United States
Department of
Agriculture

**Agricultural
Research
Service**

September 2005

U.S. Dairy Forage Research Center 2004 Research Report

U.S. Dairy Forage Research Center. 2004. U.S. Dairy Forage Research Center 2004 Research Report. U.S. Department of Agriculture, Agricultural Research Service, pp. 97.

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Appreciation is expressed to Peggy Carroll for her dedication to the task of typing and assembling this research summary.

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Issued
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Preface

It is a pleasure to bring you these summaries of research conducted over the past year at the U.S. Dairy Forage Research Center (USDFRC). The Center's mission is to build a knowledge and technology base for the dairy industry to fully exploit the use of forages in the production of milk. The Center was established in 1980 on the University of Wisconsin-Madison campus in Madison, WI, but is a federal unit of the Agricultural Research Service, U.S. Department of Agriculture (USDA). We employ agricultural engineers, plant and soil scientists, microbiologists, ruminant nutritionists, and chemists, who all work together to increase the efficiency of forage production and utilization by dairy farmers. At present, we have 22 scientists: 17 at Madison, two at Marshfield, two cluster scientists at the University of Minnesota in St. Paul, MN, and one cluster scientist at Cornell University in Ithaca, NY. Many of these scientists hold faculty appointments in university departments and provide supervision for approximately six to eight graduate students and four postdoctoral fellows. We function in close cooperation with the agricultural experiment stations of several states.

The Center's 2,000-acre research farm is located in Prairie du Sac, WI and has facilities for housing and feeding 320 milking cows and 350 replacement heifers and dry cows. On September 29, 2004, the USDA received custody of 1,943 acres of Badger Army Ammunition Plant (BAAP) land that had been previously leased by the USDFRC research farm. This acreage is divided into cropland, pasture, small-plot research, woodland, buffer strips, and non-cropped open areas. On August 6, 2005, we celebrated the transfer of land from BAAP to USDFRC with stakeholders and citizens of the communities surrounding Prairie du Sac. On behalf of the USDFRC staff, I want to thank all that have supported the justification for this significant land use.

Regarding staff updates, we hired William E. Jokela, Research Soil Scientist, in July 2005. Dr. Jokela received a B.A. degree in Biology from Carlton College in 1969, and M.S. and Ph.D. degrees in Soil Science from the University of Minnesota in 1978 and 1985, respectively. He had been a Professor at the University of Vermont since 1985, conducting research and extension activities on soil and crop management, manure and fertilizer nutrient management, and nutrient transformation and fate. His research has spanned scales ranging from the laboratory to small plots, large plots, farms, and watersheds. He brings training and knowledge in soil and nutrient management with first-hand knowledge of issues and management concerns facing dairy managers with emphasis on soil, water, and nutrient management and associated nutrient transformations from dairy manure. He is the first hire at our new work site, The Institute for Environmentally Integrated Dairy Management, Marshfield, Wisconsin.

Our second scientist hire, Dr. Wayne K. Coblentz, also fills a new position at Marshfield as a Research Dairy Scientist/Agronomist. Dr. Coblentz earned a B.A. degree in Chemistry from Western Maryland College in 1977, an M.S. in Dairy Science from The Pennsylvania State University in 1982, and a Ph.D. in Forage Agronomy from Kansas State University in 1994. He was a Research Associate in Agronomy and Animal Science at Kansas State University from 1994 to 1997. He had been an Assistant and Associate Professor of Animal Science, University of Arkansas since 1997. Both Wayne and Bill will write our newest CRIS project: "Research to Address Environmental Problems Faced by the U.S. Dairy Industry."

Our newest staff member, who was hired in July 2005, is Lori Ward Bocher, Agricultural Information Specialist. Ms. Bocher earned both a B.A. in Journalism and a B.S. in Dairy Science from the University of Wisconsin-Madison. She brings 25 years of experience in journalism and marketing.

She served several years as an associate editor for *Hoards Dairyman* and has successfully operated an agricultural communications business. Ms. Bocher comes well qualified to position USDFRC as the flagship dairy forage research facility by interpreting our research into a problem solving framework for dairy operators, dairy farm advisors, and industry businesses.

The FY 05 budget provided partial funding to support the research of our two new scientists at the newly created Institute for Environmentally Integrated Dairy Management in Marshfield. This facility, which is located at the Agricultural Research Station for the University of Wisconsin, will eventually house six USDA-ARS scientists, as well as four USDA-Natural Resources Conservation Service (NRCS) specialists. The Institute is a collaborative effort among USDA-ARS, USDA-NRCS, the University of Wisconsin, and the National Farm Medicine Center of the Marshfield Medical Clinic. Research at the Institute will be conducted to improve nutrient management, minimize emissions, and control pathogens at all stages in the dairy production system. Research objectives of the unit are: 1) improved nutrient management and manure handling, storage, and application strategies; 2) reduced degradation of air, water, and soil quality by manure; 3) controlled pathogen transmission between livestock, wildlife, humans, and the environment; and 4) education, outreach, and technical assistance. FY 03, 04, and 05 funding have been appropriated to construct animal housing and manure containment facilities and part of the new laboratory. FY 06 Senate mark-up appropriates funds to complete all Institute facilities plus $1.67 million to initiate plans to enhance research capacity at our Prairie du Sac farm.

We continue to recruit for a Research Agricultural Engineer and a Research Agricultural Scientist (Dairy Systems Specialist).

I want to thank scientists, support staff, students, visiting scientists, and stakeholders for making 2004-2005 a tremendous success. USDFRC stakeholders are currently developing a plan to enhance the dairy forage research capacity to meet the needs of the industry in the 21st century.

This collection of research summaries illustrates the progress that scientists and staff are making in developing information to help dairy farmers utilize their resources more effectively. The research is intended to benefit forage growers, dairy farmers, and the consumers of dairy products.

Sincerely,

Neal P. Martin, Director
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#Member of Agricultural Research Stations Department in the College of Agricultural and Life Sciences, and supported by the U.S. Dairy Forage Research Center.

Forage Genetics and Production

Differences in Sward Structure and Quality Among Temperate Perennial Grasses

G.E. Brink and M.D. Casler

Introduction

Reduced dry matter (DM) intake is a factor contributing to lower milk production in dairy cattle on pasture. Intake of grazing dairy cattle is less than that of confinement-fed cattle due in part to reduced bite weight. Bite weight is a product of the bite volume and the bulk density of the sward. Sward bulk density may be influenced by sward height, tiller density, grass species, proportion of leaf and stem, and physical characteristics of the leaf such as angle of attachment to the tiller, dimensions, and rigidity. Taken together, these characteristics constitute the sward structure, or the vertical distribution and arrangement of above ground plant parts within a plant community. Studies have demonstrated that sward height is the principal determinant of bite weight in lactating dairy cows grazing perennial ryegrass (*Lolium perenne* L.), but that the influence of sward bulk density on intake becomes greater as sward height declines. Depending on the composition of the pasture, producers practicing managed intensive rotational grazing typically have a target height at which grazing begins and ends. For a given sward height, a wide variation in sward density and forage quality may exist among common temperate grasses which could impact intake. Our objective was to determine differences in sward density and forage quality among temperate perennial grasses typically used in rotational grazing systems.

Methods

'Park' Kentucky bluegrass (*Poa pratensis* L.; KBG), 'Mara' perennial ryegrass (PRG), 'Bronc' orchardgrass (*Dactylis glomerata* L.; OGR), 'Itasca' timothy (*Phleum pratense* L.; TIM), 'Johnstone' tall fescue (*Festuca arundinacea* Schreb.; TFK), 'Barolex' soft-leaf tall fescue (TFS), 'Bartura' meadow fescue (*Festuca pratensis* Huds.; MDF), 'Lincoln' smooth bromegrass (*Bromus inermis* Leyss.; SBG), 'Rival' reed canarygrass (*Phalaris arundinacea* L.; RCG), and common quackgrass [*Elytrigia repens* (L.) Nevski; QGR] were broadcast-seeded at recommended rates on 4.5- x 6.1-m plots in spring, 2003 at the University of Wisconsin Arlington Agricultural Research Station (43.30EN, 89.35EW) on a Plano silt loam (fine silty, mixed, superactive, mesic Typic Argiudoll. Sward structure was measured in May (spring), July (summer), and September (fall), 2004. Plots were fertilized with 56 kg ha^{-1} before sampling and clipped to a 10-cm stubble after the spring and summer sampling, and again 21 days later. Sward structure was measured when each grass reached 25 cm height (nonextended leaf) using a 25 x 100 cm aluminum quadrat that slid vertically on four legs at 2.5-cm increments. The frame was placed randomly in two locations in each plot and DM determined for three canopy layers: 20 to 25, 15 to 20, and 10 to 15 cm. Forage from each layer was analyzed for crude protein (CP), neutral detergent fiber (NDF), and neutral detergent fiber digestibility (NDFD) concentration.

Results and Discussion

Ranking for DM by sward layer among the grasses was strongly influenced by the sampling period. Grasses that typically exhibit vigorous early spring growth, such as timothy, smooth bromegrass, and quackgrass, produced more DM in the upper and middle portions of the canopy than orchardgrass, perennial ryegrass, and meadow fescue (Fig. 1). Unlike the more rigid leaves of orchardgrass and

meadow fescue, leaves of timothy, smooth bromegrass, and quackgrass began to bend and droop before sampling, thus requiring more time to reach 25-cm height (20 to 25 days vs. 16 to 17 days) and adding DM to the upper canopy. Dry matter distribution in perennial ryegrass was opposite that in most of the other grasses, increasing incrementally from the top to the bottom of the canopy. In the summer, grasses that ranked lower in upper canopy DM in the spring (soft-leaf tall fescue, meadow fescue, and reed canarygrass) were ranked at or near the top, while those ranked higher in the spring (timothy, smooth bromegrass, and quackgrass) were ranked near the bottom. In the fall, DM generally increased from the top to the bottom of the canopy.

Forage quality differences among grasses and canopy layers were similar in the spring, summer, and fall. From the top to the bottom of the canopy, mean CP and NDFD of all grasses decreased approximately 5% and 3%, respectively, and mean NDF increased 10% (Table 1). Within perennial ryegrass, timothy, and meadow fescue, however, NDFD remained relatively unchanged. Within the upper portion of the canopy, CP of quackgrass and reed canarygrass was greater than all grasses except orchardgrass, while CP of smooth bromegrass was lowest. In the middle and lower canopy, orchardgrass had the greatest CP. Because bluegrass was sampled at a greater height and a more mature stage than it would normally be grazed, NDF of each layer was greater than the other grasses. Timothy NDF was among the lowest of the grasses throughout the canopy, ranging from 40.6 to 47.4%. In terms of digestibility, NDFD of orchardgrass at each layer was greater than all grasses except quackgrass. Digestibility of tall fescue (conventional and soft-leaf) and reed canarygrass was generally lowest in all canopy layers.

Conclusions

Temperate perennial grasses exhibit a wide range in DM distribution within swards and differences depending on the season, which may influence grazing management and utilization. Forage quality differences between canopy layers would permit more complete utilization of some grasses than others without forcing the animal to consume poorer quality forage.

Table 1. Crude protein (CP), neutral detergent fiber (NDF), and neutral detergent fiber digestibility (NDFD) concentration by canopy layer of temperate grasses grown to 25 cm height in the summer.

	CP			NDF			NDFD		
Grass	20-25	15-20	10-15	20-25	15-20	10-15	20-25	15-20	10-15
	---------- gm kg^{-1} ----------								
Bluegrass	195	175	153	514	563	605	633	611	590
Ryegrass	249	215	181	447	499	546	650	642	639
Orchardgrass	262	263	236	432	485	530	706	676	655
Timothy	223	201	177	406	436	474	608	612	602
Tall fescue	234	226	201	473	515	562	601	584	564
Tall fescue (soft leaf)	214	194	169	452	514	553	594	582	568
Meadow fescue	230	204	174	410	472	512	641	650	652
Smooth bromegrass	188	174	160	418	474	532	630	614	582
Reed canarygrass	268	230	198	473	526	567	604	583	564
Quackgrass	272	239	206	459	512	549	707	669	626
LSD (0.05)	15	19	19	24	33	27	21	21	21

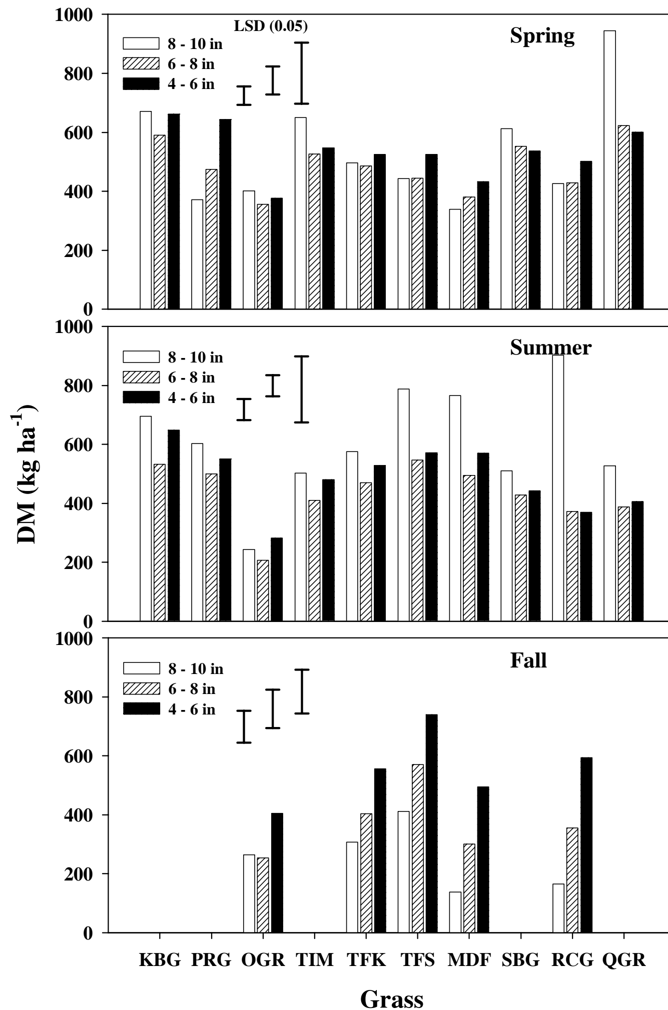

Figure 1. Dry matter (DM) by canopy layer of temperate grasses grown to 25 cm height in the spring, summer, and fall.

Improved Procedure for Genetic Transformation of Red Clover

M.L. Sullivan

Introduction

Plants that have been genetically modified by the insertion of transgenes can be powerful experimental tools. For example, gene expression patterns and protein localization studies can be carried out using reporter genes, expression of endogenous genes can be altered (up- or down-regulated) to test hypotheses regarding gene product function, and expression of foreign genes can be used to develop model systems. Red clover (*Trifolium pratense* L.) is a widely used and versatile forage legume. To take advantage of the opportunities of genetic transformation for this forage crop, a system of regeneration of red clover plants from transformed plant cells had previously been developed. This original procedure utilized aseptically grown red clover as the plant source material. In my laboratory, we have modified this procedure to use greenhouse grown plant material, which is more easily maintained and provides abundant amounts of explant material with little lead time compared to aseptically grown material.

Methods

Our improved transformation protocol is based on a procedure developed for genotypes derived from NewRC red clover germplasm (PI578052) and is provided in detail on our web site http://www.dfrc.ars.usda.gov. Briefly, petiole explants are harvested from greenhouse grown red clover plants, surface sterilized with bleach solution, and introduced into tissue culture, where they are infected with *Agrobacteria* harboring a binary vector-based transformation construct consisting of a selectable marker gene (in our case a gene conferring resistance to the antibiotic kanamycin) and other desired transgenes in the T-DNA region. Following co-cultivation, explants are placed on a series of selective media with hormone compositions to stimulate callus formation, embryo induction, and embryo development to plantlets. Plantlets are then placed in a medium to promote rooting prior to transfer to soil.

Results and Discussion

Despite surface sterilization of red clover petiole explants, in initial transformation experiments utilizing greenhouse grown plants large numbers of explants (20-50%) were lost to bacterial and fungal contamination in the first few weeks of tissue culture. To reduce the level of fungal contamination of explants, we began treating red clover plants with a systemic fungicide approximately 1 week prior to harvesting explants for transformation. To reduce the level of bacterial contamination, we replaced the antibiotic carbenicillin (used in the original protocol to select against *Agrobacteria* following the initial transformation-inducing infection) with the antibiotic Timentin. Together, these modifications have reduced the number of explants lost to contamination to less than 1%, with no significant effect on transformation efficiency. Since endogenous contamination in explants is very often a problem with plant tissue culture, the methodologies we have employed here to reduce fungal and bacterial contamination may be applicable to a wide variety of plant tissue culture systems.

We have found that transformation efficiency is highest with petioles of young leaves (i.e. those less than 6 cm in length), at 10-15% compared to 1-3% for mature petioles, where efficiency is defined as percent of explants producing fully regenerated, viable plants expressing one or more transgenes. We have successfully used this methodology to introduce a β-glucuronidase (GUS) reporter gene into red clover (Fig. 1) and in gene silencing experiments (see accompanying report on page 20).

Conclusions

We have modified a red clover genetic transformation procedure to use more easily maintained greenhouse-grown plant material. Our modifications will greatly facilitate production of genetically modified red clover for research purposes, and may be generally applicable to reduce problems of endogenous bacterial and fungal contamination in wide variety of plant tissue culture systems.

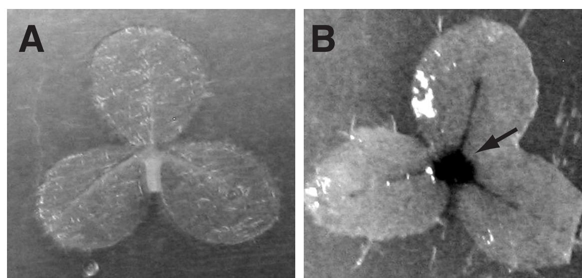

Figure 1. Histochemical staining of red clover transformed with a GUS reporter gene. Leaves from an untransformed plant (A) or a plant transformed with a GUS reporter gene (B) were incubated in a solution of X-Gluc, which forms a dark blue precipitate in the presence of the GUS gene product. An arrow points out the dark staining of the vascular tissue of the transformed plant.

Yield and Soil Nitrate Levels of Cover Crop and Living-Mulch Systems for Corn Silage Fertilized with Manure

J.H. Grabber and L.J. Massingill

Introduction

A variety of cover crop and living mulch systems for annual row crops have been developed for reducing nitrate and soil loss from cropland and for improving soil structure, soil water-holding capacity, and crop yields. Cereal rye, typically sown after harvest of corn and other annual crops, is widely used as a forage and green manure crop throughout the U.S. Italian ryegrass, overseeded as a relay crop in corn or other crops, is used for forage production and as a green manure crop in humid regions with mild winters. Red clover, overseeded as a relay crop in corn or other cereals, is occasionally used in the Midwest and Northeast as a green manure crop. Recently, Ken Albrecht and colleagues at the University of Wisconsin have developed systems for growing corn in a living-mulch of herbicide-suppressed kura clover. In this long-term study, we are evaluating how these cover crop and living-mulch systems affect yields and nitrogen-use of corn silage fertilized with manure. We are also comparing the yields of kura and red clovers the year following corn production.

Methods

In replicated plots at Prairie du Sac in southern Wisconsin, continuous corn (Roundup ready) was planted in early May and grown with relay-seeded Italian ryegrass, fall-seeded rye or no cover. Corn was also grown in a 2-year rotation with kura clover living-mulch or relay-seeded red clover followed by one year of clover production. Manure slurry was applied on a phosphorus-basis in November or April to all plots and additional fertilizer was applied to continuous corn plots at planting in early May to supply 180 kg/ha of available N. Corn was harvested for silage in September. Soil nitrate was determined in early June and in late October when cover crop and living mulch growth had ceased for the winter. Above ground biomass of cover crops and living mulch was determined in late October and again in early May at corn planting.

Results and Discussion

In 2003 (dry summer), corn silage yields ranged from 18.4 to 23 Mg/ha and were greatest with red clover and lowest with ryegrass. In 2004 (wet spring, cool summer), corn silage yields ranged from 16.6 to 20 Mg/ha and were greater with clovers and no cover than with rye and ryegrass (Table 1). Growth of cover crops and living mulch by late October was greatest with ryegrass (1 Mg/ha); spring growth by early May was similar for rye, kura and red clovers in 2003 (1 Mg/ha) and greatest for rye in 2004 (3.5 Mg/ha). June soil nitrate (0-30 cm) ranged from 21 to 43 mg/kg in 2003 and from 7 to 12 mg/kg in 2004 and was highest for corn grown with ryegrass (Table 1). In both years, fall soil nitrate levels (0-120 cm) ranged from 31 to 54 kg/ha and were greatest with clovers and lowest with ryegrass. Fall nitrate levels dropped to 10 to 37 kg/ha when a year of clover production followed corn. In the year following corn, yields of red clover were greater than kura clover in 2003 (11.4 vs. 8.6 Mg/ha); yields of both clovers were similar in 2004 (8.3 Mg/ha) even though red clover was reseeded in April due to relay seeding failure in 2003. Timing of manure application did not influence yields or soil N levels.

Table 1. Corn silage dry matter yields and soil nitrate levels for cover crop and living-mulch cropping systems.

Year and Cropping system	Corn silage (Mg/ha)	June soil nitrate (0-0.3 m) mg/kg	October soil nitrate (0-1.2 m) kg/ha
2003			
Kura living mulch	20.6bc	21.2c	50.1a
Red clover relay	23.0a	34.1b	50.8a
Italian ryegrass relay	18.4c	42.6a	34.3b
Rye fall seeded	21.4ab	40.1ab	38.0ab
None	21.2ab	38.7ab	38.3ab
2004			
Kura living mulch	20.0a	8.9b	54.1a
Red clover relay	19.3a	7.3b	53.6a
Italian ryegrass relay	16.6c	12.4a	30.9b
Rye fall seeded	17.7bc	8.2b	33.7b
None	18.8ab	11.8a	33.4b

Within years, means in a column followed by different letters are significantly different ($P < 0.05$)

Conclusions

The initial results from this long-term study indicate that corn silage yields with the red clover and kura clover systems are comparable to or better than corn grown without cover. Relay seeded ryegrass gave only modest cover and reductions in fall nitrate prior to winterkill and it depressed corn silage yields. Performance of fall-seeded rye was intermediate; corn yields were improved if rye was killed before much biomass had accumulated in spring. Crop yield, nitrogen uptake, soil quality, and soil nitrogen status of these systems will be evaluated until 2007.

Forage Handling, Preservation and Storage

Packing Practice Effects on Density in Bunker Silos

R.E. Muck, B.J. Holmes, and P. Savoie

Introduction

Crop preservation by ensiling depends on minimizing exposure to oxygen during storage and feed out. In bunker silos, packing the crop to a high density is a primary means of reducing oxygen exposure and subsequent dry matter (DM) losses. Surveys and pilot-scale experiments have reported that a number of factors affect density: packing tractor weight, packing time, layer thickness, silo height, dry matter content, and particle size. However, inconsistency in results across studies has led to uncertainty regarding which factors are the most important. So, there is a need to examine the importance of various packing practices at farm scale.

Methods

A bunker silo (37.8 x 10 x 3.5 m) with walls on three sides was filled with alfalfa. At filling, loads were placed on alternating sides of the silo. One tractor (6.6 Mg, single-wheeled, front-mounted bucket) was used to spread and pack all loads. The tractor operator was instructed to spend a similar amount of time spreading loads on both sides, but pack one side approximately twice (2X) that of the other (1X). During filling, we recorded load weight, the amount of time spent spreading and packing each load, and the distance lengthwise over which the load was spread. A grab sample from each load was analyzed for DM content. After each load was packed, the height of the crop on the side and back walls at each wall panel joint was recorded to estimate filled volume and silage density throughout filling. Final volume was more accurately estimated by elevation transects across the silo width at each panel in addition to wall heights.

Two bunker silos (21.3 x 5 x 3.5 m) were filled simultaneously with whole-plant corn with each silo receiving alternate loads. The same 6.6 Mg, single-wheeled tractor was used to spread loads in each silo. This tractor also packed one silo, but a 9.7 Mg, single-wheeled tractor was used to pack the other. The tractor operator was instructed to keep spreading and packing times similar for both silos. The measurements and procedures were the same as those used in the alfalfa trial.

During silo emptying, core samples were taken across the face on two different dates to estimate density with height. For a given treatment and date, two profiles were taken, coring at 5 heights (0.5 m from floor, 0.5 m from the top, and three equidistant intermediate points). Approximately a 30-cm deep core was taken with a 5.1 cm diameter corer, measuring the actual depth, weighing the core sample, and measuring DM content. This permitted calculation of wet and DM density.

Results and Discussion

In the alfalfa trial, spreading time was 70 s/Mg as fed on both sides whereas packing time was 48 and 86 s/Mg on the 1X and 2X sides, respectively. The estimated DM densities during filling were similar between the two sides, starting at approximately 160 kg/m^3 after packing the first load and increasing nonlinearly with additional loads to approximately 300 kg/m^3 at the end of filling. Based on final volume estimates, 166 t DM were packed into 552 m^3 on the 1X side for a DM density of 301 kg/m^3. On the 2X side, 162 t DM were packed to a density of 292 kg/m^3. These final densities assumed that no forage from a load was spread onto the opposite side. Some mixing was inevitable, but visually the packing tractor operator did not appear to favor one side. To test this, core samples were taken at two different times during emptying. Densities near the floor were similar between the two sides (Table 1); however, the densities at the two highest locations were lower on the 1X side. Averaging the core values, the 2X side had a mean DM density of 270 kg/m^3 and the 1X side a density of 256 kg/m^3.

In the two corn silage bunkers, the spreading time was similar for both bunkers (22 and 24 s/Mg as fed) whereas packing time was greater for the bunker with the heavier packing tractor (25 vs. 33 s/Mg). Despite differences in tractor weight and packing time, the final average DM densities based on volume and mass ensiled were both 236 kg/m^3. In part, this may be explained by the rear axle on the heavier tractor that extended approximately 30 cm beyond the tire, preventing the tire from running against the wall. Also, the front tires on the heavier tractor were further behind the front of the tractor so it was not able to drive as close to the back wall. Core samples taken 1.8 m from each sidewall did show differences in density between the two bunkers (Table 2). Densities 46 cm below the top were similar in both silos. However, densities at deeper locations were significantly higher in the silo where the heavier tractor was used for packing. Averaging these core densities, the average DM densities were 221 and 248 kg DM/m^3 for the lighter and heavier packing tractors, respectively.

The average core DM densities in both trials were compared with model predictions based on an earlier survey of 168 commercial bunker silos. The model predicted lower densities than measured. However, the differences in density due to the treatments were slightly underpredicted by the model, but the discrepancies were within prediction and measurement errors.

Table 1. Average core densities (kg DM/m^3) at different heights from the floor during emptying of the alfalfa bunker silo, one half packed longer than the other.

Height, m	1X[a]	2X
0.50	334	331
1.15	320	308
1.75	248	258
2.40	211	265
3.00[b]	166	191
Average	256	270

[a] 1X – 1.97 min/Mg, 2X – 2.60 min/Mg average spreading and packing time.
[b] 0.5 m below the top of the silage.

Table 2. Average core densities (kg DM/m^3) at different heights from the floor during emptying of two corn bunker silos, packed with different weight tractors.

Height, m	6.6 Mg	9.7 Mg
0.46	235	316
1.07	239	252
1.67	240	256
2.28	203	231
2.89[a]	190	187
Average	221	248

[a] 0.46 m below the top of the silage.

Conclusions

In two field trials, we tested the effects of packing time and tractor weight on density in bunker silos. In alfalfa, increasing total spreading and packing time from 1.97 to 2.60 min/Mg alfalfa increased average density from 256 to 270 kg DM/m^3. In whole-plant corn, using a 9.7 Mg packing tractor vs. a 6.6 Mg tractor increased density from 221 to 248 kg DM/m^3. These differences were consistent with expectations from earlier survey and pilot-scale research.

Inoculant Effects on *in vitro* Digestion of Alfalfa Silage

R.E. Muck, F.E. Contreras-Govea, I. Filya, D.R. Mertens and P.J. Weimer

Introduction

Inoculants (additives supplying principally lactic acid bacteria) are the most common additives used in making silage in the United States. Most inoculants contain homofermentative lactic acid bacteria that shift fermentation toward lactic acid production, reduce pH, improve dry matter (DM) recovery from the silo and may improve milk production or rate of gain. While inoculant effects on fermentation and DM recovery are understood, the effects on animal performance are often greater than expected from the shifts in fermentation. *In vitro* analyses may help uncover how inoculants may affect rumen fermentation and ultimately dairy cattle performance.

Methods

Alfalfa was ensiled in two trials [first (48% DM) and second cut (39% DM)] in 2003. Alfalfa harvested with standard field equipment was brought back to the laboratory and ensiled in 1-l and 500-ml glass Weck jars in first and second cutting, respectively, at a density of 500 g/l. Each trial had four treatments (uninoculated control, 3 inoculants), four silos per treatment. All inoculants were applied at 10^6 colony-forming units (cfu)/g crop (not label rates) to help insure domination of fermentation. Silages were stored for a minimum of 30 d at room temperature (~22°C). Silages were analyzed for pH, DM concentration and fermentation products. A portion of silage was wet ground in a Büchi mixer and frozen until analyzed. *In vitro* fermentation was carried out on 1-g samples of the wet-ground silage in 160-ml serum bottles. *In vitro* analysis was carried out at 39°C, and gas pressure was measured at 3, 6, 9, 24, 48 and 96 h. At 9 h, 4 bottles of each treatment were sampled for gas composition and then opened and analyzed for pH, volatile fatty acids, and microbial biomass yield. At 96 h, two bottles were analyzed similarly to the 9-h bottles. Duplicate sets of *in vitro* analyses were performed on all silages.

Results and Discussion

Average silage characteristics are shown in Table 1. In first cut, all three inoculants overwhelmed the natural population [5.2 $\log_{10}$(cfu/g)], producing significantly ($P<0.05$) lower pH values and residual water soluble carbohydrates and higher lactic acid concentrations. In second cut, the alfalfa contained 7.4 $\log_{10}$(cfu/g) lactic acid bacteria, and there were fewer significant differences in silage quality between the control and inoculated treatments. The 1174 treatment had a reduced pH, lower acetic acid and ethanol concentrations than the control. MTD1 had a reduced acetic acid concentration. Based on silage fermentation, larger *in vitro* fermentation effects were expected in first cut.

The majority of *in vitro* fermentation occurred in the first 9 h, and IVTD was above 700 and 780 g/kg DM in all treatments in first and second cut, respectively (Table 2). There were no significant effects of treatment on IVTD although MTD1 was numerically greater than control in both trials. Carbon dioxide production (mg/100 mg truly digested DM) was reduced in the inoculated silages relative to control silages except for 1174 in the second cut. There was a trend for lower methane production in inoculated silages in first cut, but inoculated silages produced significantly higher levels of methane in the second cut. In first cut, total VFA production (mg/100 mg TDDM) was similar across treatments, but all three inoculated treatments produced *in vitro* fermentations with a shift toward propionic acid production. In second cut, there was a trend for reduced VFA production in the inoculated silages although acetic-to-propionic acid ratio was not affected by treatment. Microbial biomass yield (MBY) was measured by the difference between true and apparent digestibility. It was also estimated by subtracting gas and VFA production from the total DM digested. In first cut, MBY was unaffected by treatment when measured by the differences in true and apparent digestibility, but MTD1 and *L. pentosus* treatments had higher MBYs than the control estimated by digestion products. In second cut, MTD1 and *L. pentosus* produced higher MBYs than control for both estimation techniques.

Conclusion

In two alfalfa silage trials, silage fermentation was substantially affected by inoculant treatment in one trial but not the other. In the trial where silage fermentation was substantially altered, *in vitro* VFA production was shifted to propionic acid in the inoculated silages. However, in both trials two of three inoculated silages produced *in vitro* fermentations with increased microbial biomass yields and reduced total gas production relative to controls. This provides evidence that inoculants can produce ruminal effects in absence of substantial effects on silage fermentation.

Table 1. Silage characteristics (g/kg DM except as noted) from the two trials.

Treatment	DM, g/kg	pH	Lactic	Acetic	Ethanol	WSC*	CP	NDF
First Cut								
Control	480	5.08	40.5	14.2	3.1	18.4	215	419
1174	476	4.50	83.5	17.3	1.3	9.9	220	436
MTD1	463	4.51	72.6	16.3	2.0	8.8	215	434
L. pentosus	463	4.66	76.2	36.8	3.6	7.2	231	421
s.e.	3.0	0.016	2.17	1.05	0.41	0.72	3.3	6.7
Second Cut								
Control	368	4.42	86.5	29.0	4.5	6.8	238	307
1174	377	4.34	81.3	18.4	3.4	9.9	240	293
MTD1	363	4.40	80.5	19.0	4.1	8.8	247	292
L. pentosus	365	4.46	86.0	31.7	5.5	7.2	239	309
s.e.	5.1	0.022	1.13	1.19	0.32	0.61	2.8	5.5

* WSC – water soluble carbohydrates; CP –crude protein; NDF – neutral detergent fiber.

Table 2. *In vitro* digestion characteristics (mg/100 mg truly digested DM, except as noted) after 9 h incubation from the two trials.

Treatment	IVTD* g/kg DM	Gas, ml/100 mg TDDM	CH_4	CO_2	VFA	MBY	MBY_{est}	Acetic: Propionic, Mol/Mol
First Cut								
Control	715	17.9	1.56	26.4	34.5	34.8	37.5	3.07
1174	713	16.9	1.55	24.8	35.9	34.0	37.7	2.68
MTD1	723	15.8	1.47	23.2	34.2	32.5	41.1	2.68
L. pentosus	703	15.9	1.49	23.3	34.4	32.8	40.8	2.65
s.e.	4.7	0.34	0.036	0.50	0.69	0.75	0.94	0.039
Second Cut								
Control	786	18.9	1.46	28.5	37.5	37.7	32.5	2.64
1174	786	19.1	1.73	28.1	35.2	39.4	34.9	2.66
MTD1	804	18.2	1.73	26.6	34.1	41.4	37.6	2.65
L. pentosus	807	17.6	1.66	25.7	33.9	43.4	38.7	2.66
s.e.	8.5	0.27	0.030	0.41	1.22	1.25	1.47	0.058

* IVTD – *in vitro* true digestibility; VFA – total volatile fatty acids; MBY – Microbial biomass yield determined by the difference in *in vitro* true digestibility and apparent digestibility; MBY_{est} – Microbial biomass yield as estimated by 100 – CH_4 – CO_2 –VFA.

Plant Chemistry/Biochemistry

Polyphenol Oxidase Activity and *In Vitro* Proteolytic Inhibition in Grasses

J.M. Marita, R.D. Hatfield, G. Brink

Introduction

Harvesting and storing high quality forage in the cool humid regions of agricultural production remains a challenge due to the potential high degree of protein degradation during ensiling. Red clover is an exception with high protein levels at harvest that are maintained during ensiling. Decreased proteolytic activity in red clover is due to polyphenol oxidase (PPO) activity and appropriate *o*-diphenol substrates. This project was undertaken to determine if PPO activity is present in a range of grasses and the potential role in proteolytic inhibition in the presence of the *o*-diphenol caffeic acid.

Methods

Fifteen grass species were established in the greenhouse under a 14/10 h (day/night) lighting regime. Leaf blades were harvested from plants at the vegetative stage, frozen in liquid nitrogen and stored at -80°C until processed. Individual samples were processed and analyzed following established laboratory methods. PPO activity was determined in duplicate using *o*-diphenol substrates (caffeic acid, chlorogenic acid and catechol; 2mM final concentration). To determine the potential impact of PPO and *o*-diphenols on proteolytic activity leaf blades were prepared similar to above. Two samples

were prepared with one processed through a G-25 sephadex spin column to remove low molecular weight materials and the other clarified by centrifugation and removal of the supernatant. At time zero, caffeic acid (3 mM final concentration) was added to the eluted protein (spin column). Aliquots were removed from each sample at specific time intervals (t_0, t_1, t_2, t_4 and t_{24} hours) and soluble amino acids and small peptides were quantified to assess the degree of proteolysis.

Results and discussion

The amount of PPO activity measured in each grass varied significantly depending upon the species and upon the specific type of *o*-diphenol used as the primary PPO substrate (Table 1). Orchardgrass, meadow fescue, ryegrass, and smooth bromegrass exhibited the highest PPO activities with 10- to 1000-fold increased activity compared to the remaining species. Chlorogenic acid and/or caffeic acid were the preferred substrates, although there were differences among the most active grasses as to which was the best utilized. These results suggest potential differences in PPO enzymes among the individual grass species. Generally in the grasses with substantial PPO activity, the addition of caffeic acid to the isolated grass extracts resulted in proteolytic inhibition. Such results suggest that several of the important grass species that were examined contain PPO activity, but may lack the appropriate *o*-diphenol substrates *in vivo* to effectively inhibit proteolysis. Initial results suggest that proteolytic inhibition can be achieved with the addition of caffeic acid.

Conclusion

PPO activity is present in a variety of grass species; however, the level of PPO activity varies up to 1000-fold depending on the *o*-diphenol substrate supplied. *In vitro* proteolytic assays indicate that there is an inhibition of proteases in the presence of specific *o*-diphenols in several grass species. Together these results suggest a potential for improvement in ensiling of specific grass species with the addition of an appropriate *o*-diphenol substrate.

Table 1. PPO activity in 15 grass species in the presence of three representative *o*-diphenol substrates and the percent reduction in *in vitro* proteolysis with the addition of caffeic acid after 24 h.

Grass Species	Polyphenol Oxidase Activity (mmoles/mg/min) x10^{-4}			Reduction in *In Vitro* Proteolysis (24 h)
	Caffeic Acid	Chlorogenic Acid	Catechol	
Orchardgrass	110.00	260.00	17.00	60%
Ryegrass	26.00	130.00	7.90	78%
Smooth bromegrass	8.20	41.00	8.70	99%
Meadow fescue	35.00	41.00	9.00	90%
Tall fescue (soft)	3.20	2.90	0.89	5%
Timothy grass	1.50	2.20	1.10	6%
Quackgrass	1.00	1.60	2.10	20%
Tall fescue	1.10	0.56	0.45	55%
Reed canary grass	0.51	0.34	0.31	49%
Kentucky bluegrass	0.60	0.33	0.70	24%
Winter wheat 1	0.25	0.18	0.25	16%
Rye	0.26	0.17	0.42	0%
Oat	0.26	0.17	0.19	0%
Spring wheat	0.21	0.10	0.26	8%
Winter wheat 2	0.11	0.10	0.21	15%

A Plate-Based Browning Assay to Screen Alfalfa Germplasm for Polyphenol Oxidase Activity and *o*-Diphenols

M.L. Sullivan and A. Bringe

Introduction

We have recently demonstrated that the action of polyphenol oxidase (PPO) on *o*-diphenols can significantly inhibit post-harvest proteolysis both in plant extracts and in ensiled macerated tissue. Although PPO is nearly ubiquitous among plants, the level of expression and the tissues in which it is expressed can vary significantly. One or more PPO genes are present in *Medicago sativa* cv. Saranac, but their expression appears to be limited to flowers and seedpods. Further, we have failed to detect significant amounts of PPO *o*-diphenol substrates in the leaves of a limited number of examined alfalfa cultivars. Although we have produced genetically modified alfalfa plants that produce PPO in their leaves, and the required biosynthetic pathways to produce foliar *o*-diphenols could potentially be engineered into alfalfa, identification of alfalfa germplasm expressing PPO and/or *o*-diphenols in its leaves would allow these traits to be introduced by conventional breeding and avoid the technical and regulatory burdens associated with genetically modified crops. Identification of PPO- and *o*-diphenol-producing germplasm in other forage crops (e.g. grasses) would be of benefit as well. Here we report the development of a simple but sensitive assay for PPO and *o*-diphenol PPO substrates based on extract browning and/or color changes associated with this enzyme/substrate combination.

Methods

Transgenic alfalfa expressing a red clover PPO gene (PPO-alfalfa), non-transformed alfalfa (control-alfalfa), and red clover silenced for foliar PPO expression were used to develop our assay and assess its sensitivity. Additionally, four plants from each of nearly 200 accessions of a core collection of perennial *Medicago* germplasm were screened with the validated assay. All plants were grown under controlled conditions in a growth chamber. Approximately 200 mg of leaf tissue was harvested, frozen in liquid nitrogen, and powdered using a bead-beater. The tissue was extracted with 50 mM Tris-Acetate, pH 7.5 (5 mL per g fresh weight) and clarified by centrifugation. A typical assay for each extract utilized five wells of a flat-bottom 96-well plate. Each of the five wells contained 100 µL of extract and one of the following additions: 5 µL of 100 mM caffeic acid, catechol, or (-)-epicatechin in ethanol; 5 µL ethanol; 10 µL PPO-alfalfa extract (to provide a source of PPO to test for endogenous *o*-diphenols in the absence of endogenous PPO). Every reaction plate also had PPO-alfalfa and non-transformed alfalfa extracts subjected to the treatments as positive and negative controls, respectively. The reaction plate was incubated at 30°C for 4 h then scanned on a flatbed scanner. Extent of browning for each well was analyzed by determining image intensity (sometimes called image volume, the integration of pixel value over the area of each well) for the yellow channel of the CMYK plate images using image quantification software. Each well was assigned a browning value (BV) based on the image intensities of the experimental wells and their corresponding positive and negative controls using the following formula:

$$BV=(Intensity_{Experimental}-Intensity_{Negative})/(Intensity_{Positive}-Intensity_{Negative})$$

Results and Discussion

Although we had previously used extract color changes or browning as a crude measurement of PPO and *o*-diphenol content, we sought to make the methodology more amenable to screening large numbers of plant extracts under various conditions and to develop a more objective measure of extract color change. We utilized extracts of PPO-expressing transgenic and control alfalfa to develop an assay utilizing a 96-well flat-bottom plate with data collection by scanning on a flatbed scanner and subsequent image analysis with image analysis software to quantify extract color changes. Analysis of scanned images proved superior to our initial attempts using spectroscopic methods because much of color change observed in extracts was associated with insoluble material that was not easily solubilized (data not shown). To assess the sensitivity of the assay for PPO activity, we made a series of dilutions of PPO-alfalfa extract, whose PPO activity is comparable to that of red clover extracts, in control-alfalfa extract and assessed the color change produced with three different *o*-diphenols or an ethanol control (Fig. 1A). The selected *o*-diphenols are diverse in chemical structure and how efficiently they are utilized by various characterized PPO enzymes, and in the case of caffeic acid (and its derivatives) and epicatechin, represent *o*-diphenols that might be reasonably available for practical applications of the PPO/*o*-diphenol system for preservation of protein in silage. As shown in Fig 1A, a color change was easily detected by simple visual inspection with caffeic acid and epicatechin at dilutions of 100-, and 10-fold, respectively, of the PPO-extract (BV=0.53 and BV=1.00, respectively). With catechol, color change was only apparent by visual inspection in undiluted PPO-alfalfa extract (BV=1.00), likely due to both poor utilization of this substrate by this particular PPO and formation of a less highly colored product. Relying on BV instead of visual evaluation alone may add slightly to the sensitivity of the assay, since for catechol and epicatechin, BV of 0.42 and 0.23 were obtained for the 10 and 100-fold dilutions of the PPO-alfalfa extracts, respectively. We also assessed the sensitivity of the assay for detection of *o*-diphenols in plants lacking endogenous PPO activity by making a series of three-fold dilutions of either PPO-silenced red clover extract (to represent a mixture of *o*-diphenols that might be found in a plant) or caffeic acid in control-alfalfa extract. We added 10 µL of PPO-alfalfa extract to provide a source of PPO, or control-alfalfa extract as a negative control. As shown in Fig. 1B, by simple visual inspection, *o*-diphenol content approximately one-third that found in red clover (BV=0.74) or 1 mM caffeic acid (BV=0.37) can be easily detected in our assay. This level of caffeic acid would correspond to approximately 5 µmol caffeic acid per g fresh weight. In several replicates of these experiments, we found we could consistently detect PPO activity approximately 10-100-fold lower than that seen in red clover (depending on the substrate used) and *o*-diphenols approximately three-fold lower than that seen in red clover.

We next used the assay to screen a core collection of perennial *Medicago* germplasm. This collection is comprised of approximately 200 accessions selected to represent the genetic diversity of alfalfa and sexually compatible relatives. For each accession, extracts were prepared from four individuals and assayed as described above for PPO activity with caffeic acid, catechol, and (-)-epicatechin, and for *o*-diphenols with exogenously added PPO. Each assay plate also contained extracts of PPO- and control-alfalfa as positive and negative controls, respectively for PPO activity. For our primary screen, individuals with a browning value ≥0.20 were considered positive. This relatively low threshold was selected to avoid discarding true PPO- or *o*-diphenol-positive plants, even though it was likely many false positives would be identified. From this primary screen, fewer than 20 putative PPO-positive individuals and no putative *o*-diphenol-positive individuals were identified. No browning was detected, however, in repeat assays of the putative positive individuals. This result indicates there are no useful levels of foliar PPO or *o*-diphenols in the tested alfalfa germplasm, and that conventional breeding approaches will likely not be useful for introducing the PPO/*o*-diphenol system into alfalfa.

Conclusions

We have developed a quick, relatively sensitive plate-based browning assay for screening plant materials for PPO and *o*-diphenols. Using the assay, we have failed to find useful levels of foliar PPO and *o*-diphenols in a collection of perennial *Medicago* germplasm, indicating these traits will not easily be introduced into alfalfa by conventional breeding approaches.

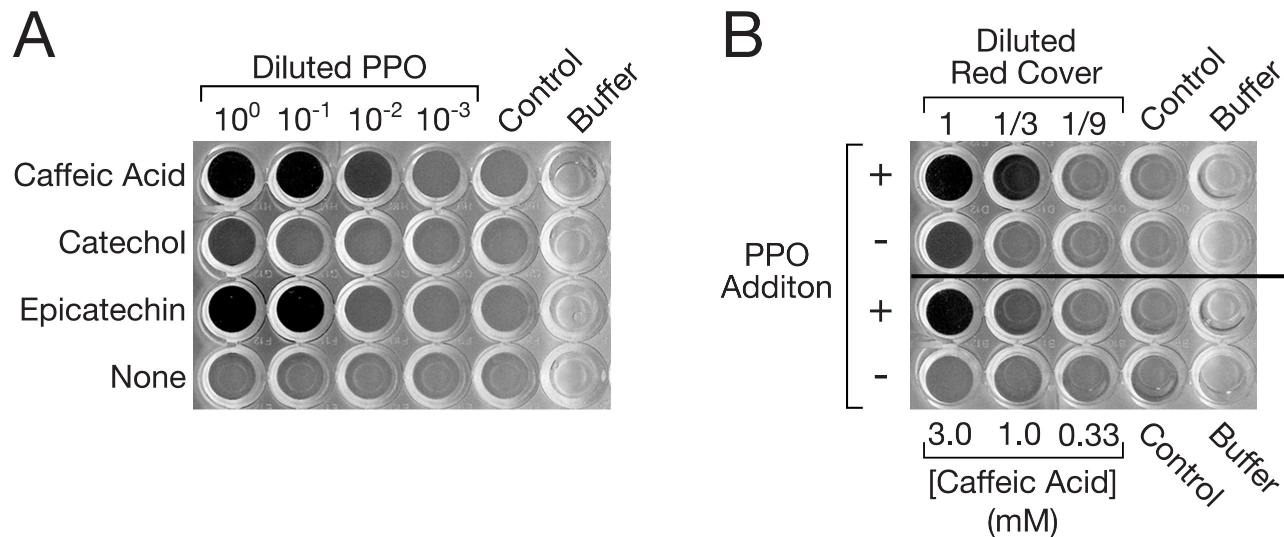

Figure 1. Sensitivity of a plate-based browning assay for PPO and *o*-diphenols. Browning reaction assay plates were incubated at 30°C for 4 hours. Following incubation, plates were imaged on a flatbed scanner. The yellow channel of the resulting CMYK image is shown. (A) Ten-fold serial dilutions of PPO-alfalfa extract in control-alfalfa extract, control-alfalfa extract, or extraction buffer were incubated in the presence of 5 mM *o*-diphenol as indicated. (B) Three fold serial dilutions of PPO-silenced red clover extract (top) or caffeic acid (bottom) in control alfalfa extract, control alfalfa extract, or extraction buffer were incubated with and additional 10 µL of either PPO-alfalfa (+) or control-alfalfa (-) extract as indicated.

Evaluation of Forage Grasses for *o*-Diphenols

J.M. Marita, R.D. Hatfield, G. Brink

Introduction

In many agricultural regions preservation of high quality forages for livestock feed is a challenge due to limited drying conditions. This results in the use of ensiling techniques to preserve high quality forage. Forage grasses and ensiling are frequently used in the cooler northeastern and upper northwestern regions of the US and in the humid regions of Europe. One of the drawbacks of ensiling forage is the typical high loss of protein during the ensiling process. Protein degradation is due to native plant proteases being released once the forage has been cut and partially dried. The proteolysis can continue into the ensiling process depending upon how fast and how low the pH can be decreased by the ensiling process. There are post harvest treatments, mostly addition of silage additives or direct application of acid solutions, to aid in a rapid decrease in pH. Red clover is an exceptional forage that proteolytic activity is inhibited during ensiling irrespective of pH levels. This loss of proteolytic activity is due to active polyphenol oxidase (PPO) and non-limiting levels of *o*-diphenols (substrates for the PPO). A project was undertaken to determine the presence of PPO activity and *o*-diphenols in ten grass species. This is a report in the identification of *o*-diphenols caffeic acid and chlorogenic acid in these grasses.

Materials and Methods

Ten forage grasses (Table 1) were established in the greenhouse and in replicated field plots at Arlington, WI. Fresh leaf blades were harvested, frozen in liquid nitrogen, and stored at -80 °C until processed. Processing consisted of grinding 5g samples in a mortar and pestle under liquid N_2. Ground samples were immediately extracted with a solution of deionized water and methanol (H_2O:CH_3OH, 80:20, 3 mL/1g) and transferred to Oakridge tubes. Capped tubes were shaken at 45 °C for 20 min before centrifuging to pellet insoluble materials. Supernatants were removed and immediately passed through a ENVI-18 solid phase extraction column (20 mL capacity). The columns were washed with 10 mL of acidified H_2O (pH adjusted to < 3 with TFA) to remove unbound materials and the wash collected. Bound phenolics were eluted with 6 mL of methanol. Total UV spectra (250 nm to 450nm) were taken of the original extracts, flow through from the ENVI-18 columns, water washes, and the methanol eluted phenolics. Subsamples were taken of the methanol recovered phenolics and analyzed as silylated derivatives by gas chromatography-mass spectroscopy for the presence of caffeic and chlorogenic acids.

Results and Discussion

Previous work with the ten grass species used in this study (Table 1) revealed that buffer extracts of ground leaf tissue of some species undergo browning reactions. Browning reactions are typically associated with polyphenol oxidase activity (PPO) and presumably the presence of *o*-diphenols needed by the enzyme. This work was undertaken to determine if grasses with PPO activity contained *o*-diphenols that could be involved in the browning reaction. Numerous *o*-diphenols have been identified in plants, but the most prominent are caffeic acid and chlorogenic acid (a quinic acid ester of caffeic acid). Methanol:water extracts contained a wide range of phenolic materials based on UV absorption spectra (data not shown). However, only timothy and tall fescue contained high levels of chlorogenic acid (Table 1). Caffeic acid was detected in several grasses (perennial ryegrass, Kentucky bluegrass, tall fescue, and timothy), but only at the trace level. Interestingly those grasses with medium to high levels of PPO activity contained either no detectable or only trace levels of caffeic and chlorogenic acids. All of the PPO containing grasses typically brown rapidly upon extraction and exposure to air

suggesting that either other *o*-diphenols (as yet unidentified) are present or there are other factors operating to cause the browning reaction.

Conclusions

Caffeic acid and chlorogenic acid are common *o*-diphenols found in plants and are typical substrates for polyphenol oxidase. Of forage grasses analyzed in this study only tall fescue and timothy contained significant levels of chlorogenic acid. Trace amounts of caffeic acid were detected in four of the remaining grasses. This may not be too surprising as caffeic acid is an important intermediate in phenylpropanoid metabolism and one would not expect large pools of it to accumulate in plants. The lack of chlorogenic and caffeic acid in those grasses with good PPO activity (low-medium) indicates other compounds may be the primary substrates for this enzyme.

Table 1: Comparison of ten forage grasses for polyphenol oxidase activity (PPO) and *o*-diphenol substrates (caffeic and chlorogenic acids).

Forage	Scientific Name	PPO Activity[1]	Caffeic Acid[2]	Chlorogenic Acid[2]
Orchardgrass	*Dactylis glomerata* L.	high	ND	ND
Bromegrass	*Bromus inermis* Leyss.	medium	ND	low
Meadow fescue	*Bromus biebersteinii* Reom&Schult	medium	ND	Trace
Perennial Ryegrass	*Lolium perenne* L.	medium	Trace	Trace
Reed Canary grass	*Phalaris arundinacea* L.	trace	ND	ND
Kentucky Bluegrass	*Poa pratensis* L.	trace	Trace	Trace
Tall Fescue	*Festuca arundinacea* Schreb.	trace	Trace	high
Tall Fescue (soft)	*Festuca arundinacea* Schreb	trace	ND	ND
Timothy	*Phelum pratense* L.	trace	Trace	high
Quackgrass	Elytricga repens L.	trace	ND	ND

[1] PPO activity is based on scale comparable to red clover. High PPO activity being equal to or greater than (0.1 mmoles/mg/min), medium is 35-65% of this level, low is 10-30% ofof this level, and trace is detectable activity, but is similar to what is found in most plants (<1-2%). [2]Levels of caffeic acid and chlorogenic acid is on a relative scale, high=10-40µmoles/gram fresh weight, medium= 3-8µmoles/gFW, low=<2 µmoles/g FW. Trace= detectable but at levels that are difficult to accurately quantify. ND= not detected.

Relationships of Lignin and Ferulate Cross-Linking with Cell Wall Degradability of Maize Stem Internodes

H.G. Jung

Introduction

Scientists at the USDFRC have conducted extensive research on the impact of ferulate cross-linking of lignin to arabinoxylan on the potential digestibility of grasses by ruminants. Research using a model cell wall system has shown that increased ferulate cross-linking is associated with reduced susceptibility of the cell wall polysaccharides to enzymatic breakdown. Smooth bromegrass (*Bromus inermis* Leyss.) plants identified as being low in ferulate ether cross-linking had higher in vitro ruminal neutral detergent fiber (NDF) digestibility. However, other studies have found poor correlations of ferulate cross-linking with cell wall degradability, particularly with more mature forage samples. Recently it was shown that ferulate cross-links are deposited in the potentially degradable secondary wall material of maize (*Zea mays* L.) as well as in the non-degradable primary wall, further complicating the topic. The objective of the current study was to describe how the impact of ferulate cross-linking on cell wall degradability may change during the course of stem development.

Methods

The fourth elongated, above-ground internode was collected from three unrelated maize hybrids (A632 x A619, A679 x FR481, and Mycogen 2677) in a replicated field trial conducted at St. Paul, MN in 1998 and 1999. Samples were harvested at 10 stages of development beginning in June when the internode was immature and actively elongating through full physiological maturity of the maize in October. Ground internodes were analyzed for cell wall polysaccharides, Klason lignin and ferulate ethers, the only form of ferulate cross-links that can currently be quantified. The internodes were also incubated in vitro for 24- and 96-h with rumen fluid and the proportion of cell wall polysaccharides degraded was calculated from the residues.

Results and Discussion

The cell wall polysaccharides of immature, elongating maize stem internodes were degradable after 24- and 96-h in vitro incubations with rumen fluid for the first three harvests. Degradability began to decline by the fourth harvest date and continued to decline until the eighth harvest. There was a strong negative relationship of cell wall polysaccharide degradability with both Klason lignin and ferulate ether concentrations in the cell wall; however, the pattern of these relationships differed (Fig. 1). From harvests four through 10, Klason lignin was linearly related to cell wall polysaccharide degradability, but internodes from the first three harvests did not follow this pattern (Fig. 1a). Ferulate ether concentration was related to cell wall polysaccharide degradability in a curvilinear pattern, increasing in negative effect at later maturity stages (Fig. 1b). In direct contrast to the pattern seen for Klason lignin, it was the most mature internodes from harvests eight through 10 that did not follow the ferulate ether trend with degradability. When frequency of ferulate cross-linking was calculated as ferulate ethers per unit Klason lignin, a very unique pattern with cell wall polysaccharide degradability was observed (Fig. 2). During the elongation phase of internode development in maize, frequency of ferulate cross-linking was negatively related to cell wall polysaccharide degradability. Immediately after internode elongation ended, this relationship reversed direction and became positive. As reported in the USDFRC 2003 Research Report, the bulk of both Klason lignin and ferulate ethers were deposited after elongation ended and during tissue maturation in maize internodes. However, the rate of lignin addition was higher than the deposition rate for ferulate cross-links, accounting for the reduced

frequency reported here. Obviously the impact of ferulate cross-linking on cell wall degradation is complex and not easily predicted.

Conclusion

The results of this study suggest that the earlier success in identifying smooth bromegrass plants with higher in vitro NDF digestibility by selecting individuals with low concentrations of ferulate cross-links was due to the selection protocol employed. In the previous study smooth bromegrass plants were evaluated during the vegetative stage of development when plant tissues were relatively immature. The current results suggest that selection for low ferualte cross-linking in mature grasses will not be successful in identifying plants with higher cell wall degradability. The poor correlations of cell wall degradability with ferulate cross-linking found in other studies support this conclusion.

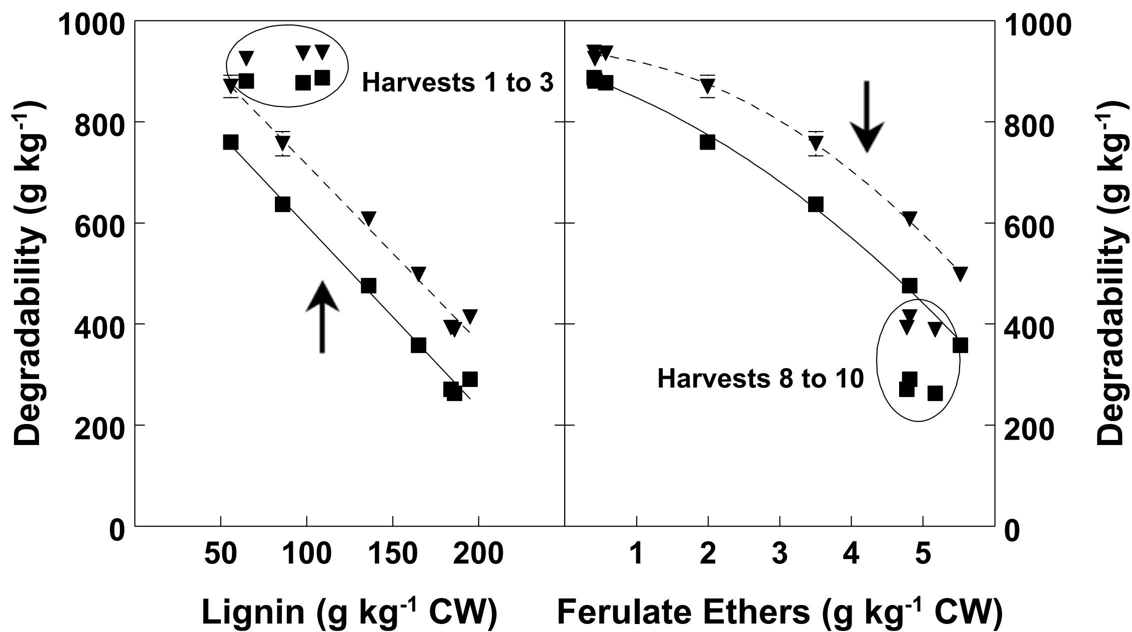

Figure 1. Relationships of in vitro ruminal degradation of cell wall polysaccharides with concentrations of Klason lignin (**a**) and ferulate ether cross-links (**b**) for maize stem internodes harvested at 10 stages of development. The arrows indicate the approximate time when internode elongation ceased.

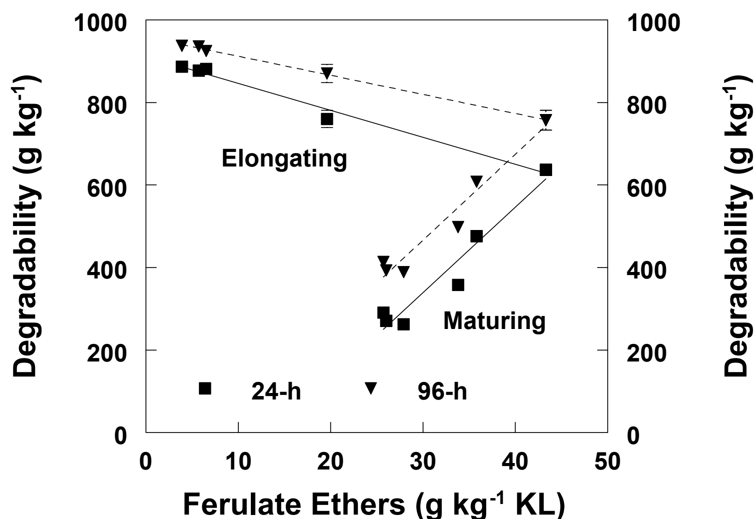

Figure 2. Relationships of cell wall polysaccharide degradability with the frequency of ferulate cross-links (ethers) to Klason lignin (KL) in a developing maize stem internode. The line breakpoints are near the end of internode elongation.

Simplified Preparation of Coniferyl and Sinapyl Alcohols

H. Kim and J. Ralph

Introduction

Synthesis of high-quality monolignols, the 4-hydroxycinnamyl alcohols (coniferyl and sinapyl alcohols), has been an important step for successful preparation of lignans, synthetic lignin model compounds, and synthetic lignins or dehydrogenation polymers (DHPs).

Since 4-hydroxycinnamaldehydes (coniferaldehyde and sinapaldehyde) have become commercially available, several borohydride reagents have been used to prepare 4-hydroxycinnamyl alcohols by one-step reductions instead of the multi-step syntheses previously used. However, all of the methods require handling moderately reactive and toxic reagents, and the quality of the product depends on non-trivial work-up steps. Time consuming reactions can also be a frustration. Since Gibson and Baily first reported the preparation and use of borohydride exchange resin (BER) in 1977, it has been used to reduce a variety of functional groups. Regioselective reduction of carbonyl compounds in alcoholic solvents is one of its most important uses. BER provides many of the practical advantages of other polymer-supported reagents in organic reactions. BER is more stable than sodium borohydride itself and easier to handle. Coniferyl and sinapyl alcohols are commercially available now, but are 8 to 10 times more expensive than 4-hydroxycinnamaldehydes. Even considering the expenses for resin and solvents, the total cost of synthesizing the 4-hydroxycinnamyl alcohols is 2 to 3 times lower than for the commercial products. More importantly, the purchased products are sometimes of vastly inferior quality, often containing degradation products, particularly in the case of sinapyl alcohol. Obliviously using these compounds without carefully checking their purity first has caused problems for several researchers.

Here we report a simple protocol that produces clean coniferyl or sinapyl alcohol from coniferaldehyde or sinapaldehyde using BER. This method is efficient, with an unfortunately required but simple filtering step, and can be safely performed without complication within short periods of time to produce fresh, clean 4-hydroxycinnamyl alcohols.

Materials and Methods

Synthesis of Coniferyl Alcohol 2a. Borohydride exchange resin (BER; 22.5 g, 56.1 mmol, 2 eq.) was introduced into a round-bottom flask (300 mL), and washed with methanol (150 mL × 3) before the reaction. Methanol was decanted from the resin after each wash cycle. Fresh methanol (200 mL) was added to the flask and then solid coniferaldehyde **1a** (5 g, 28.1 mmol, 1 eq.) was added directly to the mixture. The reaction mixture was stirred at room temperature for 1 h. The initial intense yellow color of the reaction solution faded to a pale yellow and the color of resin darkened during the reduction. The reaction may be monitored by TLC ($CHCl_3$: EtOAc, 1:1; R_f = 0.32). The resin was filtered through a plug of cotton wool in the stem of a funnel (or through a sintered glass filter) and washed with methanol (100 mL × 3). The filtrate was concentrated to a yellow oil with a rotary evaporator. Clean-up by passing through silica gel was accomplished as follows. Silica gel (11 g) was introduced into a 60 mL sintered glass filter funnel (medium porosity) and washed with ethyl acetate (50 mL x 3), discarding the eluant. The crude product was dissolved in ethyl acetate (30 mL), and the cloudy solution transferred via pipette onto the silica gel surface. Without allowing it to dry, the product was eluted with ethyl acetate (50 mL x 3). The collected eluant was evaporated, and spontaneously yielded a pale yellow crystalline mass (2.36 g, 13.1 mmol, 47 %). NMR showed that the product contained only a trace of 1,4-reduction product **3a** which was also detected at low levels (less than 0.03%) by GC-MS. Recrystallization may be carried out from dichloromethane/petroleum ether and afforded pale yellow needles: mp 73-75 °C.

Small-scale preparation (coniferaldehyde **1a**, 205 mg, 1.15 mmol, 1 eq.; BER, 920 mg, 2.3 mmol, 2 eq.) was performed as for the large-scale synthesis. Silica-gel clean-up in this case was most conveniently carried out using commercial 3 mL silica gel solid phase extraction (SPE) tubes (first washing with 5 mL ethyl acetate, then applying the crude product in 5 mL ethyl acetate, and eluting with 25 mL ethyl acetate). A pale yellow crystalline mass (128 mg, 0.71 mmol, 62%) was obtained.

Synthesis of Sinapyl Alcohol 2b. Sinapaldehyde **1b** (5 g, 24 mmol, 1 eq.) was reduced by BER (19.2 g, 48 mmol, 2 eq.) in methanol, as described for coniferaldehyde **1a**. It was stirred for 3 h at room temperature to complete the reaction, and the reaction was monitored by TLC ($CHCl_3$: EtOAc, 1:1; R_f = 0.29). Following analogous filtering, sinapyl alcohol **2b** (3.64 g, 17.32 mmol, 72%) was obtained as a pale yellow oil. In this case, no 1,4-reduction product **3b** was observed in the ^{1}H NMR spectrum, but a trace (less than 0.05%) could be detected by GC-MS. The product was pure enough to use without further purification or crystallization. Crystallization of sinapyl alcohol **2b** is difficult but possible. The pale yellow oil was dissolved in dichloromethane (5 mL) and kept in a freezer for several days. Pale yellow crystals formed.

Small-scale preparation (sinapaldehyde **1b**, 200 mg, 0.96 mmol, 1 eq.; BER, 770 mg, 1.92 mmol, 2 eq.) required stirring for 3 h to effect complete reduction, as shown by TLC monitoring, and yielded a pale yellow oil (147 mg, 0.70 mmol, 73%).

Results and Discussion

Simple Procedure. Coniferyl alcohol **2a** and sinapyl alcohol **2b** were prepared from commercially available coniferaldehyde **1a** and sinapaldehyde **1b** using borohydride exchange resin (BER) in methanol. The reactions needed to stir for an hour for coniferaldehyde **1a** and 3 hours for sinapaldehyde **1b** to complete the reduction. The reaction scaled proportionally; the procedures for large-scale and small-scale reactions were analogous. The resin changed color, from pale yellow to red-brown, and the yellow color of the reaction solution discharged when the reduction was complete. The reduction can be monitored easily by TLC ($CHCl_3$: EtOAc, 1:1). A particularly attractive aspect of this method is that conventional work-up extractions are not required. Moderate yields are the only disadvantage — 47% (61% for small-scale) for coniferyl alcohol **2a** and 72% (73% for small-scale) for sinapyl alcohol **2b**. Absorption of 4-hydroxycinnamyl alcohols into the coarse polymer resin, and binding to phenolic groups by the quaternary ammonium group of the resin, are among possible reasons, especially for the large-scale reaction of coniferyl alcohol. Changing work-up solvent to dichloromethane was not beneficial. Attempts to increase yields by adding acetic acid produced products contaminated with degraded products (which were revealed in NMR spectra but the structures were not identified). The yield detriment is offset by the simplicity of the procedure. The products are extremely clean and ready to use without further purification. Coniferyl alcohol **2a** can be crystallized, and stored at room temperature for years. Sinapyl alcohol **2b** is difficult to crystallize, and may degrade easily at room temperature. In our experience, sinapyl alcohol **2b** containing residual solvent is less likely to degrade. It must be kept in a freezer for long term storage. Alternatively, adding 0.1% BHT (butylated hydroxytoluene; 3,5-di-*tert*-butyl-4-hydroxytoluene) in acetone to the product solution before drying may prevent oxidation and prolong its life. Even crystals should be stored in the freezer.
This protocol is a safe and efficient method but there are two precautions that need to be taken to ensure success. First, the BER needs to be washed with methanol before use. This results in much less of the troublesome 1,4-reduction products (see below), presumably due to removal of unbound borohydrides. Washed resin can be stored for several days, but it is not recommended. Second, the crude products should be passed through pre-washed silica gel to remove contaminants from the resin. This procedure is also beneficial to crystallize both 4-hydroxycinnamyl alcohols.

Advantages. BER exhibits a high regioselectivity between aldehydes and ketones, reducing aldehydes faster than ketones, and reducing α,β-unsaturated aldehydes without 1,4-reduction. Commercially available BER is strong enough to complete the required reduction within hours. The products from reduction of either of the 4-hydroxycinnamaldehydes were remarkably clean as evidenced by ^{1}H NMR spectra; the (un-recrystallized) coniferyl alcohol **2a** product contained about 0.03% dihydroconiferyl alcohol **3a,** and the sinapyl alcohol **2b** product contained about 0.05% dihydrosinapyl alcohol **3b**. There were essentially no differences between large-scale and small-scale preparations. Large-scale preparation does not require longer reaction times. Contaminants from the polymer supported reagent can be readily removed from the desired products by simple "plug-filtration" through silica gel, and no extensive work-up is needed. The products were ready to use without further purification or crystallization, but both 4-hydroxycinnamyl alcohols can be recrystallized to enhance its quality and shelf-life.

The used resin can be recovered and recycled. Since the resin is relatively non-toxic and non-volatile, it is easy to handle by non-chemists.

Conclusions

Coniferyl alcohol and sinapyl alcohol can be synthesized from commercially available coniferaldehyde and sinapaldehyde using Borohydride exchange resin (BER). This method is efficient, and can be safely performed without complication within short periods of time to produce fresh, clean 4-hydroxycinnamyl alcohols. Contaminants from the polymer supported reagent can be readily removed from the desired products by simple “plug-filtration” through silica gel, and no extensive work-up is needed.

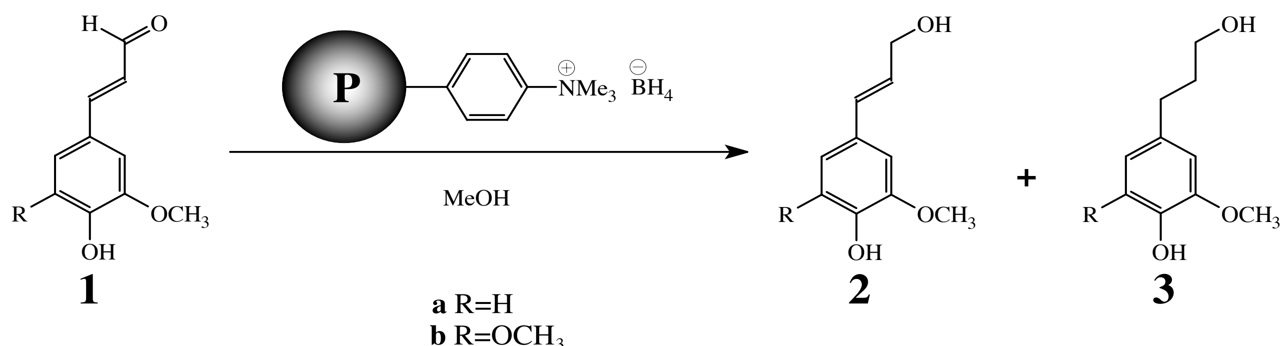

Figure 1. Reduction of coniferaldehyde 1a and sinapaldehyde 1b by borohydride exchange resin (BER).

Complete Silencing of Polyphenol Oxidase Gene Expression in Red Clover

M.L. Sullivan

Introduction

Post-transcriptional gene silencing (PTGS) is a method of specifically reducing expression of a selected gene by genetic transformation of anti-sense, co-suppression, or hairpin RNA-producing constructs and provides a powerful tool to analyze in vivo gene function in plants. Although this is a powerful technology, its effectiveness has not been evaluated in the forage crop red clover (*Trifolium pratense* L.). We have used PTGS to reduce expression of polyphenol oxidase (PPO), the enzyme associated with post-harvest browning, in red clover. Since we have several tools available to analyze polyphenol oxidase gene expression (gene-specific nucleic acid probes, anti-PPO antibodies, and easy enzyme activity assays), the PPO system provides an excellent way to evaluate PTGS as a tool for analyzing gene function in red clover. Additionally, red clover plants with reduced PPO levels will be valuable in evaluating the roles this enzyme plays in vivo and in post-harvest processes.

Methods

We prepared a gene-silencing construct that would produce an intron-spliced hairpin RNA corresponding to the major expressed PPO gene of red clover leaves. This type of silencing construct has been shown to be effective in several other plant species. The PPO-silencing construct was transformed into two red clover genotypes, NewRC27 and NewRC30, and several regenerated plants resistant to kanamycin (conferred by the marker gene cotransformed with the silencing construct) were obtained. PPO expression was evaluated in the transgenic plants by immunoblotting with a PPO-specific antibody, a quantitative assay for PPO activity using caffeic acid as the substrate, and a qualitative extract browning assay.

Results and Discussion

We have assessed silencing in four transgenic plants, each of which showing marked reductions in PPO expression compared to nontransformed control plants of the same genotype (Fig. 1). In quantitative assays of PPO activity, all the transformed plants (numbered in Fig. 1) showed <10% of the PPO activity of nontransformed controls. Two plants (NewRC27-1 and NewRC30-0) had no detectible PPO protein or enzyme activity based on immunoblotting and quantitative activity assays, respectively. Extract browning, a hallmark of PPO activity, was noticeably delayed for plants with reduced, but detectible, PPO activity (NewRC27-0 and NewRC27–6) compared to control plants (onset of browning in 1 to 4 h for the silenced plants versus <5 min for control plants). Extracts of the red clover plants with no detectible PPO activity (NewRC27-1 and NewRC30-0) exhibited no browning, even after 24 h.

These results indicate that gene silencing by expression of intron-spliced hairpin RNAs in red clover is highly effective, both in terms of percentage of plants displaying silencing (4/4, 100%), and the degree to which the target gene is silenced (two plants with <10% PPO activity and two plants with no detectible PPO activity). Recovery of plants that appear to be completely silenced for foliar PPO expression suggests that PPO does not serve any essential function in red clover leaves. Additionally, these PPO-silenced plants will greatly facilitate our evaluation of the role of PPO in preventing post-harvest protein loss in forage crops, the role PPO plays in other aspects of post-harvest quality, and the in vivo roles of PPO enzymes. Red clover plants silenced for PPO will also be useful in gene expression experiments utilizing β-glucuronidase (GUS) reporter genes, since PPO-mediated browning can significantly obscure the histochemical stain used to detect the GUS gene product.

Conclusion

Post-transcriptional gene silencing in red clover is highly effective, and will be a powerful molecular genetic tool for experiments involving this forage. Using PTGS, we have created plants with greatly reduced or undetectable levels of the browning-associated enzyme PPO, which will be useful in evaluating the role this enzyme plays in in vivo and post-harvest processes

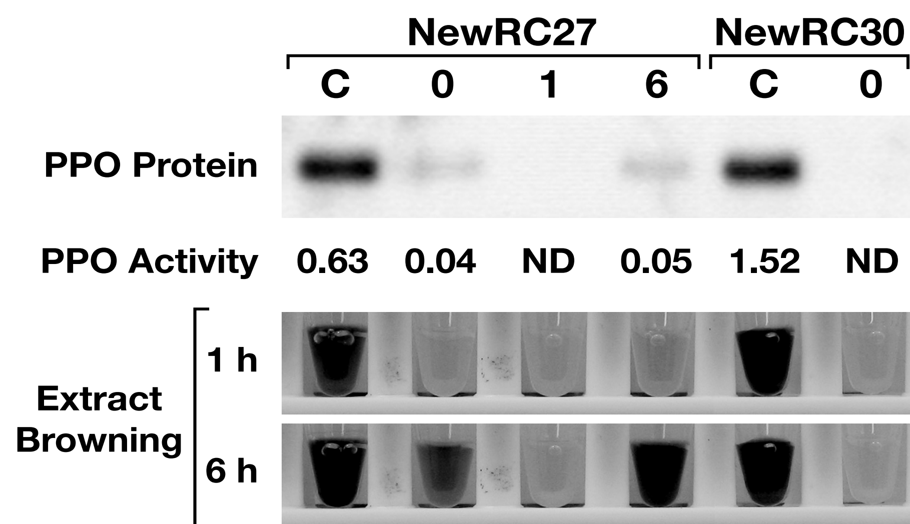

Figure 1. Silencing of PPO gene expression in red clover. Extracts of mature leaves of red clover plants transformed with a PPO gene silencing construct (numbered) were used to assess PPO protein levels by immunoblotting, PPO enzyme activity in a quantitative assay, and extract browning following 1 and 6 h incubations at room temperature. Extracts of nontransfomed plants of the same genotype (C) served as controls. Enzyme activity is expressed as μmol min^{-1} mg^{-1} or ND for none detected.

Polyphenol Oxidase (PPO) and *o*-Diphenols are Responsible for Post-Harvest Proteolytic Inhibition in Red Clover

M.L. Sullivan and R.D. Hatfield

Introduction

Ensiling is a common method of preserving forages, especially in humid regions of the US. Unfortunately, for many forages stored as silage large amounts of the forage protein are partially degraded by plant proteases and the released amino acids and peptides are readily degraded by microbes in the rumen of dairy cows. The nitrogen is excreted as urea and is no longer available to the animal. Proteolytic losses in alfalfa are especially high. In contrast, red clover has up to 90% less proteolysis than alfalfa when ensiled. Several observations had suggested that this reduction in proteolysis is due to polyphenol oxidase (PPO) and *o*-diphenol PPO substrates, both of which are abundant in red clover leaves. Using chromatographic separation to remove *o*-diphenols from red clover extracts with PPO activity and a molecular genetic approach to silence PPO gene expression in red clover plants, we are now able to definitively demonstrate post-harvest proteolytic inhibition in red clover is due to the action of PPO on *o*-diphenols.

Methods

Clarified extracts of leaves of red clover or transgenic red clover silenced for PPO expression by post-transcriptional gene silencing (see accompanying report) were made in 50 mM MES pH 6.5. Where indicated, extracts were desalted using a Sephadex G-25 gel spin column equilibrated with 50 mM MES, pH 6.5, to remove low molecular weight molecules including *o*-diphenols. Extract protein concentrations were measured by Bradford assay using a BSA standard and were adjusted to 2 mg ml^{-1} with buffer. Where indicated, the *o*-diphenol caffeic acid was added to a final concentration of 3 mM. Extracts were incubated at 37 ºC for four hours. Duplicate samples of each extract were removed after 0, 1, 2, 3 or 4 h of incubation at 37 °C. Once removed, trichloroacetic acid (TCA) was added to the samples to 5% (w/v) to precipitate nondegraded proteins and release of free amino acids (a measure of proteolytic activity) into the TCA-soluble supernatant was determined by ninhydrin assay.

Results

To test the role of *o*-diphenols in inhibition of post-harvest proteolysis in red clover, leaf extracts were passed through spin columns of Sephadex G-25 to remove *o*-diphenols and other low molecular weight molecules. We analyzed the extent of proteolysis in the resulting desalted and unfractionated crude extracts by measuring the release of free amino acids over time. As shown in Fig. 1A, proteolysis was substantially increased in red clover extracts from which low molecular weight compounds had been removed (desalted) compared to crude red clover extracts. By the end of the four-hour assay, desalted red clover extracts had nearly five-fold more proteolysis than crude extracts. The extent of proteolysis measured in the desalted extracts at 4 h, 0.73 µmol mg^{-1} protein, represents degradation of a significant amount of the protein present in the extract and is comparable to the extent of proteolysis seen in alfalfa extracts (data not shown). Assuming an average protein molecular weight of 5×10^4, the observed proteolysis corresponds to release of about 8% of the amino acids originally present as protein. This may be an underestimation of the extent of proteolysis taking place, since our assay will only measure release of free amino acids and short peptides. Proteolytic events that result in larger peptides (i.e. endoproteolytic cleavages) would not be detected. Compared to desalted extracts, proteolysis in crude extracts was inhibited by about 70% at 1 h (the earliest time point analyzed) consistent with several previous observations that proteolytic inhibition in red clover occurs rapidly

following tissue disruption and release of PPO, *o*-diphenols, and proteases. To rule out the possibility that the gel filtration spin column procedure had removed some other low molecular weight protease inhibitor from the red clover extract, we added a purified *o*-diphenol, caffeic acid, back to the desalted extract at a concentration of 3 mM. Addition of *O*-diphenol resulted in extract browning, indicating the presence of active PPO, as well as inhibition of proteolysis to an extent similar to that seen for crude red clover extract. This result indicates that *o*-diphenols are required for post-harvest proteolytic inhibition in red clover.

Using post-transcriptional gene silencing, we created red clover plants that had no detectible PPO protein or PPO activity in mature leaves which allowed us to test directly the role of PPO in inhibiting post-harvest proteolysis. We made extracts of leaves of the PPO-silenced plants and nontransformed control plants of the same genotype and analyzed the extent of proteolysis in each extract as described above. As shown in Fig. 1B, virtually no amino acids were released over the four-hour assay period in extracts of the non-transformed control plants. In contrast, amino acid release in extracts of the leaves of the PPO-silenced plants was comparable to that seen for red clover extracts from which *o*-diphenols were depleted (Fig. 1A). Although the absolute level of amino acid release in extracts of the silenced plants was slightly lower than that seen for desalted extracts, the difference in amino acid release compared to the corresponding controls was almost identical in the two experiments. Together, these results indicate that the action of PPO on endogenous *o*-diphenols in red clover plays a central role in preventing post-harvest protein loss in ensiled red clover.

Conclusion

Our results indicate that the post-harvest proteolytic inhibition seen in red clover is the result of the action of the enzyme polyphenol oxidase (PPO) on endogenous *o*-diphenols. Future studies using PPO-silenced red clover as well as transgenic alfalfa expressing red clover PPO should help to elucidate the mechanisms of PPO-mediated proteolytic inhibition and provide insights into how this natural system of protein protection can be adapted to alfalfa and other forage crops.

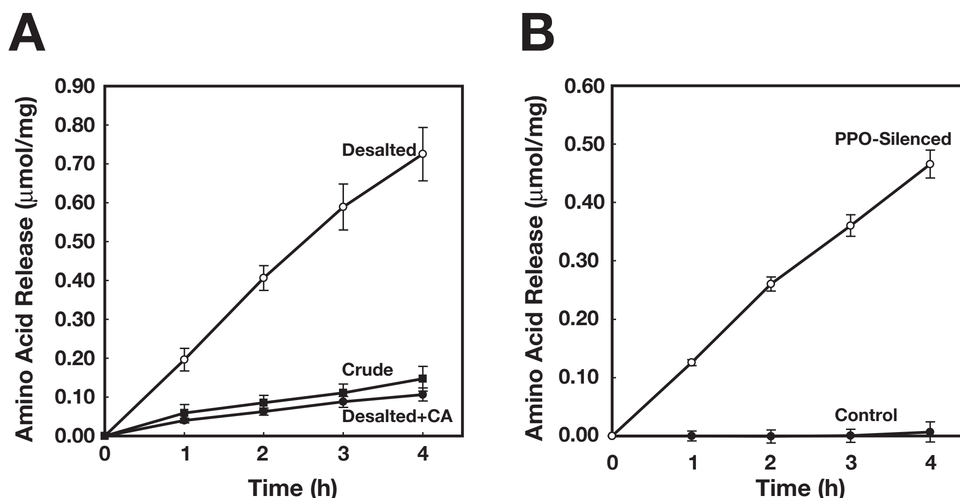

Figure 1. *O*-diphenol- and PPO-dependence of post-harvest proteolytic inhibition in red clover. Proteolysis in various red clover extracts was measured and expressed as release of free amino acids (in µmol) per mg of extract protein over time. Data are the average of three experiments using independent tissue samples. Error bars represent standard error. (*A*) Proteolysis in unfractionated leaf extracts (Crude) or leaf extracts from which low molecular weight molecules were removed (Desalted). Caffeic acid (CA) was added as indicated. (*B*) Proteolysis in leaf extracts of PPO-silenced and control red clover.

Methyl Esterification Divergently Affects the Degradability of Pectic Uronosyls in Nonlignified and Lignified Maize Cell Walls

J.H. Grabber and R.D. Hatfield

Introduction

Galacturonic acid comprises 80% or more of the total uronosyls in primary cell walls and they are the major component of pectins in forage legumes and grasses. Polygalacturonic acids deposited into cell walls are highly methyl esterified but most of these groups are subsequently removed by pectin methylesterase (PME) as tissues complete elongation and undergo lignification. Although galacturonans are among the most rapidly and extensively degraded of cell wall polymers, their enzymatic hydrolysis can be enhanced by demethylation via PME. Demethylation of galacturonans in lignifying tissues may, however, limit degradability because of enhanced formation of benzyl uronate cross-links. These cross-links are formed by the nucleophilic addition of uronosyl acid groups to quinone methide intermediates, which are formed by β–*O*–4 coupling of monolignols and lignin radicals during lignin polymerization. In this study, the potential impact of benzyl-uronate cross-linking on pectin and cell wall hydrolysis was modeled by using PME to vary the methylation of uronosyls prior to artificial lignification of primary maize cell walls.

Methods

Primary cell walls isolated from suspension cultures of corn (*Zea mays* L.) were incubated with and without PME to vary the methylation of pectic uronosyls. A portion of PME treated and untreated walls were suspended in pH 4 buffer type concnetration and artificially lignified with coniferyl alcohol and hydrogen peroxide. Nonlignified controls were stirred in buffer without additions. Cell walls were then washed thoroughly with water followed by acetone to remove non-incorporated coniferyl alcohol. Cell walls were analyzed for methanol, acid-insoluble (Klason) lignin, and uronosyls. Cell walls were also incubated with a fungal enzyme mixture containing pectinase, hemicellulase, and cellulase activity to estimate the degradability pectic uronosyls and the overall degradability of cell walls.

Results and Discussion

Treatment with PME reduced uronosyl concentrations from 97 to 92 mg/g, reduced uronosyl methylation from 57 to 21%, and increased Klason lignin concentrations in artificially lignified cell walls from 99 to 116 mg/g (Table 1.). Although PME treatment slightly enhanced uronosyl release from nonlignified cell walls, it reduced uronosyl release from artificially lignified cell walls by 55% after 4 h and by 7% after 72 h of enzymatic hydrolysis (Table 2). Pectin hydrolysis in PME treated cell walls was probably impaired by enhanced benzyl ester cross-linking of uronosyls to lignin via quinone methide intermediates. Variations in uronosyl methylation had little effect on the overall release of total sugars from cell walls.

Conclusions

Although these data provide good indirect evidence of enhanced benzyl uronate cross-linking in PME-treated cell walls, additional studies are needed to directly confirm the existence of such cross-links in our cell-wall model system and to assess whether uronosyl demethylation increases their abundance in cell walls and in Klason lignin residues. Additional studies are also needed to confirm their role in limiting pectin degradation in our cell wall model system as well as natural plant systems. The ultimate

aim of this work is to identify means of improving the digestibility of forage grasses and legumes.

Table 1. Concentration (milligrams per gram of cell wall) of Klason lignin (KL), methanol (ME), uronic acids (UA), and proportion of methylated UA (Me-UA) in nonlignified maize cell walls incubated with 0 or 3000 units of commercial pectin methylesterase (PME) and then artificially lignified with 0 or 200 mg of coniferyl alcohol (CA), added per gram of cell wall.

Treatment					
PME	CA	KL	Me	UA	Me-UA
0	0	ND	9.4	100.0	0.57
3000	0	ND	2.8	96.6	0.18
0	200	99.3	9.0	94.7	0.58
3000	200	115.9	3.4	87.3	0.23
Analysis of variance					
PME		*	*	*	*
CA		–	NS	*	*
PME x CA interaction		–	*	NS	†
Coefficient of variation (%)		3.4	3.4	2.1	5.7

ND, not determined
*, †, NS, significant at the 0.05 and 0.10 levels of probability and not significant, respectively.

Table 2. Release of uronosyls (%) and total sugars (milligrams per gram of cell wall) from maize cell walls during incubation with Viscozyme and Celluclast, a fungal enzyme mixture containing pectinase, hemicellulase, and cellulase activity. Nonlignified maize cell walls were incubated with 0 or 3000 units of commercial pectin methylesterase (PME) and then artificially lignified with 0 or 200 mg of coniferyl alcohol (CA), added per gram of cell wall.

Treatment		Uronosyls		Total sugars	
PME	CA	4 h	72 h	4 h	72 h
0	0	66.2	87.7	468.6	773.0
3000	0	68.7	95.0	467.5	768.0
0	200	48.4	87.8	157.8	519.1
3000	200	21.9	81.8	129.5	501.9
Analysis of variance					
PME		*	NS	NS	NS
CA		*	*	*	*
PME x CA interaction		*	*	NS	NS
Coefficient of variation (%)		8.9	1.9	5.7	2.9

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

Rumen Microbiology

Enrichment of Fusobacteria from the Rumen that can Utilize Lysine as an Energy Source for Growth

J. B. Russell

Introduction

Lysine stimulates production levels in livestock, and this amino acid is routinely added to the rations poultry and swine. Lysine is also a limiting amino acid for lactating dairy cows. The study of amino acid metabolism in ruminants is confounded by the fact that ruminal microorganisms degrade feed amino acids, and microbial protein is the primary source of amino acids for the animal. However, experiments with post-ruminally cannulated cattle indicate that microbial protein does not always supply enough lysine to maximize milk production.

Ruminant nutritionists have employed two strategies of enhancing amino acid availability in cattle. When feed proteins are subjected to heat or harsh chemical treatments, solubility and degradation can be decreased. 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 either growth or 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 that have been called hyper-ammonia producing bacteria. *Clostridium sticklandii* grew rapidly with lysine as an energy source, but it is very sensitive to acidic pH and could not be isolated from wild and domestic ruminants in New Zealand. The following experiments re-examined the degradation of lysine by ruminal bacteria.

Materials and Methods

Cows were fed timothy hay or a commercial ration. Two hours after feeding, ruminal contents were centrifuged to remove feed particles and protozoa. The mixed ruminal bacteria were transferred anaerobically to serum bottles that contained: 1) no addition, 2) 50 mM lysine, 3) 5 mg ml^{-1} Trypticase or 4) 5 mg ml^{-1} Trypticase plus 50 mM lysine. Mixed ruminal bacteria enriched with Trypticase and lysine were streaked onto agar plates in an anaerobic glove box and incubated at 39° C for 72 h. Colonies were transferred anaerobically to basal broth. Fermentation acids were analyzed by high-performance liquid chromatography. Chromosomal DNA was purified using the FastDNA Spin Kit. PCR products were cloned using the primers M13F and M13R. The 16S rDNA sequences were deposited with GenBank. Stationary phase cells were washed in potassium phosphate buffer (pH 6.7) and the cells were diluted 50-fold to initiate transport. Transport was terminated by rapid filtration. The transport assays typically had 3 μM L-[U-^{14}C] lysine, (pH 6.7 to 4.7)

Results

Mixed ruminal bacteria did not deaminate lysine at a rapid rate, but lysine degrading bacteria could be enriched if Trypticase was also added. Lysine degrading isolates produced acetate, butyrate and ammonia, were non-motile, stained gram-negative and could also utilize lactate, glucose, maltose

or galactose as an energy source for growth. Lactate was converted to acetate and propionate, and 16S rDNA indicated that their closest relatives were *Fusobacterium necrophorum*. Growing cultures produced ammonia at rates as high as 2400 nmol mg protein^{-1}min^{-1}. Washed cells took up ^{14}C lysine at an initial rate of 6 nmol mg protein^{-1} min^{-1}, and glucose addition did not affect the transport. Cells washed aerobically had the same transport rate as those handled anaerobically, but only if the transport buffer contained sodium. The affinity constant for sodium was 8 mM, and sodium could not be replaced by lithium. Cells treated with monensin did not take up lysine, but a protonophore that inhibited growth had no effect. An artificial membrane potential created by potassium diffusion did not increase the rate of lysine transport, and the transport rate was directly proportional to the lysine concentration. Decreasing the pH from 6.7 to 5.5 caused an 85% decrease in the rate of lysine transport. The addition of *F. necrophorum* JB2 to mixed ruminal bacteria increased lysine degradation 10-fold, but only if the pH was 6.7 and monensin was not present.

Discussion

Analysis of16S rDNA indicated that our isolates were closely related to strains of *F. necrophorum*, but lysine transport and utilization by *F. necrophorum* per se had not been previously reported. The rumen is a sodium rich environment that has been compared to an inland sea, and many ruminal bacteria cannot grow without sodium. *F. necrophorum* JB2 could not take up lysine unless sodium was added. Based on the fact that lysine is a positively charged species at physiological pH, we had originally speculated that the lysine transport of *F. necrophorum* JB2 would be driven by a membrane potential. This hypothesis was incorrect. The addition of glucose as an energy source or the creation of an artificial membrane potential did not increase lysine transport activity, and TCS, a protonophore that inhibited growth, did not decrease transport.

Carrier-mediated systems of facilitated diffusion are not as common as primary or secondary active transport. However, some secondary amino acid transport systems operate as facilitated systems when the substrate concentration is high. Because the velocity of lysine transport was always directly proportional to substrate concentration, it appeared that the lysine gradient was the only driving force. The lysine carrier is inhibited by even a small decrease in extracellular pH. Based on these results, it appeared that there would be little degradation of lysine at acidic pH, and this conclusion was supported by mixed culture experiments. The addition of *F. necrophorum* JB2 to ruminal fluid from a cow fed timothy hay greatly increased ammonia production from lysine, but little deamination was observed if the pH was 5.5 .

Conclusion

Ruminal amino acid degradation is a particular problem in cattle fed fresh forage. Under these conditions, ruminal pH is not highly acidic, there is often an abundance of soluble protein, and the bacteria have insufficient carbohydrate (energy) to incorporate these amino acids into microbial protein. The present work indicates that *F. necrophorum* is able to degrade lysine a rapid rate, but further work will be needed to see if dietary lysine enriches *F. necrophorum* in vivo and if monensin or acidic pH can counteract this enrichment.

Effect of a Bacteriocin (bovicin HC5) on *Clostridium Sporogenes* MD1, a Bacterium that has the Ability to Degrade Amino Acids in Ensiled Plant Materials

M.D. Flythe and J.B. Russell

Introduction

Researchers have developed silage inoculants to augment the natural microflora of plant materials. These inoculants are typically lactobacilli, but R.E. Muck and his colleagues noted that the ruminal bacterium *Streptococcus bovis* might also be useful. *S. bovis* "grew faster than any of the commercial species tested and resulted in the most homolactic fermentation," but bacteriocin production was not determined. Recent work indicated that many *S. bovis* strains from the bovine rumen produced bacteriocins, and the strain with the greatest activity was designated as HC5. Later work revealed that the bacteriocin of *S. bovis* HC5 (bovicin HC5) inhibited a variety of Gram-positive bacteria, but its effect on silage clostridia was not determined. The following experiments sought to: 1) isolate clostridia from fresh plant materials and silages, 2) examine the ability of these bacteria to ferment amino acids at low pH, and 3) determine their susceptibility to bovicin HC5.

Methods

Fresh, immature finely chopped alfalfa plants, freshly chopped corn, corn silage and alfalfa haylage samples (1 g) were added to tubes containing an anaerobic amino acid containing medium. The tubes subjected to a heat shock (80 ˚C, 20 min). Once the tubes had cooled to room temperature, the sub-samples (1 ml) were serially diluted (10-fold increments) into sterile basal medium. The dilutions were spread on plates and incubated at 39 ˚C. Large colonies appeared after approximately 48 h. All of the colonies had the same morphology and all of the isolates were rod-shaped bacteria with spores. The bacteria were grown in continuous culture and pH was adjusted by adding HCl to the culture vessel via a pH-controlled peristaltic pump. Ammonia was assayed by a colorimetric method. The 16S rRNA gene region was amplified and the sequences were subjected to an NBLAST. A phylogram was created using the neighbor-joining function of Clustal X. *S. bovis* HC5 and JB1 were grown in basal medium that was supplemented with glucose (10 mg ml^{-1}) and Tween 80 (1 μl ml^{-1}) to release bovicin HC5 activity from the cells after they reached stationary phase. Cultures were assayed for toxins by Dr. R. H. Whitlock (University of Pennsylvania, Kennett Square, PA) using a mouse protection bio-assay that has been previously described. Fermentation acids in cell-free supernatant samples were analyzed by high-performance liquid chromatography.

Results and Discussion

Fresh plant materials can be fermented and preserved as silage for cattle, but clostridia that deaminate amino acids increase pH. If the pH of the silage rises, spoilage microorganisms proliferate, and undesirable products accumulate. Rod-shaped, anaerobic bacteria with spores were isolated from fresh alfalfa, fresh corn, and silages. Strain MD1 had the highest specific activity of amino acid deamination, and it was most closely related to *Clostridium botulinum* A and B. However, MD1 did not produce a toxin. Opinion 69 of the Judicial Commission of the International Committee on Systematic Bacteriology states that the *C. botulinum* designation should be reserved for toxin producing strains, and *C. sporogenes* should be reserved for "non-toxigenic strains." Based on these results, MD1 was classified as *Clostridium sporogenes*.

Washed cell suspensions of *C. sporogenes* MD1 had specific activities as great as 690 nmol ammonia mg $protein^{-1}$ min^{-1}, and this rate did not decrease until the pH was less than 4.5. Batch cultures of *C.*

sporogenes MD1 did not initiate growth if the initial pH was less than 5.0, but continuous cultures (0.1 h^{-1} dilution rate) persisted until the pH of culture vessel was 4.6. When *C. sporogenes* MD1 was co-cultured with a bacteriocin producing *Streptococcus bovis* strain (HC5), ammonia production was greatly reduced. The ability of *S. bovis* HC5 to inhibit MD1 was pH-dependent. When the pH was 5.5 or less, MD1 could no longer be detected. Further work will be needed to assess the impact of bacteriocin producing bacteria on silage fermentations. However, all of our amino acid fermenting isolates were inhibited by bovicin HC5.

Properly prepared silages are not typically contaminated with *C. botulinum*, but those contaminated with animal carcasses (e.g. dead birds) often have *C. botulinum* type C. The impact of *S. bovis* HC5 on *C. botulinum* was not determined, but it should be noted that *C. sporogenes* MD1 is very closely related to *C. botulinum* type A and B. If *S. bovis* HC5 inhibits *C. botulinum*, the use of *S. bovis* HC5 as a silage inoculant could have yet another positive role.

Conclusion

Bacteriocin-producing bacteria may be used to improve silage quality.

Differential Gene Expression in *Clostridium thermocellum* ATCC 27405 Grown in Cellulose- or Cellobiose-Limited Continuous Culture

D.M. Stevenson and P.J. Weimer

Introduction

Clostridium thermocellum is a thermophilic anaerobic bacterium capable of fermenting cellulose and hemicelluloses to produce ethanol as a major fermentation product, along with a novel cellular residue that has adhesive properties of potential commercial use. The regulation of expression of genes involved in synthesis of the cellulolytic complex and the adhesive materials largely unknown. We describe here the expression of seventeen genes involved in cellulosome synthesis and primary catabolism by this organism, as a function of both growth substrate (insoluble cellulose versus soluble cellobiose) and growth rate of the organism.

Methods

C. thermocellum ATCC 27405 was grown at 55 °C in a modified Dehority medium in a continuously stirred reactor (working volume 875 ml) containing Sigmacell 50 microcrystalline cellulose or cellobiose as energy source. The medium was delivered to the reactor as a segmented slurry. Both the growth vessel and the medium reservoir were continuously sparged with sterile CO_2 . The reactor vessel was fitted with a water-cooled 0.5 m Allihn condensor to reduce evaporation of the culture liquid. For each dilution rate in the range of 0.013 to 0.16 h^{-1}, chemostats were operated to steady state (minimum turnover of three reactor volumes) prior to sampling. RNA isolated freshly from culture samples was used to prepare cDNA used for real-time quantitative PCR (RT-PCR) reactions, which were carried using the Applied Biosystems Prism® 7000 sequence detection system, with PCR product detected by SYBR Green fluorescent dye. PCR primer pairs were designed using the Applied Biosystems Primer Express software, from putative gene sequences obtained from the unfinished *C thermocellum* genome. Data were calculated relative to two calibrator genes, *recA* and 16S rDNA. RT-PCR data were analyzed using the General Linear Model of the SAS statistical software package,

v.7.0 (SAS Institute, Cary, NC). The model equation used substrate (S) and dilution rate (D) for main effects, S by D as a linear interaction term. Mean separations were determined using the LS means protocol at $P < 0.05$.

Results and Discussion

Similar expression patterns were observed with either *rec*A or 16S rDNA were used as calibrator genes. Genes encoding essential catabolic enzymes cellobiose phosphorylase (*cbp*), cellodextrin phosphorylase (*cdp*), lactate dehydrogenase (*ldh*), hydrogenase (*hyd*), acetate kinase (*ack*) and an alcohol dehydrogenase (*adh*Z) displayed slightly elevated activities on cellulose, but this enhanced expression was not sufficient to account for large differences in fermentation end products observed as a function of growth rate, a pattern that was similar for both substrates. Putative genes for carbon catabolite repression (CCR; *crp*Y, *crp*Z, *hpr*X, *hpr*Y, *hpr*Z, *hpr*K) did not exhibit dramatic changes with growth substrate or growth rate, suggesting that CCR is not controlled at the transcriptional level in this organism. By contrast, four genes were repressed at high dilution rates during growth on cellobiose (Table 1 and Fig.1): *cip*A (cellulosomal scaffolding protein), *cel*S (major exoglucanase, *man*A (putative cellulosomal mannanase), and *adh*X (an alcohol dehydrogenase). The data indicate that these genes are differentially regulated by growth substrate independent of growth rate, and that repression of these genes by cellobiose can be relieved at low growth rates. The gene encoding cellulosomal scaffoldin-dockerin binding protein did not display regulation by growth rate or substrate type.

Conclusion

This study represents, to our knowledge, the most complete analysis of the simultaneous effect of substrate identity and growth rate on gene expression in any bacterium. The demonstration that differences in the expression of certain genes on cellulose versus cellobiose are due to substrate identity rather than to differences in growth rate provides strategies for enhancing expression of specific genes involved in cellulose degradation by this organism.

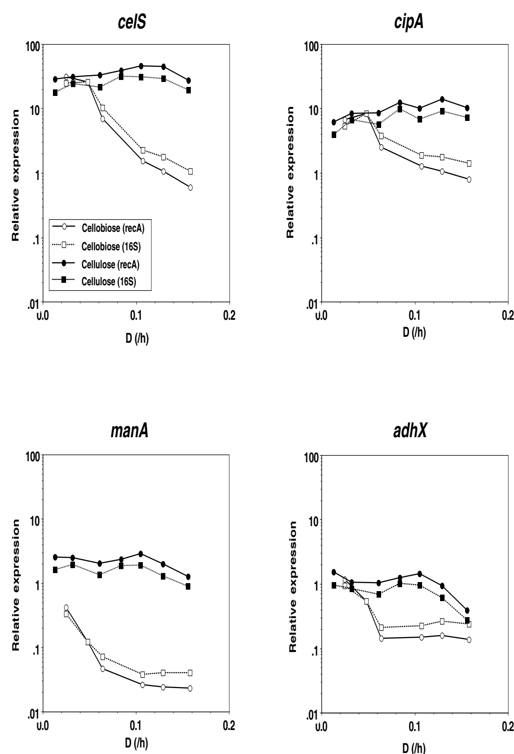

Figure 1. Gene expression as a function of growth rate of *C. thermocellum* ATCC 27405 cultures grown in cellobiose- or cellulose-limited continuous culture. Expression is shown relative to two calibrator genes, *recA* and 16S rDNA. In order to permit visualization of the relative *recA* and 16S rDNA expression on the same graph, values for expression relative to 16S rDNA were multiplied by a constant (5589.9), calculated as the ratio of the average *recA* expression divided by the average 16S rDNA expression. *celS*, exoglucanase; *cipA*, scaffoldin protein of the cellulosome; *manA*, mannanase; *adhY*, putative type IV alcohol dehydrogenase.

Table 1. Linear regression analysis of gene expression data from cellobiose-limited or cellulose-limited continuous cultures of *C. thermocellum* ATCC 27405 as a function of substrate (S) and dilution rate (D). Regression equations were determined based on expression of the indicated gene relative to mean expression of *recA*.

	Main effects (P>F)			Regression coefficients [a]					H/L ratio [b]	
				Cellobiose		Cellulose		Model		
Gene/ORF	S	D	S x D	Slope	Y-Intercept	Slope	Y-Intercept	fit (R^2)	Cellobiose	Cellulose
celS	0.003	0.124	0.010	-236.45 **	32.16**	55.75	31.14**	0.838	0	1.25
cipA	<0.001	0.745	0.003	-54.30 *	8.27**	37.66**	6.91**	0.855	0	1.72
manA	<0.001	0.054	0.352	-2.25	0.308	-6.17**	2.72**	0.935	0	0.66
sdbA	<0.001	0.070	0.006	-1.66	1.51**	4.57**	1.80**	0.930	0.84	1.35
cbp	<0.001	<0.001	<0.001	12.65**	1.92	2.40	4.69**	0.978	1.86	1.07
cdp	<0.001	<0.001	0.482	1.91**	0.118**	1.62**	0.245**	0.921	2.85	1.86
ack	0.318	0.010	0.493	2.91	0.86**	4.62**	0.86**	0.572	1.46	1.71
*adh*Y	0.003	0.014	0.721	-6.25**	0.94**	-4.91	1.50**	0.748	0	0.50
*adh*Z	0.011	<0.001	0.034	1.70	0.322**	4.74**	0.351**	0.870	1.70	2.60
*hyd*A	0.004	0.001	0.084	11.22	3.70**	29.34**	3.96**	0.815	1.42	1.95
ldh	0.004	0.010	0.156	1.14	0.393**	3.47**	0.482**	0.756	1.40	5.82
*crp*Y	<0.001	<0.001	0.917	0.048**	0.007**	0.050**	0.013**	0.894	1.89	1.52
*crp*Z	0.017	0.199	0.370	0.010	0.023**	0.062	0.026**	0.556	1.06	1.33
*hpr*X	0.630	0.002	0.959	1.01**	0.080**	0.981**	0.083**	0.688	2.51	2.43
*hpr*Y	0.093	0.072	0.131	0.208	0.194	3.58*	0.095	0.537	1.15	4.39
*hpr*Z	<0.001	<0.001	0.351	0.251**	0.035**	0.341**	0.051**	0.883	1.93	1.87
*hpr*K	0.408	<0.001	0.406	0.652**	0.024	0.461**	0.052**	0.749	3.73	2.12

[a] The linear regression model was used, as the quadratic (S x D^2) term was insignificant (P>0.05) for all 34 combinations of gene and S except *ldh*/cellobios
*, ** indicates significant parameter estimates via a two-tailed t-test at $P < 0.05$ and $P < 0.01$, respectively.
[b] Ratio of predicted expressions from the regression model at high (H, 0.16 h^{-1}) and low (L, 0.016 h^{-1}) dilution rates.

[a] The linear regression model was used, as the quadratic (S x D^2) term was insignificant (P>0.05) for all 34 combinations of gene and S except *ldh*/cellobiose. *, ** indicates significant parameter estimates via a two-tailed t-test at $P < 0.05$ and $P < 0.01$, respectively.
[b] Ratio of predicted expressions from the regression model at high (H, 0.16 h^{-1}) and low (L, 0.016 h^{-1}) dilution rates.

Forage Quality

Cell Wall Traits of Alfalfa Clones Selected for Low or High In Vitro Ruminal Neutral Detergent Fiber Digestibility

H.G. Jung and J.F.S. Lamb

Introduction

Concentration and digestibility of neutral detergent fiber (NDF) are important forage quality traits that contribute to animal performance. Feed intake by ruminants tends to decline as the NDF concentration of forages increases. Poor NDF digestibility limits energy available from forages for milk and meat production. Alfalfa (*Medicago sativa* L.) is generally considered to be an excellent forage for dairy cows because of its low NDF concentration and rapid NDF digestibility. However, alfalfa's reputation for high quality is primarily a function of its low NDF, rapidly digestible leaf fraction. The stem fraction of alfalfa is actually very high in NDF concentration and the potential extent of NDF digestibility is low. The USDA-ARS Plant Science Research Unit in St. Paul, MN has undertaken a breeding project to increase the in vitro ruminal NDF digestibility (IVNDFD) of alfalfa stems. Here we report on shifts in cell wall concentration and composition observed for groups of alfalfa clones that were previously selected as having low or high stem IVNDFD.

Methods

Seed from six commercial alfalfa varieties (5312, Rushmore, Magnagraze, Wintergreen, Winterstar, and WL 325HQ) adapted to the Minnesota environment was mixed and planted at Becker, MN in the summer of 1997. Spring growth and the first summer regrowth of ~2000 individual plants was harvested in 1998 and 1999. In vitro NDF digestibility of each sample after 16- and 96-h of incubation was estimated using near infrared reflectance spectroscopy (NIRS) and calibration equations developed for this project. Means for 16- and 96-h IVNDFD were calculated for individual alfalfa plants across years and harvests. The five most extreme individual plants (clones) were identified for each of four selection groups; low 16-h IVNDFD, high 16-h IVNDFD, low 96-h IVNDFD, and high 96-h IVNDFD. These 20 selected clones were vegetatively propagated and planted at Becker and Rosemount, MN in replicated plots in 2001. Stem samples were harvested in the spring and for the first summer regrowth in 2002 and 2003. Fiber digestibility, and the concentration and composition of stem cell wall material (using the Uppsala Dietary Fiber procedure) were estimated by NIRS.

Results and Discussion

Limited winter-hardiness resulted in the loss of six clones from the study. Also, severe stand loss at the Rosemount location during the winter of 2002/2003 prevented data collection from this location in 2003. One clone was excluded from the data set because it's IVNDFD results did not match the IVNDFD phenotype for which it had originally been chosen, both in the current study and another study in which this particular clone was included. The selection group means for the remaining 13 clones indicated that the low and high selection groups differed for IVNDFD in accordance with their original selection phenotype (Fig. 1). The high 16-h IVNDFD selection group was also greater in 96-h IVNDFD than the corresponding low group; however, the two 96-h IVNDFD selection groups did not differ for 16-h IVNDFD (Fig. 1). Cell wall concentration and composition differed between the low

and high 16-h IVNDFD selection groups (Table 1). The high 16-h IVNDFD group had ~ 3 % less cell wall material than the corresponding low group. Composition of cell wall material for the high 16-h IVNDFD group was ~1% lower in Klason lignin, and ~ 3 and 4 % higher in hemicellulose and pectin (respectively). Cell wall concentration did not differ between the 96-h IVNDFD selection groups, but composition of the cell wall was altered (Table 1). Klason lignin and hemicellulose content of the cell wall were ~ 9 and 3 % lower (respectively), and cellulose and pectin were ~ 5 and 2 % higher (respectively) in the high 96- IVNDFD selection group compared to the low group.

Conclusion

Alfalfa clones selected for low or high stem IVNDFD had similar shifts in cell wall concentration and composition within selection groups. The high 16-h IVNDFD phenotype was associated with clones that had less cell wall material in their stems and these walls were lower in lignin and had more pectin. Based on the stem tissue development pattern of alfalfa, we would hypothesize that these clones had less xylem tissue development. In contrast, the high 96-h IVNDFD phenotype was associated with altered cell wall composition without changes in cell wall concentration. This suggests that these high 96-h IVNDFD clones had normal amounts of xylem tissue deposition, but that the composition of xylem walls was different.

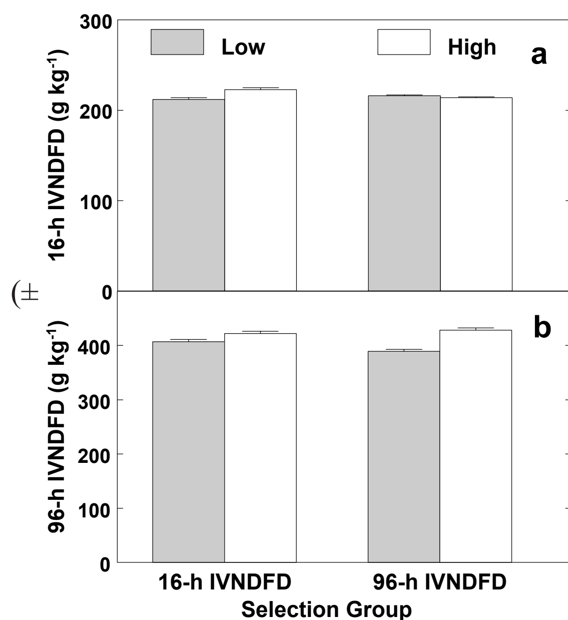

Figure 1. In vitro ruminal neutral detergent fiber digestibility (IVNDFD) after 16-h (**a**) or 96-h (**b**) incubations of alfalfa stems from groups of clones previously selected for low or high IVNDFD. Means (± 1 SE) were calculated over clones, environments, and harvests.

Table 1. Cell wall concentration and composition of stems from groups of alfalfa clones previously selected for low or high in vitro ruminal neutral detergent fiber digestibility (IVNDFD).†

Selection Group	Clones‡	Cell Wall	Klason Lignin	Polysaccharides§ Cellulose	Hemicellulose	Pectin
		g kg^{-1} DM	---------------g kg^{-1} cell wall ---------------			
16-h IVNDFD						
Low	4	688*	214*	411	169*	206*
High	4	667	212	409	164	215
SEM		1	1	1	0.3	1
96-h IVNDFD						
Low	2	682	228*	395*	170*	207*
High	3	684	208	414	165	212
SEM		2	1	1	1	1

† Least square means were calculated over clones, environments, and harvests.
‡ Number of clones included in each selection group.
§ Cellulose was estimated as cell wall glucose; hemicellulose was estimated as the sum of cell wall xylose, mannose, and fucose residues; pectin was estimated as the sum of cell wall uronic acids, arabinose, galactose, and rhamnose residues.
* Least square means for low and high clonal groups differ ($P < 0.05$) within IVNDFD selection groups.

Composition and Response to Dilute-Acid Pretreatment and Enzymatic Saccharification of Alfalfa, Reed Canarygrass, and Switchgrass

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Introduction

Increased use of ethanol as a renewable fuel is advocated to limit net CO_2 emissions. Ethanol is produced by fermentation of starch from maize (*Zea mays* L.) grain; however, maize grain can only replace at most 4 to 5 % of our gasoline needs. As an alternative, it has been estimated that use of the carbohydrates in vegetative tissues (biomass) from crop residues and dedicated energy crops could easily provide enough ethanol to replace 15 % of gasoline requirements. The primary carbohydrates in biomass are the cell wall polysaccharides (cellulose, hemicellulose, and pectin) and, as we know from feeding livestock, their susceptibility to enzymatic degradation and fermentation is limited. While not cost-effective for livestock feeding, pretreatment of biomass to increase its conversion efficiency is the accepted model for proposed biomass-to-ethanol systems. Numerous forage species have been suggested for use in biomass-to-ethanol systems, but surprisingly little is known about the compositional differences among forages species and how these impact their response to pretreatment and hydrolysis to fermentable sugars. This study is an initial attempt to characterize three high-yielding forage species for their chemical composition and potential sugar yields for subsequent fermentation to ethanol.

Methods

Alfalfa (*Medicago sativa* L.) stem material was harvested at bud and full flower stages of maturity from breeding nurseries in Minnesota. Whole herbage of reed canarygrass (*Phalaris arundinacea* L.) was harvested in Wisconsin at vegetative and ripe seed maturity stages. Pre-boot, anthesis, and post-frost maturity stages of switchgrass (*Panicum virgatum* L.) herbage were harvested in Nebraska. These biomass samples were dried, ground, and analyzed for crude protein (N x 6.25), lipids (ether extract), ash (combustion at 450°C), organic acids (HPLC of water extract), non-structural carbohydrates (glucose, fructose, sucrose, raffinose, stachyose, and starch by HPLC; fructans colorimetrically), cell wall carbohydrates (glucose, xylose, arabinose, galactose, rhamnose, and fucose by GC; uronic acids colorimetrically), Klason lignin (gravimetric), and gross energy (bomb calorimetry). The biomass samples were pretreated with dilute sulfuric acid at 121°C in an autoclave and 150°C in a fluidized heating bath. Pretreated residues were hydrolyzed with cellulase and β-glucosidase. Glucose, total non-glucose sugars, and acetate released by the pretreatments and enzymatic hydrolysis were quantified by HPLC.

Results and Discussion

Composition of the biomass samples is shown in Table 1. On average the alfalfa stem samples contained the most protein and organic acids. Reed canarygrass had the highest ash and non-structural carbohydrate concentrations, and least lignin. Organic acids were very low in switchgrass, but this species had the greatest cell wall carbohydrate content. All three species contained some starch, but only reed canarygrass had fructans. The ratio of cell wall glucose-to-non-glucose sugars was greater for the alfalfa samples than the grasses. Little difference was noted among the biomass samples in gross energy content. Forage maturity increased lignin and cell wall carbohydrate concentrations, and depressed protein and organic acids content.

Table 1. Protein, lipids, ash, organic acids, lignin, carbohydrates, and gross energy content of bulk biomass forage samples.

† Data are for alfalfa stems only; reed canarygrass and switchgrass data are for whole herbage.

Species†				Organic	Klason	Carbohydrates		Gross
Stage	Protein	Lipids	Ash	Acids	Lignin	Non-Structural	Cell Wall	Energy
	g kg^{-1} DM							kcal kg^{-1} DM
Alfalfa								
Bud	127	9	81	32	158	58	505	4412
Full Flower	88	7	58	24	175	51	547	4479
Reed Canarygrass								
Vegetative	88	22	128	24	109	116	402	4230
Ripe Seed	45	13	95	10	148	99	498	4216
Switchgrass								
Pre-Boot	65	10	89	9	133	45	524	4352
Anthesis	32	10	57	9	154	115	540	4447
Post-Frost	30	16	57	3	173	34	616	4465

The grass samples required 1.5% wt/vol acid loading to achieve maximal glucose and non-glucose sugar yields, whereas alfalfa needed more acid (2.5% wt/vol). Table 2 shows the yields of glucose, non-glucose sugars, and acetate from the 121°C pretreatment, and glucose release by cellulase hydrolysis. Notable differences among the biomass species included lower non-glucose sugar yields from alfalfa after pretreatment, reflective of the lower non-glucose sugar content of this species, and higher total glucose yield for switchgrass. For all species, glucose yields declined slightly with increased maturity. In general, there was a small increase in cell wall sugar yields when pretreatment temperature was increased to 150°C (data not shown). Of interest was a reduction in non-glucose sugar yields that was directly proportional to the fructose (and fructan) content of the biomass samples (r = 0.97) due to acid-catalyzed degradation of the fructose. Efficiency of glucose release by pretreatment and enzymatic hydrolysis was negatively correlated (r = -0.93) with Klason lignin concentration.

Table 2. Yields of monosaccharides and acetate after dilute-acid pretreatment at 121°C and cellulase hydrolysis of biomass samples.

† Data are for alfalfa stems only; reed canarygrass and switchgrass data are for whole herbage.

Species†	Released by Acid Pretreatment			Released by	Total Glucose	Glucose Release
Maturity	Glucose	Non-Glucose	Acetate	Cellulase	Released	Efficiency
	------------------------------ g kg^{-1} DM			------------------------------		%
Alfalfa						
Bud	46	121	29	176	223	71.6
Full Flower	44	137	34	173	217	63.9
Reed Canarygrass						
Vegetative	58	250	13	168	226	91.4
Ripe Seed	49	261	20	151	200	66.8
Switchgrass						
Pre-Boot	52	238	19	191	243	81.6
Anthesis	112	243	24	146	258	71.4
Post-Frost	49	252	24	184	233	67.9

Conclusion

Significant differences were discovered among these biomass samples, indicating the need for further research. Specifically further work is needed to analyze replicated biomass samples to ensure the differences observed are reflective of the compositional diversity in these forage species across growth environments. A general pattern that emerged from the data was that increased lignin content of more mature forages was correlated with reduced efficiency of glucose release from cell walls. Alfalfa appeared to be more resistant to the effects of pretreatment and subsequent cellulase treatment (e.g. lower glucose yield), and reed canarygrass conversion efficiency suffered from high sugar loses associated with high concentrations of fructose and fructans. These results suggest that processing conditions may need to be customized for individual species and to a lesser extent for harvest maturity. As to which is the most optimal biomass for ethanol production, it should be noted that this choice will also depend upon adaptation of the crops to local growing conditions and development of valuable co-products to partially defray the cost of ethanol production, such as high-protein leaf meal from alfalfa.

Effect of Forage Source and Dietary Crude Protein on Microbial Protein Synthesis and Ruminal Fermentation In Vitro

J. J. Olmos Colmenero and G. A. Broderick

Introduction

Alfalfa silage (AS) serves as a major source of dietary crude protein (CP) for dairy cows. However, much of the CP in AS is present as NPN that must be utilized via microbial protein synthesis in the rumen. Corn silage (CS) is a low CP forage that is a good source of fermentable energy because of its high starch content. A recent study (Olmos Colmenero and Broderick, 2003 USDFRC Research Summaries) showed that, on diets containing (DM basis) 25% AS and 25% CS, flow of bacterial nonammonia N (NAN) was maximal at 16.5% CP. Widely different proportions of dietary AS and CS are often fed to lactating cows. Therefore the objective of this trial was to measure the effect of 3 combinations of dietary AS and CS at each of 5 CP levels on in vitro ruminal fermentation and microbial NAN synthesis.

Material and Methods

Fifteen diets were formulated in a 3x5 factorial arrangement: 3 forages (50% AS, 25% AS plus 25% CS, and 50% CS in dietary DM) and 5 CP levels (13.5, 15.0, 16.5, 18.0, and 19.5% in dietary DM) and were incubated along with blanks in duplicate flasks in 2 separate ruminal in vitro incubations. High moisture feeds were freeze-dried; all ingredients were ground (1-mm Wiley mill) before mixing diets. The AS averaged (DM basis) 24.0% CP and 32% NDF; CS averaged 7.6% CP and 34% NDF. Due to its unusually high CP content, it was not possible to formulate diets with 13.5 and 15.0% CP with AS as sole forage. Thus, NDF isolated from AS was incorporated at 15% of DM in all 5 of those diets to dilute CP in AS. Diet compositions are in Table 1. Inoculums contained ruminal fluid and McDougall's buffer plus 5 mg ammonia-N/dl and ^{15}N-ammonia (added as ammonium sulfate). Incubations were sampled at 4 and 8 h then centrifuged to obtain supernatants, total solid pellets, and isolated microbial pellets. Supernatants were analyzed for ammonia, total amino acids and VFA. Total solid and microbial pellets were analyzed for DM, total NAN and ^{15}N to estimate DM digestibility and microbial NAN synthesis. Data were analyzed using Proc Mixed in SAS and significance declared at $P < 0.05$. Least squares means from 4-h only are reported.

Table 1. Composition of diets.

	Forage														
	Alfalfa silage					Alfalfa silage + Corn silage					Corn silage				
Item[1] CP, % of DM	13.5	15.0	16.5	18.0	19.5	13.5	15.0	16.5	18.0	19.5	13.5	15.0	16.5	18.0	19.5
	(% of DM)														
Alfalfa silage	35.0	35.0	35.0	35.0	35.0	25.0	25.0	25.0	25.0	25.0	0	0	0	0	0
Corn silage	0	0	0	0	0	25.0	25.0	25.0	25.0	25.0	50.0	50.0	50.0	50.0	50.0
Isolated NDF	15.0	15.0	15.0	15.0	15.0	0	0	0	0	0	0	0	0	0	0
RHMSC	50.0	46.7	43.4	40.1	36.8	46.5	43.2	39.9	36.6	33.3	37.6	34.3	31.0	26.6	24.4
SSBM	0.0	3.3	6.6	9.9	13.2	3.5	6.8	10.1	13.4	16.7	12.5	15.8	19.1	23.5	25.7
Chemical composition															
OM	96	96	96	96	95	96	96	96	96	95	97	97	97	97	96
CP	13.5	15.0	16.5	18.0	19.5	13.5	15.0	16.5	18.0	19.5	13.5	15.0	16.5	18.0	19.5
NDF	29	29	29	29	29	20	20	20	20	20	20	20	20	20	20
ADF	19.2	19.3	19.3	19.4	19.5	10.9	11.0	11.1	11.2	11.3	9.9	9.9	10.0	10.1	10.2
NFC[2]	53	52	50	48	47	61	59	58	56	54	60	59	57	55	54

[1]Isolated NDF = NDF isolated from alfalfa silage, NFC = nonfiber carbohydrate, RHMSC = rolled high-moisture shelled corn, SSBM = solvent-extracted soybean meal.
[2]NFC = 100 - NDF - CP - Ether exctract - Ash + NDFCP.

Results and Discussion

The only significant ($P = 0.03$) forage-by-CP interaction was a small difference in acetate to propionate ratio for AS+CS (2.59) versus the other two diets (mean 2.62). Therefore, it was possible to summarize results as least squares means for each forage source (Table 2) and CP level (Table 3). Although mean pH was lower for AS+CS, it was only 0.05 unit below that for AS and CS. Mean ammonia concentration was higher on CS versus AS and AS+CS (Table 2); this surprising effect may have resulted from sustained degradation of the greater quantity of soybean meal CP on that diet. Concentrations of total amino acids, as well as isovalerate and isobutyrate (VFA produced from degradation of branched-chain amino acids), were higher for AS and AS+CS than for CS (Table 2). This probably resulted from the higher NPN content in AS-containing diets. Concentrations of acetate, propionate, butyrate and total VFA all were higher on the two AS diets (Table 2). Alfalfa contains from 10 and 15% total pectin, which is rapidly fermented by ruminal microbes to acetate and propionate. That total VFA were lower on CS, despite similar true DM digestibility, likely occurred because microbial NAN synthesis and efficiency were significantly higher ($P<0.01$) on that diet compared to AS and AS+CS. Diets with similar digestibilities, but greater efficiencies of energy utilization, result in greater capture of microbial biomass with less dietary carbon appearing in fermentation end-products.

Table 2. Effect of forage source on ruminal fermentation and microbial NAN synthesis after 4-h in vitro incubations.

		Forage[1]			*P*>F[2]	
Item	AS	AS+CS	CS	SE[3]	Forage	Forage x CP
pH	6.73[a]	6.68[b]	6.73[a]	0.01	0.01	0.69
Ammonia-N, mg/dl	4.26[b]	4.42[b]	4.80[a]	0.10	<0.01	0.13
Total free AA, mM	3.38[a]	3.37[a]	2.59[b]	0.06	<0.01	0.93
Acetate, mM	51.6[a]	52.9[a]	44.9[b]	1.4	<0.01	0.96
Propionate, mM	19.7[a]	20.5[a]	17.1[b]	0.6	<0.01	0.84
Acetate:Propionate	2.62	2.59	2.62	0.01	0.06	0.03
Butyrate, mM	7.98[a]	8.26[a]	7.09[b]	0.20	<0.01	0.85
Isobutyrate, mM	0.82[a]	0.85[a]	0.67[b]	0.03	<0.01	0.55
Valerate, mM	0.99	1.01	0.90	0.06	0.43	1.00
Isovalerate, mM	1.34[a]	1.43[a]	1.12[b]	0.05	<0.01	0.17
Total VFA, mM	82.4[a]	84.9[a]	71.9[b]	2.3	<0.01	0.93
True DM digestibility, %	50.1	50.9	49.0	1.1	0.40	0.99
Microbial NAN, mg/dl	22.1[c]	23.3[b]	25.2[a]	0.4	<0.01	0.84
Net Microbial NAN, mg/dl	15.7[b]	16.9[b]	18.8[a]	0.5	<0.01	0.88
Microbial efficiency[4]	31.6[b]	33.2[b]	38.5[a]	1.1	<0.01	0.84

[a,b,c]Means in the same rows without a common superscript differ ($P<0.05$).
[1]AS = alfalfa silage, AS+CS = alfalfa silage plus corn silage, CS = corn silage.
[2]Probability of a significant effect of forage source or of a forage x CP interaction.
[3]Standard error of the least squares means.
[4]Net efficiency, mg microbial NAN/g of DM truly digested.

Mean pH, concentrations of all VFA, true DM digestibility, and microbial efficiency all were unaffected by dietary CP content. Concentrations of ammonia and total amino acids increased linearly; this was expected because soybean meal replaced corn to increase dietary CP and soybean meal protein has relatively high ruminal degradability. However, a quadratic response in microbial NAN synthesis to increasing dietary CP also was observed (Table 3). The maximal yield of microbial NAN occurred at 16.5% CP before declining at 18.0 and 19.5% dietary CP. Because DM digestibility was unaffected by CP, this response was reflected in a quadratic trend for microbial efficiency (g net NAN/g of DM truly digested). It is well established that, if RDP supply is lower than required, microbial growth will be restricted. Although the NRC (2001) model indicates that 10.2% degraded CP is adequate for microbial protein synthesis in the rumen, recent in vitro and in vivo reports indicate that greater amounts may be required to maximize microbial growth.

Table 3. Effect of dietary CP content on ruminal fermentation and microbial NAN synthesis after 4-h in vitro incubations.

	CP, % of DM						P>F[1]		
Item	13.5	15.0	16.5	18.0	19.5	SE[2]	CP	L	Q
pH	6.72	6.71	6.71	6.71	6.71	0.02	0.97	0.58	0.71
Ammonia-N, mg/dl	4.01[c]	4.39[b]	4.54[ab]	4.68[ab]	4.85[a]	0.12	<0.01	<0.01	0.35
Total amino acids, mM	2.98	3.10	3.09	3.15	3.24	0.08	0.29	0.04	0.99
Acetate, mM	49.9	49.7	49.7	49.6	50.3	1.8	1.00	0.91	0.81
Propionate, mM	19.2	19.0	19.1	19.1	19.1	0.7	1.00	0.98	0.93
Acetate:Propionate	2.60	2.61	2.61	2.61	2.64	0.01	0.43	0.18	0.38
Butyrate, mM	7.92	7.82	7.85	7.62	7.67	0.26	0.91	0.39	0.98
Isobutyrate, mM	0.78	0.79	0.80	0.78	0.76	0.04	0.95	0.60	0.55
Valerate, mM	0.99	0.98	0.96	0.95	0.95	0.08	0.99	0.63	0.90
Isovalerate, mM	1.28	1.31	1.37	1.27	1.26	0.07	0.79	0.70	0.34
Total VFA, mM	80.0	79.7	79.8	79.2	80.0	2.9	1.00	0.97	0.89
True DM digestibility, %	49.7	49.2	50.4	49.6	51.1	1.4	0.87	0.47	0.69
Microbial NAN, mg/dl	22.2	23.7	24.3	24.0	23.8	0.6	0.06	0.04	0.03
Net Microbial NAN, mg/dl	15.7	17.3	17.9	17.6	17.4	0.6	0.10	0.06	0.05
Microbial efficiency[3]	32.0	35.2	35.6	35.4	34.1	1.4	0.30	0.29	0.06

[a,b,c]Means in the same rows without a common superscript differ (P<0.05).
[1]Probability of a significant effect of dietary CP or of linear (L) or quadratic (Q) effects of dietary CP.
[2]Standard error of the least squares means.
[3]Net efficiency, mg microbial NAN/g of DM truly digested.

Conclusions

Under the conditions of this in vitro study, similar ruminal DM digestion was observed, but diets containing 50% CS gave rise to greater microbial growth at higher efficiency of energy utilization compared to diets containing either 50% alfalfa silage or 25% alfalfa silage plus 25% corn silage. Moreover, microbial NAN synthesis and efficiency of energy utilization were maximal at 16.5% CP with no further improvement at higher levels of dietary CP.

Measuring Particle Size, Fragmentation Index, and Effective Fiber in Corn Silages

D.R. Mertens and G. Ferreira

Introduction

Chemical composition establishes the amounts of components in corn silage that affect nutritive value, but physical properties can affect ensiling and the utilization of nutrients and the physiological responses by cows. Physical characteristics of corn kernels affect the availability of starch, which is a major contributor to the digestible energy in corn silage. Although chopping can disrupt corn kernels if the chopping length is short, processing through rollers during chopping is typically used to increase kernel damage and by implication increase starch digestibility. However, the actual impact of chopping and processing is variable depending upon the chopper setting for theoretical length of cut (**TLC**), the sharpness of the knives, roller clearance, and rate of flow of chopped material through the processor.

Physical properties can affect the packing density of the silage, which influences ensiling fermentation and storage stability. Particle size also affects chewing activity and the effectiveness of fiber in meeting the minimum fiber requirements of dairy cows. Both chopping length and processing alter the physical characteristics of corn silage that affect kernel disruption, mean particle size and effective fiber. Given the variation in chopping and processing that can occur, it is evident that methods for measuring physical characteristics of corn silage are needed. The objective of this research was to develop a laboratory method for simultaneously measuring the particle size, extent of kernel fragmentation, and physically effective fiber of corn silages.

Recommended Method for Measuring Particle Size, Fragmentation Index and Effective Fiber

Vigorous, vertical shaking of dried silages is needed to dislodge fine particles of starch that adhere to larger particles in silage when the sample is moist. To measure corn silage nominal mean particle size (**MPS**), fragmentation index (**CSFI**) and physically effective neutral detergent fiber **(peNDF)**:

1) Mix the undried silage sample thoroughly and dry a representative sample of 600 ±100 mL volume for particle size separation and a separate complete sample of 100 to 200 g wet weight for routine analysis. Dry under conditions that will not affect starch or fiber analyses (55 to 60 °C).
2) Record the tare weight of a set of sieves with square apertures of 19.00, 13.20, 9.50, 6.70, 4.75, 3.35, 2.36, 1.18, 0.60 and 0.30 mm, in addition to the bottom pan. Stack sieves in ascending order of aperture. Use 20-cm diameter brass or stainless steel sieves with 5-cm height for apertures of 4.75 mm and above and 2.5-cm height for all other apertures (ATM Corporation, Milwaukee, WI). Using a large number of sieves prevents accumulation and bridging of material on any one sieve and allows shorter shaking times.
3) Transfer the entire 600 ml of dried test sample on the top sieve of a stacked series of sieves, cover with lid, and place on a vertical shaker (RoTap or equivalent, W.S. Tyler Incorporated, Mentor, OH).
4) Shake for 10 min and record the weight of retained material plus sieve for each sieve in the series.
5) Composite all retained residues on sieves with apertures of 4.75 and larger. Grind the >4.75-mm composite and complete samples suitable for starch analysis. Determine starch concentrations of the >4.75-mm composite and total samples using an appropriate method.
6) Composite all residues passing through the sieve with 1.18-mm aperture and analyze the <1.18-mm composite and total samples for aNDF.

7) To determine MPS (minimal cross-sectional dimension) of corn silage calculate the weight of residue retained on each sieve and determine geometric mean particle size as described by ANSI (1993) using the square dimension of sieve apertures (Table 1).
8) Calculate CSFI = 100 × [(Total sample starch concentration) – (fraction of total particle size sample retained on >4.75-mm composite sample sieves) × (>4.75-mm composite sample starch concentration)] / (Total sample starch concentration) (Table 2).
9) Calculate peNDF (NDF distribution) = (Total sample aNDF concentration) – (fraction of total particle size sample passing through the 1.18-mm sieve) × (<1.18-mm composite sample aNDF concentration) (Table 2) or
   Calculate peNDF (DM distribution) = (Total sample aNDF concentration) × (fraction of DM passing through the 1.18-mm sieve)

Table 1. Calculation of nominal mean particle size in corn silage.

Aperture Dimension[a]	Sieve weight	Sieve + Sample weight	Sample weight	Fraction retained[b]	Log (GM)[c]	Fraction × Log(GM)[d]
(mm)	(g)	(g)	(g)	(%)	(log_{10})	
26.400[e]						
19.000	568.77	570.28	1.51	0.020	1.350	0.027
13.200	502.87	504.98	2.11	0.028	1.200	0.034
9.500	492.95	498.30	5.35	0.071	1.049	0.075
6.700	473.32	481.78	8.46	0.113	0.902	0.102
4.750	771.73	784.46	12.73	0.169	0.751	0.127
2.360	450.42	467.58	17.16	0.228	0.525	0.120
1.180	337.49	347.34	9.85	0.131	0.222	0.029
.600	288.42	297.04	8.62	0.115	-0.075	-0.009
(Pan) .038[f]	255.95	265.29	9.34	0.124	-0.821	-0.102
Totals			75.13	1.000		0.403[g]
MPS[h]						2.528[h]

[a] Square dimension of sieve aperture.
[b] (Sample weight / Total sample weight).
[c] Logarithm (base 10) of the geometric mean dimension of particles retained by the sieve. Log(GM) = Log_{10} [√ (aperture dimension of sieve above × aperture dimension of the sieve retaining particles)].
[d] Fraction retained × Log(GM) for each sieve.
[e] To calculate the geometric mean dimension of particles on the top sieve (19000 nm) it is assumed they would have passed through the aperture of the next largest geometric progression of apertures. To minimize error associated with this assumption the top sieve should contain < .05 of the total sample weight.
[f] To calculate the geometric average dimension of particles in the pan it is assumed they have minimum size of 38 nm. To minimize error associated with this assumption the pan should contain < 10% of the total sample weight.
[g] Weighted logarithmic normal mean = Average Log(GM) of particles.
[h] Nomical mean particle size in mm (minimal cross-sectional dimension) = $10^{[\text{Average Log(GM)}]}$.

Table 2. Calculation of corn silage fragmentation index (CSFI) and physically effective NDF (peNDF).

Fraction	Aperture size	Fraction of Total[a]	Starch	aNDF
Coarse composite	>4.75 mm	.401	20.90	
Medium composite	1.18-4.75 mm	.360		
Fine composite	<1.18 mm	.239		37.50
Complete corn silage		100.00	27.02	42.06
CSFI[b]			68.95	
peNDF[c] (NDF dist.)				33.09
pef[d] (NDF dist.)				0.79
pef[e] (DM dist.)				0.76
peNDF[f] (DM dist.)				32.00

[a] Sums of percentage retained on sieves from Table 1.
[b] CSFI =100 × [(Total starch concentration) – (fraction retained on sieves >4.75-mm) × (coarse composite starch concentration)] / (Total sample concentration).
[c] peNDF (NDF distribution) = (Total aNDF concentration) – (fraction passing through the 1.18-mm sieve) × (<1.18-mm composite sample aNDF concentration).
[d] Physical effectiveness factor (pef) = 100 × [(Total aNDF concentration) – (fraction passing through the 1.18-mm sieve) × (<1.18-mm composite aNDF concentration)] / (Total sample aNDF concentration).
[e] Physical effectiveness factor (pef) based on DM distribution = 100 – (Fraction <1.18 mm).
[f] peNDF (DM distribution) = (Total aNDF concentration) × (fraction passing through the 1.18-mm sieve).

Results and Discussion

Chemical composition. Thirty-two corn silages were obtained from a commercial feed analysis laboratory (Dairyland Laboratories, Inc., Arcadia, WI), which were diverse in chemical and physical characteristics. The range in minimum and maximum chemical and physical characteristics of the 32 selected materials indicate that they represent a wide diversity of corn silages (Table 3). Average chemical composition was similar to that reported by NRC (2001) for normal corn silage, except for ash which was higher than that reported by NRC (5.1 versus 4.0%).

Table 3. Chemical and physical characteristics of 32 diverse corn silages.

Characteristics	Average	Standard Deviation[a]	CV[b]	Minimum	Maximum
DM, %	34.7	±7.8	22.5	19.2	48.1
ANDF[c], % DM	44.2	±6.4	14.5	30.0	56.3
NFC[d], % DM	41.6	±7.1	17.1	27.4	57.7
Starch, % DM	25.2	±5.7	22.5	12.2	36.2
Crude Protein, % DM	7.8	±1.4	17.4	5.7	12.5
Ash, % DM	5.1	±1.5	29.9	3.1	9.6
MPS[e], mm	4.2	±1.4	33.5	2.1	7.3
DM <1.18 mm, %	86.2	±8.0	9.3	67.5	96.5
peNDF[f], % DM	38.3	±7.1	18.5	25.8	50.9
CSFI[g], % of starch	47.8	±20.6	43.1	0.0	91.3

[a] The range of average minus one standard deviation to average plus one standard deviation will include about 2/3 of the samples.
[b] Coefficient of variation = 100*Standard deviation / Average.
[b] Amylase-treated NDF.
[c] Nonfibrous carbohydrates = 100 – ash – CP – EE – aNDF (EE estimated to be 3.2% of DM).
[d] Mean particle size of the minimal cross-sectional dimension.
[e] Physically effective NDF estimated from DM particle size distribution.
[f] Corn silage fragmentation index, percentage of starch retained on sieves with apertures >4.75.

Mean Particle Size. There are numerous methods of measuring particle size, which generate different estimates of MPS. Separation of undried feeds with gentle shaking is often incomplete because small particles adhere to large particles and this is especially true for small starch particles. The time needed to obtain complete separation varies with the dryness and amount of sample, number of sieves, and shaking motion and intensity. With our vertical shaker and dried samples it typically takes 15 to 20 minutes to obtain maximum separation using a 600 ml volume of test sample. However, >90% of the separation occurs in the first 10 minutes and this time was selected for the recommended method to maximize sample throughput. Too minimize bridging of material on sieves, no sieve should retain more than 25% of the total sample.

Gentle, horizontal shaking keeps particles in a length-wise plane and separates them by the length (largest cross-sectional dimension). Conversely, vigorous, vertical shaking of particles tends to bounce them on end and they pass through sieve apertures based on their width or diameter. In our method, nominal MPS is calculated as described by using the square instead of the diagonal dimension of apertures. This method of sieving results a determination MPS that measures the smallest cross-sectional dimension of particles. The length of sieved corn silage particles were about 3.4 times their width and the diagonal dimension of square apertures is $\sqrt{2}$ times the square dimension. Multiplying our nominal MPS by 4.8 should approximate the mean particle length, which varied from 3/8 to 1 3/8 inches with an average of ¾ inch for the 32 diverse silages.

Corn Silage Fragmentation Index. We observed that sieves with square apertures of 6.25 mm and larger retained most whole corn kernels in corn silage and those with apertures of 4.75 mm retained kernel fragments bigger than one fourth of a kernel. The in vitro dry matter disappearance (**IVDMD**) of the kernels and fragments retained on the sieves > 4.75 mm was about 30% of that obtained when these kernel fragments were finely ground. It was concluded that starch in particles that are less than ¼ of a kernel would be readily digested, and that the proportion of total starch that is in fragments < 4.75 mm could be used as a corn silage fragmentation index (CSFI) to provide a quantitative measure

of kernel fragmentation in corn silage. We observed that CSFI was positively correlated with IVDMD of whole, unground silages, which confirms that fermentation of starch in small fragments would be digested without chewing by the animal.

For our diverse set of corn silages, the CSFI ranged from 0 to 91% and was negatively correlated with MPS (r = -0.46). The low correlation between CSFI and MPS is related to different degrees of kernel disruption within the same chop length (Fig. 1) Although we have no information about the chopping length and processing of the selected corn silages other than visual appraisal, we speculate that the upper-right and lower-left boundaries of the data in Figure 1 represent corn silages that were maximally processed or unprocessed, respectively, at a given MPS. We are planning an experiment to confirm these relationships.

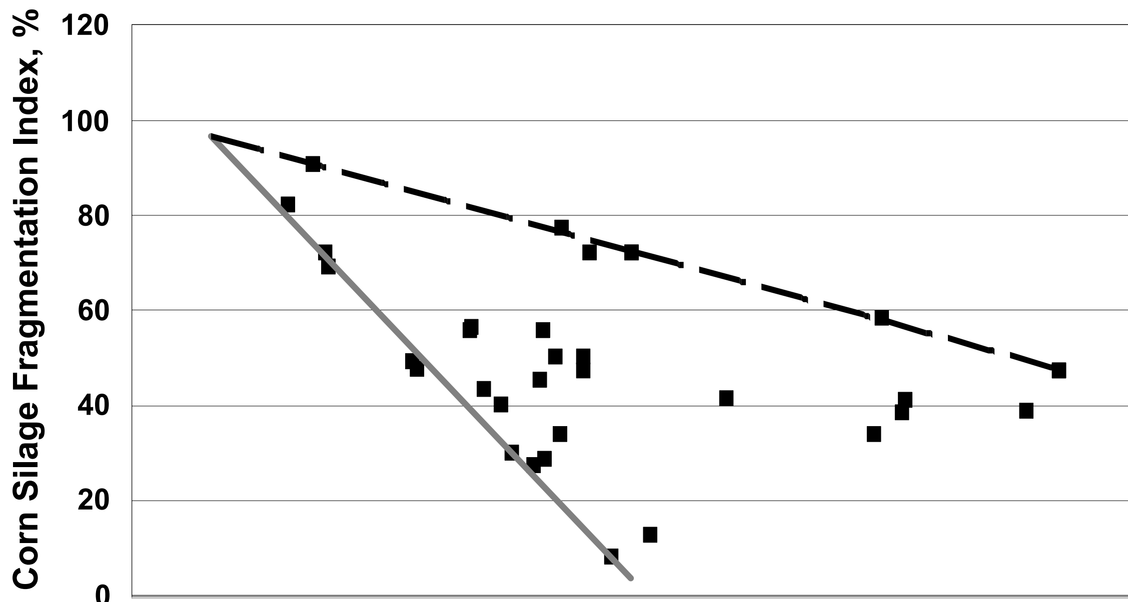

Figure 1. Relationship of corn silage fragmentation index to the mean particle size of corn silages. Upper dashed line is the proposed boundary of maximally processed corn silages at each mean particle size and the lower solid line is the proposed boundary of unprocessed corn silages.

Figure 1 suggests that longer chopping lengths will also result in less fragmentation with processing, which indicates there is a conflict between chopping length and CSFI. Currently we can speculate that a CSFI of 70% would require a MPS of 2.4 mm (about 7/16 inch particle length) without processing, but could be achieved with maximal processing at a MPS of 4.7 mm (about 7/8 inch particle length). The merging of these lines at a MPS of about 1.5 mm indicates the point at which corn processing has minimal impact because fine chopping already fragments kernels. The high variation in CSFI at a given mean particle size, especially at large MPS, confirms the importance of having a quantitative index of kernel fragmentation in corn silage.

Physically Effective Neutral Detergent Fiber. It is well known that dairy cows require a minimum amount of insoluble dietary fiber that is of adequate particle size to stimulate chewing activity and promote ruminal function and cow health. Chewing activity is related to both NDF concentration and particle size, and the concept of physically effective NDF (peNDF) combines these properties in a single measurement. Based on the stimulation of chewing per unit of NDF intake, the physical effectiveness of coarse, medium and finely chopped corn silage ranged from .90 to 1.00, .85 to .95, and .80 to .90,

respectively. The variation in physical effectiveness within and among chopping lengths indicates that a quantitative method for measuring the peNDF of corn silage directly would be useful.

Particles passing through a 1.18-mm sieve do not stimulate chewing, suggesting that a simple laboratory method for measuring particle size distribution using vigorous vertical shaking could be used to estimate physical effectiveness factors. By assuming that NDF is evenly distributed among particles, the fraction of DM retained on sieves with apertures of 1.18 mm and larger could estimate the physical effectiveness factor. Thus, peNDF (based on DM distribution) would equal aNDF X (fraction of DM retained on >1.18-mm sieves). Using this approach, the physical effectiveness factors (DM distribution) of the 32 diverse corn silages ranged from .675 to .965 (Table 3). The average physical effectiveness factor was .862, which compares favorably with the average standardized value of .85 estimated from chewing activity.

Because fiber is the feed component that requires extensive chewing, it would be most accurate to estimate peNDF based on the distribution of NDF in particles. This approach could easily be adopted by collecting the residue passing through the 1.18-mm sieve and analyzing it for aNDF. Then peNDF based on NDF distribution could be calculated as total amylase-treated NDF (**aNDF**) minus NDF passing through the 1.18-mm sieve. There is a slight difference in peNDF determined by DM or NDF distributions (Table 2); therefore the method of determining peNDF should be indicated when results are reported.

Conclusion

The proposed method for simultaneously measuring MPS, CSFI and peNDF provides a tool for assessing the physical properties of corn silage that are important in ensiling, starch utilization and ruminal health and function. Mean particle size is probably related to potential density of the silage and provides information about the relative impact of chopping length and processing on kernel fragmentation. Corn silage fragmentation index provides a quantitative measure of the extent of kernel disruption, which should be related to the rate of fermentation and total digestion of starch in corn silage. Given the important contribution that grain in corn silage makes to total dry matter digestion, information about starch utilization should be a valuable tool in adjusting the energy value of corn silage. When corn silage is a major contributor of fiber in dairy rations, it is important that its physical effectiveness be measured and taken into account when formulating diets that are borderline in fiber.

Measure Dry Matter Routinely Using a Food Dehydrator

D.R. Mertens, K. Bolton and M. Jorgensen

Introduction

Dry matter (**DM**) of silages can change from one day to the next, especially when we move to forage that was cut at different times of the day or from different fields or cuttings. These changes affect the ration that is mixed and fed, resulting in altered milk production. Changes in DM can have dramatic impacts on health and productivity of dairy cows, if the formulated ration was designed to be at the upper or lower limits of fiber concentration. For example, assume that alfalfa silage changes from 45 to 35% DM and we did not adjust the mixing ration. The 3333 lb of silage added to the total mixed ration (**TMR**) only contains 1167 lb of DM from alfalfa silage containing 35% DM instead of 1500 lb of DM when the silage was 45% DM. Not only have we have shorted the group of cows 333 lb of DM, but also we have lowed the fiber content of the diet.

We sampled the silages being fed to the non-research herd at the U.S. Dairy Forage Research Center farm every 3 to 4 days for several months (Fig. 1). The range within lots of silage varied from 3 to 8% DM and there were 6 to 10% DM differences between lots of both alfalfa and corn silages (Fig. 1). Although changes in forage DM can be gradual, rain and snow can result in abrupt changes in forage DM in exposed bunker silos of 5 to 15% DM, especially if there is loose silage on the floor of the silo.

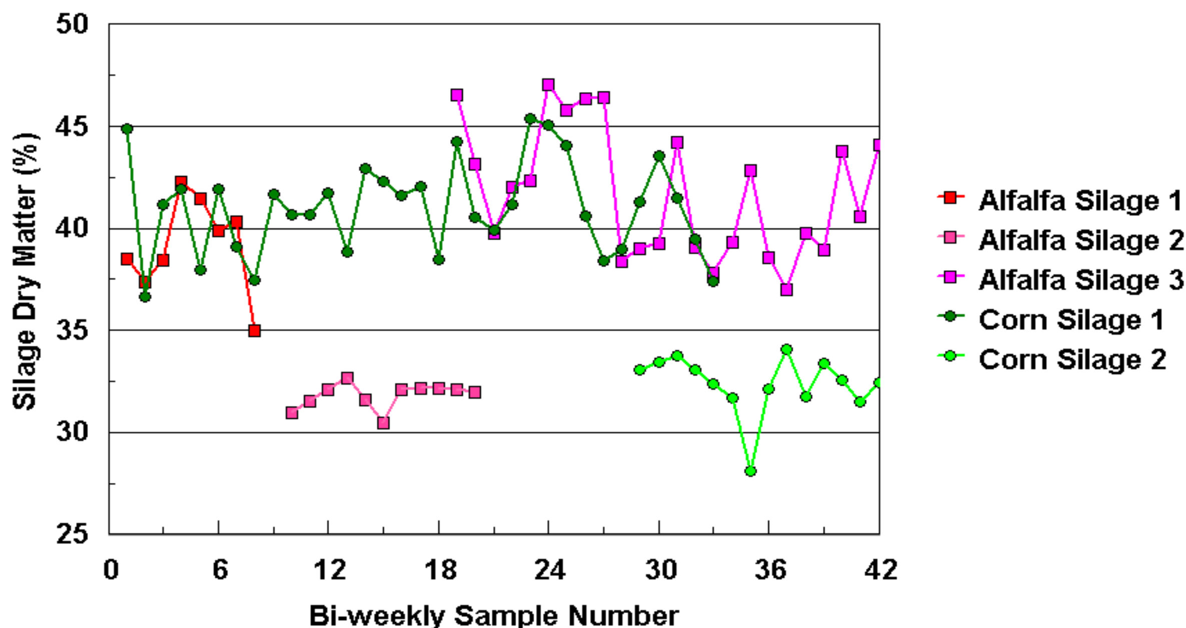

Figure 1. Variation in dry matter (determined by drying in a convection oven at 55 °C for 24 hours) of silages fed at the U. S. Dairy Forage Research Center over several months.

We observed that 25% of the TMR fed on dairy farms were significantly different in DM from the formulated diet. These difference indicate that dairy rations can be inconsistent due to changes in DM of the forage. Dairy farmers need a simple, rapid method of measuring DM that can be used routinely. Koster testers and microwave ovens provide rapid methods of measuring DM, but they dry only one sample at a time and must be monitored during the drying process. It would be ideal if a method could dry multiple samples, would be somewhat automated, and could be worked into the routine flow of daily feeding activity. Food dehydrators are designed to dry moist foods at relatively low temperatures, and they are readily available and have the capacity to dry multiple samples. The

objective of this research was to evaluate the use of a commercial food dehydrator to measure DM of multiple samples routinely.

Materials and Methods

We evaluated an Excalibur Model 3926T (26 hour timer) food dehydrator containing nine shelves with the variable temperature control set at the maximum (155 °F). By removing every other shelf, this unit can dry four samples simultaneously in pans with suitable surface area. To ensure that a single sample of forages would be representative for DM determination, we selected a 200-gram test sample amount. This test sample amount was dried in several pans with dimensions (lengthxwidthxdepth) of 21.1×11.0×7.0, 30.2×20.6×5.2, 38.5×25.7×3.0 or 26.5×16.7×3.8 cm. Recommended Procedure:

1. Collect a representative sample of all moist feeds (a total of about 2 gallons collected from 10 locations).
2. Turn on the dehydrator to preheat it and leave the front open.
3. Place a labeled 10×15, 11×14 or 12×13 inch baking pan on the scale and record the pan weight (P_wt) to the nearest 0.1 g.
4. Tare the pan to 0.0 and add 200-205 g of test sample for the first feed.
5. Remove the pan + sample from the scale, spread it evenly over the surface of the pan
6. Weigh pan + sample and record the weight (S_wt) to the nearest 0.1 g. (If any sample is dislodged from the pan when spreading the sample, it is not weighed.)
7. Place one of the fine mesh screens that come with the dehydrator on the back portion of the pan (closest to the fan) to minimize loss of dried sample by air flow. Load the pan in the dehydrator.
8. Repeat steps 2 through 6 for each additional feed.
9. After all pans are loaded, replace the front cover of the dehydrator. It will not close completely when 15-inch pans are used but this is OK.
10. Timer can be set to the appropriate time for drying. If drying for 2 hours to obtain a rapid estimate of DM, it is recommended to reload the samples and dry for an additional 2 to 6 hours to confirm the DM determination.
11. After the samples have dried, remove each sample from the dehydrator and remove the mesh screen. Weigh each pan + dried sample and record the final weight (F_wt) to the nearest 0.1g.
12. Calculate DM: % DM = 100 X (F_wt – P_wt) / S_wt. If DM was determined after 2 hours of drying, estimate DM using the equation: % DM 24h = -17.4 + 1.21 X (% DM 2h).

Drying at 55 °C for 24 hours closely matches the DM determined by measuring true water loss using Karl Fischer or toluene distillation and was selected as the reference DM value for comparison to the dehydrator method. Drying at 55 °C (131 °F) volatilizes some non-water components in feed but also leaves some residual water. These two errors seem to compensate for one another. We evaluated the food dehydrator method using 24 silages (including 8 alfalfa silages and 11 corn silages) that ranged from 22.2 to 54.2% DM and four concentrates (including high moisture corn, distiller's grain and brewer's grains) that ranged from 24.5 to 73.4 % DM.

Results and Discussion

Measuring DM is not as easy as it appears. Some water is on the surface of particles and is easy to evaporate, other water is inside of particles and must migrate to the surface to be evaporated, and some water is tightly bound to molecules in the feed. Thus, it takes time for moisture to migrate and evaporate from feeds. Oven drying is also complicated because compounds other than water can evaporate. In fermented feeds, like silages or wet distiller's or brewer's grains, there are significant amounts of short

chain fatty acids that are volatile. In addition, residual alcohols and protein degradation products are also volatile. Even carbohydrates can lose weight when dried at temperatures that are too high.

Forages are very heterogeneous, and preliminary studies indicated that 200 g test samples reduced variation among DM measurements. Initial experiments demonstrated that the surface area of the pan affected the rate at which samples would dry. Drying rate was considerably slower when there was more than 1.3 to 1.4 grams of moist sample per square inch of pan area. Thus, a 10í15, 11í14 or 12í13 inch pan would be acceptable for a 200 g test sample. We recommend cookie or baking pans because they are inexpensive, durable, and easy to obtain.

After selecting the test sample amount and pan size, we evaluated the effect of drying time in the food dehydrator. The dehydrator could dry four samples in the first 4 hours at a low temperature that would minimize evaporation of non-moisture matter and not char the sample without supervision. We suspect that this efficient drying is due to the combination of shallow test sample depth and high flow rate of heated air in the dehydrator. It is obvious that some volatile fatty acids and alcohols are evaporated by the dehydrator method, but their loss is probably compensated by the incomplete removal of all water at 155 °F, similar to the laboratory reference method for DM. After 4 hours in the dehydrator, average DM values were typically within 99% (slightly lower for wetter samples) of those from our reference oven method. Drying in the dehydrator for 6 to 8 hours equaled our reference method DM and drying for 24 hours resulted in DM that were slightly lower than the reference values. The standard deviations among replicated samples was higher for shorter drying times, but were similar to the reference method when drying time exceeded 6 hours (Table 1). It appears that the dehydrator can dry replicated 200-g test samples within 1% DM of one another, which is similar precision to the reference laboratory method.

Table 1. Change in dry matter determination and its standard deviation with drying time in a food dehydrator (155 °F) for three forages each dried on all four shelves on two different days.

Drying time	Dry Matter	Standard deviation[a]
2 hours	45.7	±1.80
4 hours	35.8	±1.41
6 hours	34.8	±1.16
8 hours	34.5	±1.04
24 hours	34.3	±1.03
24 hours, Reference Method[b]	34.6	±0.99

[a]Standard deviation is the + and – spread in the average value that would include 2/3 of the replicates.
[b]Reference method dry matter determined in a forced-air draft convection oven at 131 °F for 24 h.

On average, alfalfa silages dried more quickly than corn silages, but they also differed in DM (40.1 and 34.6%, respectively). Most of the difference in drying rate is related to the moisture content of the silage. As expected, feeds with higher moisture dried at a slower rate. When the moisture contents were similar, alfalfa silage had only a slightly faster drying rate than corn silage (Fig. 2).

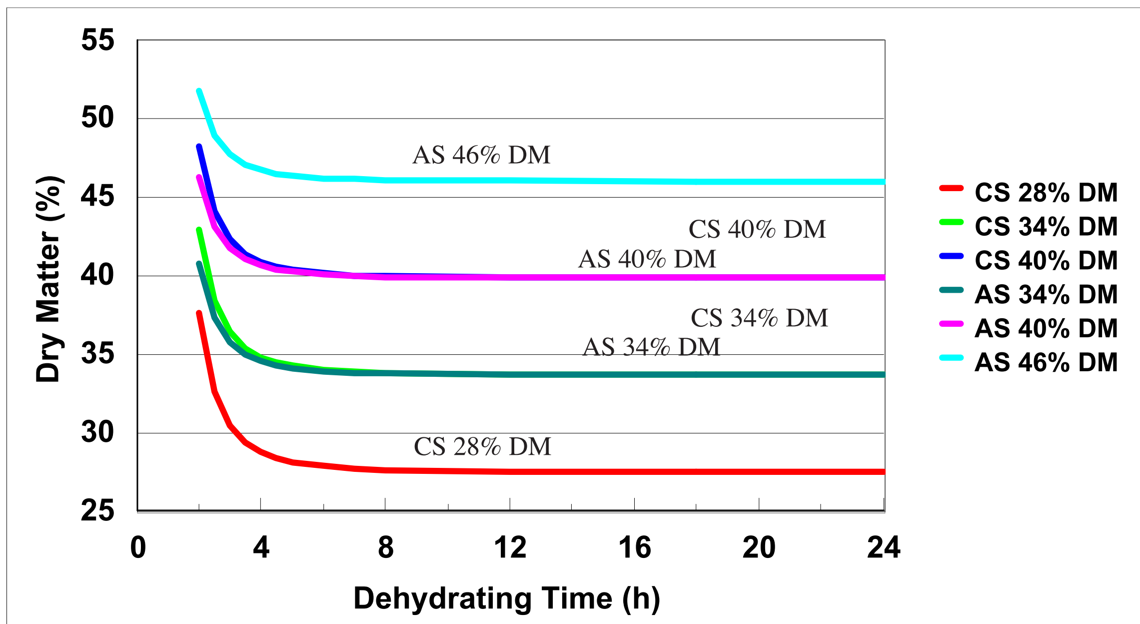

Figure 2. Effect of drying time on dry matter determination of alfalfa and corn silages of different dry matter concentrations using a food dehydrator at 155 °F.

There are times when a rapid determination of DM is needed to adjust the mixing ration for moisture added by rain or snow. Although it appears that samples cannot dry completely in 2 hours, it was possible to develop an equation that can be used to estimate the 24-hour DM with from 2-hour DM with acceptable accuracy: 24h DM = -17.4 + 1.21 X (2h DM). This equation is only valid for the specific dehydrator and method we used. Although using the equation is not as accurate as drying for 6 to 8 hours (variation that is about 2.5 times that of the reference method), it is acceptable for quickly checking DM.

We tested the food dehydrator method to ensure that it could handle four very wet samples and observed that they dried at similar rates compared to when they were dried as single sample with three other materials in the dehydrator. We also evaluated the effect of shelf location. We observed that the average DM for all drying times that was determined on the bottom and top shelves (36.7 and 36.6%, respectively) were slightly higher than DM on the middle shelves (35.9 and 36.2%). Because the bottom and top shelves had higher DM it indicates that samples on them did not dry as quickly. Most of this difference in DM was related to short drying times and by 24h there was less than 0.5% DM difference among shelves. The variation in DM among shelves is small relative to the variation among replicated test samples and should be of little concern. However, most efficient drying of samples can be obtained by putting the samples with most moisture on the middle shelves.

Conclusions

A simple DM method was developed using a food dehydrator that could dry four samples rapidly with a minimum of attention. This method could be used on dairy farms to routinely monitor the changes in DM of feeds and provide the necessary information need to adjust dairy rations. Providing more consistent with rations will minimize fluctuations in milk production and negative impacts on animal health.

Feed Utilization by Cattle

Effect of Amount and Ruminal Degradability of Dietary Soybean Meal Protein on Performance of Lactating Dairy Cows

J. J. Olmos Colmenero and G. A. Broderick

Introduction

Dietary crude protein (CP) should supply sufficient rumen-degraded protein (RDP) to support optimal microbial protein formation plus sufficient rumen-undegraded protein (RUP) with the proper amino acid (AA) balance to complement the microbial protein. Solvent-extracted soybean meal (SSBM) is the most common protein source fed to dairy cows in the U.S. However, SSBM has a relatively high RDP content and replacing some SSBM with supplemental RUP may allow equivalent production of milk and protein at lower dietary CP content. Under this scenario, there would be more efficient conversion of dietary N into milk protein and economic advantage would be gained through reduced feed costs rather than increased production. Therefore, the objective of this study was to determine whether production could be maintained at lower dietary CP content by partially replacing SSBM with expeller soybean meal (ESBM; SoyPlus, West Central Coop., Ralston, IA; a heat-treated soybean meal), thereby reducing N excretion.

Material and Methods

Twenty-eight multiparous Holstein cows averaging 129 days-in-milk and 656 kg BW were blocked by days-in-milk into 7 groups of 4 (one block with ruminal cannulas). Cows within blocks were randomly assigned to balanced 4x4 Latin squares and fed total mixed rations that differed in source and level of CP: A) 15.5% CP with RUP added as ESBM; B) 16.5% CP without ESBM; C) 16.5% CP plus ESBM; and D) 17.5% CP without ESBM (Table 1). Experimental periods lasted 28 d--14 d for adaptation and 14 d for data collection. Feeds were analyzed weekly for CP and DM to adjust to the desired CP contents as a proportion of dietary DM. Fecal and urinary N excretion were estimated from spot fecal and urine samples using indigestible ADF as fecal maker and creatinine as urine volume marker. Rumen metabolites were determined in cannulated cows at various times from 0 to 24 hours after feeding. Data were analyzed using the Proc Mixed procedures in SAS. Contrasts were used to compare the effects of: 1) Diet A versus B; 2) Diet B versus C; and 3) Diet B versus D. Significance was declared at $P < 0.05$; least squares means are reported.

Results and Discussion

Dietary NDF contents were similar (average 27% of DM), indicating that diets were of high energy and typical of those fed to early lactation cows (Table 1). Actual dietary CP contents were 15.6, 16.6 and 17.6%, so dietary CP targets were nearly achieved in the trial. Generally, there were no significant effects ($P > 0.05$) of diet on production traits in this trial (Table 2). However, there was a trend ($P = 0.08$) for lower milk yield in cows fed diet A versus diet B, suggesting that feeding RUP from soybean meal could not be used to reduce dietary CP below 16.6%. Moreover, trends were detected for higher DM intake ($P = 0.09$) and weight gain ($P = 0.10$), and lower milk yield/DM intake ($P = 0.06$), in cows fed diet D versus diet B (Table 2), suggesting that increased dietary CP increased intake but without affecting production. Another interesting finding was significantly ($P \leq 0.03$) higher milk lactose concentrations with the feeding of RUP as ESBM (Table 2). Milk urea N was increased ($P < 0.01$) by

each increment of dietary CP; adding RUP to the 16.6% CP diet also increased milk urea (Table 2).

Apparent N efficiency (milk-N/N-intake) declined 2.5 percentage units ($P < 0.01$) when dietary CP was increased from 16.6% (diet B) to 17.6% (diet D) (Table 2). This lower N efficiency was paralleled by increased ruminal concentration of ammonia-N as well as increased urinary excretion of urea-N and total-N (Table 3). Increasing dietary RUP at 16.6% CP (diet B versus diet C) resulted in greater ($P < 0.01$) urinary excretion of urea-N; this suggested that the extra RUP from ESBM was limited by insufficient methionine absorption, the amino acid most often found limiting in Mid-Western diets (Broderick et al., 2004, USDFRC Res. Sum.). Urinary excretion of purine derivatives (a measure of microbial protein formation) and fecal N excretion (Table 3), as well as nutrient digestibility (data not shown), were not altered ($P \geq 0.14$) by diet in this trial.

Conclusion

Results from this experiment suggested that inclusion of RUP from ESBM in a diet with 15.6% CP (diet A) did not sustain milk production as well as a diet containing 16.6% CP with greater RDP from SSBM (diet B). Diets with the same CP content but higher RDP (diet B) or higher RUP (diet C) yielded similar production and nutrient efficiencies. The highest CP diet supplemented with only SSBM (diet D) did not improve production of milk, but increased urinary N excretion, resulting in depressed N efficiency. Under the conditions of this trial, 16.6% CP was adequate to support milk and protein yields of 40 and 1.25 kg/d.

Table 1. Composition of diets.

	Diets			
Item	A	B	C	D
	---------------------% of DM------------------------			
Alfalfa silage	20.0	20.0	20.0	20.0
Corn silage	35.0	35.0	35.0	35.0
Rolled high moisture shelled corn	32.9	31.5	30.6	29.4
Solvent soybean meal	3.7	9.6	4.6	11.7
Expeller soybean meal	4.5	0.0	5.9	0.0
Roasted beans	2.5	2.5	2.5	2.5
Sodium bicarbonate	0.6	0.6	0.6	0.6
Limestone	0.5	0.5	0.5	0.5
Salt	0.2	0.2	0.2	0.2
Vitamin premix and trace minerals[1]	0.1	0.1	0.1	0.1
Chemical composition				
Crude protein	15.6	16.6	16.6	17.6
Neutral detergent fiber	27.3	26.9	27.4	26.9

[1]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.

Table 2. Effect of rumen-undegraded protein (RUP) or crude protein (CP) supplementation on production of lactating dairy cows.

		Diets							
		A	B	C	D				
	Dietary CP, %	15.6	16.6	16.6	17.6		Contrasts (*P*-value)[2]		
Item	Dietary ESBM, %	4.5	0	5.9	0	SE[1]	A vs. B	B vs. C	B vs. D
DM intake, kg/d		25.2	25.6	25.5	26.4	0.7	0.39	0.81	0.09
BW gain, kg/d		0.68	0.53	0.53	0.73	0.09	0.23	1.00	0.10
Milk yield, kg/d		38.8	40.0	40.3	40.1	1.3	0.08	0.68	0.91
Milk/DM intake		1.53	1.56	1.58	1.51	0.04	0.23	0.61	0.06
3.5% FCM, kg/d		41.1	42.2	42.9	42.7	1.4	0.21	0.44	0.59
Milk fat, %		4.03	3.98	4.00	4.00	0.10	0.59	0.79	0.80
Milk fat, kg/d		1.50	1.54	1.57	1.56	0.06	0.37	0.43	0.54
Milk protein, %		3.23	3.24	3.20	3.28	0.04	0.56	0.14	0.26
Milk protein, kg/d		1.21	1.26	1.24	1.27	0.04	0.11	0.54	0.76
Lactose, %		4.87	4.83	4.89	4.85	0.04	0.03	<0.01	0.34
Lactose, kg/d		1.87	1.90	1.92	1.92	0.07	0.44	0.71	0.74
SNF, %		8.99	8.97	8.98	9.01	0.05	0.45	0.67	0.12
SNF, kg/d		3.42	3.52	3.51	3.54	0.11	0.26	0.97	0.75
Milk urea N, mg/dl		8.9	9.8	10.4	12.0	0.2	<0.01	0.01	<0.01
Milk protein N, % of N intake		30.1	29.3	28.8	26.8	0.7	0.27	0.50	<0.01

[1]Standard error of the least squares means.
[2]A vs. B = effect of feeding 1% more CP without supplemental RUP; B vs. C = effect of feeding more RUP at equal CP; B vs. D = effect of feeding 1% more CP.

Table 3. Effect of rumen-undegraded protein (RUP) or crude protein (CP) supplementation on ruminal metabolites and excretion in lactating dairy cows.

		Diets							
		A	B	C	D				
	Dietary CP, %	15.6	16.6	16.6	17.6		Contrasts (*P*-value)[2]		
Item	Dietary ESBM, %	4.5	0	5.9	0	SE[1]	A vs. B	B vs. C	B vs. D
Ruminal metabolites									
pH		6.61	6.49	6.57	6.57	0.07	0.13	0.33	0.30
Ammonia-N, mg/dl		7.16	6.87	7.03	9.18	0.99	0.79	0.88	0.04
Total free AA, mM		6.84	8.58	8.45	8.51	1.05	0.25	0.93	0.96
Estimated urinary excretion									
Urine volume, L/d		26.8	27.8	29.0	32.1	2.1	0.67	0.62	0.07
Urea N, g/d		134	139	165	201	5	0.41	<0.01	<0.01
Total N, g/d		208	225	241	279	10	0.15	0.17	<0.01
Total N, % of N intake		33.3	33.2	35.7	37.6	1.3	0.97	0.12	<0.01
Purine derivatives, mmol/d		610	652	632	674	35	0.30	0.62	0.58
Estimated fecal excretion									
N, g/d		214	222	218	237	10	0.41	0.67	0.14
N, % of N intake		33.9	32.7	32.3	31.4	1.0	0.32	0.76	0.30

[1]Standard error of the least squares means.
[2]A vs. B = effect of feeding 1% more CP without supplemental RUP; B vs. C= effect of feeding more RUP at equal CP; B vs. D = effect of feeding 1% more CP.

Effect of Supplementing Rumen-Protected Methionine on Production and Nitrogen Excretion in Lactating Dairy Cows

G. A. Broderick, M. J. Stevenson, R. A. Patton, N. E. Lobos, and J. J. Olmos Colmenero

Introduction

Over-feeding of crude protein (CP) adds expense and can contribute to environmental pollution from excess N excretion. This excess N is largely excreted as urinary urea, the most labile form of manure N and the form that can be lost as volatile ammonia into the air. Methionine has been reported to be the essential amino acid most frequently limiting for milk and protein yield in dairy cows fed typical Mid-Western diets. This suggests that supplementing with rumen-protected Methionine (RP-Met) may allow feeding less CP without loss of production but with reduced urinary N excretion. We conducted a lactation trial to test whether feeding RP-Met could be used to reduce the dietary CP and N excretion but without losing milk production.

Material and Methods

Twenty-four multiparous Holstein cows averaging 598 kg BW were blocked by days-in-milk into 6 groups of 4 (one block with ruminal cannulas). Cows were randomly assigned within blocks to balanced 4x4 Latin square sequences and fed total mixed rations in which dietary CP was reduced in steps of 1.3 percentage units by replacing soybean meal with high moisture shelled corn plus incremental RP-Met (as Mepron®; Degussa Corp., Kennesaw, GA) (Table 1). Each experimental period lasted 28 d--14 d for adaptation and 14 d for data collection. Feeds were analyzed weekly for CP and DM to adjust to the desired CP contents as a proportion of dietary DM. Apparent urinary excretion of urinary N and total N was estimated from spot urine samples (taken from 4 squares of 16 total cows) using creatinine as volume marker. Rumen metabolites were determined at various times from 0 to 24 hours after feeding. Data were analyzed using the Proc GLM procedures of SAS. Significance was declared at $P < 0.10$; least squares means are reported.

Table 1. Composition of diets.

	Diets			
Item	A	B	C	D
	---------------------% of DM------------------------			
Alfalfa silage	20.9	20.9	20.9	20.9
Corn silage	28.1	28.1	28.1	28.1
Rolled high moisture shelled corn	28.0	30.7	33.3	36.0
Solvent soybean meal	11.7	8.9	6.2	3.5
Roasted soybeans	4.5	4.5	4.5	4.5
Soy hulls	5.8	5.8	5.8	5.8
Sodium bicarbonate	0.6	0.6	0.6	0.6
Dicalcium phosphate	0.2	0.2	0.2	0.2
Salt	0.2	0.2	0.2	0.2
Vitamin premix and trace minerals[1]	0.1	0.1	0.1	0.1
Mepron[2]	0	0.04	0.07	0.11
<u>Chemical composition</u>				
Crude protein	18.6	17.3	16.1	14.8
Neutral detergent fiber	27.1	27.1	27.0	27.0

[1]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.

[2]Ruminally protected methionine product from Degussa Corp., Kennesaw, GA.

Results and Discussion

Dietary NDF contents were similar (average 27% of DM), indicating that diets were of high energy and typical for those fed to early lactation cows (Table 1). The target of 1.3% decrease in CP with each reduction in solvent soybean meal, and increment of high moisture corn and RP-Met, was achieved in the trial. Dry matter intake, weight gain, as well as yields of protein, lactose and SNF, were unaltered, despite an overall reduction in dietary CP of 3.8 percentage units (Table 2). However, there were significant effects ($P \leq 0.08$) on milk/DM intake and on yields of milk, 3.5% FCM and fat (Table 2). Production was greater at 17.3% CP plus 8 g/d of RPM and 16.1% CP plus 17 g/d of RPM, than on the other 2 diets. Moreover, there were significant ($P \leq 0.02$) quadratic effects for all 5 of these variables when analyzed against CP concentration (data not shown). This means that production was greater at 16.1 and 17.3% CP, with supplemental RP-Met, than at higher or lower dietary CP. Apparent N efficiency (milk-N/N-intake) improved nearly 8 percentage units from highest to lowest CP and was greatest ($P < 0.01$) on lowest CP diet (containing the most RP-Met); however, this.was accompanied by losses in yield of milk and milk components.

The typical large reductions ($P < 0.01$) in milk urea-N (Table 2), and in ruminal ammonia-N and estimated total urinary-N excretion (Table 3), were observed as dietary CP was decreased. Urinary urea-N fell from 78 to 53% of total urinary-N and appeared to account for virtually all of the decline in urinary-N excretion as CP intake decreased. Under the conditions of this trial, feeding lower CP diets supplemented with RP-Met resulted in improved N-efficiency and reduced urinary N excretion.

Table 2. Effect of replacing CP from soybean meal with high moisture corn plus rumen protected methionine (RP-Met) on production of lactating dairy cows.

		Diets					
		A	B	C	D		
	Dietary CP, %	18.6	17.3	16.1	14.8		
Item	RP-Met, g/d	0	8.2	16.6	25.0	SE[1]	$P > F$[2]
DM intake, kg/d		23.4	23.4	23.8	23.7	0.5	0.88
BW gain, kg/d		0.14	0.42	0.55	0.42	0.17	0.41
Milk yield, kg/d		39.7[ab]	41.6[a]	41.7[a]	39.7[b]	0.7	0.06
Milk/DM intake		1.72[ab]	1.80[a]	1.77[ab]	1.69[b]	0.03	0.06
3.5% FCM, kg/d		38.9[b]	42.0[a]	41.2[ab]	38.6[b]	1.0	0.04
Milk fat, %		3.55	3.58	3.40	3.31	0.10	0.17
Milk fat, kg/d		1.37[ab]	1.49[a]	1.43[ab]	1.32[b]	0.05	0.08
Milk protein, %		3.02	2.98	2.96	3.04	0.04	0.39
Milk protein, kg/d		1.15	1.23	1.23	1.20	0.03	0.19
Lactose, %		4.81	4.82	4.78	4.81	0.03	0.78
Lactose, kg/d		1.90	2.00	1.99	1.92	0.04	0.20
SNF, %		8.74	8.72	8.68	8.71	0.02	0.31
SNF, kg/d		3.43	3.61	3.61	3.46	0.07	0.17
Milk urea N, mg/dl		14.5[a]	11.8[b]	9.4[c]	7.9[d]	0.3	< 0.01
Milk N/N-intake		0.262[d]	0.299[c]	0.317[b]	0.340[a]	0.007	< 0.01

[1]SE = standard error of the least squares means.
[2]Probability of a significant effect of diet.
[a,b,c,d]Least squares means in rows with different superscripts are different ($P < 0.05$).

Table 3. Effect of replacing CP from soybean meal with high moisture corn plus rumen protected methionine (RP-Met) on ruminal N metabolites and estimated urinary excretion.

		Diets					
		A	B	C	D		
	Dietary CP, %	18.6	17.3	16.1	14.8		
Item	RP-Met, g/d	0	8.2	16.6	25.0	SE[1]	$P > F$[2]
Ruminal concentrations							
Ammonia-N, mg/dl		9.9[a]	7.7[b]	6.5[bc]	5.5[c]	0.5	< 0.01
Total amino acids, mM		14.7[a]	11.2[b]	10.4[b]	10.5[b]	0.5	0.03
Urinary excretion							
Urine volume, L/d		27.9[a]	29.4[a]	24.8[b]	24.2[b]	0.03	0.06
Urea-N, g/d		205[a]	148[b]	115[c]	80[d]	4	< 0.01
Total-N, g/d		260[a]	207[b]	188[c]	150[d]	6	< 0.01
Urea-N/Total-N		0.78[a]	0.72[b]	0.62[c]	0.53[d]	0.01	< 0.01

[1]SE = standard error of the least squares means.
[2]Probability of a significant effect of diet.
[a,b,c,d]Least squares means in rows with different superscripts are different ($P < 0.05$).

Conclusion

Results from this experiment indicated that RP-Met could be used to replace part of the dietary CP that is normally fed as solvent soybean meal. By supplementing with RP-Met, it was possible to reduce dietary CP from 18.6 to as little as 16.1% CP without losing production of milk and milk components. This reduced urinary-N excretion, potentially the most polluting form of manure N. When feeding RP-Met, production and production efficiency were optimized between 16.1 and 17.3% CP. Reducing dietary CP to 14.8% could not be compensated for by supplementation with RP-Met.

Effect of Supplementing Rumen-Protected Methionine at Two Levels of Dietary Crude Protein in Lactating Dairy Cows

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Introduction

Methionine may be the essential amino acid most often limiting for milk and protein yield in dairy cows. Feeding rumen-protected Methionine (RP-Met) should allow dietary CP to be reduced without losing production. Leonardi et al. (J. Dairy Sci. 86:4033, 2003) reported that supplementing with RP-Met increased milk protein concentration at both 16.1 and 18.8% dietary crude protein (CP), with no interaction. This would be unexpected if methionine were a typical first-limiting amino acid. We conducted a lactation trial to determine whether there were similar or differential responses to RP-Met on production in lactating dairy cows fed 2 levels of dietary CP.

Material and Methods

Thirty-two multiparous Holstein cows averaging 604 kg BW were blocked by days-in-milk into 8 groups of 4 (1 block with ruminal cannulas). Cows were randomly assigned within blocks to balanced 4x4 Latin square sequences and fed total mixed rations in a 2x2 arrangement of diets: 17.3 or 16.1% CP, with or without supplementation of about 15 g/d of RP-Met (fed as Mepron®; Degussa Corp.,

Kennesaw, GA) (Table 1). Each experimental period lasted 28 d--14 d for adaptation and 14 d for data collection. Feeds were analyzed weekly for CP and DM to adjust to the desired CP contents as a proportion of dietary DM. Rumen N metabolites were determined at various times from 0 to 24 hours after feeding. Data were analyzed using the Proc GLM procedures of SAS; least squares means are reported. Orthogonal contrasts were used to assess the effects of CP and RP-Met, and their interaction; significance was declared at $P < 0.05$.

Table 1. Composition of diets.

	Diets			
Item	E	F	G	H
	---------------------% of DM-----------------------			
Alfalfa silage	20.9	20.9	20.9	20.9
Corn silage	28.1	28.1	28.1	28.1
Rolled high moisture shelled corn	30.7	30.6	33.4	33.3
Solvent soybean meal	8.9	8.9	6.2	6.2
Roasted soybeans	4.5	4.5	4.5	4.5
Soy hulls	5.8	5.8	5.8	5.8
Sodium bicarbonate	0.6	0.6	0.6	0.6
Dicalcium phosphate	0.2	0.2	0.2	0.2
Salt	0.2	0.2	0.2	0.2
Vitamin premix and trace minerals[1]	0.1	0.1	0.1	0.1
Mepron[2]	0	0.07	0	0.07
Chemical composition				
Crude protein	17.3	17.3	16.1	16.1
Neutral detergent fiber	27.1	27.1	27.0	27.0

[1]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.
[2]Ruminally protected methionine product from Degussa Corp., Kennesaw, GA.

Results and Discussion

Dietary NDF contents were similar (average 27% of DM), indicating that diets were of high energy and typical for those fed to early lactation cows (Table 1). The targets of 17.3 and 16.1% CP, with or without about 15 g/d of RP-Met, were achieved in the trial. There were no significant effects ($P \geq 0.27$) of supplementing with RP-Met on any production variable measured in this trial (Table 2). However, there were significant increases ($P \leq 0.03$) in yield of milk, protein and SNF, and trends ($P \leq 0.09$) for greater DM intake and increased lactose secretion, at the higher level of dietary CP (Table 2). As expected, milk urea-N was higher and apparent N efficiency (milk-N/N-intake) was lower on 17.3% CP, indicating better (P < 0.01) N utilization on the lower CP diet. There was no effect of diet on ruminal concentrations of either ammonia or total amino acids.

Results from this trial differed from those reported by Leonardi et al. (J. Dairy Sci. 86:4033, 2003) because there were no responses to RP-Met, regardless of protein level. Also, trends were detected for the interaction of dietary CP and RP-Met ($P \leq 0.10$) for protein and SNF yield. Cows in the Leonardi study were calculated to be in positive energy balance while those in the present experiment were calculated to be in negative energy balance due to lower than expected DM intake on all diets. Although apparent weight gain averaged 0.35 kg/d in this trial, the mean ratio of milk yield to DM intake was 1.87, indicating that there was considerable mobilization of tissue nutrients—both amino acids and fat—to support milk synthesis. Production likely was limited by energy supply and the small

but significant effects of CP level may have been driven by greater DM intake at 17.3% CP (Table 2). The present findings were also unlike those from the other protected methionine study (Broderick et al., 2004 Research Summaries) in that the effect of RP-Met was not greater at lower dietary CP. Average DM intake in the other trial was 2.1 kg/d greater.

Table 2. Effect of supplementing rumen-protected methionine (RP-Met) at 2 levels of crude protein (CP) on production of lactating dairy cows.

	Diets							
	E	F	G	H				
Dietary CP, %	17.3	17.3	16.1	16.1		Contrasts (*P*-value)[2]		
Item / RP-Met, g/d	0	15.4	0	14.8	SE[1]	CP	RP-Met	CP*RP-Met
DM intake, kg/d	21.6	21.8	21.6	20.9	0.3	0.09	0.44	0.14
BW gain, kg/d	0.48	0.24	0.33	0.36	0.10	0.90	0.27	0.15
Milk yield, kg/d	39.8	40.1	39.2	38.7	0.4	0.01	0.87	0.34
Milk/DM intake	1.86	1.87	1.84	1.89	0.03	0.98	0.31	0.39
3.5% FCM, kg/d	39.7	40.7	39.9	39.1	0.7	0.33	0.96	0.24
Milk fat, %	3.57	3.59	3.65	3.62	0.09	0.55	0.94	0.81
Milk fat, kg/d	1.40	1.43	1.42	1.38	0.04	0.71	0.94	0.35
Milk protein, %	3.08	3.09	3.07	3.06	0.02	0.37	0.98	0.67
Milk protein, kg/d	1.21	1.23	1.19	1.17	0.01	0.01	0.94	0.08
Lactose, %	4.84	4.84	4.84	4.83	0.02	0.92	0.66	0.99
Lactose, kg/d	1.91	1.96	1.89	1.86	0.03	0.06	0.76	0.13
SNF, %	8.85	8.85	8.83	8.83	0.02	0.24	0.79	0.99
SNF, kg/d	3.47	3.57	3.44	3.38	0.05	0.03	0.67	0.10
Milk urea N, mg/dl	12.4	12.1	10.2	10.2	0.2	< 0.01	0.38	0.45
Milk protein N, % of N intake	31.9	32.4	34.3	34.7	0.5	< 0.01	0.37	0.91
Ruminal concentration								
Ammonia-N, mg/dl	6.0	6.1	6.1	5.9	0.7	0.99[3]		
Total amino acids, mM	4.8	4.4	5.2	4.2	0.9	0.86[3]		

[1]Standard error of the least squares means.
[2]Orthogonal contrasts: CP = diets E and F vs. diets G and H; RP-Met = diets E and G vs. diets F and H; CP*RP-Met interaction = diets E and H vs. diets F and G.
[3]Effect of diet on ruminal concentration.

Conclusion

Under the conditions of this trial, reducing dietary CP from 17.3 to 16.1% reduced yield of milk, protein and SNF and this reduction was not reversed by supplementing with RP-Met. The results suggested that RP-Met may be ineffective when feed intake is inadequate to support milk yield.

Effect of Source of Rumen-Degraded Protein on Production and Ruminal Metabolism in Lactating Dairy Cows.

S. M. Reynal and G. A. Broderick

Introduction

Understanding the factors influencing efficiency of microbial protein synthesis is essential to improve the precision and accuracy of ration formulation, to maximize feed efficiency in dairy cows, and ultimately to minimize N excretion. Increasing evidence, mostly from in vitro studies, supports the idea that amino acids (AA) and peptides stimulate both growth rate and yield of ruminal microorganisms. In vivo experiments are needed to determine the sources and amounts of rumen-degraded protein (RDP) required to optimize microbial protein yield in the rumen and to improve N utilization by dairy cows. This trial was conducted to study the effects on production and ruminal metabolism of replacing true protein RDP from solvent soybean meal (SSBM) with urea RDP, while holding dietary rumen-undegraded protein (RUP) constant.

Materials and Methods

Twenty-eight lactating dairy cows (8 with ruminal cannulas) averaging 137 DIM were assigned to seven 4x4 Latin squares and fed diets differing in the proportions of RDP coming from urea and non-urea sources. Proportions of ingredients (DM basis; Fig. 1) in the four concentrate mixes in diets A to D were changed in equal increments to replace RDP from SSBM with RDP from urea, while replacing RUP from SSBM with RUP from lignosulfonate-treated soybean meal (SoyPass; Ligno-Tech, Rothchild, WI) (Table 1). Diets contained on a DM basis 16.0% CP, 25.5% NDF, and 14.2% ADF. Estimated (NRC, 2001) dietary concentrations (DM basis) were 1.60 Mcal NEl/kg, 50% nonfiber carbohydrate, 10.6% RDP, and 5.7% RUP and were held constant during the trial.

Table 1. Composition of diets

	Diet			
Item	A	B	C	D
	% of DM			
Ingredients				
Corn silage	15.0	15.0	15.0	15.0
Alfalfa silage	40.0	40.0	40.0	40.0
Rolled HMSC	29.6	28.7	28.5	27.8
Ground shelled corn	–	1.98	4.00	6.26
Solvent SBM	14.0	9.82	4.98	–
Lignosulfonate-treated SBM	–	2.52	5.12	8.00
Urea	–	0.41	0.84	1.31
Sodium bicarbonate	0.50	0.52	0.52	0.54
Salt	0.30	0.31	0.31	0.33
Limestone	0.30	0.31	0.31	0.33
Dicalcium phosphate	0.20	0.21	0.21	0.22
Vitamin-mineral mix[1]	0.20	0.21	0.21	0.22
Chemical composition				
CP, % of DM	16.1	15.9	16.0	16.1
NDF, % of DM	24.8	25.4	25.9	25.7
ADF, % of DM	14.1	14.2	14.4	13.9
NDIN, % of total N	6.23	8.28	9.46	11.1
ADIN, % of total N	2.49	2.55	2.59	2.49

[1]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.

Results and Discussion

As urea replaced SSBM in diets A to D, there were linear decreases in DM intake, body weight gain, and yields of milk, 3.5% FCM, milk protein, milk fat, and SNF by, respectively, 1.3, 0.23, 3.3, 2.6, 0.10, 0.09, and 0.27 kg/d (Table 2). Concentrations of fat, protein, and SNF in milk were not significantly affected by RDP source and averaged, respectively, 3.05, 3.19, and 9.04% across diets. Replacing true protein RDP with RDP from urea resulted in linear increases in the concentrations of urea-N in milk (from 6.8 to 9.1 mg/dl; $P < 0.01$) and blood (from 8.9 to 12.8 mg/dl; $P < 0.01$) and of ammonia-N in the rumen (from 8.4 to 10.8 mg/dl; $P < 0.01$). Total free AA averaged 18.5 mM and mean pH was 6.53 in the rumen and were not significantly affected by treatment.

Table 2. Effect of source of dietary rumen-degraded protein (RDP) on production and ruminal metabolism.

	Diet and % urea in diet DM					$P > F$[1]			
	A	B	C	D					
Item	0	0.41	0.84	1.31	SE	RDP source	Linear	Quadratic	Cubic
Production									
DM intake, kg/d	23.6[a]	23.2[a]	23.0[ab]	22.3[b]	0.4	0.02	<0.01	0.68	0.67
Milk yield, kg/d	39.3[a]	38.6[a]	38.5[a]	36.0[b]	0.9	<0.01	<0.01	0.18	0.26
3.5% FCM, kg/d	38.0	36.6	36.6	35.4	1.1	0.21	0.04	0.92	0.49
Milk fat, %	3.05	3.17	2.92	3.05	0.09	0.15	0.47	0.98	0.03
Milk fat, kg/d	1.20	1.19	1.10	1.11	0.04	0.07	0.02	0.80	0.22
Milk protein, %	3.22	3.18	3.18	3.18	0.04	0.85	0.50	0.63	0.72
Milk protein, kg/d	1.27[a]	1.22[b]	1.21[b]	1.17[b]	0.03	<0.01	<0.01	0.90	0.32
SNF, %	9.09	9.02	9.03	9.00	0.05	0.39	0.13	0.54	0.59
SNF, kg/d	3.59[a]	3.48[a]	3.45[ab]	3.32[b]	0.08	<0.01	0.06	0.16	0.10
MUN,[2] mg/dl	6.77[d]	7.45[c]	8.13[b]	9.08[a]	0.31	<0.01	<0.01	0.48	0.75
BUN,[2] mg/dl	8.87[d]	9.89[c]	11.39[b]	12.78[a]	0.45	<0.01	<0.01	0.61	0.72
Body weight gain, kg/d	0.57	0.53	0.34	0.34	0.10	0.18	0.04	0.84	0.43
Ruminal metabolism									
pH	6.51	6.52	6.51	6.59	0.05	0.24	0.14	0.28	0.39
NH_3-N, mg/dl	8.35[b]	9.32[ab]	10.27[a]	10.75[a]	0.73	0.05	<0.01	0.69	0.86
Total free AA, mM	16.9	19.5	19.4	18.0	1.7	0.62	0.63	0.22	0.84

[a,b,c]Least square means within the same row without a common superscript differ (P<0.05).
[1]Probability of a significant effect of RDP or of linear, quadratic or effects of RDP level in the diet.
[2]Milk (MUN) and blood (BUN) urea-N determined using a colorimetric assay.

Conclusion

Results from this experiment indicated that urea RDP cannot replace true protein RDP from SSBM without negatively affecting production of milk and milk components and body weight gain in lactating dairy cows. Feeding RDP from SSBM may have stimulated greater microbial growth and uptake of ammonia in the rumen, resulting in improved N utilization when compared with feeding urea.

Omasal Flow of Soluble Proteins, Peptides, and Free Amino Acids in Dairy Cows Fed Diets Supplemented with Proteins of Varying Ruminal Degradabilities

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Introduction

In vivo and in vitro evidence indicates that peptides and free amino acids (AA) stimulate growth rate and yield of ruminal microorganisms. Moreover, a high proportion of the total AA absorbed in the intestines of ruminants appears to be in the form of peptides, whereas peptide-bound Met and Lys have been shown to support protein synthesis by mammalian tissues grown in vitro. On the other hand, the new systems of ruminant ration formulation rely on rate constants for ruminal protein degradation and passage that are most commonly estimated using in situ incubations. Rates of ruminal degradation of the soluble N fraction (fraction A) from all feeds incubated in situ are assumed to be infinite. However, if a portion of the dietary N escapes ruminal degradation as solutes, the validity of this assumption may not hold true. Despite the nutritional and metabolic importance of soluble AA, most of the information on their degradation and metabolism is based on methodologies that may be unreliable. Peptide-bound AA are usually determined by quantitation of free AA (FAA) concentrations before and after hydrolysis of samples previously deproteinized by acid precipitation. However, extent of deproteinization is dependent on the type and concentration of the acid used, as well as on the structure of the proteins. Alternatively, soluble N fractions can be separated using ultrafiltration with membranes of different molecular weight (MW) cut-offs without addition of acids. The following study was conducted to study the effect of feeding diets differing in the concentration, ruminal degradability, and source of

protein supplement on the omasal flows of soluble proteins, peptides and free AA fractionated using ultrafiltration.

Materials and Methods

Three ruminally and duodenally cannulated cows were assigned to an incomplete 4 x 4 Latin square with four 14-d periods and fed diets supplemented with urea, solvent soybean meal (SSBM), lignosulfonate-treated soybean meal (LSBM), or corn gluten meal (CGM) (Table 1) to study the effects of protein source and degradability on omasa flows of soluble AA. Soluble AA in omasal digesta was fractionated by MW using ultrafiltration into soluble proteins greater than 10 kDa (10K), oligopeptides between 3 and 10 kDa (3-10K), peptides smaller than 3 kDa (small peptides), and free AA (FAA). Digesta flow leaving the rumen was quantified using the omasal sampling technique. The external microbial marker ^{15}N was used to measure microbial N flows from the rumen. Homoarginine was used as an internal standard to substract the FAA contents from the 10K, 3-10K, and small peptide fractions. Soluble fractions were analyzed for 16 AA after hydrolysis in 6 *N* HCl, using norleucine as an interal standard and ion-exchange chromatography with ninhydrin detection.

Table 1. Ingredient and chemical composition of experimental diets.[1]

	Diets			
	A	B	C	D
Item	Basal	SSBM	LSBM	CGM
	% of DM			
Ingredients				
Alfalfa silage	25.0	25.0	25.0	25.0
Corn silage	35.0	35.0	35.0	35.0
Shelled corn, ground	34.8	32.1	32.7	28.6
Urea	2.3	---	---	---
Solvent soybean meal	---	5.2	---	---
Lignosulfonate-treated SBM	---	---	4.6	---
Corn gluten meal	---	---	---	8.5
Minerals and vitamins	2.87	2.68	2.74	2.81
Chemical composition				
DM, %	50.4	50.4	50.7	49.6
CP, % of DM	19.7	15.8	15.3	18.2
NDF, % of DM	31.8	28.6	30.0	28.1
ADF, % of DM	20.0	17.8	17.5	17.6
Starch, % of DM	28.1	28.1	27.2	26.1
TAAN, % of TN	35.5	68.8	64.4	82.3
NPN, % of TN	64.5	31.2	35.6	17.7
NFC, % of DM[2]	41.2	44.8	45.5	42.5
NE_L, Mcal/kg[2]	1.57	1.59	1.56	1.58

[1]CGM = corn gluten meal, LSBM = lignosulfonate-treated soybean meal, NFC = non-fiber carbohydrates, SSBM = solvent soybean meal, TAAN = total AA-N.
[2]Estimated using the NRC (2001) model.

Results and Conclusions

Small peptides isolated by ultrafiltration contributed between 13 and 20% of the total AA in the soluble fraction but less than 3% of the total AA flowing at the omasal canal of cows fed diets differing in the concentration, source, and ruminal degradability of the protein supplement (Table 2). Similarly, soluble proteins greater than 10 kDa contributed between 7 and 13% of total soluble AA and less than 2% of total AA flowing at the omasal canal. When averaged across diets, AA present in soluble 3-10K oligopeptides averaged 71% of total soluble and 9.4% of total AA flows, whereas free AA accounted for 1.6% of total soluble and 0.23% of total AA flows at the omasal canal. Therefore, our results suggest that hydrolysis of oligopeptides was the rate-limiting step during microbial degradation of soluble proteins and that small peptides and free AA were rapidly utilized by ruminal microbes and did not accumulate in the rumen. On average, soluble AA of dietary origin accounted for 74% of total

soluble AA and 10% of total AA flows (Table 3).

Table 2. Effect of dietary treatment on omasal flows of AA in soluble proteins, peptides, and in their free form.

	Diets					Contrast		
	A	B	C	D				
Item	Basal	SSBM	LSBM	CGM	SEM	A vs. D	B vs. C	C vs. D
						--------------- *P*-value ---------------		
In soluble proteins of MW greater than 10 kDa								
g/d	29.9	30.3	30.6	26.1	4.8	0.53	0.95	0.48
% of TSAA flow	13.2	10.0	11.0	7.0	2.4	0.13	0.74	0.27
% of TAA flow	1.57	1.15	1.07	1.06	0.17	0.12	0.76	0.98
% microbial	70.2	74.1	71.5	76.6	5.8	0.46	0.75	0.55
% dietary	29.8	25.9	28.5	23.4	5.8	0.46	0.75	0.55
In soluble oligopeptides of MW between 3 and 10 kDa								
g/d	171	213	186	297	43	0.13	0.7	0.16
% of TSAA flow	65.4	67.9	69.9	79.3	3.9	0.09	0.74	0.19
% of TAA flow	10.79	7.93	6.40	12.54	3.05	0.68	0.72	0.21
% microbial	35.7	18.9	18.7	27.2	5.8	0.07	0.9	0.08
% dietary	64.3	81.1	81.3	72.8	5.8	0.07	0.9	0.08
In soluble peptides of MW smaller than 3 kDa								
g/d	47.3	59.6	45.5	49.1	5.6	0.12	<0.01	0.05
% of TSAA flow	19.5	20.3	17.5	12.7	2.1	0.11	0.42	0.21
% of TAA flow	2.80	2.34	1.63	2.13	0.47	0.20	0.18	0.30
% microbial	7.53	5.01	5.47	9.43	5.80	0.27	0.77	0.07
% dietary	92.5	95	94.5	90.6	5.8	0.27	0.77	0.07
In soluble free AA								
g/d	5.31	5.68	3.73	4.69	1.80	0.66	0.24	0.45
% of TSAAN flow	1.87	1.83	1.59	1.04	0.44	0.08	0.49	0.18
% of TAAN flow	0.34	0.21	0.14	0.22	0.11	0.25	0.50	0.44
% microbial	7.53	5.01	5.47	9.43	5.8	0.15	0.38	0.17
% dietary	92.5	95	94.5	90.6	5.8	0.27	0.77	0.07

[1]CGM = corn gluten meal, LSBM = lignosulfonate-treated soybean meal, SSBM = solvent soybean meal.
[2]TAA = total AA, TSAA = total soluble AA.

Table 3. Effect of dietary treatments on the omasal flows of total and soluble AA.

	Diets					Contrast		
	A	B	C	D				
Item	Basal	SSBM	LSBM	CGM	SEM	A vs. D	B vs. C	C vs. D
	--------------------- g AA/d ---------------------					--------------- *P*-value ---------------		
Total	2006	2608	2824	2573	161	<0.01	0.10	0.04
Microbial	1011	1312	1238	1083	68	0.48	0.52	0.20
Dietary	995	1295	1584	1491	124	<0.01	<0.01	0.05
Soluble AA flow								
Total	254	308	266	377	48	0.14	0.56	0.17
% of TAA flow	15.5	11.6	9.2	15.9	3.7	0.93	0.63	0.23
Microbial	90.3	70.7	62.4	109.5	21.8	0.29	0.57	0.20
% of TSAA flow	33.2	20.6	21.7	28.8	2.6	0.29	0.74	0.22
% of TAA flow	5.40	1.77	2.10	5.34	2.10	0.98	0.92	0.45
Dietary	168	242	206	271	25	<0.01	0.03	<0.01
% of TSAA flow	66.8	79.4	78.3	71.2	2.6	0.29	0.74	0.22
% of TAA flow	10.7	8.0	8.2	13.1	2.8	0.56	0.97	0.40

[1]CGM = corn gluten meal, LSBM = lignosulfonate-treated soybean meal, SSBM = solvent soybean meal.
[2]TAA = total AA, TSAA = total soluble AA.

Conclusion

Our results suggest that hydrolysis of oligopeptides likely was the rate-limiting step during microbial degradation of soluble proteins. Moreover, small peptides and free AA appeared to be rapidly utilized by ruminal microbes and did not accumulate in the rumen. A substantial proportion of dietary AA flowed from the rumen as solutes. This calls into question the validity of using the in situ technique for estimating the kinetics of ruminal protein degradation.

Effect of Two Forms of Lauric Acid on Ruminal Protozoa and Fermentation Pattern in Dairy Cows

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Introduction

Microbial protein synthesized in the rumen and rumen-undegraded protein are the main sources of metabolizable protein for ruminants. The ciliate protozoa in the rumen may decrease outflow of both rumen-undegraded protein and microbial protein. Ruminal protozoa are major contributors to bacterial protein turnover within the rumen, thus reducing N utilization and contributing to the negative environmental impact of ruminant production. Most defaunation techniques are harmful to animals and not applicable under practical feeding conditions. Medium chain fatty acids such as lauric acid (LA; C12:0) have been shown to have potent anti-protozoal effects. Furthermore, they are not harmful and can be fed routinely. We hypothesized that LA, either as LA or sodium laurate (NaLA) would have similar anti-protozoal effects. Hence, the aim of this study was to test the effectiveness of these two forms of LA for suppressing protozoa in the rumen.

Materials and Methods

Six multiparous Holstein cows, averaging 660 kg of BW, 102 DIM, 40 kg/d of milk and fitted with ruminal cannulas, were grouped into 2 blocks of 3 by DIM in a randomized complete block design. Cows were fed a diet composed of (DM basis) 15% alfalfa silage, 40% corn silage, 29.5% rolled high moisture corn, 14% solvent soybean meal, 0.5% sodium bicarbonate, 0.3% limestone, 0.3% salt, 0.2% dicalcium phosphate, 0.2% vitamin and trace mineral supplement, 16.6% crude protein and 29% NDF. This diet was fed for a 2-week covariate period plus a 3-week experimental phase. Cows within blocks were then randomly assigned to one of 3 treatments: 1) Control (no supplement); 2) 160 g/d of LA; or 3) 178 g/d of NaLA (equimolar to LA) dissolved in 1,600 ml of water. Both LA and NaLA were given as a single daily dose via ruminal cannula immediately before feeding. Dry matter intake was measured daily. Digesta was collected from 4 ruminal sites at 0, 1, 2, 4, 8, 12, 18 and 24 h after feeding on the last day of the covariate period (day 0), and on day 1, 2, 3, 7, 10, and 16 of the experimental phase, and strained through two layers of cheesecloth. Determinations were made of pH, ammonia, total free amino acids, VFA, and total protozoal counts. Data were analyzed using Proc Mixed in SAS with significance declared at $P \leq 0.10$. Least squares means are reported.

Results and Discussion

Giving LA as an intraruminal dose of 160g/day did not affect intake; however, the equimolar dose of NaLA reduced DM intake by 3.6 kg/d (Table 1). This may be related to the fact that NaLA was given in solution and may have dispersed more rapidly throughout the ruminal fluid phase while more LA became associated with the particulate phase. Also, more NaLA may have flowed out of the rumen and rapidly absorbed, stimulating greater CCK release, which could account for the depression in feed intake. The main effect observed in this study was the rapid reduction of protozoal populations when both LA and NaLA were dosed daily into the rumen, approaching the final populations within 2-3 days (Fig. 1). Protozoal numbers averaged 6 and 9% of Control on LA and NaLA, respectively (Table 1). Others have reported the inhibitory action of LA on ruminal protozoa. It is well accepted that lipids, in general, possess strong anti-protozoal activity. However, the mechanisms by which this occurs is unclear. Ruminal ammonia decreased sharply with both treatments and was lowest on LA (Table 1). This effect was probably associated with reduced protozoal ingestion and degradation of bacterial proteins. In the present study, both treatments reduced concentration of ruminal total free

amino acids. The active protozoal peptidases and proteases act on dietary and microbial proteins to release free amino acids into the medium. Moreover, ciliates utilize only about half of their ingested N; the rest is expelled as short-chain peptides and free amino acids. This may explain why free amino acid concentration in the rumen decreased with defaunation.

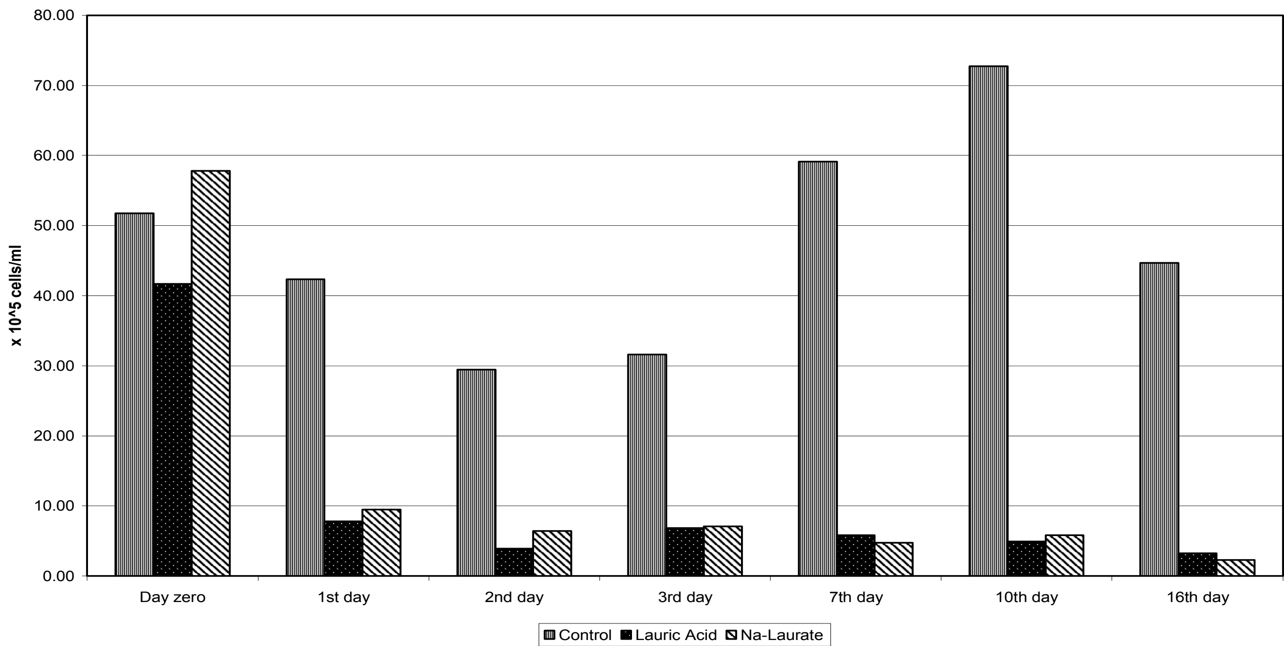
Except for butyrate, there were no significant effects of LA or NaLA on concentrations of total or individual VFA. Most literature has reported a decrease in total VFA production as a consequence of defaunation through reduced carbohydrate fermentation. Effects on the individual VFA have been variable, although experiments frequently have shown increased molar proportions of propionate at the expense of butyrate and acetate. This may explain the reduced butyrate. Defaunation studies often report that a lack of ciliates leads to ruminal pH instability due a loss of the ability of protozoa to slow fermentation of sugars and non-structural carbohydrates by reducing availability of those carbohydrates to bacterial fermentation. The reason why NaLA increased pH was not clear but this preparation may have acted to buffer ruminal acidity.

Table 1. Effects of lauric acid (LA) or sodium laurate (NaLA) on intake, ruminal metabolism and protozoal counts.

Item	Control	LA	NaLA	SE	P > F
DM intake, kg/d	25.3[a]	23.8[a]	21.7[b]	0.5	0.07
Ammonia, mM	6.6[a]	2.6[c]	4.6[b]	0.5	<0.01
Total free amino acids, mM	10.4[a]	6.6[b]	3.9[c]	0.8	<0.01
pH	6.25[b]	6.38[b]	6.69[a]	0.09	0.08
Total VFA, mM	75.6	71.6	63.6	13.7	0.83
Acetate, mM	42.6	38.0	38.6	7.6	0.90
Propionate, mM	18.7	20.0	15.4	5.13	0.82
Butyrate, mM	10.0[a]	8.0[ab]	5.7[b]	0.8	0.09
Isobutyrate, mM	0.98	0.91	0.92	0.10	0.89
Isovalerate, mM	1.53	1.92	1.71	0.13	0.18
Valerate, mM	1.76	2.66	1.23	0.37	0.15
Protozoa, x 10^6 cells/ml	5.90[a]	0.37[b]	0.51[b]	0.34	<0.01

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

Figure 1. Effects of lauric acid or sodium laurate on protozoal counts over time



Conclusion

Both LA and NaLA showed high anti-protozoal activity when single doses of 160 g/d were given daily via ruminal cannulas. These treatments reduced the ruminal ciliate population by 90% within 2-3 days. Dosing with LA reduced ruminal ammonia concentration by 60% without reducing DM intake. Both agents reduced ruminal free amino acid concentration. LA did not affect ruminal pH or reduce total ruminal VFA concentration. Thus, LA was shown to be a potent defaunation agent, obviating the need to use the more costly NaLA.

Dietary Manipulation in Dairy Cattle to Reduce Ammonia Emissions

T. H. Misselbrook,· J. M. Powell, G. A. Broderick and J. H. Grabber

Introduction

Dairy cows utilize feed nitrogen (N) much more efficiently than many other livestock, but they excrete three times more N in manure than in milk. An average cow producing 8,200 kg of milk annually excretes 21,000 kg of manure containing about 110 kg N, in approximately equal proportions in feces and urine. The majority of urinary N (depending on diet and animal condition) is in the form of urea, which is readily hydrolyzed by fecal urease to ammonia (NH_3). About 25% of dairy manure N is lost as NH_3 under current U.S. practices ammonia losses begin directly after urine deposition in the dairy barn and continue throughout manure handling, storage, and land application. Most efforts to reduce nutrient loss from dairy operations have focused on land application of manure, where a large impact can be made at relatively low cost. However, reducing N excretion through dietary manipulation represents another opportunity where large impacts could potentially be made, as subsequent losses would be reduced throughout the manure management continuum, particularly if combined with other abatement strategies such as at manure application. The objectives of this study were to assess the influence of manipulating dairy cattle dietary protein level and form on NH_3 emissions from urine and fecal deposits to a concrete floor and from fresh and stored slurries applied to soil.

Materials and Methods

Dietary treatments

Urine and feces were collected from lactating Holstein cows, housed in a tie-stall system, from two dietary trials varying in either dietary protein level or protein form. In the first trial, cattle were fed diets with a high (19%) or low (14%) CP content (treatments referred to hereafter as HCP and LCP, respectively), with two cows per dietary treatment. In the second trial, cows were fed diets of similar composition, with the exception of the forage legume component which was either alfalfa (*Medicago sativa*), or birdsfoot trefoil of low (ca. 2% DM basis) or high (ca. 4% DM basis) tannin content (treatments referred to hereafter as ALF, BFTL and BFTH, respectively), with three cows per diet.

The laboratory set-up consisted of 6 chambers in which the manure was exposed to a constant airflow. Air was drawn through the system by means of a vacuum pump, with the airflow rate through each chamber being controlled at 4 L min^{-1}. An acid trap before each chamber removed any NH_3 from inlet air and a second acid trap on the outlet side of each chamber collected any NH_3 emitted during the measurement period.

Emissions from Simulated Deposits to Barn Floor

Deposits of urine and feces to a barn floor would normally be scraped, leaving a thin layer from which emission occurs. In the simulation experiments, therefore, a constant mass of feces and volume of urine were applied to the chambers in order to achieve a thin emitting layer of approximately 1 mm above the cement surface. Immediately after adding the urine, the chamber lid was closed and sealed with silicon grease and the air flow through the system started. Acid traps were changed after 1, 3, 6, 12, 24 and 36 h and measurement stopped at 48 h.

Emissions from Slurry Applied to Soil

For simulated emissions from land applications, the urine and feces from each selected treatment were mixed in the proportions in which they were excreted to produce slurries, which were then standardized at 7% dry matter (DM) content by the addition of water. Two experiments were conducted in which

NH_3 emission measurements were made from either fresh (stored for 24 h at 4°C) or stored (two weeks at ambient temperature, mean 20°C) slurries applied to soil in the laboratory chambers.

Results

Nitrogen excretion

From the dietary protein level trial, urine N concentration in HCP was almost twice that in LCP (Table 1). There were no significant differences between the protein level treatments in fecal N concentrations or in the volumes of urine and mass of feces collected over the collection period. The greater N concentrations in urine for HCP resulted in a shift in the relative proportion of N excreted in urine or feces from approximately equal amounts in LCP to a much greater proportion in the urine for HCP. Based on the concentration and volume outputs, mean hourly total N excretion per cow over the collection period was 30% lower for LCP than HCP.

From the protein form trial, urine N concentration was greatest in BFTL, with no significant differences between that of ALF and BFTH (Table 1). There were no significant dietary effects on total N concentration in the feces or in the volumes of urine and mass of feces collected over the collection period, although there was a suggestion in the data that fecal N contents and outputs were greater with BFTH. This, combined with the lower urine N concentration for that treatment resulted in there being a significant shift in the relative proportion of N excreted in the urine and feces from a greater proportion in the urine for ALF and BFTL to a greater proportion in the feces for BFTH.

Table 1. Proportion of nitrogen excretion in urine and feces as influenced by dietary protein level or form in lactating dairy cattle. Values are the mean for the two (protein level trial) or three (protein form trial) cows on each diet.

	Dietary protein level trial		Dietary protein form trial		
	Low crude protein	High crude protein	Alfalfa	Birdsfoot trefoil low CT	Birdsfoot trefoil high CT
Urine [total N] ($g\ L^{-1}$)	4.5^{b}	8.5^{a}	7.5^{b}	8.8^{a}	6.7^{b}
Feces [total N] ($g\ kg^{-1}$ DM)	20.6	24.4	26.7	29.5	33.1
†Urine volume ($L\ h^{-1}$)	1.09	1.05	0.93	1.09	1.07
†Feces volume (kg DM h^{-1})	0.21	0.17	0.20	0.21	0.30
Relative proportion (%) of total N in:					
…urine	52^{b}	68^{a}	55^{a}	60^{a}	40^{b}
…feces	48^{a}	32^{b}	45^{b}	40^{b}	60^{a}

Within each trial, values with different superscripts are significantly different ($P < 0.05$).
†Based on total amount collected per cow over the 60 – 100 h collection period which excluded times when cows were being milked.

Ammonia Emissions from Simulated Deposits to Barn Floor

Protein level. Cumulative NH_3 emissions from the urine and feces in the chambers over the 48 hour measurement period were not significantly different between LCP and HCP (Fig. 1a), although when expressed as a proportion of either the total N (Fig. 1b) or urea N (Fig. 1c) applied losses were significantly greater from LCP, as the urine from the LCP treatment had a lower total N and urea N concentration. ***Protein form***. Cumulative NH_3 emission over the 48 hour measurement period was significantly greater from ALF than BFTL and BFTH (Fig. 2a), which were not significantly different, in absolute terms or when expressed as a proportion of the total N or urea N applied (Fig. 2b and 2c).

Figure 1. Influence of dietary crude protein (CP) content on ammonia emissions from dairy cattle urine (8 ml) and feces (8 g) deposited on a simulated barn floor: a) expressed as g N m^{-2}; b) as a % of total N applied; c) as a % of the urea N applied. Dietary CP contents: 13.6% (●) and 19.4% (○). Error bars show ± 1 standard error (n=3).

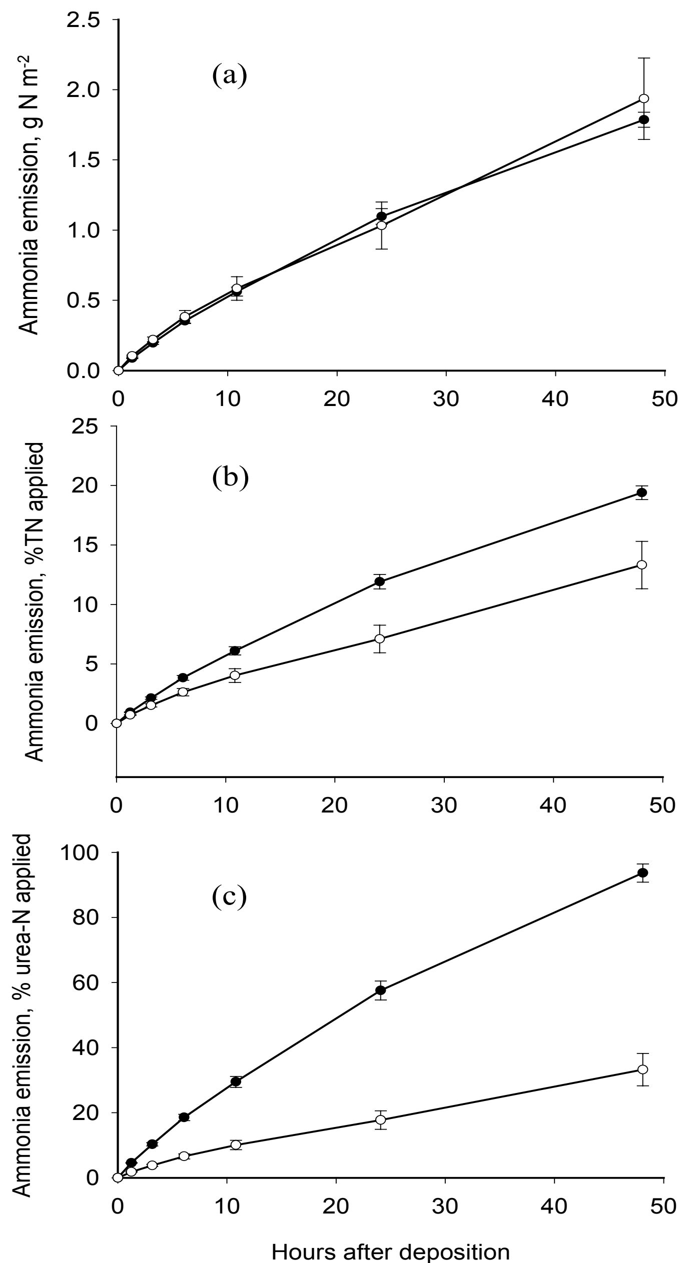


Figure 2. Influence of dietary protein form on ammonia emissions from dairy cattle urine (8 ml) and feces (8 g) deposited on a simulated barn floor: a) expressed as g N m^{-2}; b) as a % of total N applied; c) as a % of the urea N applied. Dietary forage legume component: alfalfa (●); birdsfoot trefoil with low tannin content (○); birdsfoot trefoil with high tannin content (▼). Error bars show ± 1 standard error (n=3).

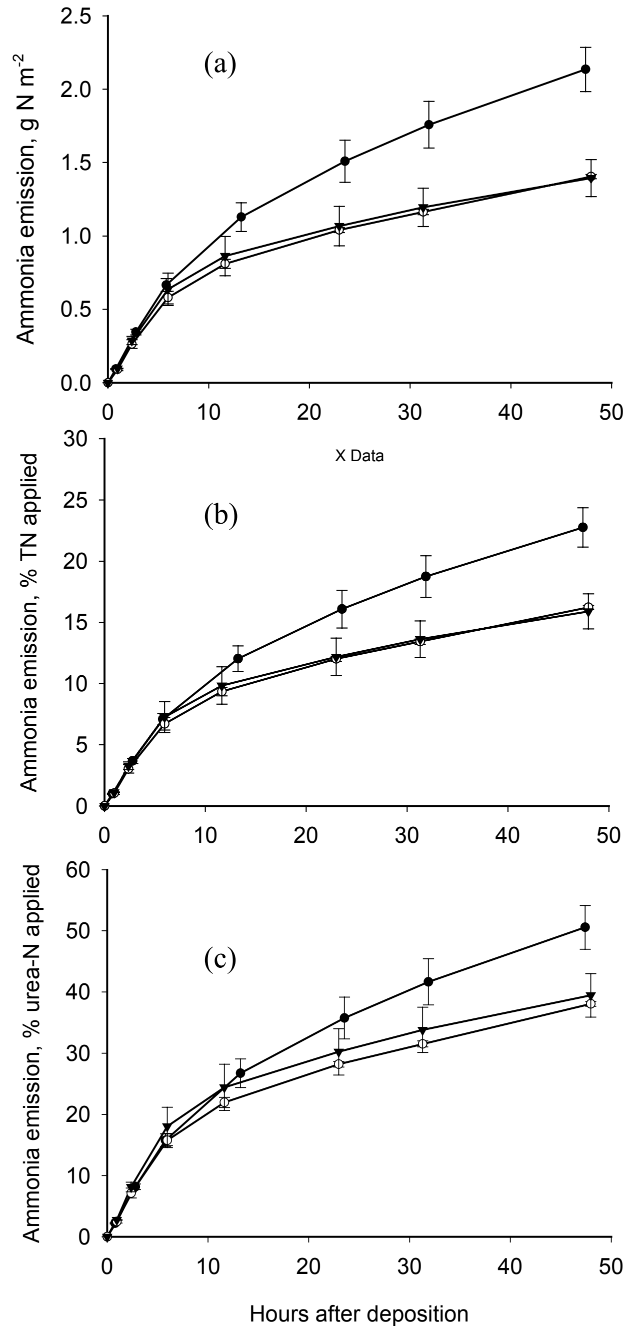


Figure 3. Influence of dietary crude protein (CP) content on ammonia emissions from fresh slurry applied to soil: a) expressed as g N m^{-2}; b) as a % of total N applied; c) as a % of the total ammoniacal N applied. Dietary CP contents: 13.6% (●) and 19.4% (○). Error bars show ± 1 standard error (n=3).

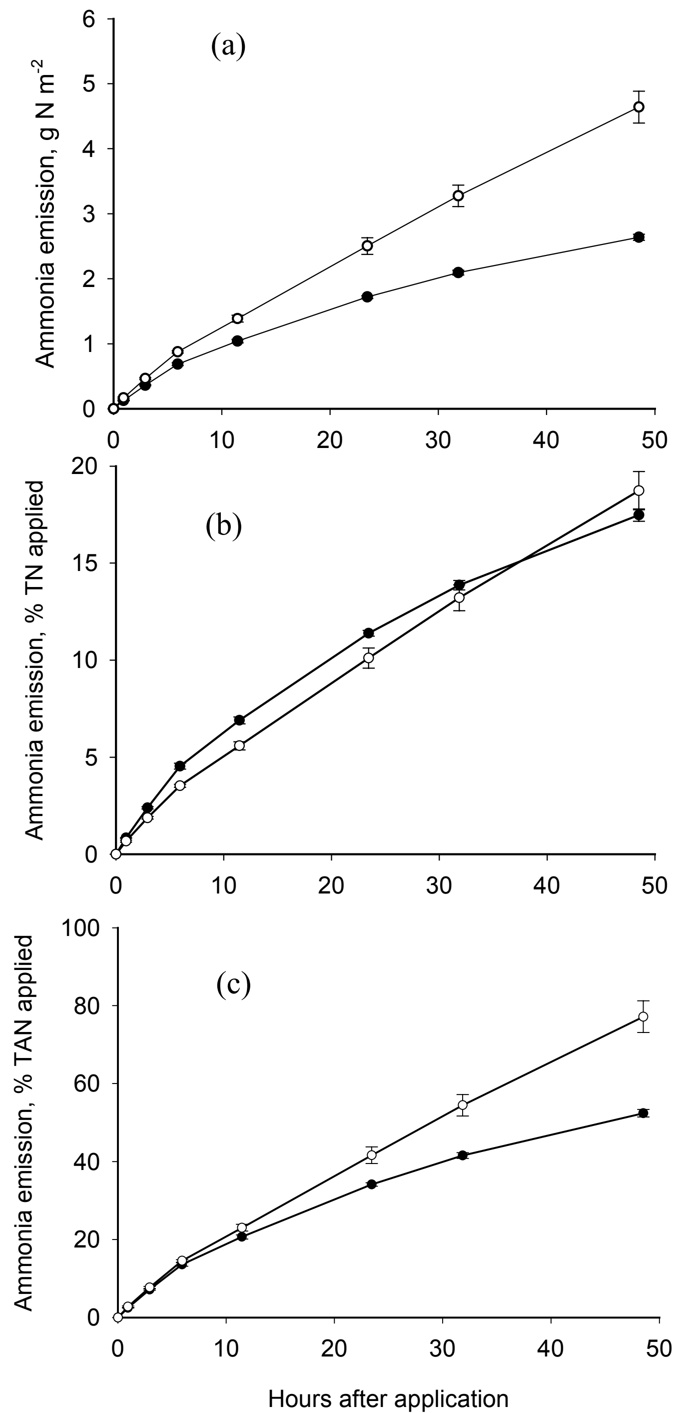
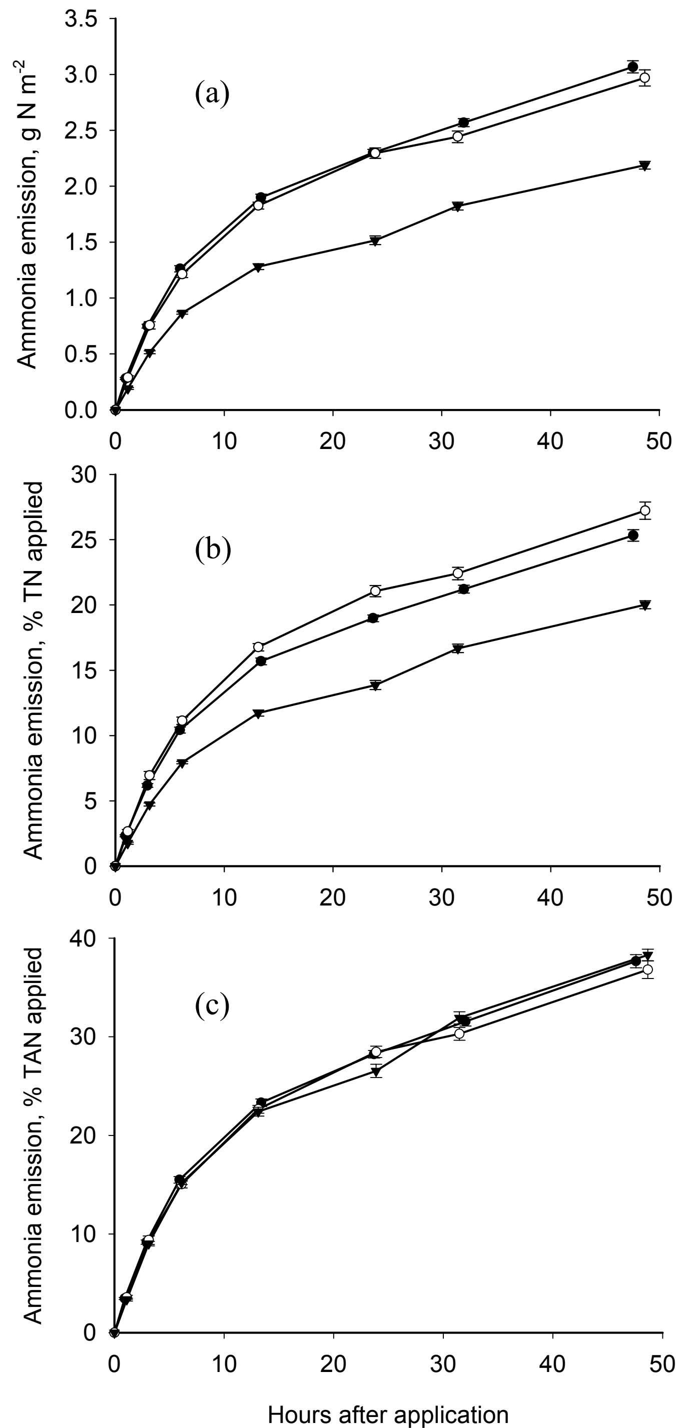


Figure 4. Influence of dietary protein form on ammonia emissions from fresh slurry applied to soil: a) expressed as g N m^{-2}; b) as a % of total N applied; c) as a % of the total ammoniacal N applied. Dietary forage legume component: alfalfa (●); birdsfoot trefoil with low tannin content (○); birdsfoot trefoil with high tannin content (▼). Error bars show ± 1 standard error (n=3).



Ammonia Emissions from Slurry Applications to Soil

Protein level. Cumulative NH_3 emissions over 48 hours from the application of fresh slurries to soil were significantly greater for HCP than LCP, both in absolute terms and when expressed as a percentage of the initial TAN concentration, but not when expressed as a proportion of the total slurry N content (Fig. 3). Cumulative emissions over 48 hours from the application of stored slurries to soil were also significantly greater for HCP, in absolute terms and as a proportion of the initial total N or TAN (data not shown). ***Protein form***. Cumulative NH_3 emissions over 48 hours following application of the fresh slurries to soil were significantly greater for ALF and BFTL than BFTH in absolute terms and as a proportion of the total N applied, but there were no treatment differences as a proportion of TAN applied (Fig. 4). Following application of the stored slurries, cumulative emissions over 48 hours were significantly greater from ALF than either BFTL or BFTH in absolute terms and as a proportion of total N applied, but again, there were no significant differences when expressed as a proportion of the TAN applied (data not shown).

Discussion

Nitrogen excretion was reduced by 30% and urinary N excretion by 45% when dietary crude protein content was lowered from 19.4 to 13.6%. These values are not based on a full daily collection of urine and feces and the possibility that differences in excretal volumes while the cows were away from the stalls cannot be excluded. In addition, the mean hourly rate of excretal output may also have been different while the cows were being moved and milked, so mean daily output values have not been given. However, these results confirm the work of others that N excretion can be reduced by lowering dietary CP content and that the reduction is predominantly in the urea-N content of the urine.

Measurements from the simulated barn floor trials indicated that cumulative NH_3 emissions would continue to increase beyond the 48 h measurement period (Figs. 1 and 2). This is consistent with the time required for complete hydrolysis of the urea content of the urine, which has to occur before NH_3 volatilization can take place.

Urea hydrolysis also appeared to be a limiting factor controlling emission rates from the fresh slurries applied to soil in the protein level trial. Slurry TAN content was only 20% greater for HCP compared with LCP whereas a much greater difference would be expected based on differences in urine urea N concentrations.

Conclusion

Manipulating the level and form of protein in the diet of lactating cows influenced the amount and form of N excretion and subsequent NH_3 emissions from the barn floor and manure management. Reducing dietary CP content from 19 to 14% reduced total N excretion and resulted in a greater proportion of the N excretion in urine, with an increase in urine N concentration of 90%. Surprisingly, losses from a simulated barn floor were similar from both treatments in the short term (48 h), presumably because urease activity was limiting, but losses from slurries applied to soil were lower for the LCP treatment both in absolute terms and as a proportion of the TAN applied. Increasing the concentration of CT in the forage legume component of the diet shifted N excretion from urine to feces and led to reduced losses from the barn floor (in absolute terms and as a proportion of urine urea-N applied) and slurries applied to soil (in proportion to the reduction in the TAN content of the slurries).

Manure Nutient Management

Nitrogen Availability From Dairy Manure

P.R. Cusick, J.M. Powell, K.A. Kelling, G.R. Muñoz, and R.F Hensler

Introduction

Dairy manure is a valuable source of nitrogen (N) for crop production. Accurate estimates of dairy manure N availability in the first- and subsequent residual-years after application are needed to maximize crop N uptake and reduce N losses to the environment. This study examined dairy manure N availability and mineralization of dairy manure components using various methods.

Materials and Methods

Experiment 1: A field trial was conducted at the West Madison Agricultural Research Station, Madison, WI on a Plano silt loam (fine-silty, mixed, mesic, Typic Argiudoll) during the 1998 to 2003 cropping seasons. Corn (Zea mays L., c v Lemke 6063) was planted all years of the study. Treatments include five fertilizer N treatments (40, 80,120,160, and 200 lb acre^{-1}) applied every year; two manure rates (estimated to provide approximately 80 and 160 lb available N/acre in the first year) applied at various intervals (every 1, 2, or 3 years) and a no fertilizer or manure control. Micro plots using manure labeled with ^{15}N were incorporated within the larger unlabeled plots. Nitrogen availability was determined using the difference, fertilizer equivalence, and ^{15}N isotope methods. The apparent recovery method compares treatment whole-plant N uptake from the manure treatments to the amount taken up by the un-manured control plots. The fertilizer equivalence method compares manurial N yield or uptake responses from where a similar response is obtained from a fertilizer N treatment. The apparent and fertilizer equivalence methods are commonly used for predicting the availability of nitrogen in manures in first year studies. The ^{15}N method is new.

Experiment 2. Dairy urine and feces were labeled with ^{15}N. Liter bags containing ^{15}N-labled manure were randomly buried horizontally at 6 inches to the side of the corn row, spaced 9.8 inches apart. Bags were excavated during the corn growing period. At the time of bag sampling in 2002, corn plants from a non-adjacent row were sampled.

Experiment 3. An incubation trial utilized a Plano silt loam with the following initial chemical characteristics: 3.55% total C, 2.56% total organic C, 72 mg kg^{-1} Bray P1, 2220 mg kg^{-1} total Kjeldahl N, and 7.6 pH. Soils were amended with ^{15}N labeled and unlabeled dairy manure components such that only one components (feces, urine or bedding) was labeled and the others not labeled.

Results and Discussion

Experiment 1. Manure N availability estimates from this study are shown in Table 1. The apparent recovery and ^{15}N methods had the lowest estimates of manure N availability and the fertilizer equivalence method had the highest estimates. The difference method relies heavily on manure N uptake by corn in the control plots. In this study the control plots had high yields throughout all study years causing low estimates of apparent manure N availability. The recovery of ^{15}N had the lowest variability of all the methods.

Table 1. Estimates of first-, second-, and third-year manure N availability using various methods.

Manure N availability	n†	Method: ^{15}N Recovery	Apparent recovery	Fertilizer Equivalence
		%		
1st year	6	17 (3.1) ‡	14 (4.7)	25 (10.5)§
2nd year	5	6 (1.1)	8 (6.6)	12 (7.9)§
3rd year	4	2 (0.4)	1 (5.8)	3 (9.4) §

† no. of measurement years in parentheses; 4 reps/year
‡ Average (standard error)
§ Data from 2002 excluded.

This study estimated first-year nitrogen availability from dairy manure to be 19% when averaged across all methods. Currently, the University of Wisconsin Extension recommends that dairy manure N availability (based on total N) during the first year to be 30% when surface applied and 40% when incorporated within 72 hours. Our estimates appear to be lower than these other sources. One explanation for this may be the use of the ^{15}N isotope where others have also found estimates to be lower as result of a phenomena inherent to the method and can be explained by "pool substitution" whereby soil microorganisms immobilize applied ^{15}N and add ^{14}N to the total N pool.

This study found second- and third-year N availability to average 9 and 2%, respectively when averaged across all methods. The University of Wisconsin Extension recommends dairy manure residual N availability to be 10 and 5% for the second- and third-year after application. The fertilizer equivalent method produced results that were comparable to University of Wisconsin Extension recommendations for residual dairy manure N availability. Estimates using ^{15}N were lower than those obtained using the fertilizer equivalent method and may be considered a minimum residual manure N availability. Based on this and other studies, it is suggested that second and third year residual N availability from a single application of semi-solid dairy manure would be 9 to 12%, and 3 to 5%, of the original manure N application, respectively. Based on our first-year and residual availability estimates for semi-solid dairy manure, producers can be confident in University of Wisconsin recommendations for dairy manure N availability.

Experiment 2. Of the total manure N added to the litterbags, an annual average of 67% apparently mineralized (data not shown). The litterbag released 53% and 86% of the mineralizable N pool by day 21 in 2000 and 2002, respectively. Much of this mineralized N may be attributed to urine, which comprised about 42% of the total manure N. This may suggest that the 45% of N remaining in litterbags from our study may remain in soil organic pool for some time. This indicates that the manure N during the first year of application may be entering soil N organic pools.

Experiment 3. In the incubation trial, soil samples taken on day 168 from all temperatures and treatments were analyzed for inorganic ^{15}N to determine individual manure component contributions to mineralized N (Table 2). Net urine mineralization was unaffected (P<0.05) by temperature and averaged 55% at the end of the 168 day observation period. Because total ^{15}N recoveries averaged 115% (data not shown), providing evidence that manure N was not lost in this experiment, it is likely

that the remaining 45% urine ^{15}N was exchanged with unlabeled N or may have been mineralized and subsequently immobilized.

Table 2. Mineralized ^{15}N recovered from various labeled manure components over all temperatures at day 168.

Temp (ºC)	Urine	Feces	Bedding	All components
	------------------------------% of applied ^{15}N--------------------------			
11	44	13	15	24
18	60	18	24	30
25	63	26	25	36
P_t>F	0.054	0.003	<0.001	0.088
LSD, 0.05	16.2	5.7	3.5	10.4

Approximately 19% of applied fecal ^{15}N was mineralized over the 168 d observation period. Fecal ^{15}N mineralization increased significantly with increasing temperature. Other studies have also shown a positive relationship between temperature and manure N mineralization. In vessels where all applied manure components were labeled, N mineralization averaged 30% and was unaffected by temperature likely dominated by high N mineralization of urine. This is relatively close to the 35% mineralized N derived by summing the proportionate contribution of individual labeled manure N components.

Conclusion

The use of the stable isotope ^{15}N provided direct, and therefore more precise estimates of dairy manure N availability to corn the first, second and third year after manure application. Use of ^{15}N labeled dairy manure confirmed current University of Wisconsin Extension recommendation on dairy manure N availability. Conservation of dairy urine N is key to manure N availability to crops. Most urine N apparently mineralizes during the first few weeks after manure application. A large portion of applied manure N that is not taken up by a crop remains in the soil, and may be available to crops over the long term.

Livestock Manure used as Fertilizer or Fuel

J. M. Powell

Introduction

In the US, animal agriculture accounts for approximately $100 billion annually, or half of all farm sales. The manure produced by dairy and beef cattle, poultry and swine contains vast amounts of nitrogen and phosphorus (Table 1) that in some regions can be land applied at agronomic rates on farms where it is produced. Manure provides essential and secondary nutrients for crop production, increases soil organic matter levels, and enhances soil physical properties and overall soil quality. Manure also contains energy that can be converted into fuel. This paper summarizes potential agronomic benefits and pollution hazards associated with land-application of manure, followed by a brief discussion of the increasing attractiveness of using manure as fertilizer and energy in the current era of high energy costs.

Table 1. Manure nitrogen and phosphorus available for application to cropland in the US, 1997

Animal Type	Manure nitrogen	Manure phosphorus
	------------------1,000 Mg-------------------	
Dairy cattle	288.2	110.6
Feedlot beef cattle	176.8	115.2
Other cattle	59.2	49.1
Poultry	552.8	251.2
Swine	124.3	125.5
Total	1,201.3	651.6

Manure as a fertilizer for crop production

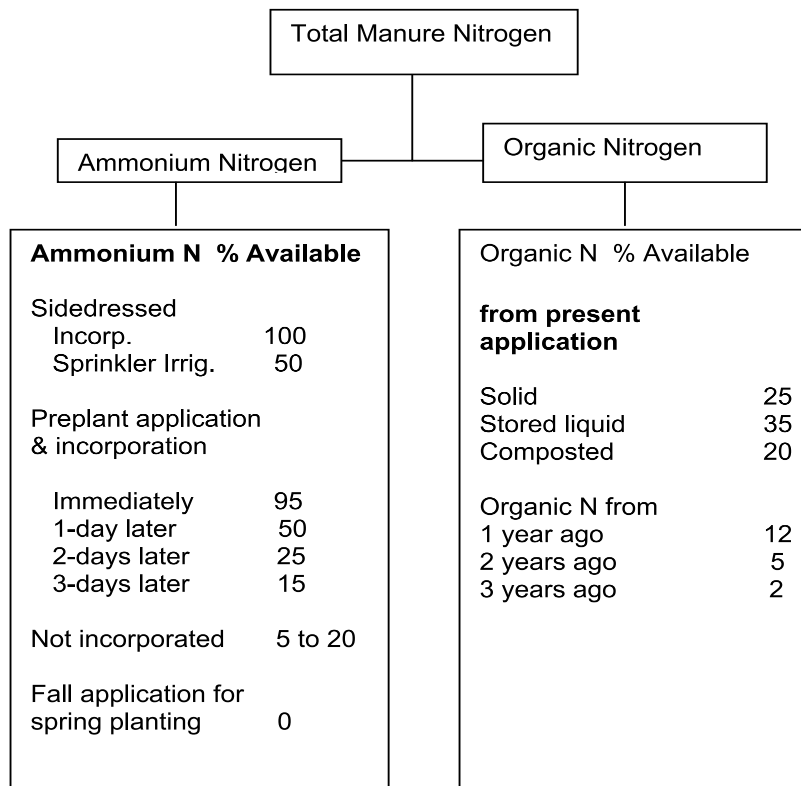
Nitrogen (N) is the most limiting nutrient to cereal crop production so the fertilizer value of manure is usually equated to its ability to provide N to a succeeding crop. Manure N availability for use by crops is highly influenced by its ammonium content (Fig. 1), which depends on the amount of urine N conserved. Organic N in feces and bedding is more slowly released than urine N, and continues to mineralize and be available for crop uptake years after application. If and when manure is incorporated also affects the availability of manure N to crops.

Two approaches are commonly used to estimate the fertilizer N value of manure; (1) apparent manure N recovery by crops (i.e., the difference method, or difference in crop N uptake in plots that received and did not receive manure); and (2) comparison of crop response with approximately equivalent rates of commercial fertilizer (i.e., the fertilizer equivalence approach). A 'decay series' is developed that predicts the proportion of manure N available the 1st, 2nd and 3rd year after application. A recent summary of approximately 90 trials conducted between 1931 and 2002 across a wide range of soils and environmental conditions and found that 1st year manure N availabilities were remarkably consistent within animal species and averaged 36% for dairy, 32% for beef, 27% for sheep, 51% for poultry and 62% for swine. However, averages tend to obscure within-study variability. For example, using the difference method and the fertilizer equivalent approach estimates of corn N uptake during the first

year after dairy manure application ranged from –60 % of applied N (a negative value obtained when crop N uptake in non-manure control plots is greater than manure-amended plots) to 148% (values > 100% obtained when crop N uptake exceeds N additions). The use of manure labeled with the stable isotope ^{15}N provides a more direct and less variable measure of manure N availability to crops.

Manure applications provide crop yields comparable or superior to yields with inorganic fertilizer. The beneficial effects of manure are due not only to nutrients, but also improvements in soil organic matter content (SOM), soil structure and tilth. Improvements in infiltration, soil aggregation and bulk density due to manure application can reduce runoff and erosion. SOM enhancement due to manure increases soil cation exchange and buffering capacities, which enables manure-amended soils to retain nutrients (and chemicals such as pesticides) for longer periods of time. SOM increases carbon sequestration in soils, which mitigates the effects of rising atmospheric CO_2 levels on global climate. However, manure may also provide soluble carbon and nitrates, which can enhance N_2O emissions from soil and contribute to global warming.

Figure 1. Availability of manure N for crop uptake



Limitations to using manure as a fertilizer

Effective recycling of manure nutrients through crops presents many challenges. For example, to achieve analytical results with a 95% confidence interval with a 10% probable error for manure N content, 1, 55 and 17 subsamples are required for dairy compost, chicken manure and stockpiled beef manure, respectively. Another factor that inhibits good manure management is the large difference in the N:P ratio of manure vs. the N:P requirements of crops. Applying sufficient manure to meet crop N requirements usually results in excessive P application, which can increase the hazard of soil P buildup and loss in runoff. The N:P ratio of manure can be aligned to the N:P requirements of crops by removing unnecessary mineral P supplements from animals' diets and through conservation of manure N.

The proximity of where manure is produced and crops are grown is key to managing manure for its agronomic benefits. During the early to mid part of the last century, crops and livestock were operationally and functionally linked enterprises. Most feed was homegrown and N provided by legumes and manure sustained crop yields. The introduction of inexpensive fertilizers and inexpensive transport costs allowed crops to be grown in one location and livestock produced in another. On many farms, manure became an undesirable byproduct and any connotation of its intrinsic fertilizer value was replaced with a 'waste' mentality. Specialization in livestock is most pronounced in feedlot cattle, swine and poultry industries. Especially since the mid-1980s, the average herd size on U.S. dairy farms has grown markedly, and milk production is becoming concentrated on the largest farms. The trend towards fewer and larger farms for all livestock types has heightened public concern about pollution.

When mismanaged, manure has the potential to pollute air, land and water resources. Over the past decade, environmental policy has focused on ways to improve manure management and arrest the build up of soil test P, runoff, and the pollution of lakes, streams and other surface water bodies. Emerging environmental policy is aimed at abating the emission of air pollutants from animal agriculture.

Major pathways of manure N losses are emissions of the gasses NH_3, N_2O and N_2, and the leaching of NO_3. Ammonia loss range from 30 to 40% of total N excreted. Nitrate losses typically range from 10 to 30% and denitrification 2 to 5% of total N applied. High nitrate leaching contaminates groundwater and increase losses of N via denitrification. Although denitrification may constitute only a small percentage of applied manure N, N_2O contributes to global warming and ozone depletion. The highly interactive nature of manure N transformations and pathways of N loss necessitate that manure management be based on an understanding of the tradeoffs involved in conservation of one N form and concomitant increases in other N losses. For example, manure injection into soil to reduce ammonia loss (and improve air quality) may increase nitrate leaching (and reduce ground water quality) and increase denitrification (greenhouse gas formation).

Nutrient management planning

Nutrient loss from agriculture, as well as natural ecosystems are inevitable. A continuous challenge is to enhance nutrient use by crops and livestock and minimize nutrient loss through good management. Animal nutrition, field, whole-farm and landscape models have been developed to improve nutrient management on crop-livestock farms. While of great use in predicting biophysical outcomes (e.g., feed nutrient use by livestock and excretion in manure, fertilizer and manure nutrient use by crops, soil nutrient buildup and loss), most models do not address social and economic factors that influence farmers' ability and willingness to adopt alternative practices. A manure management plan must be practical to be effective. A slightly imperfect, less comprehensive, but practical plan will almost always provide more desired results than a more complicated one that is not practical to implement.

Manure, fuel production and use

A principal strategy to facilitate manure management has been to improve manure handling and storage. Most dairy and swine manure is now flushed from housing, and manure is stored in outside lagoons. The widespread expansion of flush and lagoon systems was premised on labor efficiency and the notion that storage would facilitate calculation of manure nutrients available and allow for land application during favorable weather conditions and close to crop nutrient demands. However, anaerobic manure lagoons accounted for approximately 7% of the global methane emissions in 1991, a figure that has likely increased substantially. Covered lagoons, complete mix digester systems, and plug flow digester

systems can capture methane that can be converted into energy and used for electricity production, heating and cooling. The cost effectiveness of methane recovery and energy conversion is becoming increasingly attractive in areas were livestock concentrations, and therefore the supply of manure, is high enough to produced sufficient energy competitive with classical energy sources.

The use of manure as a substitute for fertilizer N may become more attractive as energy costs increase. Natural gas is used to produce a large fraction of fertilizer N, and natural gas accounts for 75-90% of the cost of making anhydrous ammonia. Conserving manure N may be of much greater importance as energy costs continue to escalate. Furthermore, it will reduce carbon dioxide (greenhouse gas) generation during the manufacture of N fertilizer.

Conclusion

Manure benefits crop production through its fertilizer value and enhancement of soil physical properties and overall soil quality. The fertilizer N value of manure depends on the conservation of urine N. Approximately 25-35% of the N contained in manure of ruminant livestock (beef and dairy cattle, sheep) is available to the plant the season following application vs 50-60% for poultry and swine. As livestock and crop production become more specialized, it becomes more difficult to conserve manure nutrients and recycle them through crops. Manure N losses via ammonia volatilization, nitrate leaching and denitrification, and manure P losses in runoff are the principal pollution concerns. The economics of practices that enhance manure's fertilizer value, and capture of methane during manure storage may become increasingly attractive in the current era of high energy costs.

Use of the Logic Model to Build Partnerships for Understanding and Abating Ammonia Emissions from Dairy Farms

J. Mark Powell, E. Taylor-Powell, R. Klemme, T. Johnson, L. Bruss, and T. Misselbrook

Introduction

Partnerships are key to understanding and solving complex issues facing agriculture. Yet, producers, agricultural research and extension, agribusiness, and policy makers often have different interests and mandates and therefore different approaches to the development and implementation of technologies that enhance farm profitability and environmental performance. All stakeholders need a common understanding of the real and perceived risks of alternative technologies and policies, and their compatibility with existing production practices. To create this common understanding and a joint action plan for abating ammonia emissions from dairy farms, a multi-stakeholder group of research, extension and policy makers used a logic model framework. The logic model provided an organizational framework that focused discussion and understanding in order to identify the inputs, activities and outputs of each partner to create an action plan and a process for achieving agreed upon desired outcomes.

The national trend towards fewer and larger livestock farms has heightened public concern about water and air pollution. Over the past several years, environmental policy related to animal agriculture has focused on land application of manure, especially how to stop or reverse soil phosphorus (P) build up, runoff, and the subsequent pollution of lakes, streams and other surface water bodies. Policy is now aimed at reducing air emissions from animal agriculture. For Wisconsin, "America's Dairyland",

air emissions relate directly to ammonia. Most of the nitrogen contained in the urine excreted by dairy cows can be transformed rapidly and emitted as ammonia gas. Two impacts of emitted ammonia are of principal concern:

(1) Emitted ammonia combines with compounds in the upper atmosphere to form particulates. These particulates have been related to haze in urban areas, and also have been attributed to a variety of adverse health effects, including premature mortality, chronic bronchitis, asthma, and hospital admissions.

(2) When deposited in natural ecosystems, ammonia contributes to ecosystem fertilization, acidification, and subsequent "ageing". This nitrogen (N) input from ammonia can cause dramatic shifts in the vegetation, enhancing grass growth and fire hazards in some areas.
Under the federal Consolidated Emissions Reporting Rule, each state will be required by 2004 to report air emissions to the US Environmental Protection Agency (EPA). These estimates will be used in air quality regulations to control the air-borne particulates and haze that affect many regions of the US.

Establishing the partnership

To respond to the need for quantitative information on ammonia emissions, the "Partnership in Understanding and Abating Ammonia Emissions from Wisconsin Dairy Farms" was created in March 2004. A number of factors came together at this particular time to cause the creation of this partnership:

1) Producer concern was rising about the impending ammonia emission regulations and effect on their business.
2) Wisconsin was required to report an ammonia emissions inventory to USEPA;
3) Funding became available to do ammonia emission research;

The partnership included four principal stakeholder groups with diverse interests:

1) Research: USDA-Agricultural Research Service, Dairy Forage Research Center and University of Wisconsin, College of Agricultural and Life Sciences;
2) Extension: Wisconsin Agricultural Stewardship Initiative (WASI)
3) Policy: Wisconsin Department of Natural Resources
4) Producers: Professional Dairy Producers of Wisconsin (PDPW)

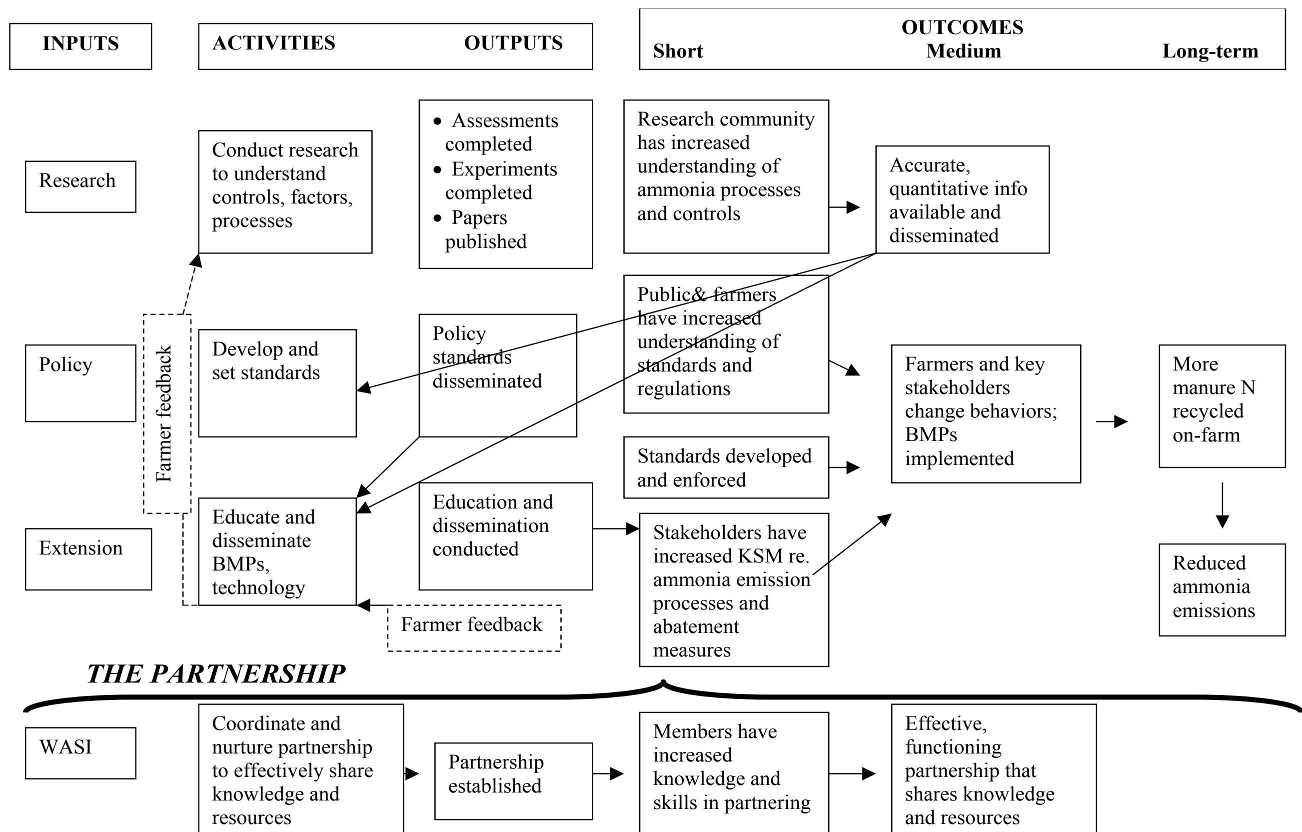
The initial purpose, bringing the partners together, was to plan an "Ammonia Emissions Workshop". During the initial planning meetings, it became clear that future workshops focusing on research, technology transfer and policy would be warranted and continued collaboration would be advantageous. Rather than an ad hoc approach, the partners decided to think more long-term and plan for ongoing collaboration. The research and extension members of the workshop planning committee had experience in using the logic model in other planning and evaluation efforts. It was felt that the logic model could provide a way for the partners to clarify their work together, articulate their joint desired outcomes and future research activities.

The logic model

The research and extension members crafted the first model. It was brought to the group for discussion and modifications were incorporated. The current partnership model is illustrated in Figure 1. It depicts outcomes for the partnership process that are necessary in order for the activities the partnership undertakes to be achieved. It shows the role of the individual stakeholders and how they interrelate and contribute to the achievement of the ultimate goal, reduction in ammonia emissions. This overall logic model has helped facilitate discussion, understanding and direction among the partners. It is considered a roadmap that highlights and communicates the end destination and route for getting there.

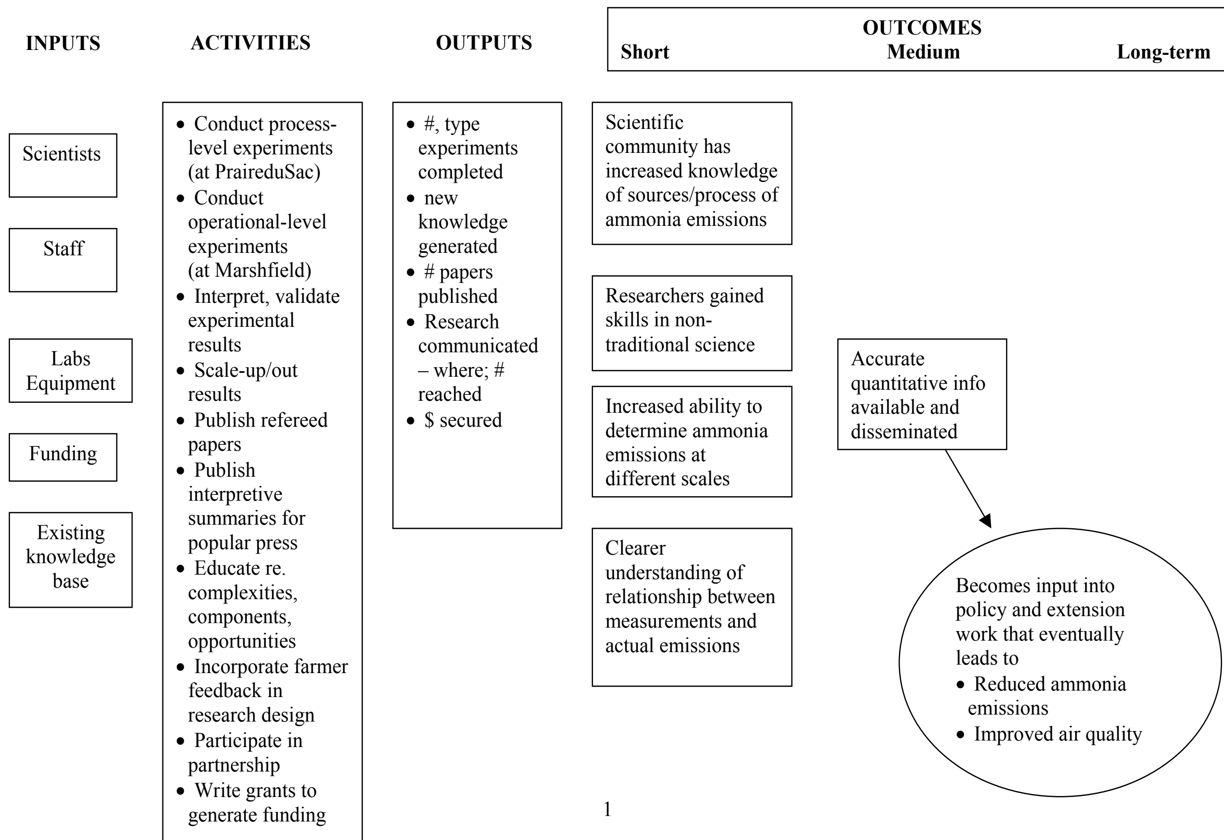
Figure 1. Logic Model: Partnership in Understanding and Abating Ammonia Emissions from Wisconsin Dairy Farms

SITUATION: Ammonia is a potential human health and environmental concern. No organized, unified effort exists to understand ammonia emissions processes and use this information to develop policy and adoptable practices that enhance ammonia capture on dairy farms.



A component research logic model (Fig. 2) was developed as a research planning exercise. It depicts, in more detail, the activities and outcomes for the research component that feeds into the overall effort (overall logic model). This component logic model was shared with the other partners and minor modifications were made as a result.

Figure 2. Research Logic Model: Partnership in Understanding and Abating Ammonia Emissions from WI Dairy Farms
SITUATION: Current research knowledge related to ammonia emissions from dairy farms is inadequate.



Conclusion

The logic model framework helped the partnership create a synopsis of initial activities and initiate a process of planning future work. It helped us understand the nature of our partnership and the value of working collaboratively. It helped us focus on and articulate an ultimate end goal and identify the contributions of each partner in achieving that desired end result. It has given us a one-page picture that communicates how we will work together. We will continue to revisit the logic models to:

- Further refine the individual contributions to the whole and the performance necessary from each partner;
- Analyze alternative strategies for achieving our desired end state;
- Refine the series of connections and plausible chains of outcomes;
- Create more specific work plans for accomplishing our goals;
- Identify ways to measure our individual contributions and the success of the whole partnership.

Factors that Impact Manure Management and Policy on Wisconsin Dairy Farms

J. Mark Powell, H. Saam, D. McCrory, D. Jackson-Smith

Introduction

Federal and state agencies have increasingly focused environmental, animal agriculture regulations on the amount and timing of manure application to cropland. The current regulatory focus is on large livestock operations under the assumption that they produce the most manure, and therefore pose the greatest environmental risk. However, it is becoming increasingly evident that farms of all sizes can generate negative environmental impacts. It often has been suggested that economies of size, more modern technologies and potentially higher management skills associated with large-scale operations may put these operations at a decreased potential to pollute in relation to smaller, outdated facilities.

Environmental policy for controlling agricultural pollution gives very little recognition to the diverse biophysical and socioeconomic conditions farmers face, and how these factors influence farmer ability to control pollution from their farms. It is often assumed that pollution is simply a matter of choice, and that policy should "examine the question of how to induce farmers who cause water quality damages through their choice of production practices to adopt pollution prevention and pollution control practices that are consistent with societal environmental quality objectives". We contend that most livestock producers do not chose to adopt practices that pollute, but rather some find themselves in environments that limit management choices. This paper summarizes recent findings related to manure collection, stocking density, feeding practices, soils and weather, and socio-economic factors that affect manure management behavior, and how this information may be considered when formulating policy aimed at improving the environmental performance of Wisconsin dairy farms.

Balancing Animal Numbers with Cropland Area for Manure Spreading

An alternative (or additive) to the current herd size or watershed indicators for targeting manure management policy is animal density, expressed in terms of the number of animals per unit area of cropland. Animal density is increasingly being used in Europe and in certain parts of the U.S. The strength of using animal density as a regulatory standard lies in its ability to provide a relatively straightforward indicator of a farm's nutrient balancing potential. By characterizing the relationship of animal numbers (and the manure they produce) to the available cropland area for manure utilization, animal density addresses the core movement of nutrients within the farm nutrient cycle. Without adequate cropland on which to recycle manure nutrients, farms of all size have an increased potential for nutrient loss.

Having sufficient cropland for agronomic manure application rates is a key aspect of nutrient management planning. There is a direct relationship between a dairy farm's ability to grow feed for its herd and the farm's manure recycling potential. A recent survey of 98 representative dairy farms in Wisconsin showed that farms having stocking rates of less than 0.70 animal units (AU = 454 kg animal) per hectare (or 1.7 acres per cow) are self-sufficient in forage and grain production, and therefore have adequate cropland area for recycling manure phosphorus (P). Approximately 50% of Wisconsin dairy farms are self-sufficient forage/grain, 68% produce 90% of their forage/grain and 80% produce 80% of their annual forage/grain requirement.

Another study of approximately 800 dairy farms showed that 95 percent of Wisconsin dairy farmers have sufficient cropland for recycling manure according to a N-based nutrient management standard (Fig. 1). Calculating animal density based on just the tilled portion of total cropland area decreases

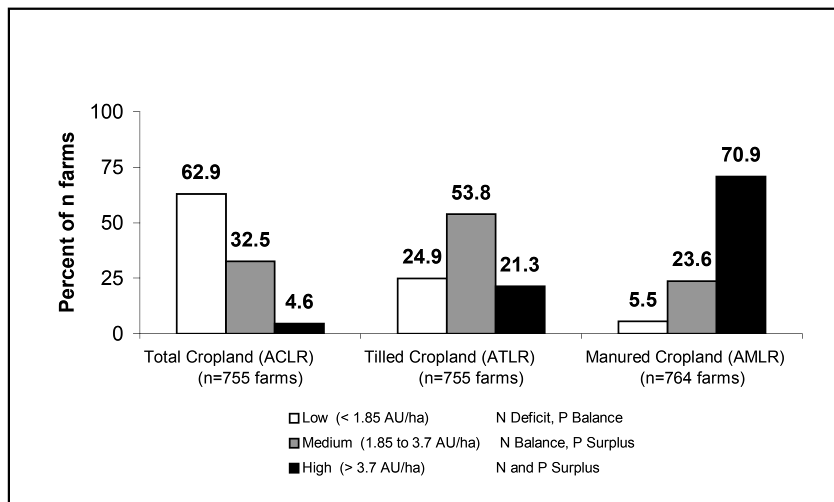
this value to 79% of dairy farms   Implementation of a P-based standard reduces the overall amount of cropland available for manure application, and a large proportion of Wisconsin dairy farms (37% based on total cropland and 75% based on tilled cropland) would lack sufficient land area for recycling manure P. When the area of cropland on which manure is actually spread is used to calculate animal density, it is clear that the majority of farms do not currently meet either manure N- or P-based land application standards.

Linkage Between Feeding Practices and Manure Nutrient Recycling

While the concept of using animal density as an indicator of nutrient balancing potential is fairly transparent, certain assumptions about feeding practices and the land base available for manure application need to be considered. For example, feeding practices can have a dramatic effect on manure P levels and the amount of land needed to recycle manure P. Research shows that excessive P feeding to dairy cows increases total and water-soluble P in manure and the amount of P in runoff after manure has been land-applied.

Over the past 2-3 years, many Wisconsin dairy farmers have reduced the dietary P content of their dairy cows' diet without sacrificing milk production, milk quality, or the herd's reproductive performance. Further reductions in dietary and therefore manure P can be attained by restricting the use of mineral P supplements, and encouraging the use of protein supplements that have a more favorable crude protein:P balance. The drawback to this approach may be cost. Since environmental improvements do not currently have direct economic benefits, it is continues to be rational for farmers to select protein supplements based on least cost, regardless of their P content.

Figure 1. Distribution of Wisconsin dairy farm abilities to meet manure nitrogen and phosphorus standards based on cropland used for manure spreading.

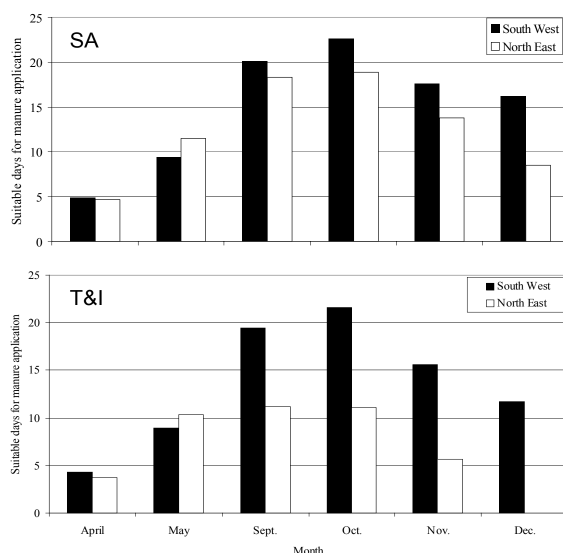


Regional Differences in Manure Management

Although most Wisconsin dairy farms have sufficient cropland for spreading manure, there is a large "manure gap" between cropland areas available for manure application and the cropland area that actually receives manure, and this "manure gap" varies depending on the region of the state. For example, dairy farmers in the northeast (NE) spread manure on only 23% of their total operated cropland area versus 30% in the south central (SC) and 44% in the southwest (SW) regions of the state. Farmers' inability to use a greater proportion of their cropland area for manure spreading may be due to various factors, such as (1) the presence or absence of manure storage facilities; (2) labor availability and machinery capacity for manure spreading; (3) the amount of manure actually collected, and therefore that needs to be spread on cropland; and (4) variations in the manure "spreading window", or days that manure can be spread given regional differences in weather and soil conditions. Manure spreading is also related to distances between where manure is produced and fields where manure is applied and to land ownership; as the percent of land owned increases so does percent of total cropland that receives manure.

The ability of farmers to utilize as much of their cropland as possible for manure application is likely to be crucial in complying with future state-wide nutrient management regulations. Inherent regional soil and climate differences may exist which hinder farmers from one area in achieving this goal as readily as those in another region. In the study of manure spreading differences between the NE and SW regions of Wisconsin, it was postulated that regional differences in soil type and weather may affect manure spreading behavior. The SW region is characterized by silt loam soils of relatively high permeability and drier field conditions in the spring and fall. In contrast, the NE is characterized by more finely textured and less permeable clay and red loam soils. A recent analysis of the impact of regional differences in soil type and weather conditions on the number of suitable days for manure application via surface spreading and tillage and/or manure injection indicated that farmers in the SW may have approximately 28% more Fall days for surface application of manure and 60% more days available for tillage and injection operations than the NE region of the state (Fig. 2). This may enable them to access greater portions of their cropland and more readily comply with manure management regulations than their NE counterparts. The major reason for differences was due to soil type, rather than weather.

Figure 2. Suitable days for manure surface application (SA), tillage and injection (T&I) for the southwest (SW) and northeast (NE) regions of Wisconsin



Spatial Arrangement Between Barns and Fields

The spatial arrangement between where the animals are housed (and where the manure is produced) and the location of fields can greatly affect manure management. Farmers apply manure more often to fields that are close to the barn, than to distant field, which are often rented land. Spreading on distant fields may be very time and energy consuming, and hence not an economically viable option.

As dairy farmers expand herd size, access to close-by or contiguous rented land parcels is becoming increasingly difficult. This appears to be particularly true in the NE region of Wisconsin where farmers have greater difficulty than in other regions in finding access to close-by rented land parcels. Farmers in northeast Wisconsin spread manure on a much smaller fraction (23%) of their total cropland base than farmers in the southwest region of the state.

Evidence from several on-farm follow-up visits suggest that farms in the NE are more likely than farms in other regions of the state to be renting fields that are more distant from the barn and more difficult to access without transporting manure on heavily traveled commuter roads. This could create additional disincentives for farmers in this region to spread on their rented cropland, compared to areas with lower development pressure. Policy can create incentives that promote closer linkages between crops and livestock. For example, manure contracts among crop and dairy farmers can reduce manure hauling distances. Such area-wide integration of crops and livestock is the most energy and nutrient efficient form of crop and livestock production.

Conclusions

Current policy approaches may not fully consider the environmental implications of important manure management options, such as the balance between cow numbers and cropland available for manure land-spreading, optimal feeding, and farmer ability to collect and land-spread manure under the diverse biophysical and socioeconomic conditions they face. Being able to match livestock numbers with an adequate land base for manure application is an integral part of good nutrient management. It creates a balance between livestock numbers, the amount of forage and grain they need and the amount of manure produced, thereby reducing the need for off-farm feed purchases, manure exportation, and the overall likelihood of nutrient accumulation and loss. From a policy perspective, unlike water quality-based indicators that are difficult to measure and attribute to farm practices, animal density can be accurately assessed on each farm at low costs.

Animal density may provide a useful, and size-neutral indicator for targeting regulatory oversight in Wisconsin, and perhaps be an effective way to target limited cost-sharing monies toward high-density operations situated in landscapes that are at greatest risk for nutrient loss. These high-density operations will likely require the greatest assistance in adhering to nutrient management standards. An animal density standard would also provide a much-needed framework for planning dairy herd expansions.

Value Added Product Processing

Novel Bio-Based Adhesives Produced by Fermentation of Alfalfa Fiber

P.J. Weimer, R.G. Koegel, L.F. Lorenz, C.R. Frihart, and W.R. Kenealy

Introduction

Fermentations of cellulosic biomass to produce fuel ethanol are not yet commercially viable, due to the high cost of enzymes for hydrolyzing cellulose to fermentable sugars, and to the lack of additional co-products that add value to the fermentation. A promising route to ethanol production is "consolidated bioprocessing" (CBP), in which the cellulosic materials are fermented by anaerobic bacteria that produce their own polysaccharide-hydrolyzing enzymes, and that ferment the resulting oligomers to ethanol. We have previously reported that the residues from cellulose fermentation by the ruminal bacterium *Ruminococcus albus*, a potential CBP organism, displays adhesive properties that may be useful for wood-bonding applications. The present study was carried out to determine i) if the fermentation residue from a more commonly available biomass, alfalfa fiber, could also serve as a bio-based adhesive; and ii) if another CBP organism, *Clostridium thermocellum*, can also produce such a residue.

Methods

Alfalfa fiber was obtained by a wet-fractionation process that separated expressed juice (containing most proteins, sugars, and other soluble materials) from a residual fiber fraction (largely stem material with some leaf material), as follows: Freshly-cut alfalfa (*Medicago sativa* L.) was passed, sequentially through a hammer mill and a multi-cone press and the retained solids (alfalfa fiber) were air-dried indoors on a tarpaulin. Approximately 75% of the original dry matter was retained in the fiber fraction. The resulting fiber chopped and then was ground through a Wiley mill (1 mm screen size). No additional pretreatment of this fiber fraction was performed prior to their use in the culture media.

R. albus 7 and *C. thermocellum* ATCC 27405 were grown under CO_2 at 300 liter scale in Modified Dehority medium(MDM) containing 1.5 kg of alfalfa fiber. After 48 h incubation, the fermentation residue (containing incompletely fermented alfalfa fiber, adherent bacterial cells, and their extracellular polysaccharide) was collected on a plate-and-filter press and freeze dried. The solids were ground to various particle sizes in a Wiley mill (0.5 mm screen), then used to formulate adhesive pastes in the presence or absence of various additives commonly used in plywood manufacture. Adhesive formulations were used to prepare duplicate three-ply aspen panels, which were pressed at 1.14 MPa (165 lb in^{-2}) and 180 ^{o}C for 5 min, yielding a three-ply panel 9.5 mm thick. After conditioning, the panels were tested for shear strength and wood failure, using standard tests of the National Institute for Standards and Technology.

Results and Discussion

Testing of the resulting plywood panels (Fig.1) revealed that the dried fermentation residue from either bacterium displayed poor shear strength and wood failure under wet conditions, typical of bio-based adhesives. However, the fermentation residues were able to serve as an effective co-adhesive in the presence of phenol-formaldehyde (PF). When formulated to contain 30% of its total dry weight as fermentation residue the fermentation residue/PF composite displayed shear strength and wood failure

values under both wet and dry conditions that were comparable to those of industry standards for PF that contained much smaller amounts of fillers or extenders. By contrast, PF adhesives prepared with 30% of dry weight as either unfermented alfalfa fiber or conventional fillers or extenders, rather than as fermentation residues, displayed poor performance, particularly under wet conditions.

Testing of fermentation residue of various particle size classes in adhesives formulated at 30% fermentation residue (dry weight basis, balance PF resin) revealed that particle size had little effect on shear strength of the resulting panels. However, particle size strongly affected wood failure values, with optimum adhesive performance obtained with fermentation residue ground to 75 μm or less in particle size. Because only this fraction represented only 19% of the dry weight of 0.5 mm-Wiley milled fermentation residue used in Fig.1, it is likely that substantial performance advantages would be gained from more vigorous size reduction prior to use of the fermentation residue in the adhesive formulation.

Conclusions

The residue from fermentation of wet-fractionated alfalfa by *R. albus* or *C. thermocellum* displays potential as a co-adhesive with phenol-formaldehyde in plywood manufacture that could substantially reduce the use of this petroleum-derived material. Consequently, the fermentation residue thus has potential to add value to a CBP fermentaion of alfalfa fiber to ethanol. Additional research should focus on economical particle size reduction of the residue, along with modification of pressing conditions to improve performance.

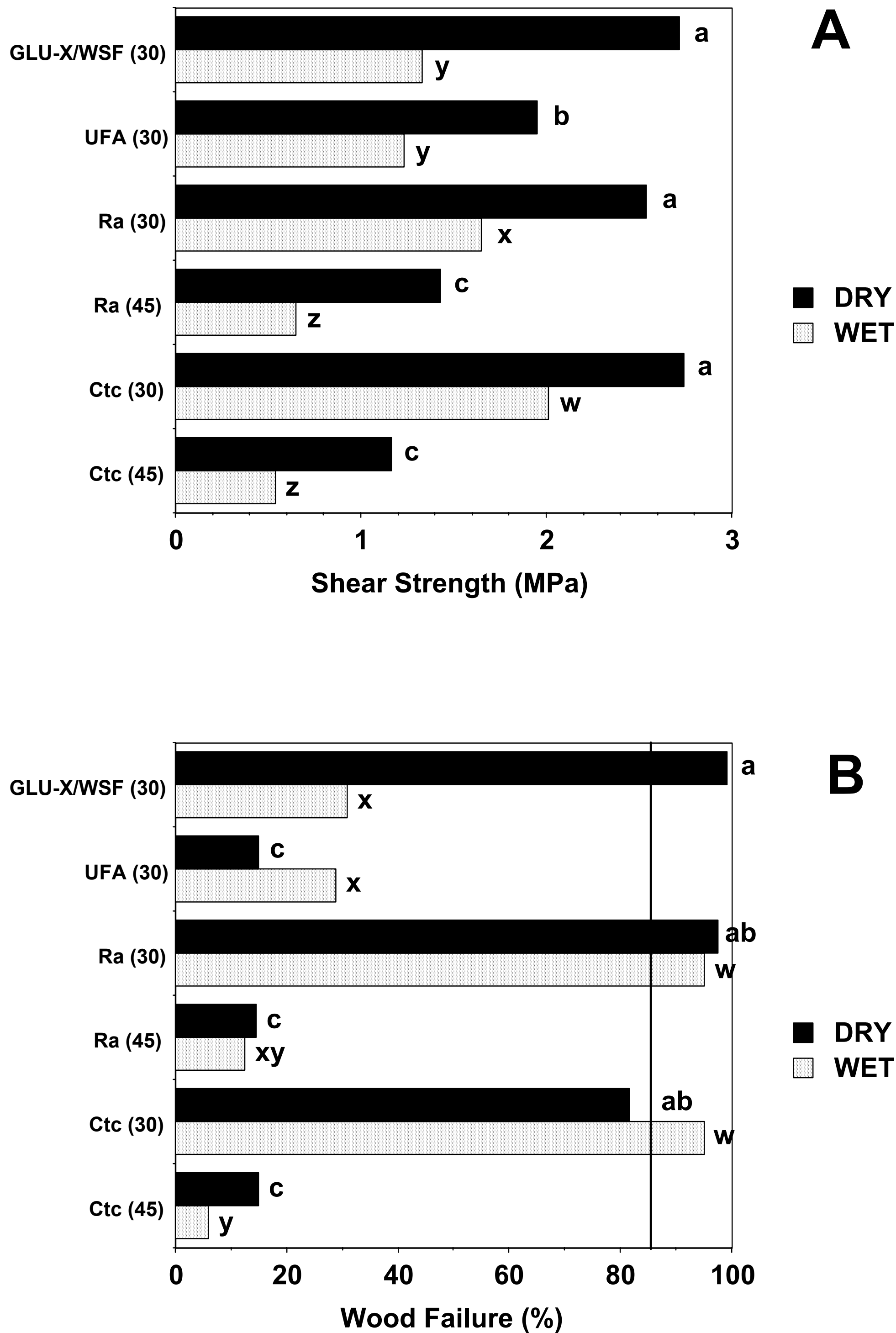


Figure 1: Shear strength (panel A) and wood failure (panel B) data for plywood adhesives formulated with phenol-formaldehyde resin and various additives: GLU-X/WSF, equal weights of GLU-X wheat bran flour and walnut shell flour, UFA, unfermented alfalfa fiber; Ra, residue of *R. albus* alfalfa fiber fermentation; Ctc, residue of *C. thermocellum* alfalfa fiber fermentation. Values in parentheses on the vertical axis indicate percentages (dry weight basis) of additive, with remainder being alkaline phenol-formaldehyde. abc: Different letters indicate significant differences ($P<0.01$) in shear strength or wood failure under dry conditions. wxyz: Different letters indicate significant differences ($P<0.01$) in shear strength or wood failure under wet (vacuum/pressure/soak) conditions. The industry standard for wood failure is 85%.

U.S. DAIRY FORAGE RESEARCH CENTER
ANNUAL DAIRY OPERATIONS REPORT
JANUARY 2005 (for 2004)

JILL A. DAVIDSON - HERD MANAGER

HERD STATISTICS			**CHANGE FROM PREVIOUS YEAR**
Herd Inventory			
Milking cows		274	- 30
Dry cows		52	- 1
average cow age	45 months		0
percent first lactation	42 %		- 1
percent second lactation	29 %		+ 2
percent third or greater lactation	29 %		- 1
Herd replacements		350	+ 4
Total		**676**	**- 26**
Rumen fistulated cows		18	- 6
Herd Performance			
Cows calved		380	+ 9
Heifer calves born live		185	- 2
Heifer calves born dead		19	+ 5
Bull calves born live		168	+ 13
Bull calves born dead		20	0
Heifer calves died < 1 year old		8 (4.3%)	+ 5
Number of sets of twins		21	0
DHIA rolling herd average			
milk		24,884 lbs.	+ 366
protein		739 lbs.	+ 11
fat		921 lbs.	- 7
Milk sold in 2003		7,908,510 lbs.	- 4002
Average Mailbox Milk Price/cwt		$15.95	+ 3.92
Heifer calves sold		5	+1
Bull calves sold		168	+15
Cows Sold			
Cows culled for:			
Reproduction Problems		48	- 7
Poor Production		15	- 4
Poor Feet and Legs		25	0
Mastitis		38	+ 24
Other		53	+ 22
Cattle Sales Revenue		133,451	+ 46,052
Herd Reproduction			
Average days open		130	- 10
Average calving interval		13.8 months	+ 0.2
Average services per conception		2.9	+ 0.5
Average age at first calving		24 months	- 1

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