In vitro bacterial growth and in vivo ruminal microbiota populations associated with bloat in steers grazing wheat forage

B. R. Min,* W. E. Pinchak,2* R. C. Anderson,† and M. E. Hume†

*Texas Agricultural Experiment Station, P.O. Box 1658, Vernon 76385; †United States Department of Agriculture, Agricultural Research Service, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit, 2881 F & B Rd., College Station, TX 77845

ABSTRACT: The role of ruminal bacteria in the frothy bloat complex common to cattle grazing winter wheat has not been previously determined. Two experiments, one in vitro and another in vivo, were designed to elucidate the effects of fresh wheat forage on bacterial growth, biofilm complexes, rumen fermentation end products, rumen bacterial diversity, and bloat potential. In Exp. 1, 6 strains of ruminal bacteria (Streptococcus bovis strain 26, Prevotella ruminicola strain 23, Eubacterium ruminantium B1C23, Ruminococcus albus SY3, Fibrobacter succinogenes ssp. S85, and Ruminococcus flavefaciens C94) were used in vitro to determine the effect of soluble plant protein from winter wheat forage on specific bacterial growth rate, biofilm complexes, VFA, and ruminal H2 and CH4 in mono or coculture with Methanobrevibacter smithii. The specific growth rate in plant protein medium containing soluble plant protein (3.27% nitrogen) was measured during a 24-h incubation at 39°C in Hungate tubes under a CO2 gas phase. A monoculture of M. smithii was grown similarly, except under H2:CO2 (1:1), in a basal methanogen growth medium supplemented likewise with soluble plant protein. In Exp. 2, 6 ruminally cannulated steers grazing wheat forage were used to evaluate the influence of bloat on the production of biofilm complexes, ruminal microbial biodiversity patterns, and ruminal fluid protein fractions. In Exp. 1, cultures of R. albus (P < 0.01) and R. flavefaciens (P < 0.05) produced the most H2 among strains and resulted in greater (P < 0.01) CH4 production when cocultured with M. smithii than other coculture combinations. Cultures of S. bovis and E. ruminantium + M. smithii produced the most biofilm mass among strains. In Exp. 2, when diets changed from bermudagrass hay to wheat forage, biofilm production increased (P < 0.01). Biofilm production, concentrations of whole ruminal content (P < 0.01), and cheesecloth filtrate protein fractions (P < 0.05) in the ruminal fluid were greater on d 50 for bloated than for nonbloated steers when grazing wheat forage. The molecular analysis of the 16S rDNA showed that 2 different ruminal microbiota populations developed between bloated and nonbloated animals grazing wheat forage. Bloat in cattle grazing wheat pastures may be caused by increased production of biofilm, resulting from a diet-influenced switch in the rumen bacterial population.

Key words: bacterial diversity, frothy bloat, gas production, methane, rumen

©2006 American Society of Animal Science. All rights reserved.

INTRODUCTION

Frothy bloat results from complex interactions among plant, animal, and environmental factors (Clarke and Reid, 1974; Min et al., 2005b). Total and soluble plant proteins have been identified as precursors to bloat on wheat pasture (Bartely et al., 1975; Min et al., 2005b). Gutierrez et al. (1963) reported that the rapid release of soluble protein into rumen environment caused the capture of ruminal gases in a low-gas permeable polysaccharide slime (referred to as biofilm) layer that promotes frothy bloat.

Considerable research on the rate of ruminal fermentation and ruminal bacterial populations (Hungate et al., 1955; Howarth et al., 1981, 1991) has documented many unique differences in bloating and nonbloating cattle fed legume forages and showing microbial differences due to frothy bloat. The potential role of ruminal microorganisms in frothy bloat in cattle grazing wheat forage has not been well established. Recently, RNA- and DNA-based approaches for characterizing ruminal
bacterial diversity have been evaluated such as competitive- and real-time-PCR, and denaturing gradient gel electrophoresis (DGGE; Quwerkerk et al., 2002; Reilly et al., 2002; Hume et al., 2003). Both real-time PCR and DGGE are the methods of choice research involving large sample sizes. The DGGE used in present study allows for rapid screening of bacterial populations and visualization of PCR products representing predominant ruminal bacterial communities (Hume et al., 2003), while not being subject to selective pressures inherent to traditional medium-based culturing techniques. A series of in vitro and in vivo experiments were designed to elucidate the effect of wheat forage on the bacterial growth, biofilm complexes, ruminal fermentation end products, ruminal bacterial diversity, and bloat potential.

**MATERIALS AND METHODS**

**Experiment 1: Microorganisms**

Six strains of ruminal bacteria were used in an in vitro experiment to determine the effect of soluble plant protein from wheat forage on specific bacterial growth rates, biofilm complexes, VFA, and H2 and CH4 profiles in mono and coculture with a methanogenic bacteria. Six strains of ruminal bacteria (Streptococcus bovis strain 26, Prevotella ruminicola strain 23, Eubacterium ruminantium B1C23, Ruminococcus albus SY3, Fibrobacter succinogenes S85, and Ruminococcus flavefaciens C94) in mono and cocultures with Methanobrevibacter smithii were used. J. Yanke, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada, generously provided these organisms.

In the current study, M. smithii was chosen as a coculture strain because ruminal gases, particularly CH4, H2, and acetate, are related to dietary energy loss (Johnson and Johnson, 1995; Miller, 1995). Strain purity was ensured by determining single colony morphology on agar plates and by a single consistent cellular morphology in liquid cultures examined under the microscope (personal communication, J. Yanke). All isolates were inoculated from their respective long-term storage (lyophilization) vials into anaerobic basal medium in Hungate tubes for 24 h at 39°C. The isolates were reinoculated into anaerobic plant protein medium and incubated for 24 h at 39°C. At the end of the incubation period, concentrations of VFA, H2, CO2, and CH4 gases present in the headspace of the tubes were determined as described later.

**Preparation of Soluble Plant Protein**

Preparation, distribution, and inoculation of a basal growth medium containing soluble plant protein (referred to as plant protein medium) were carried out under a stream of CO2 or in an anaerobic hood (Coy Laboratory Products Inc., Grass Lake, MI) with a 95% CO2/5% H2 atmosphere. Total soluble plant protein was extracted from fresh wheat forage by blending 100 g of plant material in 300 mL of artificial saliva (pH 6.8; McDougall, 1948), and squeezing the extract through 4 layers of cheesecloth. The filtrate was collected and centrifuged at 16,000 × g for 20 min at 5°C, and the supernatant fraction was filter-sterilized by passing through a 0.45-µm syringe filter (Millipore Co., Bedford, MA). All preparation and handling procedures for plant protein medium were conducted anaerobically and filter-sterilized before addition to the autoclaved basal growth medium.

**Growth of Organisms**

Six individual strains, in monoculture or in coculture with M. smithii, were grown in vitro in plant protein medium. The composition of plant protein basal medium was resazurin (Eastman Kodak), 0.2 mL; clarified rumen fluid, 40 mL; mineral solutions numbers 1 and 2, 8 mL each; glucose, 0.2 g; cellobiose, 0.2 g; starch, 0.2 g; xylose, 0.2 g; trypticase peptone (BBL), 0.1 g; Na2CO3, 0.8 g; distilled water, 134 mL, in a final volume of 200 mL. Mineral solution number 1 contained 0.6% K2HPO4, and mineral solution number 2 contained 0.6% KH2PO4, 0.6% (NH4)2SO4, 1.2% NaCl, 0.25% MgSO4 7H2O, and 0.16% CaCl2. The pH was adjusted to pH 6.8 with 30% NaOH before dispensing 9 mL of basal medium into test tubes under constant CO2 gas streams. Cystein-HCl solution (2.5%) was then added (0.2 mL) to each tube after autoclaving. Pure cultures of M. smithii were grown in methanogen medium (Balch et al., 1979); cocultures of M. smithii with respective test bacteria were grown under an oxygen-free H2-CO2 (50:50, vol/vol) gas mixture in plant protein medium. Pure cultures of other strains were grown under an oxygen-free CO2 in plant protein medium (9 mL of basal growth medium together with 3.5 mL of soluble protein extracted from wheat forage, which contained 3.27% soluble-nitrogen (N) as the major N source) in Hungate tubes.

The specific growth rate, based on optical density using a spectrophotometer (Milton Roy Co., Spectronic 20D, Rochester, NY) at 600 nm, in plant protein medium was measured after 0, 2, 4, 6, 8, 12, and 24 h of incubation at 39°C in Hungate tubes. The specific growth rate was calculated using the following equation:

\[
\text{Specific growth rate, } \%/h = \frac{(\log Z - \log Z_0)(\log Z)}{(t - t^0)} \times 100,
\]

where \(Z\) and \(Z_0\) correspond to the growth rate of rumen bacteria at times \(t\) (maximum) and \(t^0\) (time 0, start time), respectively (Stanier et al., 1976).

**Experiment 2: Forages and Animals**

The Texas A&M University Animal Care Committee approved the experimental protocol. Wheat pastures
were fertilized before planting at a rate of 56 and 17.8 kg of N and S/ha, respectively. Wheat seed (*Triticum aestivum* var. Cutter) was sown on September 24, 2004, at a rate of 67 kg of seed/ha. Wheat forage biomass was collected during the experimental period by hand-plucking and was collected from the same pasture grazed by ruminally cannulated steers. Samples from wheat forage and bermudagrass hay were taken twice monthly and dried in a forced-air oven at 60°C for 48 h and ground (Cyclone sample mill, Udy Co., Fort Collins, CO) to pass a 1-mm sieve for CP, NDF, ADF, and IVMD analyses.

In the in vivo experiment, 6 healthy ruminally cannulated steers (Angus × Hereford × Brangus; 375 ± 30 kg) were used to quantify biofilm complexes, ruminal microbiota profiles, and ruminal fluid protein fractions associated with frothy bloat in steers grazing winter wheat forage. Rumen samples were collected after 1 mo on a bermudagrass (*Cynodon dactylon*) hay diet (i.e., a nonbloat promoting diet) and served as a background sample (d 0) for each animal. Steers were then transferred with an additional 12 nonsteers ruminally cannulated cohorts to a wheat pasture (14.1 ha) and allowed to graze wheat forage up to 50 d.

Rumen contents (about 500 g/steer) were collected from the 6 steers on d 30 (no bloated steers observed), 40, and 50 (bloated steers observed) for analysis of biofilm complexes, ruminal microbiota populations, and ruminal fluid protein fractions associated with frothy bloat. Rumen microbiota population analyses were conducted on d 30 and 50 only. The grazing period was from December 10, 2004, to January 30, 2005. A pure sward of vegetative stage of fresh wheat forage was managed under ad libitum access [18 kg of DM/(100 kg of BW×d)] during the experimental period (Pinchak et al., 2005a). The biofilm complexes in all experiments were assayed using the method described by Min et al. (2005). Changes in predominant microbial populations were evaluated by banding patterns detected after DGGE. Amplicon melting (separation of the double-stranded DNA) domains and migration in the polyacrylamide gel, urea-formamide denaturing gradient were determined by the unique guanosine + cytosine (G+C) content, primary sequences, and interactions between associated bp (Muyzer et al., 1993). Genomic bacterial DNA was isolated from 0.5 mL of each rumen sample according to the kit instructions, 250 ng of template DNA were measured using a GeneQuant pro (GE Health Care Life Sciences, Piscataway, NJ).

The PCR amplifications were conducted using bacteria-specific PCR primers to conserved regions flanking the variable V3 region of 16S rDNA genes. Primers [50 pmol of each per reaction mixture; primer 2 and primer 3 (Integrated DNA Technologies Inc., Coralville, IA)] with a 40-bp G+C clamp (Sheffield et al., 1989; Muyzer et al., 1993) were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO), according to the kit instructions, 250 ng of template DNA from rumen digesta of individual steers, and 5% (wt/vol) acetic amide to eliminate preferential annealing (Reysenbach et al., 1992). The PCR amplifications were conducted on a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA) with the following program: 1) denaturation at 94.9°C for 2 min; 2) subsequent denaturation at 94.0°C for 1 min; 3) annealing at 67.0°C for 45 s, −0.5°C per cycle (Wawer and Muyzer, 1995); 4) extension at 72.0°C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94°C for 1 min; 7) annealing at 58.0°C for 45 s; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72.0°C for 7 min; 10) 4.0°C final.

Denaturing gradient gel electrophoresis was run according to the method of Muyzer et al. (1993). Polyacrylamide gels (8% [vol/vol] acrylamide-bisacrylamide ratio 37.5:1; BioRad Lab., Richmond, CA) were cast with a 35 to 60% urea, deionized formamide (USA Amersham Life Sciences, Cleveland, OH) gradient; 100% denaturing acrylamide was 7 M urea and 40% deionized formamide. Amplified samples were mixed with an equal volume of 2× loading buffer [0.05% (wt/vol) bromophenol blue; 100 mg/mL xylene cyanol] and heated at 95°C for 5 min. Loading buffer was added to 7 M urea in 40% formamide. The samples were loaded on a gel and electrophoresed for 7–9 h at 4°C. The gels were stained with SYBR Green I and the stained bands were visualized using a Typhoon 9200 scanner (Amersham, Piscataway, NJ). The bands were quantified using the ImageQuant software (GE Healthcare, Piscataway, NJ).
Table 1. Gas production and specific growth rate by predominantly proteolytic (3 strains) and cellulolytic (3 strains) rumen bacterial pure culture strains and in simple mixed cultures with *Methanobacterium smithii*

<table>
<thead>
<tr>
<th>Item</th>
<th>Total gas production</th>
<th>Gas composition, μM/mL</th>
<th>Specific growth rate 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL/24 h SEM</td>
<td>H₂</td>
<td>CH₄</td>
</tr>
<tr>
<td><strong>Proteolytic/methanogenic bacterial strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus bovis</em></td>
<td>10.0b 0.11</td>
<td>0.01c</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Prevotella ruminicola</em></td>
<td>3.0d 0.10</td>
<td>0.02c</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Eubacterium ruminantium</em></td>
<td>8.0c 0.10</td>
<td>0.06c</td>
<td>0.0</td>
</tr>
<tr>
<td><em>S. bovis</em> + <em>M. smithii</em></td>
<td>10.0b 0.10</td>
<td>0.01c</td>
<td>1.7b</td>
</tr>
<tr>
<td><em>P. ruminicola</em> + <em>M. smithii</em></td>
<td>4.0d 0.10</td>
<td>0.01d</td>
<td>0.7c</td>
</tr>
<tr>
<td><em>E. ruminantium</em> + <em>M. smithii</em></td>
<td>4.5d 0.50</td>
<td>0.07c</td>
<td>1.3b</td>
</tr>
<tr>
<td><strong>Cellulolitic/methanogenic bacterial strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ruminococcus albus</em></td>
<td>14.5a 0.50</td>
<td>2.09a</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Fibrobacter succinogenes S85</em></td>
<td>4.5d 0.50</td>
<td>0.01d</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Ruminococcus flavifaciens</em></td>
<td>8.5c 1.50</td>
<td>1.68b</td>
<td>0.0</td>
</tr>
<tr>
<td><em>R. albus</em> + <em>M. smithii</em></td>
<td>10.5b 0.50</td>
<td>1.94bc</td>
<td>5.0a</td>
</tr>
<tr>
<td><em>F. succinogenes S85 + M. smithii</em></td>
<td>6.0d 0.10</td>
<td>0.02c</td>
<td>0.6c</td>
</tr>
<tr>
<td><em>R. flavifaciens</em> + <em>M. smithii</em></td>
<td>6.0d 2.00</td>
<td>1.30bc</td>
<td>5.0a</td>
</tr>
<tr>
<td><strong>Methanogenic bacterial strain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. smithii</em> alone</td>
<td>2.0 0.10</td>
<td>3.08</td>
<td>1.90</td>
</tr>
</tbody>
</table>

a-dWithin a column, means without a common superscript letter differ (*P* < 0.05).

1Specific growth rate, %/h = [(log Z − log Z₀)/(log Z₀)(t − t₀)] × 100, where Z and Z₀ correspond to the growth rate of rumen bacteria at times t (maximum) and t₀ (time 0, start time), respectively.

*M. smithii* was grown under an oxygen-free H₂-CO₂ (50:50, vol/vol) gas mixture, and all other strains were grown under an oxygen-free CO₂ in 10 mL of basal medium in a Hungate tube. All strains were grown anaerobically in a plant protein medium that contained soluble protein (3.27% N) extracted from winter wheat forage as the sole nitrogen source.

nol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol, and 4 μL were placed in each sample well (16-well comb). Gels were placed in a DeCode Universal Mutation Detection System (BioRad Lab.) for electrophoresis in 0.5× TAE (20 mM Tris (pH 7.4), 10 mM sodium acetate, 0.5 M EDTA) at 59°C for 17 h at 60 V. Gels were stained with SYBR Green I (1:10,000 dilution; Sigma), and the fragment-pattern relatedness was determined with Molecular Analysis Fingerprinting Software, Version 1.6 (BioRad Lab., Hercules, CA) based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages for clustering.

The Dice coefficient (values between 0 and 1) is an arithmetic determination of the degree to which banding patterns are alike (i.e., contain the same bands). Clusters (groups) are determined by sequentially comparing the patterns and the construction of a relatedness tree (dendrogram) reflecting the relative similarities. The amount of similarity is reflected by the relative closeness or grouping and is indicated by the percentage similarity coefficient bar located above each dendrogram.

**Laboratory Measurements**

The VFA concentrations were determined by gas chromatography (580-Gow, Mac Instr. Co., Bethlehem, PN) using the method of Hinton et al. (1990), and H₂, CO₂, and CH₄ concentrations were measured by gas chromatography as described by Allison et al. (1992) and Anderson et al. (2005). The concentration of CP in the plant extract and rumen fluid was determined using the Kjeldahl digestion procedure (AOAC, 1990). The NDF, ADF, and IVMDM of dried forage samples were determined using the Filter Bag Technique (Ankom Technology Co., Fairport, NY).

**Statistical Analysis**

Data were analyzed as a repeated-measures analysis using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Data are presented as least squares means and the associated SEM. The variables in Exp. 1 included in vitro rumen gas products, VFA, and specific growth rate. The model included bacterial strains, with replicates as the random effect. Variables in Exp. 2 included biofilm complexes, ruminal microbial biodiversity, and protein fractions associated with bloat in steers grazing winter wheat. The model included sampling day (d 0, 30, 40, and 50), BS (0 vs. 1), and associated interactions. Animals were the experimental units and were treated as a random effect.

**RESULTS**

**Experiment 1: In Vitro Ruminal Fermentation Products and Specific Growth Rate**

Bacteria strains cultured individually or in combination with *M. smithii* are given in Tables 1 and 2. In monoculture, total gas production was greatest for *R. albus* (*P* < 0.01), intermediate for *S. bovis* (*P* < 0.05), and least for *P. ruminicola, F. succinogenes, E. ruminantium*. 

Frothy bloat and rumen bacteria

Table 2. Ruminal VFA production and acetate:propionate ratios by different ruminal bacterial strains cultured in a soluble protein extract from winter wheat forage

<table>
<thead>
<tr>
<th>Strains</th>
<th>VFA composition, μM/mL</th>
<th>Acetate:propionate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic/methanogenic bacterial strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. bovis</td>
<td>18.9b</td>
<td>5.1b</td>
</tr>
<tr>
<td>Prevotella ruminicola</td>
<td>17.1b</td>
<td>6.4ab</td>
</tr>
<tr>
<td>Eubacterium ruminantium</td>
<td>23.3a</td>
<td>5.7b</td>
</tr>
<tr>
<td>S. bovis + M. smithii</td>
<td>15.5b</td>
<td>4.4b</td>
</tr>
<tr>
<td>P. ruminicola + M. smithii</td>
<td>17.0b</td>
<td>9.4a</td>
</tr>
<tr>
<td>E. ruminantium + M. smithii</td>
<td>18.8b</td>
<td>4.4b</td>
</tr>
<tr>
<td>Cellulolytic/methanogenic bacterial strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruminococcus albus</td>
<td>27.4a</td>
<td>5.2b</td>
</tr>
<tr>
<td>Fibrobacter succinogenes S85</td>
<td>23.7a</td>
<td>6.4ab</td>
</tr>
<tr>
<td>Ruminococcus flavefaciens</td>
<td>16.2b</td>
<td>4.3b</td>
</tr>
<tr>
<td>R. albus + M. smithii</td>
<td>25.1a</td>
<td>4.5b</td>
</tr>
<tr>
<td>F. succinogenes S85 + M. smithii</td>
<td>11.2b</td>
<td>3.4b</td>
</tr>
<tr>
<td>R. flavefaciens + M. smithii</td>
<td>23.2a</td>
<td>4.8b</td>
</tr>
<tr>
<td>Methanogenic bacterial strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. smithii alone</td>
<td>13.0</td>
<td>4.1</td>
</tr>
<tr>
<td>SEM</td>
<td>3.63</td>
<td>1.14</td>
</tr>
</tbody>
</table>

*Within a column, means without a common superscript letter differ (P < 0.05).

1M. smithii was grown under an oxygen-free H2-CO2 (50:50, vol/vol) gas mixture, and all other strains were grown under an oxygen-free CO2 in 10 mL of basal medium in a Hungate tube.

nantium, and R. flavefaciens among strains during 24-h rumen incubation. In mixed culture with M. smithii, total gas production was also greater (P < 0.01) for R. albus and S. bovis than for other bacterial strains. Growth in the presence of the methanogenic bacteria resulted in a considerable shift in fermentation gas products. As expected, R. albus (P < 0.01) and R. flavefaciens (P < 0.05) produced the most H2 among strains and supported greater (P < 0.01) CH4 production when cocultured with M. smithii than other coculture combinations.

The specific growth rate was fastest for S. bovis (P < 0.01), intermediate for E. ruminantium and R. albus (P < 0.05), and slowest for P. ruminicola, F. succinogenes, and R. flavefaciens. In the mixed culture, the specific growth rate of P. ruminicola and F. succinogenes strains increased when cocultured with M. smithii (Table 1). In the absence of an exogenous supply of H2, the M. smithii was incapable of growth as a monoculture in the plant protein medium (data not shown).

When wheat soluble protein was added as a major fermentation substrate, E. ruminantium, R. albus, F. succinogenes, and R. flavefaciens + M. smithii produced more (P < 0.05) acetate than other strains and cocultures (Table 2). Cultures of P. ruminicola, E. ruminantium, and F. succinogenes produced more (P < 0.05) butyrate than any of the respective monocultures. Propionate production among strains was similar. When cocultured with M. smithii, E. ruminantium and F. succinogenes exhibited reduced (P < 0.05) acetate production compared with monocultures, indicating that methane was produced by methanogenesis from acetate. However, the mixed culture of R. flavefaciens and M. smithii produced more (P < 0.05) acetate than did the monoculture of R. flavefaciens (Tables 1 and 2). The acetate to propionate ratio was greater for R. albus and R. albus + M. smithii than for other respective mono or simple cocultures with M. smithii (Table 2).

Bio-Film Complexes and Genomic DNA Concentrations

In vitro production of biofilm complexes varied among bacterial strains (Table 3) and animal diets in vivo (Table 4). Biofilm production was greatest (P < 0.01) for S. bovis, intermediate (P < 0.05) for R. albus and F. succinogenes, and lowest for P. ruminicola, E. ruminantium, R. flavefaciens, and M. smithii (Table 3). In mixed cultures with M. smithii, biofilm production varied among strains. Culture of E. ruminantium with M. smithii was the only mixed culture to produce more (P < 0.05) biofilm than the respective monoculture did.

In Exp. 2, when diets changed from bermudagrass hay (d 0) to wheat forage, production of ruminal biofilm complexes increased (P < 0.01; Table 4). There was no difference in biofilm production between bloated and nonbloated steers grazed at d 40 of wheat grazing. Biofilm production, however, was greater (P < 0.05) on d 50 in steers that subsequently bloated than in those that did not, with BS by grazing date interactions (P < 0.03). There was no difference in rumen DNA concentrations between bloated and nonbloated steers on d 50. Average DNA concentrations were lower on d 30 than 50 (Table 4).

Experiment 2: Chemical Compositions of Forages and Rumen Fluid

The chemical concentrations of CP, NDF, ADF, and IVDMD in bermudagrass hay were 12, 61, 32, and 67%
Table 3. Mass of ethanol-precipitable biofilm complexes from different ruminal bacterial strains cultured in a soluble protein extract from winter wheat forage

<table>
<thead>
<tr>
<th>Strains</th>
<th>Bio-film complex, mg of DM/mL</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. bovis</em></td>
<td>2.9a</td>
<td>0.66</td>
</tr>
<tr>
<td><em>Prevotella ruminicola</em></td>
<td>0.4c</td>
<td>0.12</td>
</tr>
<tr>
<td><em>Eubacterium ruminantium</em></td>
<td>0.3c</td>
<td>0.19</td>
</tr>
<tr>
<td><em>S. bovis</em> + <em>M. smithii</em></td>
<td>0.5c</td>
<td>0.09</td>
</tr>
<tr>
<td><em>P. ruminicola</em> + <em>M. smithii</em></td>
<td>0.4c</td>
<td>0.12</td>
</tr>
<tr>
<td><em>E. ruminantium</em> + <em>M. smithii</em></td>
<td>2.0a</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Cellulolytic/methanogenic bacterial strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Bio-film complex, mg of DM/mL</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ruminococcus albus</em></td>
<td>0.9b</td>
<td>0.16</td>
</tr>
<tr>
<td><em>Fibrobacter succinogenes</em> S85</td>
<td>0.7b</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Ruminococcus flavefaciens</em></td>
<td>0.4c</td>
<td>0.13</td>
</tr>
<tr>
<td><em>R. albus</em> + <em>M. smithii</em></td>
<td>0.7b</td>
<td>0.21</td>
</tr>
<tr>
<td><em>F. succinogenes</em> S85 + <em>M. smithii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. flavefaciens</em> + <em>M. smithii</em></td>
<td>0.3c</td>
<td>0.10</td>
</tr>
<tr>
<td>Methanogenic bacterial strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. smithii</em> alone</td>
<td>0.5</td>
<td>0.06</td>
</tr>
</tbody>
</table>

a–cWithin a column, means without a common superscript letter differ (P < 0.05).

1M. smithii was grown under an oxygen-free H2-CO2 (50:50, vol/vol) gas mixture, and all other strains were grown under an oxygen-free CO2 in 10 mL of basal medium in a Hungate tube.

DM, respectively (data not shown). In the winter wheat forage, forage chemical compositions of CP, NDF, ADF, and IVDMD were 28, 44, 29, and 89% DM, respectively (data not shown).

The 5 ruminal protein fractions assayed differed between sampling day and presence of bloat (Table 5). When diets were changed from bermudagrass hay to wheat forage d 0 through 50, ruminal microbial protein fractions (whole ruminal content, particulate matter, cheese-cloth filtrate, and protozoa and plant particle fractions) increased (P < 0.01). When steers were grazing on wheat forage, particulate matter, and bacterial protein fractions were similar between the bloated and nonbloated animals (Table 5). However, whole ruminal protein content (P < 0.01) and cheese-cloth filtrate protein (P < 0.05) fractions in the rumen were greater for bloated than for nonbloated animals on d 50, suggesting that frothy bloat was related to changes in the total and soluble protein rumen pools.

Ruminal Bacterial Diversity in Steers Grazing Wheat Forage

The V3 region of 16S rDNA from the total DNA isolated from the rumen of steers grazing winter wheat forage was PCR amplified using primers with G+C-clamps and the resulting products were separated on DGGE gels (Figure 1). Amplicon profiles for ruminal bacterial samples from steers 1, 2, 3, 4, 5, and 6 on d 30 varied from 67 to 87% similarity among animals (Figure 1). Cluster analysis of DGGE bands between d 30 and 50 showed that there were low (<83%) similarities between sample periods (data not shown).

To further understand the effect of bloat on ruminal microbial diversity, ruminal fluid was collected and analyzed on d 50 from bloated and nonbloated animals. Molecular analysis of the 16S rDNA revealed 2 distinct bacterial clusters were present in ruminal fluid from bloated and nonbloated animals (Figure 1). The nonbloated group clustered from 90 to 97% similarity, with greater proportions of high-G+C-containing bacterial strains and a few low-G+C-containing strains than

Table 4. Effect of day and bloat score (score) on mass of ethanol-precipitable biofilm complexes and DNA concentration in rumen fluid obtained from ruminally cannulated steers grazing winter wheat forage

<table>
<thead>
<tr>
<th>Item</th>
<th>Score2</th>
<th>Bio-film complex, mg of DM/mL</th>
<th>SEM</th>
<th>DNA3, ng/μL</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 0 (Dec. 10, 2004)</td>
<td>0 (n = 6)</td>
<td>2.2a</td>
<td>0.14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d 30 (Jan. 10, 2005)</td>
<td>0 (n = 6)</td>
<td>3.7b</td>
<td>0.44</td>
<td>30.7b</td>
<td>4.92</td>
</tr>
<tr>
<td>d 40 (Jan. 20, 2005)</td>
<td>0 (n = 3)</td>
<td>3.9b</td>
<td>0.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1 (n = 3)</td>
<td>3.4a</td>
<td>0.64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d 50 (Jan. 30, 2005)</td>
<td>0 (n = 3)</td>
<td>3.3b</td>
<td>0.25</td>
<td>61.4a</td>
<td>6.84</td>
</tr>
<tr>
<td></td>
<td>1 (n = 3)</td>
<td>4.8a</td>
<td>0.75</td>
<td>63.5a</td>
<td>6.84</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>0.009</td>
</tr>
<tr>
<td>Score</td>
<td>0.03</td>
</tr>
<tr>
<td>Day × score</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 5. Effect of day and bloat score (score) on the ruminal fluid microbial protein fractions obtained from ruminally cannulated steers grazing winter wheat forage

<table>
<thead>
<tr>
<th>Item</th>
<th>d 0</th>
<th>d 40</th>
<th>d 50</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score^2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Day Score Day x Score</td>
</tr>
<tr>
<td>Number of steers</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ruminal microbial protein fraction, g/kg of DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole rumen content</td>
<td>90.6^c</td>
<td>187.0^b</td>
<td>204.6^b</td>
<td>187.3^b 239.8^a</td>
</tr>
<tr>
<td>Particulate matter</td>
<td>80.6^a</td>
<td>128.7^a</td>
<td>137.2^a</td>
<td>113.2^b 127.0^ab</td>
</tr>
<tr>
<td>Cheesecloth filtrate</td>
<td>9.9^b</td>
<td>58.3^b</td>
<td>67.4^c</td>
<td>74.2^b 113.2^b</td>
</tr>
<tr>
<td>Protozoa + plant particle, mg/mL</td>
<td>17.9^c</td>
<td>63.9^a</td>
<td>38.7^b</td>
<td>67.8^a 76.7^a</td>
</tr>
<tr>
<td>Supernatant, mg/mL</td>
<td>8.3^b</td>
<td>8.7^b</td>
<td>14.2^a</td>
<td>14.0^a 14.7^a</td>
</tr>
<tr>
<td>Bacterial, mg/mL</td>
<td>5.5</td>
<td>7.4</td>
<td>5.8</td>
<td>6.0 5.5</td>
</tr>
<tr>
<td>SEM</td>
<td>0.59</td>
<td>0.84</td>
<td>0.84</td>
<td>0.84 — — —</td>
</tr>
</tbody>
</table>

^a–cWithin a row, means without a common superscript letter differ (P < 0.05).

1Ruminally cannulated steers were used for the collection of rumen fluid on d 0 (bermudagrass hay), 40, and 50 while grazing winter wheat forage.

2Bloat scores consist of 0 = no visible signs of bloat, 1 = slight distention of left side.

found in bloated animals. Mid-G+C-containing strains were similar between bloated and nonbloated animals. Overall, the bloated group exhibited less similarity among animals than nonbloated group and clustered from 86 to 89% similarity. The data collectively suggest that ruminal bacterial populations changed when

Figure 1. Denaturing gradient gel electrophoresis of rumen bacterial 16S rDNA amplicons from steers grazing winter wheat forage on d 30 and 50 (bloated and nonbloated steers are indicated). Scaled bars above figures indicate percentage similarity coefficients. G+C = guanosine + cytosine.
steers experienced frothy bloat while grazing wheat forage.

DISCUSSION

The principal objectives of the current study were to determine how wheat forage diets affected bacterial specific growth rate, fermentation end products, polysaccharide biofilm complexes, and ruminal microbial populations associated with frothy bloat. Plant soluble protein in wheat forage is known to be rapidly digested by rumen microorganisms and is implicated as a frothy bloat precursor (Min et al., 2005b). In the current study, soluble plant protein extract from wheat forage was chosen as a suitable protein substrate for detailed studies on the potential role of microbial activities associated with frothy bloat.

When cultured in vitro with soluble protein extract from wheat forage, *S. bovis* exhibited the greatest specific growth rate and produced more biofilm than other bacteria monocultures or simple cocultures with *M. smithii*, suggesting *S. bovis* is a high producer of biofilm in cattle grazing wheat forage. In vivo whole rumen protein content, cheesecloth filtrate protein fraction, and biofilm complexes were greater in rumen fluid from bloated steers than nonbloated steers. Two distinct ruminal bacterial populations developed between bloated and nonbloated steers. Therefore, frothy bloat appears to be associated with specific changes in microbial populations in the rumen when steers graze wheat forage.

**Experiment 1: Interaction and Growth Rate of 6 Rumen Bacterial Strains**

The main sources of ruminal gasses are from microbial fermentation and acidification of bicarbonate; the major components of ruminal gases are CO₂ (45 to 70%) and CH₄ (20 to 30%), with N₂, O₂, H₂, and H₂S as minor components (Clarke and Reid, 1974). Results from the present experiment show that *R. albus* and *R. flavaeaciens* produced the most H₂ among strains and supported production of CH₄ when cocultured with *M. smithii* utilizing the H₂ to reduce CO₂ to CH₄ (Latham and Wolin, 1977) which is also consistent with reports by Miller and Wolin (1973) and Wolin et al. (1997). However, our understanding of physical and chemical associations between these gases, ruminal microorganisms, and frothy bloat interactions is not clear, and further studies are required on ruminal gases-microorganisms-frothy bloat interactions.

*Streptococcus bovis* is one of the dominant microbes in the rumen, especially in animals receiving either high soluble carbohydrate diets (Dehority, 2003) or high quality fresh forage diet (Attwood and Reilly, 1996). Attwood and Reilly (1995) reported that 61% of strains isolated from cattle grazing perennial ryegrass/white clover pasture were *S. bovis*-like strains, whereas *E. budayi*, *B. fibrisolvens*, and *P. ruminicola*-like strains made up to 23, 7, and 2% of the isolated strains, respectively. In the current study, the specific growth rate was greatest for *S. bovis* among tested strains when cultures were grown with wheat soluble protein, indicating that *S. bovis* was capable of proliferating on the wheat soluble protein substrate. The role of competitive interactions among ruminal bacterial species in the frothy bloat complex is unknown at this time.

**Experiment 2: Bloat Potential, BioFilm Complexes, and Rumen Microbial Biodiversity**

Previous research reported that the ethanol-precipitable biofilm fractions from ruminal digesta have been shown to increase during the onset of bloat in steers fed either a high grain ration (Gutierrez et al., 1959) or in cattle grazing Ladino clover forage (Gutierrez et al., 1963). In the present experiment, polysaccharide biofilm production was greatest for *S. bovis* in monoculture in plant protein medium extracted from wheat forage. This is consistent with findings of Cheng et al. (1973, 1976). A probable source of biofilm precursors are cytoplasmic granules of reserve polysaccharides that frequently occur in rumen bacteria such as *S. bovis* (Cheng et al., 1976), *R. albus* (Cheng et al., 1977), *Selenomonas ruminantium* (Wallace, 1980), *M. elsdenii* (Brown et al., 1975), and mixed rumen bacterial cells from the rumen of cattle fed a high-energy diet (Cheng et al., 1973, 1976).

In vivo, biofilm production increased when steers were switched from a bermudagrass hay diet to grazing wheat forage. There was 31% increase in biofilm production during the onset of bloat at d 50 in steers grazing wheat forage. Further studies are necessary before one can accurately assess that susceptibility to bloat is linked to microbial interactions that lead to unique production of biofilm complexes.

Differences in steer rumen microbial populations were observed among animals and between bloated and nonbloated animals. The 16S rDNA DGGE technique allowed visualization of microbial diversity patterns in the rumen of steers related to frothy bloat, while not being subject to selective pressures inherent to conventional medium-based culturing techniques (Torsvik et al., 1990; Ward et al., 1990). However, one limitation of 16S gene amplification is that several products of varied G+C content and primary sequences may comigrate in the denaturing gradient, making classification of some changes in individual microbial populations difficult and potentially resulting in an erroneous indication of assortment and abundance (Wintzingerode et al., 1997). Another restriction is that the PCR products apparent on the stained gels are representative of the most abundant bacteria in the population (Muyzer et al., 1993; Murray et al., 1996).

The in vivo study reported is the first to document differences in rumen microbial diversity patterns between bloated and nonbloated steers grazing wheat forage and suggests that bloat might be associated with specific changes in predominant microflora in the reti-
culorumen. The changes observed in rumen bacterial species are associated with differences in G+C-containing strains between bloated and nonbloated steers and implicates a significant microbial populations correlation with frothy bloat (Fletcher and Hafez, 1960). Recently, molecular diversity of rumen bacteria has been shown to be dominated by bacteria belonging to essentially 4 phyla of low-G+C Gram positive bacteria (52 to 54%), Bacteroides types (Cytophaga Flexibacter-Bacteroides; 38 to 40%), Proteobacteria (4.7%), and Spirochaetes (2.4%; Tajima et al., 1999; Edwards et al., 2004). Further isolation and phenotypic characterization are necessary for identification of individual bacterial strains associated with bloat. In companion research quantifying ingestive grazing behavior patterns associated with bloat, Pinchak and Min (2005), found that bloated steers exhibited altered grazing activities compared with nonbloated cohorts (idling time +28%, ruminating bouts −15%, and jaw movements −20%). These alterations in grazing behavior might modify meal patterns and thereby alter frequency and quantity of fermentable substrate addition to the reticulorumen and as a result affect bacterial population growth patterns and diversity and rumen pH.

The rumen microbiota is highly responsive to changes in diet, age, feed additives, health of the host animals, and season (Ogimoto and Imai, 1981; Stewart and Bryant, 1988; Min et al., 2002). In relation to diet, corn-fed animals displayed more diverse bacterial populations than grass-legume hay diets, which were mostly contributed by Bacteroides-related phylotypes (Kocherginskaya et al., 2001). Findings from our current study show that ruminal bacterial populations from within nonbloated steers grazing wheat forage (d 30) varied from 67 to 87% similarity among animals, indicating that ruminal microbial populations were heterogeneous among study animals. This is similar to reports by Kocherginskaya et al. (2001).

This study has shown that wheat pasture bloat could be related to increased production of biofilm resulting from a switch in the rumen bacterial population resulting from changes in diet composition. Consumption of wheat forage increased biofilm production over time. Experiment 1 clearly shows that some rumen microbial strains produced more biofilm than others. Further research is required to define what kind of microorganisms (bacteria, protozoa, and fungi) promote and produce biofilm in the rumen of cattle grazing wheat pasture and the relationships of these microorganisms to seasonal patterns, forage chemical composition, DMI, and grazing behavior.

LITERATURE CITED


Kocherginskaya, S. A., R. A. Aminov, and B. A. White. 2001. Analysis of the rumen bacterial diversity under two different diet condi-