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U.S. Dairy Forage Research Center 2005 Research Report

Greener Horizons for Crops, Cows, and Communities

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Preface

It is a pleasure to bring you these summaries of research conducted during 2005 at the U.S. Dairy Forage Research Center (USDFRC). Established in 1980, the Center's mission is to build a base of knowledge and technology to help the dairy industry fully exploit the use of forages in the production of milk. The USDFRC is one of about 100 units in the Agricultural Research Service (ARS) of the U.S. Department of Agriculture (USDA) – the only ARS unit with the mission of improving forage use by dairy cattle. The majority of our research is conducted at three locations: our Center offices and laboratories on the University of Wisconsin-Madison campus; our 2,006-acre, 350-cow research farm near Prairie du Sac, WI; and the new Institute for Environmentally Integrated Dairy Management in Marshfield, WI.

To solve problems that are national in scope, we take a multidisciplinary approach to our research. We employ dairy scientists, agronomists, soil scientists, agricultural engineers, plant geneticists, plant physiologists, microbiologists, and chemists. At present, we have 20 scientists: 15 at Madison; two at Marshfield; two cluster scientists at the University of Minnesota in St. Paul; and one cluster scientist at Cornell University in Ithaca, NY.

Planning future research

This past year USDFRC scientists have been busy planning for the next cycle (2007-2012) of research projects. Much work has gone into discerning the research needs of the industry and matching them to the scientific expertise at the Center. Much of our research continues to be focused on finding ways to improve the impact of manure on the environment, starting with the plants that we grow and feed to dairy cows. We are also discovering ways in which our basic plant, cell wall, and microbial research can be transferred to the growing arena of bioenergy.

We are pleased to have a very active Stakeholder group helping us to shape our research priorities. In January we held a special Stakeholder Conference, "Building a Bridge Between Today's Challenges and Tomorrow's Research." And several of our Stakeholders attended ARS National Program planning sessions where they did an excellent job of representing dairy and forage interests at the national level. We gratefully acknowledge and thank our Stakeholders for all of their support.

Breaking new ground

One of the highlights of the year was breaking ground for the new Institute for Environmentally Integrated Dairy Management in Marshfield, WI. About 70 people attended the August 23 event, including Sen. Herbert Kohl of Wisconsin. The Institute is a collaborative effort between four existing public and private entities: the USDA Agricultural Research Service/USDFRC; the USDA Natural Resources and Conservation Service; the University of Wisconsin-Madison College of Agricultural and Life Sciences; and the National Farm Medicine Center of the Marshfield Clinic Research Foundation. Research objectives of the new work site are:

- To improve nutrient management systems (crop and cropping patterns; livestock feeding and management systems; manure treatment, handling, storage, and application strategies – including grazing animals as well as mechanical application).
- To reduce degradation of air, water, and soil from livestock waste.
- To control pathogen transmission between livestock, wildlife, humans, and the environment.
- To transfer and apply the knowledge to all segments of the dairy industry.

Nitrogen Symposium

One way the U.S. Dairy Forage Research Center transferred knowledge this year was to sponsor a symposium, "Transforming Forages to Improve Nitrogen Use by Dairy Cows and Decrease Nitrogen Emissions," at the

2006 Joint Annual Meeting of the American Dairy Science Association and the American Society of Animal Science. Topics were:

- "Source, Amount, and Fate of Nitrogen on Dairy Farms"
- "Forages on Dairy Farms: Serving Cows and the Environment"
- "Preservation of Protein During Harvest and Storage"
- "Challenges in Animal Utilization of High Protein Forages by Dairy Cows,"
- "Manure Nitrogen Transformations in Crop/Soil Systems on Dairy Farms"
- "Transforming Forage Plants to Improve Nitrogen Utilization in Dairy Systems: What Are the Possibilities?"

All of the presentations are posted on our web site: http://ars.usda.gov/mwa/madison/dfrc, under "News & Events."

New to the staff

Two new scientists joined our staff this year. Dr. Peter Vadas began in April 2006 as a new research scientist. Dr. Vadas received a B.S. degree in Crop and Soil Environmental Sciences from Virginia Tech in 1993 and his M.S. and Ph.D. degrees from the University of Delaware in the area of environmental management of agricultural nutrients. For his Ph.D., Dr. Vadas refined the FHANTM nutrient/water quality model for use in the mid-Atlantic coastal plain. Upon completing his Ph.D. in 2001, he took a post-doc position with the USDA-ARS in Beltsville, MD. In 2002, Dr. Vadas joined the USDA-ARS Pasture Systems and Watershed Management Research Unit in University Park, PA. His research at the USDFRC will take a systems approach to integrate socio-economic and physical assessments of dairy forage agriculture. Ultimately, his work will help identify and develop economically, environmentally, and socially acceptable management systems to sustain agricultural ecosystems and protect environmental quality.

Matthew Digman comes to our Center through the Student Career Employment Program (SCEP), a program that allows scientists to be employed by ARS while earning their Ph.D. Digman received a B.S. degree in mechanical engineering in 2003 from the Milwaukee School of Engineering. For two summers he held an internship with Kuhn Knight, Inc. During his senior year at MSOE he worked with a team of students and Kuhn Knight, Inc., on a senior design project regarding controlled manure application. Upon graduating from MSOE he returned to Kuhn Knight, Inc. as a full-time design engineer. In 2004 he was offered a research assistantship at the University of Wisconsin-Madison under the direction of Dr. Kevin Shinners. His research included the investigation of NIRS technology to meet the challenge of predicting moisture in real time on the forage harvester. Digman's Ph.D. thesis will investigate potential on-farm pretreatments of biomass materials to enhance enzymatic or chemical hydrolysis.

I want to thank scientists, support staff, students, visiting scientists, and Stakeholders for their dedication, creativity, and cooperative spirit. And I would like to thank the dairy and forage producers that we serve, for they are the ones who are on the front line providing a healthy, safe, and economical food source for our nation's citizens.

Sincerely,

Neal P. Martin, Director
U.S. Dairy Forage Research Center

September, 2006

In Memory of
Dr. Larry D. Satter
1937 to 2006

Dr. Satter enjoying his retirement party in July of 2003.

As this Research Report was being put together, the U.S. Dairy Forage Research Center lost a great friend with the death of Dr. Larry D. Satter on August 13, 2006. Dr. Satter was one of the original research scientists hired when the USDFRC began operating in 1981. He served as Center director from 1987 to 1998, and then returned full-time to his research in dairy cattle nutrition until his retirement in 2003.

Dr. Satter, a native of western Minnesota, earned his B.S. degree at South Dakota State University. He enrolled at the University of Wisconsin-Madison in 1960 as a graduate student in dairy science. After receiving a doctorate in biochemistry and dairy science in 1964, Dr. Satter joined the faculty of the dairy science department at the UW. He retained his appointment on the dairy science faculty while working for the USDFRC.

Dr. Satter's research focused on improving the economic and environmental sustainability of livestock farming systems, particularly of dairy operations. He played a central role in developing our current understanding of how protein is digested in ruminant animals. His work also led to more accurate dietary recommendations for nitrogen and phosphorus in ruminant diets. Both lines of study have helped reduce feeding costs for farmers.

Later in his career, Dr. Satter expanded his field of study to include environmental impacts of livestock farming. To that end, his research sought to refine the use of nitrogen and phosphorus supplements in dairy and beef diets in order to minimize the amount of nitrogen and phosphorus excreted in livestock manure.

Throughout his career, Satter was willing to reassess and correct strongly held beliefs about ruminant nutrition. "Larry was known for his 'moral authority.' He could be trusted to tell you the truth about what the data did and didn't say," says Glen Broderick, a USDFRC research dairy scientist who worked with Dr. Satter. "This is one of the most important things he brought to the Dairy Forage Research Center as a scientist and then as a director. Our center still enjoys a reputation for high-quality science derived at least in part from the moral authority of our long-time leader."

Dr. Satter was involved in many professional organizations and received a variety of awards and accolades throughout the course of his career, including the Outstanding Teacher Award from the UW College of Agricultural and Life Sciences and the Award of Honor from the American Dairy Science Association.

"To be Larry's student was to be his friend and his colleague," says Broderick. "Larry Satter was a rare and fine individual who made friends easily and whose special qualities made those friends loyal for life."

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#Member of University of Wisconsin-Madison, College of Agricultural and Life Sciences, Agricultural Research Stations and supported by the U.S. Dairy Forage Research Center.

Forage Genetics and Production

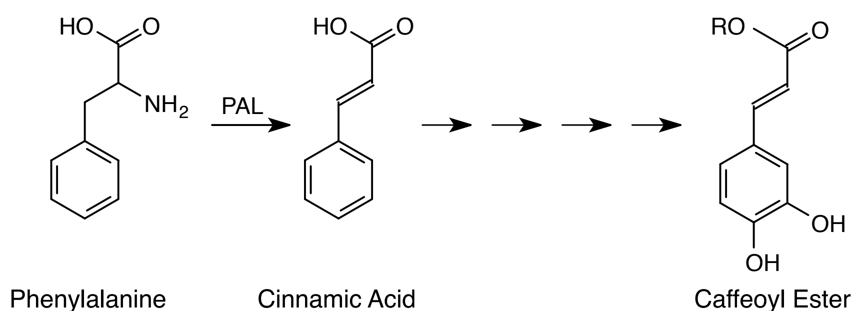
Cloning and Characterization of Phenylalanine Ammonia Lyase (PAL) cDNAs from Red Clover

M.L. Sullivan, B. Hesprich, and S.A.M. Zerbel

Introduction

Phenylalanine ammonia lyase (PAL) is responsible for the deamination of phenylalanine to yield cinnamic acid, a first step in the production of a wide range of natural products based on the phenylpropanoid skeleton (Figure 1). In red clover, *o*-diphenols, a class of phenylpropanoids, accumulate to high levels and are a substrate for oxidation by an endogenous polyphenol oxidase (PPO). When red clover is preserved by ensiling, post-harvest oxidation of *o*-diphenols by PPO prevents degradation of forage protein to non-protein nitrogen, which is poorly utilized by ruminant animals. To exploit this natural system of protein protection in other forage crops, we are trying to understand how red clover is able to accumulate the required levels of *o*-diphenol compounds. Here we report the isolation and characterization of red clover genes encoding PAL, the first enzyme in the phenylpropanoid biosynthetic pathway.

Figure 1. Deamination of phenylalanine by PAL is the first step in the biosynthesis of many phenylpropanoid compounds, including *o*-diphenol caffeic acid derivatives.



Methods

cDNA prepared from young red clover leaves was used as a template for PCR using primers designed from conserved regions of PAL genes from *Arabidopsis thaliana* and the model legume *Medicago truncatula*. The resulting red clover PAL gene fragment was used to screen a red clover cDNA library derived from unexpanded and young leaves. Isolated red clover PAL cDNAs were sequenced and the data analyzed using GCG software. Expression of the red clover PAL genes was analyzed using quantitative real time PCR (qPCR).

Results and Discussion

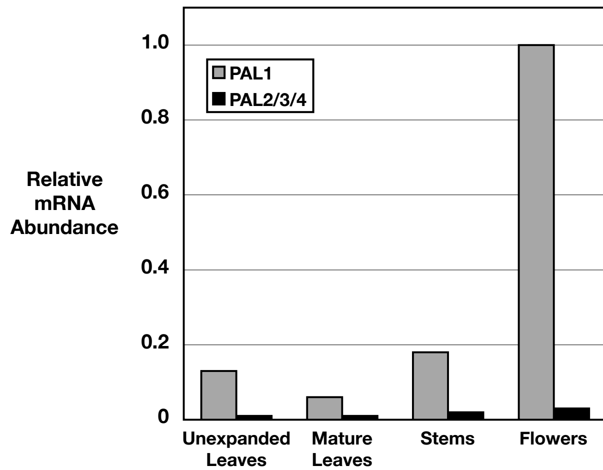
Because young red clover leaves accumulate substantial levels of *o*-diphenols, we screened a cDNA library derived from this tissue to obtain PAL cDNAs that might be most relevant to their biosynthesis. Our screen of the cDNA library yielded more than twenty putative PAL clones. Limited sequence analysis of these cDNA clones revealed they correspond to four unique red clover PAL genes, which we have designated PAL1-4 (Table 1). Full-length copies of the PAL1 cDNA were recovered far more frequently (seventeen clones) than those of PAL2/3/4 (one clone each), suggesting PAL1 is the most highly expressed of the genes in unexpanded and young leaves, the tissue from which the cDNA library is derived. The longest cDNA corresponding to each gene was sequenced in entirety on both stands and the sequence data deposited in the NCBI Genbank database. PAL2 and PAL3 encode nearly identical proteins (99%) that are very similar to that encoded by PAL4 (97% iden-

Gene	Genbank Accession	Number of Clones	Peptide Length (Residues)
PAL1	DQ073809	17	712
PAL2	DQ073810	1	725
PAL3	DQ073808	1	724
PAL4	DQ073811	1	725

tity). The proteins encoded by PAL2/3/4 are only about 89% identical to that encoded by PAL1, but have high sequence identity to an elicitor-induced PAL gene from alfalfa, suggesting a role in pathogen defense.

We used quantitative real time PCR (qPCR) to evaluate tissue-specific expression of the red clover PAL genes. The high degree of sequence similarity among PAL2/3/4 prevented analysis of the individual genes in this group. Consequently, qPCR primers specific for PAL1 or PAL2/3/4 were designed, validated using the cloned PAL cDNAs, and used to analyze PAL mRNA levels in unexpanded leaves, mature leaves, stems, and flowers. Taking into account differences in efficiency of the primer pairs used, PAL1 is expressed to much higher levels than PAL2/3/4 in all tissues examined (Figure 2), a finding consistent with the high number of PAL1 clones recovered in the library screen. PAL1 expression is highest in flowers, followed by stems and unexpanded leaves. Expression is lowest in mature leaves. The high level of PAL expression in flowers is not surprising given the enzyme's involvement in biosynthesis of flavanoid flower pigments. Like PAL1, PAL2/3/4 expression is highest in flowers.

Figure 2. Relative mRNA abundance of PAL1 and PAL2/3/4 in red clover tissues by quantitative real time PCR.



Conclusions

PAL is encoded by a small multigene family in red clover. Red clover PAL1 is expressed to much higher levels (up to sixty-fold) than PAL2/3/4 in all tissues examined suggesting the encoded enzyme plays the major role in biosynthesis of phenylpropanoid compounds under normal conditions. Sequence similarity of PAL2/3/4 to an elicitor-induced alfalfa PAL gene suggests they may be involved in pathogen defense.

Use of Hygromycin Resistance as a Selectable Marker in Red Clover Transformation

M.L. Sullivan and S.A.M. Zerbel

Introduction

Genetic modification of plants by the insertion of transgenes can be a powerful experimental approach to answer basic questions about gene product function. This technology can also be used to make improved crop varieties for use in the field. To apply this powerful tool to red clover (*Trifolium pratense*), germplasm with increased frequency of regeneration in tissue culture was developed and utilized as the explant source for development of an *Agrobacterium*-mediated transformation protocol (Quesenberry *et al.*, 1996, *Crop Sci.* 36:104). We have been testing several variables within this original protocol to increase the efficiency and flexibility of the red clover transformation system. Here we report the use of hygromycin resistance as an alternative selectable marker for red clover transformation.

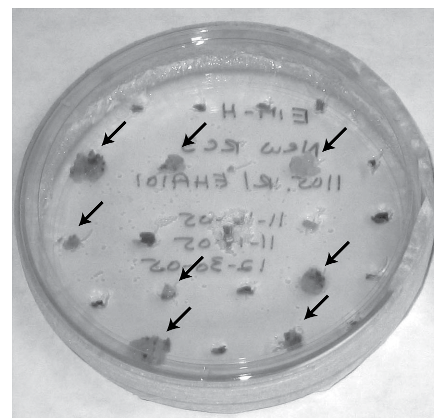
Methods

Media, tissue culture methods, and transformation were essentially as described in the detailed protocol on our web site (http://www.dfrc.ars.usda.gov/DFRCWebPDFs/2006-31_Sull_369_384.pdf). Red clover tissue explants were transformed with *Agrobacteria* harboring pCAMBIA1105.1, a binary transformation plasmid containing a gene encoding hygromycin resistance (hph) linked to a GUS reporter gene. Presence of the GUS gene in transgenic plant materials was confirmed by staining with the chromogenic substrate X-Gluc, which turns blue in the presence of the GUS gene product.

Results and Discussion

Determination of Effective Hygromycin Concentration. Approximately twenty petiole explants from a non-transformed red clover plant were incubated on media containing 0, 12.5, 25, and 50 mg/L of the antibiotic hygromycin. In the absence of the antibiotic, all explants formed healthy undifferentiated callus tissue as expected. Hygromycin at 12.5 mg/L resulted in necrosis of nearly all explants, although two formed a small amount of unhealthy brown callus. On media with 25 and 50 mg/L hygromycin, all explants became necrotic and no callus tissue formed. Based on these results, we chose 25 mg/L hygromycin in subsequent transformation experiments, since this was the minimum level of antibiotic required to prevent callus tissue formation.

Figure 1. Embryos form on several red clover petiole explants following Agrobacterium-mediated transformation with an hph gene. Explants with embryos are marked with arrows.



Transformation and Selection on Hygromycin. To test the efficacy of hygromycin selection in transformation, red clover petiole explants were cultured with *Agrobacteria* harboring the plasmid pCAMBIA1105.1 prior to transfer to antibiotic-containing selective medium. Many of the explants (5 to 50% in multiple experiments) co-cultivated with the *Agrobacterium* formed callus tissue and subsequently green embryos in the presence of 25 mg/L hygromycin (Figure 1). In many cases, the embryos developed into plantlets that were transferred to soil. Transformation efficiency was similar to that seen when using kanamycin resistance as the selectable marker. Greater than 70% of the transformed plants had detectible GUS activity, indicating most, if not all, of the recovered plants had received a transgene. In some experiments, we were able to super-transform a plant previously transformed with a kanamycin resistance marker.

Conclusions

Hygromycin resistance (hph) is an effective selectable marker for red clover transformation with few, if any, escapes. This provides added flexibility for experiments involving red clover transformation and allows super-transformation of previously transformed plants.

Forage Handling, Preservation and Storage

Alternate Covering System for Bunker Silos

R.E. Muck

Introduction

The most common covering on bunker and pile silos is 150 - 220 µm thick polyethylene plastic held in place by used tires. The plastic may be black or white. Typical practice in recent years at our research farm has been to use 220 µm white plastic because it is easier to handle on warm and/or breezy days and produces less surface spoilage than 150 µm black plastic. The handling of used tires has also been improved by using a combination of half tires at the edges and down the middle of the bunker and tire sidewalls in between. These are easier to handle than full tires and do not accumulate water. Even so, bunker covering is not a favorite farm task. An alternative system (Silostop) has been developed that uses a new plastic formulation (45 µm thick but more impermeable to oxygen), plastic on the walls as well as the top, a reusable woven plastic tarp to protect the plastic, and sand bags only at the walls and seams to hold the plastic and tarp in place.

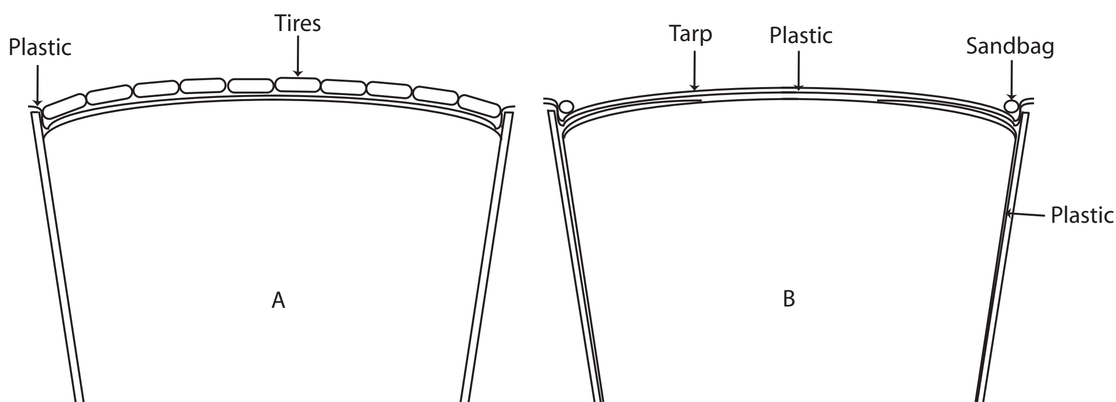
Methods

The Silostop system was compared to our standard system of 220 µm white plastic and tires over three years on six bunker silos, two with alfalfa silage and four with corn silage. Schematics of the two systems are shown in Fig. 1. Each bunker was split in half, front to back. One covering system was used on the back half and the other on the front half. Immediately before covering, core samples were taken on the top of the silo at 0-15, 15-30, 30-45 and 45-60 cm depth and at various locations (60 cm from the wall and in the middle) for each half. When the silos were opened for feeding, samples were again taken at similar locations in each half by cutting holes in the plastic and taking cores at each of the four depths. The holes were sealed with plastic tape immediately after sampling.

Results and Discussion

The Silostop system in five of six cases substantially improved the quality of silage immediately beneath the plastic (0-15 cm) near the bunker walls. This is shown by the higher pH (Fig. 2) and higher ash content (Fig. 3) for the white plastic at the wall. As oxygen infiltrates into a silo, spoilage microorganisms will consume

Figure 1. Diagrams of bunker covering systems. A) Conventional plastic weighted with used tires, B) Silostop system – plastic sheets on side walls folded over crop, plastic top sheet above side sheets, reusable tarp on top of plastic, sandbags at walls to anchor plastic and tarp.

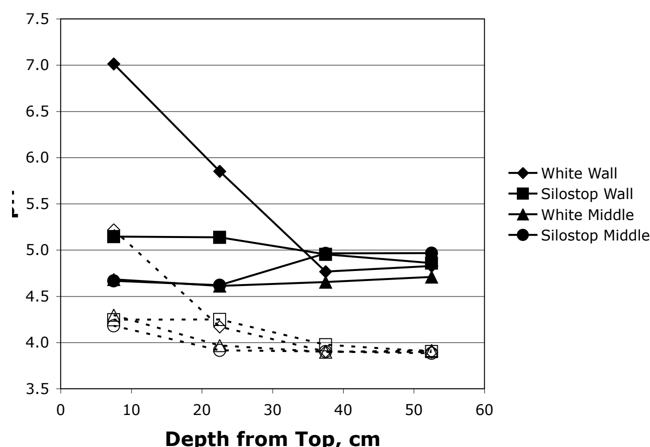


the lactic and acetic acids in silage, producing carbon dioxide and water. The loss of acids raises pH, and the loss of carbon dioxide gas increases ash content. Consequently the Silostop system did a better job of reducing oxygen infiltration on the shoulders of the bunker. Estimating dry matter loss based on the increase in ash content during ensiling, the dry matter loss in the top 15 cm near the wall was on average 17 and 13 percentage units higher under the white plastic than the Silostop system for the two alfalfa and three corn silage bunkers, respectively.

At deeper locations or in the middle of the top surface, the two systems generally had similar pH values and ash contents, indicating little difference in dry matter recovery. Even where pH and ash content were not significantly affected by the covering system, lactate/acetate ratio was higher or tended to be higher in the top 30 cm under the Silostop system, both at the wall and in the middle of the top surface. This is additional evidence that the Silostop system is more effective at keeping oxygen out.

In one corn silage bunker, the Silostop system did not provide an advantage over the white plastic. This occurred because of an inadequate overlap and seal between the wall sheet and top sheet of plastic film in the Silostop treatment. This indicates that careful management is needed for the Silostop system to work properly.

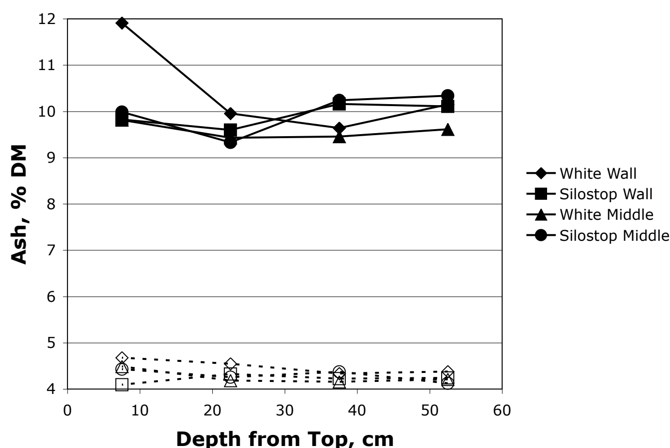
Figure 2. Average pH values at the top of two alfalfa (solid symbols, lines) and three corn silage (open symbols, dashed lines) bunkers when covered by white polyethylene or the Silostop system.



Conclusions

The Silostop system produced noticeably less spoilage on the top 15 cm of bunker silos near the walls in both alfalfa and corn silage. Differences in dry matter recovery in the middle of the top or at deeper levels were much smaller, but fermentation analysis suggested better oxygen exclusion in those areas by the Silostop system. Economic analysis is needed to assess the returns on this more costly covering system.

Figure 3. Average ash concentrations at the top of two alfalfa (solid symbols, lines) and three corn silage (open symbols, dashed lines) bunkers when covered by white polyethylene or the Silostop system.



Plant Chemistry and Biochemistry

Influence of Lignin on the Adsorption of Heterocyclic Aromatic Amines by Insoluble Fiber

C. Funk , J.H. Grabber, P. Weber, J. Thilker, H. Steinhart, M. Bunzel

Introduction

Dietary fiber may help to prevent colon cancer in humans by adsorbing carcinogens and transporting them out of the body before they become metabolically activated. Previous *in vitro* studies suggest that lignin in some types of dietary fiber might play a role in adsorbing heterocyclic aromatic amines (HAAs), a group of carcinogens found mostly in heated, protein-rich food. However, due to the complex composition of dietary fibers used in these studies, the role of lignin in HAA adsorption was not definitively demonstrated. The aim of this study was to clearly delineate how lignin concentration and composition influence the adsorption of HAAs.

Methods

Artificially lignified maize cell walls containing 5 to 20% lignin were prepared by adding various monolignols and hydrogen peroxide to nonlignified cell walls isolated from maize cell suspensions. *In-vitro* adsorption studies were carried out by incubating lignified and nonlignified cell walls with various HAAs under conditions that mimick the small intestine. The HAAs evaluated were 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC). Adsorption of HAAs was determined by HPLC-UV.

Results and Discussion

Lignification of maize cell walls enhanced the adsorption of HAAs, particularily the more hydrophobic types. For example, increasing the concentration of guaicyl lignin in the cell wall matrix from 0.4 to 11% increased adsorption of a more hydrophobic AαC from 26 to 60% whereas the adsorption of a less hydrophobic MelQx increased from only 10 to 18%. The more hydrophobic PhIP and AαC, were better adsorbed by guaiacyl-rich lignins. Among the less hydrophobic HAAs, MeIQx was best adsorbed by syringyl-rich lignins, whereas the adsorption of IQ was not clearly influenced by lignin composition. The adsorption of HAAs was also influenced to varying degrees by the incorporation of other minor 4-hydroxyphenylpropanoids (coniferaldehyde, sinapyl acetate, 5-hydroxyconiferyl alcohol, or dihydroconiferyl alcohol) into lignin. Finally, the studies demonstrated that the cell wall matrix adsorbed considerable amounts of HAAs even in the absence of lignin, however, variations in cell wall feruloylation had no effect on HAA adsorption.

Conclusions

The adsorption studies demonstrated that lignified fiber is an effective adsorber of HAAs, particularily the more hydrophobic types. Both lignin concentration and composition influenced the adsorption of HAAs to cell walls.

Formation of Syringyl-Rich Lignins in Primary Maize Walls as Influenced by Cell Wall Feruloylation and *p*-Coumaroylated Monolignols

J. H. Grabber and F. Lu

Introduction

Cell walls in maize and other grasses are atypical because their xylans are acylated with ferulate and lignins are acylated with *p*-coumarate. Lignins in maize also contain a high proportion of syringyl units, which is puzzling considering the very low capacity of maize peroxidase to oxidize sinapyl alcohol. In this study, we examined the copolymerization of monolignols and *p*-coumarate esters in feruloylated maize cell walls and in isolated maize lignin to probe the function and interactions of *p*-hydroxycinnamates during the formation of lignins in primary cell walls of maize.

Methods

Primary cell walls from maize suspension cultures (containing *in situ* peroxidases) were stirred in buffer and artificially lignified with monolignols plus varying levels of *p*-coumarate esters. In other studies, isolated maize pith walls (containing *in situ* peroxidases) or maize lignin and horseradish peroxidase were stirred in buffer with H_2O_2 and monolignols. Samples were analyzed for Klason lignin and for alkali-labile *p*-hydroxycinnamates.

Results and Discussion

Ferulate esters in cell walls enhanced the formation of wall-bound syringyl lignin more than methyl *p*-coumarate added with sinapyl alcohol, however, maximal concentrations of syringyl lignin were only one-third that of guaiacyl lignin (data not shown). Including sinapyl *p*-coumarate, the putative precursor of *p*-coumaroylated lignins, with monolignols unexpectedly accelerated peroxidase inactivation, interfered with ferulate copolymerization into lignin, and had minimal or adverse effects on cell wall lignification (e.g. Table 1). Free phenolic groups of *p*-coumarate esters in isolated maize lignin and in pith walls did not undergo oxidative coupling with each other or with added monolignols (Table 2).

Table 1. Concentrations of lignin, *p*-coumarate (*p*CA), ferulate (FA), diferulate (DFA), and total ferulate (FA plus DFA) in maize cell walls artificially lignified with monolignols and sinapyl *p*-coumarate (SA-pCA).

Precursors[a]		Lignin[b]			Alkali-labile[c]				Cross-linked[d]
Monolignol	SA-pCA	Predicted	Δ Mass	Klason	*p*CA	FA	DFA	TFA	TFA
Sinapyl alcohol									
0.75	0	151.0	108.5	113.8	0.19	0.80	1.36	2.13	18.75
0.50	0.25	179.8	81.2	90.4	11.74	4.47	4.20	8.67	12.20
Coniferyl alcohol									
0.75	0	132.0	112.1	140.8	0.25	0.58	2.12	2.70	18.20
0.50	0.25	168.4	116.2	144.5	16.88	2.24	3.09	5.33	15.55
Analysis of variance[e]									
monolignol			**	**	*	**	NS	**	**
SA-pCA			**	NS	**	**	**	**	**
monolignol x SA-pCA			**	†	*	**	**	**	**
CV %			2.9	5.7	14.3	9.3	7.8	5.3	1.6

[a] Average quantity of precursors (mmol) added to lignify 0.9 g of cell walls. Data are averaged from studies with two rates (0.6 and 0.9 mmol) of precursors addition.
[b] Lignin content (mg/g cell wall) predicted for complete polymerization of precursors into cell walls, and estimated by the Δ cell wall mass and the Klason procedure.
[c] Ester-linked hydroxycinnamates (mg/g cell wall) released by alkaline hydrolysis.
[d] Hydroxycinnamates cross-linked to lignin (mg/g cell wall) estimated as the difference in alkali-labile acids recovered from nonlignified and artificially lignified cell walls.
[e] NS, †, *, **, not significant and significant at the 0.1, 0.05, and 0.01 levels of probability, respectively.

Conclusions

The ability of maize to form syringyl-rich lignins and the functional role of extensive lignin acylation by *p*-coumarate remains a mystery. In ongoing work, we are investigating how temporal aspects of lignin formation may influence the peroxidase-mediated copolymerization of monolignols, sinapyl *p*-coumarate, and ferulates and the type of coupling structures formed. In addition, we will assess whether sinapyl *p*-coumarate contributes to the relatively low molecular weight and high extractability of maize lignins and evaluate its effect on the enzymatic degradability of cell walls. We hope these studies will provide some insight into the functional role of extensive lignin acylation by *p*-coumarate in maize and other grasses.

Table 2. Concentrations of lignin and alkali-labile *p*-hydroxycinnamates in maize pith cell walls and isolated maize lignin after H_2O_2 treatment or artificial lignification with H_2O_2 and sinapyl alcohol (SA) or coniferyl alcohol (CA).

	Maize pith walls[a]			Isolated
Treatment	Klason Lignin	Ferulate and diferulates	*p*-Coumarate	Maize lignin *p*-coumarate[b]
Untreated control	99.1a[c]	13.5a	19.8a	156.0a
H_2O_2	101.8a	11.8b	19.7a	155.2a
H_2O_2 + SA	118.0b	10.2c	19.8a	147.2a
H_2O_2 + CA	249.6c	4.6d	18.2a	148.6a
CV%	2.8	7.5	5.4	9.7

[a] Concentrations (mg/g cell wall) of ferulate, diferulate and *p*-coumarate were adjusted to account for changes in mass due to polymerization of monolignols into cell walls.
[b] Concentrations (mg/g lignin) are averaged over reactions carried out in buffer (pH 5.5) or buffer:dioxane (1:1). Solvent effects on *p*-coumarate concentrations were not significant ($P > 0.05$).
[c] Means within a column followed by a different letter are significantly different according to Fisher's protected LSD ($P = 0.05$).

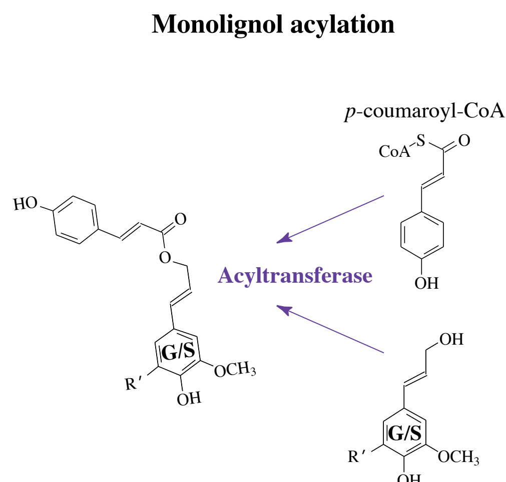
Changes in *p*CA and Lignin During Corn Stem Development

R.D. Hatfield, J.M. Marita, K. Frost

Introduction

Lignin from grasses is structurally unique from other plant lignins in that it contains up to 20% *p*-coumaric acid (*p*CA) acylated primarily to the C-9 position of syringyl units and is mediated by a p-coumaroyl transferase (Fig. 1). The function of *p*CA acylation of lignin is not understood though it has been suggested to aid in the formation of syringyl rich lignins. In most plants, lignification starts out guaiacyl rich and shifts to a syringyl rich lignin during secondary cell wall formation. If *p*CA aids in the lignification process, it seems reasonable that as the grass stem matures (increased secondary cell wall formation resulting in increased syringyl lignin) there would be a concomitant increase in cell wall bound *p*CA. A study was undertaken to test this hypothesis that followed corn stem development as the model for grasses. This is a report on the characterization of cell wall components during internode and node development in corn stems.

Figure 1. Model of sinapyl *p*-coumarate formation via the acyltransferase to form the ester linkage of *p*-coumaric acid to sinapyl alcohol.



Methods

Corn inbred B73 was grown in replicated plots at three locations, field plots at Arlington, WI, field plots at West Madison, WI, and greenhouses at the U.S. Dairy Forage Research Center, Madison, WI. Plots were established in early spring of 2005. Five plants were harvested from three replicated plots at each location when tassel emergence was visible. The seventh internode (the node just above the soil line) was cut through to remove the plants. Leaves and sheaths were carefully removed from each stem and only the stem rind of the internodes labeled from 8 to the upper most internode that had undergone some expansion (internode 16) were harvested. The stem rind material was separated into node, internode lower half, internode upper half, and node (e.g., node 8, lower internode 8 upper internode 8 and node 9). All nodes (#8-16) were processed for analysis, but only internodes #8, 10, 12, 14 and 16 were isolated and processed for analysis. Isolated cell walls were analyzed for ester-linked *p*CA, ester-linked ferulic acid, and total lignin. In addition, samples of the frozen fresh stem material were analyzed for *p*-coumaroyl transferase activity.

Results and Discussion

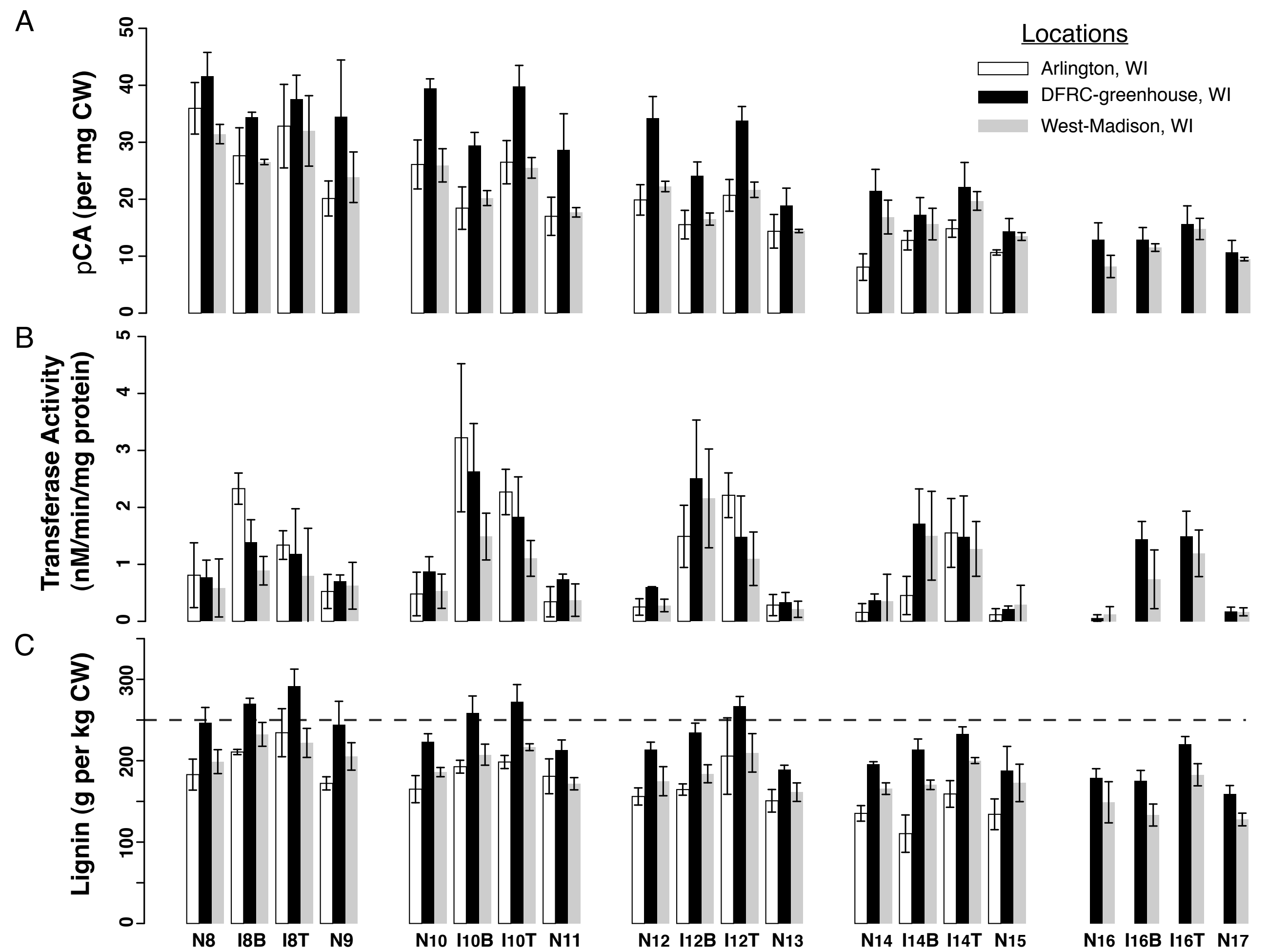
Corn stems, like all grasses, have unique developmental patterns as the plant changes from vegetative to reproductive stages. Grass stems develop from an intercalary meristem, meaning that internode cells are formed by a layer of meristematic cells at the base of the internode/top of the lower bordering node. Cell expansion accounts for the length on the fully developed internode. At tassel emergence the upper most internodes are undergoing both active cell division and cell expansion while internodes closer to the soil line have transitioned to mainly cell expansion. Within each internode there is a gradient of cells that range from fully expanded at the top to cells newly formed by cell division, depending upon the maturity of the internode. Those internodes at the base of the plant are fully elongated with little additional change occurring in the cell wall (Fig. 2). The nodes of the stem represent a complex developmental region being situated between stems cells that are fully expanded near developmental maturity and stem cells that are meristematic in nature. Developmental changes in cell

walls of corn stems (rind tissue) were followed for lignin, and ester bound phenolics (*p*-CA), and *p*-coumaroyl transferase activity (Fig. 2).

Conclusions

Lignification follows a developmental pattern up the corn stem. Generally, lignin decreases from the lowest internode (#8) to the upper most internode (#16), while within an internode, the upper half has the most lignin. Lignin levels in the nodes appear to be intermediate between the internodes it separates. As one might expect, if *p*CA is aiding in the formation of syringyl type lignins, there is a steady decrease in *p*CA that parallels the accumulation of lignin in corn stems as one moves from the lower internodes to the upper internodes. The *p*-coumaroyl transferase activity also mimics lignin formation with the highest activities in stem sections that are undergoing the most rapid lignification with syringyl units (the intermediate nodes and internodes; 10 & 12). Levels of transferase activity decrease once lignification begins to slow (lowest internodes) and is lowest in the upper most internodes that may be undergoing limited lignin accumulation or producing mostly guaiacyl-rich lignins at this stage of cell wall development. These results do not prove a role for *p*CA in lignification, but it does indicate a close relationship with formation of syringyl rich lignins produced during the latter stages of the lignification process.

Figure 2. Corn stem nodes and even numbered internodes were analyzed for lignin, *p*CA accumulation, and *p*-coumaroyl transferase activity. Deposition patterns of *p*CA (A) and lignin (C) in corn stem rind tissues harvested at three and separated into nodes and internodes (upper and lower halves). Panel B gives the transferase activity profile for nodes and internodes evaluated from the corn stem.



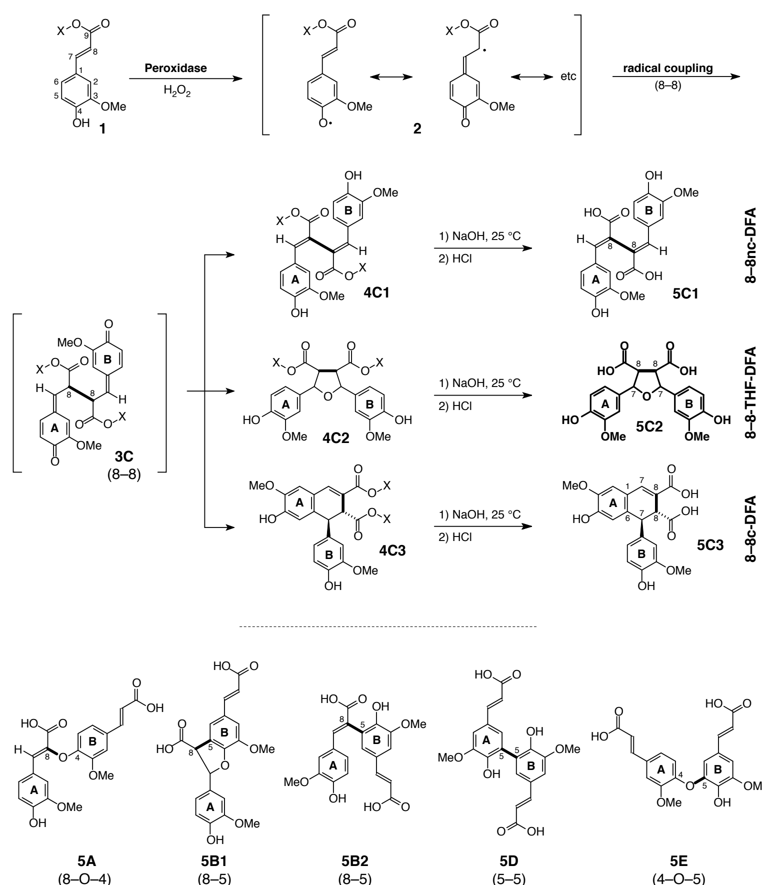
Synthesis and Identification of an Unanticipated Ferulate 8–8-Coupling Product Acylating Cereal Plant Cell Walls

P.F. Schatz, J. Ralph, F. Lu, I.A. Guzei and M. Bunzel

Introduction

Hydroxycinnamates have important roles in plant cell wall cross-linking by linking polysaccharides to each other, to lignin, and to proteins, limiting forage degradability by ruminants. The dominant mechanism for cross-linking feruloylated polysaccharides is radical coupling, leading to dehydrodiferulates and dehydrotriferulates. Radical coupling of ferulates, via the action of wall-bound peroxidases, produces several regio-isomeric dehydrodiferulates with coupling occurring at their 4–O-, 5- or 8-carbons. After saponification, the dimeric products consist of 5–5- 8–8-, 8–5-, 8–O–4-, and 4–O–5-coupled dehydrodiferulic acids (DFAs) **5**, Figure 1. In this paper we describe the synthesis of one of the six diastereomers of compound **5C2** and its unambiguous identification and quantification in different cereal grains.

Figure 1. Numbering system and radical dehydrodimerization of (cell wall bound) ferulates. The bond formed by radical coupling is bolded. In synthetic compounds X represents the methyl or ethyl ester, in the cell wall X stands for arabinoxylans, arabinans or galactans.



Methods

Synthesis. Figure 2 shows the synthetic route to the required isomer of compound **5C2**. All of the steps except the coupling step *vi* involve rather standard synthetic methods.

X-Ray crystal structure determination of 4C2-Ac. A colorless crystal with approximate dimensions 0.43 x 0.32 x 0.26 mm^3 was selected for crystal structure determination on a Bruker CCD-1000 diffractometer with Mo K_α (λ = 0.71073 Å) radiation and the diffractometer to crystal distance of 4.9 cm.

Identification of 8–8-THF-DFA in cereal grain fibers. Whole grain insoluble and soluble cereal fibers were performed according to an enzymatic preparative isolation procedure from corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), spelt (*Triticum spelta* L.), rice (*Oryza sativa* L.), wild rice (*Zizania aquatica* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), oat (*Avena sativa* L.) and millet (*Panicum miliaceum* L.). For the identification and quantification of 8–8-THF-DFA **5C2**, the internal standard 5–5-Me-DFA, the monomethyl ether of **5D**, dissolved in dioxane was added to the insoluble (40–90 mg) or soluble (60-120 mg) fiber samples weighed into a screw-cap tube, and saponification with degassed NaOH (2 M, 5 mL) was carried out protected from light for 18 h at room temperature. Samples were acidified with 0.95 mL concentrated HCl (resulting pH < 2) and

extracted into diethyl ether (4 mL, three times). Extracts were combined, evaporated under a stream of filtered air, silylated and analyzed by GC-MS and GC-FID. Some samples were also spiked following the saponification process with small amounts of the synthesized 8–8-THF-DFA **5C2**. Relative retention times of **5C2** against the internal standard 5–5-Me-DFA: GC-MS-instrumentation: 0.691, GC-FID-instrumentation: 0.683.

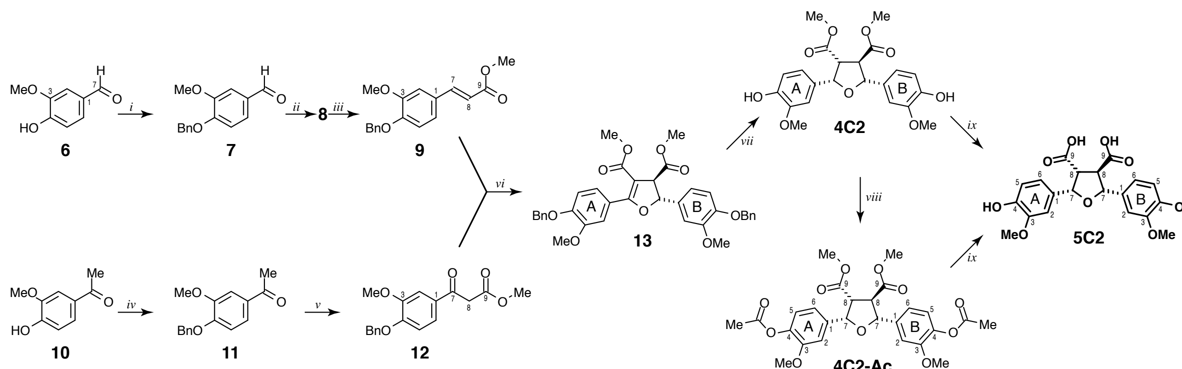


Figure 2. Synthetic pathway for **5C2**: *i*: BnCl, KI, K_2CO_3, DMF; *ii*: malonic acid, NH_4OAc, microwave; *iii*: MeI, K_2CO_3, DMF; *iv*: BnCl, KI, K_2CO_3, DMF; *v*: MeO_2CO, NaH, THF; *vi*: $Mn(OAc)_3$, HOAc; *vii*: Pd/C, H_2, EtOH; *viii*: AcCl, pyridine; *ix*: 2 N NaOH.

Results and Discussion

Synthesis. The crucial step in the synthetic pathway chosen involves the coupling of cinnamate ester **9** with a β-keto-ester **12** (Figure 2) via $Mn(OAc)_3$. The stereochemistry of the resulting dihydrofuran ring in **13** is assigned as *trans* from the NMR data. Palladium catalyzed *cis*-hydrogenation and debenzylation of the dihydrotetrahydrofuran **13** resulted in the tetrahydrofuran product **4C2**, largely as the *cis-trans-trans*-isomer. Saponification of this product, or of the derived acetate **4C2-Ac**, gave the required di-acid **5C2**. After confirming this to be the same isomer released from the natural plant sources (see below), the isomeric form was elucidated by NMR and, on **4C2-Ac**, unambiguously validated by X-ray crystal structure, Figure 4.

Identification and quantification of 8–8-THF-DFA in cereal grain fibers. In all cereal fibers, 8–8-THF-DFA (**5C2**, Figure 1) was identified by comparison of its mass spectrum and its relative GLC retention time with that of the genuine compound. Identity was further confirmed by spiking some samples with the synthesized compound. Figure 3 shows the MS-spectrum of trimethylsilylated 8–8-THF-DFA obtained via GC-MS; with originally two phenolic and two acid groups and, in comparison to the other DFAs, an additional water, its nominal molecular mass is 692.

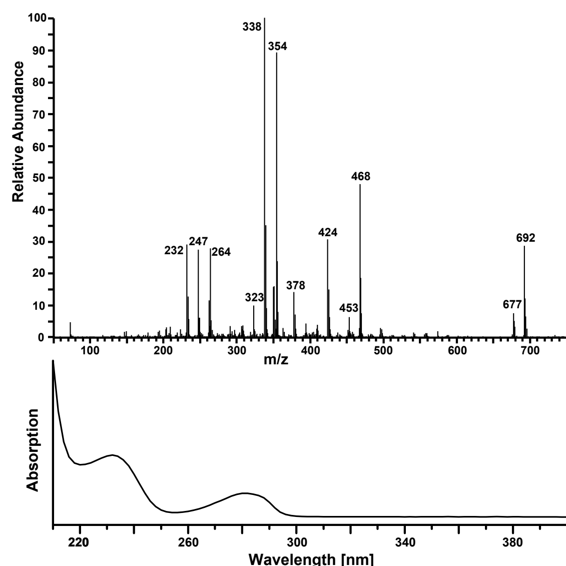


Figure 3. EI mass spectrum (top) and UV spectrum (bottom, in MeOH/1mM aqueous trifluoroacetic acid) of **5C2**.

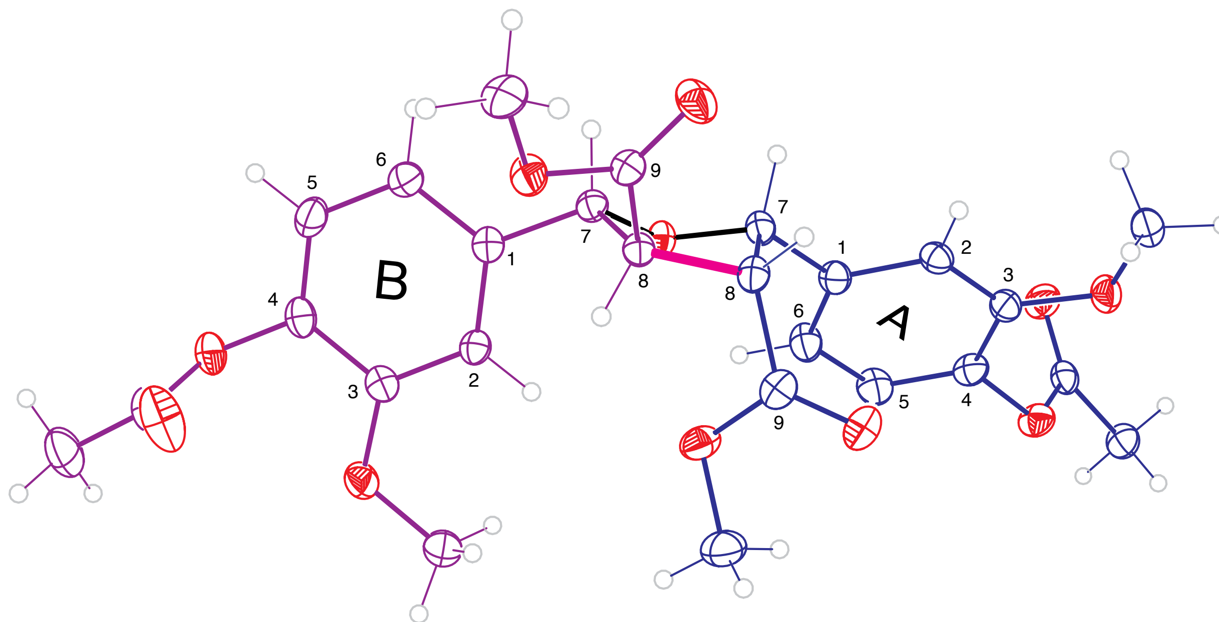
The amounts of 8–8-THF-DFA in insoluble cereal fibers were determined as follows [µg/g]: maize 692 ± 39; wheat 84 ± 8; spelt 109 ± 8; rye 132 ± 5; barley 133 ± 3; oats 149 ± 34; millet 242 ± 13; rice 109 ± 11; wild rice 115 ± 8. Regarding the DFA distribution, 8–8-THF-DFA contributes between 2.5% and 4.9% to the absolute DFA content in cereal fibers. The amounts of DFA **5C2** ranged between 50% (in rice insoluble fiber) and 90% (in rye insoluble fiber) of the amounts of DFA **5C1**. In maize insoluble fiber DFA **5C2** was even more prevalent than DFA **5C1** (about 180%).

Nature of dehydrodiferulates in the plant. We have already established that saponification of esters **4C1** and **4C3** yield only their corresponding acids **5C1** and **5C3** respectively; for example, the ester **4C1** does not produce acid **5C3**. Therefore, if these esters are in the wall, both forms need to be present to explain the saponification products. Saponification of synthesized methyl ester of **5C2** (**4C2**, X = Me, Figure 1, or the ethyl analog) in a cell-wall-like matrix consisting of cellulose/xylan 2/3 or in pure cellulose yielded predominantly the tetrahydrofuran **5C2**. It therefore now appears that 8–8-coupling in the plant cell wall leads to three distinct 8–8-diferulates (**4C1**, **4C2**, **4C3**, X = arabinoxylan, Figure 1) that are the precursors for the detected acids **5C1**, **5C2** and **5C3**. There is an urgent need to investigate the nature of the dehydrodiferulates *in vivo* and not only in alkaline hydrolysates.

Conclusions

Alkaline hydrolysates of cereal grain and grass walls contain a previously unauthenticated component derived from ferulate. Strictly DFA **5C2** is not a true dehydrodimer since an additional oxygen (from water) is incorporated. Its higher molecular mass is responsible for its being missed as a dimeric ferulate product previously. It is still formed via radical coupling, but the post-coupling steps are different from those in the previously identified DFAs. DFA **5C2** is a substantial component that should also be quantified as resulting from 8–8-dehydrodimerization. The finding of this 8–8-tetrahydrofuran DFA **5C2** along with the previously identified **5C1** and **5C2** implicates at least three 8–8-coupling products of ferulate in the cell walls of these plants and suggests that cell wall cross-linking may occur under acidic conditions.

Figure 4. 3D structure of compound **4C2-Ac** (Fig. 2) from the X-ray crystal structure, demonstrating the assigned *cis-trans-trans*-stereochemistry. The 8–8-bond formed (*in planta*) via the radical coupling step is bolded. *[This figure is in color for easier visualization of the two ferulates in the online version -- http://ars.usda.gov/mwa/madison/dfrc -- go to research, click on annual research summaries].*



Effects of Coumarate 3-Hydroxylase (C3H) Down-regulation on Lignin Composition

J. Ralph, T. Akiyama, H. Kim, F. Lu, P.F. Schatz, J.M. Marita, S.A. Ralph M.S.S. Reddy, F. Chen and R.A. Dixon.

Introduction

The effects on lignin composition and structure of perturbing one crucial step in the monolignol biosynthetic pathway remain to be addressed. Genes encoding all of the enzymes in Fig. 1 have been identified, and the effects of perturbing (by down- and/or up-regulation in transgenic plants, or via their knockouts in mutants) all but the C3H/hydroxycinnamoyl transferase (HCT) steps have been studied in some detail. Downregulation of some genes, particularly those early in the pathway, may limit the overall flux of metabolites into lignin. In other cases, the distribution of units resulting from the primary monomers (the three monlignols *p*-coumaryl **1a**, coniferyl **1b**, and sinapyl **1c** alcohols, differing in their degree of methoxylation, Fig. 1) may be dramatically altered, sometimes far beyond the limits that have been observed in nature. Such studies are not only providing rich insights into the lignification process, but are also opening up opportunities for improving the utilization of plant cell walls in a range of natural and industrial processes, e.g. ruminant digestion and chemical pulping.

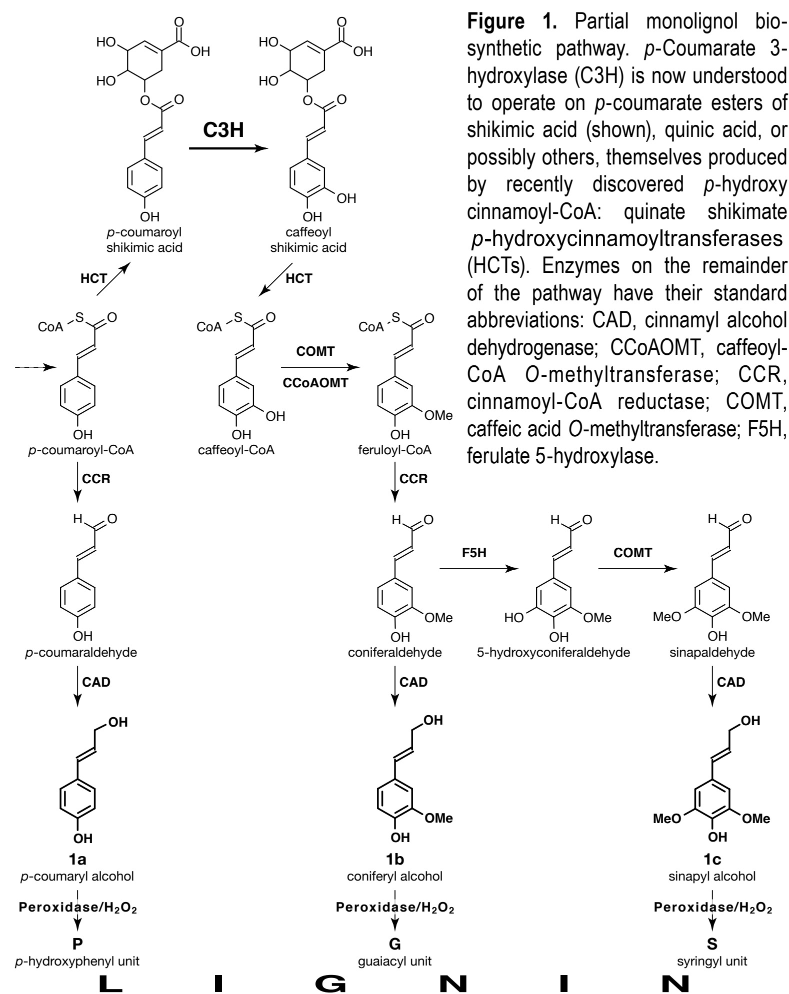


Figure 1. Partial monolignol biosynthetic pathway. *p*-Coumarate 3-hydroxylase (C3H) is now understood to operate on *p*-coumarate esters of shikimic acid (shown), quinic acid, or possibly others, themselves produced by recently discovered *p*-hydroxy cinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferases (HCTs). Enzymes on the remainder of the pathway have their standard abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase.

Aromatic hydroxylation steps are considered key reactions in plant secondary metabolism, in part due to their irreversibility. Although it is likely that perturbing C3H only affects the monolignol distribution, the monolignol *p*-coumaryl alcohol **1a** does not normally contribute to high levels of *p*-hydroxyphenyl (P) units in normal lignins. Rather, these are minor components, typically just 1-3%, in the lignins of both gymnosperms and angiosperms. *p*-Hydroxyphenyl units have long been thought to be significantly higher in grasses. They may be, but much of the data has come from incorrectly interpreting the products of degradative methods as deriving from *p*-hydroxyphenyl units in lignin whereas substantial proportions may derive from *p*-coumarate ester moieties adorning the lignins of grasses; such *p*-coumarate moieties are not involved in the backbone of the polymer and should not be confused with lignin monomers. Softwood compression wood fractions have the richest *p*-hydroxyphenyl unit content, reportedly ranging as high as 30%. Nevertheless, even this is below the levels that might be expected via C3H-downregulation. The Noble Foundation has successfully generated transgenic plants of the forage legume alfalfa (*Medicago sativa*) in which C3H levels have been reduced to as low as 5% of the wild-type level, in the absence of seriously impaired growth phenotypes. Compositional analysis of the unusual lignins in these plants is described here.

Methods

NMR Spectroscopy. The NMR spectra presented here were acquired on a Bruker Biospin (Rheinstetten, Germany) DMX-750 instrument fitted with a sensitive cryogenically-cooled 5-mm TXI $^{1}H/^{13}C/^{15}N$ gradient probe with inverse geometry (proton coils closest to the sample). Acetylated lignin preparations (30-60 mg) were dissolved in 0.5 ml $CDCl_3$; the central chloroform solvent peak was used as internal reference (δ_C 77.0, δ_H 7.26 ppm). HSQC experiments had the following parameters: acquired from 8.6-2.4 ppm in F_2 (^{1}H) using 1864 data-points (acquisition time 200 ms), 160-40 ppm in F_1 (^{13}C) using 512 increments (F_1 "acquisition time" 11.3 ms) of 16 or 32 scans with a 1 s inter-scan delay, total acquisition time of 2 h 48 min, or 5 h 34 min; the d_{24} delay was set to 1.72 ms (~1/4J). Processing used typical matched Gaussian apodization in F_2 and squared sine-bell in F_1. Volume-integration of contours in HSQC plots used Bruker's TopSpin 1.3 software. For quantification of P:G:S ratios, only the carbon-2 correlations from guaiacyl units, and the carbon-2/6 correlations from syringyl or *p*-hydroxyphenyl units were used, and the guaiacyl integrals were logically doubled. No correction factors were deemed necessary after noting only slight deviations from 1:1:1 volume integral ratios in a range of model dimers and trimers with mixed P/G/S units.

Plant Materials. Transgenic alfalfa (*Medicago sativa* cv Regen SY) plants downregulated in C3H transcripts and corresponding enzyme activity were generated. The C3H-4a line had 5% residual C3H-activity compared to the wild-type (WT) control. Lignins were isolated.

Results and Discussion

The NMR spectra reveal both massive and subtle structural differences between the syringyl/guaiacyl lignins in normal wild-type alfalfa vs the *p*-hydroxyphenyl-rich lignins in the heavily C3H-down-regulated plants. Changes in the P:G:S distribution in the lignins are most readily visualized from the aromatic region of NMR spectra, particularly the 2D ^{13}C–^{1}H correlation (HSQC) spectra correlating protons with their attached carbons. As established previously, alfalfa lignin is a typical, slightly guaiacyl-rich, syringyl-guaiacyl lignin. Syringyl and guaiacyl aromatic resonances are well separated at 750 MHz, but also at lower field. Traces of the *p*-hydroxyphenyl component are visible in this spectrum (Fig. 2a). The anticipated effect of C3H-deficiency, an enhancement of the relative level of *p*-hydroxyphenyl (P) units in the lignin, is compellingly demonstrated in the aromatic profiles, Fig. 2. The lignin from the C3H-deficient alfalfa is strikingly unlike any lignin seen by these investigators. Relatively weak, but diagnostic, syringyl (S) and guaiacyl (G) correlations remain in a spectrum that is overwhelmed by the *p*-hydroxyphenyl (P) correlations. The most severely down-regulated C3H-4a line (Fig. 2b) was G- and S-depleted and strikingly P-rich, about 65% P (Table 1). Volume-integration (Table 1) allows reasonable quantification of the differences that are plainly visible. The lignins in all three types of fractions analyzed (the solvent-soluble lignin ML, the acidolysis lignin AL on the residue, and the crude enzyme lignin EL following our cell wall dissolution procedure) all showed similar distributions (see Table 1). This suggests that only minimal partitioning of structure types between the fractions has occurred, and that the isolated solvent-soluble lignin from ball milled material is representative of that from the whole ball-milled cell wall.

Conclusions

Alfalfa lignins strikingly rich in *p*-hydroxyphenyl (P) units are produced by C3H down-regulation. Although the total lignin level appears to be lower in C3H-deficient plants, it is apparent that the plants are substituting the more available *p*-coumaryl alcohol for the normally higher levels of coniferyl and sinapyl alcohols; thus P levels that are typically 1-3% of the lignin rise to ~65% of the lignin in the most heavily down-regulated line.

Figure 2. Partial short-range ^{13}C–^{1}H (HSQC) correlation spectra (aromatic regions only) of milled lignins (ML) isolated from a) the wild-type control and b) the most highly C3H-deficient line, C3H-4a. Traces of *p*-hydroxyphenyl (P) units are seen in the typically syringyl/guaiacyl (S/G) lignin in the wild-type alfalfa, whereas P-units entirely dominate the spectrum in the transgenic. Semi-quantitative volume integrals are given in Table 1. *[This figure is in color for easier interpretation in the online version -- http://ars.usda.gov/mwa/madison/dfrc -- go to research, click on annual research summaries].*

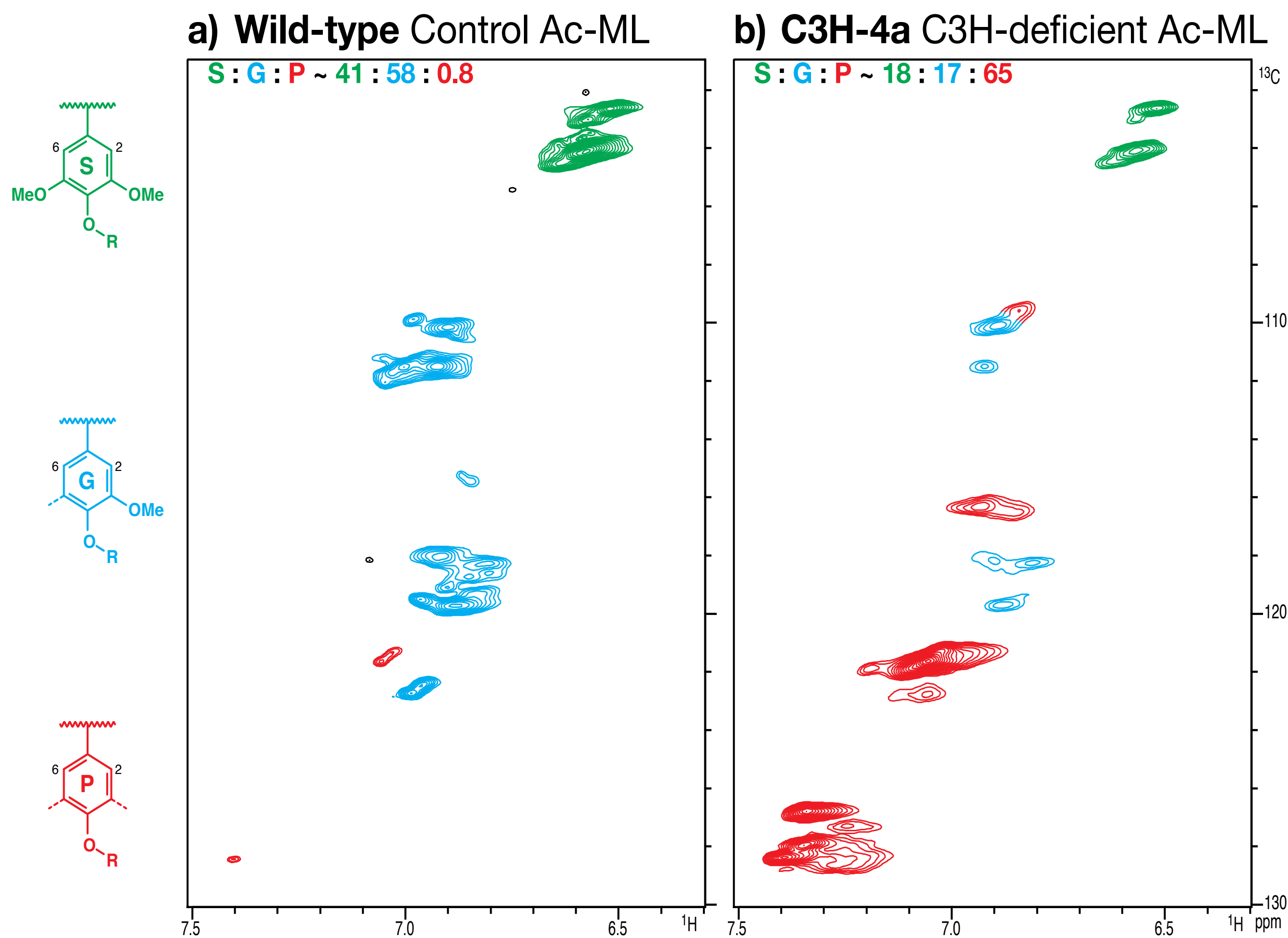


Table 1. NMR-derived *p*-Hydroxyphenyl:Guaiacyl:Syringyl (P:G:S) Data for Stem Lignins from Control and C3H-Deficient Plants

Sample	%P	%G	%S
Control Ac-ML	0.8	58	41
Control Ac-AL	0.7	61	39
Control Ac-EL	0.8	58	41
C3H-4a Ac-ML	65	17	18
C3H-4a Ac-AL	68	17	15
C3H-4a Ac-EL	66	16	18

Fractions are defined in the Materials and Methods Section; ML = dioxane:water-soluble milled lignin, AL = acidolysis lignin (from the ML residue), EL = enzyme-digested cell wall; Ac- indicates acetylated samples.
Control is the Wild-type
C3H-deficient transgenic, C3H-4a, has 5% residual C3H levels.

Effects of Coumarate 3-Hydroxylase (C3H) Down-regulation on Lignin Structure

J. Ralph, T. Akiyama, H. Kim, F. Lu, P.F. Schatz, J.M. Marita, S.A. Ralph M.S.S. Reddy, F. Chen and R.A. Dixon.

Introduction

As introduced in the previous article, the effects on lignin composition and structure of perturbing the C3H/ hydroxycinnamoyl transferase (HCT) steps are being revealed by structural analysis of C3H-downregulated alfalfa lignins.

Methods

The plant materials and methods are the same as those in the previous article, with the following additions.

Quantification of unit types via NMR spectroscopy. For quantification of the various interunit linkage types, the following well-resolved contours (see Fig. 1) were integrated: **A**α, **B**α, **C**α, **D**α, **S**α, **X1**γ and **X7**β, as well as **B**β, **C**β, **D**β, and **S**β as checks. Integral correction factors were determined by acquiring spectra from a range of mixed-unit trimers and tetramers. Such models have an exact integral molar ratio of units, usually 1:1, making this approach superior to using mixtures of more simple models. The determined relative response factors were: **A**α 1.00, **B**α 0.71, **C**α 1.06, **D**α 0.87, **S**α not determined, **X1**γ possibly 2.0 (not reliably determined), and **X7**β 0.77. These values were used to correct the volume integrals to provide the semi-quantitative estimates of unit ratios in Table 1, but note that these values can only be used here and should not be considered universal — they are dependent upon the spectrometer and acquisition conditions.

Results and Discussion

The NMR spectra reveal both massive and subtle structural differences between the syringyl/guaiacyl lignins in normal wild-type alfalfa vs the *p*-hydroxyphenyl-rich lignins in the heavily C3H-down-regulated plants. The sidechain region only peripherally reflects the changes in the P:G:S distribution, but is rich in detail regarding the types and distribution of interunit bonding patterns present in the lignin fraction.

The control lignin spectrum, Fig. 1a, is typical of a guaiacyl/syringyl lignin containing residual polysaccharides. The HSQC spectrum resolves most of the correlations for the various linkage types in the polymer, the exception being in the complex γ-region, where only the correlations from the phenylcoumarans **B** and the cinnamyl alcohol endgroups **X1** are fully resolved. The lignin is seen as being rich in β-aryl ether units **A**, with more modest amounts of phenylcoumaran **B** and resinol **C** units, as is typical for all lignins. Arylglycerol units **X7**, not normally reported, are identified here; it is suspected that they may arise from β-ether units during ball-milling, but can also be produced under oxidative coupling reaction conditions. Their 5-hydroxyguaiacyl analogs have recently been documented in COMT-deficient

Table 1. NMR-derived Interunit Linkage Data for Stem Lignins from Control and C3H-Deficient Plants

Sample	%A	%B	%C	%D	%S	%X1	%X7
Control Ac-ML	75	9	9	1.1	0.6	4.8	0.5
Control Ac-AL	80	8	7	0.6	0.2	3.7	0.4
Control Ac-EL	77	8	8	0.7	0.6	4.7	0.6
C3H´ Ac-ML	56	18	16	2.6	-	2.9	4.6
C3H´ Ac-AL	56	16	14	2.1	-	5.3	6.0
C3H´ Ac-EL	53	17	16	1.8	-	5.6	6.6

Fractions are defined in the Materials and Methods Section; ML = dioxane-water-soluble milled lignin, AL = acidolysis lignin (from the ML residue), EL = enzyme-digested cell wall; Ac- indicates acetylated samples. Control is the Wild-type.
C3H-deficient transgenic, C3H-4a, has 5% residual C3H levels.
A = β–O–4 (β-aryl ether); **B** = β–5 (phenylcoumaran); **C** = β–β (resinol); **D** = dibenzodioxocin; **S** = β–1 (spirodienone);
X1 = cinnamyl alcohol endgroup; **X7** = arylglycerol endgroup.

alfalfa lignins. Spirodienone structures **S**, β–1-coupled units only recently authenticated in lignin spectra, are readily seen in alfalfa. The diagnostic dibenzodioxocins **D** are also relatively newly discovered 8-membered ring structures resulting from radical coupling of a monolignol with a 5–5-coupled end-unit. Since syringyl units cannot be involved in 5-coupling, dibenzodioxocins have previously been considered to be most prevalent in guaiacyl-rich lignin fractions. Finally, the cinnamyl alcohol endgroups **X1**, like the resinols **C**, arise from monomer-monomer coupling and are therefore relatively minor; the deceptively strong **X1** γ-C/H correlation peak is due to the sharpness caused by the relative invariance of proton and carbon chemical shifts in such structures where the bonding is on the aromatic ring, well distant from the γ-position.

The C3H-deficient lignin has a spectrum that has several conspicuous differences. In addition to the relative intensity differences (seen more easily from the volume integral data in Table 1) are two notable structural changes. First, there are no apparent spirodienones **S**, even at contour levels closer to the noise level. Second, there are now several types of dibenzodioxocins **D**, with considerable differences in chemical shifts. The inset in Fig. 1b shows lower contour levels from an experiment run at higher ^{13}C-resolution. Sets of contours labeled **D**1, **D**2 and **D**3 are clearly visible. Elucidating the exact nature of these will require considerably more work. The **D**1 correlations match those in a GG/G-dibenzodioxocin model and in the wild-type lignin, so are logically attributable to dibenzodioxocins **D** formed by coupling of a monolignol with 5–5-linked units derived from coupling of two guaiacyl units, i.e. GG/G-, GG/S- or, possibly, GG/P-dibenzodioxocins. The data for the dibenzodioxocin peaks labeled **D**3 (**D**α3 and **D**β3 for the α- and β-C/H correlations) match those for a synthesized PP/P-dibenzodioxocin model in which all three aromatic nuclei are *p*-hydroxyphenyl. The nature of the large **D**2 correlations remains equivocal. We assumed, from the high levels of P-units in this lignin. The shifted correlations may be due to 3-substitution on P-units involved in 5–5-coupled structures. Thus PP′/P (where P′ is a 5-linked P-unit) or possibly PG/P dibenzodioxocins seem likely candidates, to be confirmed by future studies.

The other differences in unit-type distribution can be visualized in the spectra but are more readily revealed from data reported in Table 1. The lower proportion of β-ether units **A** (~53-56% vs ~75-80% of the units quantified) is clearly a major reason for the lower thioacidolysis yields (on a lignin basis) for the C3H-deficient plants vs the wild-type. It also suggests that alkaline pulping efficiency will be lower, since pulping depends on ether cleavage reactions to depolymerize the lignin and render its fragments soluble in the pulping liquor. However, since lignin-polysaccharide cross-linking can occur via trapping of intermediate β-ether quinone methides during lignification, reducing the β-ether content may reduce lignin-polysaccharide cross-linking and produce cell walls that are more enzymatically degradable, as demonstrated for the C3H down-regulated lines via their improved digestibility in ruminant animals. Much of the decrease in β-ether **A** levels appears to be due to the two other major units, phenylcoumarans **B** and resinols **C**, each of which nearly doubles in relative proportion. The higher resinol concentration particularly suggests that more monomer-monomer coupling reactions are occurring during the lignification in the P-rich lignins. Although still quite low, the relative dibenzodioxocin **D** level is about double that in wild-type plants. Quantification of cinnamyl alcohol endgroups **X1** was the most variable, but relative levels seem to be similar. Finally, the glycerol structures **X7** are at substantially higher (~8-11-fold) levels. Since it is not known whether glycerol sidechains derive from β-ether units during ball-milling or are produced during lignification, we do not speculate on their relevance at this time. However, we have noted that oxidative coupling reactions using *p*-coumaryl alcohol produce substantial levels of glycerols (in synthetic polymers that have not been ball-milled) so we are beginning to suspect that they may be, at least in part, authentic units in the native lignins.

Comments Regarding the Mechanism of Lignification. For decades the accepted theory for lignification is one in which monolignols polymerize largely by radical cross-coupling reactions with the growing polymer in a purely chemical reaction. A challenge (68) hypothesizes that lignin primary structure could be absolutely dictated by synthesis on arrays of dirigent coupling sites, and replicated by template polymerization. The existing theory explains the current facts and readily explains how massive alterations in the monomer profile may drastically

affect lignification and the resulting lignin structure but are readily accommodated by the lignification process. Essentially, since it is simply a chemical process, any phenol finding itself in the cell wall's lignifying zone is capable of entering into the combinatorial free-radical coupling process to the extent allowed by simple chemical concerns such as structural compatibility, and influenced by typical physical parameters such as pH, temperature, ionic strength, and the matrix in general. This is the case here for the at least 20-fold increased levels of the typically minor monolignol, *p*-coumaryl alcohol, but is also seen in more extreme cases. For example, COMT-deficient plants supplant sinapyl alcohol with the non-traditional monomer 5-hydroxyconiferyl alcohol during lignification. The dirigent array hypothesis encounters difficulty with monomer substitution. The challenge for the new hypothesis remains to explain how a template allows monomer substitution, and to provide direct evidence for ordered macro-structures in lignins. The plant materials examined in the present work may provide materials for such studies.

Conclusions

Alfalfa lignins strikingly rich in *p*-hydroxyphenyl (P) units are produced by C3H down-regulation. NMR analysis of the lignins suggests that *p*-coumaryl alcohol undergoes coupling and cross-coupling reactions that are for the most part analogous to those of the normally dominant monolignols, coniferyl and sinapyl alcohols. The absence of β–1-structures and a considerable shift in the proportions of others demonstrate that the lignification profile is, however, significantly different. The compositional and structural changes in the polymer noted here remain consistent with the existing theory of lignification based on combinatorial radical coupling reactions under simple chemical control.

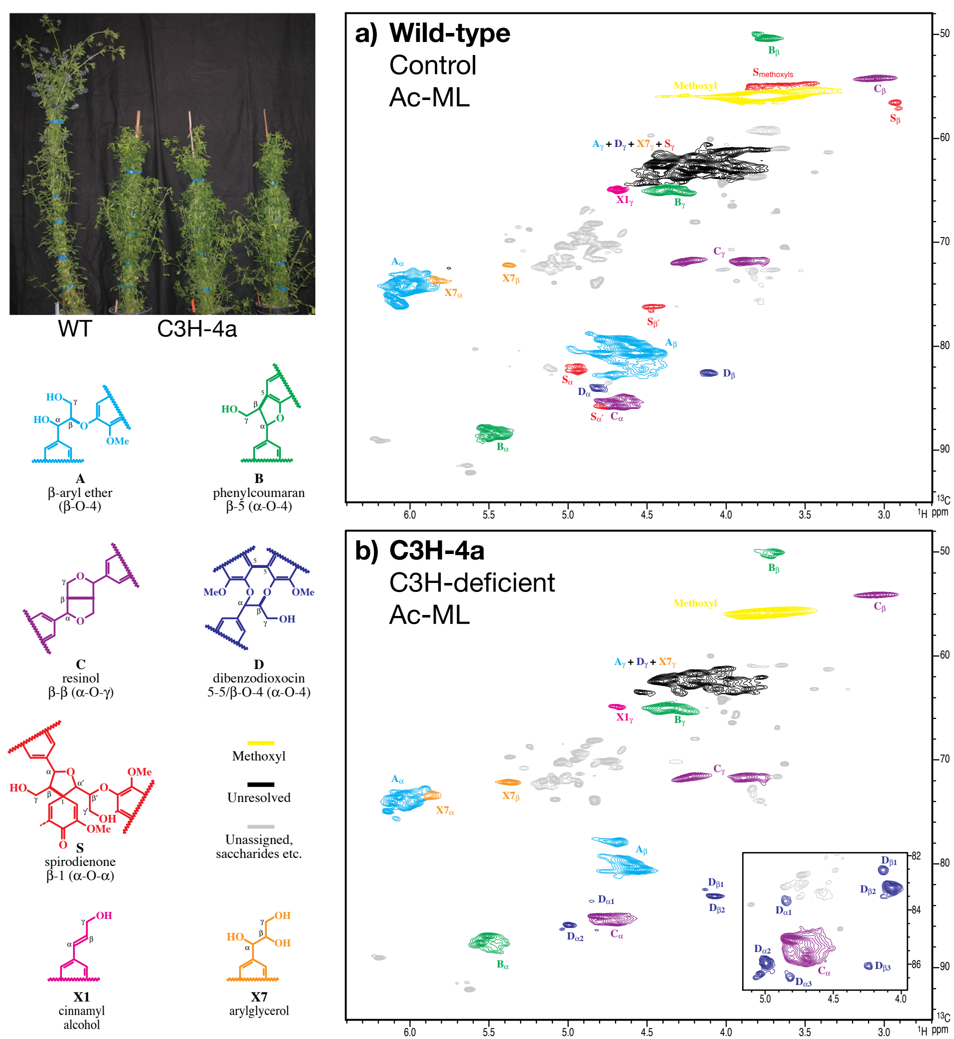


Figure 1. Partial short-range $^{13}C–^{1}H$ (HSQC) spectra (sidechain regions) of milled lignins (ML) isolated from a) the wild-type control and b) the most highly C3H-deficient line, C3H-4a. C3H-deficiency, and the incorporation of higher levels of *p*-coumaryl alcohol into the lignin, produces substantial changes in the distribution of interunit linkage types. The absence of spirodienone S units in the transgenic reveals that *p*-coumaryl alcohol does not apparently favor β–1-cross-coupling reactions. Several types of new dibenzodioxocins **D** are more readily seen at the lower contour levels in the more highly resolved partial spectrum in the inset. Note that the contour levels used to display the two spectra were chosen to highlight the structural similarities and differences; with no internally invariant peaks, interpretation of apparent visual quantitative differences needs to be cautious. The upper-left corner photograph shows WT and C3H-4a transgenic plants at the WT flowering stage; pictures of the C3H-9a transgenic and histochemical staining are provided elsewhere. Volume integrals and semi-quantitative data are given in Table 1. Interunit type designations **A-D**, **S**, **X1** and **X7** follow conventions established previously. *[This figure is in color for easier interpretation in the online version -- http://ars.usda.gov/mwa/madison/dfrc -- go to research, click on annual research summaries].*

Structural Elucidation of New Ferulic Acid-Containing Phenolic Dimers and Trimers Isolated from Maize Bran

M. Bunzel, J. Ralph, C. Funk and H. Steinhart

Introduction

Grasses and cereals contain substantial amounts of cell wall-bound hydroxycinnamate esters linked to polysaccharides. Particularly ferulates play an important role in cross-linking cell wall polysaccharides. Ferulates dehydrodimerize via a radical, oxidative mechanism to form mainly 8-5-, 8-O-4-, 5-5-, 8-8-, and 4-O-5-coupled dehydrodiferulates (often simply referred to as diferulates), thus cross-linking two polysaccharide chains. Polysaccharide cross-linking plays a significant role in the plant and plant derived products, e.g. by effecting cell wall strength, fiber degradability and food textural and processing properties. Furthermore ferulates and diferulates act as cross-links between polysaccharides and lignin. Recently, three dehydrotriferulic acids have been isolated and identified for the first time, indicating that also higher ferulate oligomers are involved in cross-linking cell wall polysaccharides as proposed by Fry. However, to date it is not possible to decide whether triferulates cross-link three polysaccharide chains or whether their cross-linking capacity is limited to two polysaccharide chains due to difficulties in the approach of three unwieldy polymer chains in a limited space.

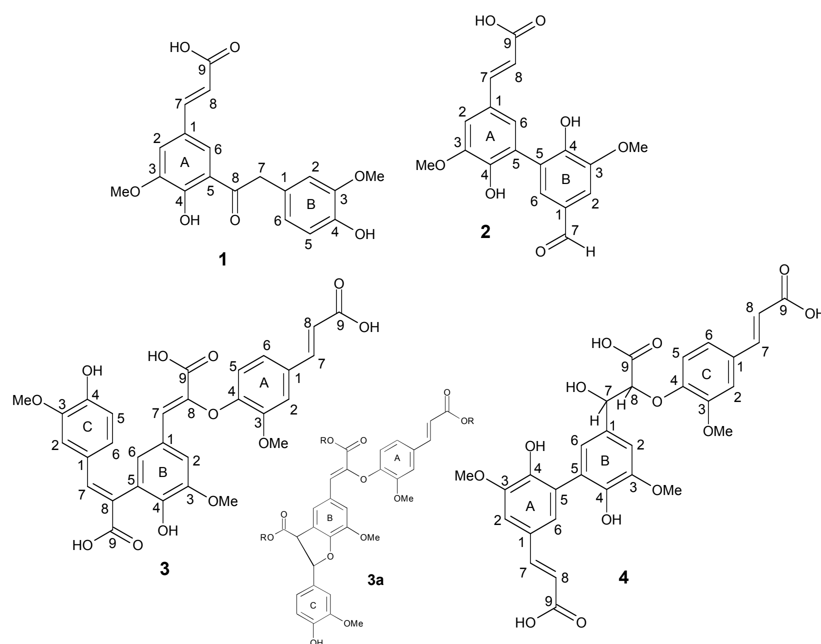


Figure 1. Structures, numbering systems, and trivial names of the new ferulic acid-containing phenolic dimers **1** and **2** (5-5-vanillin-ferulic acid cross-product) and trimers **3** (8-O-4/8-5(non-cyclic)-dehydrotriferulic acid) and **4** (5-5/8-O-4(H_2O)-dehydrotriferulic acid) and of the hypothetical cell-wall-native component **3a** (8-O-4/8-5(cyclic)-dehydrotriferulate, R = arabinoxylan chain)

Here we describe the isolation and structural identification of four new ferulic acid-containing dimers and trimers from maize bran. These studies aim to characterize the range of such products to be found in cereal grains (and grass cell walls in general), and begin to understand their role and impact on grain properties.

Methods

Compounds **1**-**4** (Fig. 1) were isolated from maize bran according to a recently published method developed by our group to isolate dehydrodiferulic acids (DFA) on a semipreparative scale. Structural analysis was via HPLC-ESI-MS, NMR and hi-resolution MS.

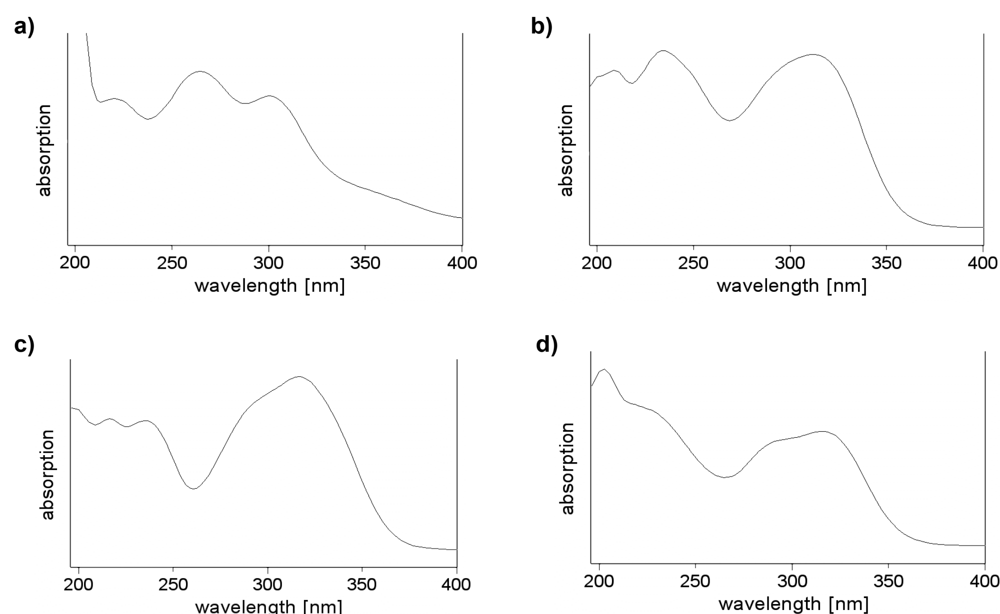
Results and Discussion

The biological significance of compound **1** in the plant remains unclear. This compound is obviously not involved in polysaccharide cross-linking since it has only one carboxylic acid group. To date, compounds related to **1** are not known in grasses. Although we cannot exclude the possibility that compound **2** is a natural prod-

uct, we rather assume that compound **2** derived from the corresponding 5-5-coupled diferulate by oxidative degradation. Oxidative degradation from ferulic acid to vanillin is a known reaction and we assumed a similar process for the 5-5-DFA although we tried to exclude oxygen during the saponification process. To confirm this theory we simulated worst case conditions and stirred 5-5-DFA in 2 M NaOH in an open pyrex tube for 138 h. The reaction mixture was analyzed by HPLC-DAD, Figure 2. Under this conditions several reaction products were found, one of them being compound **2**. The peak area at 280 nm of compound **2** represented 17% of the peak area of the parent 5-5-DFA.

Both newly identified triferulates **3** and **4** represent possible polysaccharide cross-links. Although these compounds are minor compounds in maize bran fiber, they indicate that cross-linking of polysaccharides via triferulates is an important mechanism within the plant cell wall, strengthening the polymer network. However, we suppose that compound **3** does not naturally occur in the plant but derives from an 8-O-4/8-5(cyclic)-coupled triferulate (**3a**, Figure 1). This assumption is based on studies on 8-5-coupled diferulates. The cyclic form of the 8-5-dehydrodiferulate is the only one formed under biomimetic conditions using H_2O_2/peroxidase. Saponification of the ester (e.g. the ethyl ester) gives rise to three compounds, the cyclic form, the non-cyclic (open) and the decarboxylated form of the 8-5-coupled DFA. We reason by analogy that not compound **3** but the related cyclic form **3a** should be the natural product in the plant.

Figure 2. UV-spectra of a) compound **1**, b) compound **2**, c) compound **3** and d) compound **4** recorded in 1 mM TFA/MeOH 50/50 (v/v).



Regarding the structure of triferulate **4**, the addition of water to the side chain of unit B is surprising. In the formation of 8-O-4-diferulates, the elimination of the acidic 8-proton of the quinone methide directly resulting from the coupling reaction proved to be faster than the addition of water. Consequently no analogous 8-O-4-diferulate with an additional water was found from plant extracts so far. Although such a dimer can not be excluded it is also possible that addition of water to the 7-position of the quinone intermediate during the radical coupling process is some peculiar feature of the trimerisation process.

Conclusion

The four new products identified here from maize bran expand the number of products potentially involved in cell wall cross-linking. Compound **2** is the least novel, likely arising via degradation of the known 5-5-diferulate. Trimer **3** indicates the presence of an (8-O-4/8-5-triferulate (never identified previously), and trimer **4** is a novel variation on the 5-5/8-O-4-trimer observed previously. The derivation and likely role of compound **1** remains uncertain. In total, however, these findings imply that cross-coupling reactions in the cell wall are more extensive and more varied than previously recognized.

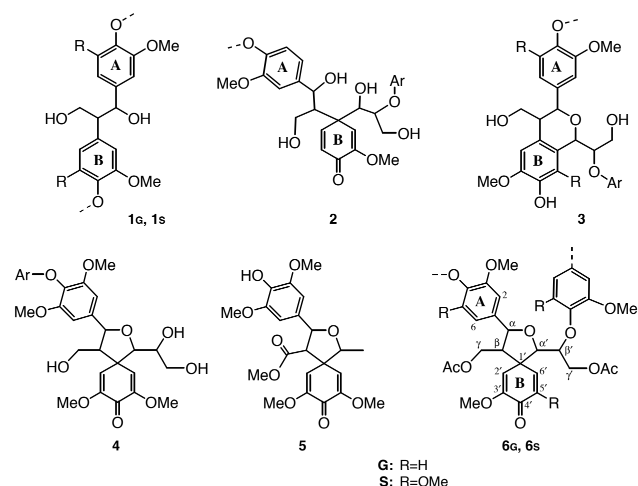
Spirodienone Structures in Lignins

L. Zhang, G. Gellerstedt, J. Ralph and F. Lu

Introduction

The presence of β–1-structures **1** (1,2-diarylpropane-1,3-diols) in lignins was first noted by the isolation of dimeric and oligomeric lignin fragments containing these structures from spruce and beech wood after mild acidic hydrolysis in dioxane-water, and by the isolation of degradation products that could be derived from them after acid hydrolysis. A major fraction of the dimers resulting from the widely applied thioacidolysis/Raney-Ni degradative analysis were β–1-derived, as were the dimers from the DFRC method. However, such structures have been difficult to detect by NMR. To explain the conundrum it has been suggested that a precursor of the β–1-unit **1**, a cyclohexadienone structure **2** or a spirodienone structure **6** might be present in native lignins, Figure 1. On acid hydrolysis, the dienone structures would be converted to the conventional β–1-structures **1**, which would explain the observation that lignin fragments containing β–1-structures are obtained after acid hydrolysis of plant fiber.

Figure 1. The conventional β–1-structures **1**, proposed dienone structures **2**, aryl isochroman structures **3** found in pine lignins, isolated natural sesquineolignan woorenol **4**, synthetic spirodienone **5**, and guaiacyl **6G** and syringyl **6S** spirodienone structures in lignins. **G** = guaiacyl, **S** = syringyl. Note: the parent compounds implicated in the lignins are shown; only 4–O-attachment is indicated but guaiacyl units may also be 5-linked.



Methods

Milled lignins (ML) were isolated from woody plants, alfalfa and other forage plants. NMR spectra were obtained by published methods.

Results and Discussion

Spirodienone structures can now be found to be present in most lignins. Originally authenticated in woody plants, spruce, birch, and kenaf, Figures 2-3, they are now also readily found in forage plants including alfalfa and a range of grasses. Their corresponding NMR signals were unambiguously assigned by a combination of NMR methods. In spruce lignin, the guaiacyl spirodienone **6G** structure was observed, whereas in kenaf lignin the syringyl spirodienone structure was predominant. In birch lignin, both the guaiacyl **6G** and syringyl **6S** spirodienones were observed, with the syringyl type being the more abundant.

The NMR observations unambiguously demonstrate the occurrence of spirodienone structures in spruce, birch and kenaf isolated lignins, and now in many other samples. The observed NMR data for both guaiacyl and syringyl spirodienone structures **6G** and **6S** are summarized in Table 1. The proportions of the dienone structures in the lignins were determined by signal integration of the carbonyl signal (C4′) in the quantitative ^{13}C NMR spectra; spruce and the birch lignins contained about three spirodienone units per 100 phenylpropanoid (C9) units and the kenaf lignin about four. To our knowledge, compounds containing the guaiacyl spirodienone structure have so far been neither synthesized nor isolated from plants. Attempts to synthesize appropriate model compounds bearing all the important functionalities in **6** are currently proving elusive.

Conclusions

Spirodienone structures are present in spruce, birch and kenaf and other isolated lignins, and presumably in all lignins. Spruce lignin contains spirodienones of the guaiacyl type. In birch and kenaf lignins, syringyl

spirodienones dominate; small amounts of guaiacyl spirodienones are detected in birch lignin. The spirodienone structures are present in the lignin polymer in two different stereoisomeric forms, with one of the isomers more abundant than the other. Assigning these isomers awaits the synthesis of appropriate model compounds. The spruce and birch lignins contained about three spirodienone structures per 100 phenylpropanoid units, and the kenaf lignin contained about four.

Figure 2. Side-chain regions of 2D HSQC NMR spectra of acetylated lignins from: (a) spruce, (b) birch, and (c) kenaf. Signals from **6G** and **6S** are marked and identified. A signal from **1G** can also be seen in spectrum (a). *[Spectra for the web version are colored to make identification easier, **6** in red, **1** in cyan and, for reference, resinol structures in purple -- http://ars.usda.gov/mwa/madison/dfrc -- go to research, click on annual research summaries].*

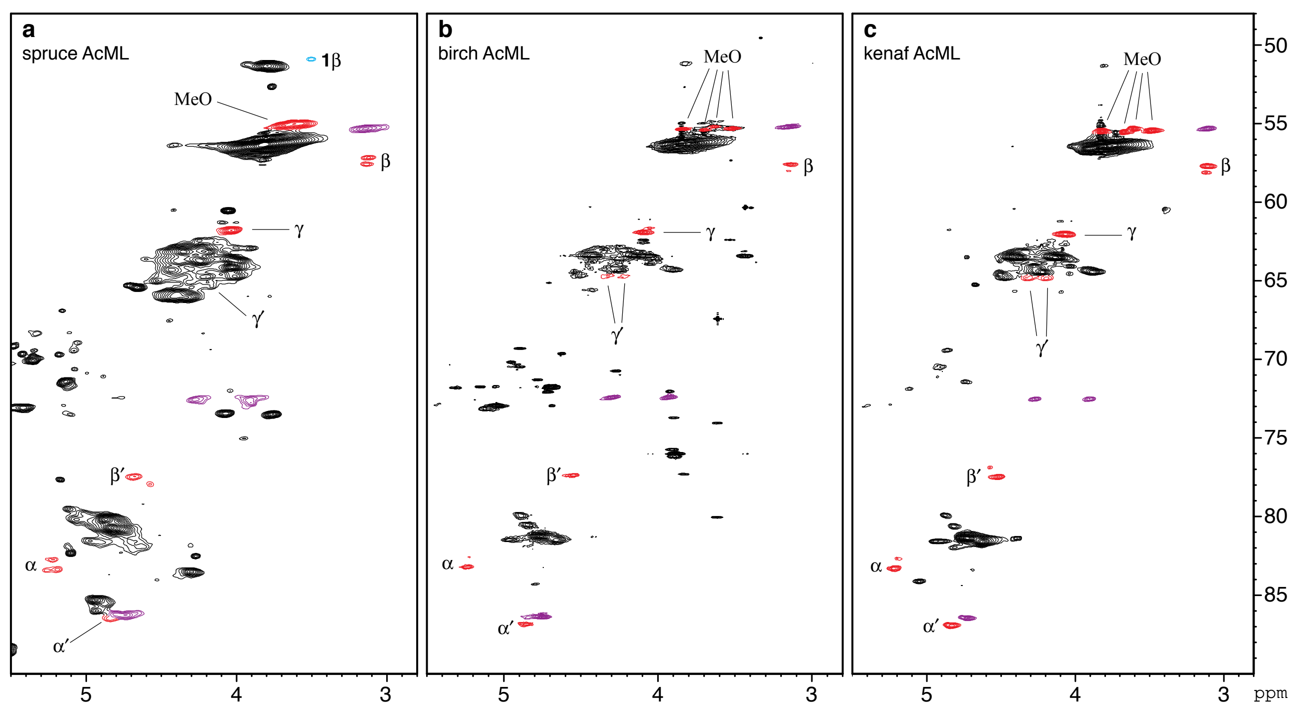


Figure 3. Aromatic/ethylenic regions of 2D HSQC NMR spectra of acetylated lignins from: (a) spruce, (b) birch, and (c) kenaf. Signals from **6G** and **6S** are marked and identified. Note: contour levels have been chosen to readily visualize the peaks of interest; the birch lignin is shown nearer the baseplane level to reveal the **6G** components. *[Colorized in the web version -- http://ars.usda.gov/mwa/madison/dfrc -- go to research, click on annual research summaries].*

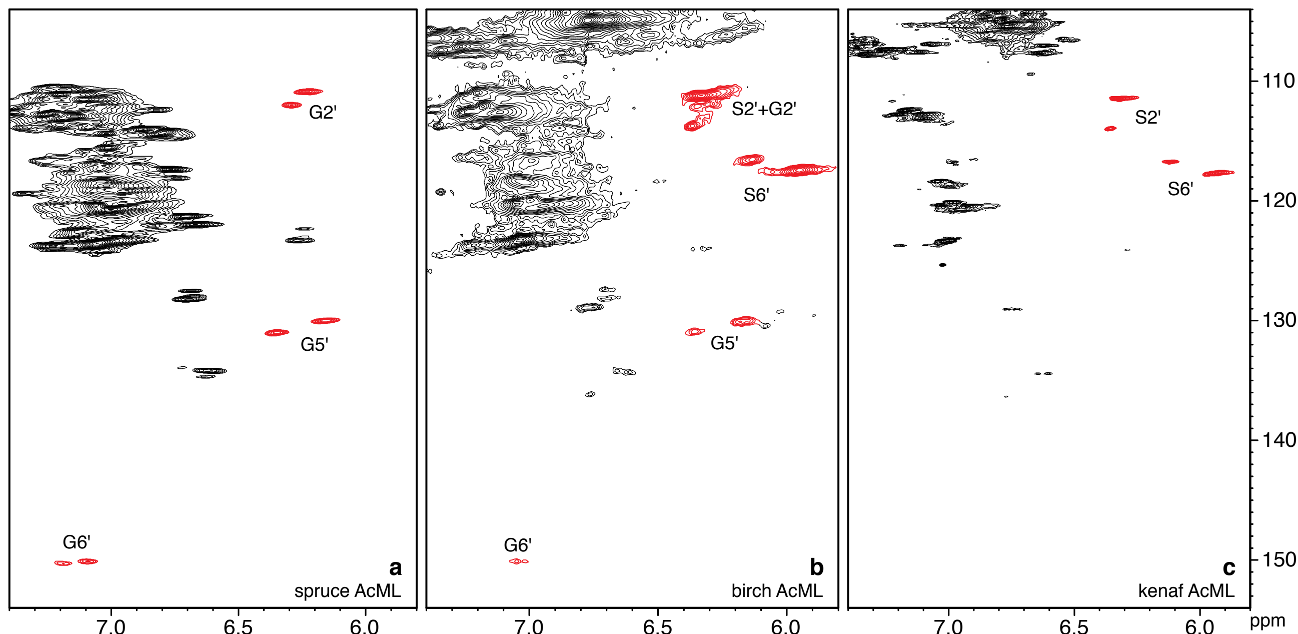


Table 1. ^{1}H and ^{13}C NMR data for **II** and **III** in acetone-d_6

Guaiacyl spiro-dienone (**II**)		Syringyl spiro-dienone (**III**)	
Position	δ_H/δ_C (ppm)	Position	δ_H/δ_C (ppm)
α	5.22/82.7, 5.19/83.3	α	5.21/83.3, 5.19/82.7
β	3.12/57.2, 3.13/57.6	β	3.10/57.8, 3.11/58.2
γ	4.03/61.8	γ	4.07/62.1
α′	4.84/86.5	α′	4.81/87.1, 4.85/87.0
β′	4.68/77.5, 4.57/78.0	β′	4.53/77.6, 4.59/77.0
γ′	4.19, 4.31/65.1	γ′	4.21, 4.32/64.9
1′	55.4	1′	53.2
2′	6.23/110.9, 6.29/112.0	2′	6.36/113.9, 6.33/111.4
3′	153.5, 154.1	3′	154.0, 152.3
4′	180.8	4′	176.4
5′	6.15/130.0, 6.35/131.0	5′	153.0, 153.9
6′	7.09/150.1, 7.18/150.3	6′	5.94/117.7, 6.12/116.7
MeO	3.57/55.0, 3.70/55.2	3′-MeO	3.82/55.5, 3.60/55.4
		5’-MeO	3.50/55.5, 3.67/55.6

Rumen Microbiology

Factors Affecting Lysine Degradation by Ruminal Fusobacteria

J.B. Russell

Introduction

Lysine is the amino acid most apt to limit the milk production of dairy cattle, but simple dietary supplements cannot counteract this deficiency. Ruminal microorganisms have the ability to take up lysine and deaminate it. Ruminant nutritionists have employed two strategies to circumvent this degradation. When feed proteins are subjected to heat or harsh chemical treatments, solubility and degradation can be decrease. Another approach has used capsules or other barriers that are stable at ruminal pH but can be liberated by the acidic pH of the abomasum. Both avenues have increased milk production, but the cost of these modifications can be prohibitive.

For many years it had been assumed that carbohydrate fermenting bacteria were primarily responsible for wasteful ruminal amino acid deamination, but the rumen also has a highly specialized group of obligate amino acid fermenting bacteria. *Clostridium sticklandii* grows rapidly with lysine as an energy source, but this bacterium is very sensitive to acidic pH and could not be isolated from wild and domestic ruminants in New Zealand. Fusobacteria capable of utilizing lysine as an energy source for growth were enriched from cattle fed timothy hay or a commercial dairy ration, and 16S rDNA indicated that these isolates were closely related to strain *F. necrophorum.*

The following experiments were designed to see if: 1) the addition of dietary lysine could enrich lysine degrading fusobacteria *in vivo*, and 2) define *in vitro* factors that might either enhance or prevent this enrichment.

Materials and Methods

Mature, non-lactating, fistulated, Holstein cows (n = 3) were fed medium quality timothy hay (12% crude protein, 45% neutral detergent fiber) ad libitum. The same animals were then given a commercial pelleted grain and protein mix (1 kg, 16% crude protein) and lysine (70 g) per day prior to the timothy hay for 21 days prior to the second sampling. Two hours after feeding, ruminal contents were squeezed through cheesecloth and pH was determined. Large feed particles and protozoa were removed by slow speed centrifugation (150 x g, 10 min, 22˚ C).

F. necrophorum JB2 was grown with lysine (50 mM) or lactate (45 mM) as an energy source. Lysine was assayed by a method based on ninhydrin. Ammonia was assayed by a colorimetric method. Fermentation acids were analyzed by high-performance liquid chromatography (HPX-87H BioRad column, 0.5 ml/min, 0.013 N H_2SO_4, refractive index detector, 50 ˚C). Cell protein from NaOH-hydrolyzed cells (0.2 M NaOH, 100°C, 15 min) was determined using bovine serum albumin as a standard.

Results

When mixed ruminal bacteria were incubated with increasing amount of lysine in vitro, the ability of the bacteria to deaminate lysine could be saturated, and bacteria from cattle supplemented with dietary lysine did not have a greater capacity to degrade lysine than those fed only hay. Serial dilutions (10-fold increments, n =3) of the mixed ruminal bacteria into basal medium that had lysine as an energy source had dense growth

(> 0.7 optical density) and high concentrations of ammonia (>50 mM) but only at dilutions less than or equal to 10^{-6}. Dietary lysine supplementation did not enhance the numbers of bacteria that could utilize lysine as a sole energy source.

When *F. necrophorum* was added to the mixed ruminal bacteria, nearly all of the lysine was degraded in 24 h, but only if the volume of ruminal fluid was less than 20%. If more ruminal fluid was present in the incubation medium, lysine degradation declined. Pure cultures of *F. necrophorum* deaminated lysine as well at pH 6.1 as 6.6, but only if acetate was not present. Sodium acetate addition had little effect on lysine deamination at pH 6.6, but sodium acetate concentrations greater than 25 mM caused a large decrease in ammonia production at pH 6.1.

Discussion

Previous work indicated that *F. necrophorum* strains capable of utilizing lysine as an energy source could be readily isolated cattle fed timothy hay or a commercial dairy ration. However, these experiments indicated that dense growth on lysine was only observed at serial dilutions less than or equal to 10^{-6}. Some growth and ammonia were detected in higher dilutions, but a sharp transition from growth to no growth was not observed. Many bacteria can be inhibited by fermentation acids if the pH is acidic, and ruminal bacteria differ greatly in this sensitivity. Because our cattle had ruminal pH values greater than 6.0, we did not initially suspect that fermentation acids would inhibit *F. necrophorum*. However, in vitro experiments indicated that *F. necrophorum* JB2 was inhibited by autoclaved ruminal fluid that had a pH of 6.1. Because the addition of sodium acetate to the basal medium had virtually no effect at pH 6.6 but was highly inhibitory at pH 6.1, *F. necrophorum* JB2 appears to be as susceptible to fermentation acid toxicity as ruminal methanogens and cellulolytic bacteria. Extrapolations of pure culture experiments to the rumen should always be made with caution, but the idea that lysine-utilizing fusobacteria are not apt to be significant in cattle fed commercial rations was supported by two other observations. The lysine degradation of mixed ruminal bacteria was inhibited by the same conditions as *F. necrophorum,* and the addition of lysine to the diet did not enhance the ability of the mixed population to degrade lysine. Mixed ruminal bacteria from supplemented cattle could be saturated with lysine, but it is unlikely that these concentrations of lysine would ever be cost effective means of delivering lysine to the small intestine.

Conclusion

Further work will be needed to see if an unidentified bacterium or the combined action of many species is responsible for ruminal lysine degradation.

The Effect of Calcium and Magnesium on the Activity of Bovicin HC5 and Nisin

A. J. Houlihan and J. B. Russell

Introduction

Many Gram-positive bacteria produce small, ribosomally-synthesized, antimicrobial peptides, and a least one of these bacteriocins, nisin, has been used commercially. However, many previously-sensitive bacteria have the ability to become nisin-resistant. Nisin-resistant bacteria were sensitive to the bacteriocin of *S. bovis* HC5, and this result indicated that bovicin HC5 might have superior characteristics. Bovicin HC5 has been purified, but the presence of post-translationally modified amino acids blocked complete Edman degradation. Because the partial sequence (80% of the total) had multiple basic amino acid residues, bovicin HC5 appeared to be a positively charged peptide, but further work was needed to confirm this hypothesis. The following experiments sought to: 1) determine the effect of divalent cations (calcium and magnesium) on the cell surface charge of *S. bovis* JB1 cells, and 2) to determine if this binding increases resistance to bovicin HC5 and nisin. The involvement of charge in resistance was strengthened by comparing nisin-resistant and nisin-sensitive *S. bovis* JB1 cells.

Materials and Methods

S. bovis JB1 and HC5 were routinely grown anaerobically in a basal medium containing salts, yeast extract and Trypticase. Glucose (5 mM) was added as a growth substrate and growth was monitored via changes in optical density. Highly purified nisin was obtained from Aplin and Barrett Ltd (Trowbridge, UK). Bovicin HC5 was liberated and partially purified from S. bovis HC5 using acidic NaCl (pH 2.0, 100 mM). Clostridium sticklandii SR was used to determine bacteriocin activity and the basal medium was modified to accommodate its growth requirements. Glucose was deleted and Trypticase was increased to 10 mg ml^{-1}. The bacteriocins were added to agar wells and diluted (2-fold) until a zone of clearing was no longer observed. Activity units (AU ml^{-1}) were calculated from the sample volume and the reciprocal of the highest serial dilution showing a visible zone of clearing. The intracellular potassium of stationary phase S. bovis JB1 cultures was determined by flame photometry. The anionic dye, Congo red, was used to test if calcium and magnesium could alter the surface charge of S. bovis JB1. Stationary phase cultures were harvested anaerobically by centrifugation, and re-suspended in potassium phosphate buffer (10 mM), with or without calcium or magnesium, that contained Congo red. Assays were carried out at pH 5.0 to prevent precipitation of metals with Congo red. Samples were centrifuged and the dye remaining in the supernatant was measured at 500 nm.

Results

S. bovis JB1 grew rapidly in basal medium, and large additions of either calcium or magnesium chloride (100 mM) caused a short lag before growth was observed, but there was only a small decrease in maximum specific growth rate and final optical density. The nisin-resistant *S. bovis* JB1 cultures also grew rapidly in the presence of large amounts of either calcium or magnesium. Stationary phase, nisin-sensitive *S. bovis* JB1 cells bound very little Congo red, an anionic dye, but significant binding was observed if magnesium or calcium were added to the buffer. Magnesium stimulated Congo red binding to a greater extent than calcium. Both effects were saturable, and the difference between calcium and magnesium could be explained by maximum binding capacity. Stationary phase, nisin-resistant *S. bovis* JB1 cells bound 3-fold more Congo red than nisin-sensitive cells before magnesium or calcium were added. The binding of Congo red to nisin-resistant cells was also enhanced by magnesium or calcium, but maximum Congo red binding capacity was less. Stationary phase *S. bovis* JB1 cells that were washed in basal medium lacking a nitrogen source had as much intracellular potassium as those growing exponentially, but only if glucose was provided as an energy source. When glucose was

not added, the intracellular potassium pool was only 100 nmol mg protein^{-1}. Nisin-sensitive cells began to lose potassium as soon as either bacteriocin (50 AU ml-1) was added. Nisin-resistant cells did not lose potassium when the same dose of nisin was added. The nisin-resistant cells were more resistant to bovicin HC5 than the nisin-sensitive cells, but they still lost approximately 50% of their potassium after 60 min of exposure. Calcium or magnesium counteracted bacteriocin-mediated potassium loss from nisin-sensitive *S. bovis* JB1 cells. Nisin-resistant cells did not lose potassium when nisin was added even if additional calcium or magnesium were not present. Calcium and magnesium counteracted potassium loss from nisin-resistant cells that were treated with bovicin HC5.

Discussion

Divalent metals can bind bacterial cell surfaces, and these cations likely interact with the ribitol and glycerol phosphates of lipoteichoic acids. Total cell-associated calcium and magnesium can be measured by a variety of methods, but these determinations cannot differentiate ions at the cell surface from those located intracellularly. Therefore, Congo red interaction with the cell surface was measured. *S. bovis* JB1 cells bound little Congo red until calcium or magnesium was added, and cells that had simply been washed with calcium or magnesium also bound more. These results support that idea that calcium and magnesium were binding negatively charged residues on the cell surface.

Previous work indicated that bovicin HC5 inhibited the growth of nisin-resistant *S. bovis* HC5 cultures, but potassium efflux was not examined. In accordance with previous work, our experiments indicated that nisin-resistant cells retained their potassium after nisin was added. Nisin-resistant cells lost nearly half of their potassium after 60 min of bovicin HC5 exposure. This result indicated that the nisin-resistant cells were also more resistant to bovicin HC5, but nisin resistance was not a complete protection from bovicin HC5.The observation that divalent cations decrease nisin and bovicin HC5 activity supports the idea that changes in cell surface charge can be a common mechanism of bacteriocin resistance.

Conclusion

Nisin and bovicin HC5 react differently with the cell surfaces of Gram-positive bacteria.

Factors Affecting the Release, Stability and Binding of Bovicin HC5 to Target Bacteria

A.J. Houlihan and J. B. Russell

Introduction

Cattle are often fed antibiotics to improve feed efficiency. However, in recent years there has been an increased perception that adding antibiotics to animal feed causes the proliferation of resistant pathogens. These fears have prompted some groups to call for a widespread ban of antibiotics as growth promotants. Many Gram-positive bacteria produce bacteriocins, and these peptides are not considered classical antibiotics. Some ruminal bacteria produce bacteriocins, and they have been proposed as an alternative to antibiotics currently used in animal feed. The ruminal bacterium, *Streptococcus bovis* HC5*,* produces a small antimicrobial peptide (bovicin HC5) that has the same spectrum of activity as monensin, the most commonly used antibiotic in cattle. Experiments were designed to determine factors affecting the release, stability and binding of bovicin HC5 to sensitive bacteria.

Materials and Methods

Bacteria were routinely grown anaerobically in a basal medium containing salts, yeast extract and Trypticase. Growth was monitored via changes in optical density. Bovicin HC5 was liberated from *S. bovis* HC5 with acidic NaCl (pH 2.0, 100 mM). Cell-free bovicin HC5 activity was assayed by serially diluting extracts in distilled water (2-fold increments) and spotting these dilutions (10 µl) on a lawn of *Clostridium sticklandii SR* (approximately 10^5 cfu ml^{-1}). *S. bovis* HC5 was grown in basal medium that contained various amounts of glucose (2 to 10 mg ml^{-1}), and then incubated at 39 ˚C for 40 days. Samples were removed periodically, pH was determined, the cells were removed by centrifugation (10,000 x g, 5 min, 22 ˚C) and the cell free supernatants were assayed. Peptidase activity of stationary phase cultures was measured using L-alanine-*p*-nitroanilide (Sigma Chemical Co., St Louis, MO). Stationary-phase target bacteria (*S. bovis* JB1, *Clostridium aminophilum* F, *Butyrivibrio fibrisolvens* A38, *Listeria monocytogenes* 10403S, *Megasphaera elsdenii* B159, *Selenomonas ruminantium* D, *Escherichia coli* K12 and *E. coli* 3TF4) were harvested anaerobically by centrifugation (4,000 x g, 10 min, 22 ˚C) and the cell pellets (200 µg cell protein) were re-suspended anaerobically in cell-free *S. bovis* HC5 culture supernatant (grown with 10 mg ml^{-1} glucose, 16 h, 39 ˚C, final pH 4.7) that had been adjusted to pH 4.7 to 6.7 with NaOH. After 20 min of incubation at 39 ˚C, the cells were removed by centrifugation (10,000 x g, 5 min, 22 ˚C), and the cell free supernatants were assayed as described above. Binding was calculated from the decrease in activity caused by the target cells. Cells were washed and incubated in phosphate buffer adjusted to pH values ranging from 6.7 to 4.7 with HCl and bovicin HC5 binding was assessed at each pH.

Results

Agar well diffusion assays using *C. sticklandii* SR had large zones of clearing (as great as 6 mm radius), and the basal medium was so well buffered that the zone was unaffected by the pH of the *S. bovis* HC5 culture. Because controls using *S. bovis* JB1, a non-bacteriocin producing strain, never inhibited *C. sticklandii* SR, the possibility of lactic acid inhibition could be excluded. This was true even if *S. bovis* JB1 was grown with 10 mg ml^{-1} glucose, and the final pH was 4.7. When *S. bovis* HC5 was cultivated with increasing amounts of glucose, cell-free bovicin HC5 activity increased, but the cell density was also greater. By correcting for differences in cell protein, it was possible to determine the effect of pH on release more precisely, and most of the activity remained cell-associated until the culture pH dropped below 5.0. Highly acidic conditions (pH 2.0) were needed to promote maximal bovicin HC5 release. NaCl caused a small increase in cell-free bovicin HC5 activity, but only if the pH was greater than 2.0. Tween 80 increased cell-free bovicin HC5 specific activity, but Tween 80 added to the cell-free supernatant after growth was even more effective. This latter comparison demonstrated

that Tween 80 was affecting bovicin HC5 activity to a greater extent than release. *S. bovis* JB1 cells (200 µg cell protein) bound bovicin HC5 and decreased cell-free activity, but this binding was much greater at pH values less than 6.0. Other sensitive bacteria were as effective as *S. bovis* JB1 at biding bovicin HC5 at pH 4.7, but little binding was observed if Gram-negative bacteria were used. Stationary phase *S. bovis* HC5 cultures had significant peptidase activity, but the rate of peptide hydrolysis declined dramatically if pH decreased. When the glucose concentration was high enough to cause a significant decline in culture pH, the bovicin HC5 activity did not decrease appreciably for more than 35 days. If less glucose was added, and the final pH was greater than 5.4, bovicin HC5 activity declined.

Discussion

Previous work indicated that bovicin HC5 was not degraded by either proteinase K or chymotrypsin, but some degradation (inactivation) was observed if Pronase E or trypsin was added. Our work showed that the bovicin HC5 activity of stationary phase cultures did not decline appreciably when pH was acidic. The ability of acidic pH to prevent degradation could be correlated with the effect of pH on *S. bovis* HC5 peptidase activity. Cell-associated peptidase activity decreased dramatically at acidic pH values. Further work will be needed to see if bovicin HC5 has commercial value, but it has a broad a spectrum, only binds significantly to sensitive cells, can be easily dissociated from the *S. bovis* HC5 cell surface and is highly stable in acidic environments. Because *S. bovis* is a very rapidly growing, aerotolerant bacterium that has simple nutritional requirements, bovicin HC5 production would not be problematic. The potential usefulness of bovicin HC5 is enhanced by the observation that viable *S. bovis* HC5 cells were never detected in the acidic NaCl treatment used to liberate activity from the cell surface.

Conclusions

Bovicin HC5 remains cell associated until the culture pH is < 5.0, but it can be easily dissociated from the cell surface with acidic NaCl. It is highly stable in acidic environments and only binds sensitive bacteria at pH values less than 6.0.

Relative Quantification Real-Time PCR of Bovine Ruminal Contents Reveals a Low Aggregate Abundance of Classical Ruminal Bacterial Species

D.M. Stevenson and P.J. Weimer

Introduction

Much of our knowledge of the metabolism of feedstuffs in the rumen has been gained through *in vitro* study of approximately two dozen bacterial species, most of which were isolated in the early decades of rumen microbiology. Techniques of molecular microbial ecology, particularly real-time polymerase chain reactions (RT-PCR), provide an opportunity to quantify these ruminal species with great sensitivity and precision. Such studies have shown, for example, that *Prevotella ruminicola* and *P. bryantii* far outnumbered 11 other described species of ruminal bacteria. However, the abundance of individual species, as a fraction of the total bacterial population in the rumen, has not been clarified. In most natural environments, known species typically represent less than 10 per cent, and often on the order of 1 per cent, of the bacterial population. The purpose of this study was to determine the relative contribution of the classical species of ruminal bacteria to the total bacterial population of the rumen.

Methods

DNA Isolation. Ruminal contents were collected from two lactating, fistulated Holstein cows maintained in adjacent indoor tie stalls. Cows were allowed *ad libitum* access to feed and water, and feed was supplied once daily (3 h after morning milking) as a total mixed ration that contained alfalfa silage, corn silage, corn grain, soybean meal, roasted soybeans, blood meal, whole cottonseed, and supplemental vitamins and minerals. The TMR component composition was 27.5% neutral detergent fiber and 18% crude protein (N x 6.25), and forage represented 37% of TMR dry matter. Ruminal samples were collected 3 hours after feeding on two consecutive days (30 and 31 d after adaptation to the TMR), were squeezed through 4 layers of cheesecloth within 10 min of collection. Ruminal solids (25 g) blended with ice-cold extraction buffer (100 mM Tris/HCl, 10 mM EDTA, 0.15 M NaCl pH 8.0) in two stages to recover bacterial cells, which were combined with squeezed ruminal liquid (25 mL). Differential centrifugation was used to recover cell pellets, from which DNA was extracted by a method developed for ruminal contents that included bead-beating, phenol/chloroform extraction and ethanol precipitation.

PCR Primer Design. Primer pairs for quantitative analysis were designed using the Applied Biosystems Primer Express software to 16S rRNA gene sequences. Primer pairs were selected for regions conserved among all known examples of a particular species or genus, and for as much sequence divergence as possible from those of other closely related species. In addition, pairs were selected to have T_m values near 60° C, to be within 2° C of each other, and to amplify a region of between 60 and 150 base pairs (bp) in total. An exception was the domain-level eubacterial pair, which was designed by Yu *et al.* (2005), to amplify a total of 468 bp and to have lower T_m values. Inevitably, a few primers designed to include all strains of a target species displayed identity to a few strains of closely-related species Table 1). Primer pairs for an individual taxon were also tested directly against all non-target taxa in Table 1, and showed no significant amplification (> 12 PCR cycles difference between the test strain of target taxon and all test strains of the non-target taxon).

RT-PCR. Reactions were performed using the Applied Biosystems Prism 7300 sequence detection system with fluorescence detection of SYBR Green dye. DNA from the target species was quantified using a relative quantification method using an experimentally-derived amplification efficiency where:

E = efficiency of the PCR reaction (theoretical maximum = 2.0 per cycle; experimental range in this study = 1.92 to 1.98) and ΔC_t = difference in number of cycles to crossing threshold.

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(control - sample)}}{(E_{\text{reference}})^{\Delta Ct_{\text{reference}}(control - sample)}}$$

In this study we used, as the reference primer set, a non-degenerate, domain-level primer set designed by Yu et al., that amplified all bacterial species. This allowed direct quantification of a target species relative to the total bacterial content.

Results

Despite substantial differences in the productivity of the two cows ruminal populations between the two were remarkably similar, with none of the individual species displaying significant differences (P>0.05) between cows or between sample days. Consequently, the data from both cows on both sampling days were combined within a target taxon for comparison of population sizes across taxa. Two genus-level primers for *Prevotella* sp. revealed a clear dominance (42 to 60 % of the ruminal bacterial populations) in the ruminal samples from both cows (Table 1). However, quantification with species-specific primers to the three *Prevotella* species considered most common in the rumen together accounted for only 2 to 4 % of the bacterial population. After *Prevotella*, the most common species were found to be *Fibrobacter succinogenes*, the *Ruminococcus flavefaciens* group, the *Selenomonas ruminantium* group and the *Succinivibrio dextrinosolvens* group, each of which were generally in the 0.5 to 1% range. Populations of *Ruminobacter amylophilus* and *Eubacterium ruminantium* were each generally in the 0.1 to 0.2% range. The *Butyrivibrio fibrisolvens* group, the *Streptococcus bovis* group, *Ruminococcus albus,* and *Megasphaera elsdenii* were even less abundant, each comprising <0.03% of the ruminal bacterial population. In aggregate the 13 individual species accounted for only 5 to 7% of the total bacteria.

Conclusions

While RT-PCR has shown that 42 to 60% of the bacterial population in two cows comprised a single genus, *Prevotella*, only 2 to 4 % of the bacterial population was represented by the three most commonly isolated species of ruminal *Prevotella* (*P. bryantii, P. ruminicola* and *P. brevis*), and an additional ~5% of the population was distributed among 10 non-*Prevotella* species classically regarded as important agents of the rumen fermentation. Thus, the vast majority of the ruminal bacterial population awaits isolation in pure culture, a prerequisite to their physiological characterization. The uncultured bacteria probably includes both fibrolytic and starch-degrading populations, as the aggregate populations of each group is ~2 to 4 %, values probably too low to account for the rates of starch and cellulose degradation observed in modern dairy rations.

Table 1. Percentages of target species in ruminal samples relative to total Eubacterial content.

Target taxon	Cow 4884		Cow 4991			P > F [f]	P > F [f]
	Day 30	Day 31	Day 30	Day 31	Mean ± S.E.M.	Cow	Day
Butyrivibrio fibrisolvens [a]	0.022	0.027	0.022	0.024	0.024 ± 0.003	0.584	0.256
Eubacterium ruminantium	0.171	0.158	0.163	0.213	0.176 ± 0.025	0.584	0.658
Fibrobacter succinogenes	0.838	0.889	0.615	0.995	0.835 ± 0.160	0.783	0.416
Megasphaera elsdenii	0.0010	0.0001	0.0003	0.0004	0.0005 ± 0.0004	0.728	0.564
Prevotella brevis	0.162	0.099	0.152	0.128	0.135 ± 0.028	0.693	0.266
Prevotella bryantii	1.226	0.730	1.942	1.830	1.432 ± 0.56	0.133	0.359
Prevotella ruminicola	1.600	1.582	1.756	2.032	1.743 ± 0.208	0.288	0.541
Ruminobacter amylophilus	0.170	0.141	0.392	0.189	0.223 ± 0.115	0.364	0.410
Ruminococcus albus	0.003	0.001	0.004	0.007	0.004 ± 0.003	0.361	0.811
Ruminococcus flavefaciens [b]	0.757	0.336	0.558	0.799	0.613 ± 0.212	0.759	0.831
Selenomonas ruminantium [c]	0.706	0.342	0.468	0.688	0.551 ± 0.176	0.883	0.345
Streptococcus bovis [d]	0.008	0.002	0.003	0.002	0.004 ± 0.003	0.525	0.477
Succinivibrio dextrinosolvens [e]	0.715	0.656	1.071	0.799	0.810 ± 0.184	0.257	0.365
Sum of individual species	6.19	4.92	6.90	7.21	6.30 ± 1.02	0.308	0.654
Genus *Prevotella*	49.60	42.44	58.12	59.93	52.52 ± 8.09	0.211	0.658

[a] BLAST search results included 1 strain of *Pseudobutyrivibrio ruminis*
[b] BLAST search results included 4 strains of *Ruminococcus callidus* and 3 strains of *Lachnospiraceae*
[c] BLAST search results included 2 strains of *S. sputigena* and 3 strains of *Mitsuokella*
[d] BLAST search results included a total of 7 strains of 3 other *Streptococcus* species
[e] BLAST search results included 2 strains of *Anaerobiospirillum* species and 1 strain of *Mycobacterium* species
[f] Probability of a greater value in an F-test, from reduced statistical models in which cow or sampling day were analyzed as the sole independent variable. The reduced models were applied after an initial statistical model, in which cow and sampling day were treated as independent variables, did not yield statistical significance for either variable at *P*<0.10.

Forage Quality

Polyphenol and Conditioning Effects on Forage Protein Solubility and Degradability

J. H. Grabber

Introduction

Excessive proteolysis during ensiling and ruminal fermentation greatly reduces the quantity of alfalfa protein digested and absorbed by cattle. In some forage legumes, binding of condensed tannins and *o*-quinones to protein hinders proteolysis, but the effectiveness of these polyphenols may be limited by their sequestration in plant organelles away from protein. Therefore, severe mechanical maceration of forages at cutting may enhance polyphenol protection of protein. In this study, we examined whether mechanical maceration could enhance polyphenol action in forages by reducing protein solubility and shifting its degradation from the rumen to the gastrointestinal tract of cattle.

Methods

Established stands of polyphenol-free alfalfa, red clover (with *o*-quinones formed by the action of polyphenol oxidase on *o*-diphenols), and three birdsfoot trefoil populations (selected for low, moderate, and high tannin levels) were grown near Prairie du Sac, Wisconsin. First and second harvests from 2002 and 2003 were conditioned with rolls or severely macerated, wilted in a forced-draft oven at 32 ^{o}C, and then conserved as silage or hay. Forages were analyzed for condensed tannins, crude protein, soluble protein, protease "rumen-undegradable" protein, and acid-detergent insoluble protein. Intestinal-available protein was estimated as rumen-undegradable protein minus acid-detergent insoluble protein.

Results and Discussion

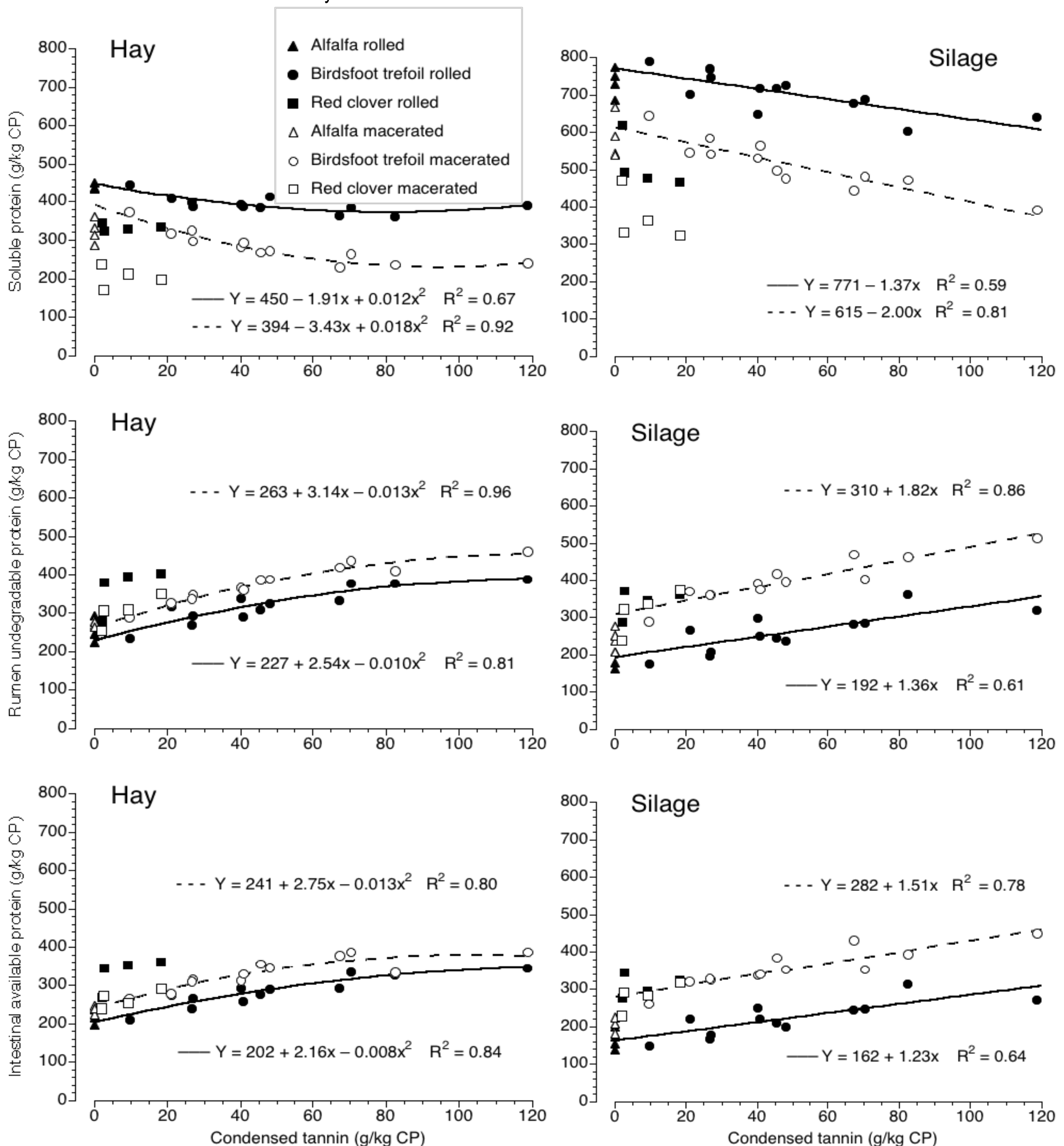
Crude protein (CP) levels varied little between treatments, averaging 207 g/kg of dry matter. Condensed tannins, absent in alfalfa, ranged from 9 to 119 g/kg CP in trefoil populations and from 2 to 18 g/kg CP in clover. Rolled hays and silages of trefoil and clover had lower soluble protein levels and greater rumen-undegradable and intestinal-available protein levels than alfalfa (Fig. 1). In rolled trefoil, tannins accounted for 67 to 84% of the variation in protein fractions for hay and ~60% of the variation in protein fractions for silage. Presumably due to *o*-quinone protection of protein, rolled hays and silages of clover had lower soluble protein levels and higher rumen-undegradable and intestinal-available protein levels than trefoil.

Shifting from rolls to maceration reduced soluble protein levels in hays and especially silages (Fig. 1). Maceration of alfalfa and trefoil increased rumen-undegradable and intestinal-available protein in hays and especially silages. Protein fractions were highly related to tannin levels in macerated trefoil, accounting for 80 to 96% of the variation in hays and ~82% of the variation in silages. Surprisingly, shifting from rolls to maceration had little effect on regression slopes, suggesting that maceration altered protein fractions in trefoil by mechanisms independent of tannin. Therefore, sequestration of tannin in vacuoles does not limit its action on protein in silage and hay. Maceration unexpectedly decreased rumen-undegradable and intestinal-available protein levels in clover hay and silage. Interestingly, these fractions in macerated hays and silages of clover responded identically to variations in tannin levels as trefoil, suggesting that severe tissue disruption inactivated *o*-quinone protection of proteins, leaving tannins as the only polyphenolic component limiting the degradability of protein in clover.

Conclusions

With conventional roll-conditioning, condensed tannins in trefoil and *o*-quinones in clover increased intestinal-available protein by up to 50% in hays and 90% in silages compared to alfalfa. Mechanical maceration also increased estimates of the intestinal-available protein in alfalfa and trefoil hays and especially silages, but disruption of tissues unexpectedly had little effect on protein protection by tannin. By contrast, maceration disrupted *o*-quinone protection of protein from proteases, yielding less intestinal-available protein in clover. The impact of these shifts on protein utilization by dairy cattle will be evaluated with nutrition and whole-farm models.

Figure 1. Forage crude protein (CP) fractions in hays and silages as influenced by condensed tannins and conditioning method. Data are from two harvests taken in two years.



Rate of Yield and Quality Change in Alfalfa

G. E. Brink, M. H. Hall, G. E. Shewmaker, N. P. Martin, D. J. Undersander, and R. P. Walgenbach

Introduction

The history of alfalfa harvest management research is one of changing goals. Early research conducted in the midwestern United States primarily addressed the effect of harvest frequency on yield as it relates to persistence; increased harvest frequency reduced yield the following year even when alfalfa was cut at full bloom. As varieties with improved winterhardiness such as Vernal became available, it was found that harvest frequency could be increased from two to three cuts per season without impacting yield and persistence. With the advent of more persistent varieties, the relationship between harvest management and forage quality expressed as nutrient yield began to receive more attention. Harvesting three times at first flower or 1/10 bloom before September 1 was recommended for maximum nutrient yield. A greater understanding of the effects of environment on plant survival suggested that alfalfa could be harvested four times per season to improve nutrient yield, although persistence could be negatively impacted.

Livestock dietary requirements ultimately dictates how alfalfa should be harvested to obtain feed of a given quality. As alfalfa matures through several identifiable morphological stages, feeding value declines with the rate of change being strongly influenced by temperature. Thus, harvesting at more immature stages (vegetative to bud) dictates a shorter cutting interval. The widespread culture and utilization of alfalfa across regions differing in environment, and its marketing as a cash crop for animal feed requires a greater understanding of the relationship between forage yield and quality. Our objective was to determine the rate of change in alfalfa forage quality relative to yield with increasing maturity for each harvest period.

Methods

The experiment was conducted in southcentral Idaho near Kimberly on a Portneuf silt loam (coarse_silty, mixed, superactive, mesic Durinodic Xeric Haplocalcid), in central Pennsylvania near State College on a Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalf), and in southcentral Wisconsin near Prairie du Sac on a Richwood silt loam (fine_silty, mixed, superactive, mesic Typic Argiudoll). 'Standfast', 'WL346', 'Affinity' alfalfa were seeded in the fall of 2003 (PA and WI) or the spring of 2004 (ID) at 20 lb/A.

In the spring, early summer, late summer, and fall of 2004 (PA and WI) and 2005 (PA, WI, ID), alfalfa was cut initially at late vegetative stage (stem length > 12 inches; no buds, flowers, or seed pods). Different plots of each variety were cut every 5 days thereafter to 20 days of maturity. Forage yield and quality were measured for each harvest. Plots not harvested for yield and quality analysis for a particular harvest period were cut at 1/10 bloom. Forage samples from each sequential harvest were dried for 48 hr at 150 F and ground through a 1-mm Wiley mill screen before quality analysis by near infrared reflectance spectroscopy. Prediction equations for crude protein (CP) and neutral detergent fiber digestibility (NDFD) were developed after laboratory analysis of samples from one replicate from each location, harvest period, and year. Data were subject to analysis of variance and regression equations were developed using SAS.

Results and Discussion

Despite differences in regrowth and dormancy, all three alfalfa varieties exhibited similar yield and forage quality responses as they matured during each harvest period after an initial cut at late vegetative stage. Total seasonal yield was not measured in this study, but these results are in general agreement with those reported by California researchers, who found that cutting interval, which directly impacts maturity, had a stronger influence on yield and quality than did variety.

At all three locations, initial dry matter yield at late vegetative stage (day 0) was greatest during the first harvest period, and declined at successive harvest periods until the last harvest period when it increased (Table 1; Fig. 1). Initial NDFD was also greatest during the first harvest period in Pennsylvania and Wisconsin. These results support previous findings that digestibility of first-cut alfalfa is usually superior to that of later cuttings. Initial CP concentration was greatest during the first harvest period in Wisconsin, but was greater at later harvest periods in Idaho and Pennsylvania.

Table 1. Regression equations describing trends for the change in yield ad quality of alfalfa harvested initially at late vegetative stage and every 5 days thereafter to 20 days of maturity during four harvest periods at three locations in 2005.

Harvest period	Yield (lb/A)	R^2	NDFD (%)	R^2	CP (%)	R^2
Idaho						
1	y = 3990 + 120x	0.84	y = 55.8 - 0.3x	0.70	y = 26.8 - 0.2x	0.97
2	y = 1590 + 180x	0.93	y = 60.9 - 0.6x	0.98	y = 27.1 - 0.3x	0.86
3	y = 2110 + 180x	0.95	y = 54.3 - 0.5x	0.98	y = 28.2 - 0.4x	0.92
4	-		-		-	
Pennsylvania						
1	y = 2640 + 290x	0.79	y = 59.4 - 0.2x	0.49	y = 24.8 - 0.1x	0.83
2	y = 1340 + 60x	0.93	y = 56.1 - 0.7x	0.88	y = 26.5 - 0.4x	0.84
3	y = 720 + 100x	0.99	y = 53.1 - 0.2x	0.59	y = 28.5 - 0.3x	0.98
4	y = 930 + 40x	0.58	y = 40.5 - 0.4x	0.83	y = 23.7 - 0.2x	0.86
Wisconsin						
1	y = 3240 + 130x	0.97	y = 58.3 - 0.4x	0.79	y = 26.1 - 0.3x	0.92
2	y = 1790 + 250x	0.91	y = 51.2 - 0.3x	0.44	y = 21.9 - 0.1x	0.30
3	y = 1760 + 90x	0.96	y = 52.4 - 0.4x	0.59	y = 24.8 - 0.3x	0.97
4	y = 2360 - 20x	0.85	y = 49.2 - 0.4x	0.88	y = 23.7 - 0.2x	0.93

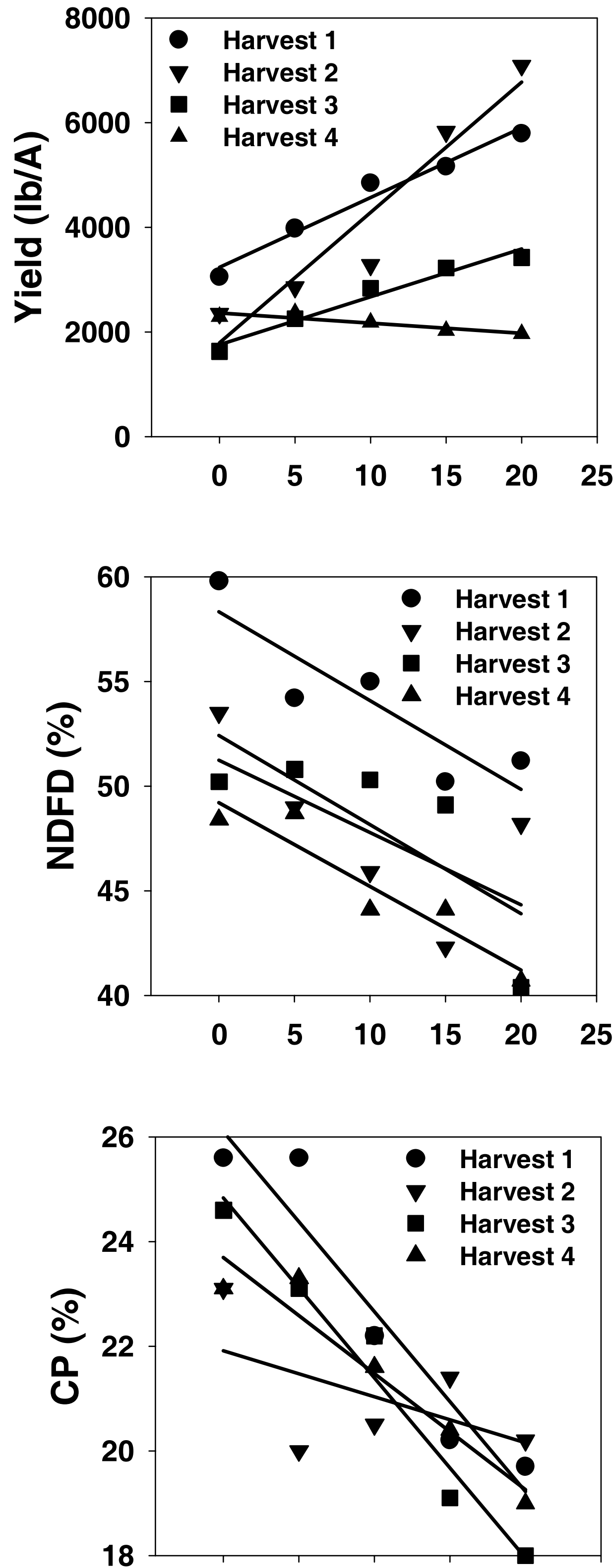
During 2005, yield increased more rapidly in Idaho during the second and third harvest periods than during the first harvest period, increasing a mean of 180 lb/acre/day compared to 120 lb/acre/day. In Pennsylvania and Wisconsin, however, yield increased most rapidly during the first and second harvest periods (290 and 250 lb/acre/day, respectively). Growing conditions in the east and midwest are generally more favorable during the spring and early summer than in the summer when temperatures are higher and soil moisture may be limiting. During the fall, declining day length and temperatures account for the fact that the rate of dry matter accumulation after the initial cutting is lowest during the fourth harvest period (Table 1).

Previous investigations have reported that the decline in alfalfa forage quality is more rapid in the summer than in the spring because higher temperatures increase the rate of morphological development. Results from Idaho and Pennsylvania generally support those findings, where both NDFD and CP concentration declined more rapidly during later harvest periods than during the first (Table 1). In Wisconsin, however, the rate of decline in NDFD and CP during the second, third, and fourth harvests periods was similar to or less than the first harvest period, indicating the influence of environmental conditions on forage quality throughout the growing season.

Conclusions

Due to known differences in climate and cultural practices (the use of irrigation at the Idaho site), location differences in dry matter accumulation and forage quality decline among harvest periods were expected. A finding common to all locations was that initial yield was greatest at the first harvest period. Whether the rate of decline in forage quality during the first harvest period is greater or less than that for subsequent harvests, a larger proportion of the annual yield is negatively impacted, particularly in more temperate areas where irrigation is not available. Harvest management considerations are thus most critical for the first harvest period.

Figure 1. Change in yield, neutral detergent fiber digestibility (NDFD), and protein concentration (CP) of alfalfa cut initially at late vegetative stage (day 0) for each of three harvest periods in Wisconsin during 2005 (mean of three varieties). See Table 1 for regression equations.



Feed Utilization by Cattle

Measurement of In Vitro Fiber Digestibility Is Affected By Fermentation Method

M. B. Hall and D. R. Mertens

Introduction

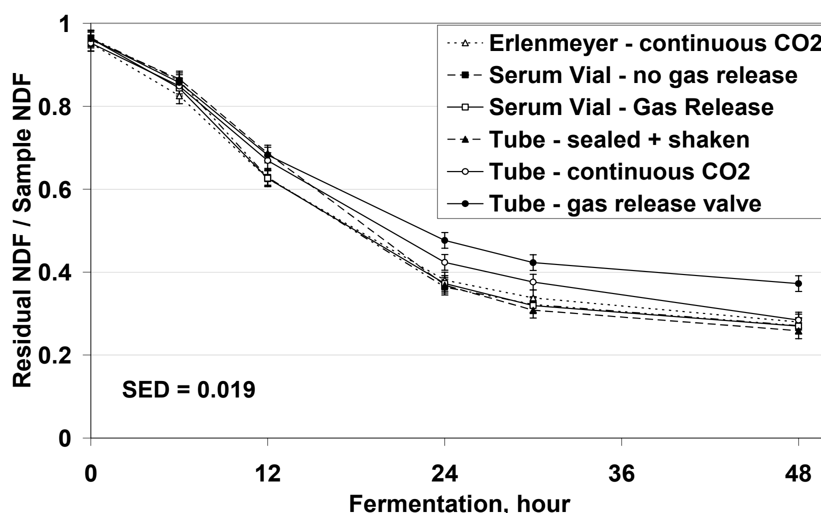
Dairy farmers and their nutritionists use analyses of neutral detergent fiber (NDF) digestibility (NDFD) of forages to better estimate the value of the feeds to support production in dairy cattle. A number of different methods to measure NDFD are currently in use, but these methods differ in type of fermentation vessel used and methods of gassing with carbon dioxide. The objective of our study was to evaluate how a number of commonly used fermentation methods (as well as some other options) compare in the NDF fermentation values they provide.

Methods

Fermentation Methods. The three vessel types and the treatments used were Erlenmeyer flasks (125 mL) with continuous CO_2 pressure maintained, serum vials (125 mL) sealed with butyl rubber stoppers and crimp seals with or without periodic release of the fermentation gas produced, and polyethylene centrifuge tubes (50 mL) sealed with rubber stoppers fitted with gas release valves, or with continuous gas pressure applied through a single gas line inserted through a rubber stopper which sealed the tube, or were sealed with rubber turnover septum stoppers and subjected to continuous shaking (60 strokes / min) on the long axis of the tube with periodic release of gas. Erlenmeyer flasks were incubated in a water bath, and all other treatments were incubated in a warm room, all at 39°C.

Alfalfa hay, corn silage, ryegrass hay, and soyhulls were used as substrates and fermented with Goering and Van Soest medium. Sample, medium, and inoculum amounts in each vessel were proportional to the amounts used in the original Goering and Van Soest procedure. Sample amounts of 0.5, 0.3, and 0.25 g were used for Erlenmeyer flasks, serum vials, and tubes respectively. Strained, blended inocula from four cows was used in each of two replicate fermentations. Samples were harvested at 0, 6, 12, 18, 24, and 48 hours of fermentation and analyzed for residual NDF. NDFD was calculated as (residual NDF – NDF in fermentation blank)/original NDF from sample, all on a dry matter and organic matter basis.

Figure 1. Proportion of NDF remaining after fermentation by different methods. Values are the average of all substrates.



Results and Discussion

NDF Disappearance & Digestibility. The disappearance of NDF across fermentation hours (Figure 1) showed differences among treatments. Closer appraisal of the 24, 30 and 48 h values which are most commonly used

to evaluate NDFD showed that substrate (30 hour values, Table 1) and fermentation hour affected whether differences were noted. Overall, vials with or without gas release, Erlenmeyer flasks with continuous gassing and continuously shaken sealed tubes did not differ from each other and gave the highest NDFD values. Tubes with gas release valves gave the lowest values for NDFD compared to other treatments, and underestimated NDFD by up to 16%. Tubes with continuous gassing gave lower NDFD values than other treatments for soyhulls at 24 and 30 h. Medium pH did not decline below 6.3 for any treatment, so it was not likely that pH had any negative effect on fiber fermentation.

Substrates differed in their sensitivity to differences among fermentation vessel treatments. In order of fewest to most treatment differences detected, alfalfa was followed by ryegrass, corn silage and soyhulls. No differences among treatments were detected at 24 and 30 h when alfalfa hay was the substrate, whereas soyhulls were far more sensitive to differences among treatments in this same timeframe.

Table 1. Neutral detergent fiber digestibility by treatment at 30 hours of fermentation. Values are least squares means[1].

Hour	30 h			
Substrate[2]	AH	CS	RG	SH
Treatment				
Flask - continuous gas	0.383	0.683[a]	0.710[a]	0.873[xg]
Vial - sealed	0.428	0.692[x]	0.703	0.888[xg]
Vial - gas release	0.430	0.695[x]	0.695	0.902[xg]
Tube – sealed + shaken	0.435	0.714[x]	0.720[x]	0.897[xg]
Tube - continuous gas	0.392	0.673[a]	0.688	0.743[h]
Tube - gas release valve	0.369	0.585[yb]	0.617[yb]	0.737[y]
SED	0.025	0.024	0.024	0.024

[1] Treatments only differed from tubes with gas release valves, x y z $P < 0.05$, a b c $0.05 < P < 0.15$, and tubes with continuous gassing, g h i $P < 0.05$, q r s $0.05 < P < 0.15$. Values in columns with different superscripts differ.
[2] AH = alfalfa hay, CS = corn silage, RG = ryegrass hay, SH = soyhulls
[3] SED = standard error of the difference

The behavior of substrates in vessels offers insight into why vessel form may affect NDFD. All vessels showed some evidence of floating layers of dry or moistened substrate through most of the fermentation, with the exception of the sealed, shaken tubes. In serum vials and Erlenmeyer flasks, the floating layer was generally thin and covered the entire surface of the medium. In the stationary tube treatments, formation of 1 to 2 cm thick, floating mats of substrate may have been a contributing factor to the lower NDFD in these treatments. Even with swirling at sampling hours, thick layers of substrate filled with gas bubbles tended to reform in the tubes and may have limited interaction of the rumen microbes and the substrate, or led to altered pH within the mat. In the case of tubes with gas release valves, although there was no indication that the medium had become aerobic, the tubes had a foul odor of rotting material at sampling. It is possible that air could have been admitted through the gas release valve, but, due to the floating mat of substrate, only part of the mat was oxygenated and not the greater portion of the medium.

Conclusions

It appears that all fermentation methods and vessels are not equivalent or perhaps even suitable for evaluation of all substrates. The differences among the systems give different estimates of fiber digestibility. The common and perhaps not unreasonable bias is to utilize the system(s) that allows for greatest digestibility to be achieved. A key aspect that these results do point out is that, because the values can vary among methods, we need to take care in how we use currently available NDFD values quantitatively, and that one should probably stay within a single fermentation system for qualitative comparisons among feedstuffs. The relationship between in vitro estimate and fiber digestion in the cow needs to be explored and established.

Effect of Dietary Crude Protein, Rumen-Undegraded Protein and Rumen-Protected Methionine on Milk Production in Lactating Dairy Cows

G. A. Broderick, M. J. Stevenson and R. A. Patton

Introduction

When cows are fed more crude protein (CP) than is needed to meet their requirement for metabolizable amino acids (AA), the excess N will be excreted in the urine and can contribute to environmental pollution. Supplementing diets with rumen-undegraded protein (RUP) may permit the reduction of dietary CP. Methionine is usually the essential AA that limits milk production in dairy cows fed typical diets based on legume forages, corn, and soybean meal. An earlier study showed that supplementing rumen-protected Methionine (RP-Met) in the form of Mepron® (Degussa Corp., Kennesaw, GA) improved the yield of milk and protein (Broderick et al., 2004 USDFRC Research Summaries). In a follow-up lactation trial, we fed diets formulated to contain 15.5 or 16.8% CP, with or without supplemental RUP, to determine whether there were similar or differential production responses to RP-Met at varying levels of dietary CP and RUP.

Materials and Methods

Thirty-two multiparous and 16 primiparous Holstein cows averaging 596 kg body weight were blocked by parity and days-in-milk into 6 groups of 8. Cows were randomly assigned within blocks to incomplete Latin square sequences and fed total mixed rations in a 2x2x2 arrangement of diets: 15.5 or 16.8% CP (DM basis), with or without RUP (added as 5% expeller soybean meal; ESBM), and with or without supplementation of 15 g/d of RP-Met (fed as Mepron®) (Table 1). Each experimental period lasted 28 d--14 d for adaptation and 14 d for data collection. The experiment had 3 periods (total 12 weeks). Data were analyzed using the Proc GLM procedures of SAS; the statistical model included square, period, cow(square), diet, and diet*period. Orthogonal contrasts were used to assess the effects of CP level, RUP supplementation, and dietary RP-Met addition, and their interactions. Probability was set at 0.05; least squares means are reported.

Results and Discussion

Mean CP contents of the diets were 15.8 and 17.1%, 0.3 percentage units higher than formulated (Table 1), because alfalfa silage averaged 23.8% CP, rather than the 22% CP measured before the trial began. Diets averaged 27.4% NDF and 16.5% ADF, indicating they were high in energy and typical of those fed to early lactation dairy cows. Supplementing with RUP from expeller soybean meal increased dietary content of Cornell protein fraction B3 from 9.0 and 8.7% of dietary CP to 11.5 and 11.1% of dietary CP (Table 1). This would have amounted to 90-100 g/d at the intakes occurring in this trial; most of this protein would be expected to escape the rumen undegraded.

Generally, there were no interactions among the main effects, so production results are reported separately for level of dietary CP, RUP, and RP-Met (Table 2). Contrasts indicated that higher dietary CP increased feed intake as well as the yield of milk, 3.5% fat-corrected milk (FCM), fat and protein, with a trend for greater yield of SNF. However, milk urea N (MUN) and estimated urinary excretion of urea-N and total-N also increased substantially at greater dietary CP. Apparent N efficiency (Milk-N/N-intake) fell from 33 to 30% ($P < 0.01$) when dietary CP increased from 15.8 to 17.1%.

The only significant effects of feeding more RUP (Table 2) were slightly increased feed efficiency and elevated MUN, although there was a strong trend for greater fat yield with more RUP. Also, apparent N efficiency was 31% at both levels of RUP. Supplementing with RP-Met increased feed intake and yield of FCM and milk fat

Table 1. Composition of diets.

	Diets							
Dietary CP, %	15.5	15.5	15.5	15.5	16.8	16.8	16.8	16.8
Expeller SBM, %	0	0	5.0	5.0	0	0	5.0	5.0
RP-Met, g/d	0	15	0	15	0	15	0	15
Ration ingredients	% of DM							
Alfalfa silage	20.7	20.7	20.7	20.7	20.7	20.7	20.7	20.7
Corn silage	33.9	33.9	33.9	34.0	33.9	33.9	33.9	33.9
High moisture shelled corn	26.0	26.0	25.3	25.3	23.1	23.1	22.4	22.4
Solvent soybean meal	10.0	10.0	5.7	5.7	12.9	12.9	8.6	8.6
Expeller soybean meal[1]	0.0	0.0	5.0	5.0	0.0	0.0	5.0	5.0
Soy hulls	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Energy Booster[2]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Ground shelled corn	2.13	2.07	2.13	2.07	2.13	2.07	2.13	2.07
Sodium bicarbonate	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Dicalcium phosphate	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Salt	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Limestone	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Dynamate	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Vitamin premix and trace minerals[3]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mepron[4]	0.00	0.06	0.00	0.06	0.00	0.06	0.00	0.06
Chemical composition								
Crude protein, % of DM	15.8	15.8	15.8	15.8	17.1	17.1	17.1	17.1
Neutral detergent fiber, % of DM	27.2	27.2	27.6	27.6	27.2	27.1	27.6	27.6
Acid detergent fiber, % of DM	16.4	16.4	16.6	16.6	16.5	16.5	16.6	16.6
NDIN, % of total N	12.6	12.6	15.7	15.6	12.1	12.1	14.9	14.9
ADIN, % of total N	3.6	3.6	4.1	4.0	3.4	3.4	3.8	3.8
B3,[5] % of CP	9.0	9.0	11.6	11.5	8.7	8.7	11.1	11.1

[1]SoyPlus, West Central Coop, Ralston, IA.
[2]Energy Booster 100, MS Specialty Nutrition, Dundee, IL.
[3]Provided (/kg DM): 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E.
[4]Ruminally protected methionine (RP-Met) product from Degussa Corp., Kennesaw, GA.
[5]B3 = NDIN (% of total N) - ADIN (% of total N) (Sniffen et al., J. Anim. Sci. 70: 3562-3577, 1992).

Table 2. Production results.

	Crude protein, % of DM			**SBM-RUP, % of DM**			**Mepron, g/d**	
Variable	15.8	17.1	*P* > F	1.9	2.6	*P* > F	0	15
DM intake, kg/d	24.3	25.4	< 0.01	25.1	24.7	0.13	24.6	25.2
BW gain, kg/d	0.37	0.38	0.96	0.42	0.33	0.50	0.35	0.40
Milk, kg/d	40.0	41.6	0.01	40.8	40.9	0.88	40.5	41.2
Milk/DMI	1.65	1.64	0.62	1.63	1.67	0.03	1.65	1.64
3.5% FCM, kg/d	44.9	47.2	< 0.01	45.4	46.6	0.16	45.1	46.9
Fat, %	3.57	3.68	0.08	3.54	3.71	< 0.01	3.56	3.69
Fat, kg/d	1.43	1.52	< 0.01	1.44	1.51	0.07	1.44	1.51
Protein, %	3.20	3.19	0.81	3.23	3.16	0.01	3.17	3.22
Protein, kg/d	1.28	1.32	0.04	1.31	1.29	0.24	1.28	1.32
SNF, %	8.91	8.88	0.62	8.92	8.87	0.31	8.87	8.92
SNF, kg/d	3.58	3.68	0.07	3.64	3.62	0.72	3.59	3.67
MUN, mg/dl	9.8	11.5	< 0.01	10.4	11.0	< 0.01	10.6	10.8
Fecal N, g/d	250	257	0.46	253	254	0.92	250	257
Urinary urea-N, g/d	130	176	< 0.01	149	157	0.30	149	157
Urinary total-N, g/d	177	213	< 0.01	194	197	0.67	191	199

content and yield; there was a strong trend for increased protein yield. Apparent N efficiency was 31% at both levels of RP-Met. Neither RUP nor RP-Met significantly altered estimated excretion of fecal N and urinary urea-N and total-N.

We were particularly interested in whether supplementation with RP-Met would allow similar yields of milk and milk components at lower dietary CP and, thus, reduced N excretion. Over both levels of RUP, yield of milk, FCM, fat and protein averaged 41.5, 46.5, 1.49 and 1.30 kg/d at 17.1% CP without RP-Met versus 40.6, 46.0, 1.48 and 1.30 kg/d at 15.8% CP with supplemental RP-Met. Excretion of urinary urea-N also was 40 g/d lower at 15.8% CP plus added RP-Met. The fact that essentially the same production of FCM, fat and protein occurred under both circumstances indicated that RP-Met could be used to lower dietary CP without loss of milk and component yield.

Conclusions

Under the conditions of this trial, increasing dietary CP from 15.8 to 17.1% increased yield of milk and milk components but also increased urinary N excretion and depressed N efficiency. Supplementing with soybean meal RUP slightly improved feed efficiency and fat yield. Adding RP-Met to the diet allowed similar production of FCM, fat and protein at 15.8% CP as was obtained at 17.1% CP without RP-Met. Thus, supplementing RP-Met in typical lactation diets based on alfalfa and corn silages, high-moisture corn, and soybean meal can be used to improve N efficiency and reduce excretion of environmentally labile urinary N.

Effect of Source of Rumen-Degraded Protein on Ruminal Digestion in Lactating Dairy Cows

S.M. Reynal, G.A. Broderick and J. Leibovich.

Introduction

Efficiency of N utilization by dairy cows may be improved by providing adequate amounts and sources of rumen-degraded protein (RDP) to the microorganisms in the rumen. Preformed amino acids (AA) have been shown to stimulate bacterial growth and fermentation in vitro. Ammonia requirements for rumen bacteria, estimated in vitro, are lower than those observed in situ and in vivo. The objective of the present study was to determine the optimum levels of preformed AA from soybean meal and ammonia from urea as RDP sources to maximize digestion of nutrients and efficiency of N utilization in lactating dairy cows.

Materials and methods

Twenty-eight lactating dairy cows (8 with ruminal cannulas; RC), averaging 114 DIM were used in seven (2 with RC) 4 x 4 Latin Squares with 4-wk periods. Diets were formulated to have similar NEL, nonfiber carbohydrate, CP, RDP, and rumen-undegraded protein (RUP), but to differ in the source (urea vs. true protein) of RDP. Solvent soybean meal (SSBM), lignosulfonate-treated SBM (LSBM), and urea were adjusted to replace RDP from SBM with that from urea (Table 1). Digesta flow from the rumen was quantified in the 8 RC cows using an omasal sampling technique (Huhtanen et al., 1997; J. Anim. Sci. 75:1380) with 3 markers to quantify digesta flow (Ahvenjärvi et al., 2000, J. Anim. Sci. 80:2176). Milk urea-N (MUN) and blood urea-N (BUN) were determined in all cows by an automated colorimetric assay (Broderick and Clayton, 1997; J. Dairy Sci. 80:2964). Fecal grab and urine spot samples were taken from all 28 cows 6 h before and 6 h after feeding on d-22 of each period. Indigestible-ADF and creatinine were used as internal markers for feces for urine, respectively. Microbial protein was estimated from urinary excretion of purine derivatives (PD) (Vagnoni et al., 1997; J. Dairy Sci 80:1695). A mixed model procedure of SAS for a Latin Square design was utilized for statistical analyses.

Table 1. Composition of diets.

	Diets			
	A	B	C	D
	(% of DM)			
Alfalfa Silage	15	15	15	15
Corn Silage	40	40	40	40
Rolled HMSC	29.6	28.7	28.5	27.8
Ground shelled corn	---	1.98	4.00	6.26
Solvent SBM	14.0	9.8	5.0	---
Lignosulfonate-SBM	---	2.5	5.1	8.0
Urea	---	0.41	0.84	1.31
Sodium Bicarbonate	0.50	0.52	0.52	0.54
Dical	0.20	0.21	0.21	0.22
Limestone	0.30	0.31	0.31	0.33
Salt	0.30	0.31	0.31	0.33
Vit. & Min. Conc.	0.20	0.21	0.21	0.22
	Chemical Composition			
Crude Protein	16.1	16.1	16.1	16.1
Total N, % of DM	2.58	2.57	2.56	2.58
Non-urea N, % of DM	2.58	2.38	2.18	1.98
NDF	24.8	25.4	25.9	25.7
ADF	14.1	14.2	14.4	13.9
RDP[1]	10.6	10.5	10.4	10.5
RUP[1]	5.5	5.6	5.7	5.6
NPN, % of total N	20.3	27.8	35.5	43.6
NDIN, % of total N	6.2	8.2	9.5	11.1
ADIN, % of total N	2.6	2.7	2.8	2.6
NEl,[1] Mcal/kg	1.58	1.58	1.58	1.57

[1]Calculated by NRC 2001 based on actual composition of diets fed.

Results and discussion

Replacing RDP from SSBM with that from urea resulted in a small, linear decline in DM intake (about 1 kg/d from 0 to 1.3% dietary urea) and substantial reductions in yield of milk and protein (Table 2). Ruminal VFA production and profile were not affected by RDP source; however, inclusion of urea in the diets increased concentrations of ruminal ammonia (Table 2), as well as MUN, and BUN, and urinary excretion of urea-N (Table 3), indicating a poor utilization

of urea-N by ruminal microorganisms. However, microbial N flow from the rumen, estimated from urinary PD excretion, was not significantly affected by source of RDP. Apparent ruminal digestion of OM, NDF, and ADF also was not affected by dietary RDP source (Table 4). The linear increase in apparent total tract digestibility of NDF with incremental replacement of SSBM with lignosulfonate-treated SBM and urea may have been an artifact of the NDF analysis.

Conclusions:

Replacing RDP from urea with that from soybean meal in diets fed to lactating dairy cows reduced potential environmental pollution by improving milk production and reducing N losses from the rumen. However, no significant effects of RDP source on ruminal digestion and metabolism were detected in this trial.

Table 2. Effect of dietary RDP source on DMI and milk production (28 cows), and mean concentrations of ruminal metabolites (8 RC-cows).

	----------Diet and % urea in diet DM-----------							
	A	B	C	D			*P* > F[1]	
	0	0.41	0.84	1.31	SE	Linear	Quadratic	Cubic
DM intake, kg/d	23.6	23.2	23.0	22.3	0.4	<0.01	0.68	0.67
Milk yield, kg/d	39.3	38.6	38.5	36.0	0.9	<0.01	0.18	0.26
3.5% FCM, kg/d	38.0	36.6	36.6	35.4	1.1	<0.05	0.92	0.49
Milk true protein, kg/d	1.27	1.22	1.21	1.17	0.03	<0.01	0.90	0.32
pH	6.51	6.52	6.51	6.59	0.05	0.12	0.30	0.42
NH3-N, mg/dl	8.18	9.32	10.27	10.75	0.86	<0.01	0.52	0.92
Total free-AA, mM	16.8	19.5	19.4	18.0	1.3	0.52	<0.05	0.67
Acetate, mM	83.0	78.8	86.4	86.0	3.6	0.20	0.61	0.13
Propionate, mM	29.4	30.6	33.4	31.6	1.6	0.20	0.28	0.42
Isobutyrate, mM	1.88	1.72	1.90	1.74	0.08	0.42	0.81	<0.05
Butyrate, mM	17.3	16.6	17.5	17.3	1.1	0.75	0.77	0.48
Isovalerate, mM	2.72	2.28	2.98	2.60	0.16	0.41	0.96	<0.01
Valerate, mM	2.76	2.90	3.30	2.78	0.28	0.72	0.16	0.31
Total, mM	137	133	146	142	5.8	0.21	0.97	0.14

[1]Probability of a significant linear, quadratic, or cubic effect of dietary RDP source.

Table 3. Effect of dietary RDP source on N efficiency and excretion, microbial N flows from the rumen, MUN and BUN (28 cows).

	Diet and % urea in diet DM					$P > F$[1]		
	A	B	C	D				
Item	0	0.41	0.82	1.31	SE	Linear	Quadratic	Cubic
Nitrogen								
Intake, g/d	564	556	548	537	10	<0.01	0.85	0.97
Urinary excretion, g/d								
Total N	191	192	195	203	6	0.10	0.53	0.98
Urea-N	136	136	152	150	6	<0.01	0.62	0.11
Fecal excretion, g/d	179	170	167	151	7	<0.01	0.59	0.43
Total excretion								
g/d	370	361	363	355	11	0.26	0.98	0.59
% of N intake	65.3	64.6	65.5	65.8	1.5	0.69	0.73	0.75
Microbial N flow,[3] g/d	498	435	393	435	48	0.21	0.16	0.75
N efficiency								
Milk/DMI	1.68	1.67	1.69	1.63	0.04	0.18	0.17	0.26
Milk N/N intake	0.36	0.33	0.36	0.35	0.01	0.96	0.61	<0.05
Milk/N excreted[4]	112	112	113	106	3	0.13	0.16	0.47
MUN, mg/dl	6.77	7.45	8.13	9.08	0.31	<0.01	0.48	0.75
BUN, mg/dl	8.87	9.89	11.39	12.78	0.45	<0.01	0.61	0.72

[1]Probability of a significant linear, quadratic, or cubic effect of dietary RDP source; [2]Apparent total tract digestibility, %; [3]Microbial N flows from the rumen estimated from urinary PD excretion; [4]Milk yield (kg) per kg of N excreted.

Table 4. Intake, omasal flow, and apparent ruminal digestibility of OM, NDF, ADF, and N (8 RC-cows).

	Diet and % urea in diet DM						$P > F$[1]	
	A	B	C	D				
Item	0	0.41	0.84	1.31	SEM	Linear	Quadratic	Cubic
DM intake, kg/d	24.1	23.0	23.9	22.5	0.9	0.17	0.64	0.15
OM								
Intake, kg/d	21.0	19.7	20.4	19.2	0.6	0.02	0.90	0.03
Omasal flow, kg/d	12.4	11.5	12.1	11.3	0.6	0.10	0.99	0.05
ARD,[2] %	40.4	41.5	40.4	41.1	2.2	0.90	0.96	0.56
NDF								
Intake, kg/d	5.54	5.52	5.95	5.46	0.22	0.90	0.07	0.05
Omasal flow, kg/d	3.77	3.23	3.71	3.48	0.18	0.59	0.50	0.02
ARD,[2] %	32.5	40.6	36.0	35.4	2.8	0.80	0.14	0.15
ADF								
Intake, kg/d	3.18	3.12	3.36	2.97	0.14	0.33	0.06	0.05
Omasal flow, kg/d	2.04	1.72	1.91	1.76	0.09	0.11	0.46	0.03
ARD,[2] %	36.5	43.7	40.9	39.6	2.9	0.64	0.13	0.31
Nitrogen								
Intake, g/d	569	545	561	534	18	0.09	0.71	0.10
Omasal flow, g/d								
Total N	603	559	573	509	27	<0.01	0.37	0.08
NDIN	18.9	17.9	22.0	16.2	1.7	0.45	0.05	0.03
ADIN	5.18	5.55	6.41	5.35	1.52	0.85	0.55	0.69
ARD,[2] %	-5.76	-2.98	-2.28	4.95	4.1	0.02	0.37	0.49

[1]Probability of a significant linear, quadratic, or cubic effect of dietary RDP source; [2]Apparent ruminal digestibility, %.

Effects of Different Levels of Lauric Acid on Ruminal Protozoa, Fermentation Pattern, and Milk Production in Dairy Cows.

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Introduction

Overfeeding protein to dairy cows is uneconomical and results in excessive urinary N, the most labile form of excreted N. Although protozoa appear to stabilize the rumen environment, there is evidence that protozoa also reduce microbial protein production in the rumen. Partial suppression of protozoal populations, rather than complete defaunation, has received little attention. Moreover, most defaunation techniques are not applicable under practical feeding conditions. Previously, lauric acid, a saturated 12-carbon fatty acid, had potent anti-protozoal effects and may have improved N utilization in the rumen (Faciola et al., 2004 USDFRC Research Summaries). The aims of this study were: 1) to evaluate lauric acid as a practical defaunating agent, and 2) to assess the effects of partial defaunation on fermentation patterns and milk production in dairy cows.

Materials and Methods

Fifty-two Holstein cows (8 with ruminal cannulas) averaging 607 kg of BW, 140 DIM, and 42.1 kg milk/d, were blocked into groups of 4 by DIM to give 13 blocks (2 with ruminal cannulas) in a randomized complete block design. Prior to starting the experimental phase, all cows were fed the same diet (control) for a 2-wk covariate period and production of milk and milk components was determined for use in statistical analysis. Cows within blocks were then randomly assigned to one of the 4 levels of lauric acid (LA) supplementation: 0, 80, 160 or 240 g/d, during the remaining 8 weeks of the study (Table 1). Diets were adjusted weekly to reflect changes in DM and CP of forages and concentrate mixtures by drying weekly composites of each ingredient and analyzing for total N. About 100-200 ml of strained ruminal fluid was collected from 4 different sites of the rumen of the cannulated cows over 24 h after the feeding on the last day of wk-4 and wk-8. Samples were analyzed for ammonia, total free amino acids, VFA, and total protozoal counts. A single mean value was computed for each cow over the experimental phase of the trial for DM intake, milk production, and protozoal counts. Data were analyzed with the Proc Mixed procedure of SAS using models that included the covariate mean for each cow. Significance in each trial was declared at $P \leq 0.05$, and separation of least squares means was conducted using pdiff.

Table 1. Composition of diets.

Ingredients	------Lauric acid supplement (g/d)------			
	0	80	160	240
Alfalfa Silage	28.81	28.81	28.81	28.81
Corn Silage	35.72	35.72	35.72	35.72
Rolled HMSC	14.10	14.10	14.10	14.10
48% Soybean meal	7.59	7.59	7.59	7.59
Sodium bicarbonate	0.75	0.75	0.75	0.75
Limestone	0.36	0.36	0.36	0.36
Salt	0.19	0.19	0.19	0.19
Dicalcium phosphate	0.24	0.24	0.24	0.24
Vitamins & Minerals	0.08	0.08	0.08	0.08
Ground shelled corn	12.16	8.11	4.05	0.00
Premix[1]	0.00	4.05	8.11	12.16
Composition				
Crude Protein	15.5	15.7	15.9	16.1
NDF	28.7	28.8	28.8	28.8
ADF	17.4	17.5	17.5	17.6

[1]Premix made of 92% ground shelled corn and 8% lauric acid.

Results and Discussion

There was only a trend ($P = 0.10$) for an effect on DM intake in this trial so it appeared that inclusion of these levels of LA in the TMR did not have adverse effects on nutrient intake (Table 2). No other significant effects on yield of milk or milk components were observed in this experiment. Ruminal pH and ruminal metabolites, including NH_3, total free amino acids, and VFA concentrations were not different among treatments (Table 2). Earlier, we observed significant reduction in ruminal NH_3 and total free amino acids with the supplementation of 160 g of LA once/d via the ruminal cannula (Faciola et al., 2004 USDFRC Research Summaries). The lack of

Table 2. Dry matter intake, milk production, ruminal traits and protozoal counts.

Item	----------Lauric acid supplement (g/d----------				SEM	$P > F$
	0	80	160	240		
Production						
DM intake, kg/d	26.6	25.5	25.3	25.0	0.55	0.10
Milk, kg/d	35.3	36.1	35.8	36.5	0.78	0.73
Fat, %	3.99	3.89	3.71	3.72	0.14	0.42
Fat, kg/d	1.46	1.47	1.35	1.28	0.08	0.25
Protein, %	3.20	3.15	3.08	3.16	0.06	0.57
Protein, kg/d	1.16	1.19	1.12	1.09	0.05	0.47
SNF, %	8.93	8.90	8.85	8.89	0.07	0.89
SNF, kg/d	3.23	3.37	3.26	3.08	0.14	0.51
Ruminal traits						
pH	6.56	6.60	6.51	6.40	0.06	0.27
Ammonia, mM	6.11	6.24	6.57	7.51	0.61	0.40
Total free AA, mM	11.6	9.5	12.4	9.4	1.03	0.23
Total VFA, mM	101.5	82.7	93.9	97.3	7.65	0.44
Acetate, mM	64.7	55.2	60.0	60.8	4.15	0.52
Propionate, mM	20.7	15.6	19.6	21.6	2.05	0.31
Butyrate, mM	10.6	8.33	9.77	9.91	1.00	0.52
Isobutyrate, mM	1.54	1.12	1.31	1.35	0.13	0.28
Valerate, mM	1.88	1.15	1.56	1.66	0.27	0.39
Isovalerate, mM	1.99	1.31	1.71	2.05	0.28	0.34
Protozoa, x 10^6/ml	5.05[a]	5.16[a]	3.81[b]	3.43[b]	3.77	0.05

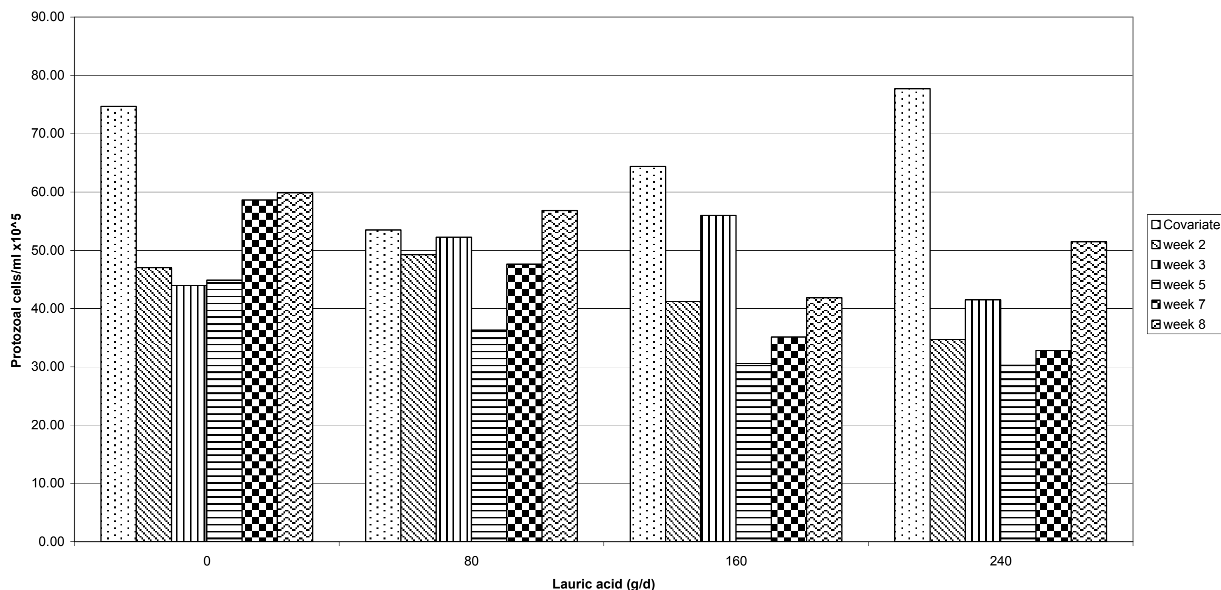
[a,b]Means in rows without common superscripts are different (P < 0.10).

significant effect may have been due to the low suppression of the protozoal population in this trial. Mean protozoal numbers were reduced only by 25 and 30%, respectively, at 160 and 240 g/d of LA over the course of the trial (Table 2). It appears that these doses of LA, when included in the TMR, were not high enough to reach the concentrations necessary to suppress ruminal protozoa. In our previous study, the protozoal population was suppressed by 90% after 2 days when 160g of LA was dosed intra-ruminally via the cannula. The slow decline in protozoa at 160 and 240 g/d of LA feeding is apparent in Figure 1. It is possible that, without the critical level of LA required to achieve a certain degree of protozoal suppression, the remaining protozoa may be able to reestablish the population by replication. Thus, it will be necessary to test different approaches for suppressing the protozoal population.

Conclusions

Feeding lauric acid in the TMR at 80, 160, or 240 g/d did not reduce DM intake and did not adversely affect production or ruminal traits. However, 160, or 240 g/d of lauric acid only reduced the ruminal protozoal population by 25 and 30%, respectively, showing that these levels in the diet were not adequate to have a significant anti-protozoal effect. Further studies are necessary to determine the concentration of lauric acid in the TMR required to obtain substantial protozoal suppression and to determine if this will improve N efficiency in the lactating dairy cow.

Figure 1. Effects of lauric acid feeding over time on ruminal protozoal counts.



Optimal Nutrient Intake and Digestion for Ruminal Microbial Protein Synthesis and Milk Yield in Lactating Dairy Cows

S. M. Reynal and G. A. Broderick.

Introduction

Optimization of N utilization for milk production relies on accurate predictions of the partitioning of dietary protein for metabolic processes in the tissues. Ruminal N metabolism dictates the fate of N compounds between productive processes and excretion by the whole animal. However, prediction equations derived from in vivo data on ruminal N metabolism and its effect on milk production and N excretion are lacking. The objective of this data analysis was to study the relationships among nutrient intake, ruminal digestion, milk production, and N excretion in high-producing dairy cows using individual-cow data.

Materials and Methods

Data from 6 in-vivo experiments conducted at the US Dairy Forage Research Center were used to study the responses of microbial efficiency and protein flow from the rumen, milk protein yield, N utilization efficiency, and N excretion of lactating dairy cows to several dietary and digestion factors. The data set (195 cow-periods from 6 Latin square experiments) included lactating dairy cows in early to mid lactation fitted with rumen cannulas. The omasal sampling technique (Huhtanen et al., 1997; J. Anim. Sci. 75:1380; Ahvenjärvi et al., 2000, J. Anim. Sci. 80:2176) was used to quantify nutrient flow from the rumen. Microbial flows at the omasal canal were measured using ^{15}N (5 trials) and total purines (1 trial) as microbial markers. Urinary N excretion was estimated in 5 trials from spot samples of urine using creatinine as internal marker. Milk urea-N (MUN) was determined by an automated colorimetric assay (Broderick and Clayton, 1997; J. Dairy Sci. 80:2964). All diets were based on high-moisture corn, corn silage, and legume silage (mainly alfalfa), and ranged from 13.5 to 20.3% CP (mean = 17.4%), from 43 to 55% nonfiber carbohydrate (NFC) (mean = 49%), and from 22 to 30% NDF (mean = 25%). Urea, heated and unheated soybean meal, corn gluten meal, blood meal, canola meal, and cottonseed meal were fed as protein supplements. Data from cows fed urea as the only protein supplement were omitted from the analyses presented here. Cows averaged 113 days in milk, consumed between 14 and 34 kg of DM/d (mean = 23 kg/d), and produced between 14 and 55 kg milk/d (mean = 36 kg/d).

Regression analyses were executed using PROC MIXED of SAS (1999), with the effect of study included as a random variable in the model, following the methodology described in detail by St. Pierre (2001, J. Dairy Sci. 84:741). Residual

Table 1. Description of variables.

Variable	N	Mean	Std. Dev.	Min.	Max.
DM intake, kg/d	195	23.4	3.7	15.0	32.5
Diet CP, %	190	17.9	2.0	13.3	24.7
Diet RDP, %	185	12.4	2.4	7.5	18.7
Diet RUP, %	185	5.42	1.25	2.11	8.14
Diet RDP, % (NRC)[1]	195	12.3	2.3	7.5	18.6
Diet RUP, % (NRC)[1]	195	5.69	1.41	4.02	11.96
Diet NDF, % of DM	195	26.5	3.9	20.6	36.0
Diet NFC, % (NRC)[1]	195	48.5	3.1	43.2	55.0
Diet NEL, Mcal/kg (NRC)[1]	195	1.57	0.05	1.48	1.81
Diet CP:NFC ratio	190	0.37	0.06	0.24	0.56
Microbial CP flow, kg/d	185	2.70	0.50	1.35	4.33
Microbial efficiency,[2] g/kg	185	29.7	4.0	16.7	40.3
Rumen N balance, g/d	184	30	80	-163	268
ENU,[3] %	183	96	18	51	154
Mean ruminal NH3-N, mg/dl	195	8.46	3.23	1.46	16.81
Daily minimum, mg/dl	195	3.09	2.23	0.32	10.07
OMTDR,[4] kg/d	185	14.6	2.4	8.9	20.7
TOMD,[5] %	185	67.3	5.5	55.2	87.6
Ruminal NDF digestibility, %	185	38.1	10.7	13.8	63.1
Milk yield, kg/d	189	36.8	7.25	20.1	55.3
Milk protein yield, kg/d	187	1.15	0.20	0.58	1.64
Milk N efficiency,[6] %	188	27.2	5.3	15.8	45.1
MUN, mg/dl	195	15.8	5.1	4.4	28.4
MUN secretion, g/d	189	5.83	2.21	1.51	14.63
Urinary N excretion, g/d	156	200	55	92	375

[1]Predicted by NRC model (2001); [2]g microbial NAN/kg OM truly digested in the rumen; [3]Microbial efficiency of N use, g of microbial NAN/g rumen-available N; [4]OM truly digested in the rumen; [5]True OM digestibility, % of OM intake; [6]Milk N as % of N intake

and inter-study standard errors (SE) are reported for each equation. The description of the variables studied is presented in Table 1.

Results and discussion

Inclusion of measured–but not NRC-predicted–rumen-undegraded protein (RUP) (% of diet DM) in a bivariate model with DM intake marginally improved precision of the predictions of milk protein yield (Table 2). Moreover, response to measured RUP was only 0.022 kg per percentage-unit increase in dietary RUP concentration. A meta-analysis of literature data on milk protein production responses to dietary RUP concentrations yielded a coefficient of 0.041 kg per percentage-unit RUP (NRC, 2001). When this same data set was analyzed using trial as random in the model (Firkins et al., 2006; J. Dairy Sci. 89:E31), the resulting coefficient was 0.020, similar to the one obtained in the present study. These findings suggest that the NRC overestimates production responses to RUP supplementation. Efficiency of dietary N utilization for milk protein production was best predicted using a bivariate model including milk yield and MUN concentration. The negative effect of dietary CP content on milk N efficiency (slope = -1.67) was mainly due to the contribution from dietary RDP (slope = -1.35). Increasing microbial efficiency of N use in the rumen resulted in increased efficiency of N use for milk protein secretion.

Table 2. Equations for predicting milk protein yield (kg/d) and N efficiency (Milk N, % of N intake) based on diet composition, nutrient intake, and ruminal metabolism; $Y = A + BX_1 + CX_2$, where A is the intercept, and B and C are the regression coefficients (*N*=195).

Y/ X1, X2	A	SE	*P*	B	SE	*P*	C	SE	*P*	Interstudy SE	Residual SE
Y = milk protein yield (kg/d)											
DMI[1], RUP[2] (measured)	0.16	0.11	0.20	0.037	0.003	<0.001	0.022	0.009	0.02	0.05	0.14
Y = milk N efficiency (milk N, % of N intake)											
CP[3]	56.9	4.2	<0.001	-1.67	0.23	<0.001				2.3	4.1
RDP[4] (measured)	44.0	2.3	<0.001	-1.35	0.17	<0.001				1.9	4.0
Milk yield, kg/d	15.1	2.0	<0.001	0.33	0.05	<0.001				2.1	4.1
CP[3], milk yield (kg/d)	44.0	3.8	<0.001	-1.63	0.19	<0.001	0.33	0.04	<0.001	1.7	3.5
RDP[4], milk yield (kg/d)	30.9	2.5	<0.001	-1.23	0.14	<0.001	0.32	0.04	<0.001	1.1	3.5
MUN, mg/dl	34.4	1.7	<0.001	-0.45	0.10	<0.001				1.5	4.5
MUN, milk yield (kg/d)	21.1	1.9	<0.001	-0.44	0.07	<0.001	0.36	0.04	<0.001	0.6	3.9
Rumen N balance, g/d	28.0	0.9	<0.001	-0.022	0.005	<0.001				2.0	4.4
ENU[5]	17.5	2.2	<0.001	0.102	0.021	<0.001				2.1	4.4
Ruminal NH3-N, mg/dl	32.0	1.4	<0.001	-0.58	0.11	<0.001				2.4	4.3

[1]DM intake, kg/d; [2]RUP, % of diet DM; [3]CP, % of diet DM; [4]RDP, % of diet DM; [5]ENU = microbial efficiency of N use, microbial N as % of rumen-available N.

As expected, the best single predictors of microbial protein yield in the rumen were DM and NEL intake (Table 3). The positive linear response of microbial yield to measured-RDP and mean–but not minimum–ruminal NH_3-N suggested that amino acids and peptides, rather than NH_3-N, stimulated microbial growth as dietary RDP increased. Microbial efficiency of N use increased linearly with increased efficiency of energy use (slope = 2 percentage-units/g) and intake of NFC (slope = 4 percentage-units/kg). A 100% efficiency of microbial N use was obtained at a CP:NFC ratio of 0.344 (i.e., 15.5% CP in a diet containing 45% NFC). Microbial efficiency (g N/kg OMTDR) was negatively associated with ruminal OM digestibility (-0.45 g/percentage-unit).

Urinary N excretion was best predicted from mean NH_3-N concentration in the rumen and decreased linearly with increased microbial efficiency of N use (slope = -0.70 g/percentage-unit; Table 4). These findings highlight the importance of improving N use efficiency in the rumen for reducing N excretion and environmental pollution. Milk urea-N secretion was a better predictor of urinary N excretion than MUN concentration when compared across studies.

Conclusions

Efficiency of N use for milk production in dairy cows can be substantially improved by improving microbial efficiency of N use in the rumen. More research should be focused on determining the appropriate sources and amounts of RDP and energy for optimal microbial growth and minimal N excretion without compromising milk production.

Table 3. Equations for predicting microbial CP flow (kg/d) and efficiencies based on diet composition, nutrient intake, and ruminal metabolism; $Y = A + BX_1 + CX_2$, where A is the intercept, and B and C are the regression coefficients (*N*=195).

X1, X2	A	SE	*P*	B	SE	*P*	C	SE	*P*	Interstudy SE	Residual SE
Y = Microbial CP flow, kg/d											
DMI, kg/d	-0.02	0.19	0.93	0.116	0.007	<0.001				0.16	0.32
NELI[1] (NRC)	-0.17	0.20	0.44	0.078	0.005	<0.001				0.21	0.32
RDP[2] (measured)	1.68	0.27	0.002	0.082	0.020	<0.001				0.24	0.47
DMI[3], RDP[2] (measured)	-0.68	0.23	0.03	0.112	0.007	<0.001	0.060	0.013	<0.001	0.14	0.30
OMTDR[4], kg/d	0.50	0.16	0.03	0.151	0.011	<0.001				0.05	0.35
Ruminal NH3-N, mg/dl	2.43	0.12	<0.001	0.032	0.012	0.007				0.13	0.48
Y = ENU[5]											
Microbial efficiency[6]	34.4	7.5	0.01	2.05	0.21	<0.001				10	11
RDP[2] (measured)	162	6	<0.001	-5.39	0.47	<0.001				6	11
RDP[2] (NRC)	121	10	<0.001	-2.13	0.74	0.004				9	14
RDPS[7] (measured), NFCI[8]	106	6	<0.001	-19.7	1.8	<0.001	4.03	0.66	<0.001	5	11
CP:NFC[9]	166	10	<0.001	-192	26	<0.001				7	12
Ruminal NH3-N (mg/dl)	108	5	<0.001	-1.55	0.33	<0.001				10	13
Y = Microbial efficiency[6]											
Rumen N balance (g)	30.7	0.5	<0.001	-0.033	0.004	<0.001				1.2	3.3
TOMD[10] (%)	60.4	4.0	<0.001	-0.46	0.06	<0.001				1.7	3.4

[1]NELI = NEL intake, Mcal/d; [2]RDP, % of diet DM; [3]DMI, kg/d; [4]OMTDR = OM truly digested in the rumen, kg/d; [5]ENU = microbial efficiency of N use, microbial N as % of rumen-available N; [6]g of microbial NAN/kg OM truly digested in the rumen; [7]RDP supply, kg/d; [8]NFCI = NRC-predicted NFC intake, kg/d; [9]CP:NFC = dietary CP to NFC ratio; [10]TOMD = true OM digestibility, % of OM intake.

Table 4. Equations for predicting urinary N excretion (g/d) based on diet composition, nutrient intake, MUN, and ruminal metabolism; $Y = A + BX_1$, where A is the intercept, and B is the regression coefficient (*N*=156).

X1, X2	A	SE	*P*	B	SE	*P*	Interstudy SE	Residual SE
RDP[1] (measured)	62	29	0.10	11.7	2.0	<0.001	40	40
RDP[1] (NRC)	104	50	0.09	10.7	3.3	0.001	75	57
CP intake, kg/d	94	27	0.02	25.7	5.2	<0.001	35	41
MUN, mg/dl	123	22	0.005	5.34	0.95	<0.001	37	40
MUN secretion, g/d	152	19	0.001	8.83	2.08	<0.001	35	42
Ruminal NH3-N, mg/dl	136	18	0.002	8.14	0.98	<0.001	36	36
Efficiency of N use, %	266	30	<0.001	-0.70	0.24	0.005	38	42

[1]RDP, % of diet DM.

Replacing Alfalfa Silage with Red Clover Silage or Birdsfoot Trefoil Silage in the Diets of Lactating Dairy Cattle

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Introduction

Previous research at our Center showed that N-efficiency of lactating dairy cattle was greater with red clover (RC) silage than with alfalfa (Alf) silage because *o*-quinones in RC formed through the action of polyphenol oxidase limit proteolysis during ensiling and possibly in the rumen. Condensed tannins (CT) bind to plant proteins and also reduce protein degradation in both the silo and the rumen. Commercial cultivars of birdsfoot trefoil (BFT) contain modest levels of CT. Miller and Ehlke (Euphytica 92:383-391, 1996) used recurrent phenotypic selection to develop forage lines with low and high levels of CT from a BFT cultivar with intermediate CT content. The objective of this trial was to compare silages made from RCS, and these BFT with low, medium and high levels of CT, to Alf for milk production and N-efficiency in dairy cows.

Table 1. Composition of silages.

Item	Alf	RC	LBFT	NBFT	HBFT	SE	$P > F$[2]
	Silage source[1]						
DM, %	41.2[b]	60.2[a]	40.1[b]	33.9[d]	37.1[c]	1.2	< 0.01
	% of DM						
CP	22.0[a]	18.1[c]	21.7[a]	20.4[b]	20.1[b]	0.3	< 0.01
Ash	11.2[b]	13.4[a]	9.9[c]	10.1[c]	10.1[c]	0.3	< 0.01
NDF	35.3[b]	42.7[a]	32.4[c]	32.4[c]	32.2[c]	0.4	< 0.01
ADF	25.1[ab]	26.1[a]	24.0[b]	23.7[b]	23.5[b]	0.6	< 0.01
ADICP	2.36[b]	3.00[a]	1.61[b]	1.58[b]	1.75[b]	0.27	0.01
ADICP, % of CP	10.7[b]	16.6[a]	7.5[b]	7.7[b]	8.6[b]	1.4	< 0.01

[a-c]Means in the same row with different superscripts differ ($P \leq 0.05$).
[1]Alf = Alfalfa; RC = red clover; LBFT = low CT birdsfoot trefoil; NBFT = normal CT birdsfoot trefoil; HBFT = high CT birdsfoot trefoil.
[2]Probability of a significant effect of silage source.

Table 2. Composition of diets.

Item	Alf	RC	LBFT	NBFT	HBFT
	Silage source[1]				
	% of DM				
Alfalfa	59.4	0	0	0	0
Red Clover	0	50.5	0	0	0
Low CT BFT	0	0	60.5	0	0
Normal CT BFT	0	0	0	60.3	0
High CT BFT	0	0	0	0	60.7
Corn Silage	15.6	15.6	15.2	15.2	15.1
High moisture shelled corn	21.9	24.4	21.2	21.3	21.2
Soybean meal	3.0	9.0	3.0	3.0	3.0
Sodium bicarbonate	0.3	0.3	0.3	0.3	0.3
Salt	0.2	0.2	0.2	0.2	0.2
Dicalcium phosphate	0.1	0.1	0.1	0.1	0.1
Vitamin and trace mineral premix[2]	0.1	0.1	0.1	0.1	0.1
Composition					
CP	18.0	17.8	17.3	16.6	17.0
NDF	29.2	28.6	26.2	26.9	27.1

[1]Alf = Alfalfa; RC = red clover; LBFT = low CT birdsfoot trefoil; NBFT = normal CT birdsfoot trefoil; HBFT = high CT birdsfoot trefoil.
[2]Provided (/kg DM): 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E.

Methods

Alf, and BFT containing low (LBFT), normal (NBFT) or high (HBFT) concentrations of CT, were harvested and ensiled at about 40% dry matter (DM) using conventional methods. RC silage was harvested at about 60% DM. Silage composition is shown in Table 1. Twenty-five Holstein cows (5 fitted with ruminal cannulae) averaging 634 kg of body weight, 148 days-in-milk and 46 kg/d of milk were blocked by days-in-milk into 5 groups and were randomly assigned within blocks to 5 incomplete 5 x 5 Latin squares. Each trial period lasted 4 weeks—2 weeks for adaptation and 2 weeks for data collection. The trial had a total of 4 periods (16 weeks). Four diets contained 60% silage DM and 40% DM from corn silage, high moisture corn and soybean meal but the RC diet contained only about 50% silage DM because of the higher NDF content of that silage. Dietary components were analyzed weekly for DM to maintain the desired proportions of dietary DM. Rations were of similar composition except that more soybean meal was fed with RC because of its lower CP content. Diets averaged 17% CP and 28% NDF (Table 2). Apparent excretion of urinary urea-N and fecal-N

and digestibility were estimated from spot samples of urine (using creatinine as volume marker) and feces (using indigestible ADF as fecal marker). Ruminal pH and ammonia were determined at various time points from 0 to 24 hours after feeding. Data were analyzed using the Proc GLM procedures of SAS. Significance was declared at $P \leq 0.05$; least squares means are reported.

Results and Discussion

There were substantial composition differences among the silages (Table 1). Alf and LBFT were highest, NBFT and HBFT intermediate, and RC lowest in CP. The 3 BFT silages were similar and lowest in ash, NDF and ADF, while Alf was intermediate and RC highest in these components. RC silage was higher than the other 4 silages in ADICP, expressed as either a proportion of DM or total CP, suggesting that *o*-quinones may have limited intestinal digestion of RC protein. The 5 diets were not greatly different in CP and NDF (Table 2), although those containing NBFT and HBFT had slightly less CP and all 3 BFT diets were slightly lower in NDF (reflecting the composition of the BFT silages).

There were no differences in DM intake, milk composition, ruminal pH or fecal-N excretion due to silage source. However, milk yield was higher on diets containing NBFT and HBFT than on that containing LBFT, which was higher than diets with RC and Alf (Table 3). Fat yield was 0.19 kg/d higher on NBFT than on Alf, with the other 3 diets being intermediate. Yield of FCM and SNF and feed efficiency (milk/DM intake) were highest on NBFT and similar on HBFT, while these 3 traits were intermediate on LBFT, and lowest on Alf and LBFT. Feed efficiency and was lower on RC and Alf than on NBFT and HBFT. Protein yield on all 3 BFT diets, regardless of CT level, was higher than on ALF and RCS, despite the fact that the BFT diets contained about 1% less CP. Concentration of MUN were lower on LBFT and HBFT than on LBFT, RC and Alf, suggesting improved N efficiency on those 2 diets. As has been observed in a number of trials, ruminal ammonia was lower, and apparent digestibility of OM and NDF was greater, on RC than on the other legume forages. Generally, ruminal ammonia and apparent digestibility were similar on the BFT and Alf diets although ruminal ammonia was lower on LBFT than Alf. Excretion of urinary urea-N was lowest on RC and HBFT. Differences in milk yield may have been confounded by the BFT diets being lower in fiber (27% NDF) then the Alf and RC diets (29% NDF). However, these results suggest the presence of CT was directly related to improved utilization of CP in the BFT silages.

Table 3. Experimental results.

Item	Silage source[1] Alf	RC	LBFT	NBFT	HBFT	SE	$P > F$[2]
DM intake, kg/d	24.4	25.6	25.2	23.3	24.5	0.7	0.41
Milk, kg/d	30.2^{c}	31.1^{c}	32.9^{b}	34.6^{a}	34.3^{a}	0.5	< 0.01
Milk/DM intake	1.24^{c}	1.24^{c}	1.34^{bc}	1.50^{a}	1.40^{ab}	0.04	< 0.01
3.5% FCM, kg/d	31.4^{d}	32.6^{cd}	33.8^{bc}	36.3^{a}	35.3^{ab}	0.6	< 0.01
Fat, %	3.77	3.71	3.64	3.80	3.62	0.08	0.27
Fat, kg/d	1.13^{c}	1.17^{bc}	1.20^{bc}	1.32^{a}	1.24^{ab}	0.03	< 0.01
Protein, %	3.16	3.02	3.18	3.16	3.14	0.04	0.14
Protein, kg/d	0.94^{b}	0.96^{b}	1.04^{a}	1.09^{a}	1.07^{a}	0.02	< 0.01
SNF, %	8.59	8.60	8.74	8.73	8.53	0.12	0.61
SNF, kg/d	2.59^{c}	2.72^{bc}	2.91^{ab}	3.03^{a}	2.95^{a}	0.07	< 0.01
MUN, mg/dl	10.8^{a}	11.0^{a}	10.8^{a}	9.3^{b}	9.2^{b}	0.3	< 0.01
Rumen pH	6.40	6.52	6.50	6.34	6.38	0.05	0.07
Rumen NH_3-N, mg/dL	12.3^{a}	8.3^{c}	10.5^{b}	11.1^{ab}	11.3^{ab}	0.6	< 0.01
Urinary Urea-N, g/d	134^{ab}	123^{b}	131^{ab}	139^{a}	120^{b}	1.5	0.02
Fecal-N, g/d	212	228	234	216	217	9	0.64
OM digestibility, %	72.3^{c}	76.9^{a}	71.4^{c}	72.3^{c}	74.6^{b}	0.5	< 0.01
NDF digestibility, %	47.4^{bc}	59.9^{a}	43.1^{d}	45.6^{c}	49.0^{b}	0.8	< 0.01

$^{a-d}$Means in the same row with different superscripts differ ($P \leq 0.05$).
[1]Alf = Alfalfa; RC = red clover; LBFT = low CT birdsfoot trefoil; NBFT = normal CT birdsfoot trefoil; HBFT = high CT birdsfoot trefoil.
[2]Probability of a significant effect of silage source.

Conclusions

Overall, production was greatest on the diets containing NBFT and HBFT, intermediate on LBFT, and lowest on RC and Alf. The results indicated that forage legumes with CT can enhance N utilization by lactating dairy cows fed total mixed rations.

Impact of Alfalfa Hay Neutral Detergent Fiber Concentration and Digestibility on Holstein Dairy Cow Performance and Diet Digestibility

M.L. Raeth-Knight, J.G. Linn, H.G. Jung, D.R. Mertens and P.R. Peterson

Introduction

The concentration of fiber, measured as neutral detergent fiber (NDF), and the digestibility of NDF are important factors used to establish forage quality. Currently, there is increased interest in quantifying the effects of forage fiber digestibility on animal performance. This can be attributed to the inclusion of fiber digestibility in the 2001 Nutrient Requirements of Dairy cattle model, as part of the calculation for total digestible nutrients of feeds, and in the new hay quality index, Relative Forage Quality, used to determine the relative economic value of forages. Previous research to determine the impact of fiber digestibility has provided results that are difficult to interpret because forage fiber digestibility was often confounded by the level of NDF in the diet. The objective of this study was to determine the effect of alfalfa fiber digestibility, when compared within relatively high and low NDF concentration hays, on dairy cow performance.

Materials and Methods

Twenty-two commercial alfalfa hay lots were analyzed for NDF concentration and 48-h in vitro NDF digestibility (IVNDFD). Four alfalfa hay lots were selected: low NDF, high IVNDFD (LH); low NDF, low IVNDFD (LL); high NDF, high IVNDFD (HH); high NDF, low IVNDFD (HL). Sixty multiparous Holstein cows were blocked by calving data and randomly assigned to one of these four alfalfa hays included at 15% of the diet dry matter (DM) for 133 d starting immediately after calving. Diets, fed as total mixed rations, contained 14.8% alfalfa hay, 35.0% corn silage, 26.5% grain mix, 15.5% corn, 5.2% roasted soybeans, and 3.0% molasses, on a DM basis. Diets were formulated to contain 18.5% crude protein and 30% NDF. Milk yield and dry matter intake data were collected daily. Milk components (fat, protein, lactose) and SCC were analyzed weekly. Cow observations of time spent eating and ruminating were done for each cow every 15 min for 2 d and body weights were recorded every 28 d. During the digestibility sampling period cows averaged 67 ± 20 d in milk (mean ± SD). Nine fecal grab samples were collected per cow over 72 h and fecal samples were composited by cow for analysis. Apparent total tract DM and NDF digestibility were measured using the acid insoluble ash technique. Data was analyzed as a randomized complete block design using the PROC MIXED procedures of SAS with repeated measures analysis for production and body weight data.

Results and Discussion

The NDF concentration and 48-h in vitro NDF digestibility (IVNDFD) of the treatment hays used in this study are shown in Table 1. Alfalfa hay NDF and IVNDFD did not significantly impact DM intake, milk or energy corrected milk (ECM) production (Table 2). Milk true protein percentage was significantly lower for the LH treatment (2.7 %) as compared to the HL treatment (3.0 %); however, fat, protein and lactose yields were not affected by hay NDF or IVNDFD (Table 2). Feed efficiency was similar across treatments, averaging 1.9 kg of 3.5% fat-corrected milk per kg of DM intake (Table 2). There was no significant difference in body weight change across treatments with cows losing a similar amount of body weight (-27.5 kg). Chewing activity was similar across treatments. Time spent eating averaged 206, 217, 211, and 239 min per 24 h and time spent ruminating averaged 495, 441, 467, and 468 min per 24 h for the LH, LL, HH and HL treatments, respectively. Alfalfa hay NDF concentration and digestibility did not result in any difference in apparent total tract dry matter digestibility (DMD) or NDF digestibility (Table 3). However, within low NDF hays, there was a trend for decreased IVNDFD to increase DMD; and within the high NDFD hays, increased hay NDF increased DMD.

Conclusion

Fiber concentration and in vitro digestibility of alfalfa hay did not have a significant impact on lactation performance, apparent DMD or NDFD when fed to Holstein dairy cows at 15% of the diet DM. Although the reasons for the lack of any treatment effects in this study is unclear, a contributing factor may have been the small differences in the NDF concentration and IVNDFD of the hays. The high and low NDF hays differed by only 5 percentage units and within each NDF level NDF digestibility differed by ~ 3 percentage units. An additional factor may have been the low inclusion rate of the hay in the diet (15% of the diet DM).

Table 1. Neutral detergent fiber (NDF) and 48-h in vitro NDF digestibility (IVNDFD) of treatment hays.

	Treatment[1]			
	LH	LL	HH	HL
NDF, % DM	37	36	42	41
IVNDFD, % NDF	41	38	45	41

[1]LH= low NDF, high IVNDFD (LH); LL= low NDF, low IVNDFD; HH= high NDF, high IVNDFD (HH); HL= high NDF, low IVNDFD hay.

Table 2. Dry matter (DM) intake, energy corrected milk (ECM) yield and milk component percents from calving through 133 days in milk.

	Treatment[1]					
	LH	LL	HH	HL	P-Value	SE
DM Intake, kg/d	22.8	21.7	22.1	22.8	0.77	0.80
ECM, kg/d	34.2	35.4	36.3	36.3	0.48	1.20
Milk Fat, %	3.4	3.7	3.7	3.7	0.33	0.10
Milk Protein, %	2.7	2.9	2.9	3.0	0.04	0.06
Milk Lactose, %	4.8	4.8	4.8	4.8	0.24	0.05
Feed efficiency[2]	1.7	1.9	1.9	1.9	0.53	0.09

[1]LH= low NDF, high IVNDFD (LH); LL= low NDF, low IVNDFD; HH= high NDF, high IVNDFD (HH); HL= high NDF, low IVNDFD hay.
[2]Feed efficiency = kg 3.5% fat corrected milk per kg DM intake.

Table 3. Digestibility period milk yield, dry matter (DM) and neutral detergent fiber (NDF) intake and apparent and total tract DM digestibility (DMD) and NDF digestibility (NDFD).

	Treatment[1]					
	LH	LL	HH	HL	P-Value	SE
Milk, kg/d	40.4	39.5	41.2	41.0	0.88	1.68
DM intake, kg/d	20.6	19.2	20.3	20.6	0.90	1.52
NDF intake, kg/d	6.5	5.7	5.9	6.1	0.62	0.46
DMD, %	65.5	72.1	68.6	65.9	0.10	2.13
NDFD, %	49.3	56.3	52.0	51.9	0.23	2.51

[1]LH= low NDF, high IVNDFD (LH); LL= low NDF, low IVNDFD; HH= high NDF, high IVNDFD (HH); HL= high NDF, low IVNDFD hay.

Within-Laboratory Variation for In Vitro Neutral Detergent Fiber Digestibility Determinations of Forages and High-Fiber Byproduct Feeds

J. Wakker, H.G. Jung, and J.G. Linn

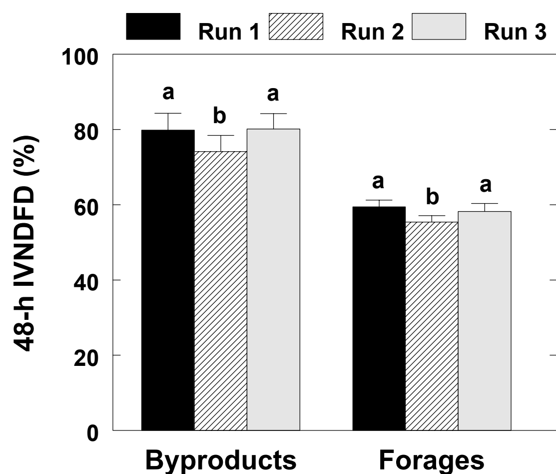
Introduction

Digestibility of fiber in dairy cow diets impacts the supply of digestible energy available for milk production. The 2001 Dairy NRC has included 48-h in vitro neutral detergent fiber digestibility (IVNDFD) as part of the energy calculation for diets. The recently developed Relative Forage Quality Index also includes 48-h IVNDFD as a variable in determining forage quality. As a result, dairy producers are increasingly requesting IVNDFD analysis as part of their forage testing program. While in vitro digestibility procedures using rumen fluid have been applied with great success in research, it is known that this measurement is influenced by numerous intrinsic factors that can be difficult to control. The current study was undertaken to evaluate within-laboratory variation of the 48-h IVNDFD procedure when used to rank forages and high-fiber byproduct feeds for fiber digestibility.

Materials and Methods

A diverse set of 12 forage and 10 high-fiber byproduct feed samples were included in the study. Each feed sample was analyzed in duplicate for neutral detergent fiber (NDF) concentration using the Ankom bag method. Duplicate subsamples of each feed were subjected to a 48-h in vitro digestion with a 20% rumen fluid:80% McDougall's buffer media at 39˚C in a Daisy oven incubator. The digestibility experiment was repeated a total of three times, at bi-weekly intervals, using rumen fluid collected 20-h post feeding from a lactating, rumen-fistulated Holstein cow fed a total mixed ration containing corn silage, alfalfa hay, and a concentrate mixture. The same donor animal was used for all rumen fluid collections. Diet, time of feeding, and time of rumen fluid collection were kept constant throughout the 6 wk study. IVNDFD was calculated for each subsample. Data were analyzed separately for forages and byproduct feeds in a completely randomized design with a factorial arrangement of run (rumen fluid collection) and feed treatments.

Figure 1. Differences among in vitro runs (rumen fluid collections) on 48-h in vitro neutral detergent fiber digestibility (IVNDFD) of high-fiber byproduct feeds and forages.



Results and Discussion

Table 1 lists the feeds used in this study and their NDF concentrations. For both byproduct and forage feeds, the main effects of run and feed were significant. Run 2 had lowered IVNDFD results across both feed types (Fig. 1). The byproduct feeds basically fell into two groups. Cottonseed and wheat mids had relatively low IVNDFD (< 55%) whereas all other byproducts had very high IVNDFD (> 80%). The forages ranged in IVNDFD from ryegrass at 74.0% to corn stover at 45.5%, with substantial overlap among forages. There was no run x feed interaction for the byproduct feeds, indicating that these feeds ranked the same in all runs even though Run 2 had lower IVNDFD results. In contrast, the interaction was significant for forages. As a result, ranking of forage samples for IVNDFD differed among in vitro runs. This effect is illustrated in Figures 2 and 3 for the alfalfa and corn silage samples, respectively. Similar shifts in IVNDFD ranking occurred among the four grass hays (ryegrass, mixed grass, oat, and barley).

Conclusions

These data indicate that significant variation exists among in vitro runs for IVNDFD even though donor animal management and the in vitro protocol were kept constant. This suggests that diet NRC energy calculations will differ for the same feed depending on the particular in vitro run from which the IVNDFD data were derived. The interaction of run x feed for forage samples additionally indicates that individual forage samples are digested differently among in vitro runs making it impossible rank forages for IVNDFD if only a single in vitro run is used. While we expect that combining rumen fluid from multiple donor animals would probably reduce run effects, this option increases costs due to surgical preparation and maintenance of multiple animals. A solution to the variation inherent in the IVNDFD procedure must be found if this feed quality measure is going to benefit practical dairy diet formulation.

Figure 2. Statistical differences in 48-h in vitro neutral detergent fiber digestibility (IVNDFD) among three alfalfa samples within individual runs (rumen fluid collections). NS, non-significant (P > 0.05).

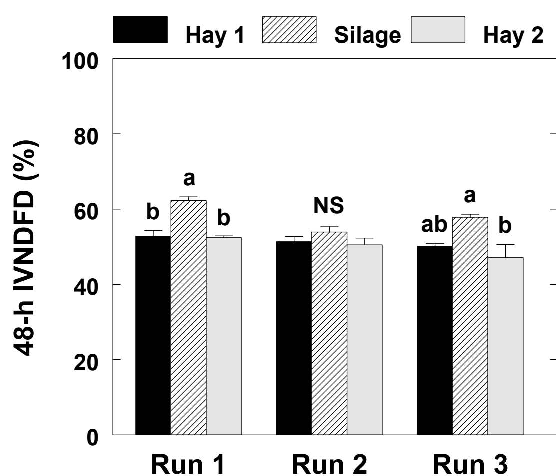


Figure 3. Statistical differences in 48-h in vitro neutral detergent fiber digestibility (IVNDFD) between two corn silage samples within individual runs (rumen fluid collections). NS, non-significant (P > 0.05).

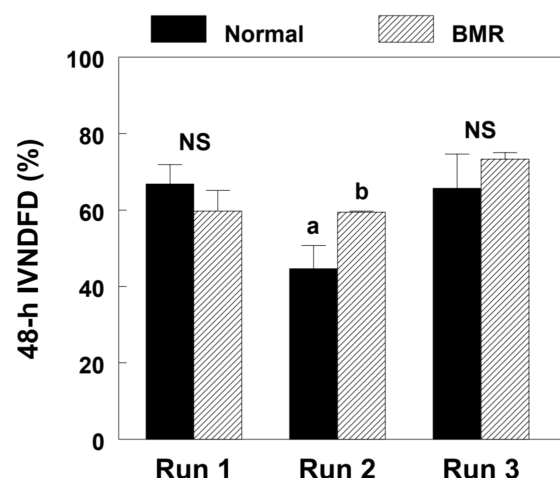


Table 1. Neutral detergent fiber (NDF) concentration and 48-h in vitro NDF digestibility (IVNDFD) of high fiber byproduct feeds and forages.

Byproduct	NDF	IVNDFD	Forage	NDF	IVNDFD
	% DM	%		% DM	%
Wet beet pulp	42.8	90.7[a]	Alfalfa hay-1	52.9	51.4[ef]
Dry beet pulp	41.6	88.2[ab]	Alfalfa hay-2	55.8	50.0[fg]
Citrus pulp	18.1	87.1[ab]	Alfalfa silage	42.1	58.0[d]
Soyhulls-1	62.8	88.2[ab]	Birdsfoot trefoil	50.7	53.9[def]
Soyhulls-2	57.6	85.2[ab]	Red clover	47.3	66.0[b]
Cottonseed	55.8	32.2[d]	Ryegrass	43.8	74.0[a]
Wet DDGS[1]	37.0	84.2[b]	Mixed Grass	65.6	56.1[de]
Dry DDGS	37.9	85.7[ab]	Barley hay	56.6	50.9[e]
Corn gluten	29.2	85.1[ab]	Oat hay	64.5	63.4[bc]
Wheat mids	37.1	53.8[c]	Normal corn silage	41.5	59.0[cd]
			BMR corn silage	39.3	64.1[bc]
			Corn stover	77.2	45.5[g]
SEM		2.1			1.8

[1] DDGS, distillers dried grains and solubles.

Validation of Feed and Manure Data Collected on Wisconsin Dairy Farms

J.M. Powell, D.B. Jackson-Smith, D.F. McCrory, H. Saam and M. Mariola

Introduction

Our ability to develop and implement sound feed and manure management strategies for dairy farms depends not only on biophysical factors (e.g., herd feed requirements, soils, weather, cropping systems), but also on socio-economic conditions that influence farmers' nutrient management behavior. Various approaches have been used to collect data on nutrient management practices on dairy farms. The "On-Farmers' Ground" (OFG) research project was established in 2002 with 54 representative Wisconsin dairy farms to evaluate impacts of regional climate and soil differences, farm size and operational features on overall nutrient use including when, where and how much manure was land-spread. The objective of the present analysis was to evaluate the accuracy of key OFG data. To accomplish this task, two basic questions were posed: (1) how well did data collected on-farm regarding cow diets, feed analyses and milk production reflect established feed-milk-manure relationships; and (2) how well did farmer-recorded data on manure spreading reflect literature values of manure phosphorus (P) excretions and losses, and other literature estimates of manure collection and spreading.

Materials and Methods

Stratified random sampling procedures were used to provide a study population of 54 dairy farms that represented the range of farm sizes, livestock densities and manure recycling capacities typical of the Wisconsin dairy industry. The hilly southwest (SW) region of Wisconsin is characterized by well-drained silt loam soils; the relatively flat northeast (NE) region has less permeable clay loam and loam soils; and the undulating south-central (SC) region has landscapes and soils somewhat intermediate to those of the SW and NE. Four to five visits per farm and data collection were done during the period September 2002 to March 2005. Survey instruments were designed to compile an overall picture of each farming operation, including herd size, cropping patterns, livestock facilities, management practices, and motivations and goals related to feed, fertilizer, and manure management. Aerial photographs validated and recored farm and field boundaries, which were coded and digitized for use throughout the study.

Table 1. State-wide and regional values, and impact of herd size, feed management and milking frequency on dietary crude protein (CP) and phosphorus (P) concentrations, dry matter intake (DMI), milk production, and feed N (FNUE) and feed P (FPUE) use efficiencies on the study dairy farms in Wisconsin.

Parameter	Variables	CP	P	DM offer	Milk Prod.	FNUE	FPUE
		g kg DM^{-1}		kg cow d^{-1}		%	%
State-wide values	Mean	172	4.1	22.7	29.6	25.4	29.0
	5th percentile	168	4.0	21.5	27.6	23.9	26.6
	95th percentile	175	4.3	23.9	31.6	27.0	31.3
Regional values	NE	172	4.2	23.8	31.8	27.1	29.5
	SC	173	4.0	22.3	29.6	25.1	29.0
	SW	172	4.3	22.1	27.3	23.8	28.4
Herd class (lactating cows $farm^{-1}$)	1-29	169	3.7b[1]	21.3	20.0c	18.2c	23.5b
	30-49	168	4.2ab	23.6	27.4b	24.2b	26.6ab
	50-99	173	4.2ab	21.8	29.7b	26.6b	32.1ab
	100-199	175	4.0ab	25.3	33.1ab	24.3b	24.4b
	200+	176	4.5a	23.0	38.7a	32.6a	34.6a
Use of TMR	Yes	172	4.0	23.1	33.5a	27.0a	28.9
	No	172	4.2	22.5	26.1b	24.1b	29.0
Balance rations ≥4x y^{-1}	Yes	171	4.1	22.6	30.6a	26.5a	30.0a
	No	175	4.3	23.2	24.7b	21.0b	24.8b
Milk thrice daily	Yes	176	4.5	23.0	40.2a	32.6a	34.6
	No	171	4.1	22.7	28.8b	24.9b	28.7
Use Posilac®	Yes	174	4.2	24.4	37.1a	29.0a	28.7
	No	171	4.1	22.4	27.7b	24.6b	29.1

[1]within a variable category, means followed by different letters differ significantly (P<0.08).

Results and Discussion

Dairy Diets and Feed Use Efficiencies. Approximately one-third of the study farms fed TMR and the remaining two-thirds did not feed TMR, but provided feeds individually. There were no significant (P<0.05) differences in diet dry matter (DM) offered, or in dietary crude protein (CP) or P concentrations

between farms feeding TMR and non-TMR. Statewide and regional statistics for feed DM, CP and P concentrations, milk production and feed N (FNUE) and feed P (FPUE) use efficiencies are presented in Table 1. No significant (P<0.05) regional differences in diet CP or P concentrations, milk production, FNUE or FPUE were observed. There were, however, farm size differences in these production parameters. Dairy farms with the largest herds (200+ lactating cows) fed diets having greater P concentrations than farms having the smallest herds (1-29 cows). Milk production was significantly higher on farms having 200 or more lactating cows than on farms having 30-99 cows. Milk production on farms having the smallest herds (1-29 cows) was significantly lower than on farms having 30 or more dairy cows. Feed N use efficiencies were highest (P<0.05) on the largest farms.

Balancing rations has the potential to reduce dietary nitrogen (N) intake, manure N excretion and therefore FNUE. On dairy farms in the present study, the use of TMR (n= 15 farms), balancing rations at least four times per year (n=37), milking thrice daily (n=3) and the use of Posilac® (n=9) all resulted in significant (P<0.05) increases in milk production. Gains in FNUE were obtained on farms that used TMR, balanced rations, and milked thrice daily. Feed P use efficiencies were similar across regions, herd sizes and whether TMR or Posilac® were used or not, or whether cows were milked twice or thrice daily. Feed P use efficiencies were significantly greater on farms (n=37) that balanced rations at least four times per year. In general, it was more common for dairy farmers in the NE to have adopted a range of productivity enhancing dairy management practices when compared to the other regions (data not shown). For example, a higher percentage of farms in the NE balanced dairy rations at least four times per year, kept records on individual cows, and fed TMR. Possibly as a result of this enhanced technology adoption, milk production per cow was also found to be highest in the NE compared to the other regions of Wisconsin.

Manure Characteristics. The chemical characteristics of semi-solid and liquid manure spread on the study farms during the period 2003-2004 are given in Table 2. There were few significant monthly differences in the chemical characteristics of semi-solid manure. Concentrations of organic matter (OM) were higher during the period January-May than other months; concentrations of N in semi-solid manure were highest during the period March-May; and concentrations of P were higher in May than January. Chemical characteristics of liquid manure were similar during the various stages of manure removal from pit storage. This indicated that farmers were able to successfully mix manure pit depths prior to manure removal and land-application. On a wet weight basis, semi-solid manure contained higher concentrations of OM, N and P than liquid manure indicating that nutrients in semi-solid would be less expensive to spread (i.e., less water) than liquid dairy manure.

Table 2. Manure characteristics on the study dairy farms in Wisconsin.

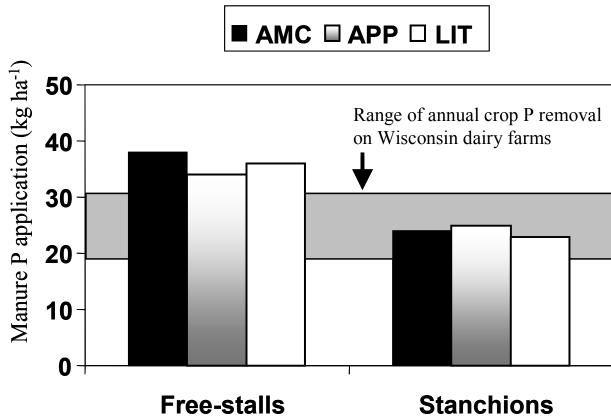
Manure type	Sampling period	Samples	Chemical characteristics			
			OM	N	P	N:P
			g kg wet^{-1}			
Semi-solid	January	44	777ab[1]	4.62c	0.87b	6.09
	March	60	797a	5.26ab	0.89ab	6.55
	May	69	740abc	5.46a	1.04a	6.02
	July	59	713c	4.83bc	0.99ab	5.98
	Sept.	66	719bc	4.97bc	1.01ab	5.59
	Nov.	42	722bc	4.85bc	0.92ab	6.19
	Mean	340	743 A[2]	5.03 A	0.96 A	6.05
Liquid	Pit full	60	646	3.76	0.64	6.36
	½ empty	16	576	3.70	0.66	5.96
	⅓ empty	36	627	3.65	0.64	6.20
	⅔ empty	36	614	3.88	0.68	5.92
	Almost empty	50	574	3.80	0.73	5.43
	Mean	198	611 B	3.77 B	0.67 B	5.98

[1]Within a manure type, chemical characteristic means followed by different lower case lett significantly (P<0.0001) different.
[2]Between manure types, chemical characteristic means followed different upper case letter significantly (P<0.0001) different.

Manure Production, Collection and Land-spreading. Manure P is much less susceptible than manure N to losses during manure handling and storage. For this reason, differences in manure P should provide comparisons

of apparent manure collection (AMC), as determined on the study farms in a previous analysis, and amounts of manure P applied, as recorded by farmers in manure application records (APP). For both stanchion and free-stall farms, manure P application rates to tilled cropland would be statistically similar ($P<0.05$) using either AMC or APP (Figure 1). These applications would also be similar if an overall average manure collection efficiency of 80% in the literature (LIT) were assumed. Average manure P application rates (39 kg ha^{-1}) to tilled cropland on free-stall farms would be significantly greater than application rates (25 kg ha^{-1}) on stanchion farms, and also perhaps in excess of the 20-30 kg ha^{-1} range of P removal rates for most crops grown on Wisconsin dairy farms. Per livestock unit, there are no differences in the amount of cropland available on free-stall and stanchion dairy farms. A principal likely reason for differences in manure P application rates is that significantly less manure is collected and therefore available for land-spreading on stanchion than on free-stall farms, that bedding and manure handling systems make it more difficult to get a representative sample on stanchion farms, etc.

Figure 1. Comparison of average manure P applications to tillable cropland: Apparent manure collection (AMC) method, farmer-kept application records (APP) and standard literature values (LIT) for free-stall and stanchion dairy farms in Wisconsin.



Conclusions

The data collected on representative dairy farms during this study provided a snap-shot of industry practices in Wisconsin, as well as information on the range of feed and manure management practices on individual farms. Improvements to data collection methods require increased skills and training of both farmers and those responsible for assisting farmers in data collection and analyses.

Manure Nutrient Management

Alfalfa and Grass Response to Summer Manure Application

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Introduction

Manure is usually applied to fields cropped to corn or other annual crops, but farmers often have more manure than should be applied to those crops. In addition, it is difficult to apply manure to annual crops during the growing season. Perennial forages, like alfalfa or perennial grasses, which are harvested several times during the growing season, could provide an alternative land base and time management strategy for manure applications.

Many farmers are reluctant to apply manure to established alfalfa, even though some research has shown no negative economic impacts. Reasons for avoiding manure applications to alfalfa include increased weed incidence, stand damage by equipment tires, lack of sufficient manure, and lower palatability of the forage. Most of these concerns can be alleviated by manure application before seeding alfalfa, but manure application during the growing season offers farmers a window of opportunity to utilize manure. Manure applications to established alfalfa usually are made as soon as possible after forage harvest to reduce damage to the crop or in winter when the plant are dormant.

Our objectives were to evaluate the response of reed canarygrass, standard N_2-fixing alfalfa, and a non-N_2-fixing alfalfa to increasing rates of swine manure slurry applied in mid-summer, and to compare commercially available alfalfa cultivars for tolerance to applications of swine manure during the growing season.

Methods

Manure Rate Experiment. Four entries, 'Venture' reed canarygrass, 'Agate' alfalfa, released in 1973, Ineffective Agate, an experimental germplasm selected from Agate that is incapable of N_2 fixation, and UMN 3097, an alfalfa cultivar blend created by mixing six recently released commercial cultivars that are adapted to the upper Midwest, were seeded at two Minnesota locations. In the first and second production years, liquid swine manure was applied at five target rates (0, 23.4, 32.7, 42.1, and 93.6 kL ha^{-1}) 3 to 4 d after the second forage harvest at Rosemount and Waseca. The manure application equipment was not driven over the control (0 kL ha^{-1}) plots.

Cultivar Experiment. In this experiment, the six alfalfa cultivars, Magnagraze, 5312, Rushmore, Wintergreen, Winterstar, and WL 325 HQ, that were blended to create UMN 3097 used in the rate experiment, were each planted in adjacent plots to those described in the rate experiments at both locations. Liquid swine manure was applied at three target rates (0, 37.4, and 93.6 kL ha^{-1}) 2 to 4 d after the second harvest as described above.

In both experiments, manure slurries were sampled at each application to determine total N, P, K, and solids concentration, and soil test levels of P and K were adequate for high yields of perennial forages. The effect of manure on forage yield and persistence was assessed in non-wheel-tracked areas in September of each year with a stand score based on ground cover.

Results and Discussion

Manure rate experiment. Third harvest forage yield, fall stand score, and total forage yield demonstrated that N_2-fixing and non-N_2-fixing alfalfa and reed canarygrass responded differently to the increasing rates of manure applied in each year and location. Differences in the manure characteristics and response to the

application procedures for the manure treatments in each year and location contributed to the complex yield and fall stand score responses we observed. Wheel traffic damage from the manure application equipment was apparent in all manure-treated plots. Based on our observations, driving over the plots a second time when the plants and soil surface were wet from the first pass of the application caused greater plant damage than the other manure rate treatments. Compensatory growth occurred in all plots with wheel traffic damage, limiting the yield decline caused by wheel traffic damage.

Yield and stand longevity of the two N_2-fixing alfalfas were unaffected by the addition of manure in midsummer at Rosemount. In contrast, yield and in some cases fall stand score declined at Waseca, even though rate of manure application at the two locations was essentially the same. Yields of non-N_2-fixing alfalfa increased with manure rate, but these entries were chlorotic (yellow-green) and stunted and eventually died at Rosemount, where the manure contained less N. A single annual application of manure at typical N application rates even during the growing season did not meet the N supply requirement for this non-N_2-fixing alfalfa to keep it productive. Reed canarygrass was intermediate in yield between Ineffective Agate and the two N_2-fixing alfalfas in nonmanured plots, but out-yielded alfalfa for all other manure rate treatments, suggesting that it was less damaged by traffic or manure solids. Reed canarygrass fall stand scores were comparable to the two N_2-fixing alfalfas at both locations in both years and did not change as manure rates increased. Total forage yield for reed canarygrass at both locations increased as manure rate increased.

Cultivar experiment. No differences in third harvest or total yield were found among the cultivars for any of the manure application treatments in any environment. Total forage yield was about 1 Mg ha^{-1} greater in the manure-treated plots than the control at Rosemount, but no differences in total forage yield were found among the manure treatments at Waseca. Forage yield may improve or at least not be compromised by applying manure slurry in mid-summer within a few days after cutting to commercially available alfalfa cultivars.

Conclusion

By combining data from both experiments, we found that rates of swine manure slurry in excess of about 3300 kg ha^{-1} resulted in loss of late summer yields. In our experiments, the effect of extra wheel traffic at the high manure rate occurred at both sites, but yield declines associated with the highest manure rate occurred only at Waseca. We conclude that extra wheel traffic was not a significant independent factor related to short-term yield decline. Regardless of the mechanisms involved, this research highlights the need to restrict rates of broadcast manure slurry on the basis of solids content, as well as nutrient content.

A Novel Approach to Regulating Organic N Mineralization

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Introduction

Soil acts as a sink for proteins from plants, animals, and microorganisms and is characterized by rapid protein hydrolysis (proteolysis) under a wide range of environmental conditions. Proteolysis is the necessary first step in nitrogen (N) mineralization and the enzymes that break down proteins are called proteases. The significance of protease production is evidenced by the importance of proteolytic microorganisms, which may comprise between 20 and 89% of total soil microbial biomass.

Proteolysis regulates inorganic N availability to soil organisms and plants and to processes that lead to N loss. Organic N sources (manure, crop residues, and soil organic matter) are the largest reservoirs of N on dairy farms. Scientists are still trying to develop methods to achieve the balance in N mineralization between providing enough N for optimum plant growth, while limiting N losses. One of the key problems is that N mineralization from these sources occurs too long before crop growth or after crops are mature, resulting in nitrate leaching, for example.

In earlier research, we found that application of purified protease inhibitors reduced proteolysis from plant residues and soil. Plants, however, produce protease inhibitors as a means of protection against disease organisms and insects. Here, we tested the hypothesis that protease inhibitors produced in a crop could reduce the initial rate of N mineralization from crop residues.

Methods

Plant materials. We used three crop plant species, tobacco (*Nicotiana tabaccum* L.), Japonicum rice (*Oryza sativa* L.), and *Brassica napus* (L.), which is the name for a wide variety of plant forms from turnip to kale to rapeseed. In each case, we had transgenic and isogenic, nontransformed types. Each was grown in soil in the greenhouse with 16-hr photoperiod, supplemental light, additional fertilizer nutrients, and a stable tracer for N, ^{15}N. Three days before harvest, at least 40% of the leaf area of each plant was wounded using needle-nosed forceps to increase protease activity. We measured protease inhibitor activity in the plant tissue at harvest by radial diffusion assay and other tissue characteristics were determined with standard procedures.

Incubation procedure. Two lab incubations were conducted with topsoil of a Hubbard loamy sand. We measured net N mineralization in leaching tubes in which plant residues were added to soil and either mixed or left on the surface. These were incubated at 25 C for 100 days and leached periodically to extract nitrate-N for analysis by conductimetric methods. A second static incubation was conducted in which we used ^{15}N-labeled plant residues, to distinguish between N derived from the soil and N derived from the plant residues. These were incubated at 25 C for 8 weeks, with moisture content adjusted about half-weekly. Mineralized N was extracted with 2 *M* KCl, analyzed by conductimetric methods, concentrated, and analyzed for ^{15}N concentration by mass spectrometry.

Results and Discussion

Plant residues. Within each crop species, transgenic and nontransgenic tissues did not differ in N concentration, lignin concentration, or C/N ratio. C/N ratio and Klasson lignin concentration increased from Brassica to rice (C/N 17.3 to 22.2 and lignin 5.4 to 11.6 mg g^{-1}, respectively). Protease inhibitor activity was 50 to 280% higher in transgenic plants than the isogenic nontransformed controls.

N mineralization. For rice and tobacco, N release during the first 30 days from surface-applied residues was less for transgenic than control tissues. Thereafter, N release was equivalent or higher from transgenic residues. Release of N from Brassica tissue followed similar patterns whether residues were left on the soil surface or incorporated in the leaching tube experiment, but there were no differences between plant treatments when rice or tobacco residues were mixed with soil. Cumulative N mineralization over 100 days was either equal or higher for control than transgenic residues. In the static incubations, about 25% less N was mineralized during the first 30 to 45 days from transgenic plant residues than from controls. No differences in plant N mineralization were apparent thereafter. In contrast, soil N mineralization was not affected by plant protease inhibitor concentration.

We used regression models of plant tissue characteristics to fit the measured results of percentage of N mineralized from the tissues. The equations with best fits for our data always contained protease inhibitor activity in addition to the typical measures of tissue N, C/N ratio, and/or lignin.

Conclusions

This is the first time, to our knowledge, that a comparison of N mineralization has been made for isogenic lines of plant species that express different protease inhibitor activity, but which do not differ in N or lignin concentration or C/N ratio. These three characteristics are those most often used to predict N mineralization of organic materials.

Increasing endogenous protease inhibitor activity reduced N mineralization from the plant tissue, but did not affect soil N mineralization. This suggests that selection and management of winter cover crops or green manures to increase protease inhibitor activity may improve control of N mineralization from these residues. Furthermore, including a measurement of protease inhibitor activity may help predict N mineralization when green plant residues are added to soil.

The plants we used were engineered to enhance serine-type protease inhibitors as a means of plant protection. A variety of proteases are present in soil. Our earlier research showed that adding leupeptin (which inhibits both serine- and cysteine-type proteases) or a mixture of protease inhibitors was more effective than serine-types alone. Longer-term effects on controlling N mineralization may be achieved by transgenic approaches to engineer enhanced expression of other classes of protease inhibitors.

Influence of Bedding Material on Ammonia Emissions from Dairy Barns

T. H. Misselbrook and J. M. Powell

Introduction

Ammonia emissions from dairy barns may be influenced by interactions between deposited urine and the materials used for bedding. Different bedding materials may influence emissions in a number of ways. Firstly, the physical structure of the material may be important, influencing the extent to which urine drains through the bedding. Secondly, bedding may separate urine from feces. Feces are one of the main sources of the enzyme urease that hydrolyses the urea contained in urine to ammonia. Thirdly, different bedding materials may have different capacities to absorb deposited urine. Absorption may reduce emissions, by increasing the resistance to gaseous transport. Lastly, the chemical composition of bedding may influence NH_3 emissions in many ways. The pH and cation exchange capacity (CEC) of the bedding materials may influence the extent to which ammonium ions are held by the bedding, and the addition of a carbon (C) source in the bedding material may promote rapid mineralization of organic to ammoniacal nitrogen. The objectives of this study were to assess 1) the capacity of commonly-used beddings to absorb urine; 2) the extent to which urine-soaked beddings retained NH_3; and 3) the importance of the physical structure of the bedding in reducing NH_3 emissions.

Materials and Methods

Six bedding materials of different physical and chemical properties (Table 1) were used in the experiment: chopped wheat straw, sand, pine shavings, chopped newspaper, chopped corn stalks and recycled dairy manure solids. Urine and feces were collected separately from lactating Holstein cows fed a diet consisting of approximately 17% crude protein and 24% neutral detergent fiber using standard components for a lactating dairy cow diet. Bedding materials were oven-dried at 60 ^{o}C overnight, allowed to cool to room temperature and then approximately 0.5 liters of each placed in a 1 liter coffee-press canister. After being submerged in urine for 4 h, the canisters were placed at a 60° angle to allow urine to drain from the bedding material. Beddings were reweighed to determine urine absorption.

Table 1. Chemical characteristics of the bedding materials used in the study.

Bedding material	pH	Total C ($g\ kg^{-1}$)	Total N ($g\ kg^{-1}$)	C:N ratio	CEC ($cmol_c\ kg^{-1}$)
Chopped wheat straw	6.9	422	4	105	9.7
Sand	6.0	1	0	-	0.3
Chopped newspaper	6.1	429	7	63	8.8
Chopped corn fodder	6.7	430	9	49	9.9
Recycled manure solids	6.0	395	33	12	16.0

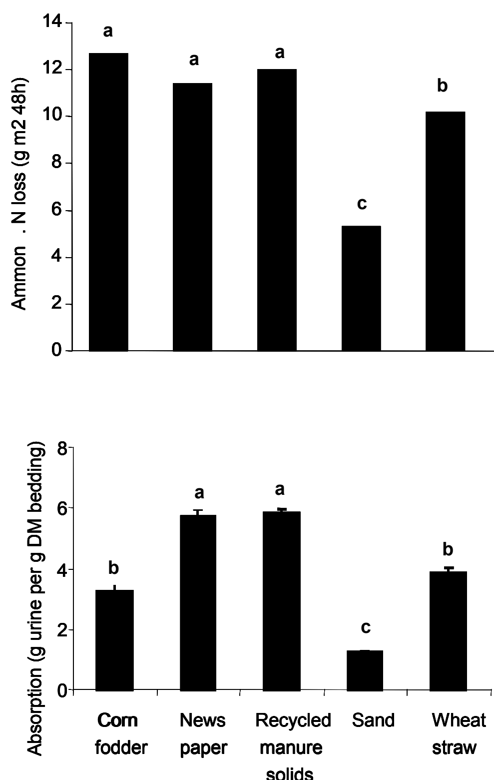
Two types of NH_3 emission experiments were conducted using a system of six small laboratory chambers. In the first, emission measurements were made from urine soaked beddings taken directly from the bedding absorbance measurements, to determine the capacity of the different bedding materials to retain the adsorbed NH_3. In the second, measurements were made from urine additions to dry bedding materials in order to simulate what occurs in practice on a dairy barn floor.

Results and Discussion

Recycled manure solids and chopped newspaper were the most absorbent of the bedding materials, retaining 6

times more urine than sand, the least absorbent, on a weight for weight basis (Figure 1). Corn fodder and wheat straw were the next most absorbent beddings. After equal volumes of dry bedding were added to each ammonia emission chamber and urine applied, ammonia emissions, both in absolute terms and expressed as a proportion of the urine N applied, were least from sand, followed by wheat straw and chopped newspaper, recycled manure solids and chopped corn fodder (Figure 1).

Figure 1. Urine absorbance capacity of different bedding materials, and cumulative ammonia emissions over 48 h from urine applied to dry bedding materials [bars with different letters have significantly (*P*<0.05) different urine absorbance or ammonia emissions].



The chemical properties of the bedding materials (Table 1) were less important than their physical structure in determining NH_3 emissions following urine addition. When urine was applied to dry beddings in the chambers, we expected the more absorbent materials to have lower emissions, but the opposite effect was observed. Results from this study suggest that the physical structure and the relative absorbance capacities of the different bedding types were the most important factors influencing NH_3 emission in these laboratory chamber studies. With sand, the urine could percolate readily through the material to pool in the bottom layer. Ammonia emission would then be reduced due to the increased resistance to transport as NH_3 emitted within the lower sand layer had to diffuse through the upper layers before release into the free air stream. For the recycled manure solids, with the much greater absorbance capacity, it is suggested that the majority of the urine was retained in the upper layers with a lower resistance to transport and therefore greater emission.

Conclusions

Bedding materials differed in their capacity to absorb urine, with sand being the least and chopped newspaper and recycled manure solids the most absorbent on a weight for weight basis. When urine was applied to dry beddings, emissions over 48 h were significantly lowest for sand, followed by wheat straw. Larger, longer scale studies are required where interactions between the animal and bedding are included as well as housing design. Choice of bedding material need to include a number of factors other than ammonia emissions, including cow comfort, hygiene and manure management considerations.

Dairy Diet Impacts on Fecal Chemical Properties and Nitrogen Cycling in Soils

J.M. Powell, M.A. Wattiaux, G.A. Broderick, V.R. Moreira, M.D. Casler

Introduction

Dairy cows and replacement heifers in the Midwest and Northeast regions of the U.S are fed primarily home-grown feed from crop rotations comprising of alfalfa, corn and soybean, and protein and mineral supplements that are purchased to balance dairy rations. Many farms are increasing herd size and replacing alfalfa with corn silage in crop rotations. Under current feeding practices for lactating cows fed in confinement, nitrogen (N) not secreted in milk is excreted about equally in feces and urine, but this can be affected dramatically by diet. Relatively little is known about relationships between the type and amount of forage and crude protein (CP) fed to dairy cows, manure N composition and cycling in soils. The objective of this study was to determine effects of typical dairy diets fed in the Midwest US on the chemical composition of dairy cow feces and, after application to soil, effects of feces derived from different diets on soil inorganic (IN) levels, plant yield, and N uptake.

Methods

Fresh fecal samples were taken directly from the rectum of dairy cows during three lactation trials that incorporated a wide range of forage and dietary CP levels (Table 1). One feeding trial used 63 cows to investigate interactive effects of dietary CP and neutral detergent fiber (F) on milk production and composition. A second trial used 24 cows to compare cow responses to corn silage (CS), alfalfa hay (AH) or alfalfa silage (AS). The third trial used 48 cows to evaluate cow responses to CS or AS as the primary forage source, each fed at low CP (LP) level of 16.7% or a high CP (HP) level of 17.8%. Feces were analyzed for total C and total N, fecal cell wall components (NDF), and total N contained in NDF (NDIN). Ground feces from the 14 diets were applied to a silt loam and a sandy loam soil in incubation cups at a rate equivalent to 350 kg total N ha^{-1}. Soil moisture content was maintained at 60% water filled pore space (WFPS) throughout the trial. Single cores were taken from incubation cups at 7, 14, 28, 56, 112, 224 and 365 d after application of feces. The same soils and 12 of the 14 fecal types used in the incubations were used in a greenhouse trial. Feces were added at a rate equivalent to 350 kg total N ha^{-1} and mixed thoroughly with 800 g of air-dried soil in 4 replicates. Pots were watered every 2 to 3 d to maintain 60% WFPS and pots were relocated randomly twice weekly. After an initial 5 d fallow period, oats were planted, and after a 45 d growth period, shoots and roots were harvested.

Table 1.

Feeding trial designation	Feed components	TC	TN	NDF	NDIN	C:N
		g kg DM^{-1}				g g DM^{-1}
CP-F[1]	LCP-HF	451	27.0	571	6.5	17.8
	HCP-HF	447	29.4	564	7.3	14.7
	LCP-MF	462	27.6	538	6.6	16.0
	HCP-MF	453	29.4	599	7.8	16.5
	LCP-LF	457	28.6	526	6.6	11.7
	HCP-LF	460	30.5	512	5.6	14.5
CS-AS-AH[2]	100CS	452	29.0	545	8.0	15.8
	75CS-25AH	461	30.3	528	7.8	16.1
	75CS-25AS	457	29.7	505	8.4	14.5
	50CS-50AS	474	32.2	501	7.6	15.0
CS-AS-CP[3]	CS-LP	448	28.4	570	8.3	15.5
	CS-HP	454	28.2	537	6.4	14.8
	AS-LP	444	24.4	545	4.8	18.3
	AS-HP	439	24.4	561	5.3	18.1

[1]Crude protein (CP) and Fiber (F) diets fed at low (L), medium (M), high (H) levels
[2]Corn silage (CS), alfalfa silage (AS) or alfalfa hay (AH) comprised 100, 75, 50 or 25% of dietary forage component
[3]CS or AS fed at low crude protein (LP) or high crude protein (HP).

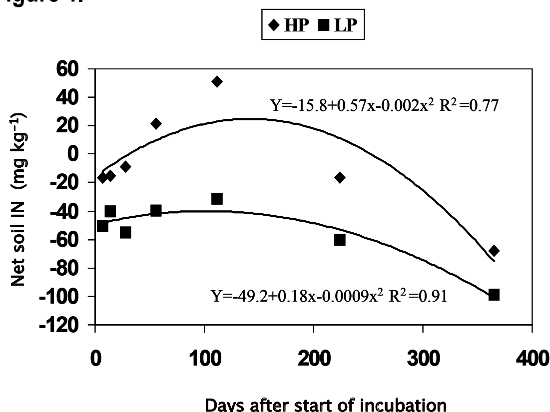
Results

Feces from different diets had somewhat narrow ranges of total C (439-474 g kg^{-1}), total N (24.4-32.3 g kg^{-1}), C:N ratios (11.7-18.3), NDF (501-599 g kg^{-1}) and undigested feed N (NDIN, 4.8-8.4

g kg^{-1}; Table 1). Highest concentrations of fecal total C, total N, and NDIN tended to come from cows fed CS-AS-AH diets, mid-range concentrations were associated with cows fed CP-F diets, and lowest concentrations came from cows fed CS-AS-CP diets.

Differences between soil IN extracted from the feces-amended and un-amended soils were deemed due to effects of diet on chemical composition of feces and subsequent fecal N mineralization in soil. Positive soil IN values indicated net mineralization of applied fecal N, and negative values indicated that applied feces immobilized soil N.

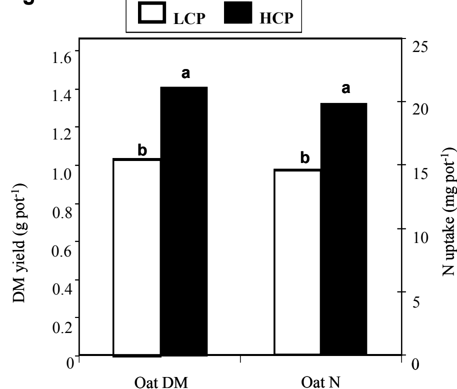
Figure 1.



After application to the silt loam, feces derived from HP diets (averaged across CS and AS) produced higher net soil IN than feces derived from LP (Figure 1). At trial's end, fecal type had significant effects on net soil IN levels. In the silt loam, feces derived from AS-HP had higher ($P<0.05$) net soil IN levels than feces from other diets. In the sandy loam, feces from AS diets averaged across CP levels produced greater ($P<0.05$) net soil IN than feces from CS diets averaged across CP levels.

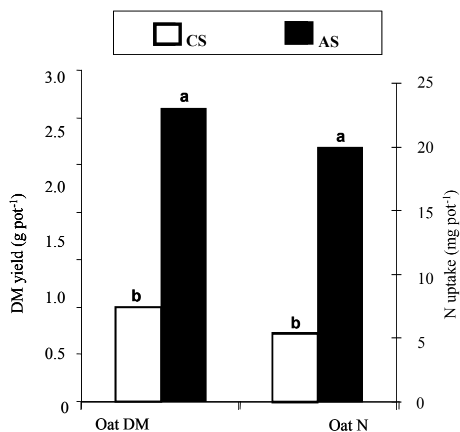
Similar to treatment effects in the incubation trial, plant DM production, and especially N uptake, were affected by fecal type. For example, application of feces from high dietary CP (averaged across soil types and fiber levels) significantly increased oat DM and N uptake compared to the application of feces from low levels of dietary CP (Figure 2). Also, oat DM and N uptake were significantly greater in pots amended with feces from AS than in pots amended with feces from CS (averaged across soil types and CP levels in the CS-AS-CP trial; Figure 3).

Figure 2.



An improved understanding of the impact of dairy diet on manure N composition and transformation in soil may assist in various short- and long-term management decisions. Over the short term, better predictions of manure N mineralization and plant availability may be made. The long-term impacts, such as soil N accumulation and associated hazards of NO_3^- leaching and denitrification, sequestration of C in soil, and impacts (e.g., fertilizer use and soil erosion) of the cropping systems designed to provide diet components (e.g., alfalfa vs. corn grain or silage) may be most important. Repeated applications of dairy manure derived from different diets could be more pronounced than this study's short-term impacts observed during 365 d incubation and 45 d greenhouse trial that followed single manure applications.

Figure 3.



Conclusions

This study determined that dietary forage and CP levels impact fecal chemical composition, fecal N mineralization, plant yield and N uptake.

Value Added Product Processing

Effect of Inoculants on the Ensiling of Corn Stover

R.E. Muck and K.J. Shinners

Introduction

Corn stover is a potentially attractive source of biomass for conversion to ethanol or lactic acid. A key issue in developing such an industry is preservation of the stover between harvest and processing at a bioconversion facility. One alternative is ensiling whereby lactic acid bacteria ferment some of the stover sugars to lactic acid and other products. The objectives of the study reported here were to investigate the ensilability of various corn stover sources ensiled at different DM contents with and without inoculant treatment.

Methods

Corn stover was harvested with single-pass stover and grain harvesters from various fields at the University of Wisconsin Arlington Agricultural Research Station on four dates in fall 2005. A different corn hybrid was harvested on each date: Pioneer Hi-Bred 34M93 on October 11, Agri-Gold A6333 Bt on October 20, Mycogen F697 on November 3 and Pioneer 35Y67 on November 4. The harvester for the Pioneer 34M93 produced two stover fractions, stalk and ear, whereas the harvester used on the other three dates produced a single whole-stover fraction.

Stover was ensiled as harvested and after rehydration to 30 and 40% dry matter (DM) for the latter three and two harvest dates, respectively. The stover was ensiled in vacuum-sealed bags (28 x 35 cm). For each DM content, six bags were uninoculated, six bags were treated with a homofermentative lactic acid bacterial corn silage inoculant (Biomax 5, Chr. Hansen Biosystems), six with *Lactobacillus buchneri* (11A44, Pioneer Hi-Bred), and six with a combination product (*L. buchneri*, *L. plantarum*, *Enterococcus faecium*; 11C33, Pioneer Hi-Bred). The ensiled stover was stored at room temperature (~22°C) with half the bags being analyzed at 21 d and the remainder at 90 d for fermentation products and quality.

Results and Discussion

The as-harvested stover ranged between 45 and 58% DM and ensiled well without inoculants. Fermentation was essentially complete at 21 d in the stovers at 51% DM or less, and pH was less than 4.4 (Table 1). The two drier stovers still were actively fermenting at 21 d, and pH continued to decline to 4.33 and 4.76 at 90 d for the Pioneer 35Y67 and Agri-Gold stovers, respectively. The lactate/acetate ratios at 90 d were generally at or above 2.0, indicating a good fermentation. Adding a homofermentative lactic acid bacterial inoculant (Biomax 5) generally reduced pH and increased lactate/acetate ratio relative to those in the as-harvested untreated control. The only exception was pH at 21 d for the Mycogen stover where the natural population of lactic acid bacteria at ensiling was more than 100 times greater than the inoculant application rate. Adding the heterofermentative lactic acid bacteria, *L. buchneri* (11A44), decreased pH relative to the control only in the two driest stovers but consistently shifted fermentation to acetate, particularly at 90 d. The combination inoculant (11C33) provided at a faster pH decline than 11A44 but generally produced similar shifts in fermentation products to acetate like 11A44 by 90 d.

Rehydrating stover to approximately 40% DM had little effect on the fermentation of the brown mid-rib stover (Mycogen) (Table 2) relative to that in the as-harvested stover (Table 1). All four treatments had similar pH at

21 d with or without rehydration. However at 90 d, the 40% DM control and Biomax 5 treatments had lower lactate/acetate ratios than their respective as-harvested stovers. In the Pioneer 35Y67 stover, all treatments had lower pH values but similar lactate/acetate ratios in the 40% DM rehydrated stovers compared with their respective as-harvested stovers.

Rehydrating the stovers to 30% DM resulted in low pH values at 21 d in all three stovers (Table 2). However, pH increased between 21 and 90 d in all stovers and treatments. In the Agri-Gold and Pioneer stovers, there was a loss of lactate and increase of acetate, even in the untreated and Biomax 5 treated stovers, that appeared to be due to naturally-present *L. buchneri* as indicated by 1,2-propanediol in all treatments. In the Mycogen stover, low concentrations of butyric acid were present at 90 d in all treatments indicating the low pH values at 21 d were not sufficient to prevent the growth of clostridia.

Table 1. Characteristics of corn stovers ensiled as harvested.

	Pioneer 34M93 Stalk	Pioneer 34M93 Ear	Mycogen F697	Pioneer 35Y67	Agri-Gold A6333
DM, %	45	51	51	54	58
pH at 21 d					
Control	4.14	4.34	4.13	4.58	5.18
Biomax 5	4.04	4.01	4.12	4.08	4.44
11A44	4.29	4.39	4.27	4.23	4.71
11C33	4.18	4.30	4.28	4.16	4.58
Lactate/Acetate at 90 d					
Control	2.7	2.0	2.3	2.2	1.9
Biomax 5	3.5	2.7	2.9	3.4	3.0
11A44	0.8	0.3	0.7	0.9	0.7
11C33	1.1	0.2	0.8	1.2	0.7

Table 2. Characteristics of ensiled, rehydrated corn stovers.

	Mycogen F697	Pioneer 35Y67	Agri-Gold A6333	Mycogen F697	Pioneer 35Y67
DM, %	40	38	31	30	32
pH at 21 d					
Control	4.12	3.97	3.98	4.15	3.96
Biomax 5	4.12	3.93	4.03	4.16	3.94
11A44	4.20	4.10	4.38	4.27	4.14
11C33	4.20	4.01	4.25	4.19	4.15
Lactate/Acetate at 90 d					
Control	1.3	2.4	0.9	1.1	0.8
Biomax 5	1.7	3.2	1.6	1.3	1.3
11A44	0.7	0.8	0.3	0.4	0.5
11C33	0.9	1.3	0.2	0.8	0.4

Conclusions

Stovers ensiled as harvested (45 to 58% DM) ensiled well with no additive. Similarly stovers rehydrated to 40% DM ensiled well and were stable over 90 d. Stovers rehydrated to 30% DM initially ensiled well (21 d) but experienced secondary fermentations by 90 d. It appears that ensiling stover at 30% DM may result in occasional clostridial activity that could reduce the recovery and quality of the stover for downstream uses. The three inoculants were successful in all trials even when the inoculant application rates were less than 1% of the natural lactic acid bacterial population. The homofermentative inoculant shifted fermentation to lactate as expected. The two inoculants with *L. buchneri* shifted fermentation toward acetate, which should help the stability of stover in transit between the farm and bioprocessing plant.

Fermentability of Eastern Gamagrass, Big Bluestem and Sand Bluestem Grown Across a Wide Variety of Environments

P.J. Weimer and T.L. Springer

Introduction

Eastern gamagrass (EG, *Tripsacum dactyloides* (L.) L.), big bluestem (BBS, *Andropogon gerardii* Vitman), and sand bluestem (SBS, *A. hallii* Hack) are relatively high-quality perennial warm-season grasses that are generally low in nitrogen and are less tolerant of grazing than are most other warm-season grasses. Because relatively low nitrogen inputs and low plant nitrogen are beneficial attributes of biomass materials for bioconversions by fermentative or thermochemical means, these grasses may be useful as a source of fuels and chemicals in cellulosic-based biorefineries. One particular biorefinery strategy, termed consolidated bioprocessing (CBP), involves direct fermentation to ethanol and other products by bacteria that produce their own fibrolytic enzymes. Some configurations of CBP allow fermentations to be conducted without chemical pretreatment of the biomass to produce a fermentation residue with beneficial characteristics as a wood adhesive. In order to examine the suitability of several cultivars of EG, BBS, and SBS grown under a wide range of environmental conditions for bioconversion by CBP, we examined the fermentability of these grasses using an in vitro ruminal assay, which simultaneously assesses ruminal fermentability and mimics fermentability in CBP-type nonruminal bacterial systems.

Methods

Forages and fermentability assays. Various cultivars of EG, BBS, and SBS were space-planted in 2000 at up to nine U.S. locations east of the 100th meridian, and were harvested from six field replicates, one to four times per year (depending on location) in the succeeding three years. Fermentability was determined using separate in vitro ruminal assays for gas production (IVG) at 24 and 96 h, and for dry matter digestibility (IVDMD) and organic matter digestibility (IVOMD). Fermentations for gas production were conducted in volume-calibrated vials (50 mL nominal volume) containing 100 mg forage,10 mL of reduced Goering-Van Soest buffer containing a 15% (v/v) of ruminal inoculum. IVG was determined by converting headspace gas pressure, measured with a digital pressure transducer, to gas volume per g of dry matter, and were normalized across runs by comparison to alfalfa fiber standards to obtain net normalized gas production at 24 h and 96 h (NNG24 and NNG 96). IVDMD and IVOMD were determined by a two-stage Tilley-Terry procedure.

Statistics. Data for EG and for the two bluestems (BBS and SBS) were analyzed separately using SAS 7.0. Correlations among fermentation parameters were determined using PROC CORR. Effects and interactions of genotype and growth environment were analyzed using PROC MIXED. Mean separations were conducted using the adjusted Tukey-Kramer test.

Results and Discussion

Comparison of fermentation methods for all forages revealed strong correlations (r2=0.88 to 0.99) within method (NNG24 vs. NNG96, or IVDMD vs. IVOMD) but weaker correlations (r^2=0.54 to 0.69) across methods (e.g., NNG96 vs. IVDMD), probably due to inclusion of the acid pepsin step in the IVDMD/IVOMD method.

Five EG varieties grown at seven different locations over three harvest years, with two to four cuttings per year, ranged from 6.0 to 7.9 Mg total dM/ha/y, 0.42 to 3.68% (mean 1.69%) N, 17.3 to 71.5 % IVDMD, 16.4 to 71.6% IVOMD. NNG24 ranged from 59.0 to 234 mL/g DM, and NNG96 ranged from 137.0 to 292.8 mL/g DM. Substantial variations in fermentative gas production were observed by variety, harvest number, harvest year, and location (Table 1).

For three varieties each of the bluestems grown at five different locations over three years, mean DM yields of SBS (5.87 to 6.39 Mg/ha) greatly exceeded those of BBS (3.92 to 4.54 Mg/ha). Harvested bluestems varied in N content (0.46 to 1.69%, mean 1.07%), IVDMD (24.0 to 63.7%), IVOMD (23.2 to 63.5%), NNG24 (69.0 to 194.7 mL/g DM), and NNG96 (146.9 to 284.8 Mg/ha). Fermentability of the three BBS varieties only slightly exceeded that of the three SBS varieties (Table 2), and the bluestems in general showed less effect of variety and growth environment (harvest number, harvest year, location) on fermentation than did the Eastern gamagrass.

Conclusions

Big bluestem and sand bluestem displayed lower nitrogen contents, similar or greater in vitro ruminal fermentability, and less variation in fermentability with environmental conditions than did eastern gamagrass. While the sand bluestem cultivars examined here showed slightly poorer fermentability than did the big bluestems, the considerably greater biomass yield of the sand bluestems suggest that this forage is a worthwhile subject for further efforts to enhance yield for use of this species as a biomass feedstock, particularly for direct fermentation (consolidated bioprocessing) applications.

Table 1. Least square mean values for net normalized gas production at 24 h (NNG24) and 96 h (NNG96) during in vitro ruminal fermentation of eastern gamagrass.

	NNG24 (ml/g DM)	NNG96 (ml/g DM)
Variety		
'Pete'	134.01 [a]	220.49 [a]
FGT I	124.46 [b]	207.81 [c]
FT II ('Verl')	134.95 [a]	217.42 [a]
FT IV	126.68 [b]	212.83 [b]
FT 94-8	112.73 [c]	185.88 [d]
Harvest year		
2001	111.27 [b]	201.67 [a]
2002	132.28 [a]	208.16 [a]
2003	136.16 [a]	216.83 [a]
Harvest number		
1	145.18 [a]	224.64 [a]
2	126.95 [b]	208.56 [b]
3	107.58 [c]	193.46 [c]
Location		
Big Flats, NY	151.95 [a]	224.19 [a]
Brooksville, FL	137.17 [a]	226.65 [a]
Coffeeville, MS	122.96 [ab]	209.13 [ab]
Elsberry, MO	137.44 [a]	217.49 [a]
Knox City, TX	95.78 [b]	178.35 [b]
Manhattan, KS	123.91 [ab]	204.52 [ab]
Woodward, OK	116.76 [ab]	201.87 [ab]

[abc] Mean values having different letters for a given class within a column differ ($p < 0.05$).

Table 2. Least square mean values for net normalized gas production at 24h (NNG24) and at 96h (NNG96) during in vitro ruminal fermentation of big bluestem (BBS) and sand bluestem (SBS).

	NNG24 (ml/g DM)	NNG96 (ml/g DM)
Variety		
'Kaw' BBS	147.82 [a]	228.78 [a]
Syn-1 BBS (early maturity)	147.52 [a]	231.38 [a]
Syn-1 BBS (medium maturity)	150.70 [a]	232.08 [a]
'Woodward' SBS	139.78 [b]	218.55 [b]
Syn-1 SBS (tall stature)	137.91 [b]	220.25 [b]
Syn-1 SBS (medium stature; 'Chet')	136.81 [b]	217.92 [b]
Harvest year		
2001	143.40 [a]	230.30 [a]
2002	137.63 [a]	211.25 [a]
2003	149.24 [a]	232.63 [a]
Location		
Knox City, TX	143.34 [a]	212.05 [c]
Manhattan, KS	135.15 [a]	216.89 [bc]
Nacogdoches, TX	142.91 [a]	238.02 [a]
Perkins, OK	146.45 [a]	226.21 [ab]
Woodward, OK	149.26 [a]	230.79 [ab]

[abc] Mean values having different letters for a given class within a column differ ($p < 0.05$).

Impact of Germplasm Source and Harvest Management System on Yield of Alfalfa Stem Cell Wall Polysaccharides for Conversion to Ethanol

J.F.S. Lamb, H.G. Jung, C.C. Sheaffer, and D.A. Samac

Introduction

Alfalfa is cut frequently at an immature developmental stage for feeding livestock to provide high concentrations of protein and low concentrations of fiber in the forage. An alternative use for alfalfa would be as a biomass crop for the production of ethanol from the stem cell wall polysaccharides. The leaf fraction would be a supplemental protein feed that contributes to economic efficiency of the production system. To maximize stem yield, less frequent harvesting of more mature alfalfa has been proposed. The impact of alfalfa germplasm source and harvest management was investigated in a field trial comparing these alternative systems for production of leaf protein and stem cell wall polysaccharides for biomass-derived ethanol from alfalfa.

Materials and Methods

Four alfalfa germplasms (MP2000, MWNC-2, New Europa, and ORCA-WTS) were planted at two densities in replicated plots at Becker and Rosemount, MN. MP2000 and MWNC-4 are conventional hay-type germplasms whereas the New Europa and ORCA-WTS germplasms are of Flemish origin with a lodging-resistant growth habit (biomass-type). Seeding densities were 450 and 180 plants m^{-2} for the hay and biomass harvest management systems, respectively. The hay management system was harvested three or four times per season at early bud stage and the biomass management system was harvested at the green pod stage of development twice yearly. Plots were harvested in 1997 and 1998. Yields of total dry matter, leaf crude protein, and stem cell wall polysaccharides were determined on a seasonal basis. Concentrations of leaf crude protein and stem cell wall components were calculated as yield-adjusted seasonal means.

Results and Discussion

The four alfalfa germplasm sources differed in leaf crude protein concentration, and stem cell wall concentration and composition. The hay-type alfalfa, MP2000, had a higher leaf crude protein concentration and lower concentrations of total cell wall and cell wall polysaccharides than the biomass-type ORCA-WTS, with the other two germplasms being intermediate (Table 1). Lignin and xylose composition of stem cell wall material did not differ among germplasms, but the biomass-type germplasms generally had more cell wall glucose

Table 1. Yield-adjusted mean concentrations of leaf crude protein and stem cell wall material and cell wall composition of alfalfa stems as influenced by germplasm source and harvest management system.

	Germplasm					Management System		
Trait	MP2000	MWNC-4	New Europa	ORCA-WTS	LSD[†]	Hay	Biomass	LSD
Concentration	------------------------- $g\ kg^{-1}$ Dry Matter -------------------------							
Leaf crude protein	275	270	269	266	7	298	242	7
Stem cell wall	667	676	683	686	5	657	700	10
Stem polysaccharide	524	531	535	539	4	520	545	7
Composition	------------------------- $g\ kg^{-1}$ Cell Wall -------------------------							
Stem Klason lignin	214	215	217	214	NS[‡]	209	222	3
Stem glucose	424	428	428	433	3	429	428	NS
Stem galactose	26.4	26.0	25.6	25.3	0.3	26.9	24.7	0.6
Stem mannose	27.3	27.3	27.3	27.1	0.2	27.8	26.7	0.2
Stem xylose	136	137	136	136	NS	134	139	1
Stem arabinose	26.7	26.1	25.5	25.0	0.5	26.8	24.9	0.9

[†] LSD, Least significant difference ($P < 0.05$).
[‡] NS, Non-significant ($P > 0.05$).

Figure 1. Variation in leaf crude protein yield (CP) between harvest management systems depending on growth environment (site x year).

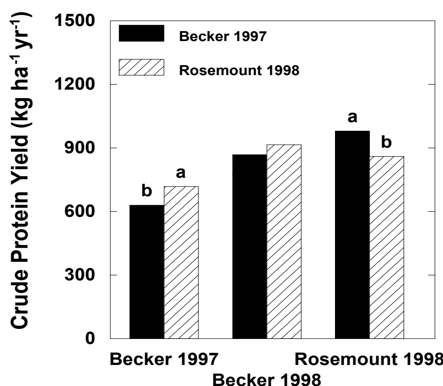
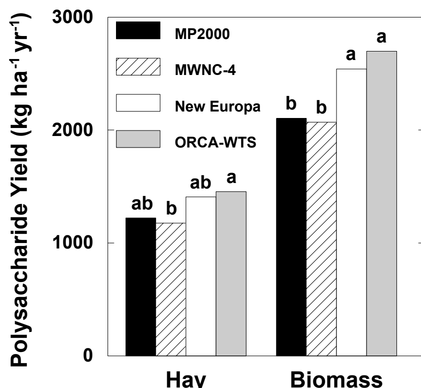


Figure 2. Differences among alfalfa germplasms, within harvest managements system, for total cell wall polysaccharide yield on a yearly basis.



and less galactose, mannose, and arabinose than the hay-type germplasms. Harvest management system impacted concentrations of leaf crude protein and cell wall material, and cell wall composition (Table 1). The biomass management system resulted in lower leaf crude protein and higher stem cell wall concentrations. Compared with the hay management system, cell wall material from the biomass management system alfalfa stems was more lignified and contained more xylose, similar glucose, and less of the other polysaccharide components. Yield of leaf crude protein varied between the two harvest management systems depending on growth environment, but was not consistently lower for the biomass management system (Fig. 1). Yield of cell wall polysaccharides was affected by germplasm x harvest management system interactions. The germplasms differed only marginally for cell wall polysaccharide yield under the hay system, but substantial germplasm differences were observed under the biomass management system (Fig. 2). The biomass management system yielded more cell wall polysaccharides than did the hay management system.

Conclusion

Both alfalfa germplasm source and harvest management system impacted composition and concentration of leaf crude protein and stem cell wall polysaccharides. Using the National Renewable Energy Laboratory's theoretical ethanol yield calculator for lignocellulosic biomass (http://www1.eere.energy.gov/biomass/ethanol_yield_calculator.html), potential ethanol yield increased 11.4% per kg of stem biomass due to the differences in stem cell wall concentration and composition resulting from germplasm and harvest management (MP2000 under hay system vs. ORCA-WTS under biomass system). However, theoretical ethanol yield increased from 1533 L ha^{-1} yr^{1} for the forage-type alfalfa grown under the hay management system to 3048 L ha^{-1} yr^{1} for the biomass-type alfalfa under the biomass management system. Clearly, the increased total dry matter yield from the biomass management system was the dominant factor in this doubling of ethanol yield. Equivalent leaf protein yield, reduced harvesting costs, and potentially longer stand life under the less frequent harvesting biomass management system would contribute additional economic efficiencies.

USDFRC Dairy Operations Report

for 2005

J.A. Davidson, Herd Manager

The current dairy herd personnel include Orie Eilertson, Marsha Key, Mark Hintz, Deidre Kannenberg, Kelly Miess, Rich Campbell, Kurt Pickar, Ken Lazzara, Frank Klemm, John Biech, Bob Breunig, Dick Hager, Jim Lukken, Greg Schultz, Mike Wilson, Ann Julson, Tina Melvin, Sarah Omvedt, and Sebastain Baxter. Stephanie Jicinsky, a University Wisconsin-River Falls student, was employed during the summer break as an intern. Three undergraduate students will have intern experience at the research farm in 2006. These individuals are the keys to a successful research program at the farm and the increased number of experiments that have been conducted during the past year. They have been instrumental and cooperative during the on-going renovations to the facility.

The operation had a very successful year maintaining a DHIA rolling herd average over 25,000 pounds along with steady production of milk components. Facility updates nearing completion include the addition of a drover's lane to our lactating cow free-stall wing; altering the feed alley and side walls of the heifer and lactating cow free-stalls; and installing a feed pad with headlocks to our dry cow and pregnant heifer lots. Our research capacity in the heifer wing was increased with additional water tanks and stalls for each pen. We will soon begin renovating the tie-stall wings to improve labor efficiency and ergonomics of collecting intake data. Continued efforts will be made to improve cow performance and health, cow comfort, and labor efficiency with an increase in our research efforts.

During the past year, cows and heifers committed to research experiments ranged from 4 to 120 at any given time. The scope of research projects was to increase nitrogen efficiency in rumen, forage utilization, and environmental impacts of dairy systems. We were able to renovate a tie-stall wing and conduct ammonia emission research (8 replicates) in

HERD STATISTICS

		Change from previous year
Herd Inventory		
Milking cows	294	+ 20
Dry cows	35	- 17
average cow age (months)	44	- 1
percent first lactation	46	+ 4
percent second lactation	25	- 4
percent third or greater lactation	29	0
Herd replacements	335	- 15
Total	**664**	**- 12**
(Rumen fistulated cows)	23	+ 5
Herd Performance		
Cows calved	369	- 11
Heifer calves born live	178	- 7
Heifer calves born dead	17	- 2
Bull calves born live	179	+ 11
Bull calves born dead	12	- 8
Heifer calves died < 1 year old	8 (4.4%)	0
Number of sets of twins	27	+ 7
DHIA rolling herd average		
lbs. milk	25,261	+ 377
lbs. protein	754	+ 15
lbs. fat	912	- 9
Milk sold in 2005 (lbs.)	7,427,164	- 481,346
Average mailbox milk price/cwt.	$15.54	- $0.41
Cattle Sales		
Heifer calves sold	10	+5
Bull calves sold	176	+8
Cows sold, culled for		
Reproduction problems	42	- 6
Poor production	16	+ 1
Poor feet and legs	19	- 6
Mastitis	29	- 11
Other	42	- 11
Cattle sales revenue	$110,704	- $22,747
Herd Reproduction		
Average days open	138	+ 8
Average calving interval (months)	13.6	- 0.2
Average services per conception	2.5	- 0.4
Average age at first calving (months)	24	0

chambers looking at the influence of diet and bedding materials on emissions.

The farm continues to increase involvement in state and multi-state functions hosting many national and international visitors, meetings, and veterinary, technical school, and undergraduate students throughout the year. The continued development of the intern programs is a priority for the next year. Other future targets and goals include the development of long-term farmstead plans for the center, improved milk production, reduction of involuntary culling, improved reproductive performance, and continued plans for updating the facilities for greater cow comfort and improved labor efficiencies.

USDFRC Field Operations Report

for 2005

R.P. Walgenbach, Management Agronomist and Farm Manager

The 2005 crop year began with fairly cool but very dry conditions during April. This allowed for some very good planting conditions for alfalfa, corn and soybeans. Corn planting started on April 18 and was 80 percent completed by April 28. Typically we start planting corn around the 24th of April.

A fairly open winter and some very cold temperatures in late December of 2004 produced some scattered winter kill in alfalfa. No stands were complete losses, but small pockets of total winter kill were evident in most fields. The cool temperatures in spring slowed alfalfa growth, but plants seemed to recover from the winter stress and produced reasonable first-crop yields. For our second year we utilized a custom operator to help harvest the first and second alfalfa crops. This was done in part to facilitative a study to evaluate the impact of storage structure type on dry matter losses and feed quality.

For corn, the early planting was followed by some frequent rain in mid May and a dry June. Temperatures in July and August were quite hot. The pattern of rain and high temperatures helped the corn plants to develop an extensive root system that was needed to obtain water. A few timely rains really helped to prevent yield losses and to produce the highest average corn grain yield (194.9 bushels/acre) at the research farm. Soybean yields were good as well, but I think they had some yield loss due to dry periods that did not impact the corn as much.

The big weather event for the 2005 season was a series of tornadoes that hit the Badger Army Ammunition Plant (BAAP) where many of our fields are located. Three buildings that we had planned to use for storage, several hundred trees, and several acres of corn sustained damage from these tornadoes. One field of corn was completely flattened by a tornado and most of the corn for silage was damaged to varying degrees. We were unable to harvest several acres of alfalfa because the fields were littered with downed tree limbs and building materials. Most of the damage occurred in rather open areas and the main farm buildings were not impacted by the tornado and, most fortunately, no one was injured.

A new agronomy research plot area is being developed on the far west side of the BAAP. An irrigation system has been installed, and future plans will provide storage space for equipment and samples, as well as additional work space. Planning and design of a manure pumping facility has also taken place. When completed, manure will be pumped from the storage pit at the farm to a temporary holding tank inside the BAAP fence. From there, trucks will be able to load and go without having to cross a busy highway or be stopped to wait for a gate to open. We have added a 7,500 bushel storage bin for roasted soybeans, along with a cooling system and augers. This serves as a completely automated system for roasting soybeans.

Table 1. Precipitation (inches) for 2005.

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2.05	1.38	1.66	0.65	3.07	1.06	4.90	2.14	2.75	0.85	3.37	0.58

Table 2. Planting and harvesting dates for 2005.

	----Planting----		--Harvesting--	
Crop	Start	Finish	Start	Finish
Oats	4/19	4/19	6/22	6/22
Corn	4/18	5/06	9/23	10/28
Soybeans	5/03	5/20	9/21	10/28
Corn silage			9/07	9/21
Oats f.b.* sorghum	7/01	7/01	8/20	10/10
Alfalfa f.b.* sorghum	6/10	6/10	8/08	10/03
Alfalfa	4/11	4/18	5/29	10/11
Winter wheat	10/05	10/08	7/11	7/18

*followed by

Ron Lipka and John Wesolowski, two previously retired full-time employees who returned to work as

limited-time employees, have decided to finally and fully retire. Both Ron and John have been excellent employees. John is moving to the Wausau area to be closer to family. Ron just wants to take it easy for a change. I and fellow employees very much appreciated the excellent work and good friendship from Ron and John. Some of us will also miss Ron's sometimes colorful wardrobe and his barely successful attempts as a comedian. We wish them both the best.

Overall, 2005 was a good year in terms of research projects supported and completed, and in terms of the amount and quality of feed and bedding produced for the dairy operation. It was also the year we celebrated with our Sauk County neighbors to mark the transfer of xx acres from the BAAP to the U.S. Dairy Forage Research Center. Our accomplishments are possible because of the efforts of our employees; we appreciate their work and thank them for it.

Table 4. Crop yield data for 2005.

		Yields			
Crop	**Acres**	**Low**	**High**	**Mean**	**Total**
		Bushels per acre			
Winter wheat	90.5	76.7	77.4	76.9	6,961
Soybeans	347.3	47.6	71.0	62.3	21,636
Corn grain +	354.1	148.7	231.4	194.9	69,027
		Tons DM per acre			
Corn silage – DM basis	161.5	6.9	9.8	7.2	1,163
(Corn silage -- as is basis)	(161.5)	(22.1)	(30.5)	(22.0)	(3,557)
Alfalfa, established ++	260.3	2.9	5.49	4.5	1,165
Alfalfa, seeding year	110.2	0.6	2.1	1.8	202
Oats	20.0			1.6	30.4
Sorghum after alfalfa	22.0			3.0	67.4
Sorghum after oats	20.0			2.0	38.6

+ 24,563.5 bushels (896.6 tons wet) harvested as high moisture cracked shell corn.
++ The 22.4 acres of alfalfa followed by forage sorghum is not included in this data (it produced 1.3 tons DM per acre).
Alfalfa yields were not adjusted for DM that was chopped back to fields. If there was no DM harvested from a particular cutting, a zero yield was recorded for that field and was included in the average yield for that field.

Table 3. Alfalfa cutting dates for 2005.

	Established Alfalfa			Seeding-Year Alfalfa		
Cutting	**Acres***	**Start**	**Finish**	**Acres***	**Start**	**Finish**
First	283	5/29	6/01	110	6/20	6/21
Second	260	6/26	6/28	28	7/18	7/19
Third	182	7/27	7/29	91	8/22	8/31
Fourth	283	8/21	8/31			
Fifth	78	10/09	10/09			

* Indicates actual acres harvested. After first harvest, 22 acres were planted to BMR forage sorghum. Rainy weather prevented the harvest of 78 acres of third crop established alfalfa and 82 acres of second crop seeding-year alfalfa. Unharvested alfalfa was chopped back into fields. A tornado scattered building debris and trees in 19 acres of third crop seeding-year alfalfa leaving it unharvestable.

Publications List

for 2005

A representative listing of scientific articles that USDFRC scientists have authored or co-authored. Not intended to be a complete listing.

Allerdings, E., **Ralph, J.**, Steinhart, H., Bunzel, M. 2006. Isolation and structural identification of complex feruloylated heteroxylan side-chains from maize bran. Phytochem. 67(12):1276-1286. [Log Number: 195598]

Allerdings, E., **Ralph, J**., Schatz, P., Gniechwitz, D., Steinhart, H., Bunzel, M. 2005. Isolation and structural identification of diarabinosyl 8–O–4-dehydrodiferulate from maize bran insoluble fibre. Phytochem. 66(1):113-124. [Log Number: 163636]

Braun, L.C., **M.P. Russelle**, and J.H. Gillman. 2005. Nitrogen fertilization for hybrid hazelnuts in the Upper Midwest. Poster three p. 1-10 (online). 9th North American Agroforestry Conf. Proc. 12-15 June 2005, St. Paul, Minnesota. Dept. of Forest Resources, University of Minnesota, St. Paul, MN.

Brink, G.B., Hall, M.H., Shewmaker, G.B., **Martin, N.P**., Undersander, D.J., **Walgenbach, R.P**. 2006. Rate of yield and quality change in alfalfa. p. 5-10. Proc. Idaho Alfalfa and Forage Conference. Twin Falls, ID.

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Broderick, G.A. 2005. Feeding dairy cows to minimize nitrogen excretion. Proceedings of the 2005 Tri-State Dairy Nutrition Conference, pp. 137-152.

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Broderick, G.A., Stevenson, M.J., Patton, R.A., Lobos, N.E., Olmos, J.J. 2005. Supplementing rumen-protected methionine to reduce dietary crude protein in dairy cows. J. Dairy Sci.:88 (suppl. 1):89. (Abstract)

Broderick, G.A., Stevenson, M.J., Patton, R.A., Lobos, N.E., Olmos, J.J. 2005. Effect of supplementing rumen-protected methionine at two levels of dietary crude protein in lactating dairy cows. . J. Dairy Sci.:88 (suppl. 1):319. (Abstract)

Broderick, G.A., Brito, A.F. 2005. Effects of varying dietary ratios of lucerne to maize silage on production and microbial protein synthesis in lactating dairy cows. Proceedings of XIVth International Silage Conference. p. 172. (Abstract)

Bunzel, M., **Ralph, J.** 2006. NMR Characterization of Lignins Isolated from Fruit and Vegetable Insoluble Dietary Fiber. J. Agric. Food Chem. in press. [Log Number: 196368]

Bunzel, M., **Ralph, J.,** Brüning, P., Steinhart, H. 2006. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize fiber J. Agric. Food Chem. in press. [Log Number: 195634]

Bunzel, M., **Ralph, J**., Funk, C., Steinhart, H. 2005a. Structural elucidation of new ferulic acid-containing phenolic dimers and trimers isolated from maize bran. Tetrahedron Lett. 46(35):5845-5850. [Log Number: 179634]

Bunzel, M., **Ralph, J**., Steinhart, H. 2005b. Association of non-starch polysaccharides and ferulic acid in grain amaranth (*Amaranthus caudatus* L.) dietary fiber. Mol. Nutr. Food Res. 49(6):551-559. [Log Number: 178608]

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