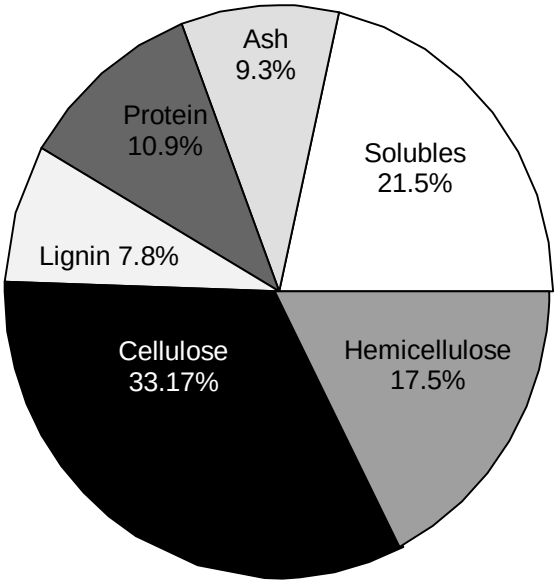

Figure 1. Representative composition of alfalfa "fiber" obtained by the wet fractionation process. N = 22 (circle area represents 100g)

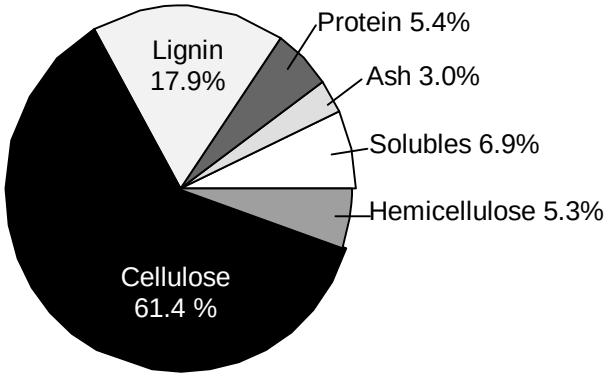

Figure 2. Representative composition of the fibrous residue resulting from the LHW treatment (circle area represents 41g, an average yield from 100g.

the sum of dry matters in the residue and the extract consistently exceeded the initial dry matter. This mass change anomaly cannot therefore be explained simply by volatilization. It could be the result of oxidation-reduction reactions, but in any case, requires further study.

The composition of the residue resulting from the LHW treatment is shown in Fig. 2. Since the circle areas in Figs. 1 and 2 are proportional to the masses of dry matter in the fiber and residue respectively, it is possible to see approximately to what degree various components were extracted. This degree of extraction of the three major fiber constituents—cellulose, hemicellulose, and lignin—is shown in Fig. 5. Hemicellulose is clearly the major constituent extracted. The results differ somewhat from those reported by Van Walsun et al. (1996) for bagasse and aspen. They reported extraction of 98-100% of the hemicellulose, 5-7% of the cellulose, and 37-65% of the lignin. The differences may be due to differences in the feedstock and/or slight processing parameter differences. For example, it is recognized that differences in cellulose crystallinity affect hydrolysis. It is also known that lignin may be solubilized and then reprecipitated under certain conditions.
The alfalfa fiber was enzymatically saccharified and fermented with and without the LHW pretreatment. In both cases,100 g of feedstock yielded 41-45 g of reducing sugars. In the case of no pretreatment, fermentation of these sugars gave an ethanol yield of about 14% rather than the "rule-of-thumb" 50%, indicating inhibition of the *Candida shehatae* used. In the case of sugars from LHW products, those resulting from the high-cellulose residue gave an ethanol yield of about 40% while the extract totally inhibited the organism, yielding no ethanol. Later work showed, however, that this extract could be fermentated to lactic acid using selected lactobacilli.

Conclusions

The LHW treatment was able to hydrolyze a large fraction of the hemicellulase (87%) and smaller fractions of the cellulose (24%) and lignin (6%). It did not appear, however, that this lead to more total fermentable sugars after enzymatic saccharification or to more ethanol. More study will be needed to determine whether the LHW treatment reduces the quantity of enzymes required. Inhibition of the fermentation organism is tentatively assumed to be caused primarily by acetic acid, a breakdown product of hemicellulose.

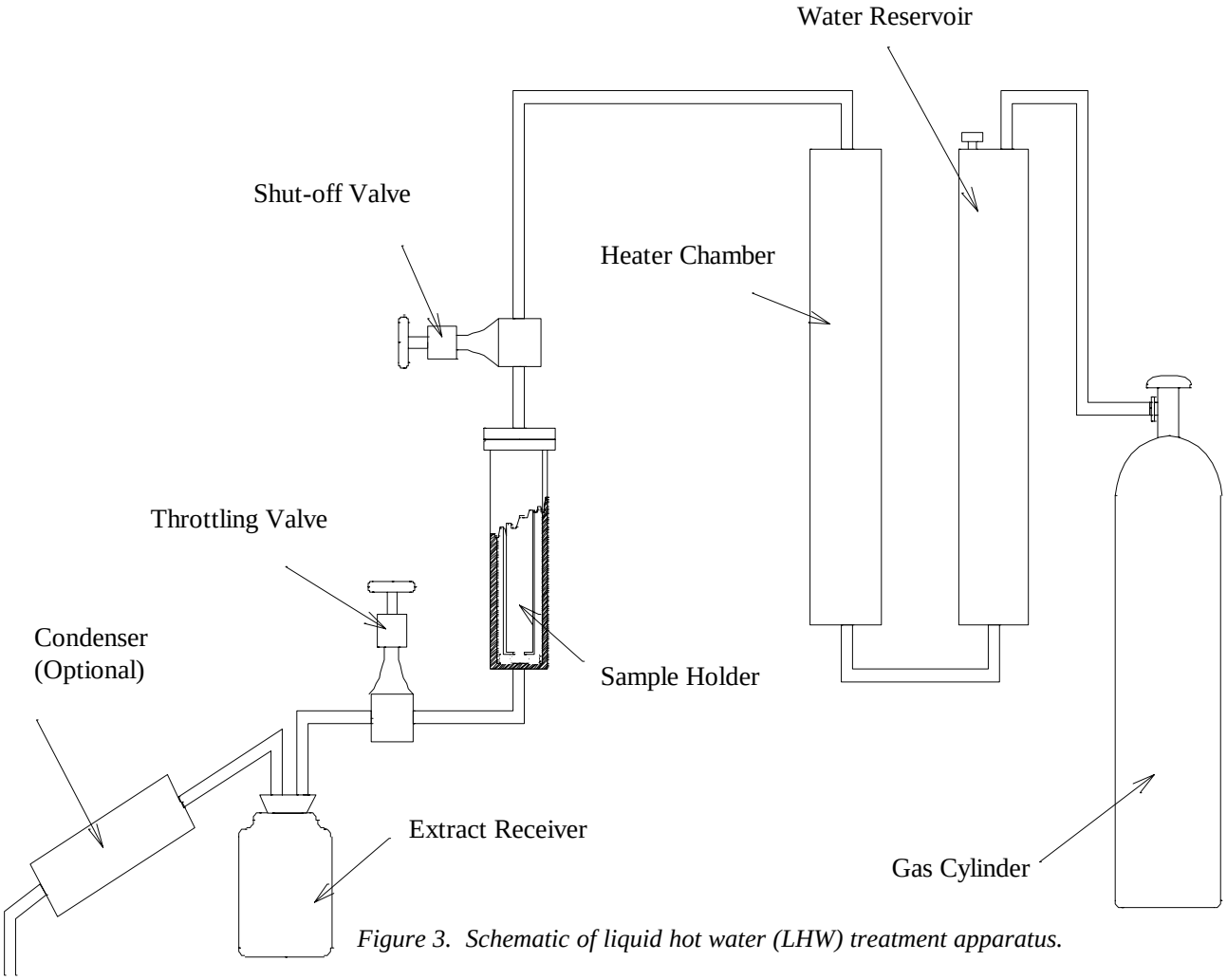

Figure 3. Schematic of liquid hot water (LHW) treatment apparatus.

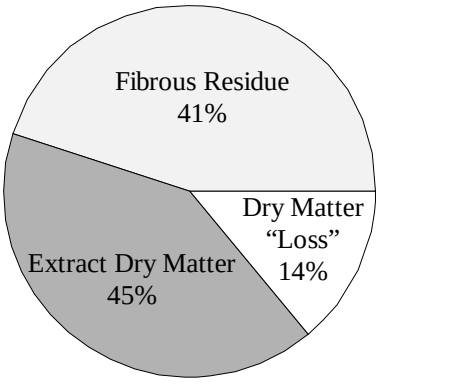

Figure 4. Division of dry matter in alfalfa fiber after LHW treatment. N = 22.

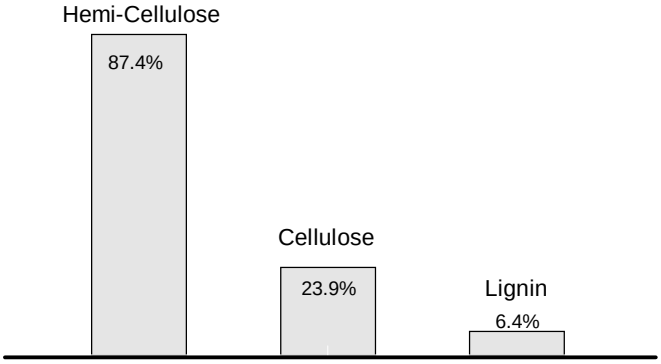

Figure 5. Percentage of the fiber constituents hydrolyzed in a LHW treatment.

References

Lu, C.D., Jorgenson, N.A. and Barrington, G.P. 1979. Wet fractionation process: preservation and utilization of pressed alfalfa forage. J. Dairy Sci. 62:1399-1407.

Mok, W.S. and Antal, M.J. 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. Ind. Eng. Chem. Res. 31:1157-1161.

Rowell, R.M., Young, R.A. and Rowell, J.K. eds. 1997. Paper and composites from agro-based resources (book). Lewis Publishers.

Van Walsum, G.P., Allen, S.G., Spencer, M.J., Laser, M.S., Antal, M.J. and Lynd, L.R. 1996. Conversion of lignocellulosics pretreated with hot water to ethanol. Appl. Biochemistry and Biotechnology. 57/58:157-170.

Forage Production

Integration of Forage in Farming Systems

C.A. Rotz

Introduction

As more knowledge is acquired on forage crop growth, harvest and use, there is an increasing need to study the crop in an integrated farming system. Forage crops are interrelated to many other parts of the farm by the flow of material and nutrients, the scheduling of operations and the priority for use of limited available funds. Animal based farming systems must be developed that have all components working together to provide profitable and sustainable food production. The growing concern over environmental impacts of farms also brings greater need to study the whole farm and its relationship to the environment. This work reviews various topics related to forage production in an integrated farming system. Direction is provided for future research to fill information gaps and modeling needs for better analysis of forages in a whole farm context.

Materials and Methods

In a crop production system, forage is grown and sold as a marketable product in conjunction with other crops. Most forage is marketed as animal feed, but opportunities exist for other uses such as industrial processing and biomass energy. Regardless of the end use, the same basic crop production system is used. Forage is linked with other crops on the farm by their individual effects on soil structure and composition and the scheduling of operations according to available machinery, labor, and suitable weather.

Animal production normally includes crop production along with feeding, animal, and manure handling components. Crops, particularly forage, are primarily produced to feed the animals. Excess crops are marketed, and supplemental feeds are purchased to meet the nutrient requirements of the animals. Feeding is linked with other components of forage production by the form, particle size, and nutrient content of the forage. Supplemental feed must be properly combined with available forage to meet the animal's requirements.

Forages produced in farming systems must be evaluated with a systematic approach. All aspects of crop production and use must be viewed in relation to one another. This approach normally leads to modeling. Models can range from pictorial diagrams and simple accounting procedures to complex numerical models that simulate farming systems on a computer. Comprehensive models available today provide excellent research tools that enable better understanding of the role of forages in farming systems. In the future, these models also may be used for better management of forage systems.

Results and Discussion

Much work has focused on modeling and analysis of forage crops in animal production systems. This work has studied either grazing systems or conserved forage systems with little work including both options on the same farm. Grazing models primarily have been applied to beef production in the US, UK, and Australia. Conserved forage system models have been developed to study dairy production in the US, Canada, and Northern Europe.

Models serve three major functions: research, teaching, and management aids. Each of these functions has different goals or requirements, which generally prevent one model from serving all three functions. Until now, most forage system models were developed and used as research tools to generate information on forages in integrated farming systems. Opportunity for further development of these research aids exists. Major areas for more work include 1) integration of forages in cropping system models that evaluate production benefits along with externality costs to society, 2) integration of more crop options

along with forages in animal production system models, and 3) integration of grazing and conserved forage systems in a more comprehensive animal production model.

Teaching aids are models that can be used alone or in a classroom or workshop to illustrate the complexity of the production system and the impact of technological or management changes. Research aids provide good teaching aids if the effort is given to creating models that are easily used by others. Few models have taken this step, but this is likely to change over the next few years. Computer technology is now available for developing extensive, but intuitive, user interfaces for models.

Many models were developed with the promise of providing management aids for producers, yet few have reached this level of practical application. Comprehensive models that provide good research tools are generally too complex for application by a general audience. Although a good teaching aid can provide useful information for decision making, training of the user is required to insure that the model is used properly and thus provides reliable information. Management aids that integrate forages in farming systems will be developed in the future, but these may not come quickly. A different modeling approach such as an expert system is needed where comprehensive models or information generated by those models are used as experts in the decision making process. Expert system models are now used in forage crop management, but more development is needed for comprehensive management of an integrated multiple crop and animal production system.

Information gaps exist for the development of all three types of models. This begins with the crop. Most available forage crop models do not adequately predict developmental stages of the growing plant and the effect of stage on nutritive content. Much more information is also needed to better predict forage utilization by ruminants. Available animal models perform adequately when predicting intake and performance for well balanced diets. There is a growing need, however, to be able to predict intake and performance when the availability or nutritive characteristics of feeds is limiting animal response. This is particularly true under grazing conditions where animals tend to select plant material and feed supplements may not be available. The recycling of manure nutrients is another area where information is lacking. These include nutrient losses during handling and application, and the transport and transformation of nutrients in the soil profile. A relatively new and unexplored area of need is in quantifying the effects of farm losses on the environment and the societal cost for dealing with those effects.

Conclusion

Opportunities for further research on forages in cropping systems include basic and applied research on system components as well as modeling and analysis of complete farm systems. Regardless of experience and training, those with an interest in contributing to the effort of evaluating forages in cropping systems can find a place to apply their expertise.

A Dairy Herd Model for Use in Whole Farm Simulations

C.A. Rotz, D.R. Mertens, D.R. Buckmaster, M.S. Allen and J.H. Harrison

Introduction

The dairy industry faces complex issues related to improving efficiency, reducing negative impact on the environment, and developing a more competitive position in the world economy. To solve economic and environmental issues, a systematic whole farm approach must be taken. All major farm components, the interaction among components, and the interaction with the environment must be considered. This requires an interdisciplinary modeling approach. Farm models require submodels of each of the major farm components. On dairy farms, a major component is the animal herd. Work was undertaken to develop a herd model that predicted feed intake, milk production and manure excretion with rations balanced to meet roughage, energy, and protein requirements, and to verify or validate the important model components by comparing predicted information to that of other widely accepted models and data bases.

Materials and Methods

The model was developed to simulate or predict performance of a dairy herd consisting of growing heifers, lactating cows, and dry cows. The model was developed in five sections. In the first section, the characteristics of the major animal groups making up the herd were established. Next, the allocation of available feeds and the characteristics of those feeds were set. The animal's requirements for roughage, energy, and protein were then determined, and a linear program was used to determine the best mix of feeds to meet the animal's requirements. Finally, based upon the diet fed, the quantity and nutrient content of the manure produced was determined.

Results and Discussion

The herd was described as six animal groups: young stock under one year old, heifers over one year old, three groups of lactating cows, and dry cows. The characteristics of each animal group were a function of the animal type and the period of the lactation cycle covered. The seven available animal types were large Holstein, average Holstein, small Holstein, Brown Swiss, Ayrshire, Guernsey, and Jersey. Feed characteristics used to balance rations and predict feed intake included crude protein, protein fractions, net energy of lactation, and neutral detergent fiber. Protein fractions were the portion of the total protein that was ruminally degradable and the portion of acid detergent insoluble protein. Total digestible nutrient, phosphorous and potassium contents were used to predict manure excretion. A feed allocation scheme was established to match available forage with the animal group that could best use forage of that nutrient content.

Animal diets and performance were modeled using a linear program that simultaneously solved five constraint equations. These constraints included 1) a limit on the rate of fiber passage through the rumen, 2) a minimum roughage requirement, 3) an energy requirement, 4) a minimum requirement of rumen degradable protein, and 5) a minimum requirement of undegradable or bypass protein. Manure production was modeled as feed dry matter consumed minus the digestible DM extracted by the animals plus urine DM and feed DM lost into the manure. Manure nutrients equaled the nutrient intake minus nutrients contained in the milk produced and in the meat produced through animal growth.

Three procedures were used to verify that the dairy herd model worked properly and produced reasonable results. Animal requirements predicted by the model were first compared to those recommended by the National Research Council. Next, diets formulated by the model for each animal group were compared to those formulated by the Spartan Dairy Ration Evaluator/Balancer. Finally, manure excretion predicted by the model was compared to measured data from calorimeter studies performed over a 30-year period at the Energy Metabolism Unit in Beltsville, Maryland.

Dry matter intakes predicted by our model were about 15% less than those from the NRC model for both the late lactation and dry cow groups. This occurred because the forage used in our model included corn silage. With this high-energy forage, requirements for these groups were met with a lower intake. Intakes for all other animal groups were very similar between the two models. Net energy requirements for the early and mid lactation groups were about 5% greater from our model, but they were essentially the same for all other groups. The small difference for the higher producing groups was likely due to a change in the energy constraint of our model that provided an added energy requirement for excreting excess degradable dietary protein. Small differences in undegradable and degradable protein requirements were due to differences in the way acid detergent insoluble and rumen influx proteins were handled in the ration constraints.

Rations were generated for each animal group using alfalfa hay, alfalfa silage, corn silage, high moisture corn grain, soybean meal, and cotton seed. Using the same forage mix and the same feed characteristics, rations were generated by the Spartan Dairy Ration Evaluator/Balancer. Rations formulated by the two models were similar, but some difference occurred. For the early and mid lactation groups, our model used less forage and more concentrate in rations than those from the Spartan program. For all other animal groups, the opposite trend occurred with our model using more forage in rations. This difference was largely due to differences in the logic for use of forage. Our model formulated rations using the maximum amount of forage in the diet while meeting the energy and protein requirements. The assumption in the Spartan program was that feeds were balanced to minimize ration costs using market prices for feeds.

The manure component was validated by comparing predicted and measured dry matter and nitrogen excretions. Our model predicted greater manure excretion than that measured in the calorimeter chambers. Dry matter intakes from our model were also proportionately greater compared to the measured data. Greater intakes, and thus greater manure excretion, might be due to genetic and feeding improvements made over the past 30 years. Values predicted by our model for lactating cows were similar to the average manure production values for dairy cows published in the ASAE Standards. In general, differences in N excretion between predicted and measured values were in proportion to differences in protein or nitrogen intake. Because animal diets could not be formulated to exactly match those fed in the many experiments, differences in N excretion were more a function of the way animals were fed than model or measurement error.

Conclusion

A model was developed that predicts nutrient requirements, feed rations, feed intake, milk production, and manure excretion for a dairy herd. The model was verified to predict feed intakes, nutrient requirements, feed rations, and manure excretion similar to those commonly recommended or expected for the various animal groups making up a dairy herd.

Performance and Economics of a Perennial Cow Dairy Farm

C.A. Rotz and D.L. Zartman

Introduction

Economic and political forces are prompting change in the dairy industry. New ideas are being explored to improve the efficiency and profitability of dairy farms while reducing potential adverse effects on the environment. One possibility is the use of perennial dairy cows. A perennial cow is defined as one that remains at a relatively high milk production level for three to five years without cycling through a dry period and calving. The potential exists to develop the genetics of this type of animal to create a full herd on this management plan. The use of BST or other techniques may further increase the production level and longevity of such a herd. There is a need to determine how such a management change would effect the profitability and environmental impact of a typical dairy farm. A comprehensive evaluation of the whole farm is needed. The objective of this work was to use DAFOSYM to compare the performance, nutrient management and economics of dairy farms using traditional and perennial lactation cycles.

Materials and Methods

DAFOSYM is a simulation model that integrates the many biological and mechanical processes on a dairy farm. Crop production, feed use, and the return of manure nutrients back to the land are simulated over many years of weather. Simulated performance is used to predict the costs, income, and net returns or profit for typical dairy farms. DAFOSYM was first used to simulate a typical farm of today. Cows were bred for calving each year and replacement heifers were raised on the farm. In the next variation of this farm, calves were sold and replacement heifers were purchased as they came into production. The need for heifer housing was eliminated, less feed was required and less manure was produced.

To model the perennial herd, the number of animals in each production group was modified. The number of first lactation animals in early and mid lactation remained the same. All other animals were placed in a late lactation group that maintained an average production level of 51 lb of milk per day. There were thus no non-lactating cows on the farm. Breeding costs were eliminated further reducing annual livestock expenses. The replacement rate was also reduced from 35% to 25%. Therefore, animals remained in production for about four years.

Results and Discussion

The 25-year average performance and economic results for our representative farm are listed in Table 1. Compared to a conventional 100-cow dairy farm, use of 100 perennial cows with purchased replacements greatly under utilized the feed production potential of the farmland and reduced the profit potential of the farm. Use of 140 perennial cows with purchased replacements adequately utilized the feed production and manure recycling potential of the farmland and increased the profit potential by $20,000 per year. Feed use, nutrient recycling, and profit potential were similar between farms that used either perennial cows or cows that followed a standard lactation cycle where all replacement animals were purchased from off farm sources.

Conclusion

The concept of using a perennial cow dairy herd appears to be a feasible option for efficient dairy production. Further development of the concept for practical application is justified.

Table 1. Effect of purchased replacement heifers and a perennial cow herd on annual feed production, feed use, nutrient balance, costs, and net return of a dairy farm in south central Michigan.

Production or cost parameter	Units	Standard farm*	Purchased heifers† 100 cows	Purchased heifers† 135 cows	Perennial herd‡ 100 cows	Perennial herd‡ 140 cows
Alfalfa silage production	ton DM	334	334	334	334	334
Alfalfa hay production	ton DM	119	119	119	119	119
Corn silage production	ton DM	172	172	172	172	172
Grain production	ton DM	196	196	196	196	196
Alfalfa purchased (sold)	ton DM	(22)	(229)	(136)	(265)	(183)
Corn grain purchased (sold)	ton DM	154	140	263	162	310
Protein mix purchased	ton DM	32	35	30	41	50
Average milk production	lb/cow	20,000	20,000	20,000	18,974	18,974
Manure produced	ton	6,178	4,180	5,682	3,842	5,417
Nitrogen exported in milk and meat	lb	12,359	12,359	15,227	10,601	14,844
Nitrogen from fertilizer	lb	5,500	16,500	8,250	16,000	8,000
Nitrogen from manure and legumes	lb	81,430	78,020	80,366	77,011	70,874
Nitrogen removed in crops	lb	50,705	50,780	50,813	50,767	50,718
Nitrogen lost to atmosphere	lb	15,289	9,192	12,239	8,628	11,881
Nitrogen lost to ground water	lb	5,597	4,932	5,526	4,852	5,454
Residue and unused soil nitrogen	lb	9,839	13,114	11,788	12,767	10,819
Phosphorous shortage (buildup)	lb	363	1,222	(55)	1,013	(575)
Potassium shortage (buildup)	lb	6,171	16,815	12,405	18,205	11,760
Field and feeding machinery cost	$	43,207	41,802	42,945	41,467	42,703
Fuel and electric cost	$	5,253	4,422	5,101	4,219	4,958
Feed and machinery storage cost	$	21,511	20,562	21,155	20,522	20,839
Labor cost	$	40,724	38,749	50,274	38,273	51,342
Seed, fertilizer, and chemical cost	$	11,486	14,734	12,505	14,757	12,571
Purchased feed and bedding cost	$	36,552	31,464	54,814	35,822	64,501
Animal and milking facilities cost	$	36,896	31,088	31,088	31,088	31,088
Livestock expenses	$	23,800	62,800	84,780	47,300	66,220
Milk hauling and marketing fees	$	17,643	17,643	23,817	16,739	23,435
Property tax	$	4,466	4,261	4,261	4,261	4,261
Total production cost	$	241,539	267,524	330,741	254,449	321,919
Milk, feed, and animal sale income	$	308,553	322,510	417,623	307,327	409,770
Net return to management	$	67,014	54,986	86,882	52,878	87,851

*100 mature cows and 85 replacement heifers on 225 acres of cropland simulated over 25 years of East Lansing, Michigan weather.

†Same as standard farm except that replacement heifers were purchased, heifer housing was eliminated and livestock expenses were reduced by $30/cow/year.

‡Perennial cows were used and all replacements were purchased. Perennial cows had a peak milk production a few weeks after calving and then drop to a consistent level for the remainder of their productive life.

Prediction of Alfalfa Forage Yield Loss due to Freezing Injury: I. Model Development and Sensitivity Analysis

V.R. Kanneganti, C.A. Rotz and R.P. Walgenbach

Introduction

Freezing injury causes extensive dry matter (DM) yield loss in alfalfa (*Medicago sativa* L.) exposed to cold winters in North America. Existing simulation models for alfalfa either do not account for freezing injury effects or do not differentiate cultivars for their varying response to freezing stress and subsequent effects on forage production. Alfalfa models must simulate freezing injury effects in a cumulative way so that forage yield can be accurately predicted over multiple years of the same crop. This is particularly important when models are used to evaluate cropping systems or management alternatives in relation to farm profitability. The objective of this project was to incorporate cultivar specific effects of freezing injury on forage yield into an existing alfalfa model (ALSIM 1, Level 2) to predict yield over the life of an alfalfa crop. The new model was titled ALFACOLD, an acronym to mean 'ALFAlfa model for yield prediction in COLD climates.' Model structure and sensitivity of predicted yield to changes in model parameters are presented in this paper. Model validation is presented in a companion paper.

Methods

Model development. Numerical functions of cold tolerance, fall dormancy and freezing injury were developed from data in the literature, and integrated with the growth processes in the ALSIM model. A schematic representation of the ALFACOLD model is shown in Fig. 1. If cold tolerance, fall dormancy, freezing injury and population components shown in Fig. 1 are excluded, the resulting diagram would represent the ALSIM model.

ALFACOLD model simulates growth as a function of air temperature, solar radiation, soil moisture, and plant density. It predicts DM growth in leaf, stem and buds on a daily basis. Material available for top growth and storage (MATS) is equivalent to photosynthate after respiration has been subtracted. Material in MATS is partitioned daily into leaves (LEAF), stems (STEM) or total non-structural carbohydrates (TNC) in the crown and root tissue. The TNC are utilized in the formation of buds (BUDS) that elongate into new leaves and stems during regrowth. While DM partitioning pathways are the same in ALSIM and ALFACOLD, rate equations defining the amount of material flow are different because of the additional processes of fall dormancy and freezing injury in ALFACOLD. Effect of fall dormancy on growth is modeled as a function of cultivar's fall growth score (FGS). Plant death due to freezing injury is modeled as a function of cultivar's potential for cold tolerance, and magnitude and duration of freezing soil temperature in the crown region. Effect of snow cover on soil temperature is estimated from air temperature and snow depth. Soil water (AW) movement and evapo-transpiration are modeled to compute water stress factor to account for the effects of limited water supply on crop growth.

ALFACOLD model simulates plant death and consequent yield loss due to freezing injury only. While freezing injury may be a dominant cause of winterkill in cold regions, other factors such as ice sheeting, poor soil aeration or low soil potassium can add significantly to winter injury when alfalfa is grown on poorly drained or infertile soils. The model assumes adequate drainage and soil fertility. The model ignores the effects of pests and diseases on plant kill. The model does not simulate growth during a seeding year.

Input data needed are: (1) daily solar radiation (W m^{-2}), maximum and minimum air temperature (°C), and precipitation (mm); (2) latitude (degrees); (3) harvest dates; (4) cultivar's Fall Growth Score (FGS); (5) initial plant density (plants m^{-2}); and (6) maximum plant available water in the root zone (mm). Plant density and FGS are not required to run ALSIM. The computer code, documentation, and sample data files are available upon request from the primary author.

Sensitivity analysis. Changes in ALFACOLD predictions of forage yield to a ± 25% change in the

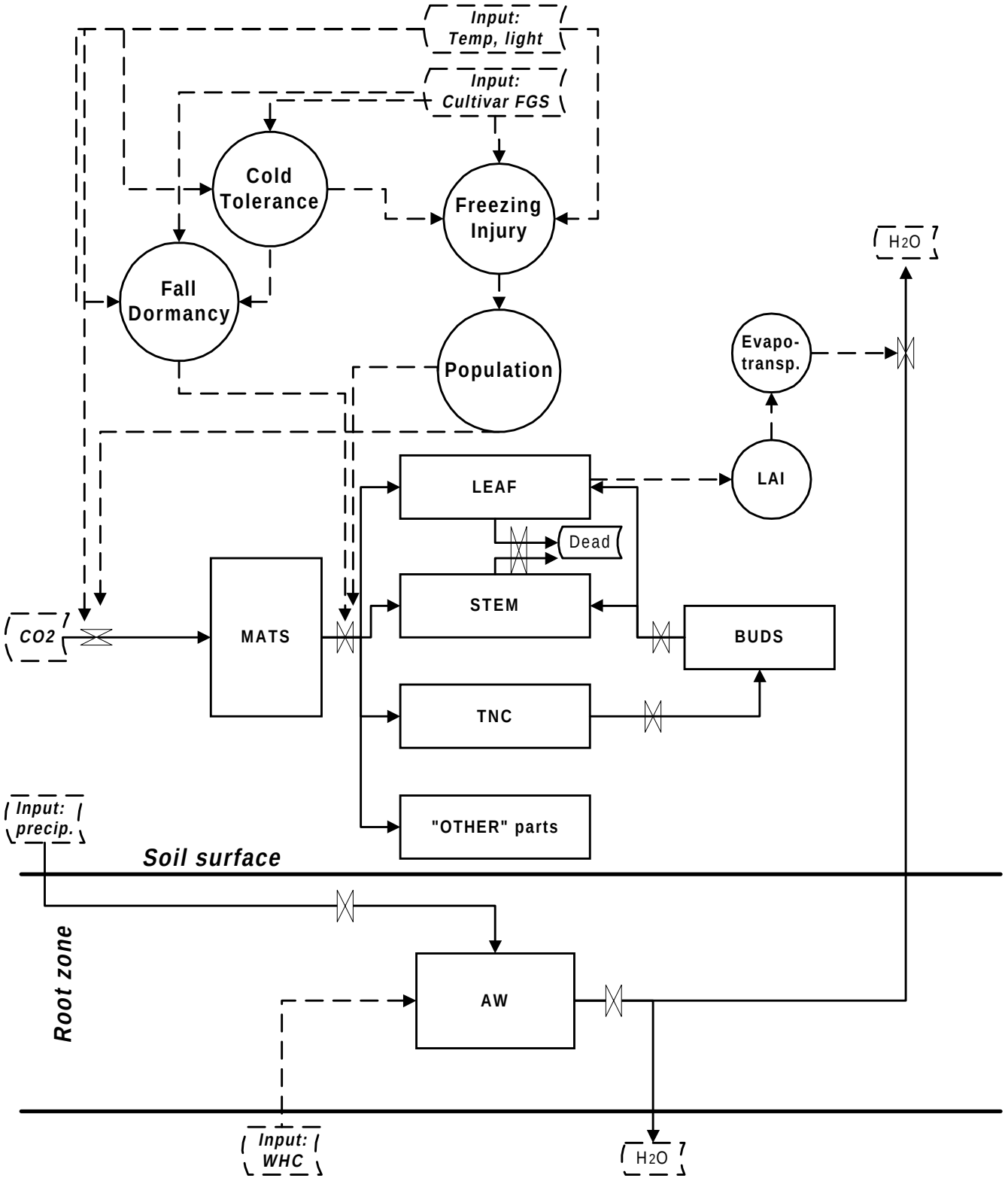

Figure 1. A simplified schematic representation of the alfalfa model, ALFACOLD. (Boxes represent model states; circles for auxiliary variables, processes or components; solid lines for material flow; dashed lines for information flow; valves for rate functions; dashed-line boxes for input/output. Acronyms are defined in the text.)

Table 1. Sensitivity Index (SI, Eq. 1) for forage DM yield to a ±25% change in selected model parameters for three different cultivars of FGS of 2, 3, and 4 during three production years: PY1, PY2, and PY3. (An absolute SI value greater than 1 indicates that the model is very sensitive to the particular change.)

				Sensitivity Index (SI)					
				Negative Change (-25%)			Positive Change (+25%)		
Variable definition	Acronym	Scenario†	Reference‡ value	PY1§	PY2	PY3	PY1	PY2	PY3
Potential crop death coefficient (d^{-1})	PDFMX	CV2	0.109	0.0	-0.05	-0.05	0.0	-0.05	-0.05
		CV3	0.121	0.0	-0.10	-0.10	0.0	-0.10	-0.10
		CV4	0.133	-0.0	-0.70	-0.80	-0.0	-1.30	-1.40
Potential rate of cold hardening (°C d^{-1})	CHRMX	CV2	0.184	0.05	0.30	0.35	-0.0	0.10	0.15
		CV3	0.162	2.45	3.85	3.95	-0.05	0.25	0.30
		CV4	0.139	4.00	4.00	4.00	0.05	1.00	1.10
Potential rate of dehardening (°C d^{-1})	CDRMX	CV2	0.820	0.20	0.20	0.30	0.05	0.05	0.05
		CV3	0.795	0.20	0.05	0.10	0.05	0.0	0.05
		CV4	0.770	0.0	-0.15	0.60	-0.0	-0.20	-0.20
Lowest temperature tolerance (°C)	CTMX	CV2	-22.4	-0.05	-0.05	-0.05	-0.0	0.0	-0.15
		CV3	-20.4	-0.05	0.0	-0.05	-0.0	0.0	-0.05
		CV4	-18.4	-0.20	1.35	1.40	0.0	0.0	0.0
Snow (mm)	SNOD	CV2	dlyinp¶	0.0	0.0	0.0	0.0	0.0	0.0
		CV3	dlyinp	0.05	0.0	0.0	0.05	0.0	0.0
		CV4	dlyinp	0.25	1.70	1.70	0.10	0.55	0.60
Initial plant density (plants m^{-2})	POP_{init}	#CV2	160	0.25	0.30	0.35	0.10	0.30	0.35
		CV3	160	0.20	0.70	0.80	0.15	0.30	0.30
		CV4	160	0.20	1.80	1.80	0.20	0.80	0.90

Eq. 1: SI = (DY/Y)/ (DP/P), where Y represented model predicted forage DM yield (kg ha^{-1} yr^{-1}), and P represented the value of a model parameter or input variable.

† Three scenarios, coded as CV2, CV3, and CV4, correspond to 3 different cultivars with FGS of 2, 3, and 4, respectively, during 3 production years.

‡ Reference value for each parameter was extracted from functions developed based on data in the literature. Each reference value was subjected to a ± 25% change.

§ PY1, PY2 and PY3 are three consecutive production years after a seeding year in Arlington, WI. Measured weather data were used as model input.

¶ Daily snow fall data (SNOD) were subjected to a ± 25% change.

Initial plant density at the start of a simulation was subjected to a ± 25% change.

newly added parameters (Table 1) were determined to test the ability of the model to respond to varying intensities of freezing injury in cultivars of contrasting FGS. Three production years of a 4-year crop grown in Arlington, WI spanned 1991-93. These years were selected because crop reports indicated substantial variation in winter injury during this period. Three different scenarios were produced (coded as CV2, CV3 and CV4) that corresponded to forage production in three different cultivars of FGS 2, 3 and 4, respectively. Most cultivars grown in this region fall in the range of 2 to 4 FGS. Each simulation was started on 1 March 1991 and ended on 31 October 1993. The simulated crop was harvested in 4 cuts on May 26, June 27, July 29 and August 27 in all three years.

Results and Discussion

Sensitivity of predicted yield to changes in the selected parameters under the three scenarios simulated for Arlington, WI is presented in Table 1. Model parameters associated with sensitivity index (SI) absolute values greater than 1.0 were considered very sensitive while SI values less than 0.5 indicated low sensitivity of model output to changes in parameter value or to variation in input data due to potential measurement errors.

Except for the crop death coefficient (PDFMX, d^{-1}), ALFACOLD was generally more sensitive to a decrease (negative change) in parameter value than to a corresponding increase. Sensitivity generally increased with crop age as plant density decreased due to winterkill following repeated exposure to winter seasons. Predicted forage yield was influenced more by a cultivar's rate of cold hardening (CHRMX, °C d^{-1}) and lowest temperature tolerance (CTT, °C) than by the rate of dehardening (CDRMX, °C d^{-1}). High values of SI for rate of cold hardening suggest that the model needs carefully measured experimental data for this parameter. Error in estimating snow cover had minor effect on predicted yield in hardy cultivars but had a greater impact on yield predicted for the less hardy cultivars. Need for good plant density in the first production year was revealed during the second and third production years as plant population declined due to winterkill following repeated exposure to cold winter seasons.

Conclusion

(1) The ALFACOLD model predicted forage yield on a daily basis while accounting for the cumulative effect of freezing injury on yield over multiple years of the same crop. (2) The model parameters added showed sensitivity to freezing injury as expected. (3) Sensitivity analysis indicated that cold hardening rate and lowest temperature tolerance influenced yield more than the rate of dehardening in spring. (4) ALFACOLD can be incorporated into whole farm simulators, such as the DAFOSYM model, to predict forage yield daily while accounting for the effects of winter injury on forage production, thus influencing farm economics.

Prediction of Alfalfa Forage Yield Loss due to Freezing Injury: II. Model Validation and Example Simulations

V.R. Kanneganti, W.L. Bland and D.J. Undersander

Introduction

A new alfalfa model called ALFACOLD was developed by expanding upon an existing model (ALSIM 1, Level 2) to predict forage yield on a daily basis while accounting for the cumulative effects of freezing injury over multiple years of the same crop. Model description and analysis are presented in a companion paper. Accurate assessment of freezing injury at the start of a growing season can help in managing a shortfall in forage production in the subsequent growing season.

The objective of this study was to test ALFACOLD model's ability to predict field measured forage DM yield and to determine the model's prediction accuracy. Example simulations were presented to show potential applications of the model.

Methods

Model Validation. Validation data consisted of 874 yield observations collected during 1990-93 from variety trials seeded in 1989 and 1990 at Arlington and Lancaster sites in WI. These years were selected because crop reports indicated substantial variation in winter injury during this period. Minimal winter injury occurred following the winter of 1990-91 while moderate to severe injury occurred during 1991-92 and 1992-93. All crops were managed under a 4-cut system, and cutting dates fell around May 26, June 27, July 29 and August 27. Only commercial varieties with at least moderate resistance to different diseases were included. Thirty-nine cultivars with fall growth scores (FGS) ranging between 2.3 and 3.8 were represented. Most cultivars grown in the region fall in the range of 2 to 4 FGS.

Example Simulations

Cold tolerance and freezing injury. Selected simulations were presented to discuss model predictions of cold tolerance and freezing injury in alfalfa. The model was set up to simulate growth and freezing injury during two contrasting years (1990-91 and 1991-92) with respect to snow cover and freezing injury observed in Arlington, WI. Two sets of simulations were run. The first set was started on 1 March 1990 and ended on 31 October 1991, and the second set was started on 1 March 1991 and ended on 31 October 1992. In each set, growth of five alfalfa cultivars with a FGS of 1.5, 2.5, 3.5, 4.5, and 5.5 was simulated. All crops were managed under a 4-cut system in both years.

Risk of yield loss due to freezing injury. The ALFACOLD model has the potential to quantify risk of yield loss due to freezing injury for a specified region. To demonstrate this application, the model was set up to simulate growth during production years 1, 2 and 3 (i.e., crop years 2, 3 and 4) of a 4-year crop, with a new crop seeded each year during 1970 to 1987 in Arlington, WI, for a total of 18 different crops. Simulation was started for each crop on 1 March in the first production year and ended on 31 October in the third production year. Two sets of 18 crops were simulated. In the first set, freezing injury simulation was disabled, so the predicted yield reflected potential yield under conditions of negligible freezing injury. In the second set, freezing injury was simulated.

Results and Discussion

Model Validation. ALFACOLD and ALSIM models were tested against measured yield from 39 cultivars seeded in two years and managed under a 4-cut system for three years after the seeding year at two sites in Wisconsin (Figs. 1, 2). The ALFACOLD model adequately predicted measured yields over multiple production years of a crop. Averaged over the sites, cultivars and years, ALFACOLD model predicted annual forage yield within 12% of the measured yield, compared to an error of 35% with the ALSIM model (Fig. 1). A regression of field measured yield of individual harvests (n=874) on the corresponding ALFACOLD predicted yield indicated that 70% of the measured variability in yield was explained by the model

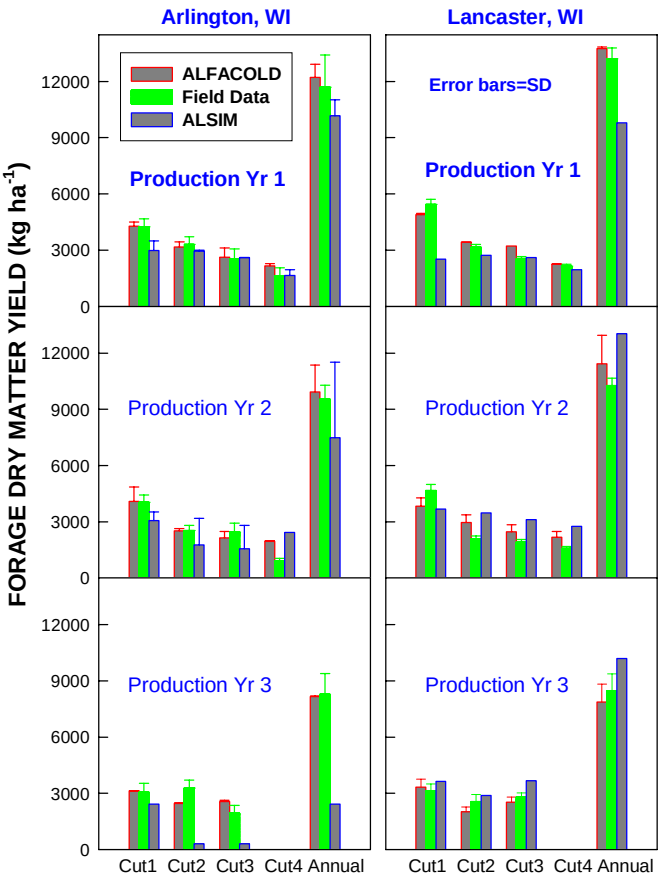

Figure 1. The ALFACOLD and ALSIM 1 (Level 2) model predictions of forage DM yield averaged over 39 cultivars harvested in a four-cut system during three consecutive years after the seeding year compared to the corresponding field measured yield. (Data: 2 sites, 39 varieties, 6 crop years: n=874)

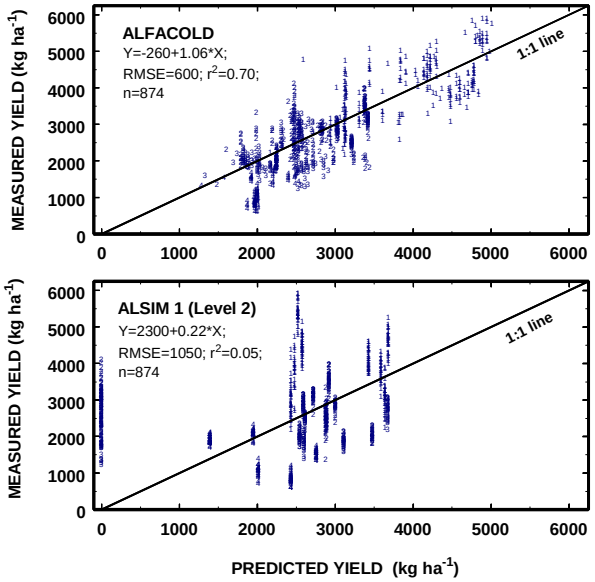

Figure 2. The ALFACOLD and ALSIM 1 (Level 2) model predictions of forage DM yield for 39 cultivars harvested in a four-cut system at two sites during three consecutive years after the seeding year compared to the corresponding field measured yield. (Numbers 1 to 4 represent harvest numbers in a year: 1 for first, 2 for second, 3 for third, and 4 for fourth harvest. Data: 2 sites, 39 varieties, 6 crop years: n=874)

(r^2, Fig. 2). The average difference between measured and predicted yield for harvests 1, 2 and 3 was less than 7%, but ALFACOLD over-predicted fourth cut yield by 38% (Fig. 1). Averaged over the sites, cultivars, years and cuts, ALFACOLD predicted yield with an average standard deviation of 600 kg ha^{-1} cut^{-1} (RMSE, Fig. 2).

Cold tolerance and freezing injury. Cold tolerance was quantified by sub-zero ℃ temperature that a crop could withstand without being killed. Cold hardening initiated in mid-September (Fig. 3). Cold tolerance increased at a faster rate in hardy cultivars (FGS 1.5) than in cold sensitive cultivars (FGS 5.5), which resulted in greater tolerance of hardy cultivars compared to non-hardy cultivars at any specified time during winter. Plants began to deharden in March and completely lost tolerance to freezing temperature by mid to late April. Non-hardy cultivars completely dehardened about 8-15 days earlier than did the hardy cultivars. Crown temperature represented the predicted soil temperature in the crown region (3 cm), and included the "insulation" effect of snow. Crown temperatures fell below the tolerance temperature of the cold sensitive cultivar resulting in its death (discontinued line, Fig. 3). Even though crown temperatures were much colder in 1990-91 than in 1991-92, the cultivar died early in 1991-92 due to a "cold snap" (rapid fall in temperature for a short duration) in autumn before the crop had a chance to develop adequate tolerance. For the same reason, more plants of medium hardy and cold hardy cultivars were killed in 1991-92 than in 1990-91. Model simulations of hardening, de-hardening, and freezing injury fit reasonably well with qualitative observations reported in the literature.

Yield loss due to freezing injury. Forage yield lost during production year 2 (PY2) and production year 3 (PY3) due to freezing injury in the preceding years was simulated for a 4-year crop of 3.0 FGS, with a new crop seeded each year over a period of 18 years (1970-87). In 3 out of 18 years, predicted yield loss due to freezing injury was 30% or greater during PY2 and 40% or greater during PY3 (Fig. 4). A third of the crops seeded during this period sustained a simulated yield loss of 5% or greater during PY2 and 10% or greater during PY3. The predicted average potential

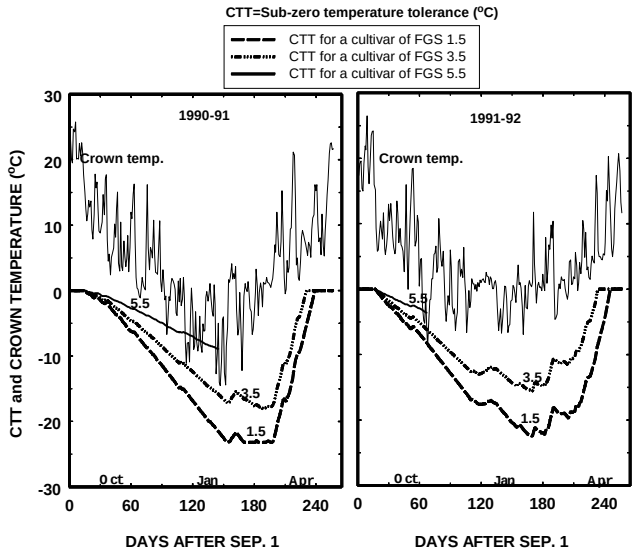

Figure 3. Simulated cold tolerance in cultivars of contrasting fall growth score (FGS) compared to predicted average daily soil temperature in the crown region (3 cm depth) during two winter seasons in Arlington, WI. (Discontinued line for FGS 5.5 indicates total winterkill due to freezing injury.)

annual yield was 11180 kg ha^{-1} (SD 1930 kg ha^{-1}) in 4 harvests, assuming zero freezing injury.

Conclusion

(1) ALFACOLD adequately predicted forage yield measured in the field over multiple production years of the same crop. (2) ALFACOLD predicted yield more accurately than did the ALSIM 1 (Level 2). (3) Breeding alfalfa cultivars for rapid rate of hardening will minimize the lethal effects of "cold snaps" (rapid fall in temperature for short periods) in autumn and winter.

Model Applications

Potential applications of ALFACOLD include: (1) forecasting yield loss due to freezing injury at the start of a growing season; (2) quantifying risk of freezing injury for a specified combination of cultivar and climatic conditions in a region; (3) estimating probability of freezing injury in autumn, winter or spring for a specified cultivar in a region; and (4) serving as a component module in decision support systems.

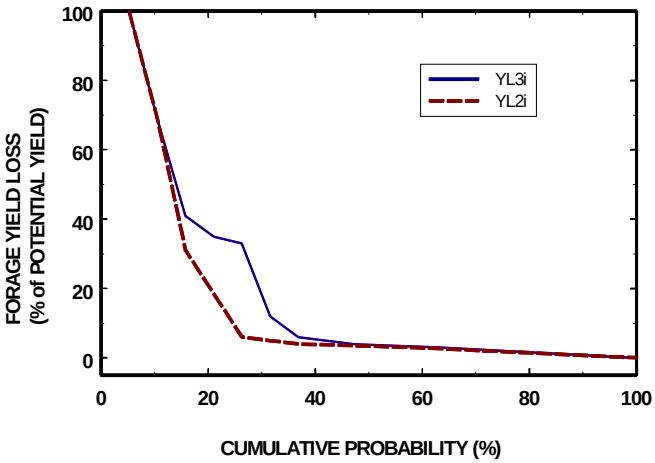

Figure 4. Cumulative probability distribution of simulated forage DM yield lost during production year 2 (YL_{2i}) and production year 3 (YL_{3i}) due to freezing injury in the preceding years in a 4-year alfalfa crop of 3.0 FGS, with a new crop seeded each year for 18 years in Arlington, WI. (Yield loss was expressed as percent of potential annual DM yield predicted with minimal freezing injury).

In-Season Applications of Dairy Manure Slurry on Reed Canarygrass

M.P. Russelle, G.W. Randall, P.D. Clayton, M.A. Schmitt, L.J. Greub, C.C. Sheaffer, R.R. Kalton and D.H. Taylor

Introduction

Available land for spreading manure is a serious constraint for some livestock producers, especially in summer. Perennial forages in the crop rotation provide land area for manure application. Reed canarygrass (*Phalaris arundinacea* L.) is widely adapted to temperate climates, and is tolerant to both flooding and drought and a wide range of soil pH. Palatable, low alkaloid varieties have been developed. It is among the highest yielding cool season grasses, has high N uptake capacity, and forms a dense sod, making it possible to carry traffic when other fields are too wet. It has been recommended for N removal from land-applied sewage effluents. Thus, reed canarygrass is an ideal candidate for manure applications during the growing season. Our objective was to evaluate reed canarygrass response to manure slurry spreading alternatives and to compare the manure N source with commercial N fertilizer.

Methods

Pure stands of 'Palaton' reed canarygrass were established on Nicollet clay loam soil at Waseca, MN and Webster City, IA, and on a Sparta loamy sand soil at River Falls, WI. After the establishment year, dairy manure slurry and fertilizer N treatments were applied in a randomized complete block design with four replications. Dairy manure slurry treatments were several combinations of rates ranging from 0 to 375 m^3 ha^{-1} (cubic meters/hectare), broadcast or surface-band application, and either split or single annual applications. Application time ranged from early spring to after the 3rd harvest and total N application rates varied with changes in N concentration of the stored slurry at each location. Ammonium nitrate was surface broadcast at 0 to 448 kg N ha^{-1}, with the two highest rates (336 and 448 kg N ha^{-1}) being applied at 224 kg N ha^{-1} in early spring and the remainder after first harvest.

Herbage dry matter (DM) yield and total N concentration (by near infrared reflectance spectroscopy) were determined for each harvest. Apparent manure N use efficiency was estimated as the slope of herbage N yield vs. total N applied. Deep soil cores were collected in some treatments in late autumn and analyzed for exchangeable NH_4-N and NO_3-N using standard methods. Soil solution samples collected in some treatments with ceramic suction cup samplers at the 1.5-m depth were analyzed for NO_3-N.

Results

Highest annual yield with slurry on the clay loam soils was 11.8 Mg ha^{-1}, using a total of 375 m^3 ha^{-1} split either after the 2nd and 3rd harvests (1130 kg N ha^{-1}) or in early spring and after 2nd harvest (1190 kg N ha^{-1}). The early spring/2nd harvest treatment applied 460 kg N ha^{-1} on the loamy sand soil and produced the highest yield observed on this site. Yield response to surface-banded and broadcast slurry was similar, except in one instance at Waseca where yield was reduced by 29% compared to broadcast-applied at the same time when 187 m^3 ha^{-1} slurry was surface-banded after the 2nd harvest. Maximum herbage DM yields were higher on clay loam soils (14.5 Mg ha^{-1}) than on the loamy sand soil (10.2 Mg ha^{-1}), and yields increased by less than 1 Mg ha^{-1} with N rates greater than 224 kg ha^{-1}, whereas yield did not increase above 224 kg N ha^{-1} on the loamy sand soil.

Herbage crude protein (CP) concentrations ranged from 78 to 270 g kg^{-1} and were increased by slurry and fertilizer applications. Topdressed slurry produced herbage CP concentrations higher than 155 g kg^{-1} only at Waseca and River Falls, primarily in the autumn harvest or with the highest manure rate. Highest CP concentration was achieved at high N fertilizer rates and generally was not equaled by slurry. Crude protein concentrations were greater than 155 g kg^{-1} with 224 kg N ha^{-1} in nearly all harvests.

Herbage N removal increased linearly with slurry additions at all locations (Fig. 1). Apparent manure N use efficiencies were 13% to 22% when slurry was

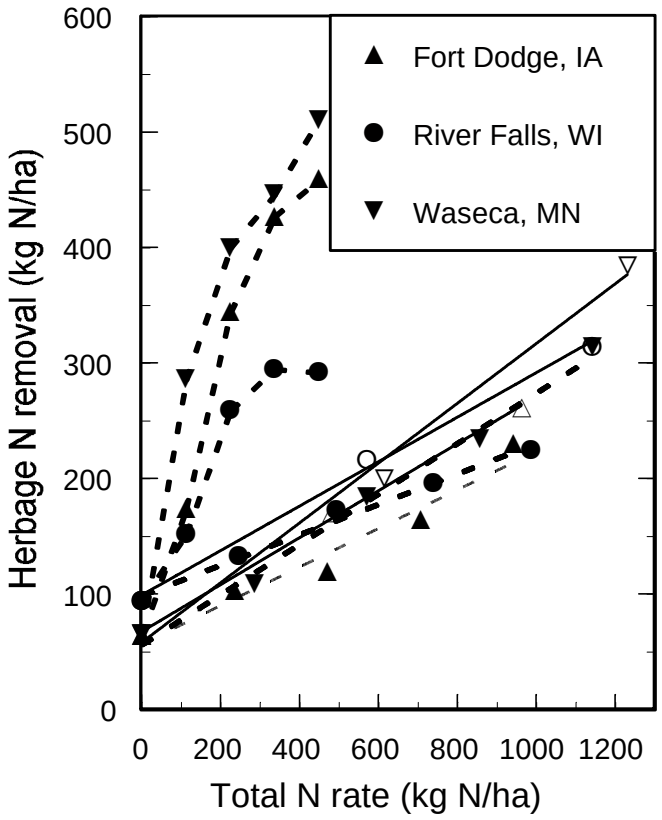

Figure 1. Herbage N removal by reed canarygrass with dairy manure slurry or fertilizer N at three sites in the Upper Midwest, USA. Slurry treatments were split-applied either in early spring and after the 2nd harvest (open symbols) or after 2nd and 3rd harvest (closed symbols); fertilizer N was applied up to 224 kg N ha^{-1} in early spring, with the remainder after the 1st harvest.

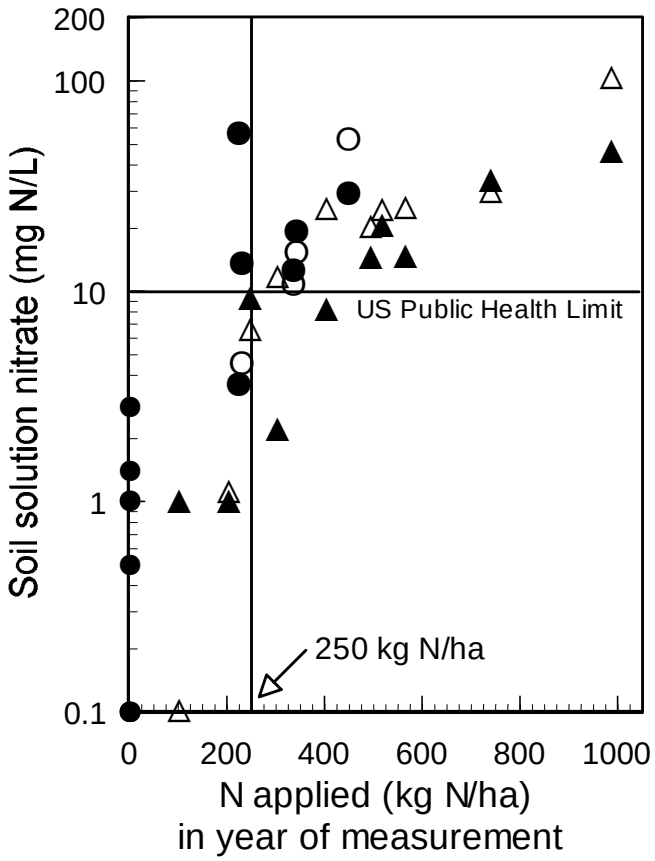

Figure 2. Dairy manure slurry (triangles) or fertilizer (circles) effect on soil solution nitrate concentration in ceramic cup samplers placed 1.5m deep in a sandy loam soil at River Falls, WI. Samples were collected in spring (open symbols) and autumn (closed symbols). Risk of ground water contamination increased at N rates exceeding 250 kg N ha^{-1}.

applied after the 2nd and 3rd harvests; efficiencies of 19% to 26% were achieved when slurry was applied in early spring and after the 2nd harvest. Herbage NO_3 concentrations generally were small (< 0.2 g NO_3-N kg^{-1} dry matter with slurry, < 2.8 g kg^{-1} with fertilizer), even with high rates of slurry addition. Surface banding after harvest did not improve N use efficiency compared to broadcast applications.

There was no evidence of excessive soil solution NO_3-N concentrations for any treatment on the clay loam soils. On the sandy loam, however, soil solution NO_3-N concentrations at 1.5 m in autumn and spring often exceeded the USA Public Health limit of 10 mg NO_3-N L^{-1} with application of more than 250 kg N ha^{-1} (Fig. 2), indicating a higher risk of significant NO_3-N leaching loss. Inorganic N did not accumulate in the soil profile at any site.

Conclusions

Low alkaloid reed canarygrass cultivars are a high yielding, palatable forage resource. Palaton reed canarygrass was responsive to application of high rates of dairy manure slurry during the growing season. Apparent N removal efficiency was good on all soils, but nitrate leaching losses may be significant when high rates of slurry (or fertilizer) are applied to sandy soils. We conclude that reed canarygrass can provide a window of opportunity for summer manure slurry applications, especially on fine-textured soils, without posing a hazard to ground water supplies.

Acknowledgment

This research was supported in part by grant no. 9301326 from the USDA Water Quality Special Grants Program.

Nitrate Losses in Pastures Grazed by Lactating Dairy Cows

M.P. Russelle, T. Dhiman, V.R. Kanneganti, L.D. Satter, D.G. Johnson, A.D. Garcia and W.S. Nord

Introduction

The practice of intensive rotational grazing of livestock is becoming popular in the USA because of lower costs, more attractive labor tasks, higher profitability, and apparent conformation to good ecological principles. Data from humid, marine climates suggest that significant ground water contamination by NO_3-N has occurred under intensive grazing.

Grazing animals harvest herbage from a broad area and return most of the ingested N to small, concentrated excreta patches; a single urination by a dairy cow applies the equivalent of 500-1000 kg N/ha on an area of 0.2 to 0.5 m^2. About 15 to 30% of dietary N typically is "excreted" in the form of milk. Milk production increases with proper feed supplementation, and N excretion in urine may decrease simultaneously. Our objective was to collect the first data on apparent N use efficiency and consequent NO_3-N leaching losses under this intensive rotational grazing in the Upper Midwest Region of the USA.

Materials and Methods

Permanent paddocks were fenced in established pastures on the US Dairy Forage Research Center Farm, Prairie du Sac, WI, and the West Central Experiment Station, Morris, MN. Pastures were comprised primarily of *Bromus inermis* Leyss and *Elytrigia repens* (L.) Nevski at the MN site, and *Poa pratensis* L., *E. repens*, *B. inermis*, and *Trifolium repens* L. (about 12% of pasture DM) at the WI site. Pasture received 65 kg fertilizer N/ha annually at WI and no fertilizer N at the MN site. In 2 years in WI and 1 year in MN, lactating dairy cows were grazed for 12 to 24 h in each paddock at intervals determined by forage regrowth.

Cows were divided into groups fed different levels of supplement. At the WI site, one-third of the cows consumed pasture (and minerals) only and other groups received supplement equal to about 0.33 or 0.67 of their daily DM intake. In 1994, this supplement averaged 5.6 and 10.5 kg/d, respectively, and contained about 155 g/kg crude protein (CP), and in 1995 it was a constant 6.0 and 11.6 kg/d containing about 195 g CP/kg. Before and after the grazing season, cows were fed diets with grain:forage ratios of 0:100, 25:75, and 50:50 for the 0, 0.33, and 0.67 supplement treatments, respectively, and daily milk production was measured for about 275 d. At the MN site, cows received either 4.5 or 9.0 kg supplement/d containing 128 g CP/kg in 1995 and daily milk production was measured for 67 d from May 19 through July 24. Milk CP concentrations were determined once per week at both sites.

The soil at the MN site was a Doland silt loam and at the WI site was a McHenry silt loam. Before grazing began, large diameter (30 cm) polyvinyl chloride drainage lysimeters were installed to depths averaging 70 cm (WI) or 115 cm (MN). Each grazed paddock contained 12 lysimeters, and adjacent mechanically harvested areas contained four lysimeters each for control and urine (3 L per lysimeter applied twice each grazing season) treatments. Leachate volume and concentrations of NH_4-N and NO_3-N were determined for each lysimeter. Soil cores were taken to 1.5 m in autumn to monitor soil NO_3-N concentrations.

Results and Discussion

Milk production increased linearly with replacement of pasture by supplemental feed at the WI site in both years, with total milk yield increasing from 17.3 kg/d on pasture alone to 26.3 kg/d with 0.67 supplement. Total milk yield was 22.0 kg/d at the MN site, with no effect of supplementation. Nitrogen excretion in milk increased with supplement addition in all three site years ($P < 0.1$). Supplement and pasture contained similar CP concentrations at the WI site. Pasture herbage samples have not been analyzed from the MN site, but we expect that the supplement contained less CP than grazed pasture. If cows substitute supplement

for pasture, then N excretion in dung and especially urine likely decreased with increased feed supplementation, implying that dietary N use efficiency increased with supplementation.

No significant leaching occurred during summer at either site. During the period from fall to spring, total water leaching losses at the WI site averaged 2.6 cm in 1994-5 and 1.4 cm in 1995-6, reflecting differences in over-winter precipitation. Essentially no drainage occurred at the MN site, due to the drier than normal spring in 1995 and the deeper volume in the lysimeters. In the absence of animals and except in years of very high rainfall, leaching losses in the Upper Midwest typically are small under perennial species grown on fine-textured soils (Randall et al., 1994).

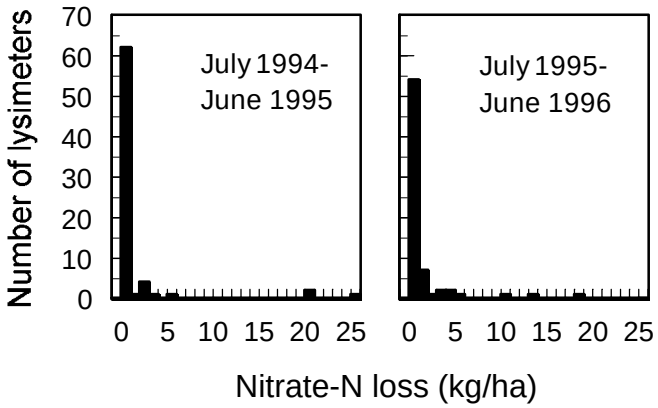

Figure 1. Frequency diagrams of NO_3-N losses in drainage lysimeters located in pastures under intensive rotational grazing management with lactating Holsteins at Prairie du Sac, WI. All three diet supplements are included, because there were no differences in NO_3-N loss among treatments.

There were no detectable differences in NO_3-N leaching losses among supplement treatments at the WI site, but losses were higher when urine was applied directly to mown grass. As expected, there was tremendous variability among lysimeters (Fig. 1). Total losses in grazed paddocks ranged up to 27 kg NO_3-N/ha from July 1994 to June 1995 and 18 kg NO_3-N/ha during the next 12 months, but 87 and 77% of these lysimeters lost less than 1 kg NO_3-N/ha over the two respective grazing seasons. Annual leaching losses directly under urine spots (applied twice during each grazing season) averaged 22 kg NO_3-N/ha.

Nitrate losses measured under pastures on these silt loam soils were insignificant over three site-years. High water and N use by the deeply rooted pasture species and low fertilizer N rates likely helped conserve N. Use of appropriate dietary supplementation with lactating dairy cows increased milk yield in 2 of 3 site years, but did not affect already low NO_3-N leaching losses in these fine-textured Upper Midwest soils. Questions remain about the potential impact of leaching losses on shallow or coarse-textured soils and in highly N-fertilized pastures, and in areas with adverse animal impact (e.g., near water tanks).

Acknowledgment

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Farm/Herd Report - Wisconsin

U.S. Dairy Forage Research Center - Annual Dairy Operations Report
January 1998

L.L. Strozinski - Herd Manager

Herd Statistics		Change from previous year
Herd Inventory		
Milking cows	280	-20
Dry cows 57	+7	
average cow age	45 months	0
percent first lactation	41%	0
percent second lactation	27%	-2
percent third lactation	17%	+2
percent greater than third	15%	0
Herd replacements	315	0
Total	**652**	-13
Rumen Fistulated Cows	22	
Herd Performance		
Cows calved	333	-35
Heifer calves born	160 live + 13 dead	(-4)
Bull calves born	181 live + 14 dead	(-4)
Heifer calves died < 1 year old	4 (2.5%)	
DHIA rolling herd average		
milk	22,468 lbs	+ 2131
protein	720 lbs.	+ 69
fat	876 lbs.	+ 129
Milk sold in 1997	6,937,771 lbs.	+ 638,850
Heifer calves sold	17	+ 8
Bull calves sold	180	+ 1
Cows sold	144	+ 13
cows culled for:		
reproduction problems	43	0
poor production	15	+ 1
poor udder	27	+ 7
poor feet and legs	11	+ 2
mastitis	31	+ 8
other	17	0
Cattle sales revenue	$72,555.43	+17,596.50
Herd Reproduction		
Average days open	125	+ 2
Average calving interval	12.99 months	-.03
Average services per conception	2.6	+ .5
Average age at first calving	24 months	0

The USDFRC herd size has decreased slightly in 1997. A review of the herd statistics shows a net reduction of 13 mature cows in the herd from one year ago. Culling pressure has increased slightly, especially for the reasons of poor udders and mastitis. In late 1996 and continuing through 1997 the herd experienced a considerable mastitis problem with average somatic cell counts of the herd peaking at 611,000 in August. Professional assistance to identify specific problem areas and recommended corrective action was obtained from the University of Wisconsin School of Veterinary Medicine. Corrective action, including culling of chronic problem cows, has resulted in a significant decline in the mastitis problem as evidenced by the present somatic cell count average of 218,000. Our herd goal is to maintain a somatic cell count of 200,000 or less. Despite the mastitis problem, our DHIA rolling herd average for milk production increased significantly in 1997 to 22,468 pounds. Our herd recently received special recognition from the Wisconsin DHIA for increasing the rolling herd average by 2355 pounds in one year. Current average production per cow per day is 70.5 pounds. The farm "mailbox" net price received per hundred weight of milk ranged from $11.736 to $14.800 with an average of $13.169 for 1997.

Cull cattle prices were somewhat stronger in 1997 but bull calf prices have remained extremely low and variable.

Research activities with the herd continued at a high level in 1997 with 355 milking animals involved in nine different trials during the year. There is an increase in numbers of cows on full and multiple lactation experiments. Very seldom does a mature cow freshen and not go onto an experiment. Most but not all first calf heifers are also used on experiments.

One full time position was added back to the dairy staff in 1997. This was a very welcome addition to ease the labor pressure and improve the overall attention to details which had slipped somewhat in the previous year. With unemployment levels in the area at record low levels it has become increasingly difficult to fill vacant positions at the farm through the cumbersome civil service system. Action has been taken to obtain approval to hire through a critical recruitment procedure which has the potential to streamline the process considerably.

This autumn the construction of the new 48 free stall facility was completed. On December 2, dry cows were moved into the new facility. This new facility will be used in the summer in connection with the grazing research and in the winter will be used by dry cows. A remodeling project to reposition and strengthen the free stall dividers in the existing facility is nearing completion. This project is being done by the field crew. It is a welcome improvement that should stand the test of time and improve the overall function of the stalls.

Two new cooperative projects were started in 1997. One is a project working with the University of Wisconsin School of Vet Medicine. The school uses our herd to teach reproductive physiology and palpation techniques. Supervised students have also taken over a portion of the routine herd health and vaccination program. This cooperative effort provides valuable hands on experience for students while easing the work load of USDFRC staff. This win-win program has worked extremely well and may be expanded in the future. A second new cooperative project just under way is with Trans-Ova Genetics in Iowa. Trans-Ova is producing transgenic calves and is in need of colostrum from a closed, vaccinated, leukosis free herd for the newborn calves. USDFRC has agreed to collect colostrum, bleed colostrum donors and maintain appropriate records for a compensatory fee.

The farm continues to be a popular place to visit and we continue to host the return visits of many national and international organization tours.

U.S. Dairy Forage Research Center - Annual Field Operations Report January 1998

R.P. Walgenbach

The 1997 growing season began with cold temperatures. This is the third year in a row that the growing season started with cooler than normal temperatures. The first crop of alfalfa grew at a very slow rate which adversely affected first crop yields. Very little winter injury occurred to alfalfa plants and all stands were good to excellent this past season. The temperatures for most of the growing season were below normal and clouds covered the sky during much of August. Accumulated growing degree days were well below normal and corn developed at a slow pace. Fortunately, frost held off and above normal temperatures in September and October allowed most corn in the area to reach or nearly reach the black layer stage of maturity. But most corn had moisture levels higher than typically seen at harvest. Rainfall in April and May were below normal. This allowed us to get an early start on planting. Rainfall recorded at the farm entrance rain gauge in inches was 1.4 in April, 1.26 in May, 3.33 in June, 7.74 in July, 3.63 in August, 1.27 in September, 1.62 in October and 0.6 in November. The precipitation in December was equivalent to .66 inches of rain. We planted 60 acres of barley, 204 acres of soybeans, 396 acres of corn, 78 acres of spring and 46 acres of summer seeded alfalfa and 14 acres of summer seeded red clover. The prior year seedings of alfalfa totaled 292 acres. Barley planting started on 4 April and was completed on 18 April. All barley was no-till planted at 110 pounds per acre into soybean stubble with a John Deere 750 no-till drill. We spread about 9,000 gallons per acre of liquid manure on all barley fields. We no-till seeded 19 acres of alfalfa at 10 lbs per acre on 20 April. This seeding followed corn harvested as silage and was spread with 15,000 gallons per acre of liquid manure. A second field of alfalfa (60 acres) was seeded following minimum tillage to prepare a seedbed needed for equipment travel. Both methods produced excellent stands of alfalfa. In mid August we no-tilled seeded alfalfa and red clover into harvested barley fields. We planted corn at about 33,500 seeds per acre for grain and at 40,000 seeds per acre for corn silage. About 15 acres of grass sod were planted to corn following conventional tillage, 40 acres of heavily manured corn residue were planted following one pass with a disk and 341 acres were planted with little or no-tillage. Some fields were rotary hoed and others were tilled with an aerway implement to improve surface drying of soils. We applied 10,000 to 18,000 gallons of liquid manure to most corn ground. All corn received 85 pounds per acre of 5-14-42 starter fertilizer and 160 pounds per acre of nitrogen (N) from a combination of soybean, alfalfa and manure credits and surface applied 28% liquid N. We no-till seeded soybeans in 7.5 in rows at about 225,000 seeds per acre from 6 to 16 May.

Early planting and cool growing temperatures produced barley yields that averaged 86 bushels per acre and ranged from 85.6 to 87.4 bushels per acre. We harvested barley from 22 to 29 July and stored part of it as high moisture grain and part was sold to the Arlington research farm. Soybeans produced an average yield of 56.7 bushels per acre with a range of 42.7 to 63.7 bushels per acre. The total harvest of soybeans was 11,571 bu. Soybean harvest occurred from 3 to 15 October. In spite of the cool weather, soybeans grew very well this season. Weed control was excellent in most fields. White mold was present in all soybean fields; however, it was less severe than in 1996. It was estimated that yields were reduced 3 to 7% as a result of white mold.

About 2,213 wet tons of corn silage were harvested from 92.3 acres between 25 September and 8 October. Yields ranged from 5.6 to 7.6 tons of dry matter (DM) per acre and averaged 7.2 tons of DM per acre. We harvested about 250 tons of wet corn silage with a chopper equipped with a kernel processor. We harvested approximately 162.2 acres of high moisture ground ear corn (HMGEC), 61.4 acres of HMG shelled (S) corn, and 80.4 of SC from 17 October to 19 November. The shelled corn (85% DM) equivalent yields averaged 170.4 bushels per

acre and ranged from 139.8 to 186.4 bushels per acre. The total amount of HMGEC harvested was 1,011 tons adjusted to 29% moisture content. The total amount of HMGSC harvested was 351 tons adjusted to 29% moisture content. First crop alfalfa yields ranged from 1.85 to 2.3 tons DM per acre. The second crop yields were estimated at 1.3 tons DM per acre and third crop yields were estimated at 1.2 tons DM per acre. Total seasonal yield on established fields was estimated at between 4.3 and 4.5 tons of DM per acre. In early July and mid August, we had very severe lightning that damaged our drive over scale, so accurate weights were not available on most of the second and some of the third crops of alfalfa. All alfalfa was harvested three times this past season. The first cut started on 6 June and the last cut was made on 27 August.

We have received final approval for the close out of the fuel oil contamination located behind the milk house. Because this area has an impermeable clay layer that prevents any downward movement and because of the relatively small amount of contamination, the site requires no additional remediation.

The August lightning that damaged our drive-over scale also did major damage to our electronic gate at the Badger Army Ammunition Plant. We are planning on replacing this system with an intercom gate-opening system. This system should be less susceptible to lightning. The Secretary of the Army is currently deciding the future of the BAAP. We have been told by Dave Fordham, Commander's Representative at BAAP that it is likely that the BAAP will be declared excess. If this occurs, the land and facilities of BAAP will be turned over to GSA for relocation of these lands and facilities for use by other agencies and the private sector. As a federal agency, USDA-ARS would have a priority for lands and facilities currently used by or needed by U.S. Dairy Forage Research Center programs. The current permit for use of BAAP land expires in February 1999. We are currently working to re-establish these permits.

As part of the new pasture cattle housing facility, a concrete manure collection pad with retaining bump walls was constructed. A new cooperative project was initiated this year with the U.S. Geologic Survey and the Wisconsin Department of Natural Resources. As part of this project, three mini watersheds were identified in our pasture. These watersheds were equipped with flumes, weirs and automated sampling devices to collect runoff from these pastures. Electric lines were installed underground to the three collection locations. As part of this project, a water line and cattle waterers also were installed. Part of this project will involve outwintering of cattle and their impact on the environment. This installation adds a unique dimension to the facilities and research capabilities of the USDFRC.

The vacant Ag Project Supervisor position has been evaluated this past season and will be re-filled as soon as possible.

I want to again express my appreciation to all employees at the research farm for their efforts and accomplishments this past year. They have much to be proud of.

Effect of Supplementing Low Levels of Monensin to Lactating Dairy Cows Fed Alfalfa Silage

G.A. Broderick

Introduction

When alfalfa is ensiled, typically 50-60% of the CP is converted to NPN; most of this NPN is in the form of free AA and small peptides. Microbial deamination of free AA and peptides usually exceeds incorporation into microbial protein in the rumen and most of the amino N in silage is converted to NH_3. Ammonia overflow from the rumen contributes to the excessive loss of urinary N by dairy cows fed alfalfa silage. Yang and Russell (1993) found that feeding Monensin reduced in vitro and in vivo ruminal NH_3 formation from protein hydrolysates by suppressing certain bacteria with high deamination activity. Thus, feeding Monensin may improve N efficiency by increasing gut absorption of amino acids (AA). Phipps et al. (1995) reported that feeding Monensin at 150 and 300 mg/d, but not 450 mg/d, improved milk yield in cows averaging 26.7 kg milk/d. The objective of this trial was to determine if supplementing Monensin to dairy cows fed all their forage as alfalfa silage would improve the yield of milk and milk components, improve N efficiency and reduce the need to supplement proteins resistant to ruminal degradation.

Materials and Methods

Second-cutting alfalfa was field wilted to 40% DM and ensiled in two bunker silos. In trial 1, two sets of 24 multiparous Holstein cows were blocked by DIM and parity each into six groups and randomly assigned to one of four diets. The first set of 24 was fed the diets during part 1 of the trial (9/14/95 to 12/7/95) and the second during part 2 (from 11/2/95 to 1/25/96). The four diets were: 1) Control; 2) Control plus fish meal; 3) Monensin; and 4) Monensin plus fish meal (Table 1). Cows were fed the same diet for all 12-wk of the trial. Rumensin pre-mix was blended with ground shelled corn and included at 1% of the diet; ground shelled corn was used in the Control. The 20 weekly samples of ground corn (Control) and ground corn plus Rumensin (Monensin) were analyzed for Monensin. Monensin averaged 1062 mg/kg DM in the supplement and 10.2 mg/kg DM in the two Monensin diets (Table 1). Control diets were intended to have no Monensin; however, the ground shelled corn fed in part 2 of the trial was contaminated, containing 238 mg Monensin/kg DM. Thus, Monensin content of the Control diets averaged not 0, but 1.1 mg/kg DM during the trial (Table 1). In trial 2, eight ruminally cannulated lactating cows were blocked into two groups by DIM, randomly assigned to one of two balanced 4 X 4 Latin squares with 4-wk periods (9/28/95 to 1/18/96) and fed the same diets as trial 1 (Table 1). On d-27 of each period, strained ruminal fluid was taken from each cow at 0, 1, 2, 3, 4, and 6 h after feeding and analyzed for NH_3, total AA and VFA. On d-28 of each period, strained ruminal fluid was taken only at 4 h after feeding, mixed with buffer, then dispensed to tubes containing buffer only (blanks), or buffer plus casein, acid-hydrolyzed casein (CHA), or enzymatically-hydrolyzed casein (CHE). Tubes were incubated at 39°C for 0 to 3 h. Rates of release of NH_3 and total AA from casein, and NH_3 from CHA and CHE, were determined using linear regression.

Results and Discussion

Alfalfa silage fed in these trials contained 21.0% CP (DM basis); NH_3, free AA plus peptides, and NPN accounted for 5, 49, and 54% of total N. Free AA plus peptide N from alfalfa silage NPN comprised 33 and 36% of total N intake in diets with and without fish meal. Thus, these diets were appropriate for testing whether Monensin reduced ruminal AA and peptide catabolism. Dry matter intake and BW gain

were increased by fish meal, but there was a trend (P = 0.11) for Monensin to reduce DMI (Table 2) and a significant Monensin by fish meal interaction due to the low BW gain on the Monensin only diet (with lowest DMI). Milk yield was not influenced by diet but there was a significant (P = 0.07) reduction in fat yield with Monensin. Fish meal increased protein yield 90 g/d on Control but only 30 g/d on Monensin (Table 2). The smaller milk protein response to fish meal feeding may have been due to improved protein status of the cows fed Monensin; however, mean protein yield was 1.12 on Control and 1.10 kg/d on Monensin. During part 2 of the trial, ground shelled corn fed in the Control diets was contaminated by Monensin. Thus, the net difference in Monensin intake between cows fed Monensin and Control diets during part 1 (247 mg/d) was greater than during part 2 (193 mg/d). There was no difference (P = 0.37) in milk yield between Control and Monensin diets without fish meal during part 1; however, there was a trend (P = 0.15) for milk yield on Control without fish meal to be lower than on Monensin without fish meal during part 2. This suggested that contamination of Control diets during part 2 did not mute detection of a Monensin response. BUN was increased by fish meal but unaffected by Monensin; there was a large increase in MUN with fish meal (from 11.6 to 15.0 mg N/dL; $P < 0.01$) and a small increase in MUN with Monensin (from 12.9 to 13.7 mg N/dL; P = 0.08). Blood glucose was increased 2.6 mg/dL by Monensin and reduced 2.8 mg/dL by fish meal.

Although there were no effects of diet on ruminal NH_3, pH and total VFA, feeding fish meal reduced total AA (Table 3). Monensin feeding decreased acetate, butyrate and acetate: propionate ratio, and increased propionate, all commonly observed with Monensin feeding. However, these changes were small in magnitude. Fish meal slightly reduced butyrate and appeared to reduce propionate on the Control diet and to increase propionate on the Monensin diet. A Monensin by fish meal interaction also was detected for acetate: propionate ratio. In vitro degradation of casein to NH_3 and total AA, and NH_3 release from CHE (a mixture of free AA and small peptides) were not influenced by feeding either Monensin or fish meal (Table 3). However, in vitro NH_3 release from CHA (a mixture of free AA) was increased 6% by Monensin and 20% by fish meal. Increased ruminal deamination of free AA with fish meal was surprising and may account for the depressed total AA observed in vivo (Table 3). Overall, there was no evidence that Monensin fed to dairy cows at 250 mg/d depressed ruminal AA and peptide catabolism. Although significant, the small alterations in ruminal acetate, propionate and butyrate suggested that the amount of Monensin fed was insufficient to alter ruminal catabolism of free AA and peptides. Yang and Russell (1993) fed 52 mg Monensin/kg DM when they observed a 30% reduction in ruminal NH_3 in vivo.

Summary and Conclusion

Except for a reduced fat yield, supplementing an alfalfa silage based diet with 250 mg Monensin/d did not alter yield of milk and milk components. Fish meal feeding increased protein yield on both Control and Monensin diets, indicating that the basal ration was limiting in absorbable protein. There was a nonsignificant trend for lower protein response to fish meal on Monensin. Monensin increased blood glucose and ruminal propionate, and decreased ruminal acetate, butyrate and acetate: propionate ratio. That these changes were smaller than is usually observed suggested that Monensin was fed at too low a level. There was no evidence that feeding Monensin at 250 mg/d (10 mg/kg DM) reduced ruminal AA and peptide catabolism. Additional trials with alfalfa silage based rations are needed to confirm that feeding Monensin improves ruminal N metabolism and milk yield in lactating dairy cows and to identify a possible optimal level of dietary Monensin.

References

Phipps, R. H., B. A. Jones, J.I.D. Wilkinson, and M. D. Tarrant (1995). Effect of monensin on milk production of Friesian dairy cows in the United Kingdom. J. Dairy Sci. 78 (Suppl. 1): 268 (Abstr.).

Yang, C.M.J., and J. B. Russell (1993). Effect of monensin on the specific activity of ammonia production by ruminal bacteria and disappearance of amino nitrogen from the rumen. Appl. Environ. Microbiol. 59: 3250-3254.

Table 1. Composition of diets (Trials 1 and 2).

Item	Control	Control + Fish Meal	Monensin	Monensin + Fish Meal
		(% of DM)		
Alfalfa silage	56.4	56.4	56.4	56.4
High moisture ear corn[1]	38.8	35.8	38.8	35.8
Solvent soybean meal	2.8	2.8	2.8	2.8
Fish meal[2]	- - -	2.9	- - -	2.9
Dicalcium phosphate	0.6	0.6	0.6	0.6
Trace mineral salt (+ Se)	0.3	0.3	0.3	0.3
Dynamate	0.06	0.06	0.06	0.06
Vitamins A, D, E Premix	0.1	0.1	0.1	0.1
Ground shelled corn[3]	0.96	0.96	- - -	- - -
Monensin supplement[4]	- - -	- - -	0.96	0.96
Mean composition (DM basis)				
CP (%)	16.8	18.6	16.8	18.6
NE (Mcal/kg)	1.67	1.66	1.67	1.66
NDF (%)	29	29	29	29
Monensin (mg/kg)[3]	1.1	1.1	10.2	10.2

[1]High moisture ear corn was ground through a 3/8" screen.

[2]Low solubles fish meal ("Sea-Lac," Zapata Proteins, Hammond, LA).

[3]Control, ground shelled corn fed during the second half of the trial (weeks 11 to 20) was contaminated with Monensin at the UW Feed Mill at Arlington. Control diets contained 0 Monensin during weeks 1 to 10 and 2.28 mg Monensin/kg DM during weeks 11 to 20 (mean 1.14 mg/kg DM).

[4]Monensin supplement was mixed with ground shelled corn and commercial Rumensin pre-mix, blended at the UW Feed Mill at Arlington to contain 1,200 mg Monensin/kg DM and found to contain a mean of 1062 mg Monensin/kg DM.

Table 2. Effect of Monensin and fish meal supplementation on DMI, BW gain, milk composition and yield, milk urea and blood urea and glucose (Trial 1)[1].

						$P > F$[2]		
							Contrasts	
Item	Control	Control + 3% FM	Monensin	Monensin + 3% FM	SE	Diet	Mon	FM	Mon x FM
Mon intake, mg/d	28	29	243	257	. . .	. . .	. . .	. . .	. . .
DMI, kg/d	24.7	25.4	23.8	25.2	0.6	0.100	0.109	0.068	0.532
BW gain, kg/d	0.57	0.52	0.33	0.68	0.07	0.010	0.569	0.038	0.007
Milk, kg/d	35.4	38.2	36.0	37.3	1.1	0.287	0.308	0.118	0.612
3.5% FCM, kg/d	33.2	34.4	32.2	33.0	1.1	0.341	0.102	0.428	0.932
Milk composition, %									
Fat	3.26	2.92	2.95	2.88	0.12	0.089	0.110	0.133	0.174
Protein	3.07	3.05	3.07	3.03	0.05	0.959	0.624	0.862	0.875
Lactose	4.74	4.70	4.83	4.78	0.05	0.699	0.309	0.948	0.558
SNF	8.53	8.44	8.60	8.52	0.08	0.957	0.858	0.955	0.602
Yield, kg/d									
Fat	1.12	1.10	1.04	1.05	0.04	0.306	0.071	0.918	0.573
Protein	1.07	1.16	1.08	1.11	0.03	0.113	0.163	0.060	0.447
Lactose	1.65	1.79	1.72	1.77	0.06	0.362	0.533	0.134	0.459
SNF	2.97	3.22	3.05	3.14	0.10	0.260	0.343	0.107	0.473
Efficiency,									
Milk/DMI	1.45	1.51	1.53	1.49	0.02	0.692	0.583	0.808	0.300
MUN, mg N/dL	11.35	14.41	11.90	15.51	1.85	< 0.001	0.079	< 0.001	0.530
BUN, mg N/dL	13.06	16.66	12.99	16.85	0.81	< 0.001	0.918	< 0.001	0.804
Blood Glc, mg/dL	52.8	49.9	55.3	52.6	5.7	0.079	0.077	0.053	0.947

[1]BUN = Blood urea N, FM = fish meal, Glc = glucose, Mon = Monensin, MUN = milk urea N.

[2]Probabilities of significance for diet and orthogonal contrasts.

Table 3. Effect of Monensin and fish meal supplementation on DMI, ruminal ammonia, total AA, pH, VFA and rates of in vitro N catabolism (Trial 2)[1].

						$P > F$ [2]			
							Contrasts		
Item	Control	Control + 3% FM	Monensin	Monensin + 3% FM	SE	Diet	Mon	FM	Mon x FM
Monensin intake, mg/d	27	26	227	227	. . .	. . .	. . .	. . .	. . .
DMI, kg/d	22.3	21.4	22.5	22.5	1.0	0.820	0.381	0.813	0.784
Ruminal concentrations									
NH_3, mM	15.03	15.02	15.14	14.56	0.63	0.914	0.782	0.644	0.658
Total AA, mM	3.59	2.36	3.41	2.48	0.39	0.096	0.951	0.016	0.699
pH	6.15	6.20	6.24	6.20	0.04	0.593	0.322	0.911	0.362
Total VFA, mM	135.0	131.4	130.7	135.4	2.6	0.470	0.952	0.831	0.130
Individual VFA, mol/100 mol									
Acetate	63.2	64.4	63.5	62.6	0.3	0.008	0.023	0.646	0.004
Propionate	20.4	18.1	20.1	20.4	0.4	0.003	0.024	0.027	0.006
Acetate: propionate ratio	3.16	3.65	3.21	3.12	0.09	0.006	0.026	0.051	0.010
Butyrate	11.2	12.0	10.9	11.6	0.1	< 0.001	0.012	< 0.001	0.842
Isobutyrate	1.25	1.33	1.35	1.30	0.02	0.019	0.098	0.421	0.007
Isovalerate + 2-Methyl butyrate	1.98	2.21	2.16	2.10	0.07	0.187	0.607	0.261	0.070
Valerate	1.95	2.01	2.01	2.05	0.02	0.098	0.061	0.088	0.687
In vitro N catabolism rates, nmol/(hr*mg protein)									
NH_3 release from casein	86.0	85.1	80.0	79.9	7.1	0.917	0.606	0.917	0.627
TAA release from casein	17.8	18.1	8.2	11.0	3.7	0.330	0.137	0.641	0.401
NH_3 release from CHA	299.1	356.9	312.9	380.3	15.7	0.053	0.059	0.039	0.689
NH_3 release from CHE	324.5	397.2	350.7	388.7	20.1	0.315	0.211	0.194	0.717

[1]CHA = Acid-hydrolyzed casein, CHE = enzymatically-hydrolyzed casein, FM = fish meal, Mon = Monensin, TAA = total AA.

[2]Probabilities of significance for diet and orthogonal contrasts.

Value of Treating Alfalfa Silage With Fibrolytic Enzymes Prior to Feeding the Silage to Lactating Dairy Cows

G.A. Broderick, R. Derosa and S. Reynal

Introduction

Increasing energy intake in dairy cows enhances utilization of NPN in alfalfa silage by stimulating ruminal protein synthesis. Increased fiber digestion in the rumen also would be beneficial by increasing the supply of both energy and protein. Beauchemin et al. (1995) reported improved fiber digestibility and growth rate when steers were fed alfalfa hay or timothy hay, but not barley silage, that had been treated with a mixture of fibrolytic enzymes. Higginbotham et al. (1995) observed little effect of treating a TMR containing alfalfa hay and corn silage with another source of fibrolytic enzymes prior to feeding to lactating dairy cows. The objective of our research was to determine if treating alfalfa silage with a mixture of xylanases and cellulases would enhance fiber and DM digestibility, and milk yield, in lactating dairy cows when the enzymes were applied to the silage just prior to feeding.

Materials and Methods

Second-cutting alfalfa was field wilted to 40% DM, chopped to a theoretical length of 1.0 cm and ensiled in bunker and upright tower silos. Forty multiparous (eight with ruminal cannulae) and eight primiparous Holstein cows were blocked by parity and DIM into 12 groups of four and randomly assigned to one of the four enzyme levels used to treat alfalfa silage. Means for all 48 cows at the start of the trial were 573 kg BW, parity 2.7, 89 DIM, and 37 kg/d of milk. All cows were injected every 14 d with rBST (Posilac). The basal diet contained (DM matter basis): 50% alfalfa silage, 43% rolled high moisture ear corn, 6% solvent soybean meal, 0.5% sodium bicarbonate, supplemental minerals and vitamins, 16.9% CP, and 28% NDF. The four diets differed only in level of enzyme applied to alfalfa silage; cows were fed the same diets for all 14 weeks of the trial. Enzyme stock solutions of xylanase and cellulase, provided by FinnFeeds, were stored at 5°C. Enzymes were diluted daily with water and sprayed on at 1.0% of the as-fed silage as it mixed in a Rissler mixer. Alfalfa silage and enzymes were mixed for 10 min before adding the other TMR ingredients. The four treatments were: Control (water only); 0.7x (234 ml xylanase/ton plus 468 ml cellulase/ton); 1.2x (400 ml xylanase/ton plus 800 ml cellulase/ton); and 1.7x (567 ml xylanase/ton plus 1134 ml cellulase/ton). Body condition score (BCS) as well as milk yield and concentrations of rumen and blood metabolites were determined.

Results and Discussion

Composition of alfalfa silage was typical of good quality alfalfa, containing (DM basis): 19.8% CP, 45.9% NDF, 36.5% ADF, pH 4.78 and 57% NPN (percent of total N). Because it was higher than average in fiber, this silage should have been more amenable to improvement in nutrient value with treatment with fibrolytic enzymes. The silage was treated at four enzyme levels: 0x (Control), 0.7x, 1.2x, and 1.7x; cellulase was applied at two times the volume of xylanase. Level 1.2x was that recommended by FinnFeeds. Feed DMI, BW change and BCS, apparent digestibility, and milk yield data are in Table 1. There was no effect of enzyme treatment on apparent digestibility of DM, NDF or ADF. Although there was no effect at the lowest enzyme level, feeding diets containing alfalfa silage treated at 1.2x and 1.7x increased DMI by 1.0 and 2.4 kg/day ($P < 0.001$) versus Control. Despite increased DMI, there were no differences in BW gain, BCS or milk yield. Similar milk yields at greater DMI resulted in reduced efficiency (milk:DMI) for diets containing enzyme treated silage ($P < 0.001$). There were no differences in yield of fat, protein, lactose or SNF, but milk content of fat, protein and SNF were increased in cows fed the enzyme treated silages (P^2 0.04). Milk protein content, particularly on the 1.7x level of enzyme treatment, was unusually high. Average milk protein concentration found in 20 trials at the Dairy Forage Center in cows fed 60 different diets

Table 1. Effect of treating alfalfa silage with four levels of a mixture of cellulase and xylanase enzymes on DMI, BW gain, change in body condition score (BCS), apparent digestibility, production of milk and milk components, and somatic cell count (SCC).

	Enzyme level					
Item	0x (Control)	0.7x	1.2x	1.7x	SEM[1]	$P > F^2$
DMI, kg/d	23.1[c]	23.1[c]	24.1[b]	25.5[a]	0.3	< 0.001
BW change, kg/d	0.68	0.54	0.60	0.52	0.10	0.637
Change in BCS	0.32	0.25	0.38	0.20	0.09	0.595
DM digestibility, %	65.5	65.3	65.2	65.1	0.4	0.980
NDF digestibility, %	37.4	38.2	37.5	37.5	0.6	0.898
ADF digestibility, %	40.2	41.2	40.8	40.1	0.5	0.777
Milk yield, kg/d	35.1	34.2	33.6	35.2	0.6	0.762
3.5% FCM, kg/d	33.9	33.4	32.7	34.4	0.6	0.534
Fat, %	3.24[b]	3.36[ab]	3.28[ab]	3.46[a]	0.07	0.040
Fat, kg/d	1.14	1.15	1.11	1.19	0.03	0.237
Protein, %	3.28[b]	3.24[b]	3.30[ab]	3.41[a]	0.04	0.004
Protein, kg/d	1.16	1.10	1.12	1.17	0.02	0.062
Lactose, %	4.86	4.79	4.90	4.83	0.03	0.147
Lactose, kg/d	1.74	1.65	1.67	1.69	0.04	0.370
SNF, %	8.87[ab]	8.77[b]	8.93[a]	8.96[a]	0.05	0.011
SNF, kg/d	3.16	3.00	3.03	3.11	0.06	0.318
Efficiency[3]	1.53[a]	1.47[b]	1.40[c]	1.39[c]	0.03	< 0.001
SCC (x 10^3)	302[b]	430[ab]	708[a]	735[a]	128	0.032
Log10 SCC	2.01	2.11	2.24	2.25	0.08	0.101

[a,b,c]Means within the same row without a common superscript differ ($P < 0.05$).
[1]SEM = Standard error of the mean.
[2]Probability of a significant effect of enzyme treatment.
[3]Milk yield: DMI.

with all of the forage from alfalfa silage was 2.99% (Broderick, unpublished). Increased protein and SNF content of milk is now more valuable in the U.S. with the advent of component pricing. Mean SCC was higher ($P < 0.03$) and there was a trend ($P = 0.10$) for log of SCC to be elevated in cows fed the 1.2x and 1.7x diets (Table 1). This suggested that, by chance, cows assigned to the enzyme treatments had increased incidence of subclinical and clinical mastitis, obscuring possible positive effects of the enzymes. Cows averaged 89 DIM and 37 kg/d at the start of the trial; mean milk yield was 34.5 kg/d during the trial, despite bi-weekly injections of rBST. That increased DMI did not alter yield of milk or milk components of cows on treatments 1.2x and 1.7x indicated that production in this trial was too low for the cows to respond to improved nutrient supply. No significant effects due to enzyme treatment were detected in concentrations of blood glucose and blood and milk urea, in ruminal ammonia and total amino acids, or in ruminal pH (Table 2). There was a highly significant ($P < 0.005$) effect of time after feeding on ruminal pH, but no differences due to enzyme treatment. Slight elevations of ruminal ammonia at 15 h (1.7x) and at 18 h (0.7x and 1.2x) after feeding (data not shown) suggested that those cows had substantial feed consumption at these times.

Beauchemin et al. (1995), using a similar preparation of FinnFeed fibrolytic enzymes, observed a positive response with enzyme treatment of alfalfa and timothy hays, but not barley silage. If the mixtures of xylanase and cellulase enzymes predigested some of the fiber, then NDF content of the alfalfa silage should have declined with time. Two 24-h time studies were conducted in which samples of the alfalfa silage were collected at the research farm immediately after enzyme treatment; these samples were held at room temperature (24°C) for 24 h. No pattern of decline in NDF content was observed over that time. Mean NDF contents of alfalfa silage samples were (DM basis): 42.5 (Control), 42.3 (0.7x), 42.3 (1.2x), and

42.0% (1.7x); these were not different ($P > 0.53$). In a laboratory study conducted later, two samples of alfalfa hay and two of alfalfa silage (including that fed in the lactation trial) were treated at the 1.2x enzyme level. After 16 h at 24°C, mean NDF content of the alfalfa hays declined (DM basis) from 48.7 to 43.8% with enzyme treatment; mean NDF contents of the two alfalfa silages were 43.2 and 42.4% without and with enzyme treatment. Alfalfa silage may have been unresponsive to the fibrolytic enzymes under the conditions of our experiment. Of course, effects of the enzyme treatment on intra-ruminal fiber digestion would not have been detected in these time studies.

Summary and Conclusion

Feeding lactating dairy cows diets containing alfalfa silage treated with a mixture of xylanases and cellulases elevated DMI. However, this increased feed intake was not accompanied by increased BW gain, BCS, or milk yield. Treating the alfalfa silage with the fibrolytic enzymes elevated milk content of fat, protein and SNF, but yields of these milk components were not increased. Blood glucose and urea, milk urea, and ruminal pH, ammonia and free amino acids were not influenced by the enzymes. Neither apparent digestibility of DM, NDF or ADF, nor content of NDF in alfalfa silage over time, was influenced by enzyme treatments. However, enzyme treatment decreased NDF content of alfalfa hay. Lack of response to fibrolytic enzymes in this trial may mean that alfalfa silage is not amenable to enhanced fiber digestion.

References

Beauchemin, K. A., L.M. Rode, and V.J.H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641-644.

Higginbotham, G.E., E.J. DePeters, S.L. Berry, and A. Ahmadi. 1995. Effect of adding a cell wall degrading enzyme to total mixed ration for lactating cows. The Professional Anim. Sci. 12:81-85.

Table 2. Effect of treating alfalfa silage with four levels of a mixture of cellulase and xylanase enzymes on concentrations of blood glucose, blood and milk urea, ruminal ammonia and total amino acids, and ruminal pH.

	Enzyme level					
Item	0 (Control)	0.7	1.2	1.7	SEM[1]	$P > F$[2]
Blood glucose, mg/dL	68.2	67.3	66.1	64.4	0.8	0.647
Blood urea, mg N/dL	16.9	15.9	18.0	16.3	0.3	0.155
Milk urea, mg N/dL	14.3	13.8	14.0	14.2	0.4	0.855
Ruminal ammonia, mM	8.77	9.79	9.38	9.80	0.39	0.760
Ruminal total amino acids, mM	2.03	1.48	1.83	1.56	0.19	0.320
Ruminal pH	6.14	6.09	6.32	6.31	0.03	0.570

[1]SEM = Standard error of the mean.
[2]Probability of a significant effect of enzyme treatment.

Effect of Feeding Corn Hybrids Selected for Leafiness or Grain to Lactating Dairy Cattle

C.S. Kuehn, J.G. Linn, D.G. Johnson and H.G. Jung

Introduction

Corn silage is a valuable source of energy and fiber for lactating dairy cattle. Corn silage in dairy cattle diets has received more interest lately because of the shortage and high price of good quality alfalfa. There are many corn silage hybrids on the market that producers must choose from. However, little data exist which compare hybrids in side-by-side feeding and production trials. The general recommendation to producers has been to plant the highest grain yielding hybrid. Recently corn hybrids that possess traits specifically targeted for silage have entered the marketplace. Therefore, the objective of this study was to compare three divergent sources of corn hybrids used as corn silage in the diets of early lactation dairy cows and measure the production response of these animals.

Material and Methods

The three corn hybrids used in this study were a hybrid that has been selected for silage yield and leafiness (L), a high-grain yielding hybrid in the west central region of Minnesota (G), and a blended combination of various hybrids (B) which was selected because it was the lowest priced seed corn that could be purchased in this area in 1996. Corn was planted in May of 1996 according to specifications for each variety. Corn was chopped for ensiling between September 12 and 14, 1996. The theoretical length of chop for the corn at harvest was .635 cm. Varieties were ensiled in silage bags and allowed to ferment for 14 d prior to opening. Prior to harvest, four representative corn plants were collected from each hybrid for dissection and evaluation of specific plant parts. The three silage hybrids were fed to a group of early lactation dairy cows. Sixty-two Holstein cows (39 primiparous and 23 multiparous) were used in this experiment. Cattle were housed in a tie-stall barn at the West Central Experiment Station in Morris, MN. Cows were placed on their dietary treatment 3 d after calving and remained on the diet for 154 days. Diets were formulated to contain 40.6 % corn silage, 10.2 % alfalfa haylage, 23.5 % corn grain, 7.4 % whole fuzzy cottonseed, 7.2 % soybean meal (48% protein), 5.3 % dried distiller's grains, 1.3 % blood meal, and 4.5 % vitamin and mineral supplement on a dry matter (DM) basis.

Results and Discussion

Silage yields were 13.79, 14.93, and 14.48 metric tons of DM/ha for the G, L, and B hybrids, respectively. Dry matter, nutrient content, in vitro digestibility, and proportion of each of the dissected plant parts for the three corn hybrids are shown in Table 1. Nutrient content of dissected plant parts exhibited similar trends for all three hybrids. The most pronounced nutrient differences were in the stalk, sheath, and tassel. In this fraction, the B hybrid was highest in NDF and ADF content, and lowest in IVDMD and IVNDF value. The IVDMD content differed by over 14.3 percentage units between the B and G hybrid and the IVNDF content differed by over 14.7 percentage units between the B and L hybrid. Leaf IVNDF was highest in the L hybrid and was over 8.0 percentage units higher than the G hybrid. Average DM and nutrient content, and *in vitro* digestibility of the three silage hybrids during the trial are shown in Table 2. Daily DM and nutrient intakes did not differ among diets (Table 2). Average daily milk, 3.5 % fat corrected milk, fat, and protein production did not differ across dietary treatments.

Conclusion

The three corn hybrids evaluated for silage were different in their chemical composition and in vitro digestibility. However, these differences did not affect feed intake, milk production, or milk composition of lactating dairy cows. It appears that in properly formulated dairy diets, differences in corn silage composition must be greater than those observed in this trial in order to affect cow performance.

Table 1. Average dry matter (DM) content, nutrient composition[1], digestibility, and percent of various plant parts for three corn hybrids.

Plant Part		DM	CP	NDF	ADF	IVDMD	IVNDF	% of Plant
	%	- - - - - - - - - - % DM - - - - - - - -						
Husk, Silk and Shank								
	L	30.0	3.1	73.5	33.9	57.9	45.0	6.8
	G	31.5	3.8	76.7	38.0	50.8	51.2	7.0
	B	33.9	4.1	76.1	37.9	52.0	50.6	8.1
Stalk, Sheath and Tassel								
	L	24.7	2.5	69.1	40.8	47.8	42.3	30.4
	G	22.9	1.9	69.1	40.8	52.3	32.7	29.5
	B	24.4	2.3	75.2	45.6	38.0	27.5	24.7
Cob								
	L	47.2[2]	1.8	80.5	38.7	47.5	30.0	8.9
	G	52.1[2]	1.7	85.7	43.8	44.5	35.5	7.9
	B	54.9[2]	1.5	85.6	44.7	44.6	33.6	11.0
Grain								
	L	—	9.1	12.8	2.4	88.7	44.9	41.0
	G	—	9.2	11.7	2.1	93.5	48.5	43.7
	B	—	8.4	12.8	3.1	86.6	43.1	46.2
Leaves								
	L	28.9	10.0	57.1	30.9	63.1	53.7	13.0
	G	25.4	10.2	56.4	30.2	58.2	45.7	11.9
	B	24.9	10.6	57.8	30.8	60.1	48.5	10.1

[1]CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), IVDMD (in vitro dry matter disappearance), and IVNDF (in vitro NDF digestibility).
[2]DM value includes cob and grain.

Table 2. Silage nutrient measures and cow performance.

Item	L	G	B
Silage			
DM, %	34.6	36.7	38.7
	- - - - - - - - % DM - - - - - - - -		
CP	6.9	7.0	7.3
NDF	45.6	43.7	45.1
ADF	24.3	23.6	24.4
IVDMD	69.2	66.8	66.7
IVNDF	38.0	34.6	34.4
Cow Performance	- - - - - - - - - - kg/d - - - - - - - - - -		
DM Intake	22.4	22.3	21.8
Milk	35.2	35.1	36.3

Alfalfa Leaf Meal: Evaluation as a Hay Replacement in Dairy Diets

J.-M. Akayezu, M.A. Jorgensen, J.G. Linn and H.G. Jung

Introduction

Alfalfa leaf meal (ALM) is a new feed ingredient that may become available to the dairy industry in the near future. A farmer cooperative has been organized in southwest Minnesota to produce alfalfa hay for use in a biomass electricity project. The concept is to produce electricity by gasification of the stems from alfalfa hay. The leaves will be separated from the hay prior to combustion to reduce nitrous oxide pollution and provide another source of revenue to the project. The ALM may be a valuable feedstuff for inclusion in dairy diets. Because the initial batches of ALM were relatively low in protein and high in fiber, a trial was conducted investigating partial replacement of forage in lactating cow diets with ALM. The advantage of replacing alfalfa hay with ALM might be the assurance of consistency in the quality of the product and ease of handling when incorporated in TMR diets. But because the particle size of ALM is inherently fine in comparison with long or chopped hay, it was not known what effect replacing hay with ALM would have on rumen function and, thus, on milk yield and composition. A study was conducted to evaluate the effect of partial substitution of good quality chopped alfalfa hay with ALM pellets of similar composition on milk yield, milk composition, and rumen function.

Materials and Methods

Eighteen multiparous cows were used in a replicated 3 x 3 Latin square design. Treatments consisted of diets in which ALM partially replaced chopped alfalfa hay. Alfalfa leaf meal used in this study was in the form of pellets and contained 23.6% crude protein (CP) and 44.5% neutral detergent fiber (NDF). The alfalfa hay contained 22.0% CP and 43.9% NDF. Treatment diets contained 0, 8 or 16% ALM pellets. Diets were fed twice a day, ad libitum. The study consisted of 3 experimental periods with 11 to 14 d for adaptation to dietary treatments and 5 to 7 d for data collection. Feed intake and digestibility, and milk yield and composition were measured. The impact of feeding ALM pellets on rumen function was assessed by monitoring eating and chewing activities of cows for 24 h at the end of each experimental period.

Results and Discussion

Chemical composition of the diets was similar across treatments (Table 1). Dry matter (DM) intake of cows fed a diet containing ALM pellets at 8% of the diet DM were similar to those of cows fed no ALM pellets (Table 2). However, when cows were fed ALM pellets at 16% of the DM, dry matter intake tended to decrease compared with cows fed no ALM pellets, and was significantly reduced compared with cows fed ALM pellets at 8% (Table 2). Digestibility of diet DM and fiber was not significantly affected by treatment (Table 2), suggesting that partial substitution of ALM pellets for hay did not affect rumen fermentation. Also, feeding ALM pellets in partial replacement of good quality hay had no effect on milk yield or milk composition (Table 3). Replacing alfalfa hay with ALM pellets did not affect the time cows spent eating (Table 4). However, the time cows spent ruminating decreased as the amount of ALM pellets in the diet increased. Conversely, the time spent resting (not eating or ruminating) increased as the proportion of ALM pellets in the diet increased. This observation could be interpreted to suggest that ALM pellets may not be equivalent to chopped alfalfa hay for stimulation of rumination. However, in this experiment, DM and NDF digestion were not affected by treatment (Table 2) and milk fat test was not different across treatments (Table 3). In addition, pH measurements indicated that regardless of the diet fed, rumen pH did not drop below 6 (data not shown) and stayed within the range of values where fiber digestion is not greatly compromised. Also, total volatile fatty acid production and the ratio of acetate plus butyrate to propionate did not decrease when two rumen cannulated cows were fed the diets containing ALM (data not shown).

Conclusion

The results of this study suggest ALM pellets can be included in the diets of dairy cows up to 16% of the

DM in replacement of an equivalent amount of high quality alfalfa hay without compromising production or rumen health. However, these results should not be interpreted to suggest ALM could totally replace all hay in dairy cow diets. It is not known if ALM in the form of meal rather than pellets could partially replace alfalfa hay and, because this was a short trial, the impact of extended ALM feeding in replacement of hay on rumen function, body condition, and milk production is still unknown. However, it does appear that ALM may have a role in dairy rations as a partial replacement for alfalfa hay.

Table 1. Composition of experimental diets.

	Alfalfa leaf meal, % of diet DM		
Item	0	8	16
Ingredient Composition:	- - - - % of diet DM - - - -		
Corn silage	25.8	26.0	26.0
Alfalfa hay, chopped	25.9	18.5	11.2
ALM pellets	—	7.9	15.8
Grain mix	48.3	47.6	47.0
Chemical composition[1]			
CP	18.8	18.7	18.6
NDF	31.2	31.4	31.7
ADF	16.8	17.1	17.4
EE	3.4	3.4	3.5
NFC	37.4	37.0	36.5

[1]CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), EE (ether extract), and NFC (non-fiber carbohydrates).

Table 2. Effects of feeding alfalfa leaf meal on intake, and dry matter and fiber digestibility.

	Alfalfa leaf meal, % of diet DM				
Item	0	8	16	SE	*P*
Dry matter intake, kg/d	27.9[ab]	29.3[a]	26.7[b]	0.60	0.04
DM digestibility, %	66.4	65.8	65.0	0.95	0.57
NDF digestibility, %	51.0	52.3	52.7	1.52	0.72

[a, b]Means in the same rows not sharing a superscript differ at $P \leq 0.05$.

Table 3. Yield and composition of milk from cows fed alfalfa leaf meal in partial replacement of alfalfa hay.

	Alfalfa leaf meal, % of diet DM				
Item	0	8	16	SE	*P*
Milk, kg/d	38.8	39.7	39.5	0.66	0.56
Fat, %	3.69	3.49	3.64	0.08	0.34
Protein, %	3.10	3.03	3.07	0.03	0.47
Lactose, %	4.77	4.72	4.75	0.06	0.78

Table 4. Eating and chewing activities of cows fed diets containing alfalfa leaf meal pellets partially replacing chopped alfalfa hay.

	Alfalfa leaf meal, % of diet DM				
Item	0	8	16	SE	*P*
Time spent eating, min.	208	213	203	5.5	0.49
Time spent chewing, min.	448[a]	425[a]	381[b]	9.0	< 0.01
Time spent resting, min.	646[b]	659[b]	719[a]	9.0	< 0.01

[a, b]Means in the same row not sharing a superscript differ at $P \leq 0.05$.

Alfalfa Leaf Meal: Use as a Source of Supplemental Protein

M.A. Jorgensen, J.-M. Akayezu, J.G. Linn and H.G. Jung

Introduction

As described in the previous report by Akayezu et al. (this volume), alfalfa leaf meal (ALM) is potentially a new feed ingredient for use by the dairy industry. Alfalfa leaves contain approximately 30% crude protein (CP) and 25% neutral detergent fiber (NDF). This composition suggests that ALM might be a useful supplemental protein feed for lactating dairy cows. Our initial research indicated that ALM can partially substitute for alfalfa hay in lactation diets. The objective of this study was to determine if ALM could replace soybean meal as a supplemental protein in lactating cows.

Materials and Methods

Twenty multiparous Holstein cows were used in a replicated 4 x 4 Latin square design. Cows were grouped based on days in milk, milk yield, and body weight. Within group, cows were randomly assigned to one of four treatments. Treatments consisted of a control diet (ALM0) in which soybean meal (SBM) was the main source of supplemental protein, and three diets containing ALM formulated to contain 11 (ALM11), 22 (ALM22), or 33 (ALM33) % of the total dietary CP from ALM, in replacement of SBM. In ALM0, SBM constituted about 24% of the total CP in the diet. For diets ALM11, ALM22, and ALM33 the SBM contribution decreased to 17, 9, and 0% of total dietary CP. The ALM used in this study was in meal form and contained 22.4% CP and 39.5% NDF. The composition of the experimental diets is given in Table 1. Diets were fed as total mixed rations. Cows were fed twice daily, ad libitum, and milked twice daily. Periods consisted of 14 d for adaptation and 7 d for sample collection. Measurements were taken for nutrient intake, and milk yield and composition.

Results and Discussion

When ALM supplied 0, 11, 22, or 33% of the total dietary CP, then diet DM contained 0, 7.5, 14.6, and 23.5% ALM, respectively. Dry matter intakes (DMI) of cows decreased linearly as ALM content increased in the diet (Table 2). Despite decreases in DMI, the yield of milk or fat-corrected milk from cows fed ALM were similar to those cows fed ALM0 (Table 3). Milk composition was not affected by treatment except milk protein percentage, which was slightly reduced when ALM supplied 22% of dietary CP, compared with the ALM0 diet. In this study, the lack of treatment effect on milk yield, along with decreased DMI, as ALM is increased in the diet suggests that cows fed diets containing ALM were more efficient in converting nutrients into milk and milk components. However, in this short-term study, it is impossible to detect if cows were, in fact, drawing on body reserves to maintain milk production while decreasing intake. A longer lactation trial would be required to determine if ALM fed cows were truly more efficient.

Conclusion

The results from this trial indicate that ALM can provide supplemental protein in lactating cow diets. Whether the improved efficiency of milk production compared to SBM-based diets for ALM fed cows can be sustained over a full lactation is unclear. It must be noted that the ALM available for this study was lower in quality (lower protein, higher fiber) than could be anticipated based on the quality of alfalfa leaves. As the separation technology for producing ALM matures it might be expected that the protein concentration of the ALM could be raised. Such a change should make ALM a more attractive supplemental protein source.

Table 1. Composition of experimental diets.

	Diet			
Item	ALM0	ALM11	ALM22	ALM33
Ingredient Composition	- - - - - - - - - - % of diet DM - - - - - - - - —			
Hay	17.3	17.5	17.4	14.5
Corn Silage	32.0	32.9	32.7	26.9
Grain Mix	50.7	49.6	49.9	58.6
Chemical Composition[1]				
DM	58.7	58.3	58.6	62.7
CP	16.3	15.9	16.0	15.9
NDF	31.8	35.4	37.2	34.3
ADF	15.2	17.1	19.4	18.0

[1]DM (dry matter), CP (crude protein), NDF (neutral detergent fiber), and ADF (acid detergent fiber).

Table 2. Effect of diet on nutrient intake.

	Diet					
Item	ALM0	ALM11	ALM22	ALM33	SE	P
	- - - - - - - - - - - - kg/d - - - - - - - - - - - -					
DM	27.6[a]	26.9[ab]	26.4[b]	25.8[b]	.44	.05
CP	4.5[a]	4.3[b]	4.3[b]	4.1[b]	.07	<.01
NDF	8.8[c]	9.3[b]	9.9[a]	9.0[bc]	.16	<.01

[abcd] Means in the same row with no common superscripts differ (P < 0.05)

Table 3. Effect of diet on milk yield and composition.

	Diet					
Item	ALM0	ALM11	ALM22	ALM33	SE	P
Milk, kg/d	34.5	33.9	34.1	34.1	.64	.91
FCM[1], kg/d	36.5	36.4	36.6	35.5	.66	.45
Fat, kg/d	1.3	1.3	1.3	1.3	.03	.27
Protein, kg/d	1.1	1.1	1.1	1.1	.02	.21
Lactose, kg/d	1.6	1.6	1.6	1.6	.03	.73
Fat, %	3.9	3.9	4.0	3.8	.07	.23
Protein, %	3.2[a]	3.2[a]	3.1[b]	3.1[ab]	.02	.05
Lactose, %	4.6	4.6	4.6	4.6	.02	.81

[1]FCM = 3.5% fat-corrected milk.

[ab]Means in the same row not sharing superscripts differ (P < 0.05).

Effect of Corn Silage Chop Length on Intake, Milk Production, and Rumen Function in Lactating Dairy Cows

C.S. Kuehn, J.G. Linn and H.G. Jung

Introduction

Particle length of forages is important in the diets of lactating dairy cows because it can impact numerous aspects of rumen function. A minimal amount of long particles is important in the diet to insure proper rumen health by promoting rumination and salivation. Diets with small particles may spend less time in the rumen, resulting in less microbial digestion, but may result in increased dry matter (DM) intake. A reduced chop length of corn silage may improve its digestibility because of increased attachment sites for the microbes. In addition, cracked corn kernels may be rendered more digestible because the starch might be more accessible. The objective of this study was to determine the effects of corn silage chop length on DM intake, milk production, rumen fermentation, and nutrient digestion in mid-lactation dairy cows.

Material and Methods

A 92 to 96 d relative maturity hybrid corn was harvested at a theoretical chop length of .87 cm (L) or .32 cm (S). Approximately five and one-half metric tons of each chop length were packed into a 25 cu m steel refuse container lined with plastic. Corn was allowed to ferment for 28 d before feeding. The Penn State Forage Particle Separator (NASCO, Fort Atkinson, WI) was used to evaluate particle length. Mesh sizes of the top and middle screens were 1.9 and .8 cm, respectively. Smaller material passed through the screens to the collection box. This experiment was conducted as a 2 x 2 Latin square (2 periods and 2 treatments) replicated across 4 blocks. Eight mid-lactation Holstein cows were assigned to blocks according to days in milk and milk production. The DM content of the L and S silages were similar (33.9 vs. 34.7 %, respectively). Chemical composition of the two silages was similar and averaged 9.0, 48.1, and 24.3% for crude protein, neutral detergent fiber (NDF), and acid detergent fiber, respectively. Diets were formulated to contain 40.0 % L or S silage, 37.8% grain mix, 9.9% chopped alfalfa hay, 8.8 % protein mix, and 3.5% soyhulls on a DM basis. Adaptation periods were 5 to 7 d, followed by 4 d sample and data collections. Chewing activity was monitored at 5 min intervals for a 24 hr period during each period. Two rumen fistulated cows were fed the L diet during period 1 and the S diet during period 2. Rumen fluid samples were taken to observe pH changes after feeding.

Results and Discussion

Particle size distribution of the L silage was 16.5, 57.3, and 26.2% (wet basis) on the top, middle and bottom screens, respectively. The S silage particle size distribution was skewed toward smaller particles (14.5, 37.9, and 47.7% on the top, middle, and bottom screens, respectively). Intake, milk yield, and milk composition did not differ between the L and S silage diets (Table 1), although intake did trend higher for cows fed the S silage diet. Rumen pH of the fistulated cows declined during the first 2-h post-feeding for cows fed both silage chop lengths, but the S silage treatment continued this decline in pH until 4-h post-feeding whereas the L silage treatment cows had already stabilized at 2-h post-feeding (Fig. 1). The ratio of volatile fatty acids (acetate plus butyrate to propionate) showed the same pattern as pH with time after feeding. These rumen measurements suggest that the S silage treatment may have resulted in less rumination and saliva flow to maintain rumen pH at optimal levels. Observations on all cows indicated trends toward less time spent ruminating (431 vs. 427 min/d) for L and S silage treatments, respectively. Similar trends were observed in rumination time per kg of DM and NDF consumed (data not shown); however, rumination times were not statistically different. Digestibility of DM and NDF also tended to be lower on the S silage treatment (76.1 and 68.7% vs. 73.8 and 65.9%, for L and S silages, respectively), but these differences were also not significant.

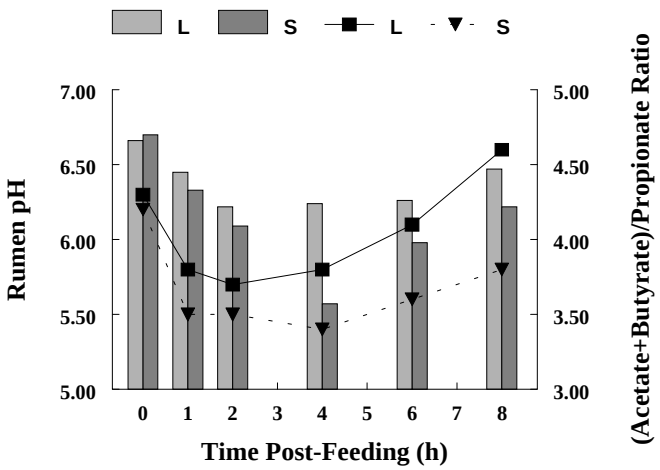

Figure 1. Rumen pH (bars) and volatile fatty acid ratio (lines) for fistulated cows fed long (L) or short (S) chop-length corn silage.

Conclusion

Trends for higher intakes and lower pH in the S diet, as well as higher digestibilities for the L diet, suggest that differences in the chop length of corn silage affects intake and feed digestibility by lactating cows. The short sampling period of 4 d, in addition to low cow numbers, limited the power of this study to detect differences in cow performance and behavior. Further research on corn silage chop length using longer collection periods and more cows is warranted.

Table 1. Dry matter intake, and production of milk, fat, and protein of cows fed long or short chopped corn silages.

	Silage Chop Length		
Item	Long	Short	Probability
	- - - - kg/d - - - -		
Dry Matter Intake	26.1	26.8	.14
Milk	29.6	29.1	.25
Fat	1.21	1.20	.91
Protein	1.04	1.02	.45
Lactose	1.42	1.36	.12

Effect of Mechanical Conditioning on In Situ DM and NDF Disappearance of Alfalfa

T.J. Kraus, R.G. Koegel and D.R. Mertens

Introduction

The physical form of forage may have an effect on rate of digestion. Several studies found severely conditioned macerated forage had greater rates and extents of fiber digestion than conventionally conditioned forages. Koegel et al. (1992) found the average dry matter digestibility of macerated alfalfa silage in sheep was 15.9% greater than conventionally conditioned silage. Hong et al. (1988) determined macerated alfalfa fed as dry hay had a significantly higher extent of neutral detergent fiber (NDF) digestion compared to conventionally treated alfalfa. Suwarno et al. (1997) found that DM digestibility of macerated hay fed to beef steers was 6.6% greater than roller conditioned hay. Mertens and Koegel (1996) found that maceration of alfalfa resulted in greater milk production and a more positive energy balance.

Not all research, however, has reported increased forage utilization as a result of severe conditioning. Conflicting results on the rate and extent of digestibility may have been attributable to different levels of conditioning. It is possible that in those studies where improved digestion and fermentation properties were found, the conditioning level was greater than in those studies where no such differences were found. However, level of conditioning was not measured in any of these studies. Therefore, the objective of this research was to determine the correlation between conditioning level, as measured by the leachate conductivity, and digestibility of DM and NDF of alfalfa (*Medicago sativa*).

Methods

Third crop late vegetative alfalfa at 84% moisture w.b. was harvested at the University of Wisconsin Madison Experimental Station in Madison, WI on August 18, 1994. This alfalfa was conditioned at 4 different levels. Four treatment 1, the alfalfa was not conditioned. For treatment 2, the alfalfa was conditioned with a pair of intermeshing rubber-covered rolls similar to those used in conventional mower-conditioners. For treatment 3, the alfalfa was conditioned using a crushing-impact conditioning mechanism (Kraus et al. 1993). This mechanism severely crushed and shredded the alfalfa stems into long fibrous pieces. For treatment 4, the alfalfa was conditioned using the rotary-impact macerator. This unit had several blunt blades attached to a high speed electric motor which was mounted inside a cylindrical tube. As the alfalfa was metered into the center of the rotating blades, it was impacted numerous times by the blunt blades before it exited the mechanism. This process extremely macerated the alfalfa. After the alfalfa was conditioned, the leachate conductivity of 10 samples from each treatment was measured according to the methods described in Kraus (1997).

Two fistulated Holstein dairy cows having similar milk production and days in lactation were selected for this study. Both cows were fed daily at 8:00 A.M. to obtain *ad libitum* intake. Diets consisted of approximately 72% alfalfa silage, 20% cracked corn, 7% soybean meal, 0.5% dicalcium phosphate, and 0.5% trace mineral salt. The polyester bags were inserted into the rumen of each cow 1 h after feeding.

Typically, in situ digestibility is measured on ground forage samples. Grinding homogenizes the samples and reduces the volume of each sample, allowing many samples to be placed into the rumen simultaneously. Because the effect of conditioning was being examined, the forage samples of this experiment were not ground. Consequently, the volume of each sample was relatively large. To prevent over-filling the rumen, 12 samples, 3 of each treatment, were placed in each rumen 1 h after feeding and all were removed at the same time. The digestion times, 6, 12, 24, and 48 h were carried out over successive days.

At the end of each digestion period, the samples were removed, submerged in ice water to suppress microbial activity, and washed with cool tap water until

the washings appeared clean. The washed samples were oven dried at 60°C for 72 h and weighed to determine the final DM contents. After weighing, each polyester bag was opened, and the sample was ground through a 2 mm mesh screen using a small Wiley mill. The neutral detergent fiber (NDF) of each digested sample was measured. The NDF of an undigested oven dried sample was also measured to provide an estimate of the initial NDF concentration of each digested sample.

The kinetics of DM digestion were modeled using a simple first-order kinetic equation with the addition of a discrete instantly soluble fraction (Mertens, 1997). An independent instantly soluble fraction was estimated for each treatment; however, a common digestion rate constant and a common fraction of indigestible residue was estimated for all treatments.

The kinetics of NDF digestion were analyzed using a first-order kinetic equation with the addition of a discrete lag time based on that of Mertens and Lofton (1980). An independent lag time was estimated for each treatment; however, a common digestion rate constant and a single fraction of indigestible residue were estimated for all treatments, and lag times were not allowed to be less than zero.

Results

The degree of conditioning of the four treatments, as measured by leachate conductivity, were as follows:

Treatment	Leachate conductivity (µS/cm)
Control	28
Mower-Conditioner Rolls	60
Crushing-Impact	518
Rotary-Impact	992

Figure 1 is a "best fit" plot of DM disappearance vs. digestion time for each treatment. Figure 2 is a "best fit" plot of NDF disappearance as a fraction of total DM vs. digestion time for each treatment. Compared to the unconditioned material (28 µS/cm), conditioning with intermeshing rubber rolls (60 µS/cm) increased the instantly solubilized DM fraction from 0 to approximately 11%. Similarly, the crushing-impact treatment (518 µS/cm) increased the instantly solubilized DM fraction to nearly 34%.

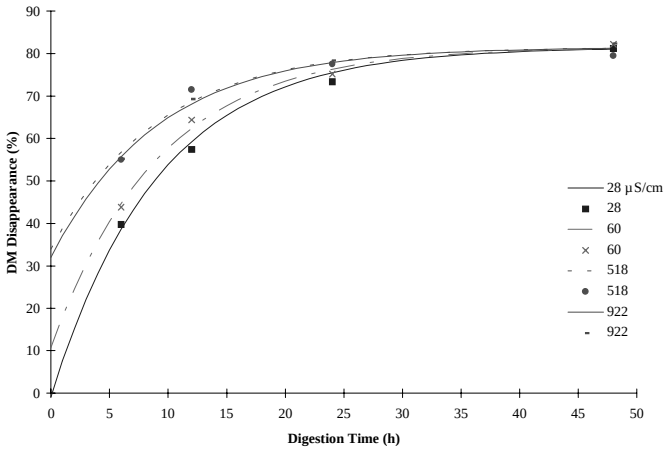

Figure 1. Level of conditioning vs. DM disappearance of alfalfa.

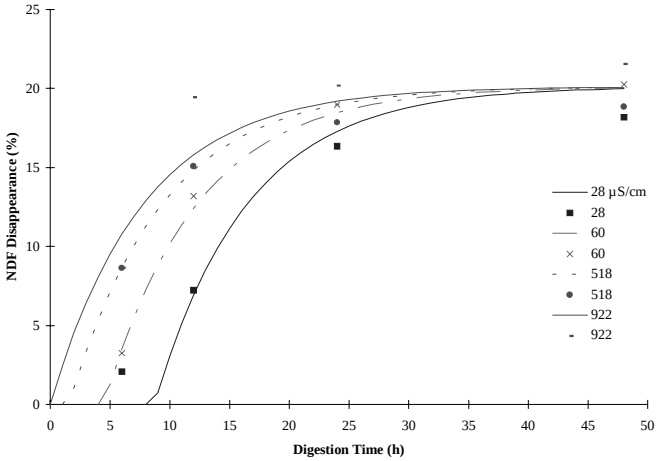

Figure 2. Level of conditioning vs. NDF disappearance of alfalfa.

The DM digestion model suggests that mechanical conditioning increased the instantly soluble DM fraction; however, mechanical conditioning did not increase the DM digestion rate constant. These results suggest that mechanical conditioning of plant tissue ruptured cells which allowed intercellular constituents to be solubilized instantly. Compared to the unconditioned material (28 µS/cm), conditioning with intermeshing rubber rolls (60 µS/cm) reduced the lag time from 8.7 to approximately 4.5 h. The crushing-impact treatment (518 µS/cm) decreased the lag time to approximately 1.5 h. The rotary impact treatment (992 µS/cm) allowed digestion of fiber to begin immediately.

The NDF digestion model suggests that mechanical conditioning decreased the lag time associated with the digestion of fiber but did not change the NDF digestion rate constant. The lag time associated with fiber digestion in the model may reflect the time required for microbes to penetrate cells and begin digestion from the interior surface of plant cell walls. Mechanical conditioning allowed rumen microflora which produce the enzymes for fiber digestion easier access to the inside of the cell wall and thereby allowed them to attach and begin digestion more quickly. This would explain why the lag time decreased as conditioning level increased.

Conclusions

There was a direct correlation between level of conditioning as measured by leachate conductivity and the disappearance of DM and NDF from nylon bags for fermentation times less than 12 h.

A first-order kinetic equation with the addition of a discrete instantly soluble DM fraction indicated that increasing the level of conditioning increased the fraction of DM instantly solubilized in the rumen but hat no effect on the rate of digestion or the indigestible DM fraction.

A first-order kinetic equation with the addition of a discrete lag time used to model NDF disappearance from the nylon bags indicated that increasing the level of conditioning decreased the lag time associated with the digestion of fiber in the rumen but had no effect on the rate of digestion or the magnitude of the indigestible fraction.

References

Hong, B.J., G.A. Broderick, R.G. Koegel, K.J. Shinners and R.J. Straub. 1988. Effect of shredding alfalfa on cellulolytic activity, digestibility, rate of passage, and milk production. J. Dairy Sci. 71:1546-1555.

Koegel, R.G., R.J. Straub, K.J. Shinners, G.A. Broderick and D.R. Mertens. 1992. An overview of physical treatments of Lucerne performed at Madison, Wisconsin, for improving properties. J. Agric. Engineering Res. 52:183-191.

Kraus, T.J., R.G. Koegel, K.J. Shinners and R.J. Straub. 1993. Evaluation of a crushing-impact macerator. Trans. ASAE. 36(6):1541-1545.

Kraus, T.J.1997. Forage conditioning and its effect on forage quality. Unpublished PhD thesis. Agricultural Engineering Dept., Univ. of Wisconsin - Madison.

Mertens, D.R. and J. R. Loften. 1980. The effect of starch on forage fiber digestion kinetics in vitro. J. Dairy Sci. 63(9):1437-1446.

Mertens, D.R. and R.G. Koegel. 1996. Maceration of alfalfa and silage improves milk production. U.S. Dairy Forage Research Center 1996 Research Summaries. U.S. Dept. of Ag., Agricultural Research Service. P. 35-36.

Mertens, D.R. 1997. Personal communication. Research animal nutritionist. U.S. Dairy Forage Research Center, Madison, WI.

Suwarno, K.M., K.M. Wittenberg and W.P. McCaughey. 1997. Intake, digestion and performance comparisons for cattle fed macerated vs. roller-conditioned alfalfa (*Medicago sativa* 1.) forage. Proc. of the XVIII International Grassland Congress. June 8-19, 1997. Winnipeg, Manitoba, Saskatoon, Saskatchewan, Canada. Session 14. P. 9-10.

Effect of Feeding Macerated Alfalfa Silage on Digestibility and Milk Production by Dairy Cows

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Introduction

Ease of mechanization, lower field losses and reduced weather damage have made ensiling the method of choice for harvesting alfalfa in the Midwest U.S. Increasing the ruminal fermentability of alfalfa would be as beneficial to dairy cows as feeding higher concentrate because it would increase both the supply of energy from VFA and protein from microbial protein synthesis. Several small studies showed that macerating alfalfa before ensiling reduced NPN formation in the silo, improved ruminal fiber digestion, reduced ruminal protein degradation, and improved milk yield in lactating cows. The objectives of this research were to determine whether the macerated alfalfa produced with a prototype, capable of producing sufficient forage for large feeding studies has the same improved nutritional value as that obtained using previous machines and to confirm, in larger and statistically more powerful studies, the benefits of macerating alfalfa silage.

Materials and Methods

Alfalfa was harvested using either a conventional mower-conditioner (Control) or the prototype maceration-mat machine (Macerated), field wilted to 40 to 50% DM and ensiled in upright concrete stave silos (first-cutting), or in upright concrete stave silos plus AgBags (second-cutting). In a 2 x 2 Latin square digestibility trial (Trial 1), 20 cows, including four with ruminal cannulas, were blocked by DIM and randomly assigned to diets containing (DM basis) 29% high moisture corn plus 70% of either Control or Macerated second-cutting alfalfa silage from AgBags. Diets were fed for two week periods before switching. Digestibility of DM, organic matter (OM), NDF and ADF was estimated using five fecal grab samples/cow per period; indigestible ADF was used as the internal marker. Ruminal samples were analyzed for ammonia and total amino acids. In the lactation trials (Trial 2 and Trial 3), negative control (Control alfalfa) and test (Macerated alfalfa) diets were formulated with high forage contents; both diets were to be identical except for forage source. A third diet was formulated containing Control alfalfa but with a greater proportion of concentrate. In Trial 2 (second-cutting), 45 multiparous cows were fed a high energy standard diet for two weeks and milk yield data collected for use in covariate analyses. Cows then were blocked by DIM into 15 groups of three and randomly assigned to one of the three experimental diets (Table 1). Two of the cows fed each diet were dropped from the trial because they developed mastitis or because of low production. After Trial 2 was completed, the 11 lowest producing cows were replaced and cows also were fed the standard diet for two weeks to collect covariate milk yield data. Trial 3 (first-cutting) was begun by re-randomizing and re-assigning the 42 highest producing cows to one of the three experimental diets. These also differed from those in Trial 2 in that diets A and B contained less forage and all three diets were supplemented with ruminant-grade fish meal (Table 1). Cows were fed their diets for all 10 weeks of Trial 2 and 3. Cows were injected biweekly with rBST; intake, milk yield, apparent digestibility using two fecal grab samples/cow (indigestible ADF as internal marker), and BW changes were measured in both trials.

Results and Discussion

The second-cutting alfalfa silages fed in Trials 1 and 2 contained (DM basis) 21.1% CP, 40.5% NDF and 33.2% ADF (Control), and 21.3% CP, 42.3% NDF and 34.5% ADF (Macerated). In Trial 1 (70% dietary forage), although DM and OM digestibility were not influenced by treatment, apparent digestibility of NDF and ADF was greater on Macerated than Control alfalfa silage (Table 2). Also, ruminal concentrations of ammonia ($P < 0.10$) and total amino acids ($P < 0.02$) were lower on Macerated alfalfa, perhaps because its greater digestibility stimulated microbial growth. However, in Trial 2, neither milk composition nor yield were altered by feeding Macerated alfalfa compared to Control when this silage was included at 64% of diet DM (Table 3). Moreover, no differences in apparent digestibility between the Macerated and Control diets were detected (Table 2). However, compared to the diet with 64% Macerated alfalfa, feeding greater concentrate (as high moisture corn)

Table 1. Composition of diets fed during lactation trials.

Ingredient	A (Negative Control)	B (Macerated)	C (Positive Control)
		(% of DM)	
Trial 2			
Control Alfalfa Silage	63.7	- - -	48.6
Macerated Alfalfa Silage	- - -	63.8	- - -
Rolled high moisture corn	31.1	31.0	40.5
Solvent soybean meal	- - -	- - -	5.4
Roasted Soybeans	4.2	4.1	4.1
Dicalcium phosphate	0.5	0.5	0.4
Sodium bicarbonate	- - -	- - -	0.45
TMS (+ Se)[1]	0.3	0.3	0.3
Dynamate[2]	0.1	0.1	0.1
Vitamin ADE concentrate[3]	0.1	0.1	0.1
Composition (DM basis)			
CP (%)	17.6	17.7	17.9
NE_L (Mcal/kg)[4]	1.62	1.70	1.69
NDF (%)	32	32	29
Trial 3			
Control Alfalfa Silage	61.3	- - -	51.8
Macerated Alfalfa Silage	- - -	59.8	- - -
Rolled high moisture corn	35.1	36.5	41.3
Solvent soybean meal	- - -	- - -	3.4
Low solubles fish meal	2.9	2.9	2.9
Dicalcium phosphate	0.5	0.5	0.4
Sodium bicarbonate	- - -	- - -	0.45
TMS (+ Se)[1]	0.3	0.3	0.3
Dynamate[2]	0.1	0.1	0.1
Vitamin ADE concentrate[3]	0.1	0.1	0.1
Composition (DM basis)			
CP (%)	18.0	17.8	18.2
NE_L (Mcal/kg)[4]	1.55	1.60	1.60
NDF (%)	34	34	31

[1]Provided (/kg of DM): Mn, 27 mg; Zn, 27 mg; Fe, 17 mg; Cu, 7 mg; I, 0.40 mg; Se, 0.30 mg; and Co, 0.10 mg.
[2]Provided (/kg of DM): Mg, 110 mg; K, 180 mg; S, 220 mg.
[3]Provided (/kg of DM): vitamin A, 3880 IU; vitamin D, 730 IU; and vitamin E, 0.73 IU.
[4]Computed from estimated NE_L contents of alfalfa silage and NRC tables.

increased protein and SNF content of milk as well as yield of milk, protein, lactose and SNF (Table 3). Fat yield was not influenced by diet in this trial. Interestingly, there were trends ($P < 0.10$) for greater digestibility of OM and NDF on the diet with Macerated alfalfa versus the higher concentrate diet (Table 2). These results suggested that, although maceration improved apparent digestibility of second-cutting alfalfa silage, this change did not result in significantly greater milk yield under the conditions of this experiment.

The first-cutting alfalfa silages fed in Trial 3 contained (DM basis) 21.3% CP, 45.9% NDF and 36.1% ADF (Control), and 21.4% CP, 46.0% NDF and 36.3% ADF (Macerated). Milk composition was not influenced by diet in this trial. However, when cows were fed diets containing about 60% silage DM, milk yield was greater ($P = 0.04$) on Macerated than on Control alfalfa and increases in yields of protein, lactose and SNF approached significance ($P < 0.10$) in this trial (Table 3). Feeding greater amounts of high

moisture corn with the Control alfalfa did not result in increased yields of milk or milk components compared to feeding 60% Macerated alfalfa (Table 3). As in Trial 1, fat yield was not influenced by diet. Comparing the two diets with about 60% forage, apparent digestibility of DM and OM was greater ($P < 0.01$) on Macerated than Control alfalfa (Table 2). Rather than roasted soybeans, fish meal was the source of undegraded protein fed in this trial; also, the higher concentrate diet contained only eight (rather than 15) percentage units more high moisture corn than did the diet with Macerated alfalfa (Table 1). The amino acid pattern of the undegraded protein in fish meal is more complementary to microbial protein than is that in roasted soybeans. The protein substitution, as well as the greater amount of high moisture corn, may have interacted with the higher fiber content of first-cutting alfalfa to improve the relative response to Macerated silage. Under the conditions of this experiment, there was a clear advantage to feeding Macerated alfalfa, compared to the Control, untreated alfalfa.

Summary and Conclusion

Processing alfalfa immediately after cutting using a prototype maceration-mat machine before the forage was ensiled increased apparent digestibility of DM and fiber in both first- and second-cutting forage. Compared to control, feeding macerated second-cutting alfalfa (42% NDF) did not significantly increase yield of milk or milk components when fed at about 64% of dietary DM. However, feeding macerated, first-cutting alfalfa (46% NDF) improved yield of milk and milk components when fed at about 60% of the DM in

Table 2. Effect of feeding Control or Macerated alfalfa silage on apparent digestibility of DM, organic matter, NDF and ADF (all trials).[1]

Diet	Dietary forage (% of DM)	Apparent digestibility of DM	Organic matter	NDF	ADF
Trial 1 (second-cutting)					
Control	70	59.5	61.4	34.3	28.6
Macerated	70	59.7	60.9	39.7	33.6
SEM[2]		0.6	0.6	0.5	0.5
$P > F$[3]		0.791	0.552	< 0.001	< 0.001
Trial 2 (second-cutting)					
A	64	61.2	62.5	41.6	41.7
B	64	62.2	63.5	41.5	40.1
C	49	60.6	61.6	39.6	38.1
SEM		0.7	0.7	0.8	0.9
$P > F$		0.304	0.152	0.144	0.021
A vs. B[4]		0.332	0.263	0.971	0.198
B vs. C[4]		0.129	0.055	0.092	0.115
Trial 3 (first-cutting)					
A	61	58.8	60.3	41.2	40.3
B	60	61.3	62.8	41.6	40.6
C	51	61.5	62.9	40.3	39.1
SEM		0.6	0.6	0.8	0.9
$P > F$		0.006	0.006	0.449	0.466
A vs. B		0.007	0.005	0.725	0.828
B vs. C		0.779	0.944	0.222	0.247

[1]Apparent digestibility was estimated using indigestible ADF as an internal marker.
[2]SEM = Standard error of the mean.
[3]Probability of a significant effect of diet.
[4]Probability of a significant difference of orthogonal contrast.

diets containing fish meal as the source of undegraded protein. We interpret these results to indicate that maceration improves utilization of both first- and second-cutting alfalfa; however, lactation responses are greater, and more easily detected, with higher fiber alfalfa and the magnitude of this response is influenced by the protein quality of supplemental undegraded protein. Milk yield on diets with 60% macerated, first-cutting forage was equivalent to that on 52% untreated forage.

Table 3. Effect of feeding Control or Macerated second-cutting (Trial 2) or first-cutting (Trial 3) alfalfa silage on DMI, BW gain, and production of milk and milk components.

	Diet[1]					Contrasts[4]	
Item	A	B	C	SEM[2]	*P* > F[3]	B vs. A	B vs. C
Trial 2							
DMI, kg/d	24.9	25.9	26.7	0.5	0.063	0.252	0.206
BW gain, kg/d	0.31	0.47	0.33	0.07	0.243	0.121	0.175
Milk yield, kg/d	37.7	38.7	42.7	0.7	< 0.001	0.893	< 0.001
3.5% FCM, kg/d	35.5	35.8	37.8	0.8	0.174	0.963	0.101
Fat, %	3.24	3.21	2.93	0.13	0.230	0.886	0.123
Fat, kg/d	1.19	1.20	1.22	0.05	0.927	0.896	0.802
Protein, %	2.99	3.03	3.21	0.06	0.015	0.319	0.050
Protein, kg/d	1.12	1.14	1.34	0.03	< 0.001	0.696	< 0.001
Lactose, %	4.94	4.98	5.00	0.05	0.571	0.403	0.891
Lactose, kg/d	1.84	1.88	2.09	0.05	0.004	0.902	0.004
SNF, %	8.65	8.74	8.93	0.09	0.043	0.221	0.174
SNF, kg/d	3.23	3.30	3.74	0.08	< 0.001	0.810	< 0.001
Milk yield : DMI	1.52	1.51	1.61	0.03	0.063	0.435	0.022
Trial 3							
DMI, kg/d	26.5	27.1	26.5	0.7	0.702	0.461	0.477
BW gain, kg/d	0.33	0.45	0.34	0.15	0.798	0.557	0.569
Milk yield, kg/d	34.4	36.5	37.6	0.8	0.019	0.042	0.445
3.5% FCM, kg/d	35.4	36.8	38.4	1.2	0.220	0.350	0.414
Fat, %	3.72	3.57	3.70	0.13	0.630	0.372	0.456
Fat, kg/d	1.27	1.30	1.37	0.06	0.434	0.689	0.388
Protein, %	3.35	3.36	3.48	0.07	0.327	0.990	0.195
Protein, kg/d	1.14	1.22	1.30	0.03	0.004	0.061	0.115
Lactose, %	4.69	4.73	4.75	0.07	0.822	0.687	0.833
Lactose, kg/d	1.62	1.74	1.78	0.05	0.084	0.080	0.738
SNF, %	8.72	8.76	8.91	0.11	0.478	0.814	0.361
SNF, kg/d	2.99	3.20	3.32	0.08	0.023	0.055	0.406
Milk yield : DMI	1.32	1.35	1.42	0.04	0.179	0.458	0.264

[1]Diets were: A = Negative Control (Trial 2, 64% and Trial 3, 61% Control alfalfa silage); B = Macerated (Trial 2, 64% and Trial 3, 60% Macerated alfalfa silage); and C = Positive Control (Trial 2, 49% and Trial 3, 51% Control alfalfa silage).

[2]SEM = Standard error of the mean.

[3]Probability of a significant effect of diet.

[4]Probability of a significant difference of orthogonal contrast.

Conjugated Linoleic Acid (CLA) Content of Milk From Cows Offered Diets Rich in Linoleic and Linolenic Acid

T.R. Dhiman, L.D. Satter, M.W. Pariza, M.P. Galli and K. Albright

Introduction

CLA is a fatty acid found primarily in the meat and dairy products of ruminant animals. It is produced by rumen bacteria and differs from linoleic acid (9 cis, 12 cis) by having a conjugated double bond with a 9 cis, 11 trans configuration. Studies with laboratory animals have demonstrated that CLA has anticarcinogenic properties, reduces atherosclerotic risk, and can alter body composition in the direction of reduced fat-increased lean. The objective of this study was to study the effect of diet on CLA content of milk.

Materials and Methods

Two experiments were conducted to determine CLA content of milk from cows offered diets rich in linoleic and linolenic acid. In Expt. 1, thirty-six cows were divided between control (CTL) and five treatment groups. Cows in the CTL group received a diet containing 34% alfalfa silage, 17% corn silage, 31.9% high moisture ear corn, 15.5% soybean meal and 1.6% minerals and vitamins (DM basis). In the treatment groups dietary corn and soybean meal were partly replaced by either 18% raw cracked soybeans (RS), 18% roasted cracked soybeans (RSB), 3.6% soybean oil (SO), 2.2% linseed oil (LO2), or 4.4% linseed oil (LO4) on a DM basis. Experimental diets were fed for 5 weeks.

In Expt. 2, thirty-six cows were assigned to control (CTL) and five treatment groups. Cows in the CTL group received a diet containing 37% alfalfa silage, 18% corn silage, 28.6% high moisture ear corn, 14.8% soybean meal and 1.6% minerals and vitamins (DM basis). In the treatment groups, dietary corn and soybean meal were partly replaced by either soybean oil at 0.5% (SO), 1% (SO1), 2% (SO2), or 4% (SO4) of diet DM, or linseed oil at 1% (LO1) of diet DM. Experimental diets were fed for 4 weeks.

Results and Discussion

Experiment 1

Average CLA contents in milk fat from wk 2 through 5 were 3.9^{d}, 3.9^{d}, 7.7^{c}, 21.2^{a}, 15.9^{b} and 16.3^{b} mg/g of fatty acids in the CTL, RS, RSB, SO, LO2, and LO4 groups, respectively. Feeding unheated full-fat soybeans had no effect on milk CLA content, whereas roasting the soybeans resulted in a small increase in CLA content. It is not clear why roasting should have this effect. Feeding of the free oil was by far the most effective way to increase milk CLA content.

Experiment 2

Average CLA contents in milk fat during wk 2 through 4 were 5.0^{d}, 7.5^{c}, 7.6^{c}, 14.5^{b}, 20.8^{a} and 7.3^{c} mg/g of fatty acids in the CTL, SO, SO1, SO2, SO4 and LO1 treatments. Soybean oil and linseed oil are good sources of linoleic and linolenic acid, respectively. Linoleic acid is a known precursor of CLA. While linseed oil contains some linoleic acid, the relatively high milk CLA concentrations obtained with feeding of linseed oil suggests that linolenic acid may also be a substrate for CLA synthesis.

Summary

Conjugated linoleic acid content of milk fat can be increased by offering diets rich in linoleic or linolenic acid, but only when oil is readily accessible to the rumen organisms. It remains to be demonstrated if the enhanced levels of CLA in milk, achieved through manipulation of the cow's diet, are sufficient for improving health status of people consuming dairy products.

Supplementation of Roasted Soybeans to Dairy Cows on Pasture

T.R. Dhiman, V.R. Kanneganti, L.D. Satter, R.P. Walgenbach and L.J. Massingill

Introduction

The protein contained in most forages is rapidly degraded by rumen microbes. This could result in a reduced supply of amino acids getting to the intestine. The objective of this study was to determine if supplementing roasted soybeans as a source of undegraded or "by-pass" protein would improve milk production in grazing cows.

Materials and Methods

Thirty-six lactating cows (primiparous and multiparous) between 21 and 109 days in milk and yielding between 29.5 to 45.4 kg of milk daily were randomly assigned to three treatments. Cows in all treatments were grazed as a single group under an intensive rotational grazing system. The experiment lasted 11 weeks. The pasture contained primarily bluegrass, quackgrass, bromegrass, and white clover. In addition to the pasture, cows were offered a concentrate mix containing 3.4% minerals and vitamins plus either 96.6% high moisture ear corn (HMEC) (Corn) or 78.4% HMEC and 18.2% roasted soybeans (RSB1), or 60.2% HMEC and 36.4% roasted soybeans (RSB2) on a DM basis. Concentrate mixes (DM basis) had CP: 7.6%, 13.7% and 19.9%; undegraded intake protein: 3.9%, 7.0% and 10.0% in the corn, RSB1 and RSB2 treatments, respectively. During the grazing season pasture contained an average of 19.9% CP, 46.7% NDF, and 29.5% ADF (DM basis).

Results and Discussion

The amount of concentrate offered was similar for the three treatments, but there was a small difference in the actual amount consumed. The two supplements containing roasted soybeans were consumed in slightly larger amounts than the corn supplement. Milk yield was not different between treatments. Milk fat percent was increased with the addition of roasted soybeans, and milk protein percent was decreased ($P < .1$). Addition of full-fat soybeans to dairy diets has often resulted in a slight increase in milk fat and decrease in milk protein. The differences in body weight change during the experiment were not different between treatments.

The lack of a milk production response to feeding of a high quality source of rumen undegraded protein (roasted soybeans) was surprising. Feeding of ensiled alfalfa as the sole forage source results in significant increases in milk production when roasted soybeans substitute for high moisture ear corn in the grain mix. Unlike the protein in grazed grass, 50% or more of the protein in alfalfa silage is typically in the form of non-protein nitrogen. More than 80% of the crude protein in grazed grass would be in the form of true protein. Even though this protein can be rapidly degraded in the rumen, it may be that rapid turnover of liquid digesta in the rumen under grazing conditions results in some "flushing" of grass protein from the rumen before it can be degraded.

Conclusions

Addition of roasted soybeans to a high corn supplement for lactating dairy cows grazing a mixed grass native pasture did not increase milk production. This suggests that grazed grass is a more effective source of protein than ensiled grass or legume forages which contain 50% or more of their crude protein in non-protein nitrogen form.

Table 1. Feed intake, milk production and body weight change in cows fed supplements differing in protein content.

Parameter	Corn	RSB1	RSB2	SD	$P =$
Concentrate intake, kg/d	8.0[b]	8.5[a]	8.3[a]	0.04	0.006
Milk yield, kg/d	28.1	28.7	27.8	0.3	0.6
3.5% FCM, kg/d	25.0	26.8	26.0	0.5	0.4
Milk fat, %	2.96	3.18	3.20	0.06	0.1
Milk protein, %	3.06	2.90	2.85	0.02	0.1
BW gain (start-end BW), kg	-5.2	-5.2	4.8	4.9	0.5

Paddocks Containing Red Clover Compared to All Grass Paddocks Support High CLA Levels in Milk

Z. Wu, L.D. Satter, V.R. Kanneganti and M.W. Pariza

Introduction

Conjugated linoleic acid (CLA), an isomer of linoleic acid, is a potent anticarcinogen. In rat models, feeding CLA inhibited epidermal and colonic tumors. In human models, CLA inhibited the proliferation of malignant melanoma, lung adenocarcinoma, and breast and colorectal cancer cells. The National Research Council (1996) stated that CLA is the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals.

The effective intake of CLA for humans is estimated to be 3.5 g/d. This is approximately 3 to 4 times what is consumed in the average American diet each day. Dairy products are the major natural dietary source of CLA. In milk, the concentration of CLA averages 5.5 mg/g of fat, ranging from 2.5 to 18.0 mg/g. Concentrations are higher in summer than in winter, coinciding with seasonal availability of fresh pasture. The highest level of CLA in milk that has been found in individual cows approaches 50 mg/g of fat. This level of milk CLA could easily result in a daily intake of 3 g of CLA or more by humans, which approximates the dosage level thought necessary for efficacy.

In a previous research summary, we (Dhiman et al., 1995) reported changes of CLA concentration in milk when pasture contributed different proportions to the cow's feed intake. Changes in concentration of CLA in milk when cows were changed from conventional feeds to pasture were determined in the present study.

Materials and Methods

Grazing began in the middle of May 1997, when pasture became available in Wisconsin. The diet before grazing consisted of 30% alfalfa silage, 20% corn silage, 28% high moisture ear corn, 8% soybean meal, 12% roasted soybeans, and 2% mineral-vitamin supplements. Forty cows (20 primiparous) were grazed, including 27 on a pasture that consisted of grasses only and 13 on a mixed pasture that consisted of 20% red clover and 80% grasses. The grasses in both pastures were primarily Kentucky bluegrass, quack grass, and smooth bromegrass. Grazing cows on both pastures were also fed a supplemental concentrate mix. The mix consisted of 75% corn, 11% corn silage, 10.6% roasted soybeans, and 3.4% mineral-vitamin supplements. The amount of the mix fed was 15.4 lb (DM) daily. This provided approximately 40% of the total feed intake. Cows were allowed to graze all of the time during the 24-h day except for 4 h/d when taken to the milking parlor and fed the supplemental mix. Electric fences were used to allocate a new paddock of fresh pasture daily. Milk yield was recorded and milk samples were taken one day before and six weeks after cows were taken to the pasture.

Results and Discussion

The concentration of CLA in milk before and during grazing did not differ between primiparous and multiparous cows (Table 1). Grazing increased the concentration nearly 2 fold (10.8 vs. 5.8 mg/g of fat, $P < 0.001$) without changing milk fat content. Pasture provided approximately 60% of the total feed intake (due to feeding of the supplemental mix). Energy supplements are usually fed to grazing cows for maximum milk yields. Grazing with little or no supplementation can increase CLA even more (see 1995 USDFRC Research Summaries). Concentration of CLA in milk was approximately 50% (14.0 vs. 9.2 mg/g) higher ($P < 0.001$) for cows grazing mixed red clover and grasses than those grazing grasses only.

The CLA in milk originates from the rumen and is formed as an intermediate in the biohydrogenation process of unsaturated fatty acids. Fatty acids in immature herbage exist mainly as galactolipids and phospholipids. These complex lipids are rapidly hydrolyzed in the rumen, resulting in release of free fatty acids. Rapidly available fatty acids are associated with increased formation of biohydrogenation intermediates rather than end products. Because the

retention time of feed particles in the rumen under grazing conditions is shorter than the retention time with dry feeds, these intermediates might be rapidly passed from the rumen before being further biohydrogenated. The fact that fresh immature pasture forages contain 6-7% lipids compared with 3-4% in hay or corn feeds makes passing of CLA from the rumen more possible.

Conclusions

Grazing lactating cows increased concentration of CLA in milk. The concentration was further increased when the pasture contained red clover. Grazing on pastures containing abundant red clover with minimum concentrate supplementation has potential to produce milk with exceptionally high CLA content.

Table 1. Concentration of fat and CLA[1] in milk of cows grazed on pasture.

	Parity		Grazing			Pasture type[2]	
	P[3]	M[3]	Before	After		Grass	Red clover
Item	(n = 40)	(n = 40)	(n = 40)	(n = 40)	SEM	(n = 27)	(n = 13)
Fat, %	4.05	3.91	4.10	3.85	0.2	3.93 $\pm$ .14	3.69 $\pm$ 0.21
CLA, mg/g of fat	8.4	8.2	5.8a	10.8a	0.5	9.2 $\pm$ 0.4[a]	14.0 + 0.6[a]

[1]Conjugated linoleic acid.

[2]The red clover pasture contained 20% red clover and 80% grasses. Values are means $\pm$ SD.

[3]P = primiparous, M = multiparous.

[a]Values before and after grazing and between grasses and red clover are significantly different at $P < 0.001$.

Milk Production and Reproductive Performance of Dairy Cows Fed Low or Normal Phosphorus Diets

Z. Wu and L.D. Satter

Introduction

The NRC (1989) recommends that dairy cow diets contain at least 0.48% P for early lactation (wk 0-3), 0.41% for 40 kg/d of FCM, and other various percentages for lower production levels. These amounts include a 10% increase over the previous recommendations (NRC, 1978). Field nutritionists recommend even higher amounts. It is common to see 0.55 to 0.60% P in herd diets.

The objective of the present study was to determine if a typical diet with no supplemental P can provide adequate P for the entire lactation. The experiment is designed to be carried out for two consecutive lactations. The first lactation has been completed, and the data are presented herein.

Materials and Methods

Forty-eight Holstein dairy cows (28 multiparous) were assigned at calving to either a low or normal P treatment. Calving was during September to October, 1996, within a seven-week period. Assignment of cows to treatments was random within parity. Cows were fed TMR in free stalls until the middle of May, 1997. Pasture provided the forage until the end of August, at which time all of the cows were dried off. The TMR was offered for ad libitum consumption during confinement and the supplement mixes at 7.0 kg/d (DM) during grazing, which accounted for approximately 40% of the total feed intake; both were fed in groups. The P content was 0.35 or 0.50% of the TMR (DM basis), and 0.35 or 0.65% of the supplements for the low and normal P treatments, respectively (Table 1). No supplemental P sources

Table 1. Ingredient and chemical composition of total mixed rations during confinement, and of supplements offered during grazing for low or normal P treatments[1].

	TMR during confinement		Supplements during grazing	
Item	Low P	Normal P	Low P	Normal P
	% of diet DM			
Ingredient				
Alfalfa silage	30.0	30.0	- - -	- - -
Corn silage	20.0	20.0	11.0	11.0
High moisture ear corn	28.4	28.0	74.7	74.1
Soybean meal	8.0	8.0	- - -	- - -
Soybean, roasted	12.0	12.0	10.6	10.6
Monosodium phosphate	- - -	0.3	- - -	- - -
Calcium carbonate	1.1	1.0	2.8	1.7
Dicalcium phosphate	- - -	0.2	- - -	1.7
Salt	0.4	0.4	0.6	0.6
Mineral and vitamin mix	0.1	0.1	0.3	0.3
Chemical analyses				
CP	16.9	16.8	9.6	9.4
NDF	33.1	34.0	24.3	25.4
ADF	24.5	24.7	12.2	13.3
Ca	0.77	0.77	1.10	1.10
P	0.35	0.50	0.33	0.65

[1]Total mixed rations during confinement were fed for ad libitum consumption and supplements during grazing at 7.0 kg/d to provide approximately 40% of the total intake.

Table 2. Lactation performance of cows fed diets containing low or normal P.

Item	Low P[1]	Normal P[1]	SEM	*P*
DMI, kg/d[2]	20.7	20.4	- - -	- - -
Milk, kg/d	31.7	29.5	1.1	0.16
3.5% FCM, kg/d	31.0	29.6	1.0	0.34
Milk fat				
%	3.41	3.61	0.09	0.12
kg/d	1.06	1.04	0.04	0.69
Milk protein				
%	3.05	3.17	0.04	0.02
kg	0.93	0.96	0.03	0.47
BW during lactation				
Beginning, kg	565	542	10	0.11
End, kg	590	592	15	0.93

[1]P content: 0.35% for the low and 0.50% for the normal P diets.

[2]Based on measurements from parturition to the time cows went on pasture (between 28 to 35 weeks in lactation)

Table 3. Concentration of P in blood serum of lactating cows fed diets containing low or normal P.

Lactation week	Low P[1]	Normal P[1]	SEM	*P*
		mg/dl		
1-5	6.0	7.3	0.2	0.001
6-15	6.5	7.4	0.2	0.001
16-25	5.8	6.4	0.1	0.009
26-34	6.1	6.9	0.2	0.005
35-44	5.6	5.6	0.1	0.880
Overall	6.0	6.7	0.1	0.001

[1]P content: 0.35% for the low and 0.50% for the normal P diets.

Table 4. Reproductive measures of lactating cows fed diets containing low or normal P.

Measure	Low P[1]	Normal P[1]	SEM	*P*
Days to first estrus	51.3	42.5	3.5	0.09
Days to first AI	74.3	75.5	3.8	0.83
Days open	112.4	120.1	11.5	0.64
Conception rate at first AI, %	29.2	37.5	- - -	- - -
Pregnancy rate				
Before 120 DIM, %	50.0	45.8	- - -	- - -
Entire lactation, %	87.5	79.2	- - -	- - -
Services per conception[2]	2.4	2.6	0.4	0.71

[1]P content: 0.35% for the low and 0.50% for the normal P diets.

[2]Including only the cows that ultimately became pregnant (21 for Low P and 19 for Normal P).

were fed to the low P group during either regimens whereas phosphates were used to raise total dietary P for the normal P group. The level of Ca was equal between the treatments during both feeding phases.

Results and Discussion

Averages of DMI during confinement were similar for the low and normal P groups (Table 2). Milk or 3.5% FCM production did not differ. The concentration of protein in milk was lower ($P < 0.05$) for low P than normal P; however, yield did not differ. The concentrations of other milk components were not different ($P > 0.12$) between P levels except for SNF, which was slightly lower ($P < 0.05$) for low P. Body weight change during lactation was similar ($P = 0.16$) for the two groups.

Concentration of blood serum P was lower ($P < 0.01$) for cows fed the low P diet than those fed the normal P diet during most of lactation, but was similar at the end of lactation (Table 3). However, all of the concentrations were within the normal range (4-8 mg/dl) typically seen in lactating cows.

Cows fed the low P diet were detected in estrus 8.3 d later ($P < 0.09$) than cows fed the normal P diet but both were served with the first AI and conceived at similar DIM (Table 4). Feeding low P did not negatively affect pregnancy rates. Overall, reproductive efficiencies were low for both groups.

Conclusion

Reducing dietary P from 0.50 to 0.35% for a complete lactation did not impair DMI, milk yield, or reproductive performance of dairy cows. Blood serum P concentration was lower, but within normal ranges. Inclusion of P supplements in diets was without apparent benefit, except possibly a small increase in milk protein percentage. Reducing P from the current NRC recommendations appears possible, and would be more environmentally friendly and more economical.

Comparison of Ground Shelled Corn and High Moisture Ear Corn of Two Particle Sizes for Their Effects on Milk Production and Rumen Fermentation When Cows Are Fed Green Chopped Grass-Legume Forage

F. San Emeterio, R.B. Reis, L.D. Satter and D.K. Combs

Introduction

Supplemental feed is required to achieve maximum profitability under most grazing conditions in the United States. Relatively little research has been done to identify optimum supplements for feeding programs based on green chopped or grazed forages. The objective of this study was to evaluate the effect of moisture and particle size of supplemental corn on milk production, milk composition, dry matter intake, and rumen fermentation when green chopped forage was fed.

Materials and Methods

Six primiparous and three multiparous Holstein cows averaging 120 and 180 days in milk, respectively, at the beginning of the trial were fitted with rumen cannulas and arranged in a 3 x 3 Latin square design replicated three times. Periods were 21 days, 15 days for adaptation and 7 days for sampling. Cows were fed 4x daily at 6 hr intervals. Feed bunks were cleaned prior to each feeding. The forage was green chopped from a mixed grass-legume pasture managed similar to an intensive rotational grazing situation. Green chop rather than grazed forage was used so an accurate measure of feed intake could be made. Pasture was chopped daily, hauled to the barn where the animals were located and divided in four parts. One part was fed to the cows immediately, and the other three parts were stored at 4 ^{o}C in individual mesh bags for the next three feedings. Average forage composition throughout the experiment was: DM-22.5%; CP-21.8%; NDF-35.4%; and ADF-24.1%.

The corn based concentrate was fed four times daily, about 30 min after the forage was offered. A total of 10 kg of concentrate DM was fed daily. The grain mix contained (DM basis) 92% corn, 6.5% soybean meal, and 1.5% of a vitamin-mineral mix. Chemical composition of the grain mix (DM basis) was CP-12.0%; NDF-10.7%; and ADF-2.7%. The ratio of forage:grain in the feed consumed was approximately 53:47.

The treatments were dry ground shelled corn (mean particle size of 1.25 mm) (DC); coarsely ground high moisture ear corn (mean particle size of 3.14 mm) (HC); and medium ground high moisture ear corn (mean particle size of 2.22 mm) (HM). Measurements made were dry matter intake, milk production and composition, and ruminal pH, NH_3, and volatile fatty acid concentration. Blood allantoin was measured and used as an indicator of rumen microbial protein synthesis. Ytterbium chloride, cobalt EDTA and chromium mordanted fiber were used as external markers to evaluate rate of digesta passage and starch digestibility.

Results and Discussion

Dry matter intake was not affected by moisture level or particle size of the corn in the supplement. Though not significant, there was a trend toward increased milk yield and milk protein percentage as the particle size of supplemental corn was reduced. Ammonia and total amino acid (TAA) concentration in rumen fluid tended to be higher with the coarsely ground high moisture ear corn than for the other two treatments. Fine grinding of high moisture ear corn improved overall starch digestibility ($P < .001$).

Conclusions

Grinding of corn is a relatively simple and inexpensive procedure that can be effective for increasing starch digestibility. The difference in mean particle size between the two high moisture ear corn treatments in this experiment (3.14 mm and 2.22 mm) may not have been enough to cause sufficient difference in rumen fermentation. Finer grinding may be necessary to see consistent changes in rumen fermentation and milk production measurements.

Table 1. Animal performance, rumen environment, blood allantoin and fecal starch concentration for cows fed green chopped grass-legume forage supplemented with ground shelled corn, or high moisture ear corn of two particle sizes.

	Treatments			
	DC	HC	HM	SEM
Dry matter intake, kg/d	21.4	20.2	20.8	0.46
Milk yield, kg/d	27.5	25.9	26.1	0.83
Milk fat, %	3.45	3.47	3.35	0.07
Milk protein, %	3.52	3.50	3.60	0.03
Ruminal pH	5.84	5.85	5.87	0.03
Ruminal NH_3, mmol	8.46[a]	10.3[b]	8.85[ab]	0.46
Ruminal TAA, mmol	1.31[a]	1.55[b]	1.37[ab]	0.07
Blood allantoin, mg/dl	53.2	57.9	57.3	3.11
Fecal starch, % DM	13.1[c]	14.0[c]	9.42[d]	0.53

[a,b]Means within the same row with different superscripts differ ($P < .05$)
[c,d]Means within the same row with different superscripts differ ($P < .001$)

A New Frontier for Plant Modification

J. Ralph and R.D. Hatfield

Should we be Trying to (Bio)Genetically Modify Plants?

Before introducing the reader to the beginning of a new era in lignin modification in particular and plant modification in general, a few comments regarding genetic engineering of plants are perhaps in order. Although the ethical issues are complex and need to be aired elsewhere, it is worth remembering that humans have exploited plants throughout history. Nearly every fruit, vegetable, and grain grown today for human consumption is far from its native cousin. Modern plants have been forced to evolve along a specific path that benefits our needs. The continued enjoyment of eating seedless fruits is not the result of natural selection but rather man's careful selection and crossing based on a sound understanding of the plants' genetics. By their very definition, such plants are doomed to rapid extinction in the natural world. While some have problems with the idea of taking a gene from one plant and putting it into another, they have no problems with grafting a prized apple stem onto a hardy root stock of some completely different variety. And let's not forget that nature, with the aid of cosmic rays and other mechanisms, has already explored many such options herself. There is little doubt that biogenetic engineering will produce a variety of more exploitable plants.

What are the Benefits of Engineering Lignin?

What benefits are to be gained from engineering lignin? In the dairy forage arena, the biggest aim is toward improved forage digestibility. Certainly, the ruminant animal needs an indigestible component, but much of the potentially digestible polysaccharides in the forage cell wall are inaccessible to that ruminant. To put cell wall digestibility gains in perspective, other factors being equal (an assumption), a 10% improvement in forage cell wall digestibility would result in some $350 million in increased U.S.milk and meat production, while producing 2.8 million tons less manure solids, and requiring 2 million tons less supplementary grain.

One of the mechanisms limiting digestibility highlighted by work in the Cell Wall Group is chemical cross-linking of polysaccharides to the indigestible lignin component. What if we could down-regulate cross-linking by genetic engineering? Hans Jung and Weting Ni are exploring such an approach. If post-harvest treatments are considered, altering the lignin or the cross-linking to make polysaccharides more available is also an option.

Modifications to lignin has benefits beyond the forage industries. The chemical pulping industry, producing paper from wood and agricultural feedstocks, would save $billions if the cooking temperature or time in the digester could be reduced. Pulping is the process by which lignin is removed from the desired cellulose component.

Current Approaches to Changing Lignin

There is considerable activity in trying to down-regulate lignification, particularly targeted toward the pulping industry. Since lignin is the polymer that must be removed to make quality white paper, its down-regulation seems attractive. Of course, lignin serves a variety of functional roles in the plant so its manipulation is not without costs in plant vigor. To date, antisense techniques have been used to down-regulate many of the enzymes on the lignin monomer biosynthetic pathway. For only a few of those has the actual amount of lignin been reduced — as will be explained below; depriving the plant of its ability to produce traditional lignin monomers does not necessarily thwart its ability to produce 'lignin.' A case can be made for the opposite approach, that of increasing lignin. Such approaches may provide

additional commodities, higher fuel-value plants, and a potential for a decreased use of pesticides in plants with improved pest resistance.

Recent Findings

We recently made some startling discoveries concerning the lignin in a natural pine mutant that have opened up a new frontier for plant modification (Ralph et al., 1997). That mutant, identified by workers at NC State (MacKay et al., 1997), had a CAD enzyme activity 1% or less of wild type. We isolated a lignin fraction to carefully examine the lignin structure by NMR. The spectra were immediately striking. In addition to the obvious decline in units derived from coniferyl alcohol, the lignin had enhanced aldehyde levels (as predicted), including new aldehyde components that had never been reported in plant lignins, and a predominant component derived from dihydroconiferyl alcohol. Dihydroconiferyl alcohol is not associated with the normal monolignol biosynthetic pathway and was not anticipated in the polymer.

Two aspects of the findings have generated considerable excitement (and controversy) and the Science article has been followed up and popularized (not always accurately!) by several publications (Boudet, 1997; Kling, 1997; Anon., 1997; Ralph, 1997). First, the findings begin to explain prior mysteries regarding the normal levels of "lignins" produced by plants whose genes were so heavily down-regulated; the down-regulated plants simply create their lignins from other available phenolic components. Second, they suggest enormously increased potential for improved utilization of plants through genetic engineering of plant lignins.

Opposing View

There are those who claim that lignification is a highly regulated process incorporating only the three hydroxycinnamyl alcohols into lignin. The evidence for this position is a tenuous extrapolation of fascinating lignan observations (Davin et al., 1997); it faces continually mounting evidence for the contrary view, i.e., that many other components incorporate into lignins in a wide variety of 'normal' plants and certainly in stressed and mutant plants. When you can take two plant materials, isolate a fraction by identical methods, and obtain polymeric fractions that are clearly different, then the plants must be metabolically different. But is this product lignin? Certainly not if you define lignin as a strict polymer of hydroxycinnamyl alcohols (see following paper). But we have sufficiently demonstrated that these "lignins" with substantial amounts of nonconventional components are formed by the plant using radical coupling methods analogous to those that typify lignification. It is reasonable to assume that the plant is utilizing this polymer for the same purposes as it uses lignin. Whether we want to continue to call it lignin or not is just semantics. Plants appear to have considerable plasticity in producing a polymeric material that functions as "lignin."

Implications for Genetic Engineering of Lignin

The ability to produce functional lignins or lignin equivalents from nontraditional monomers, Figure 1, opens up enormous new opportunities for engineering lignins for better utilization of plant materials. Researchers are no longer restricted to the enzymes and components of the traditional lignin biosynthetic pathway. The range of properties that can be engineered is not yet known, but there is reason to assume that engineering lignins to be more extractable, allowing easier pulping or providing opportunities for post-harvest treatments to improve forage digestibility, is among the likely outcomes.

Conclusions

Evidence suggests that current assumptions about the process of lignification are too narrow. A remarkable range of 'nontraditional' phenols, i.e., phenols other than the three hydroxycinnamyl alcohols, are incorporated into the lignins of a variety of plants. Various mutant plants have unanticipated responses to impositions placed on them by down-regulation, by various means, of normal pathway enzymes. Deprived of their ability to produce normal monolignols, these plants utilize other phenolic compounds to produce a modified 'lignin' that appears to provide the basic

mechanical and water transport functions that allow the plants to survive. Consequently, mutants provide a rich, largely untapped, source of insights into biosynthetic pathways and the metabolic plasticity of lignification. Exploring the breadth of the lignification process by studying the structure of lignins from lignin-biosynthetic-pathway-mutants will not only improve our understanding of lignin pathways but will provide new targets for biogenetic engineering to produce plants with exploitable characteristics.

References

Anon. 1997. On the trail of the mutant pine. *Chemistry and Industry*, (14):543.

Boudet, A.-M. 1998. A new view of lignification. *Trends in Plant Sci*. 3:67-71.

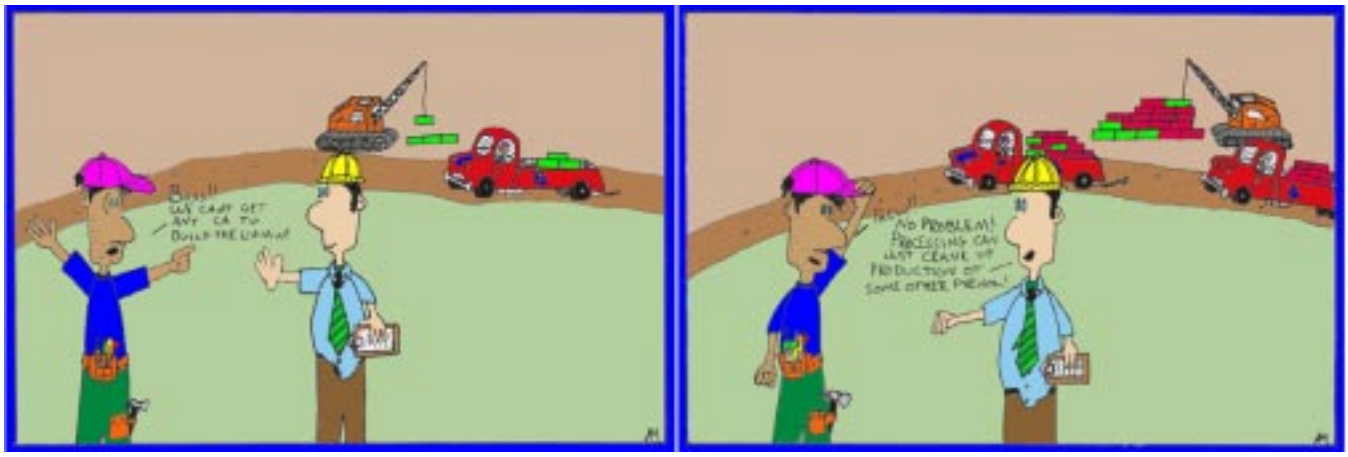
Davin, L.B.; Wang, H.-B.; Crowell, A.L.; Bedgar, D.L.; Martin, D.M.; Sarkanen, S.; Lewis, N.G. 1997. Stereoselective biomolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275:362-366.

Kling, J. 1997. Lignin variants challenge wooden thinking. *Nature Biotechnology* 15:948-949.

MacKay, J.J.; O'Malley, D.M.; Presnell, T.; Booker, F.L.; Campbell, M.M.; Whetten, R.W.; Sederoff, R.R. 1997. Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Proc. Natl. Acad. Sci. U.S.A.* 94(15):8255-8260.

Ralph, J. 1997. "Fake plastic trees." *Chemistry and Industry*, 708.

Ralph, J.; MacKay, J.J.; Hatfield, R.D.; O'Malley, D.M.; Whetten, R.W.; Sederoff, R.R. 1997. Abnormal lignin in a loblolly pine mutant. *Science* 277:235-239.



Boss!! We can't get an CA (coniferyl alcohol) to build the lignin!

No problem! Processing can just crank up production of some other phenol! Whew!

Figure 1. This cartoon, drawn by nephew Andy Muenchow, 13, was supposed to be provocative. We do not yet know what other phenols a plant may be able to incorporate into its lignin, but we do know that phenols that are not normally associated with the lignin biosynthetic pathway are incorporated into 'lignins' in a range of normal and genetically modified plants. Certainly, the currently held views of lignification (i.e., that lignins are built from only three hydroxycinnamyl alcohol monolignols) are too narrow. With modifications possible beyond the traditional monomer pathway, there is considerable new potential for biogenetically modifying lignin's components and structure to our advantage.

Should Lignin be Redefined?

R.D. Hatfield and J. Ralph

Lignin is that stuff that pulp and paper industries work hard to remove from wood in order to produce paper. It binds up wall carbohydrates in forages so they have limited digestibility, and too much of it in certain vegetables results in a poor quality food that is tough and woody in texture. Is there nothing good that we can say about this molecule? Of course, it is important in terms of adding structural strength to wood for construction; and there is evidence that lignin may be important in human nutrition for aiding digestion, lowering cholesterol, and perhaps reducing the risk of colon cancer. Perhaps it is a bit narrow to think of lignin as just some negative factor that limits our exploitation of plants.

Just what is this material called lignin that is deposited in virtually every cell wall of every vascular land plant? There are two ways to define lignin— from a chemical point of view (i.e., its chemical composition and structure) or from a functional view that stresses what lignin does within the plant. As for the chemical definition, it has been recognized for 50 years now that lignin is a polymeric material composed of phenylpropanoid units from three simple compounds (monolignols): *p*-coumaryl, coniferyl and sinapyl alcohol (Fig. 1). Until recently it was believed that only these phenolics were involved in the synthesis of lignin.

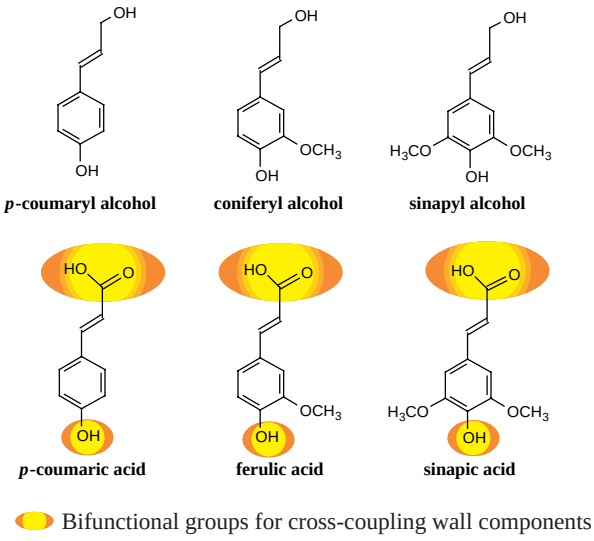


Figure 1. Major products of the phenylpropanoid pathway that are incorporated into plant cell walls.

Structurally, lignin is a polymer built from the more or less random coupling of the three monolignol units (Fig. 1) into the growing polymer.

From a functional point of view, lignin imparts strength to wall matrices, has a critical function in terms of water transport, and impedes the degradation of wall polysaccharides, thus acting as a major line of defense against pathogens, insects and other herbivores. As we have investigated the structural nature of lignin, we are understanding more about what it is doing within the plant. The functional role of lignin has not changed, but our strict compositional definition is no longer accurate. So what has changed?

As new analytical techniques have been applied to the study of lignin, evidence is rapidly mounting that forces us to critically evaluate and ultimately reject a number of accepted dogma. Lignin is not about a polymer simply composed of three monolignols!! It is a much more dynamic structure that is formed from the elegant incorporation of key wall phenolics to produce a functional molecule. The key, as far as the plant is concerned, is producing a functional polymer, but not necessarily restricting its synthesis to the three recognized monolignols. There have been hints of the dynamic nature of lignin for some time now; we have erroneously chosen the most simplistic view. Perhaps we simply wanted to deny any additional complexity to a molecule that already is impossible to investigate as a complete entity.

Let us examine the cross-linking story as an example. Cross-linking of lignin to wall polysaccharides has been speculated upon for 20 to 30 years, but the definitive proof always seemed to be just beyond reach. The discovery of hydroxycinnamates in walls (particularly of grasses) led to the speculation that they might be involved in cross-linking due to their bifunctional nature (Fig.1). As it turns out, *p*-coumarates are quite prominent yet appear to have no crosslinking role. Ferulates, on the other hand, have been shown to be covalently linked to lignin. It is interesting to note that even though ferulates are quite

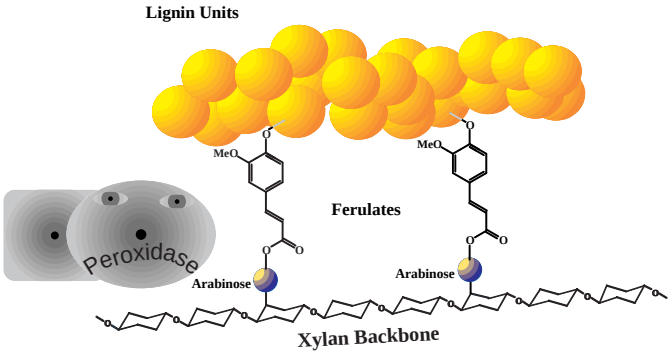


Figure 2. Schematic representation of ferulate attachment to lignin via simple ether linkages.

similar in structure to coniferyl alcohol, which incorporates nicely into lignin structures with complex non-repeating covalent bonds, it was generally believed that ferulic acid attachment was restricted to a simple ether linkage (Fig. 2). Such attachment would render ferulates completely hydrolyzable by high temperature alkaline treatment. One could picture it as ferulates attached only to the outer surfaces of the lignin molecule (whatever an outer surface would be, see Fig. 2). This assumes that the preferred reaction would be the addition of the phenolic hydroxyl to a quinone methide (an opportunistic reaction).

Although this is a nice picture that makes us feel good, we now know that it does not represent the reality of ferulate interactions with lignin or its involvement in the lignification process. Ferulates undergo extensive coupling with lignin particularly during the initial stages of lignin formation. The radical coupling that takes place parallels that of coniferyl alcohol and therefore is so extensive (both in the sense of bond types and numbers) that much of the wall-bound ferulate is no longer extractable from the lignin molecule (Fig. 3). So

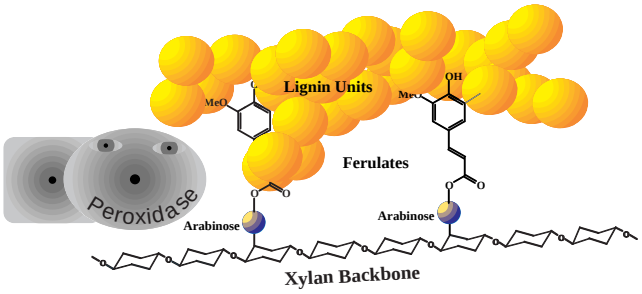
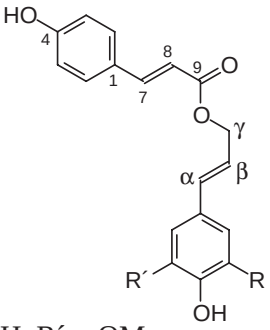


Figure 3. Schematic representation of ferulate incorporation into lignin polymers via several types of covalent linkages.

now we have a "lignin" that contains the normal monolignols but also contains this other molecule intimately incorporated into the growing polymer. Is this still lignin?

What of the *p*-coumarates? It has been recognized for some time now that *p*CA is covalently linked to lignin subunits. These molecules do indeed "hang off" the lignin structure by a simple ester bond. Early assumptions tended to take the most simplistic view, i.e., free *p*CA within the wall matrix would add to growing lignin polymers through quinone methide intermediates to form a-ester linkages. However, structural analysis some 30 years ago suggested a major portion of the *p*CA was on the g-carbon, not the a-carbon (Shimada et al., 1971). This has recently been confirmed and indeed shown that all *p*CA within detectable limits is attached to the g-carbon. Since there is no radical mechanism that would give rise to this attachment, it must be formed via enzyme activity. There are two possibilities. The first is that there is an apoplastic enzyme that can attach *p*CA to accessible Cg-OH groups after the lignin polymer is formed. Plausible, but for what function? The second possibility is a cytoplasmic enzyme that couples *p*CA to coniferyl or sinapyl alcohol (Cg-OH) forming *p*-coumarate conjugates (Fig. 4) that are transported to the wall matrix as a unit. This seems the more likely possibility in light of recent evidence indicating a potential role of hydroxycinnamates in aiding the formation of sinapyl alcohol-rich lignins. (Takahaama et al. 1996). In the latter case the plant is forming a unique molecule (sinapyl *p*-coumarate) that deviates from the "normal" lignin monolignols but is incorporated into lignin.



Coniferyl alcohol R = H, R´ = OMe
Sinapyl alcohol R = R´ = OMe

Figure 4. Ester linkage of p-coumarate acid to monolignols.

As yet another example, let us consider the maize brown midrib mutant bmr_3. It is known that this mutant contains a lower syringl/guaiacyl ratio due to reduced activity of an *O*-methyl transferase (Grand et al. 1985) involved in sinapyl alcohol formation. Lapierre et al. (1988) demonstrated that bmr_3 incorporates 5-hydroxyconiferyl alcohol (an intermediate in the formation of sinapyl alcohol, see Fig. 1) into its lignin. The mutation leading to reduced *O*-methyl transferase activity may not result in reduced lignin content but it does alter its composition.

In a similar fashion a pine mutant that has severely reduced activity of CAD (cinnamyl alcohol dehydrogenase) incorporated coniferylaldehyde into its lignin. The CAD enzyme reduces coniferylaldehyde to coniferyl alcohol as the last step of monolignol biosynthesis. With this enzyme blocked, the pine incorporates the intermediate into its lignin. The pine is also unique in that it was not a simple tradeoff of coniferylaldehyde for coniferyl alcohol in the lignin; there were also large amounts of dihydroconiferyl alcohol indicating a significant divergence from the current monolignol biosynthetic pathway (Fig. 5). The impact of these changes on the properties of lignin are not fully understood at this time, nor is their impact upon wall cross-linking or, ultimately, on wall degradation. What it does indicate is a significant latitude for lignin structure. The plant is utilizing

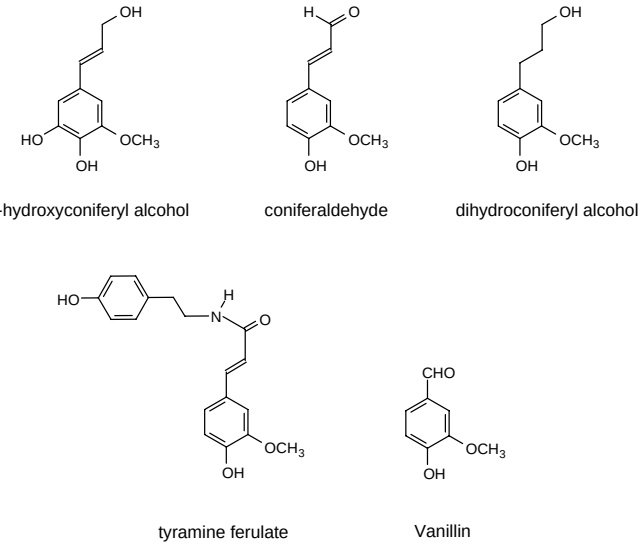


Figure 5. Non-traditional phenolics that are incorporated into lignin isolated from natural mutants and molecularly altered plants.

available molecules that polymerize into lignin efficiently even though they are not the "normal" lignin building blocks.

Attempts to alter lignin concentration in plants has focused on the genes encoding the various enzymes within the monolignol biosynthetic pathway (Boudet and Grima-Pettenati 1996; Boudet et al. 1995; Whetten and Sederoff 1995). The last two enzymes of the lignin pathway (cinnamoyl CoA reductase, CCR and cinnamyl alcohol dehydrogenase, CAD) are good candidates for genetic manipulation of lignin in that they are solely involved in monolignol biosynthesis. One, therefore, does not have to be concerned about altering some other metabolic pathway that may be crucial to plant viability (e.g., flavonoid or phytoalexin pathways). Efforts to alter lignin through the down-regulation of CAD have been successful to some degree (Baucher et al. 1996; Halpin et al. 1994; Higuchi et al. 1994). In all cases, there was a definite shift in the lignin composition towards higher levels of cinnamaldehyde units and lower coniferyl alcohol units. However, total lignin levels were virtually unchanged though there was a change in the alkaline solubility of lignin. The CCR altered plants did show reduced lignin levels (50% !!), although the plants were not as physically robust. Recent ^{13}C-^{1}H NMR work analyzing ^{13}C-enriched lignins isolated from tobacco lines with down regulated CAD (by antisense genes) and other tobacco lines with down regulated CCR (by antisense genes) revealed the incorporation of further non-traditional components into their lignins (Fig. 5; Ralph et al. 1998). The antisense CAD tobacco contained lower coniferyl alcohol and higher levels of cinnamaldehydes along with elevated levels of benzaldehydes with little change in Klason lignin levels. The results from genetically manipulated plants and from the natural mutants (bmr and pine CAD deficient) clearly indicate the metabolic plasticity involved in the lignification process. There are many unanswered questions as to the impact of these alterations on wall degradation, but it indicates the wide potential for altering lignin structure.

It is also clear that lignin can no longer be thought of as a molecule composed solely of varying ratios of coniferyl, sinapyl, and *p*-coumaryl alcohols. Like it or

not, plants produce functional lignins that are mildly or extensively contaminated with other components. These components are inextricably bound and cannot be separated from the "pure lignin" polymers because they have been intimately incorporated into the lignin structure by the radical coupling reactions that define lignification. As complex as lignin was before, it now seems structurally even more complex. However, this complexity is entirely logical from a mechanistic point of view and the metabolic plasticity may be evolutionarily wise.

Lignin — THINK DIFFERENTLY!

References

Baucher, M.; Chabbert, B.; Pilate, G.; van Doorsselaere, J.; Tollier, M.-T.; Petit-Conil, M.; Cornu, D.; Monties, B.; van Montagu, M.; Inz, D.; Jouanin, L.; Boerjan, W. Red xylem and higher lignin extractability by down-regulating cinnamyl alcohol dehydrogenase in poplar (*Populus tremula* and *Populus alba*). *Plant Physiol.* 1996, *112*, 1479-1490.

Boudet, A. M.; Grima-Pettenati, J. Lignin Genetic Engineering. *Molecular Breeding* 1996, *2*, 2539.

Boudet, A. M.; Lapierre, C.; Grima-Pettenati, J. Biochemistry and molecular biology of lignification. *New Phytol.* 1995, *129*, 203-36.

Grand, C.; Parmentier, P.; Boudet, A.; Boudet, A. M. Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (bm3) mutant. *Physiol. Veg.* 1985, *23*, 905-11.

Halpin, C.; Knight, M. E.; Foxon, G. A.; Campbell, M. M.; Boudet, A. M.; Boon, J. J.; Chabbert, B.; Tollier, M.-T.; Schuch, W. Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. *Plant J.* 1994, *6*, 339-350.

Higuchi, T.; Ito, T.; Umezawa, T.; Hibino, T.; Shibata, D. Red-brown color of lignified tissues of transgenic plants with antisense CAD gene: Wine-red lignin from coniferyl aldehyde. *J. Biotechnol.* 1994, *37*, 151-158.

Lapierre, C.; Tollier, M. T.; Monties, B. A new type of constitutive unit in lignins from the corn bm3 mutant. *C. R. Acad. Sci., Ser. 3* 1988, *307*, 723-8.

Ralph, J.; Hatfield, R. D.; Piquemal, J.; Yahiaoui, N.; Pean, M.; Lapierre, C.; Boudet, A.-M. Lignin in antisense-CAD and antisense-CCR tobacco. *Proc. Nat. Acad. Sci.* 1998, in preparation.

Takahama, N.J; Oniki, T.; Shimokana, H. A possible mechanism for the oxidation of Sinapyl alcohol by peroxidase dependent reactions in the apoplast: Enhancement of the oxidation by hydroxycinnamic acids and components of the apoplast. 1996, 37, 499-504.

Shimada, M.; Fukuzuka, T.; Higuchi, T. Ester linkages of *p*-coumaric acid in bamboo and grass lignins. *Tappi* 1971, *54*, 72-78.

Whetten, R.; Sederoff, R. Lignin Biosynthesis. *The Plant Cell* 1995, *7*, 1001-1013.

Discovery of an Abnormal Lignin in a Loblolly Pine Mutant

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Introduction

Lignins are complex phenolic plant polymers essential for mechanical support, defense, and water transport in vascular terrestrial plants. They are usually derived from three hydroxycinnamyl alcohol precursors **2a-c** in varying proportions, Figure 1. Low levels (~5%) of cinnamaldehydes and benzaldehydes are found in all isolated lignins and are responsible for the bright crimson staining of lignified tissues by phloroglucinol/HCl. Removal of lignin from wood and plant fibers is the basis of chemical pulping to produce diverse pulp and paper products. Genetic engineering of the lignin biosynthetic pathway to lower lignin concentration or construct lignins more amenable to extraction is an active area of current research.

CAD catalyzes the last step of the lignin precursor biosynthetic pathway (Fig. 1), reduction of hydroxycinnamaldehydes **1** to hydroxycinnamyl alcohols **2** (the conventional lignin monomers or monolignols). A reduction in CAD activity might lead to accumulation of hydroxycinnamaldehydes **1** which could copolymerize with normal lignin monomers. Transgenic plants, suppressed in the synthesis of CAD sometimes have red-brown xylem tissue, resembling that of grass brown midrib mutants. Such plants have increased aldehyde levels, although little of the aldehyde may actually be incorporated into the lignin.

Here we report that a viable loblolly pine, homozygous for the mutant *cad-n1* allele, incorporates novel monomers into its lignin in response to a CAD deficiency. The lignin structural changes were extensive and not predicted by the current view of the lignin biosynthetic pathway. The wood of this mutant is brown-red, similar to the color of the xylem in *brown midrib* mutants and transgenic plants suppressed in lignin biosynthetic enzyme activity. CAD activity is 1% or less of wild type, and relative abundance of *cad* mRNA transcript is greatly decreased. In mutant plants, free coniferaldehyde **1b** (the CAD substrate) accumulates to a high level.

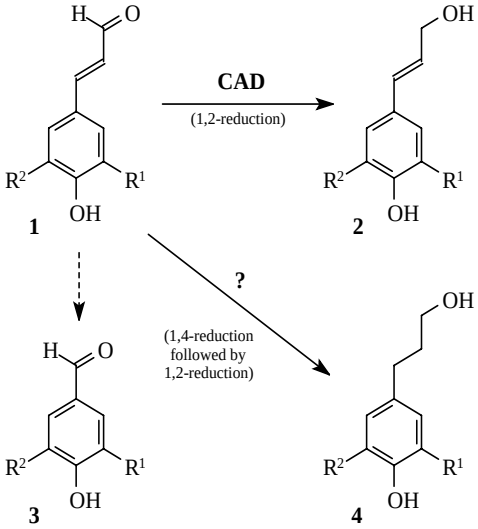

*Figure 1. Some precursors and products involved in the lignin biosynthetic pathway. The normal lignin monomers are the p-hydroxycinnamyl alcohols **2**; p-coumaryl alcohol **2a**, coniferyl alcohol **2b** and sinapyl alcohol **2c**. Coniferaldehyde **1b** is normally reduced regioselectively to produce coniferyl alcohol **2b**. When CAD activity is depressed, coniferaldehyde **2b** accumulates and could polymerize or co-polymerize into lignin. Dihydroconiferyl alcohol **4b**, observed previously only as a minor component of softwood lignins, is presumed to derive from coniferaldehyde **1b** via a 1,4- followed by a 1,2-reduction. However, no mechanism for this conversion has been reported. p-Coumaryl alcohol **2a** is readily derived from its aldehyde **1a** in the mutant, implying that different CAD enzymes are involved for **1a**→**2a** vs. **1b**→**2b**.*

Results and Discussion

Milled wood lignins were isolated for NMR analysis from the wood of a 12 year old CAD-deficient mutant and a normal sibling from the same cross. An estimate of the subunit composition of this unusual lignin fraction, based on quantitative NMR and other analytical data, is given in Table 1.

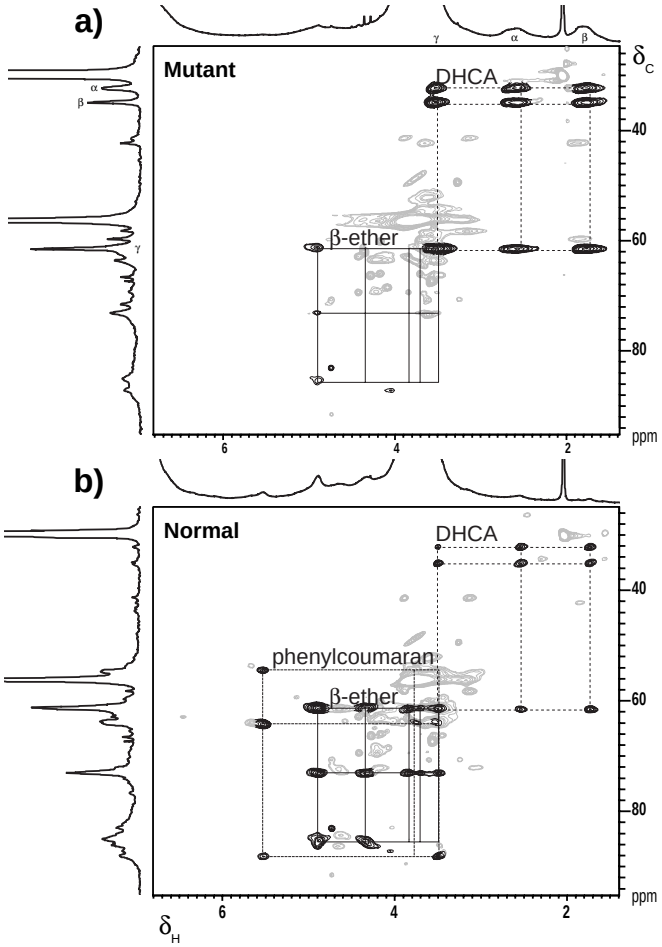

Figure 2. Regions of the HMQC-TOCSY spectra of milled wood lignins from a) the pine cad-n1 mutant, b) a cad-normal wood. In the CAD mutant, dihydroconiferyl units are dominant, displacing much of the intensity from the normal coniferyl alcohol-derived region. Some of the minor units can be seen in the pine samples when looking at lower contour levels (not shown). The normally predominant b-aryl ether and phenylcoumaran components, Figure 2b, are severely reduced in the cad-n1 mutant, with only some b-ether peaks being observable at comparable contour levels — these may also arise from p-coumaryl alcohol (in addition to coniferyl alcohol). Grey contours are from intense methoxyl signals, carbohydrate impurities, and other lignin structures not discussed here.

NMR spectra show that both coniferaldehyde and vanillin endgroups, as well as new aldehydes, are present in the lignin of the pine mutant as may be expected from the suppression of CAD. Wood from the mutant also had a higher extractable aldehydes content. The most striking feature of spectra was due to unexpected elevation of dihydroconiferyl alcohol units. The HMQC-TOCSY experiment (Fig. 2) identified the coupling network for the aryl propanol sidechain that are consistent with model compound data. Products representing hetero-coupling of dihydroconiferyl alcohol with a conventional lignin monomer/oligomer as well as products from initial 5–5-homo-coupling of dihydroconiferyl alcohol monomers are present in roughly equal amounts reinforcing the claim that dihydroconiferyl alcohol is a major monomer during lignification. The monomer was also found in wood extracts.

CAD normally effects a regioselective "1,2-reduction" (at C-9) of coniferaldehyde **1b** to produce coniferyl alcohol **2b**. Our results suggest that the loss of CAD activity activates or up-regulates pathways based on "1-4 reduction" (at C-7) and subsequent 1,2-reduction during lignin formation to produce the dihydroconiferyl alcohol monomer **4b** (Fig. 1). If the biochemical reduction is not totally regioselective, the small amounts of **4b** producing the dihydroconiferyl units seen in normal pine lignins could be explained but this rationale would not allow production of **4b** in such major proportions without a significant shift in enzyme activity or without enhanced activity of an alternate enzyme. At least one new enzyme would be required to explain these results.

The amount of subunits derived from *p*-coumaryl alcohol **2a** in the mutant is unchanged (Table 1), while the amount of coniferyl alcohol subunits **2b** is greatly reduced. These results imply that the formation of *p*-coumaryl alcohol **2a** utilizes an independent mechanism such as an additional enzyme with "1,2-reductase activity" specific for *p*-hydroxycinnamaldehyde **1a**. Furthermore, few dihydro-*p*-coumaryl alcohol **4a** units were detected (29). The 1,4-reductase activity proposed for the formation of dihydroconiferyl alcohol is therefore equally specific for coniferaldehyde **1b**.

Incorporation of novel monomers into lignin is inconsistent with a high level of enzymatic specificity in lignification. Independence from rigid enzymatic control is further supported by other examples of incorporation of non-traditional monomers into lignins — see page 30.

Conclusions

Well characterized differences in lignin subunit composition have long been known between major

taxonomic groups of higher plants, for example, between lignins of hardwood and softwood trees. However, the narrow range of variation in lignin compositions within groups has suggested structural constraints imposed for vascular function and support. The ability of this pine mutant to produce a functional lignin polymer from unexpected subunits extends the limit of "metabolic plasticity" for the formation of lignin within an individual species. Concepts of lignin function based on the previous range of lignin compositions must now be reexamined in view of the unusual structure and composition of lignin in this mutant pine. A greater understanding of these processes should increase our opportunities to modify lignin content or composition through genetic engineering — see "A New Frontier for Plant Modification" page 27 in these summaries.

For a more complete manuscript describing these findings in greater detail, see *Science*, **277**:235-239, 1997. The full manuscript in pre-publication form is on our website at: *http://www.dfrc.wisc.edu/FullTextPubs.html*

Table 1. Estimates of subunit compositions (from quantitative ^{13}C-NMR and DFRC-method data) of the normal and mutant pine isolated lignins. **2a** = *p*-coumaryl alcohol units; **2b** = coniferyl alcohol units; **1** = cinnamaldehyde units; **3** = benzaldehyde units; * aldehydes at ~188 ppm in ^{13}C-NMR spectra appear to derive from coniferaldehyde; **4b** = dihydroconiferyl alcohol (+ traces of dihydro-*p*-coumaryl alcohol **4a**), the major component of the *cad-n1* mutant lignin.

Lignin	**1**	**2a**	**2b**	**3**	**4b**	*****
cad-n1-mutant	15	10	15	15	30	15
cad-normal	7	10	73	7	3	trace

Abnormal Lignins in Antisense-CCR and Antisense-CAD Plants

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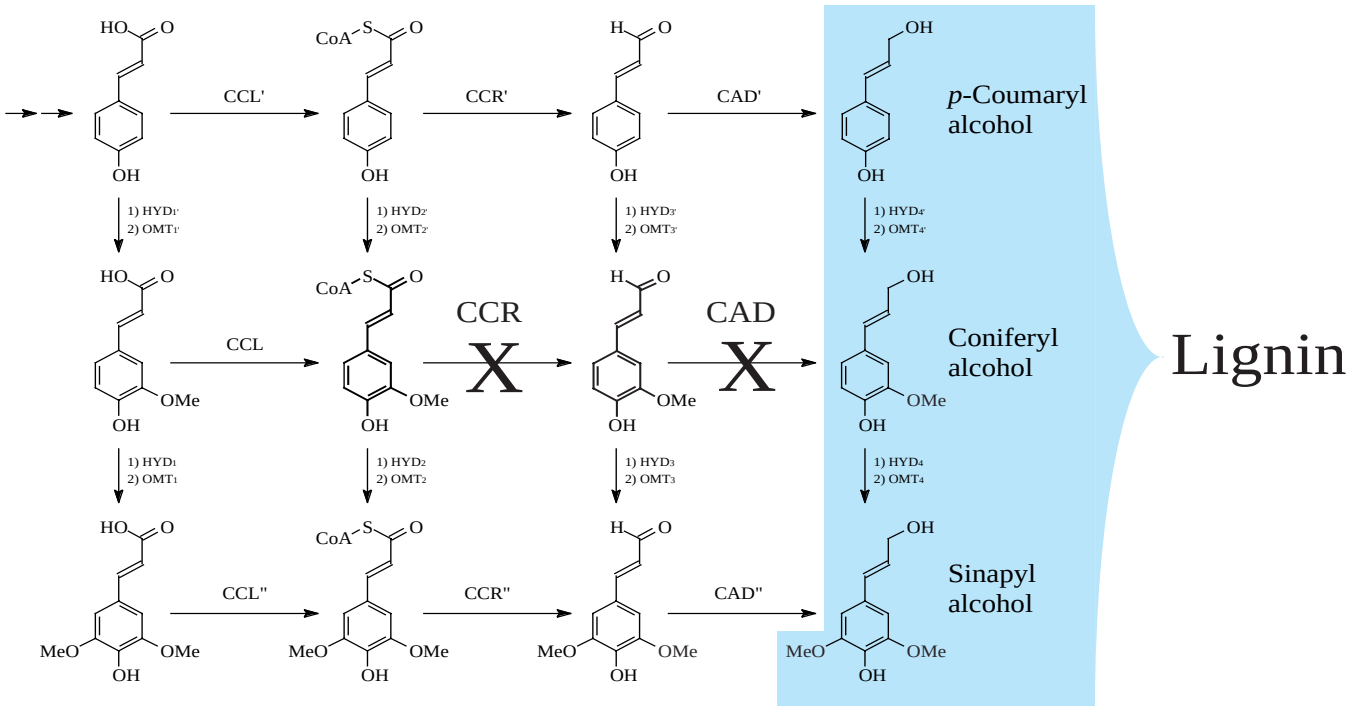
Introduction

Lignins are phenolic plant polymers essential for mechanical support, defense, and water transport in vascular terrestrial plants, but are a major obstacle to efficient utilization of plant polysaccharides in processes ranging from digestion in ruminant animals to industrial chemical pulping. Down-regulation of lignification is being actively pursued by many groups. A recent and promising approach has been the application of genetic biotechnologies targeting enzymes on the lignin monomer biosynthetic pathway, Scheme 1. Antisense applications are particularly attractive for research as they provide materials in which a single enzyme has been selectively targeted. Tobacco is an excellent model system due to the extensive literature on its analysis, chemistry, biochemistry, and the characterization of genes in the monolignol pathway. Antisense gene constructs were made to the CAD and CCR enzymes of tobacco by collaborators in France. For this study, lignins were isolated from the down-regulated CAD and CCR plants and compared to tobacco plants without the antisense constructs to examine how plants respond to the down-regulation of specifically targeted enzymes. This paper reports preliminary results obtained through the application of analytical NMR experiments.

Materials and Methods

Transgenic Tobacco Plants

(*Nicotiana tabacum* L. Cv. Samsun)



Scheme 1. Monolignol biosynthetic pathway. Not all of the enzymes are known or are necessarily discrete. Down-regulating CAD might be expected to build up coniferaldehyde; blocking CCR might build up feruloyl-SCoA, but then what?

Antisense CAD Plants. Seeds resulted from self pollination of primary transformant T37 carrying a 1kb CAD cDNA in antisense orientation, associated to the 35S CaMV promoter and the 3' terminator of the nopaline synthase.

Antisense CCR Plants. Seeds resulted from a test cross on primary transformant B3 carrying a 1.3 kb CCR cDNA in antisense orientation, associated to the 35S CaMV promoter and the 3' terminator of the nopaline synthase.

Both CAD and CCR antisense constructs also carried the neomycine phosphotransferase gene conferring resistance to kanamycin.

Lignin isolation and NMR were by normal procedures.

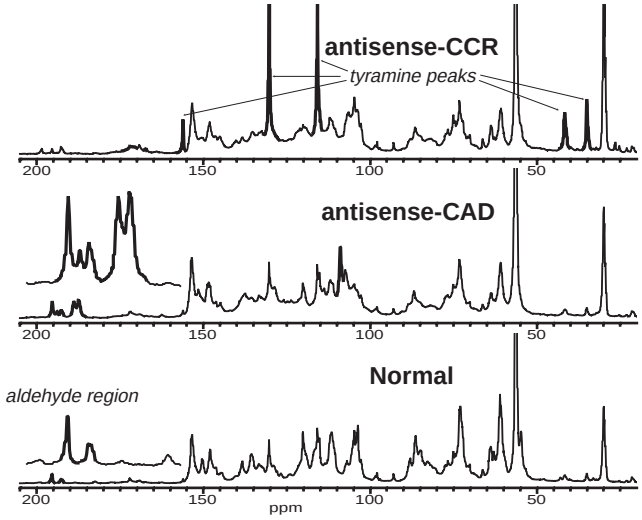


Figure 1. 13C NMR spectra of normal, antisense-CAD and antisense-CCR tobacco isolated lignins (unacetylated). The normal lignin is a syringyl/guaiacyl lignin, with guaiacyl units predominating. Aldehydes are elevated in the antisense-CAD sample; tyramine units (ex. tyramine ferulate) are markedly elevated in the antisense-CCR lignin.

Results and Discussion

Caveat

All following comments refer to the characterization of an extracted lignin. It is recognized that this is not the total lignin and may not be entirely representative of the in situ material; we will not continue to qualify each observation with that important point. However, isolation from the normal control and two antisense plants by identical procedures assures that differences noted are indicative of differences in the original plants. Additionally, the isolates characterized do have components that are not normally associated with lignin biosynthesis intimately incorporated by radical coupling processes with traditional lignin monomers into an analogous polymer. While this polymer is not a lignin by the traditional definition (...a polymer derived from three hydroxycinnamyl alcohol monomers...) it may have the properties to provide the required structural, water transport, and defense functions that the plant needs.

Antisense-CAD Tobacco Lignin

The Klason lignin level of the normal and antisense-CAD cell walls was similar (17% and 15%). Guaiacyl components (from coniferyl alcohol) were reduced as evidenced by the sharp reduction in traditional b-5 (phenylcoumaran) components visible in 2D experiments (not shown). It was the aldehyde region of the antisense-CAD lignin that displayed the most striking change, Fig. 1. Both cinnamaldehyde and benzaldehyde levels were higher (as expected from down-regulation of CAD) and new aldehydes became predominant. Benzaldehydes and cinnamaldehydes are well known components of lignins and are the agents responsible for the characteristic purple staining of lignified tissues by phloroglucinol-HCl. Whether these aldehydes are produced from normal hydroxycinnamyl alcohol sidechains by oxidative reactions following lignification, are from incorporation of monomers that have been oxidized to aldehydes by the oxidizing system, or are the result of direct incorporation of aldehydes that are exported to the wall before undergoing the final CAD-catalyzed reduction to the traditional alcohol monomers, is unknown. Direct incorporation of aldehyde monomers into free-radical polymerization reactions with normal monomers would explain their bonding patterns and relatively high contents, but other possibilities are not ruled out.

Aldehydes are assumed to be elevated components of CAD-deficient mutants, and some proof has been forthcoming, although distinguishing aldehydes that are

truly incorporated into the phenolic polymer from those present simply as extractives can be a problem. The red-brown coloration of CAD-deficient mutants has been ascribed to the presence of these aldehydes; synthetic lignins produced with coniferaldehyde incorporation have a similar red-brown color.

NMR indicates that the known benzaldehyde and cinnamaldehyde levels in the antisense-CAD isolated tobacco lignin are roughly doubled (Fig. 1). Extraction of the lignin after isolation with methylene chloride does not reduce this content — the aldehydes are an integral part of the polymer and are not simply un-extracted low molecular weight components associated with this fraction. Far more striking is the appearance of new aldehydes. They are at best barely discernible in the normal lignin but become major contributors to the antisense-CAD lignin (Fig. 1).

Antisense-CCR Tobacco Lignin

The Klason cell wall lignin content of this material was about half that of normal tobacco, but the extractable lignin was at about the same relative level. The stem material was quite flexible and plastic; unlike the more brittle normal and CAD-antisense tissues, it repeatedly lined around the grinder instead of following the normal path through the blades. The CCR mutant did not have normal development; plants severely depressed in CCR activity had a strong reduction in lignin content, reduced growth, abnormal leaf morphology, and collapsed vessels. Although its lignin content was lower, the percent of original lignin extractable was similar to the normal plant. The lignin showed clear evidence of the reduction of guaiacyl components, again by the lower levels of β-5 (phenylcoumaran) units, but the levels were not as drastically reduced as in the CAD-antisense plants. In this case, the syringyl components were relatively retained, as evidenced by the strong β-β-peaks in 2D spectra (not shown) and the $S_{2/6}$ carbons in the 1D ^{13}C NMR spectrum (Fig. 1).

Aldehyde levels were not significantly changed. The most striking change in this lignin was the dominance of tyramine units **Z** in the ^{13}C NMR spectra (Fig. 1). Huge *p*-hydroxyphenyl peaks were apparent that initially appeared attributable to *p*-coumarate or *p*-coumaryl alcohol units but were soon revealed to be tyramine-derived. They are totally free phenolic (within NMR sensitivity limits) as demonstrated by the expected shifts following acetylation of the phenol (not shown). As such they are reminiscent of *p*-coumarates in grasses. In both cases, the sharp, intense peaks result from a combination of the minuscule shift differences engendered by the moieties they are attached to, and the longer carbon relaxation times compared to main polymer carbons due to their being mobile endgroups. Other NMR experiments show that the tyramine is attached to ferulates. Tyramine ferulates are well known components in tobacco, and have been previously associated with the lignin. They are also associated with suberization, e.g., in wounded potatoes. Their levels increase with various stresses, including that produced by TMV infection. Interestingly, the levels were not notably different from the normal tobacco in the plant down-regulated by antisense-CAD methods, Fig. 1. Unfortunately, NMR spectra of tobacco lignins are sparse and other data is insufficient to determine whether the levels observed here are higher than would be observed in environmentally stressed plants.

With the above caveat that such units increase in stressed tobacco, tyramine ferulates are a logical sink for feruloyl–SCoA units that might be expected to build up when the CCR enzyme is down-regulated — CCR is the enzyme that takes hydroxycinnamoyl–SCoA's to cinnamaldehydes (Scheme 1). Tyramine derives from earlier in the pathway, from decarboxylation of tyrosine, a precursor of *p*-coumaric acid. The required transferase, hydroxycinnamoyl-coenzyme A:tyramine hydroxycinnamoyltransferase, has been found in many plants. It is obviously a leap to infer that the plant up-regulates production of tyramine specifically to provide a sink for feruloyl–SCoA and produce a derivative that can be incorporated into the lignification pathway to offset the deficit caused by decreased coniferyl alcohol. Nevertheless, it seems clear that, like the CAD-deficient mutant pine and the antisense-CAD tobacco, the antisense-CCR tobacco is making a copolymer that extracts as normal lignin and may serve many of the functions required of lignin

in the normal plant. It becomes a question of semantics whether or not to call this copolymer of tyramine ferulate and normal lignin monomers 'lignin,' but if it provides the plant with lignin's functionality, that seems appropriate. However, the CCR mutant does not have normal development. Clearly the functions required of lignin were not fully met by the reduced levels of this modified lignin. If the tyramine ferulate is intimately incorporated into the hydroxycinnamyl alcohol polymer, we have another example of the metabolic plasticity of plants to utilize other phenols to produce 'lignins' when normal monomers become limited. That the same units are used in certain stress and wounding responses perhaps indicates that this pathway can be up-regulated more quickly than the full lignin pathway to hydroxycinnamyl alcohols.

Conclusions

Down-regulating enzymes in the lignin monomer biosynthetic pathway may or may not reduce actual lignification. In the case of CCR, reduction was significant. In each down-regulated plant that has been structurally examined to date, the plant appears to compensate for its inability to produce (sufficient) normal lignin (lignin from the three hydroxycinnamyl alcohol monomers) by utilizing other phenols through up-regulation or redirection of other pathways. The materials isolated contain significant levels of new components. These 'lignin' isolations, performed equally for control and down-regulated plant samples, are relatively modest fractions of the total lignin, but clearly contain copolymers of normal lignin monomers and these new units. While we have good evidence regarding the structural claims for these modified lignins, their functional roles are not clear. The abnormality of heavily CCR down-regulated plants, which have 50% less lignin, does not clarify whether the plant's vigor is affected by lignin's quantity or its functional quality. Viability of mutants appears to be due to the plant's ability to produce lignin-like polymers with acceptable properties from unconventional components. Such a capability would appear to be evolutionarily wise and may be at least partly responsible for plants' abilities to remain viable despite mutations which severely curtail their normal biosynthetic pathways. If plants required and produced lignins exquisitely synthesized via highly controlled biochemical reactions, it would seem unlikely that they could, in a single generation, without the benefit of evolution, be viable when crucial components for that synthesis are heavily down-regulated. Although the plants' abilities to circumvent genetic obstacles by utilization of other components to make lignin interferes with our aims of significantly down-regulating lignification by genetic biotechnologies, it opens up enormous new potential for manipulation of lignin's composition and properties. Studies on lignin-biosynthetic-pathway-mutants will also provide a rich source of insight into the processes of lignification. In several instances now, down-regulation of the monolignol pathway has resulted in amplification of unusual units already present in low amounts in normal lignins.

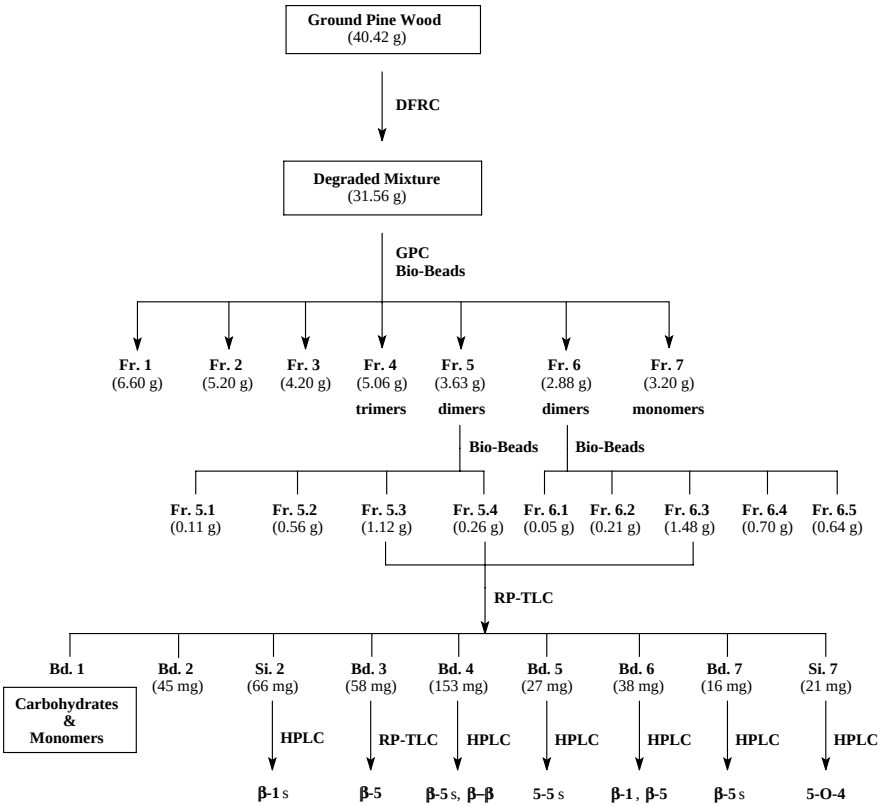


Figure 1. Separation scheme for lignin dimeric products released from DFRC-degraded pine wood.

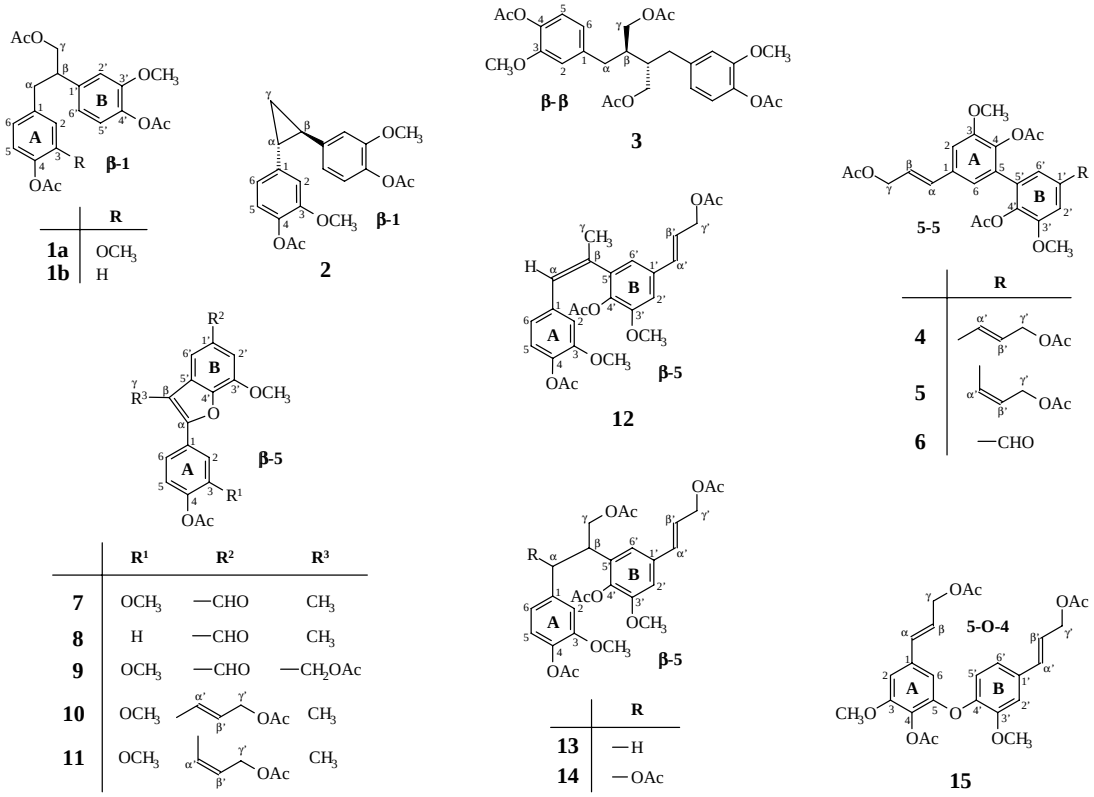


Figure 2. Structures of 16 lignin dimeric products isolated from DFRC-degraded pine wood. All of the common interunit linkages known in lignin are found in the 16 dimers.

wood mixture (about 4% of the lignin) and individual dimers accounted for from 0.01% to 0.1% of the wood.

Although there may be other minor dimers, the structures identified here provide insights into details of the pine lignin structure. Intact sidechains on most dimeric products provide information about the original linkages and where the ether linkages occur; the corresponding dimeric compounds produced by hydrogenolysis or thioacidolysis may lose such information. In the DFRC method, an unsaturated sidechain arises only if that sidechain was originally a b–ether, whereas any endgroup sidechain (–CH=CH–CH_2OH) produces quite different products following DFRC treatment. The 5–5-dimers **4**-**5** and the 5–O–4 dimer **15** can only come from two b–ether units which have coupled, i.e., from coupling of two pre-formed b–ether dimers or higher oligomers. Products from 5–5-dimerization of coniferyl alcohol could not be found — it is well known that coniferyl alcohol prefers coupling at its b–site, so such products are unlikely.

Aldehydes are well known for the famous phloroglucinol lignin staining reaction. Whether they are present in native lignins or are a product of the isolation procedure has been debated. Aldehydes are not created during the DFRC procedure. Therefore, the aldehydes in dimers **6**-**9** must have been present in the original lignin or created during the minimal preparation of the wood. We have found that cinnamaldehyde endgroups react reasonably efficiently under the DFRC procedures to give diagnostic products that can be located in small amounts in DFRC product chromatograms of lignins. Benzaldehydes come through the procedure intact or as benzyl acetates. Aldehydes appear in dimers from which they are reasonable products of radical coupling of the pre-formed aldehyde, but they could also arise from post-coupling oxidations. It is well established that vanillin favors coupling at the 5–site; 5,5-dehydrodivanillin can be prepared almost quantitatively from vanillin with peroxidase and hydrogen peroxide. The dimers isolated here contain the vanillin unit coupled at the 5–position. However, the identification of vanillin itself and vanillyl acetate in the monomers implies that it (or its cinnamaldehyde or coniferyl alcohol precursor) can also be 4–O–etherified.

Unusual benzofuran dimers **7**-**11** are also intriguing. Analogous compounds arise from ozonation reactions. While they clearly arise from b–5 units, the exact mechanism of their formation is not clear.

Currently, there is still some debate on the source of b–1 units; they can scarcely be found in NMR spectra of milled wood lignins, yet can be significant products in solvolysis dimers from the same lignins. The DFRC procedure appears to give b–1 products at similar levels to thioacidolysis.

Conclusion

The DFRC method can play a role in identifying traditional and novel or new lignin constituents. It has recently been invaluable in screening CAD-deficient pine mutants for increased levels of dihydroconiferyl alcohol-derived units. It produces extraordinarily clean products for b–aryl ether units in lignin, cleaving them efficiently, yielding low molecular weight lignin fragments. Other inter-unit structures in lignin are also altered by the DFRC conditions and, regrettably, each linkage type does not give a single product. However, the major, 5–5, b–b, b–1, b–5 and 5–O–4 structures are readily identified in the dimeric fraction, as are several minor b–5 structures with unusual benzofuran skeletons. As with the extended thioacidolysis method, quantification of these released dimers will provide useful insight into lignin structure and structural changes occurring under various chemical and biological treatments. Additionally, the minor products observed here and in the monomer fraction are interesting indicators of minor structural elements in lignins.

Now that dimers have been identified and have been shown to be reasonable products from the chemistry involved, we will optimize conditions for quantitation of major dimers from small samples. A combination of normal and reversed solid-phase extraction followed by GC-FID or GC-MS detection should allow development of a convenient method for dimers in small samples (~30-50 mg wood sample), once response factors are determined. Preliminary trials suggest that the DFRC-degraded products of loblolly pine wood, with the originally overwhelming carbohydrates and monomers removed by solid-phased extraction, give a clean profile with most dimers separated on the GC chromatogram. Hopefully, this emerging method can be utilized for identification, quantification, and comparison of dimers in wide-ranging plant materials.

Highly Selective Syntheses of Coniferyl and Sinapyl Alcohols

F. Lu and J. Ralph

Introduction

Coniferyl and sinapyl alcohols are the building blocks for lignin. Lignin is the plant cell wall polymer that provides the plant with structural integrity, water transport, and defense. It is also the polymer that limits the utilization of wall polysaccharides by ruminants, and is the component that must be removed in chemical pulping for paper production. Coniferyl and sinapyl alcohols are used in a variety of studies relating to forage chemistry as well as in wood chemistry.

A number of synthetic methods for them have been developed; every year in these Research Summaries, we come up with a better method! The alcohols were originally obtained from their corresponding cinnamates by lithium aluminum hydride reduction in moderate yield. Recently, more selective reducing agents such as diisobutylaluminum hydride have been used to improve selectivity and allow conversion of unprotected 4-hydroxycinnamates to alcohols. Other approaches have also been reported. All of them suffer from either low yields, undesirable contaminants, or difficult in handling reagents. Normally, coniferyl or sinapyl alcohol prepared from these methods is contaminated with varying amounts of the saturated alcohol which is hard to separate even by recrystallization. Now that the aldehydes are commercially available, their reductions provide the easiest way to prepare the hydroxycinnamyl alcohols. However, coniferyl or sinapyl alcohol prepared by the recently developed method, in which sodium borohydride ($NaBH_4$) is used as the reducing agent, is still contaminated with saturated alcohols. Here we report a facile and highly selective method to synthesize coniferyl or sinapyl alcohol from coniferaldehyde or sinapaldehyde by sodium triacetoxyborohydride ($NaBH(OAc)_3$) reduction in ethyl acetate in excellent yields. The method is simple and safe enough that non-chemists can readily make the requisite monolignols.

Experimental Procedures

Preparation of Sodium Triacetoxyborohydride

To $NaBH_4$ (74 mg) suspended in ethyl acetate (15 mL) and cooled with an ice water bath, was added glacial acetic acid (3.05 eq) by syringe over about 5 min. Stirring was continued for another 5 min. until a clear solution was formed. The solution is the ready-to-use reducing agent. For large scale (3.5 g), the addition of acetic acid was performed dropwise over about 30 min and stirring was continued for a further 30 min.

Coniferyl Alcohol 2a

Coniferaldehyde **1a** (134 mg, 0.753 mmol) was added to sodium triacetoxyborohydride (3.0 eq) in ethyl acetate, prepared as above. The reaction was monitored by TLC (5% CH_3OH in CH_2Cl_2). TLC showed that the starting material completely disappeared in 7 h. However, the reaction mixture was kept overnight, then diluted with ethyl acetate and quenched with water (20 mL). The organic phase was separated and the water fraction was extracted with ethyl acetate (2 x 20 mL). The combined ethyl acetate was washed with water (20 mL), sat. NH_4Cl (20 mL) and dried over $MgSO_4$. Evaporation of the solvent gave a light yellow syrup which still contained acetic acid. The residual acetic acid was removed by coevaporation with ethanol. ^1H NMR and GC of this crude **2a** (132 mg, 97%) showed no detectable 1,4-reduction product (detectability limit < 0.05%). Crystallization from CH_2Cl_2/petroleum ether gave **2a** as pale yellow plates (95 mg, 70%), mp 77.2-77.9 ° C. For large scale preparation (5 g), after addition of coniferaldehyde **1a**, the mixture was stirred overnight (~10 h) at room temperature. Work up as above afforded crude **2a** without any 1,4-reduction product detectable by GC. Crystallization from CH_2Cl_2/ petroleum ether led to pure **2a** as pale yellow plates in 77% yield.

Sinapyl Alcohol 2b

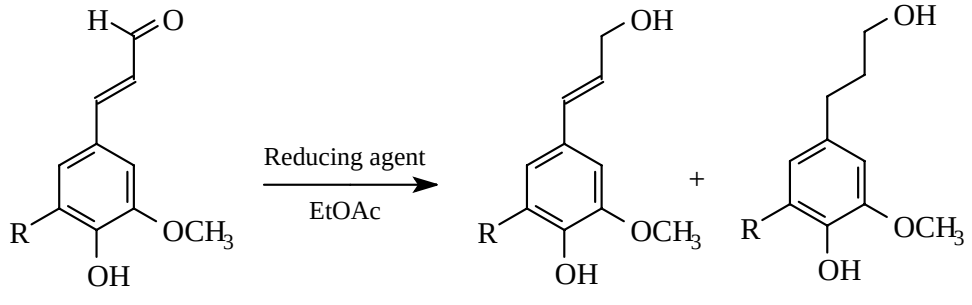
Sinapaldehyde **1b** (130 mg, 0.62 mmol) was reduced as described for **1a** to yield crude sinapyl alcohol **2b** as pale yellow syrup (120 mg, 91%). Again, no dihydrosinapyl alcohol could be detected by NMR or GC. For large scale preparation, sinapaldehyde **1b** (5.0 g, 24.0 mmol) was reduced overnight as described for **1a** to yield crude sinapyl alcohol **2b** as a pale yellow syrup (4.85 g, 96%). Crystallization of sinapyl alcohol is difficult. The product produced by this method is suitable for use without further purification.

Discussion

Sodium borohydride is a versatile and relatively mild reducing agent generally used for the reduction of aldehydes and ketones. However, reduction of conjugated aldehydes and ketones with sodium borohydride is highly solvent dependent and generally does not result in useful regioselectivity. It is not surprising that coniferyl alcohol prepared by sodium borohydride reduction of coniferaldehyde was contaminated with saturated coniferyl alcohol. GC showed that about 3% levels of saturated coniferyl alcohol were obtained when coniferaldehyde was reduced by sodium borohydride in ethyl acetate (Fig. 1).

Sodium (mono- and tri-)acetoxyborohydrides, prepared by adding controlled amounts of acetic acid to sodium borohydride in a solvent, reduced enones and enals in THF more selectively than the parent sodium borohydride. $NaBH(OAc)_3$ reduced aldehydes in the presence of ketones. $NaBH(OAc)_3$ was chosen for preparing coniferyl and sinapyl alcohols, even though it is weaker in reactivity than sodium monoacetoxyborohydride, because it is commercially available and more easily prepared *in situ*. Coniferaldehyde **1a** and sinapaldehyde **1b** were smoothly reduced to coniferyl alcohol **2a** and sinapyl alcohol **2b** by sodium triacetoxyborohydride, in ethyl acetate with no detectable 1,4-reduction product (Figure 1). The yields were 97% and 92% for **2a** and **2b**, respectively. Coniferyl alcohol **2a** was crystallized from methylene chloride/petroleum ether in 70% yield. Multigram quantities of coniferyl alcohol and sinapyl alcohol were prepared in the same way without any difficulty and coniferyl alcohol was easily crystallized in 77% yield. Attempts to crystallize sinapyl alcohol from methylene chloride/petroleum ether were unsuccessful. We and others have had this difficulty in the past and it is not necessarily indicative of less pure sinapyl alcohol. Crude sinapyl alcohol was pure enough to be used directly for making synthetic lignins or other purposes.

The major advantages of this method are: 1) the reducing agent, sodium triacetoxyborohydride, is either available from commercial sources or easily generated and used directly without requiring particular caution; 2) large scale preparations can be easily accomplished by this method with similar results; and 3) the expected products are prepared in high yields without any 1,4-reduction that produces the saturated alcohols that always exist in previously described methods. The products are used in a variety of studies ultimately aimed at enhancing the utilization of forages and improving sustainability.



1 **2** **3**

a R= H
b R= OCH_3

$NaBH(OAc)_3$	100	:	0 *
$NaBH_4$	97	:	3

*Figure 1. Reduction of coniferaldehyde **1a** and sinapaldehyde **1b**.*
The ratio was measured by GC; **3a-b were undetectable in products of $NaBH(OAc)_3$ reductions.*

Effect of Diferulate Cross-linking on the Hydrolysis of Xylans and Nonlignified Walls by a Fungal Enzyme Mixture Containing Feruloyl Esterase and Potent Xylanase Activities

J.H. Grabber, R.D. Hatfield and J. Ralph

Introduction

Ferulates are esterified to a-L-arabinose sidechains on xylans in grasses. During wall biosynthesis and lignification, xylans are cross-linked by oxidative coupling of ferulate monomers into dehydrodimers. In previous work (1996 Research Summaries), we found that diferulate cross-links reduced the rate and, to a lesser degree, the extent of cell wall hydrolysis by Celluclast and Viscozyme, a fungal enzyme mixture free of feruloyl esterase activity. Preliminary studies revealed that another fungal enzyme preparation, Biofeed Beta, contained 15 mU of feruloyl esterase activity per mg of solid, using feruloylated arabinose as a substrate. It was also observed that a mixture of Biofeed and Celluclast degraded xylans at a two-fold greater rate than Viscozyme and Celluclast. The degradation of cellulose was similar for both enzyme mixtures. Therefore, the Biofeed and Celluclast mixture was used to determine whether the inhibitory effects of diferulate cross-linking on wall hydrolysis could be mitigated by a fungal enzyme mixture containing feruloyl esterase and high xylanase activities.

Methods

Nonlignified cell suspensions of maize (*Zea mays*) were grown with 0 or 40 mM 2-aminoindan-2-phosphonic acid (AIP) to produce walls with normal (17.2 mg g^{-1}) or reduced (5.1 mg g^{-1}) ferulate concentrations. Walls were then incubated with mercaptoethanol to inhibit diferulate formation or with hydrogen peroxide to stimulate diferulate formation by wall bound peroxidases. A xylan-rich indigestible residue fraction and an 80% EtOH insoluble xylo-oligosaccharide fraction were isolated from the hydrolysate of hydrogen peroxide treated cell walls degraded for 72 h with Viscozyme and Celluclast (each added at 0.04 mL mg^{-1} of cell wall). Cell walls and wall fractions were then degraded with Biofeed Beta (CT form, 0.04 mg mg^{-1} of cell wall) and Celluclast 1.5 L (0.04 mL mg^{-1} of cell wall). Periodically, hydrolysates were clarified by centrifugation and an aliquot was analyzed for total sugars or reducing sugars by colorimetric procedures. Ferulates released from walls or wall fractions by saponification or esterases were quantified by GC-FID.

Results

Xylo-oligosaccharides and xylan-rich indigestible residue fractions (Table 1) were hydrolyzed with a Biofeed and Celluclast mixture containing 0.6 mU of feruloyl esterase activity per mg of substrate, theoretically enough activity to completely release all ferulates within 5 to 9 h of incubation. A 24 h incubation released 168 mg g^{-1} of reducing sugars from the oligosaccharide fraction and 737 mg g^{-1} of total carbohydrate from the indigestible residue fraction. Substantial quantities of ferulate monomers but only small amounts of 5-5 and 8-5 coupled diferulates were released from the fractions. As noted in earlier work with Viscozyme and Celluclast, hydrogen peroxide/peroxidase-mediated coupling of ferulate monomers into dehydrodimers reduced carbohydrate solubilization from walls after 3 h of hydrolysis with Biofeed and Celluclast. Differences were not significant after 54 h of hydrolysis (Table 2). Walls with similar cross-linking (2.62 vs 2.25 mg g^{-1} of diferulates) but substantially different ferulate substitution (17.15 vs 4.52 mg g^{-1} of total ferulates) had roughly the same degradability. Although diferulate cross-linking reduced the initial hydrolysis of walls by both enzyme mixtures, degradation was more rapid and extensive with Biofeed and Celluclast, particularly for walls with high levels of diferulate cross-linking.

Discussion and Conclusions

This study provides additional evidence that the rate of wall degradation is restricted by diferulate cross-linking of xylans. In contrast, simple feruloylation of

xylans does not appear to influence cell wall hydrolysis. The activity of feruloyl esterases on diferulates was extremely low, even on soluble substrates. Therefore, the effects of feruloyl esterases on cell wall degradation are probably nil. However, our results indicate that the inhibitory effects of diferulate cross-linking on wall hydrolysis may be partially overcome if enzyme mixtures contain high xylanase activity.

Impact Statement

Research of this kind provides a unique means of elucidating factors which limit efficient utilization of cell walls for nutritional and industrial purposes. Ultimately, these studies should allow rational approaches to maximizing plant utilization and farm sustainability while minimizing adverse impacts on the environment.

Table 1. Ferulate and diferulate composition of nonlignified walls and wall fractions recovered after a 72 h incubation with Viscozyme and Celluclast. Values in parentheses indicate the percentage of each constituent released as free acids from wall fractions after a 24 h incubation with Biofeed and Celluclast. Data represent the means of duplicate analyses.

			(*E*)-Diferulates			
	(*Z*)-Ferulate	(*E*)-Ferulate	8-8	8-5	8-*O*-4	5-5
	- mg g^{-1} -					
Cell wall	2.51	6.64	1.05	5.17	1.53	1.24
Xylo-oligosaccharides	1.69 (0)	9.74 (83)	3.08 (0)	13.83 (12)	3.65 (0)	4.27 (24)
Xylan-rich indigestible residues	7.21 (0)	21.90 (52)	3.22 (0)	17.92 (8)	6.00 (0)	5.66 (13)

Table 2. Ferulate concentration and degradability of structural carbohydrates (SC) in nonlignified cells (n = 2). Feruloylation of walls was manipulated by growing cell suspensions with and without AIP, a specific inhibitor of phenylalanine ammonia lyase. Peroxidase-mediated coupling of ferulate monomers into dimers was limited by isolating and incubating walls with mercapoethanol or stimulated by incubating walls with H_2O_2. Walls were hydrolyzed with a mixture of Biofeed and Celluclast.

		Ferulates			Carbohydrate released	
AIP	H_2O_2	monomers	dimers	total	3 h	54 h
mM	mmol	- - - - mg g^{-1} cell wall - - - - -			- - - - mg g^{-1} SC - - - -	
Normal feruloylation						
0	0	14.53	2.62	17.15	546	871
0	0.4	8.96	6.65	15.61	416	856
Low feruloylation						
40	0	3.75	1.31	5.06	570	898
40	0.4	2.27	2.25	4.52	511	916
Analysis of Variance						
AIP		*	*	*	*	*
H_2O_2		*	*	*	*	NS
AIP X H_2O_2		*	*	NS	NS	NS

*, NS Significant at the 0.05 level of probability and not significant, respectively.

A Comparison of Tifton 85 and Coastal Bermudagrass Cell Walls

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Introduction

Bermudagrass is well adapted to the warm humid climates of the southeastern states allowing good biomass production either as hay or in grazing situations. However, fiber digestibility is poor resulting in limited animal performance. Selection for improved bermudagrass resulted in the development of Tifton 85 with improved digestibilities and animal performance compared to Coastal bermudagrass. Fiber analysis indicated Tifton 85 had slightly higher NDF and ADF, yet higher digestiblity compared to Coastal bermudagrass. We evaluated these two bermudagrass lines for cell wall characterisitcs that might explain the differences routinely observed in animal performance.

Methods

Plants were grown at Tifton, GA and harvested at two stages of regrowth, 3 and 6 weeks. Cell walls were isolated from ground samples (1 mm) using a modified Uppsala Fiber Isolation procedure. Isolated walls were analyzed for total neutral sugars, total uronosyls, acid insoluble lignin, neutral sugar composition, and hydroxycinnamic acids.

Results and Discussion

A comparison of cell wall characteristics of Tifton 85 and Coastal bermudagrass revealed several differences between these two lines. Table 1 shows the total cell wall, lignin, neutral sugar and total uronosyl content of the two lines. Major wall constituents increased from 3 and 6 week harvest times for Tifton 85 and Coastal bermudagrass, as one would expect with increased maturity of the plants. However, there was no increase in the acid insoluble lignin in the Coastal line indicating that the small increase in the cell wall fraction was due to structural carbohydrates (Table 1). Total uronosyls did not change with maturity. A comparison of Coastal to Tifton 85 indicated the total cell wall fraction and acid insoluble lignin were less in the Tifton 85 line yet there were higher amounts of total neutral sugars. This result differed from earlier fiber analysis that indicated Tifton 85 had slightly higher NDF and ADF levels yet lower permanganate lignin. A comparison of the neutral sugar composition of isolated walls (Fig. 1) indicated that Tifton 85 had a higher glucose content than Coastal. The higher glucose content would support earlier findings of higher ADF levels since the major carbohydrate in ADF is cellulose. Part of the problem in comparing fiber analysis based on the detergent system with the analysis scheme used here is that hot detergent solutions (especially acid detergent) solubilize significant amounts of lignin and wall carbohydrates (most likely as lignin carbohydrate complexes). The earlier observed higher NDF values for Tifton 85 may be due to less lignin complexes and higher cellulose content that would not be solubilized by the hot detergent solutions.

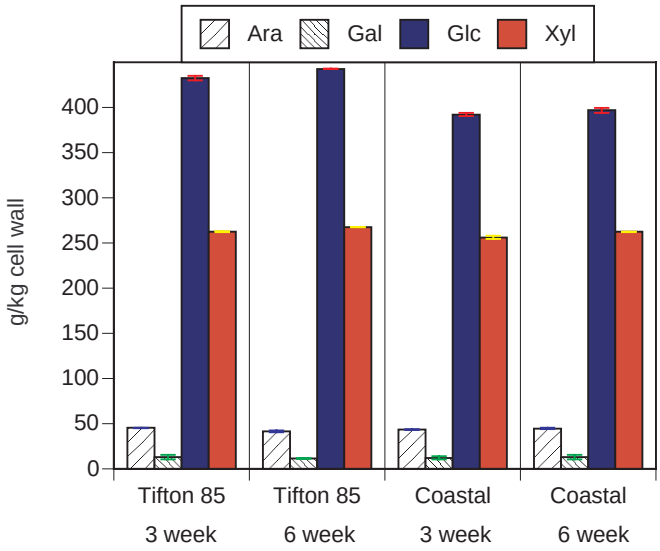

Figure 1. Neutral sugar composition of Tifton 85 and Coastal bermudagrass harvested after 3 and 6 weeks of regrowth.

The hydroxycinnamic acid composition of the walls was also similiar (Table 2) at the two harvest dates and between the two lines of bermudagrass. It is important to note there were no differences in the *p*-coumarates and ferulates (monomers and dimers) that were simply ester linked to wall components. However, ferulates that were also etherified were different between Tifton 85 and Coastal lines and appear to change with maturity of the plants. The more mature Coastal line had higher levels of total ether linked ferulates. It is important to realize that the number of possible chemical bonds between ferulates and lignin residues is not limited to simple ether linkages; therefore, it is impossible to determine the total amount of ferulates cross-linked to lignin. However, it seems likely that the portion released is indicative of the total bonding pattern within the wall matrices.

Taken together, these results indicate that the improved digestibility and animal performance of Tifton 85 is most likely due to lower lignin concentrations coupled with lower levels of crosslinked polysaccharides based on the lower levels of ferulates ether linked to lignin. In addition, Tifton 85 has a significantly higher concentration of cellulose in its wall matrices. Since there is no strong evidence that other wall components are directly cross-linked to cellulose, increased quantities of cellulose within a matrix with equal or less lignin should lead to a more digestible wall.

Table 1. General cell wall characteristics of Tifton 85 and Coastal Bermudagrass.

		g kg^{-1} DM		g kg^{-1} of cell wall		
Sample	Harvest	DM	CW	AIL	TU	Tot. NS
Tifton 85	3 wk	879.2 ± 1.3	778.9 ± 8.1	174.5 ± 0.7	20.5 ± 1.6	753.4 ± 3.2
Tifton 85	6 wk	872.3 ± 3.6	785.0 ± 2.6	177.5 ± 1.0	20.4 ± 1.0	763.2 ± 1.3
Coastal	3 wk	827.9 ± 2.8	820.8 ±10.7	202.8 ± 2.6	20.4 ± 0.5	704.1 ± 7.5
Coastal	6 wk	848.7 ± 0.9	832.1 ± 9.6	200.1 ± 2.3	20.7 ± 0.2	716.9 ± 3.9

Table 2. Total hydroxycinnamic acids released from Tifton 85 and Coastal bermudagrass. Room temperature alkaline hydrolysis will release all hydroxycinnamates linked to wall components by a simple ester linkage, while the high temperature alkaline hydrolysis (4M at 170 °C for 2 h) releases all ester and simple ether linked hydroxycinnamates. Determination of hydroxycinnamates linked to wall components by both ester and ether linkages was made by subtracting the room temperature hydrolysis from the high temperature hydrolysis. (*p*CA= *p*-coumarates, FA= ferulates, diFA= dehydrodiferulates, Tot FA = total ferulates)

		g k^{-1} CW							
		Ester Linked				Ether Linked			
Sample	Harvest	pCA	FA	diFA	Tot FA	pCA	FA	diFA	Tot FA
Tifton 85	3 wk	9.9	6.7	4.1	10.7 ± 0.7[a]	0.8	3.1	2.8	5.9 ± 0.4
Tifton 85	6 wk	10.7	6.1	4.7	10.8 ± 0.5	0.5	4.0	3.6	.5 ± 0.2
Coastal	3 wk	10.0	5.9	4.1	9.9 ± 0.4	0.5	2.9	4.6	7.4 ± 3.1
Coastal	6 wk	10.2	5.9	4.6	10.5 ± 0.9	0.9	4.5	4.8	9.3 ± 0.2

[a]Standard error of the mean for duplicate samples

Publications

Akayezu, J.M., Jorgensen, M., Linn, J.G., **Jung, H.G.** 1997. Effects of substituting alfalfa leaf meal for alfalfa hay in diets of early laction cows. J. Dairy Sci. 80(Suppl. 1):184.

Ayisi, K.K., Putnam, D.H., Vance, C.P., **Russelle, M.P.**, Allan, D.L. 1997. Strip intercropping and nitrogen effects on seed, oil, and protein yields of canola and soybean. Agron. J. 89(1):23-29.

Bidlack, D.R., **Buxton, D.R.**, Shibles, R.M., Anderson, I.C. 1997. Phenylalanine ammonia lyase and syringaldazine oxidase activities in relation to lignin deposition in legumes. Recent Res. Devel. in Agron. P. 1-8.

Bliss, D., **Jung, H.**, Lowry, A., Savik, K., Jensen, L., Lemoine, M., Werner, C. 1997. Effects of dietary fiber for fecal incontinence (FI). The Gerontologist 37:325.

Blumenthal, J.M., **Russelle, M.P.**, Vance, C.P. 1997. Localized and internal effect of nitrate on symbiotic dinitrogen fixation. Physiol. Plantarum. 101:59-66.

Blumenthal, J.M., **Russelle, M.P.**, Bernasconi, C., Vance, C.P. 1997. Ineffective nodulation modifies nitrate uptake kinetics. Plant Physiol. 114:68.

Blumenthal, J.M., **Russelle, M.P.**, Lamb, J.F.S. 1997. Subsoil nitrate and bromide uptake in constrasting alfalfa germplasms. Agron. Abstracts. P. 235.

Blumenthal, J.M., **Russelle, M.P.**, Vance, C.P. 1997. Nitrogenase activity as affected by reduced partial pressures of dinitrogen and nitrate. Plant Physiol. 114:1405-1412.

Borton, L.R., **Rotz, C.A.**, Black, J.R., Allen, M.S., Lloyd, J.W. 1997. Alfalfa and corn silage systems compared on Michigan dairy farms. J. Dairy Sci. 80:1813-1826.

Broderick, G.A., Clayton, M.K. 1997. A statistical evaluation of animal and nutritional factors influencing concentrations of milk urea nitrogen. J. Dairy Sci. 80:2964-2971.

Broderick, G.A., Albrecht, K.A. 1997. Ruminal in vitro degradation of protein in tannin-free and tannin-containing species. Crop Sci. 37:1884-1891.

Chan, S.C., Huber, J.T., Theurer, C.B., **Wu, Z.**, Chen, K.H., Simas, J.M. 1997. Effects of supplemental fat and protein source on rumen fermentation and nutrient flow to the duodenum in dairy cows. J. Dairy Sci. 80:152-159.

Chan, S.C., Huber, J.T., Chen, K.H., Simas, J., **Wu, Z.** 1997. Effect of ruminally inert fat and evaporative cooling on dairy cows in hot environmental temperatures. J. Dairy Sci. 80:1172-1178.

Chan, S.C., Huber, J.T., Theurer, C.B., **Wu, Z.**, Chen, K.H., Simas, J.M. 1997. Effects of supplemental fat and protein source on rumen fermentation and nutrient flow to the duodenum in dairy cows. J. Dairy Sci. 80:152-159.

Chen, J., **Weimer, P.J.** 1997. Competition among three strains of ruminal cellulolytic bacteria in continuous culture. 24th Conf. on Rumem Function. P. 4.

Cotta, M.A., **Russell, J.B.** 1997. Nitrogen Compounds in Gastrointestinal Environments. In: R.I. Mackie and B.A. White (eds.), Vol. 1, Gastrointestinal Ecosystems and Fermentations, Chapman & Hall, NY: P. 380-423.

Delgado, N.J., Castler, M.D., Grau, C.R., **Jung, H.G.** 1997. Do lignin and ferulic acid concentration have an impact on disease resistance in smooth bromegrass? Agron. Abstr. P. 74.

Dhiman, T.R., **Satter, L.D.** 1997. Yield response of dairy cows fed different proportions of alfalfa silage and corn silage. J. Dairy Sci. 80:2069-2082.

Dhiman, T.R., Korevaar, A.C., **Satter, L.D.** 1997. Particle size of roasted soybeans and the effect on milk production of dairy cows. J. Dairy Sci. 80:1722-1727.

Dhiman, T.R., **Satter, L.D.** 1997. Effect of ruminally degraded protein on protein available at the intestine assessed using blood amino acid concentrations. J. Anim. Sci. 75:1674-1680.

Dhiman, T.R., **Kanneganti, V.R.**, **Walgenbach, R.P.**, Massingill, L.J., Wiltbank, M.C., **Russelle, M.P.**, **Satter, L.D.** 1997. Production response to feed supplementation of dairy cows in a seasonal calving and grazing system. 18th Intl. Grassland Congress, Saskatoon, Canada. P. 71-72.

Dhiman, T.R., **Kanneganti, V.R.**, **Satter, L.D.**, **Walganbach, R.P.**, Massingill, L.J. 1997. Supplementation of roasted soybeans to dairy cows on pasture. J. Dairy Sci.(80)(Suppl. 1):222.

Dhiman, T.R., **Satter, L.D.**, Pariza, M.W., Galli, M.P., Albright, K. 1997. Conjugated linoleic acid (CLA) content of milk from cows offered diets rich in linoleic and linolenic acid. J. Dairy Sci. 80(Suppl. 1):184.

Diez-Gonzalez, F., **Russell, J.B.**, Hunter, J.B. 1997. The acetate kinase of Colstridium acetobutylicum strain P262. Arch. Microbiol. 166:418-420.

Diez-Gonzalez, F., **Russell, J.B.**, Hunter, J.B. 1997. NAD-independent lactate and butyryl CoA dehydrogenases of Clostridium acetobutylicum P262. Curr. Microbiol. 34:162-166.

Diez-Gonzalez, F., **Russell, J. B.** 1997. The ability of Escherichia coli O157:H7 to decrease its intracellular pH and resist the toxicity of acetic acid. Microbiol. 143:1175-1180.

Diez-Gonzalez, F.,.**Russell, J. B.** 1997. The effect of carbonylcyanide-m-chlorophenylhydrzone (CCCP) and acetate on Escherichia coli O157:H7: uncoupling versus anion accumulation. FEMS Microbiol. Lett. 151:71-76.

Ekinci, C., **Broderick, G.A.** 1997. Effect of processing high moisture corn on ruminal fermentation and milk production. J. Dairy Sci. 80:3298-3307.

England, M.L., **Broderick, G.A.**, Shaver, R.D., Combs, D.K. 1997. Comparison of in situ and in vitro techniques for measuring ruminal degradation of animal by-product proteins. J. Dairy Sci. 80:2925-2931.

Falkner, L.K., Ostrander, B., Coors, J.G., Kaeppler, S.M., **Hatfield, R.D.**, Shaver, R.D. 1997. Lax leaf corn: a low lignin mutant for high digestibility silage. 89th Annual Mtg., American Soc. Agron., Crop Sci. Soc. of America, Soil Sci. Soc. of America, Anaheim, CA.

Fields, M.W., **Russell, J.B.**, Wilson, D.B. 1997. A mutant of Prevotella ruminicola B14 deficient in b-1,4-endoglucanase and mannanase activities. FEMS Microbiol. Lett. 154:9-15.

Fontes, Jr., C., Meserole, V.K., Mattos, W., Barros, R.P., **Wu, Z.**, Huber, J.T. 1997. Response of Brazilian crossbred cows to varying doses of bovine somatotropin. J. Dairy Sci. 80:3234-3240.

Gardner, R.G., Wells, J.E., Fields, M.W., Wilson, D.B., **Russell, J.B.** 1997. A Prevotella ruminicola B14 operon encoding extracellular polysaccharide hydrolases. Curr. Micorbiol. 35:274-277.

Grabber, J., **Ralph, J.**, **Hatfield, R.D.**, Quideau, S. 1997. p-hydroxypenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. J. Agric. Food Chem. 45:2530-2532.

Hatfield, R., **Ralph, J.** 1997. Acetyl bromide method for quantifying lignin: a cautionary note. 9th International Symp. on Wood and Pulping Chem., Montreal, Quebec. June 9-12.

Hatfield, R.D., Grabber, J., **Ralph, J.** 1997. A potential role of sinapyl p-coumarate in grass lignin formation. American Soc. of Plant Physiologists Abstracts, Vancouver, British Columbia.

Heintzman, A.K., **Russelle, M.P.**, Johnson, M.R. 1997. Calibration of CERES-maize in the upper Midwest. Agron. Abstracts. P. 228.

Huber, J.T., **Wu, Z.**, Fontes, J., C., Sullivan, J.L., Hoffman, R.G., Hartnell, G.F. 1997. Administration of recombinant bovine somatotropin for four consecutive lactations. J. Dairy Sci. 80:2355-2360.

Huhnke, R.L., **Muck, R.E.**, Payton, M.E. 1997. Round bale silage storage losses of ryegrass and legume-grass forages. Trans. ASAE. 13:451-457.

Huhtanen, P., Brotz, P.G., **Satter, L.D.** 1997. Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. J. Anim. Sci. 75:1380-1392.

Iragavarapu, T.K., Randall, G.W., **Russelle, M.P.** 1997. Yield and nitrogen uptake of rotated corn in a ridge-tillage system. Agron. J. 89:397-403.

Irwin, J.A.G., Maxwell, D.P., **Smith, R.R.** 1997. Expression of resistance by tetraploid and diploid lucerne genotypes to Phytophthora medicaginis is not influenced by inoculum level or temperature. Aust. J. Agric. Res. 48:181-190.

Jung, H.G. 1997. Analysis of forage fiber and cell walls in ruminant nutrition. J. Nutr. 127:810S-813S.

Jung, H.G., **Mertens, D.R.**, Payne, A.J. 1997. Correlation of acid detergent lignin and Klason lignin with digestibility of forage dry matter and neutral detergent fiber. J. Dairy Sci. 80:1622-1628.

Jung, H.G., Sheaffer, C.C., Barnes, D.K., Halgerson, J.L. 1997. Forage quality variation in the U.S. alfalfa core collection. Crop Sci. 37:1361-1366.

Jung, H.G., Varel, V.H. 1997. Comparison of Klason lignin and ADL by calorimetry. J. Dairy Sci. 80(Suppl. 1):159.

Jung, H.G., **Ralph, J.**, **Hatfield, R.D.**, Grabber, J.H., Ni, W. 1997. Ferulate cross-linking in grasses: chemistry, impact on degradability, and genetic manipulation. 7th Scottish Cell Mtg. P. 1-2.

Jung, H.G., Engels, F.M. 1997. Impact of tissue maturation on cell wall degradability of alfalfa stems. Conf. of Rumen Function. 24:19.

Kanneganti, V.R., Dhiman, T.R., Massingill, L.J., **Walgenbach, R.P.**, **Russelle, M.P.**, **Satter, L.D.** 1997. Seasonal distribution of forage yield from a "natural" pasture under rotational grazing. 18th Intl. Grassland Congress, Saskatoon, Saskatchewan, Canada. P. 75-76.

Kanneganti, V.R., **Walgenbach, R.P.**, Bland, W.L., Undersander, D.J., **Rotz, C.A.** 1997. Prediction of alfalfa forage yield loss due to freezing injury. Agron. Abstracts. P. 12.

Koegel, R.G., Sreenath, H.K., Straub, R.J. 1997. Liquid hot water pretreatment of alfalfa fiber destined for ethanol production. ASAE Paper # 97-1087.

Kraus, T.J., **Koegel, R.G.**, Straub, R.J., Shinners, K.J. 1997. Leachate conductivity as an index for quantifying level of forage conditioning. ASAE Paper #97-1100.

Kraus, T.J., **Koegel, R.G.**, **Mertens, D.R.**, Straub, R.J. 1997. Intensive mechanical forage conditioning: relationship to increased animal production. ASAE Paper #97-1085.

Kuehn, C.S., Linn, J.G., **Jung, H.G.** 1997. Effect of corn silage chop length on intake, milk production, and milk composition of lactating diary cows. J. Dairy Sci. 80(Suppl. 1):159.

Kung, Jr., L., **Muck, R.E.** 1997. Animal response to silage additives. In Silage: Field to Feedbunk: NRAES-99. Northeast Reg. Agr. Engineering Ser., Ithaca, NY.

Lamb, J.F.S., Magarian, D.M., **Russelle, M.P.** 1997. Gain from one cycle of divergent selection of nitrate-N uptake in alfalfa using bromide uptake as the selection criterion. Central Alfalfa Improv. Conf. P. 39.

Lamb, J.F.S., **Russelle, M.P.** 1997. Selection for geotropic root elongation rate in alfalfa under field conditions. Central Alfalfa Improv. Conf. P. 47.

Lana, R.P., **Russell, J.B.** 1997. Effect of forage quality and monensin on the ruminal fermentation of fistulated cows fed continuously at a constant intake. J. Anim. Sci. 75:224-229.

Lana, R.P., Fox, D.G., **Russell, J.B.**, Perry, T.C. 1997. Influence of monension on Holstein steers fed high-concentrate diets containing soybean meal or urea. J. Anim. Sci. 75:2571-2579.

Lana, R.P. 1997. Effects of monensin on ruminal bacteria, ruminal fermentation and feedlot performance. Ph. D. (with **J. B. Russell**), Cornell Univ.

Lu, F., **Ralph, J.** 1997. Efficient ether cleavage in lignins: the "DFRC" method as a basis for new analytical methods. In N.G. Lewis and S. Sarkanen, (eds.). Lignin and Lignan Biosynthesis, American Chem. Soc.

Lu, F., **Ralph, J.** 1997. Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. J. Agric. Food Chem. 45(7):2590-2592.

Lu, F., **Ralph, J.** 1997. The DFRC method for lignin analysis. Part 1. A new method for b-aryl ether cleavage: lignin model studies. J. Argic. Food Chem. 45(12):4655-4660.

Lu, F., **Ralph, J.** 1997. The "DFRC" method for lignin analysis. Protocol for analysis of DFRC monomers. J. Agric. Food Chem. 45(7):2590-2592.

Lu, F., **Ralph, J.** 1997. The "DFRC" method for lignin analysis. Protocol for analysis of DFRC monomers. 9th International Symp. on Wood and Pulping Chem., Montreal, Quebec.

Luchini, N.D., **Broderick, G.A.**, **Muck, R.E.**, Makoni, N.R., Vetter, R.L. 1997. Effect of storage system and dry matter content on composition of alfalfa silage. J. Dairy Sci. 80:1827-1832.

Luchini, N.D., **Broderick, G.A.**, Hefner, D.L., Derosa, R., Reynal, S., Treacher, R. 1997. Production response to treating forage with fibrolytic enzymes prior to feeding to lactating cows. J. Dairy Sci. 80(Suppl. 1):262.

Maglione, G., **Russell, J.B.**, Wilson, D.B. 1997. Kinetics of cellulose digestion by *Fibrobacter succinogenes* S85. Appl. Environ. Microbiol. P. 665-669.

Makoni, N.F., **Broderick, G.A.**, **Muck, R.E.** 1997. Effect of modified atmospheres on proteolysis and fermentation of ensiled alfalfa. J. Dairy Sci. 80:912-920.

Mertens, D.R. 1997. Creating a system for meeting the fiber requirements of dairy cows. J. Dairy Sci. (80):1463-1481.

Mertens, D.R., **Weimer, P.J.**, Waghorn, G.C. 1997. Inocula differences affect in vitro fiber digestion kinetics. J. Dairy Sci. 80(Suppl. 1):185.

Mertens, D.R. 1997. Comparing systems of ration formulation for dairy cows. J. Dairy Sci. 80(Suppl. 1):211.

Mertens, D.R., **Weimer, P.J.** 1997. Method for measuring gas production kinetics. Inter. Symp. on In Vitro Techniques for Measuring Nutrient Supply to Ruminants. Br. Soc. Anim. Sci. Programme and Summaries. P. 44.

Mertens, D.R., **Weimer, P.J.**, Waghorn, G.C. 1997. Inocula differences affect in vitro gas production kinetics. Inter. Symp. on In Vitro Techniques for Measuring Nutrient Supply to Ruminants. Br. Soc. Anim. Sci. Programme and Summaries. P. 15.

Micard, V., Grabber, J.H., **Ralph, J.**, Renard, C.M.G.C., Thibault, J.-F. 1997. Diferulic acids from sugar-beet pulp. Phytochemistry 44(7):1365-1368.

Muck, R.E., Kung, Jr., L. 1997. Effects of silage on ensiling. In Silage: Field to Feedbunk, NRAES-99. Northeast Reg. Agr. Engineering Ser., Ithaca, NY. P. 187-199.

Nadeau, M.G., **Buxton, D.R.** 1997. Cellulase and bacterial inoculant effects on cocksfoot and lucerne ensiled at high dry matter levels. J. Sci. Food Agric. 73:369-376.

Oosterveld, A., Grabber, J.H., Beldman, G., **Ralph, J.**, Voragen, A.G.J. 1997. Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. Carbohydrate Res. 300(2):179-181.

Quideau, S., **Ralph, J.** 1997. Lignin-ferulate cross-links in grasses. Part 4. Incorporation of 5-5-coupled diferulate into lignin. J. Chem. Soc., Perkin Trans. 1:2351-2358.

Ralph, J., MacKay, J.J., **Hatfield, R.D.**, O'Malley, D.M., Whetter, R.W., Sederoff, R.R. 1997. Abnormal lignin in a loblolly pine mutant. Science. 277:235-239.

Ralph, J., **Hatfield, R.D.**, Grabber, J.H. 1997. Ferulates and diferulates as nucleation sites for lignification in grasses. Polyphenols Actualities. P. 4-6.

Ralph, J. 1997. "Fake plactic trees." In Chem. and Ind. P. 708.

Ralph, J. 1997. NMR applications: Determination of lignin structure and matrix interactions. 9th International Symp. on Wood and Pulping Chem. Presymposium workshop on Advances in NMR spectroscopy of lignocellulosic complexes, Montreal, Quebec. June 9-12.

Ralph, J. 1997. Recent advances in characterizing 'non-traditional' lignins and lignin-polysaccharide cross-linking. 9th International Symp. on Wood Pulping Chem., Montreal, Quebec. June 9-12.

Ralph, J., Han, J., **Hatfield, R.**, Ralph, S., **Jung, H.G.**, Lu, F. 1997. Kenaf's unusual lignin. Fifth Chem. Cong. of North America, Cancun, Mexico. Nov. 11-15.

Ralph, J., **Hatfield, R.D.**, Grabber, J.H., **Jung, H.G.**, Quideau, S., Helm, R.F. 1997. Cell Wall Cross-linking in Grasses by Ferulates and Diferulates. In: Sarkanes, N.G. Lewis and S. Sarkanen (eds.). Lignin and Lignan Biosynthesis, American Chem. Soc.

Randall, G.W., Huggins, D.R., **Russelle, M.P.**, Fuchs, D.J., Nelson, W.W., Anderson, J.L. 1997. Nitrate losses through subsurface tile drainage in Conservative Reserve Program, alfalfa, and row crop systems. J. Environ. Qual. 26:1240-1247.

Randall, G.W., Schmitt, M.A., **Russelle, M.P.**, Greub, L.J., Clayton, P.D., Ivers, D.R. 1997. Nitrate accumulation in the soil profile and N removal by reed canarygrass as affected by rates of dairy manure. Agron. Abstracts. P. 224.

Rotz, C.A. 1997. Intgration of forage in farming systems. Proc. XVIII Int. Grassl. Congr., Saskatoon, Saskatchewan, Canada.

Rotz, C.A., Harrigan, T.M. 1997. Economics of silage based cropping systems. Silage: field to feedbunk, NRAES-99, NRAES, 152 Riley-Robb Hall, Ithaca, NY. P. 3-12.

Rotz, C.A. 1997. Corn silage in the whole farm system. 21st Forage Production and Use Symp., Wisconsin Forage Council, DeForest, WI. P. 96-104.

Rotz, C.A. 1997. Economics of forage processing. J. Anim. Sci. 75(Suppl. 1):140.

Russell, J.B., Wallace, R.J. 1997. Energy consuming and yielding mechanisms. In: P.N. Hobson and C.S. Stewart (eds.). The Rumen Microbial Ecosystem, Blackie Academic and Professional Press, London. P. 246-282.

Russell, J.B., Wells, J.E. 1997. The ability of 2-deoxyglucose to promote the lyis if *Streptococcus bovis* via a mechanism involving cell wall stability. Curr. Microbiol. 35:299-304.

Russell, J.B. 1997. Mechanisms of action of ionophores. Cornell Nutr. Conf. for Feed Manufacturers, Rochester, NY.

Russelle, M.P. 1997. Nitrogen loss from pastures. Proc. 21st Forage Production and Use Symp. Wisconsin Forage Council, Wisconsin Dells, WI, Jan. 28-29. P. 83-92.

Russelle, M.P. 1997. Nutrient management of humid, temperate region forages: Recommendations and practices. Proc. 18th Int. Grassland Cong., Winnipeg and Saskatoon, Canada.

Russelle, M.P., **Satter, L.D.**, Dhiman, T.R., **Kanneganti, V.R.**, Johnson, D.G., Garcia, A.D., Nord, W.S. 1997. Nitrogen cycling in pastures grazed by lactating dairy cows. Proc.18th Intl. Grassland Congress, Saskatoon, Canada. P. 11-12.

Russelle, M.P., Johnson, D.G., **Satter, L.D.**, Dhiman, T.R., **Kanneganti, V.R.**, Garcia, A.D., Nord, W.S. 1997. Nitrogen cycling in management intensive grazing. Agron. Abstracts. P. 31.

Russelle, M.P. 1997. Nitrogen flow in intensively managed pasture systems. US Dairy Forage Res. Center Conf. Dairy & Forage Industries. P. 73-80.

Russelle, M.P. 1997. Nutrient cycling in pastures. International Symp. on Animal Production Under Grazing, Univ. of Vicosa, MG, Brazil. P. 235-266. Nov. 4-6.

Russelle, M.P. 1997. Nutrient management of perennial forages on Minnesota dairy farms. Agron. Abstracts. P. 228.

Russelle, M.P., Randall, G.W., Clayton, P.D., Schmitt, M.A., Greub, L.J., Sheaffer, C.C., Kalton, R.R., Taylor, D.H. 1997. Reed canarygrass (*Phalaris arundinacea* L.) response to dairy manure or fertilizer N. Proc.18th Int. Grassland Congr., Winnipeg and Saskatoon, Canada. P. 53-54.

San Emeterio, F., Reis, R.B., **Satter, L.D.**, Combs, D.K. 1997. Comparison of ground shelled corn and high moisture ear corn of two particle sizes for their effects on milk production and rumen fermentation when cows are fed green chopped grass-legume forage. J. Dairy Sci. 80(Suppl. 1):166.

Santos, F.A.P., Huber, J.T., Theurer, C.B., Swingle, R.S., **Wu, Z.**, Simas, J.M., Chen, K.H., Chan, S.C., Santos, J., Depeters, E.J. 1997. Comparison of barley and sorghum grain processed at different densities for lactating cows. J. Dairy Sci. 80:2098-2103.

Schmitt, M., Randall, G., Sheaffer, C., **Russelle, M.** 1997. Manure management for forages: Consider the possibilities. Minnesota Forage UPDATE. 22(2):6.

Schmitt, M.A., Randall, G.W., Sheaffer, C.C., **Russelle, M.P.**, Greub, L.J., Clayton, P.D. 1997. Manure application strategy effects on reed canarygrass yields. Agron Abstracts. P. 229.

Schocke, L., **Weimer, P.J.** 1997. Purification and characterization of phosphoenolpyruvate carboxykinase from the anaerobic ruminal bacterium *Ruminococcus flavefaciens*. Arch. Microbiol. 167:289-294.

Sewalt, V.J.H., Ni, W., **Jung, H.G.**, Dixon, R.A. 1997. Lignin impact on fiber degradation: increased enzymatic digestibility of genetically engineered tobacco (*Nicotiana tabacum*) stems reduced in lignin content. J. Agric. and Food Chem. 45(5):1977-1983.

Sewalt, V.J.H., Ni, W., Blount, J.W., **Jung, H.G.**, Masoud, S.A., Howles, P.A., Lamb, C., Dixon, R.A. 1997. Reduced lignin content and altered lignin composition in transgenic tabacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol. 115:41-50.

Shi, Y., **Weimer, P.J.** 1997. Formation of formate and H_2, and flux of reducing equivalents and carbon in the ruminal cellulolytic anaerobe *Ruminococcus flavefaciens* FD-1. Antonie van Leeuw. 72:101-109.

Shi, Y., **Weimer, P.J.** 1997. Competition for cellobiose among three predominant ruminal cellulolytic bacteria under substrate-excess and substrate-limited condition. Appl. Environ. Microbiol. 63:743-748.

Shi, Y., Odt, C., **Weimer, P.J.** 1997. Competition for cellulose among three predominant ruminal cellulolytic bacteria under substrate-excess and substrate-limited conditions. Appl. Environ. Microbiol. 63:734-742.

Smith, R.R. 1997. Increased longevity of red clover through selection for persistence and disease resistance. Proc. XVIII Intern. Grassl. Cong. Winnipeg, Manitoba, June 8-19. 1(4):19-20.

Steiner, J.J., **Smith, R.R.**, Alderman, S.C. 1997. Red clover production: V. root rot resistence under forage and seed production systems. Crop Sci. 37:1278-1282.

Stombaugh, S.K., Somers, D.A., **Jung, H.G.**, Killiam, A.S., Orf, J.H. 1997. Genetic variation in polysaccharide content in soybeans. Agron. Abstr. P. 120.

Vagnoni, D.B., **Broderick, G.A.**, **Muck, R.E.** 1997. Preservation of protein in wilted lucerne using formic, sulfuric or trichloroacetic acids. Grass and Forage Sci. 52:5-11.

Vagnoni, D.B., **Broderick, G.A.**, Clayton, M.K., **Hatfield, R.D.** 1997. Excretion of purine derivatives by Holstein cows abomasally infused with incremental amounts of purines. J. Dairy Sci. 80:1695-1702.

Vagnoni, D.B., **Broderick, G.A.** 1997. Effects of supplementation of energy or ruminally undegraded protein to lactating cows fed alfalfa hay or silage. J. Dairy Sci. 80:1703-1712.

Van Kessel, J.S., **Russell, J.B.** 1997. The endogenous polysaccharide utilization rate of mixed ruminal bacteria and the effect of energy starvation on ruminal fermentation rates. J. Dairy Sci. 80:2442-2448.

Wallace, R.J., Kopecny, J., **Broderick, G.A.**, Walker, G.A., Sichao, L., Newbold, C.J., McKain, N. 1997. Peptidases of the rumen bacterium *Prevotella ruminicola*. Anaerobe 3:35-42.

Weimer, P.J. 1997. Microbiology of the Dairy Animal. In E.H. Marth and J. H. Steele (eds.). Applied Dairy Microbiology, Marcel Dekker, New York. P. 1-35.

Weimer, P.J., Waghorn, G.C., **Mertens, D.R.** 1997. Relationship between ruminal fibrolytic populations, ruminal VFA concentrations, and milkfat in cows fed diets containing corn silage and alfalfa silage at two levels of fiber. J. Dairy Sci. 80(Supple. 1):166.

Wells, J.E., Krause, D.O., Callaway, T.R., **Russell, J.B.** 1997. A bacteriocin-mediated antagonism by ruminal lactobacilli against *Streptococcus bovis*. FEMS Microbiol. Ecol. 22:237-243.

Wilkerson, V.A., **Mertens, D.R.**, Casper, D.P. 1997. Prediction of excretion of manure and nitrogen by Holstein dairy cattle. J. Dairy Sci. 80:3193-3204.

Wilson, J.R., **Hatfield, R.D.** 1997. Structural and chemical changes of cell wall types during stem development: consequences for fibre degradation by rumen microflora. Aust. J. Agric. Res. 48:165-180.

Wu, Z., Fisher, R.J., Polan, C.E., Schwab, C.G. 1997. Lactation performance of cows fed low or high ruminally degradable protein prepartum and supplemental methionine and lysine postpartum. J. Dairy Sci. 80:722-729.

Wu, Z., Polan, C.E., Fisher, R.J. 1997. Adequacy of amino acids in diets fed to lactating dairy cows. J. Dairy Sci. 80:1713-1721.

Yu, P., Huber, J.T., Theurer, C.B., Chen, K.H., Nussio, L.G.**, Wu, Z.** 1997. Effect of steam-flaked or steam-rolled corn with or without *Aspergillus oryzae* in the diet on performance of dairy cows fed during hot weather. J. Dairy Sci. 80:3293-3297.

Method for Measuring Gas Production Kinetics

D.R. Mertens and P.J. Weimer

Introduction

Methodology can play a critical role in the measurement of digestion kinetics, especially when the objective is to define kinetic parameters for feeds when formulating rations or modeling animal responses. Measurement of gas production kinetics provides the opportunity to evaluate the rate of digestion of the soluble, more rapidly fermenting fractions of feeds. However, differences among procedures that have little impact on digestion of dry matter after 48 h of incubation may have dramatic effects on fermentation of soluble matter during the first 20 h. Our objective was to develop a method for measuring the kinetics of gas production that would minimize any detrimental effects associated with the in vitro system and provide estimates of digestion kinetics that can be used to both describe feeds for ration formulation systems and provide parameters for models of ruminal digestion.

Method Description and Justification

The capacity of the incubator, stirring system, and data acquisition system developed at Cornell University was increased from 16 to 40 vials to accommodate experimental designs that include multiple treatments and blanks within runs. During inoculation, vials are kept upright to prevent particles of the sample from sticking to sides of the vial and being out of contact with the fermenting media. After several uses, needles attached to the transducers became dull and often were bent when inserted into incubation vials. Attachment of needles to transducers was modified so needles could be replaced easily. Luer lock adapters were glued to the pressure transducers by using the end of a plastic tuberculin syringe as a connector. A thin coating of stopcock grease applied to the inside of the hub of each 18 gauge needle provided a gas-tight seal. Needles were replaced after 4 uses and the gas-tight seal of new needles was verified by injecting 10 mL of air into an empty vial attached to each needle and observing no drop in pressure overnight.

Transducers that measure pressure relative to ambient were used because they were more economical than absolute pressure transducers. However, relative pressure transducers are affected by building and ambient barometric pressure changes. Four sealed vials were used to monitor changes in background pressure and the data were used to correct for these changes. Pressure transducers were calibrated by injecting pre-heated (39ºC) CO_2 gas into vials containing 10 mL of in vitro media and recording voltage changes. Five injections of 10 mL of CO_2 gas were spaced at 3.5 h intervals to generate calibration curves. Voltages, adjusted for background pressure changes and differences in vial volume were regressed against the mL of CO_2 injected to obtain a calibration coefficient for each transducer. Media containing phosphate-bicarbonate buffers, trace minerals, macrominerals, ammonia, and trypticase was used that could maintain pH above 6.2 when 40-60 mg of substrate was fermented and gasses were not released during a 96 h fermentation.

On the day before inoculation, 40 to 100 mg of sample was weighed into a 50 mL serum bottle (previously weighed and calibrated for volume) containing a small (1015 mm) stirring bar of known volume. On the morning of inoculation, 400 mL of media containing rezasurin was mixed and a 0.7 mL of media was added to each vial to wet the sample and minimize floating. Vials were swirled and placed in a water bath (39ºC). Each vial was connected to a manifold and continuously purged with CO_2. An additional 5.0 mL of media was added to each vial while they were in the water bath. Reducing solution (sodium sulfide and cysteine) was prepared and 0.3 mL was added to each vial. Vials were kept in the water bath, but were disconnected from the manifold and lightly capped. Reduction of the media and sample prior to inoculation is critical to minimize the shock to strictly anaerobic micro-organisms and the resulting lag period.

While samples were reducing, 1200 mL of ruminal fluid and 800 mL of solids were collected from a fistulated cow into a warmed insulated container. In the lab, the top 20 cm of solids were discarded and the remaining fluid and solids were squeezed through 2 layers of cheesecloth. One hundred grams of squeezed solids were placed in a blender with 200 mL of previously chilled and reduced media and blended for 45 sec. Two hundred mL of strained ruminal fluid was filtered through 4 layers of cheesecloth, the contents of the blender were added and squeezed tightly. Blending of solids with chilled media instead of ruminal fluid eliminates potential damage to bacteria in ruminal fluid during blending and improves the likelihood of detaching particle-bound bacteria. The inoculum was warmed to 39ºC while being continuously stirred and purged with CO_2.

After samples were reduced as indicated by colorless rezasurin indicator, 4 mL of inoculum was added while purging each vial with CO_2. Vials were capped with flanged butyl rubber stoppers and sealed with aluminum crimps. Two people can inoculate and connect 40 samples within 20 min., and the time between collection of ruminal contents and inoculating the last sample should be less than 40 min. After 6 h, all vials were gently swirled to mix material that was floating and to loosen the ring of residue from the sides of the vial. Vials were swirled every 24 h until the end of fermentation being careful to insure that material did not creep up the side of the vial. Voltages were recorded using a computer data acquisition system every 0.01 h for the first 0.5 h, every 0.10 h for the next 5 to 6 h and every 0.5 h until 96 h of fermentation. When fermentation was complete, vials were equilibrated to room temperature for 1-2 h and centrifuged at 220Xg for 30 min. The stopper was removed, a sample of the fluid was collected for VFA analysis, the contents were mixed and pH was taken as rapidly as possible. Dry matter and neutral detergent fiber of the residue remaining were determined.

Discussion

Evaluating in vitro methodology is complicated because the reference value to be used for comparison is arbitrary and because there are many interactions between substrates and the *in vitro* system that make it difficult to insure that any system is acceptable in all situations. The criterion of maximal digestion kinetics suggests that any in vitro method that does not achieve maximal rates and extents of fermentation and minimal lag times clearly does not measure digestion kinetics that is limited by feed characteristics. In previous experiments, in vitro system using continuous gassing of flasks obtained the fastest fractional rates, shortest discrete lag times, and smallest indigestible residues for fiber digestion compared to other systems. The use of reducing agents to remove oxygen from the sample and media before inoculation, blending of ruminal solids to detach bacteria, and including trace minerals and trypticase in the media also have been observed to maximize fiber digestion.

In the in vitro gas production system, the use of chilled media to detach bacteria from ruminal solids with blending was observed to slightly decrease lag and increase rates of gas production. Complete reduction of the media and rapid inoculation of the samples resulted in no lag time for gas production. An increase in gas pressure was observed within 1 min. of inoculation. It appears that stirring at approximately 1 min. intervals with swirling 6 h after inoculation and every 24 h thereafter also maximized gas production. A small but inconsistent increase in gas production was observed when gas was manually released from the vials. The lack of a significant effect due to gas pressure build-up in this system may be related to the large head-space to sample ratio. Typically less than 60 mg of substrate is fermented in 10 mL of media with a head-space volume in the serum bottles of 48 mL which results in a maximum pressure of twice ambient after 96 h of fermentation.

Conclusions

If the objective of an in vitro method is to provide kinetic parameters that characterize the feed, it is evident that the method itself should not limit fermentation. The complete reduction and equilibration of samples and media before inoculation, the rapid and thorough preparation of an inoculum that maximizes detachment of particle-bound microbes, and the rapid inoculation of samples in the method described insures that lag time is minimized and fermentation rate and extend are maximized.

Inocula Differences Affect In Vitro Gas Production Kinetics

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Introduction

The kinetics of gas production during ruminal fermentation may provide valuable information about feeds that can be used to formulate rations and model animal responses. However, measurement of digestion kinetics is affected by methodology, and techniques must be established that provide accurate and precise estimates of kinetic parameters. Because gas production measurements provide the opportunity to estimate the digestion kinetics of both soluble and insoluble matter in feeds, it would be desirable to use this technique on a wide variety of forages, grains, supplements, and by-product feeds. Applying an in vitro technique to such a wide variety of substrates raises questions about the type of inoculum that should be used. The objective of our study was to evaluate the effects of donor animal and its diet on the measurement of gas production kinetics using both forage and concentrate substrates.

Materials and Methods

Inocula were obtained from four lactating Holstein cows in mid lactation using a balanced 4 X 4 Latin square design with a 2 X 2 factorial arrangement of treatments. The two forage sources were alfalfa and corn silage and the two fiber levels were 24 and 32% amylase-treated neutral detergent fiber (aNDF). Rations were formulated to meet National Research Council requirements for protein and minerals using corn, soybean meal, trace-mineralized salt and mineral supplements. Rations were fed twice daily at 12 h intervals to obtain > 10% refusal. Each period of the Latin square lasted 4 weeks. On day 24, rumen contents were taken from each cow and used as an inoculum for an in vitro experiment with a standard media designed to maintain pH above 6.0 at the end of fermentation. On day 28 of each period, ruminal contents were used in a second in vitro run in which the pH of the in vitro system was matched to that of the donor.

The in vitro gas production system of Mertens and Weimer was used to obtain gas production curves for alfalfa silage, corn silage, corn, soybean meal, cotton, and mixed rations using standard media or media that had pH adjusted using citric acid. About 90 mg of dry substrate was fermented. Corn and soybean meal were ground to pass a 2-mm screen using a Wiley cutter mill. Surgical absorbent cotton was cut by hand into 15-mm squares that weighed about 80 mg. Silages were not dried, but were frozen and ground through a meat grinder with a 12-mm die which resulted in a particle size distribution similar to materials ground through a 6-mm screen using a Wiley cutter mill. Amylase-treated neutral detergent fiber (aNDF) was measured using both sodium sulfite and amylase.

Results and Discussion

The rations used in this experiment resulted in differences in ruminal pH among treatments (Table 1). In addition, the ruminal pH of individual donors varied, irrespective of the ration that they consumed. When the standard buffer was used, the pH of the in vitro system after 96 h of fermentation was slightly lower than the pre-feeding ruminal pH. The in vitro pH after 96 h was similar to post-feeding ruminal pH, when the in vitro buffer was adjusted to match in vivo pH (Table 1). The diet of the inocula donors had a significant effect on the maximum asymptotic gas production, but not the rate of fermentation as indicated by the time required to reach one-half of the maximum gas production (Table 1). Inocula from donors fed alfalfa silage based rations resulted in greater average maximal gas production for the alfalfa and corn silage substrates than did the inocula from donors fed corn silage based rations. The effect of forage source was greater when pH of the in vitro buffer was adjusted to match that of the donor, but was also evident when the standard in vitro buffer was used. Although rations containing 32% aNDF resulted in greater maximal gas production than those with 24% aNDF, the effect of fiber content was not as large as that due to forage source.

Inocula from individual cows affected both rate and extent of gas production irrespective of the buffer used in vitro (Table 2). Cow 2661, which had a low ruminal pH for all diets, consistently had the lowest maximum

gas production and shortest time to one-half maximum gas production. Conversely, cow 3807 had high maximum gas production with short time to one-half maximum. The composite inocula had the largest maximum gas production in both trials, but the time to reach one-half maximum production was among the longest in the first trial and shortest in the second trial. It appears that a composite inoculum may be most desirable to obtain maximal gas production/100 mg of dry substrate assuming that this is an indication of maximal digestion.

Conclusions

Both rations and donors provided a range in inocula characteristics that influenced gas production kinetics. Differences in gas production between cows and their diets seemed to be associated with ruminal pH. Our results indicate that pH and the microbial populations associated with them have a significant impact on digestion kinetics as measured by gas production. A composite inoculum will help to minimize variations among in vitro runs and ensure an adequate population of microorganisms for diverse substrates and pH conditions. Accurate modeling of ruminal pH and its affects on digestion must be a critical component of any system that is designed to use digestion kinetic parameters to formulate rations or predict animal responses.

Table 1. Ruminal pH and effect of feeding alfalfa (AS) or corn silage (CS) rations containing 24 or 32% aNDF to inocula donors on the maximum gas production and time required to reach one-half maximum gas production in vitro when standard or matched buffers were used.[1]

	AS32	AS24	CS32	CS24
Ruminal pH				
Pre-feeding	6.07	5.78	6.30	6.14
Post-feeding	5.78	5.52	5.53	5.37
Standard in vitro buffer				
pH after 96 h	5.94	5.83	5.84	5.80
Maximum gas, ml/100 mg DM	21.98^{a}	20.32^{ab}	19.52^{ab}	19.34^{b}
Time at 1/2 maximum, h	5.59^{a}	5.96^{a}	6.57^{a}	5.63^{a}
Matched in vitro buffer				
pH after 96 h	5.59	5.46	5.40	5.35
Maximum gas, ml/100 mg DM	19.11^{a}	16.78^{a}	11.85^{b}	11.88^{b}
Time at 1/2 maximum, h	5.13^{a}	5.54^{a}	6.92^{a}	5.04^{a}

[1]Treatments with different superscripts are different at $P < .05$.

Table 2. Differences among donor cows in maximum gas production per 100 mg of substrate and time required to reach one-half maximum gas production in vitro when standard or matched buffers were used.[1]

	Cow 749	Cow 3807	Composite	Cow 3691	Cow 2661
In vivo ruminal pH					
3 h post-feeding	5.69	5.60		5.57	5.23
Standard in vitro buffer					
In Vitro pH after 96 h	5.87^{a}	5.87^{a}	5.82^{a}	5.86^{ab}	5.81^{b}
Maximum gas, ml/100mg	19.78^{b}	20.68^{ab}	22.76^{a}	21.56^{ab}	19.10^{b}
Time at 1/2 maximum, h	6.57^{a}	6.16^{ab}	6.94^{a}	6.00^{ab}	5.03^{b}
Matched in vitro buffer[2]					
In Vitro pH after 96 h	5.54^{b}	5.47^{bc}	5.84^{a}	5.44^{cd}	5.35^{d}
Maximum gas, ml/100mg	14.97^{c}	18.52^{b}	24.33^{a}	14.47^{c}	11.66^{d}
Time at 1/2 maximum, h	5.44^{ab}	5.24^{ab}	5.33^{ab}	7.01^{a}	4.93^{b}

[1]Treatments with different superscripts are different at $P < .05$.

[2]Composite inoculum was evaluated using the standard in vitro buffer when individual cow inocula were evaluated using an in vitro buffer that was matched to the ruminal pH of the donor.

Populations of Individual Species of Cellulolytic Bacteria in the Rumen of Lactating Cows Fed Different Diets

P.J. Weimer, C.L. Odt, G.C. Waghorn and D.R. Mertens

Introduction

Because the ruminal microflora are the key link between the ruminant animal and its diet, the species composition of the microflora are expected to have a great impact on the availability of VFAs and microbial cells used by the animal for energy and protein synthesis. In the case of forage-fed animals, the cellulolytic population would be expected to be major components of the microflora. *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* are three of the most abundant cellulolytic bacteria in the rumen. All three species are able to rapidly degrade crystalline cellulose, but these species differ in their growth yields and their fermentation endproducts, and thus the relative populations of these species should affect nutrient availability. Advances in the development of oligonucleotide probes specific for 16S ribosomal RNAs of individual microbial species has permitted quantification of individual species in their natural environment. This study describes the application of such probes to the assessment of population sizes of the three predominant cellulolytic species in the rumina of cows fed diets based on alfalfa silage or corn silage, at two different forage levels.

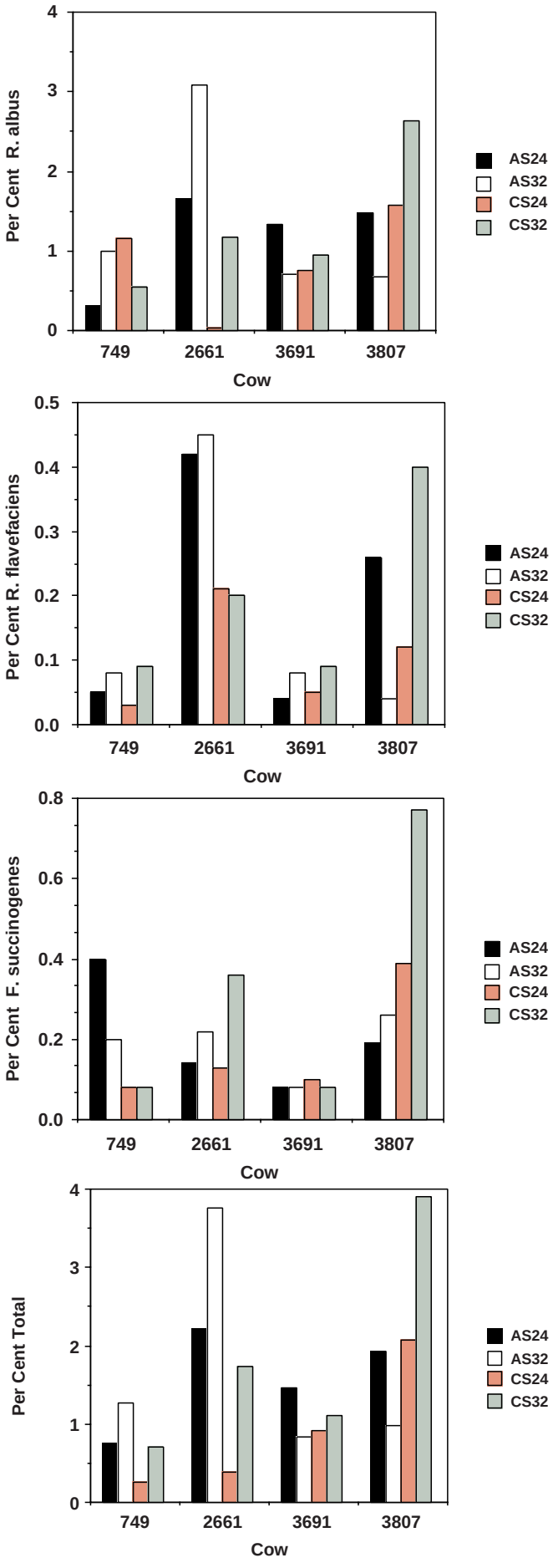
Material and Methods

Four ruminally-fistulated, multiparous Holstein cows were fed, in a Latin square design, diets differing in source (alfalfa silage or corn silage) and amount (24% or 32% NDF, determined after treatment with a-amylase) of fiber. Diets were offered at 12h intervals as total mixed rations in amounts that assured ad libitum intake. After adaptation to diet for 19 days, 13 samples were collected from each rumen over a 4-day period. The rumen contents were separated by squeezing through cheesecloth, and the strained ruminal fluid (SRF) was used for measurement of pH and VFAs , and as inocula for in vitro digestion kinetic experiments; the data has been reported previously (Weimer et al. 1997). SRF and squeezed ruminal solids were stored at -70 ^{o}C for isolation of microbial RNA.

For each cow-diet combination, RNA analyses were performed only with the five samples collected 3 h post-feed during the week of sampling. Thawed SRF (25 mL) was combined with thawed squeezed solids (25 g), and mixed in a chilled Waring blender. Microbial cells were recovered by centrifugation at 500 x g, and the solids washed with chilled saline and re-centrifuged. The second supernate was combined with the first, filtered through glass wool, and the cells were pelleted and stored at -70 ^{o}C. Methods for extraction of RNA from cells, and for hybridization with digoxigenin-labeled oligonucleotide probes, and quantitation of hybridized RNA have been described previously (Shi et al. 1997). Probes used for hybridization were: S-Ss-F.s.suc-0207-a-A-21 for *F. succinogenes*, S-S-R.fla-1176-a-A-17 for *R. flavefaciens*, and S-S-R.alb-0196-a-A-18 for *R. albus*. The amounts of RNA on each densitometer image were determined from standard curves prepared with purified RNA from *F. succinogenes* S85, *R. flavefaciens* B34b, or *R. albus* 7. The corrected RNA values were normalized to total eubacterial RNA in the samples, determined from separate hybridizations using the eubacterial domain-specific probe S-D-Bact-0338-a-A-18 and commercial *Escherichia coli* RNA as standard.

Results and Discussion

The relative population sizes of the three predominant cellulolytic species for the sixteen cow-diet combinations are shown in Fig. 1. Total RNA from the three species represented 0.3- 3.9 per cent of the total bacterial RNA, similar to the proportion of cellulolytic bacteria in the total bacterial population estimated by culture procedures (Van Gylswyk 1970, Dehority et al. 1989). *R. albus*-specific RNA was much more abundant than was that of either *R. flavefaciens* or *F. succinogenes*.



Comparison of the populations across cows is complicated by the known variation in response of different strains to species-specific probes. Moreover, the high variability of the method (CV=39.7%, averaged across 48 cow x diet x bacterial species combinations) makes comparison difficult even within diets. In general, the populations of individual species did not show obvious patterns with diet that were consistent across cows. Nevertheless, some trends are apparent. In most cases, the three cellulolytic species represented a greater fraction of the total bacterial population in the higher-fiber diets than in the lower-fiber diets, in accord with the notion that cellulose digestion in the rumen is limited by substrate (i.e., increasing cellulose concentration will increase the cellulolytic population). In cows 749 and 2661, the depressed levels of *R. albus* in the CS24 diet may have been due to the 3h-postfeed pH values (5.46 and 5.18, respectively), which were lower than those observed on other diets. This observation is in accord with the known sensitivity of ruminal cellulolytic bacteria to low pH.

Conclusions

Despite their functional importance in fiber digestion, the three predominant species of ruminal cellulolytic bacteria represent only a small fraction of the total bacterial population across a variety of cow-diet combinations. The RNA probe method, while powerful and sensitive, had limited quantitative reproducibility that made it difficult to detect changes in populations resulting from changes in diets. Changes in the populations of the three predominant cellulolytic species, when observed, differed among cows, suggesting that each cow maintains unique assemblages of ruminal microbial strains.

Figure 1. Populations of three predominant species of ruminal cellulolytic bacteria, and their sums, in four cows on four diets, determined using oligonucleotide probes, and expressed as a fraction of total bacterial RNA. Results are mean values of 3-5 samples collected 3h-postfeeding during the week of sampling. Note differences in the scale of each ordinate.

References

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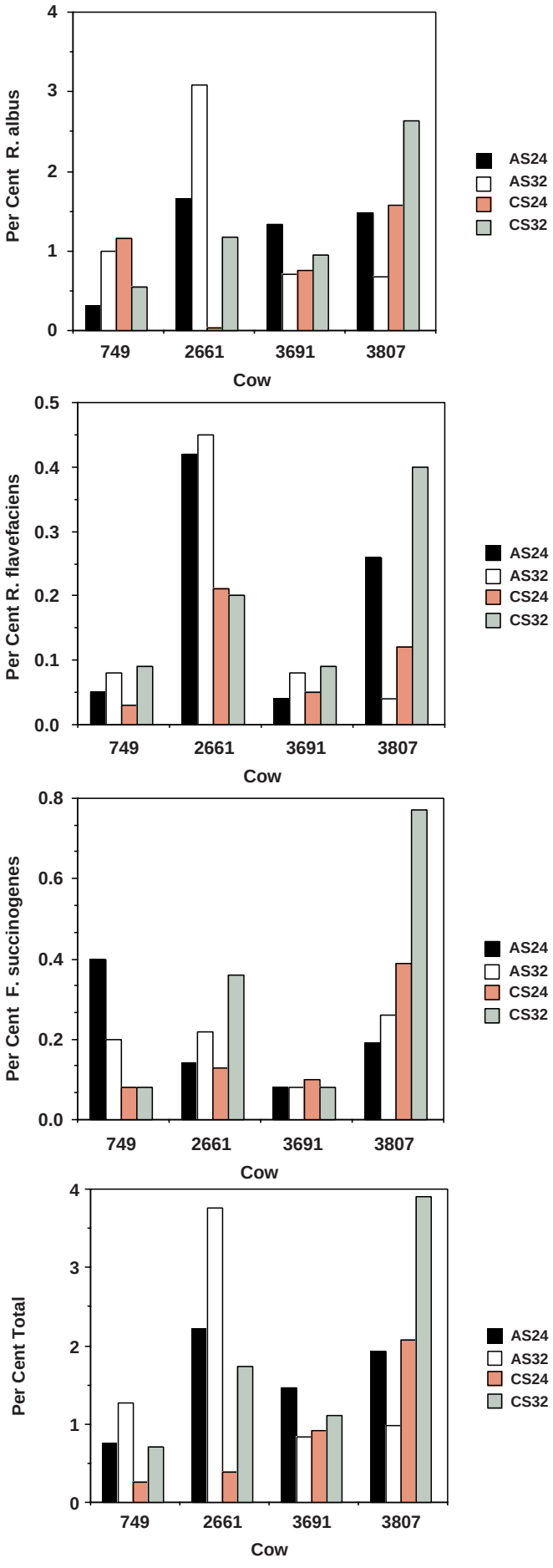

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Degradability of Alfalfa Stem Tissues by Rumen Microorganisms

H.G. Jung and F.M. Engels

Introduction

While alfalfa is recognized as a high-quality forage crop for feeding dairy cattle, the majority of alfalfa's nutritional quality is associated with the leaf fraction. Alfalfa stems contain 60+% cell-wall material and these cell walls are poorly degraded by rumen microbes. Because stems comprise 50% or more of the alfalfa crop, significant improvement in nutrient (energy) availability will require reducing the cell-wall concentration of alfalfa stems or improving cell-wall degradability. The latter approach is complicated by the heterogeneity of tissues that comprise the alfalfa stem. Grabber et al. reported in the USDFRC 1996 Research Summaries that xylem and non-xylem tissues isolated from alfalfa stems were markedly different in their cell-wall degradability. Our objective in this study was to examine differences in stem tissue degradability at the cellular level using microscopy.

Materials and Methods

Alfalfa stems were collected after 31 d of regrowth following cutting in late June 1996. The seventh internode from the base of the stems was excised and preserved in 50% ethanol. Thin sections (100 mm) were prepared from the middle of the internodes using a mirror sectioning procedure. By this method of sectioning the same cell walls which were cut could be examined in two separate sections adhered to slides with double-sided tape. One mirror section from each pair served as a non-degraded control and the other mirror section was incubated in vitro with rumen fluid. Both sets of mirror sections were examined using light microscopy (LM) and scanning electron microscopy (SEM). Degradation of tissues and specific cell-wall layers could be assessed by this procedure. Additional sections were stained with phloroglucinol or ruthenium red to detect the presence of lignin and pectins, respectively. These stained sections were examined by LM.

Results and Discussion

In vitro ruminal degradation resulted in the removal of many alfalfa stem tissues. Figure 1 illustrates the degree of degradation that was observed after 24 h of incubation with rumen microorganisms. Collenchyma, chlorenchyma, secondary phloem, cambium, and protoxylem parenchyma tissues were completely degraded in 24 h (compare Fig. 1a and 1b). Only the cuticle of epidermal tissue remained after degradation (Fig. 1b). None of the completely degradable tissues in alfalfa stems stained positively for lignin. Xylem tissue showed very little degradation and stained intensely with phloroglucinol. Cortical fiber tissue underwent extensive, but incomplete degradation. Figure 1c illustrates cortical fiber cells that had been colonized by rumen bacteria after a 4 h incubation. The thick secondary wall of cortical fibers was still clearly visible after 4 h, but after 24 h of incubation all secondary wall material had been removed from the cortical fiber cells (Fig. 1d). Only a thin primary wall remained undegraded. The primary walls of cortical fibers were observed to be thickened when viewed by LM, but these primary walls shrank in thickness due to the dehydration procedure needed to prepare sections for SEM. Under LM it was observed that the thick primary walls stained strongly for pectins, but only the luminal edge of the primary wall stained positively for the presence of lignin. The secondary wall of cortical fibers did not stain for either pectins or lignin. The use of LM revealed that the thick, non-lignified primary wall of cortical fibers was completely degraded leaving only the thin, lignified primary wall structure seen in Figure 1d.

Conclusions

Alfalfa stems contain tissues which are completely degradable, virtually undegradable, and partially degradable by rumen microbes. The presence of lignin

is a good indicator of tissues which are poorly degraded. Contrary to previous reports in the literature, all secondary wall material is not lignified as evidenced by the alfalfa cortical fiber secondary cell walls. The results of this research suggest two routes to improving alfalfa stem cell-wall degradability. One possibility would be to prevent or reduce cell-wall lignification of tissues which normally lignify. The alternative approach would be to increase the proportion of the stem comprised of those tissues which are completely degradable. The successful path to improving alfalfa stem degradation will depend on which modifications yield an agronomically viable crop.

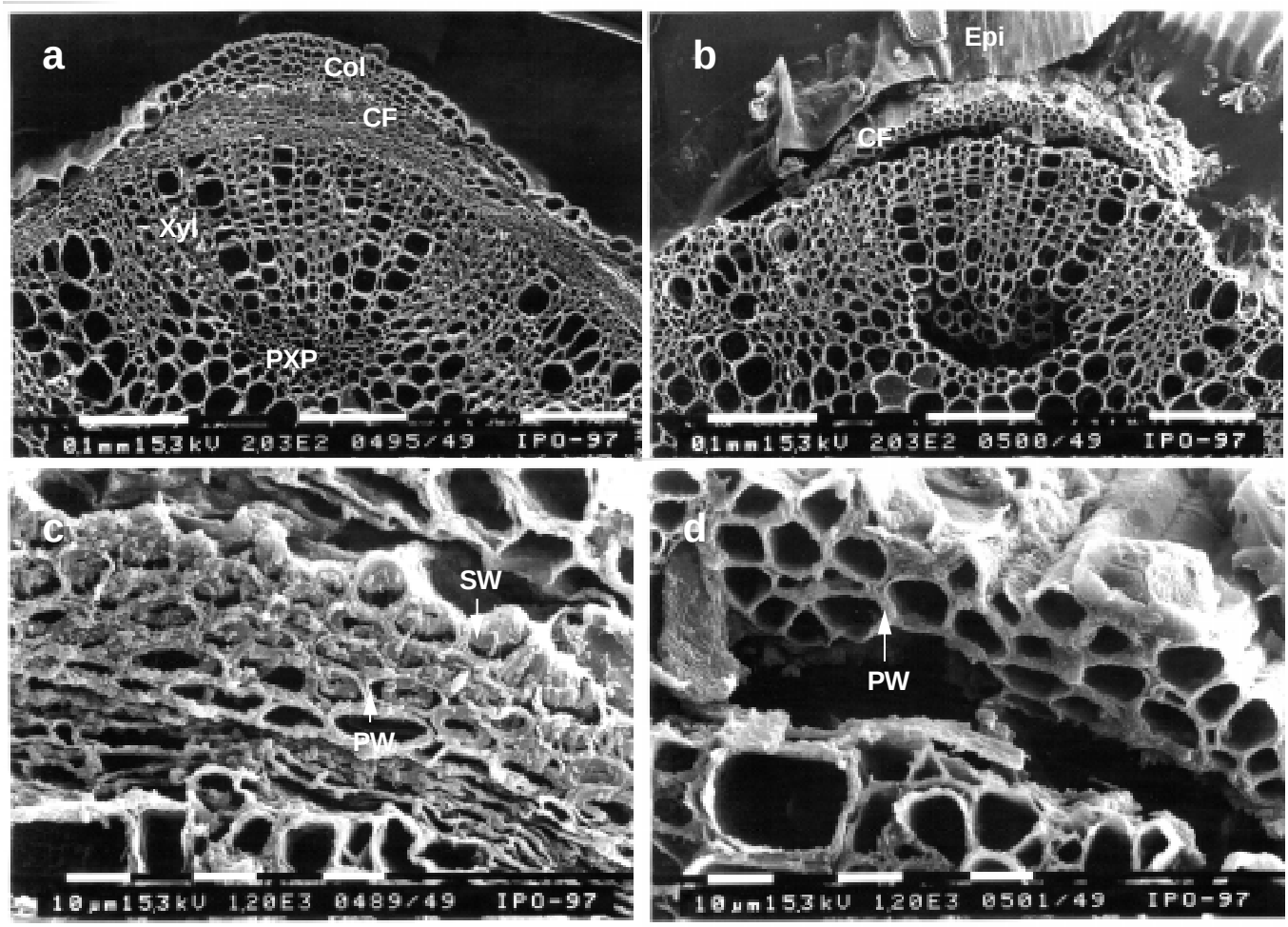

Figure 1. Scanning electron micrographs of alfalfa stem tissues before (a) and after (b) in vitro degradation by rumen microorganisms for 24 h. Collenchyma (Col), epidermis (Epi), and protoxylem parenchyma (PXP) were completely degraded, whereas cortical fiber and xylem were only partially degraded. Extensive bacterial colonization of thick secondary walls (SW) of cortical fiber was observed after 4 h of incubation (c) and only the primary wall (PW) remained after 24 h of degradation (d).

Kinetics of Cellulose Digestion by *Fibrobacter Succinogenes* S85

G. Maglione, J.B. Russell and D.B. Wilson

Introduction

Simple-stomached animals digest cellulose poorly, but ruminants, by exploiting cellulolytic microorganisms, have developed a much greater capacity for cellulose digestion. The relationship between ruminants and ruminal bacteria is clearly symbiotic. The animal provides the bacteria with a habitat for growth, the rumen, and the bacteria supply the animal with volatile fatty acids and microbial protein. The enzymology of cellulases has been confounded by variations in cellulose structure and the methodology used to measure cellulose hydrolysis. Early work was based on gravimetric procedures, but these methods are inherently insensitive. Reducing-sugar release has been used, but linear rates could only be obtained if the extent of cellulose digestion was less than 4%. Many "cellulase" assays used carboxylmethylcellulose (CMC), a soluble derivative, but many CMCases have little if any activity on crystalline cellulose. Both surface area and crystallinity can affect the rate of cellulose digestion, but Weimer concluded that surface area was a much more important parameter for ruminal cellulose digestion than was crystallinity. Surface area is sometimes estimated from particle size, but these estimates can be biased by the shape of the particle. Nitrogen absorption and solute exclusion are in theory more accurate, but both of these methods can be problematic. The following experiments were performed with *Fibrobacter succinogenes* S85, one of the most active cellulolytic bacteria isolated from the rumen. The experiments were designed to: 1) define conditions necessary to estimate rates of cellulose digestion, 2) determine the impact of cellulose surface area on rates of cellulose digestion, and 3) evaluate the relationship between culture status (growing versus stationary) and the rate of cellulose digestion.

Materials and Methods

F. succinogenes S85 was grown anaerobically in a defined medium. Exponentially growing cells were harvested by centrifugation, washed and resuspended in fresh media containing different concentrations of cellulose (from 1 to 10 mg/ml). After incubation for 60 min at 39°C, the suspensions were centrifuged. Succinate in the cell-free supernatant was measured by HPLC. Three commercial pure celluloses were used. Sigmacell 100 (Sigma Chemical Co.), Whatman no. 1 filter paper and Avicel type PH101 (FMC Co., Philadelphia). In addition two of them were pre-treated as follow: Whatman no. 1 filter paper (20 mg/ml) was ball-milled for 9 days using a 800 ml Mill Jar, "roller-type" (Norton, Chemical Process Products Division, Ohio) and microcrystalline cellulose, Avicel type PH 101, was hydrolyzed with hydrochloric acid and regenerated. Cellulose suspensions (10 mg/ml) were mixed with 2% Congo red solution to final concentrations ranging from 0.005 to 6.0 mg/ml of Congo red. The cellulose suspensions were centrifuged and the dye in the supernatant was monitored at 500 nm. The extinction coefficient of Congo red was 23,300 M^{-1} cm^{-1}. Exponentially growing cells of *F. succinogenes* S85 were centrifuged, washed and resuspended in fresh media containing 0.2 mg/ml of 4-thiocellobiose (Sigma Chemical Co.). Cellulose was added, the cells were incubated for 60 min at 39°C and cellulose-dependent succinate production was detected as described above. Cells were treated with 0.2 N NaOH (100°C, 10 min) and protein was determined by the method of Lowry et al.

Results

Growing cultures of *F. succinogenes* S85 digested cellulose at a rapid rate, but non-growing cells and cell extracts did not have detectable crystalline cellulase activity. Cells that had been growing exponentially on cellobiose initiated cellulose digestion and succinate production immediately, and cellulose-dependent succinate production could be used as an index of enzyme activity against crystalline cellulose. Cells incubated with cellulose never produced cellobiose, and cells that were pre-incubated for a short time with thiocellobiose lost their ability to digest cellulose (competitive inhibition, K_i of only 0.2 mg/ml or 0.56 mM). Based on these results, the crystalline cellulases

of *F. succinogenes* were very sensitive to feedback inhibition. Different cellulose sources bound different amounts of Congo red, and the binding capacity was HCl-regenerated cellulose > ball-milled cellulose > Sigmacel > Avicel > filter paper. Congo red binding capacity was highly correlated with the Vmax values of cellulose digestion and inversely related to Km. Congo red (250 mg/ml) did not inhibit the growth of *F. succinogenes* S85 on cellobiose, but this concentration of Congo red inhibited the rate of ball-milled cellulose digestion. A Lineweaver Burk plot of ball-milled cellulose digestion rate versus the amount of cellulose indicated that Congo red was a competitive inhibitor of cellulose digestion (K_i was 250 mg/ml).

Discussion

F. succinogenes S85 is one of the most active cellulolytic bacteria ever isolated from the rumen, but cell extracts have little or no crystalline cellulase activity. A variety of b-glucanases have been either isolated or cloned from *F. succinogenes* S85, but none of these enzymes had crystalline cellulase activity per se. Preliminary experiments indicated that cell extracts *of F. succinogenes* S85 were unable to produce reducing sugar from ball milled cellulose. Since cells limited by ammonia or branched chain volatile fatty acids also lost their ability to digest cellulose as soon as growth ceased, it appeared that only actively growing cells had crystalline cellulase activity.

When *F. succinogenes* S85 cultures that had been growing exponentially on cellobiose were harvested by centrifugation and re-suspended in medium containing cellulose, it was possible to measure the initial rate of succinate production. Experiments with ball-milled cellulose indicated that the cellulose-dependent succinate production rate was comparable to the succinate production of cultures growing on cellulose (53 versus 60 nmol succinate/mg protein/min). The rate of cellulose-dependent succinate production was linear so long as the incubation time was less than 60 min and the cell concentration was less than 150 mg protein/ml.

The cellulase activity of *F. succinogenes* S85 was directly linked to cellobiose metabolism. Little decline in "cellulase activity" was noted until the rate of cellobiose fermentation was less than 50 nmol succinate per mg protein per min, but there was a linear and proportional decrease in cellulose degradation at slower rates of cellobiose metabolism. The cellulases of non-ruminal microorganisms can be inhibited by cellobiose, but large amounts are needed to cause significant inhibition. The idea that *F. succinogenes* cellulases might be unusually sensitive to feedback inhibition was supported by the effect of thiocellobiose, a non-metabolizable cellobiose analog. Exponentially growing cells that were treated with thiocellobiose lost their ability to degrade cellulose, and there was no detectable increase in cellobiose. Thiocellobiose was a competitive inhibitor of cellulose degradation, and the K_i was only 0.2 mg/ml or 0.56 mM.

The V_{max} of cellulose digestion varied 8-fold when different cellulose sources were provided, and this rate was highly correlated with the ability of the cellulose to bind Congo red. Congo red is a polyphenolic, sulfonated azo-dye that has been used as an pH indicator, but it also binds to the surface of insoluble plant carbohydrates. Based on these results, it appeared that cellulose surface area was the primary factor affecting cellulase activity. Cellulose sources with high V_{max} values tended to have low K_m values and vice versa, and this result is not surprising. If the turnover rate of cellulose is rapid (high V_{max}), it would take less cellulose to saturate the cellulase (low K_m). Avicel is frequently used as a substrate for crystalline cellulase assays, but a plot of Congo red binding versus K_m indicated that Avicel was abnormal. Avicel is a microcrystalline cellulose, but the crystalline cellulase of *F. succinogenes* S85 is cell associated that cannot penetrate into small pores.

Conclusion

Crystallinity seems to have little impact on ruminal fiber digestion rate, but surface area can have a major impact.

The Endogenous Metabolic Rate of Mixed Ruminal Bacteria and the Effect of Energy Starvation on Ruminal Fermentation Rates

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Introduction

Grazing ruminants sometimes consume food continuously, but domestic livestock usually eat larger, less frequent meals. The impact of meal interval on ruminal fermentation has not been studied in a systematic fashion. The rumen usually has an abundance of insoluble feed materials, but soluble carbohydrates are only available 1 to 2 h post feeding. Soil and marine bacteria can remain viable for long periods of time, but long term survival is not a ubiquitous feature of bacteria. Many ruminal bacteria are very sensitive to energy starvation in vitro, and direct counts can be 10-fold higher than viable counts. Some ruminal bacteria store large amounts of polysaccharide that resembles glycogen, but glycogen does not always prevent starvation. Viability is a highly operational definition that is based solely on bacterial reproduction. Given the fact that bacteria seem to survive better in dense populations than dilute ones and can suffer from a variety of stresses including substrate accelerated death, "viable" is not always a synonym for metabolically active. The following experiments sought to: 1) measure the endogenous metabolic rate of mixed ruminal bacteria, 2) correlate the endogenous metabolic rate with fermentation capacity, and 3) assess the effect of feeding frequency on the initial rate of ruminal fermentation.

Materials and Methods

Ruminal contents were collected 2 h or 24 h post-feeding from the ventral and anterior sections of the rumen. Contents were squeezed through 8 layers of cheese cloth and the pH was determined immediately. Feed particles and protozoa were removed by centrifugation (325 x g, 5 min) and ruminal fluid was anaerobically transferred to a flask that was flushed with oxygen-free carbon dioxide (39°C). Samples were removed from the flask at 4 h intervals. The specific rate of soluble carbohydrate fermentation (final concentration per ml: 0.42 mg of soluble starch, 0.175 mg of cellobiose, 0.105 mg of sucrose, 0.125 mg of xylose, 0.125 mg of arabinose, and 0.05 mg of pectin) was estimated from the rate of heat production. The specific degradation rate for ball-milled cellulose was estimated from the reciprocal of digestion time needed for complete digestion, the amount of cellulose degraded (2 mg/ml) and the initial concentration of bacterial protein. The specific activity of ammonia production from Trypticase (15 mg/ml) was estimated from the increase in ammonia. The specific activity of methane production from hydrogen and carbon dioxide (1 atm each) was estimated from the increase in methane. ATP was extracted from the cells by perchloric acid-EDTA treatment after separation of the cells from the growth medium. ATP was measured by the luciferin-luciferase method. Total cellular polysaccharide was measured by the anthrone method. Utilizable polysaccharide was estimated from the difference in total carbohydrate and the amount of carbohydrate that was still present after 48 h of starvation. Bacterial protein was measured by the method of Lowry. RNA and DNA were measured using an orcinol reaction.

Results

When mixed ruminal bacteria were starved in vitro for 24 h, cellular ATP decreased, but there was little decline in cell protein. Starved ruminal bacteria utilized nucleic acids (primarily RNA), but the decline in ATP was more closely correlated with decreases in polysaccharide. Because polysaccharide declined at a first order rate of 23% per h, it was possible to estimate the endogenous metabolic rate at various stages of starvation. The bacteria were initially able to ferment soluble carbohydrates at a rate of 700 mg of hexose equivalent per mg of protein per h. Starvation had little impact on the rate of soluble carbohydrate fermentation until 8 to 12 h, and the endogenous metabolic rate was less than 10 mg of hexose per mg of protein per h. The bacteria digested ball-milled cellulose at a rate of 25 mg of hexose per mg of

protein per h for 8 to 12 h. Even bacteria that had been starved for 24 h digested cellulose at a rate of 16 mg of hexose per mg of protein per h. The bacteria produced methane from hydrogen and carbon dioxide at a rate of 70 nmol of methane per mg of protein per min. Short periods of starvation (< 12 h) had little impact on methane production, but longer times caused an almost complete inhibition of methanogenesis. The bacteria deaminated amino acids at a rate of 30 nmol per mg of protein per min, and the critical phase of starvation was again 8 to 12 h. Ruminal bacteria that were harvested 24 h after feeding had 10-fold less polysaccharide than bacteria 2 h after feeding, but this polysaccharide supported high rates of soluble carbohydrate fermentation, cellulose degradation, deamination and methane production.

Discussion

The maintenance rate of animals is frequently estimated from the fasting (basal) metabolic rate, but bacteria can have different rates of maintenance and endogenous metabolism. With bacteria, maintenance is defined as the non-growth energy dissipation of growing cells, and, in this case, energy is derived from exogenous sources. Endogenous metabolism is a characteristic of starved, non-growing cells, and this energy is derived from cellular reserves. Mixed ruminal bacteria have a maintenance rate of 100 mg hexose per mg of bacterial protein per h, but starved ruminal bacteria had endogenous metabolic rates that were 2 to 50 fold lower.

Based on the observation that the decline in cellular polysaccharide was highly correlated with the decrease in cellular ATP, it appeared that the specific rate of polysaccharide degradation was a reasonable estimate of endogenous metabolic rate. The endogenous metabolic rate declined logarithmically at a simple first order rate of 23% per h. Starvation did not reach its critical phase until 8 to 12 h when the endogenous metabolic rate was less than 10 mg hexose per mg of bacterial protein per h. Even highly starved (as long as 24 h) bacteria were able to ferment soluble carbohydrates at a rapid rate. Cellulose-digesting bacteria were even less sensitive to starvation than bacteria that fermented soluble carbohydrates. Methanogens only account for a small portion of the total ruminal population, and they must depend on the hydrogen that arises from the endogenous metabolism. Even ruminal bacteria that had been starved for 24 h could still deaminate amino acids at a rate of 20 nmol per mg of protein per min, and this result indicated that polysaccharide reserves were not a critical feature of deamination.

The idea that mixed ruminal bacteria were not particularly sensitive to energy source starvation in vivo was supported by the observation that mixed ruminal bacteria obtained from the rumen 24 h post feeding had nearly as high a rate of soluble carbohydrate fermentation, cellulose degradation, methane production and amino acid deamination as bacteria that were obtained soon after feeding. The 24 h post-feeding bacteria had 10-fold less utilizable polysaccharide, but even this amount of polysaccharide supported a rate of endogenous metabolism that did not compromise subsequent fermentation rates. The notion that the rumen has a large population of non-viable or dead bacteria is probably nothing more than an artifact of "culturability." If the ruminal bacteria were indeed metabolically inactive and unable to grow, they would soon be washed out of the rumen and be replaced by ones that could grow.

Conclusion

Ruminal bacteria may be more susceptible to starvation than other bacteria, but they clearly seem to have enough cellular reserves to withstand the relatively brief periods of starvation imposed by normal feeding practices.

The Effect of Nisin and Monensin on Ruminal Fermentations In Vitro

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Introduction

In ruminant animals, feedstuffs are fermented in the rumen, and this method of digestion promotes fiber and non-protein nitrogen utilization. Animals can utilize ruminal volatile fatty acids and microbial protein, but methane, heat and ammonia are also produced. When methane production is the primary mechanism of reducing equivalent disposal, the ratio of acetate to propionate increases and energy retention by the animal declines. Since the 1970's, nutritionists have sought chemical additives to decrease fermentation losses and increase the useful end-products of ruminal fermentation.

Chlorinated hydrocarbons are potent inhibitors of methane production in vitro, but these additives are inactivated in vivo . Ionophores were developed as coccidiostats, but they can alter ruminal fermentation by dissipating the ion gradients of gram-positive bacteria. Monensin, the most commonly used ionophore, was originally marketed as a methane inhibitor and propionate enhancer, but it also appears to decrease wasteful amino acid degradation and ammonia accumulation. Monensin often decreases food intake. This latter effect is often explained by increases in energy availability, but a learned aversion to monensin has been demonstrated.

Animals absorb monensin, but much is returned to the gut in bile via the enterohepatic circulation. The LD_{50} of monensin in ruminants is 20 to 40 times the recommended dose, but horses have an LD_{50} that is approximately 10-fold lower than cattle. Doses of monensin that are commonly used for cattle will kill horses, and this finding has created serious problems for the feed industry. Humans exposed to monensin during its manufacture have reported symptoms that included headache, nausea, nosebleed and skin rash, and people feeding monensin to animals have complained of headache and dizziness.

Many lactic acid bacteria produce small peptides that increase the transmembrane flux of protons, and these bacteriocins are primarily effective against gram-positive bacteria. Nisin was the first recognized bacteriocin, and it is produced by Lactococcus lactis, a common starter culture for cheese making. Nisin is not toxic to animals, and it has been approved by the FDA for use in human foods. Nisin has been used to control food-borne pathogens, but its effects on ruminal fermentation have not been examined.

Based on its ability to inhibit gram-positive ruminal bacteria, it appeared that nisin might be a useful additive for controlling or modifying ruminal fermentations. The following experiments compared effects of monensin and nisin on mixed and pure cultures of ruminal bacteria in vitro.

Materials and Methods

Mixed ruminal bacteria were incubated anaerobically in tubes (10 ml of incubation fluid) containing 0.1 g of ground alfalfa hay. Tubes were sealed with butyl rubber stoppers and aluminum crimps and incubated at 39°C. Nisin and monensin were prepared as separate anaerobic solutions dissolved in 95% ethanol. Nisin solutions were covered in aluminum foil to prevent degradation by light. All inhibitors were stored at 4°C. Nisin and monensin were added to each culture tube to achieve final concentrations needed and equal volumes of ethanol were added as controls. The ethanol concentration was never greater than 1.6% (vol/vol). A gas sample (0.5 ml) was removed from each tube after 24 h of incubation and analyzed for methane and hydrogen. The cell-free supernatant was stored at -15°C. Mixed ruminal bacteria were added anaerobically to tubes (10 ml of 1/3 ruminal fluid) containing 20 mg of commercial starch, Trypticase, or ball-milled cellulose. The tubes were incubated at 39°C in a water bath for 24 h. The cell-free supernatants were prepared and stored as described above. To see if the ruminal bacteria could adapt to monensin or nisin, the mixed ruminal bacteria were transferred successively (4 times) in fresh tubes that contained 1/3 ruminal fluid (clarified by centrifugation and autoclaved), 2/3 basal medium (see above), 20

mg starch, Trypticase, or 9 d ball-milled cellulose and either 5 mM monensin or nisin. Cultures of ruminal obligate amino acid-fermenting bacteria were grown anaerobically on Trypticase or Casaminoacids with different concentrations of nisin and monensin (as above). To study adaptation, the obligate amino acid-fermenting bacteria were transferred successively (4 times) with the highest dose of either monensin or nisin that did not inhibit growth. Fermentation acids were measured by HPLC. Ammonia was assayed by a colorimetric method.

Results

When mixed ruminal bacteria and alfalfa were incubated in vitro, monensin and nisin both inhibited methane production so long as the concentrations were greater than 1 mM. Monensin- and nisin-dependent methane depressions caused a decrease in the acetate to propionate ratio (4.5 to 3.0). Total volatile fatty acid production was decreased by both monensin and nisin addition at concentrations greater than 2 mM. Starch-digesting ruminal bacteria were initially inhibited by monensin and nisin, but this effect disappeared after 2 to 4 transfers. Nisin always inhibited cellulolytic bacteria, but the nisin-dependent inhibition of cellulose digestion was no greater than the inhibition caused by monensin. Monensin and nisin also inhibited amino acid degradation, and nisin was more effective than monensin in controlling the growth of Clostridium aminophilum, an obligate amino acid-fermenting ruminal bacterium that can tolerate low concentrations of monensin. Because nisin was as potent as monensin, bacteriocins such as nisin may have potential as feed additives.

Discussion

Monensin and nisin have very different chemistries, but both of these molecules are able to translocate ions across cell membranes. Monensin is a donut-shaped ionophore that complexes monovalent metal ions and allows them to pass across the cell membrane. Once the metal ion has been released, the carboxyl group then binds a proton and shuttles it in the opposite direction. Nisin is a short peptide that aggregates to form a pore through the cell membrane. Nisin dissipates both components of the protonmotive force, and nisin appears to produce nonselective channels for ions, amino acids and ATP.

Ionophore sensitivity is closely correlated with cell wall structure. Gram-negative bacteria have an outer membrane that keeps monensin from reaching the cell membrane, but gram-positive bacteria lack this defense mechanism and are generally more sensitive. The outer membrane also serves as a barrier to nisin, but recent work indicated that nisin was able to increase the oxygen consumption of *Escherichia coli*, a gram-negative bacterium. Ruminal bacteria produce a variety of peptidases and proteinases, but nisin is an unusual peptide. The sulfur linked alanine residues (lanthionine) of nisin are resistant to common proteinases.

Because monensin is typically fed at a daily dose of 350 mg per day and the ruminal volume of mature cattle is approximately 70 liters, the in vivo concentration of monensin would be approximately 5 ppm or 7.2 mM. These calculations, however, do not consider the fact the bacterial concentration in vivo is very high and the observation that monensin can bind to gram-negative bacteria as well as feed particles. Because the effective in vivo dose of monensin is less than 5 mM, we decided to examine the effect of monensin and nisin in a dose-dependent fashion.

Monensin has little direct effect on methanogenic bacteria, but it inhibits ruminal bacteria that produce hydrogen, a precursor of ruminal methane. Because ruminal methane can also be derived from formate, monensin never causes a complete inhibition of methane production. Our in vitro experiments indicated that monensin and nisin decreased methane production 47 and 32%, respectively. Monensin and nisin both decreased the ratio of acetate to propionate production and this result is consistent with their ability to decrease methane production and divert reducing equivalents to other disposal mechanisms (e.g., propionate).

When mixed ruminal bacteria were incubated in vitro with alfalfa hay, monensin and nisin did not cause an inhibition of volatile fatty acid production until the

concentration was greater than 2 mM, and the maximum inhibition was 18%. Purified substrates were more sensitive to monensin and nisin, and even low doses (≤ 1 mM) caused a significant inhibition. Starch was less sensitive than cellulose, but even starch-fermenting bacteria were inhibited by low concentrations of monensin or nisin. Monensin and nisin-dependent inhibitions of starch fermentation disappeared in subsequent transfers, but the cellulolytic bacteria never seemed to adapt.

Ruminal amino acids can be deaminated by carbohydrate-fermenting ruminal bacteria, but these bacteria have low specific activities of ammonia production and are in most cases monensin resistant. The rumen also has a small but highly active population of monensin-sensitive, obligate amino acid-fermenting bacteria. When cattle were supplemented with monensin, the specific activity of ammonia production and steady state concentration of ammonia decreased 30 to 50%, and there was a 10-fold decline in the numbers of obligate amino acid-fermenting bacteria. Monensin and nisin were both able to decrease Trypticase deamination, and little, if any, adaptation was observed.

Obligate amino acid-fermenting ruminal bacteria are sensitive to monensin in vitro, but *C. aminophilum* is more resistant to monensin than *C. sticklandii* or *P. anaerobius* . When animals are fed monensin, the amount of rRNA that would hybridize *with C. sticklandii* or *P. anaerobius* probes declined to undetectable amounts, but *C. aminophilum* persisted. The present work indicates that *C. aminophilum* was 8-fold more resistant to nisin than monensin and adaptation was not observed.

Conclusion

Nisin and monensin are both able to inhibit ruminal methane, decrease acetate to propionate ratios and prevent amino acid deamination. Nisin and monensin have similar effects on carbohydrate fermentation, but nisin is a more potent inhibitor of obligate amino acid-fermenting ruminal bacteria. There was little evidence that nisin was inactivated by ruminal bacteria, but in vivo feeding trials are obviously needed to evaluate more fully the use of bacteriocins as ruminant feed additives.