

Effect of Diet on Populations of Three Species of Ruminal Cellulolytic Bacteria in Lactating Dairy Cows¹

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ABSTRACT

The effects of four contrasting diets were determined on populations of three species of ruminal cellulolytic bacteria (*Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes*) using oligonucleotide probes to rRNA. Diets based on alfalfa silage or corn silage as the primary fiber source were formulated to contain either 24 or 32% neutral detergent fiber measured after α -amylase treatment. The diets were fed twice daily to four ruminally fistulated, lactating Holstein cows in a trial using a Latin square design. The cows fed the alfalfa silage diets had higher dry matter intakes and milk production and smaller pH fluctuations than did cows fed the corn silage diets (0.3 vs. 0.8 units). The total populations of the three cellulolytic species at 3 h after feeding ranged from 0.3 to 3.9% of the bacterial domain; *R. albus* was generally the most abundant of the three species. The data are in general agreement with population assessments obtained by some traditional methods of culture enumeration. Although diet and individual cows had major effects on ruminal pH and volatile fatty acid concentrations and on milk production and composition, differences in cellulolytic populations that were attributable to individual cows were larger than those attributable to diet, suggesting that each cow maintained a unique assemblage of cellulolytic species.

(**Key words:** cellulolytic, microbial populations, ribonucleic acid probes, ruminal bacteria)

Abbreviation key: aNDF = NDF determined after α -amylase treatment, A:P = ratio of acetate to propionate, AS = alfalfa silage, CS = corn silage, SRF = strained ruminal fluid.

INTRODUCTION

The ruminal microbiota (bacteria, protozoa, and fungi) form the key link between the dairy animal and diet. Volatile fatty acids and microbial protein from the degradation of feed constituents account for many of the nutrients that are utilized by the host animal, and these nutrients arise from the diverse microbiota present in the rumen (17). Each microbial species possesses a unique combination of characteristics, including substrates utilized, types and ratios of fermentation products, and growth yield (17, 43). The proportions of fermentation end products affect both milk composition and the efficiency with which feeds are utilized for production (3). As a result, the relative proportions of various nutrients that are available for absorption by the animal can be expected to change according to the population sizes and activities of individual microbial species, and these changes, in turn, can affect animal production.

Although the dependence of the ruminant on its ruminal microbiota is well known, the association of specific microorganisms with diet and production has remained elusive because of difficulties in the enumeration of individual microbial species. The attachment of microbes to feed particles (7, 13) and the morphological and physiological similarities among related microbial species (6, 17) have confounded quantitation based on microscopic or culture methods. Over the last decade, the development of oligonucleotide probes for species-specific segments of 16S rRNA has provided an unprecedented opportunity to characterize and quantify microbial populations in situ and has revolutionized the field of microbial ecology (2, 26, 35). Stahl et al. (36) were the first to apply these probes to ruminal microbes and characterized the population of *Fibrobacter succinogenes* in the rumen of a single cow over time. Briesacher et al. (4) subsequently reported that the populations of *F. succinogenes* in the rumens of three steers fed orchard-grass accounted for a fairly constant fraction (~6%) of the ruminal bacterial population at several times after feeding; May et al. (19) found a lower proportion (1.5 to 2.5%) in mixed ruminal microbes that fer-

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mented orchardgrass in vitro. Oligonucleotide probe techniques have also been used to demonstrate that another fibrolytic bacterium, *Butyrivibrio fibrisolvens*, is not an abundant member of the bovine ruminal population (14); to characterize competition among ruminal cellulolytic bacteria in a defined coculture (22, 23, 32, 33); and to examine obligate amino acid-fermenting bacteria in chemostats of mixed ruminal microflora (18). Those studies have improved our understanding of the interactions among ruminal bacteria but have not addressed the relationships among microbial populations in vivo or their relationship to diet, feed digestion, and animal production.

Our study was undertaken to determine the relationships among populations of the three predominant culturable species of cellulolytic bacteria (*F. succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*) in the rumens of lactating cows fed diets that differed substantially in the amount and source of forage. Cellulolytic bacteria were chosen for study because of their importance in fiber digestion (17, 43), their characteristic differences in fermentation end products (43), and the availability of well-characterized, species-specific oligonucleotide probes (22, 26). The experimental design emphasized the inherent variability of response to dietary differences in individual cows rather than in populations of cows in terms of microbial populations, ruminal chemistry, digestion kinetics, and production. This manuscript defines the cellulolytic populations, ruminal chemistry (pH and VFA profiles), and milk production of four cows fed four diets (16 combinations of cow and diet). The same combinations of cow and diet were used concurrently for a study of digestion kinetics (20).

MATERIALS AND METHODS

Experimental Design

Four multiparous Holstein cows (BW, 649 to 681 kg; DIM, 64 to 186) that were ruminally fistulated according to University of Wisconsin animal care and use guidelines were housed indoors for the 16-wk trial. The trial was a 4 × 4 Latin square design with a 2 × 2 factorial arrangement of dietary treatments. The diets (Table 1) contained alfalfa silage (**AS**) or corn silage (**CS**) as the principal fiber source at 24 or 32% **aNDF** (NDF determined after α -amylase treatment) in the DM. The diets were formulated to exceed NRC (21) CP requirements, and P, Ca, Mg, and Na were provided to meet requirements (21). The diets were offered for ad libitum intake at 12-h intervals (at 0730 and 1930 h) to obtain >10% orts. Orts

TABLE 1. Composition of diets that varied in source of forage and concentration of aNDF.¹

Composition	Diet ²			
	AS24	AS32	CS24	CS32
	(% of DM)			
Ingredient				
Alfalfa silage (AS)	41.60	65.47
Corn silage (CS)	44.65	72.42
Corn, coarsely ground	46.29	30.65	31.16	2.20
Soybean meal (44% CP)	10.59	2.83	21.54	22.99
Calcium carbonate	0.31	...	1.33	1.14
Dicalcium phosphate	0.59	0.12	0.48	0.53
Monosodium phosphate	...	0.62
Dynamate ³	0.07	...	0.30	0.18
Salt and trace minerals ⁴	0.47	0.26	0.48	0.48
Vitamin A, D, and E premix ⁵	0.02	0.02	0.02	0.02
Magnesium oxide	0.02	...	0.04	0.03
Chemical DM ⁶	54.9	44.7	53.5	42.5
	(% of DM)			
Ash	7.8	9.2	7.1	7.6
CP	18.5	18.3	17.6	17.5
aNDF	22.9	30.2	23.0	31.5
ADF	16.2	22.9	13.2	19.1
NE _L (calculated), Mcal/kg of DM	1.71	1.60	1.76	1.68

¹aNDF = NDF measured after α -amylase treatment.

²AS24 = AS at 24% aNDF, AS32 = AS at 32% aNDF, CS24 = CS at 24% aNDF, and CS32 = CS at 32% aNDF.

³Pitman Moore, Inc. (Mundelein, IL).

⁴Composition: 0.35% Zn, 0.34% Fe, 0.20% Mg, 0.033% Cu, 0.007% I, and 0.005% Co.

⁵Composition: 1,600,000 IU of vitamin A/kg, 300,000 IU of vitamin D/kg, and 300 IU of vitamin E/kg.

⁶Percentage of undried DM.

and DMI were determined daily as was milk volume from twice daily milkings. Cows were introduced to the new diets by mixing the old and new diets at 50:50 (wt/wt) on d 1, at 30:70 (wt/wt) on d 2, at 15:85 (wt/wt) on d 3, and at 0:100 (wt/wt) on d 4. The experimental design permitted adaptation of the ruminal microflora to the diets for 3 wk before ruminal sampling (d 24 to 28) and milk analysis (d 26 to 28). One cow had to be removed from the trial midway through the last week of the last period because of udder trauma and, consequently, mastitis. In this case, samples for ruminal chemistry and microbial populations were collected during d 24 to 26 only, and no milk was collected for analysis.

Ruminal Sampling

The trial incorporated extensive sampling of digesta for determination of feed digestion kinetics as

well as the ruminal parameters and microbial populations presented here. During each sampling period, ruminal contents were collected at 0730 h (prefeeding) on d 24 and at 2, 3, 6, 9, 12, 15, 18, 24, 27, 36, 39, 48, 96, and 99 h thereafter. The VFA and pH analyses were performed on all samples. Because of the labor-intensive nature of the RNA analyses, microbial populations were analyzed only in samples collected 3 h postfeeding (3, 15, 27, 39, and 99 h after the prefeeding sample was taken at 0730 h on d 24); these samples provided a greater number of replicates (five time points) than did any other postfeeding times.

Samples of ruminal contents were obtained by the same two operators throughout the trial after thorough hand mixing and consisted of several handfuls of solids (~300 g) and ~600 ml of ruminal fluid taken approximately 20 to 25 cm ventro-medial to the fistula. The samples were placed in sealed, insulated bottles for ~3 min during transport to the laboratory; the top 3 cm of the sample were discarded, and the pH of the remaining sample was measured. The digesta were then squeezed through four layers of cheesecloth, and a portion of strained ruminal fluid (**SRF**) was acidified (10 ml of SRF into vials containing 0.20 ml of concentrated sulfuric acid) for analysis of VFA and lactate. An additional 35 ml of SRF and ~50 g of squeezed, tightly packed solids were stored separately in 50-ml polypropylene tubes at -70°C for bacterial analysis using species-specific RNA probes.

Analyses of Ruminal Fluid and Milk

Samples for analysis of VFA and lactate were prepared in duplicate by a modification of the method of Siegfried et al. (34). Six hundred microliters of acidified SRF were combined with 600 μ l of calcium hydroxide solution and 300 μ l of copper sulfate solution (containing crotonic acid as internal standard) and then frozen. These samples were thawed and centrifuged at 12,500 $\times g$ for 10 min, and the supernatants were transferred to tubes containing 28 μ l of concentrated sulfuric acid. These suspensions were frozen and thawed twice and then centrifuged as was previously described; the clear supernatant was analyzed for VFA and lactate by the HPLC method of Weimer et al. (45). Only the major VFA (acetate, propionate, and butyrate) were quantified. Ammonia and total amino acids in the ruminal fluid were determined by the method of Broderick and Kang (5). Milk fat and milk protein were determined by the DHIA.

Microbial Populations

Populations of the three predominant culturable species of ruminal cellulolytic bacteria (*F. succinogenes*, *R. flavefaciens*, and *R. albus*) were determined using species-specific probes to 16S rRNA. All glassware was heated to 160°C overnight prior to use; plasticware was either new or was treated prior to use with RNase-Away (Molecular Bio-Products, San Diego, CA). All reagents were prepared using water that had been previously treated with diethylpyrocarbonate to destroy RNases.

Frozen ruminal solids (25 g of squeezed solids) and SRF (25 ml) were placed in a chilled 1-L blender jar with 50 ml of ice-cold saline (0.9% sodium chloride) that was used to rinse the contents of the tubes. This mixture was blended for 2 min at low speed in a commercial blender (Waring Div, Dynamics Corp. of America, New Hartford, CT) at 4°C. The homogenate was transferred to two 50-ml Oak Ridge tubes (Nalge Nunc, Milwaukee, WI) for centrifugation at 500 $\times g$ for 15 min at 4°C. The liquid phase was decanted to a single, ice-cold Oak Ridge tube. The residual solids were resuspended in 25 ml of chilled saline, vortexed, and then centrifuged as described previously. This supernatant was pooled with the first supernatant, filtered through glass wool, and centrifuged at 10,000 $\times g$ for 25 min at 4°C. The resulting microbial cell pellets were resuspended in 1 ml of water and stored at -80°C.

The RNA was isolated from thawed cell suspensions. Two 500- μ l amounts were transferred to 2-ml screw-cap microcentrifuge tubes along with 0.40 ml of 50 mM sodium acetate, 10 mM EDTA (pH 5.1) solution, 0.5 g of zirconium beads, 0.05 ml of 20% SDS, and 0.7 ml of saturated phenol (pH 4.3; AM-RESCO, Solon, OH). This suspension was shaken in an eight-place bead beater for 2 min at 4°C. The suspension was incubated to 60 to 65°C for 10 min, was beaten for an additional 2 min, and then centrifuged at 12,000 $\times g$ for 5 min. The supernatant was extracted twice with 0.50 ml of the following sequence of reagents: saturated phenol (pH 4.3); phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol; Sigma Chemical Co., St. Louis, MO); phenol:chloroform (1:1, vol/vol); and chloroform. Purified RNA was precipitated and stored as described by Shi et al. (32).

Hybridizations were conducted using oligonucleotide probes labeled with digoxigenin (National Biosciences, Plymouth, MN): S-Ss-F.s.suc-0207-a-A-21 for *F. succinogenes* (*Bacteroides succinogenes* S85 and A3c), S-S-R fla-1176-a-A-17 (RFL 1176) for *R. flavefaciens*, and S-S-R.alb-0196-a-A-18 for *R. albus* (RAL196) (26). Hybridizations were conducted as

TABLE 2. Mean values for intake and production for four cows fed diets that varied in source of forage and concentration of fiber.

	Diet ¹				Forage		aNDF ²		Cow no.				Pooled SE
	AS24	AS32	CS24	CS32	AS	CS	24%	32%	749	2661	3691	3807	
DMI, kg/d	24.5 ^a	21.8 ^{ab}	22.3 ^{ab}	20.0 ^b	23.1 ^a	21.2 ^b	23.4 ^a	20.9 ^b	22.3 ^{ab}	24.3 ^a	21.5 ^b	20.5 ^b	1.3
Milk, kg/d	34.4 ^a	26.7 ^b	27.3 ^b	27.2 ^b	30.8 ^a	27.2 ^a	31.2 ^a	26.9 ^a	27.8 ^{ab}	32.2 ^a	25.3 ^b	30.2 ^a	2.2
4% FCM, kg/d	30.6 ^a	26.7 ^{ab}	23.5 ^b	25.1 ^{ab}	29.1 ^a	24.3 ^b	27.3 ^a	25.9 ^a	26.3 ^{ab}	30.6 ^a	24.0 ^b	24.8 ^b	2.9
Milk fat, %	3.39 ^{bc}	3.99 ^a	3.11 ^c	3.50 ^b	3.68 ^a	3.30 ^b	3.17 ^b	3.74 ^a	3.81 ^a	2.84 ^b	3.74 ^a	3.60 ^a	0.23
Milk protein, %	3.40 ^b	3.44 ^b	3.74 ^a	3.50 ^b	3.46 ^a	3.62 ^a	3.62 ^a	3.47 ^a	3.30 ^b	3.65 ^a	3.46 ^{ab}	3.67 ^a	0.12

^{a,b,c}Means within the same group (diet, forage, aNDF, or cow) with different superscripts differ ($P < 0.05$).

¹AS24 = Alfalfa silage (AS) at 24% aNDF, AS32 = AS at 32% aNDF, CS24 = corn silage (CS) at 24% aNDF, and CS32 = CS at 32% aNDF.

²NDF determined after α -amylase treatment.

described by Shi et al. (32) with the following modifications: 1) hybridizations were carried out on Nytran[®] membranes (Schleicher & Schuell, Keene, NH); 2) 100 μ l (~100 to 2000 ng) of isolated RNA solution were used for each slot; 3) a heat shock of 65°C for 15 min was used after RNA denaturation; 4) after crosslinking RNA to the membrane, prehybridization was conducted in Rapid-Hyb buffer (Amersham, Arlington Heights, IL) at dissociation temperatures of 49°C for S-Ss-F.s.suc-0207-a-A-21, 48°C for S-S-R.alb-0196-a-A-18, and 41°C for S-S-R fla-1176-a-A-17; 5) these same temperatures were used for hybridizations; and 6) after hybridization, membranes were washed twice with a solution of 2× standard saline citrate and 0.1% SDS at room temperature and then washed twice with a solution of 0.5× standard saline citrate and 0.1% SDS at the probe-specific hybridization temperature.

Methods for detecting hybridization of the probe to the target RNA by use of the GENIUS[™] chemiluminescence system (Boehringer-Mannheim, Indianapolis, IN) and for subsequent quantitation by densitometry were those of Shi et al. (32), except that 1) membranes were washed twice with maleate buffer and once with detection buffer; 2) Lumi-film (Boehringer-Mannheim) was substituted for Kodak X-OMat-AR film (Eastman Kodak, Rochester, NY); and 3) ImageQuant[™] software (Molecular Dynamics, Sunnyvale, CA) was used to quantify the intensity of the bands on the developed film. The amounts of RNA on each densitometer image were determined from standard curves prepared with purified RNA from *F. succinogenes* S85 (for S-Ss-F.s.suc-0207-a-A-21), *R. flavefaciens* B34b (for S-S-R fla-1176-a-A-17), and *R. albus* 7 (for S-S-R.alb-0196-a-A-18). The corrected RNA values were normalized to total bacterial RNA in the samples, determined from separate hybridizations at 49°C using the bacterial domain-specific probe S-D-Bact-0338-a-A-18 [EUB338 (2)], and com-

mercial *Escherichia coli* RNA (Sigma Chemical Co.) as the standard. Three replicate RNA samples were slotted for each time point within each combination of cow and diet, and these analyses were repeated two or three times on separate membranes. The data from these samples were averaged, and these means were used to calculate a mean relative population size for all time points analyzed within a particular combination of cow and diet. Expressing the amounts of RNA contributed by each individual species as a fraction of the total bacterial RNA circumvents the well-known variation in efficiency of extraction of bulk RNA from environmental samples (35, 36).

Statistics

The main effects (diet, period, cow, forage source, and aNDF concentration) and interactions of treatments on milk variables, ruminal chemical variables, and microbial populations were determined by ANOVA using the general linear models procedure of SAS (30). Comparisons among means were performed using a paired *t* test at a probability level of 0.05. Correlations among parameters were performed using Statview 512+ (BrainPower, Calabasas, CA), and the significance of correlations was determined using the tables of Steele and Torrie (37).

RESULTS

Production Parameters and Ruminal Chemistry

The DMI and milk production data are shown in Table 2. The mean values for these parameters across all combinations of cow and diet over the 16-wk experimental period were 22.1 and 28.9 kg/d, respectively. Orts averaged $18.1 \pm 5.9\%$ of DM offered across all combinations of cow and diet, and DMI during the

sampling period (d 24 to 28) averaged $102.4 \pm 9.1\%$ of the DMI during the adaptation period (d 4 to 23).

Cows fed diets based on AS had higher DMI and higher production of FCM than did cows fed diets based on CS (Table 2), although this difference may have been due to the slightly higher protein in the AS diets. Milk production was affected ($P < 0.05$) by cow, period, diet, forage, and aNDF and displayed an unexpected interaction of forage and aNDF (Table 3). Milk fat percentage was lower for cows fed the low fiber diets (Table 2) and was also affected ($P < 0.05$) by cow, diet, and forage (Table 3). Milk protein was affected ($P < 0.05$) by cow, period, diet, and forage but not by aNDF concentration.

Ruminal pH values during the sampling period were maximal just prior to feeding and were minimal at 3 to 6 h after feeding. Diets based on AS yielded smaller fluctuations during the 12-h feeding cycle than did diets based on CS (range of means = 0.3 vs. 0.8 pH units; Table 4). Low fiber diets resulted in lower pH values at 3 h postfeeding than did the high fiber diets, but the pH prior to feeding did not vary with dietary fiber concentration. The mean values of pH at 3 h postfeeding (Table 4; Figure 1) revealed substantial differences among cows fed the same diet and among diets fed to the same cow. One cow (no. 2661) had a chronically depressed ruminal pH and a

high ruminal DM content but maintained adequate milk production, albeit with depressed milk fat.

For each dietary treatment, ruminal concentrations of individual and total VFA were relatively constant throughout the sampling period, which permitted the averaging of values of all samples within treatments. The mean values for the major VFA are shown in Figure 2. The diets based on AS resulted in higher acetate concentrations and higher acetate to propionate ratios (**A:P**) than did the diets based on CS (Table 4). The sum of acetate, propionate, and butyrate and A:P were affected by cow and aNDF via their effects on propionate concentration and by forage type via its effect on acetate concentration (Table 3). Substantial differences among cows in VFA and A:P were primarily due to differences in ruminal propionate concentration (Figure 2). Two cows (no. 2661 and no. 3807) showed considerable changes in these parameters according to diet; the other two cows showed little variation. Lactate was not detected in any of the ruminal samples at any time during the sampling periods. The mean ammonia concentrations, averaged for all time points, exceeded 9 mM for all combinations of cow and diet. Both ammonia and total amino acid concentrations were affected by cow, diet, forage, aNDF, and a forage \times aNDF interaction (Table 3).

TABLE 3. The probability of significance of main effects and interactions among treatments on ruminal and milk variables and the relative populations of ruminal cellulolytic bacteria in cows fed diets that varied in source of forage and concentration of fiber.¹

	Probability ²					
	Cow	Period	Diet	Forage	aNDF	Forage \times aNDF
DMI	0.031	0.008	0.146	0.058	0.170	0.207
Milk production	0.026	0.009	0.025	0.036	0.020	0.022
4% FCM	0.079	0.033	0.119	0.038	0.493	0.138
Milk fat	0.007	0.194	0.014	0.026	0.010	0.452
Milk protein	0.037	0.047	0.047	0.029	0.200	0.087
Prefeeding pH	0.052	0.793	0.064	0.033	0.074	0.580
3-h Postfeeding pH	0.004	0.246	0.014	0.015	0.011	0.410
Acetate (A), mM	0.208	0.004	0.083	0.021	0.914	0.316
Propionate (P), mM	0.035	0.265	0.332	0.782	0.092	0.766
Butyrate (B), mM	0.213	0.187	0.893	0.745	0.666	0.618
A + P + B, mM	0.005	0.002	0.069	0.048	0.091	0.223
A:P	0.007	0.759	0.048	0.060	0.023	0.905
Ammonia, mM	0.003	0.060	<0.001	0.003	0.001	0.001
Amino acids, mM	<0.001	0.117	<0.001	<0.001	<0.001	0.064
<i>Ruminococcus albus</i>	0.158	0.133	0.415	0.385	0.227	0.452
<i>Ruminococcus flavefaciens</i>	0.039	0.385	0.615	0.611	0.581	0.297
<i>Fibrobacter succinogenes</i>	0.217	0.788	0.684	0.596	0.499	0.426
Sum of three species	0.127	0.194	0.456	0.560	0.248	0.361

¹aNDF = NDF determined after α -amylase treatment. Diets contained alfalfa silage or corn silage with 24 or 32% aNDF.

²Determined from ANOVA using the general linear models procedures of SAS (30).

TABLE 4. Mean values for ruminal pH and VFA from four cows fed diets that varied in source of forage and concentration of fiber.

	Diet ¹				Forage		aNDF ²			Cow no.			Pooled SE
	AS24	AS32	CS24	CS32	AS	CS	24%	32%	749	2661	3691	3807	
Prefeeding pH	5.79 ^b	6.07 ^{ab}	6.14 ^{ab}	6.30 ^a	5.93 ^b	6.22 ^a	5.96 ^a	6.19 ^a	6.14 ^{ab}	5.81 ^b	6.35 ^a	6.00 ^{ab}	0.21
3-h Postfeeding pH	5.52 ^b	5.78 ^a	5.37 ^b	5.53 ^b	5.64 ^a	5.45 ^b	5.44 ^b	5.65 ^a	5.69 ^a	5.23 ^b	5.67 ^a	5.60 ^a	0.12
Acetate (A), mM	107.4 ^a	102.5 ^{ab}	90.1 ^b	94.2 ^{ab}	105.0 ^a	92.0 ^b	98.8 ^a	98.3 ^a	92.8 ^a	106.7 ^a	96.6 ^a	98.2 ^a	8.2
Propionate (P), mM	37.5 ^a	27.5 ^a	37.4 ^a	30.1 ^a	32.5 ^a	33.5 ^a	37.4 ^a	28.8 ^a	24.4 ^b	45.8 ^a	25.3 ^b	36.9 ^{ab}	8.6
Butyrate (B), mM	17.8 ^a	16.8 ^a	16.9 ^a	17.0 ^a	17.3 ^a	16.9 ^a	17.4 ^a	16.9 ^a	15.5 ^a	19.3 ^a	17.0 ^a	16.7 ^a	2.2
A + P + B, mM	163.2 ^a	146.7 ^{ab}	144.4 ^b	141.2 ^b	155.0 ^a	142.8 ^a	154.8 ^a	144.0 ^a	132.7 ^c	172.2 ^a	138.9 ^{bc}	151.8 ^b	9.8
A:P	3.27 ^{ab}	3.94 ^a	2.71 ^b	3.43 ^{ab}	3.60 ^a	3.07 ^b	2.99 ^b	3.69 ^a	4.08 ^a	2.47 ^b	3.89 ^a	2.91 ^b	0.46
Ammonia, mM	17.3 ^a	13.1 ^b	13.4 ^b	13.5 ^b	15.2 ^a	14.4 ^b	15.4 ^a	13.3 ^b	13.6 ^b	16.3 ^a	13.8 ^b	13.7 ^b	4.1
Amino acids, mM	1.51 ^a	1.01 ^b	1.65 ^a	1.42 ^b	1.26 ^b	1.54 ^a	1.58 ^a	1.21 ^b	1.14 ^c	1.65 ^a	1.34 ^b	1.45 ^{ab}	0.51

^{a,b,c}Means within the same group (diet, forage, aNDF, or cow) with different superscripts differ ($P < 0.05$).¹AS24 = Alfalfa silage (AS) at 24% aNDF, AS32 = AS at 32% aNDF, CS24 = corn silage (CS) at 24% aNDF, and CS32 = CS at 32% aNDF.²aNDF determined after α -amylase treatment.

The correlations among production and ruminal parameters are summarized in Table 5. Both the pH and A:P at 3 h postfeeding had a strong, positive correlation with milk fat percentage and a negative correlation with milk protein percentage. The correlation of A:P with milk fat percentage was even greater ($r = 0.94$) in the seven combinations of cow and diet in which ruminal acetate concentration exceeded 100 mM. Milk fat percentage also showed a substantial negative correlation ($r = -0.71$) with ruminal propionate concentration (Table 5).

Microbial Populations

Examination of the microbial populations collected 3 h postfeeding (Figure 3) revealed that RNA from the three predominant cellulolytic species represented only 0.3 to 3.9% of the total bacterial RNA. In most cases, the relative population size (i.e., the fraction of RNA of a single species as a fraction of the total bacterial RNA) of *R. albus* was much greater than that of either *R. flavefaciens* or *F. succinogenes*. However, extremely low populations of *R. albus* were observed for two cows (no. 749 and 2661) fed the CS diet at 24% aNDF; the pH values at 3 h postfeeding were lower (5.46 and 5.18, respectively) than those observed for other combinations of cow and diet.

Despite large differences in ruminal chemistry and production from individual cows, the relative population sizes of individual cellulolytic species showed few consistent differences across diets for individual cows (Table 6; Figure 3). The difficulty in demonstrating the effects of diet on the populations of these three species was in part due to the high variability of the RNA probe method [coefficient of variation = 39.7% averaged across 48 combinations of cow, diet, and bacterial species; (36)]. Nevertheless, ANOVA revealed that microbial populations showed a greater dependence on cow than on diet, forage type, or dietary aNDF percentage (Table 3).

Positive correlations were observed among the relative population sizes of the three species (Table 5), suggesting some similarities in response to environmental conditions. The relative populations of *R. albus* displayed significant, positive correlations with milk production and FCM. *Ruminococcus flavefaciens* displayed the strongest negative correlation with ruminal pH, and both *Ruminococcus* species displayed a significant positive correlation with ruminal butyrate concentration. Correlations of individual cellulolytic species or their sum, with either ammonia or total amino acids, ranged from $r = -0.46$ to 0.11 (NS at $P = 0.05$) (data not shown).

DISCUSSION

Molecular approaches, such as the use of oligonucleotide probes, permit the analysis of microbial populations to a degree that is not possible by conventional techniques based on culture (1, 2, 26, 28, 35). This fact, combined with the known shortcomings and biases of culture techniques, has eroded the confidence of some microbiologists (26, 35) in data obtained by these traditional methods. Therefore, it is interesting that the sums of the relative populations of the three major culturable cellulolytic species that we have measured using probes based on RNA were typically in the range of 1 to 4% of the total bacterial RNA. These values compared favorably with the relative population sizes of the cellulolytic population as a whole as determined by such traditional culture methods as roll tube colony counts and most probable number estimates (9, 39). However, these values were lower than were the proportions (3 to 28%) of randomly selected isolates (from cultivation on solid, nonselective media) that were capable of cellulose digestion when tested in pure culture (6, 24). This disparity reinforced the current view in microbial ecology (1, 2, 35) that the characterization of individual microbial isolates from natural environments is biased toward easily cultured strains (viz., the predominant ruminal cellulolytic species), and their relative population sizes in the natural environment may thus be overestimated.

The ruminal cellulolytic population might have possibly been underestimated in our study by incomplete detachment of bacteria from plant fiber during the cell recovery phase prior to RNA isolation. In addition, quantitation of the species composition

might have been skewed by the differential distribution of individual species into the solid and liquid phases of ruminal contents. However, the recovery methods we used (chilling and blending) have been reported to be the most successful in quantitative recovery of adherent bacteria (7, 8, 19). Moreover, Briesacher et al. (4), using oligonucleotide probes, have observed that the relative proportion of *F. succinogenes* in the fluid and mat phases of ruminal contents does not differ significantly, which suggests that reasonably effective cell recovery methods should not inordinately bias our population assessments.

Comparison of population sizes which was based on the proportion of species-specific RNA in ruminal contents, nevertheless must be interpreted with care because individual strains within a given species can display marked variation in response to probes specific to their species (22, 26). This interstrain variation suggests that a more meaningful assessment of the contribution of each species to total bacterial RNA could be obtained within cows (i.e., resident strains are likely to be maintained over time) than among cows (i.e., different strains of a given species are present). The apparent differences in the relative populations of both *R. albus* and *R. flavefaciens* between cows no. 2661 and no. 3807 and between cows no. 749 and no. 3691 might have been due to these interstrain differences in response to the probes. Odenyo et al. (22) have reported considerable differences in response of *R. flavefaciens* strains to probes specific for *R. flavefaciens*, although the probes used in our study were selected to minimize differences among strains of the same species based on previous studies with a variety of laboratory strains (22, 26, 36).

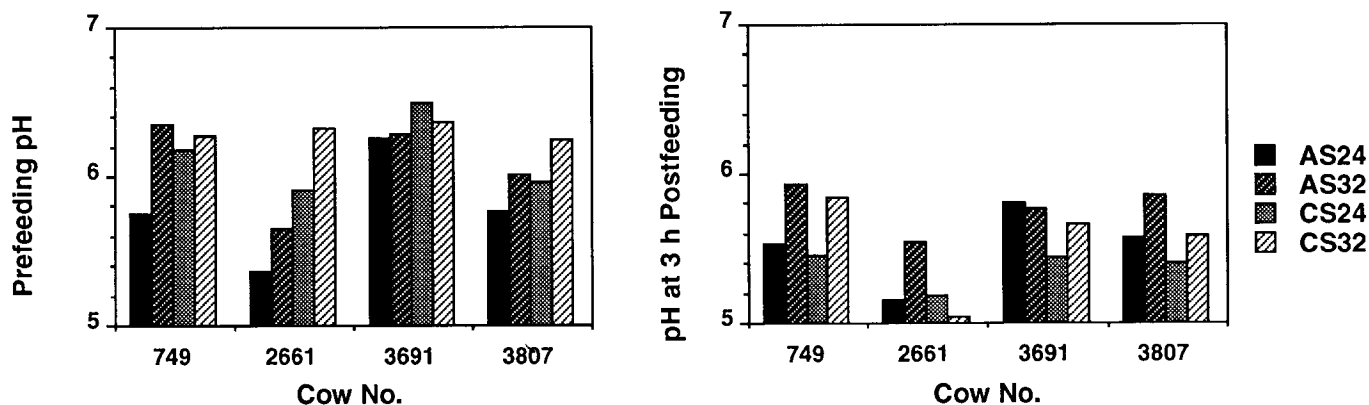


Figure 1. Mean values for ruminal pH for all combinations of cow and diet that were collected during the sampling period (d 24 to 28). Diets contained alfalfa silage (AS) or corn silage (CS) as the principal fiber source with 24 or 32% aNDF (NDF determined after α -amylase treatment). Diets: AS24, AS at 24% aNDF; AS32, AS at 32% aNDF; CS24, CS at 24% aNDF; and CS32, CS at 32% aNDF.

TABLE 5. Correlations (r) among milk, ruminal chemistry, and relative populations of cellulolytic bacterial species.¹

	DMI	Milk production	FCM	Milk fat %	Milk protein %	Pre pH ²	Post pH ³	Acetate (A)	Propionate (P)	Butyrate (B)	A + P + B	A:P	<i>Ruminococcus albus</i>	<i>Ruminococcus flavefaciens</i>	<i>Fibrobacter succinogenes</i>
Milk production	0.74**														
4% FCM	0.58*	0.90**													
Milk fat, %	0.01	-0.48	-0.32												
Milk protein, %	-0.32	-0.18	-0.34	-0.40											
Pre pH	-0.17	-0.57*	-0.51*	0.47	-0.26										
Post pH	0.28	-0.24	-0.14	0.80**	-0.64**	0.38									
A, mM	0.28	0.52*	0.63*	0.05	-0.03	-0.37	-0.18								
P, mM	0.17	0.55*	0.41	-0.71**	0.49	-0.67**	-0.77**	0.37							
B, mM	0.15	0.53*	0.64**	-0.27	0.21	0.34	-0.46	0.69**	0.41						
A + P + B, mM	0.27	0.65**	0.66**	-0.34	0.24	0.25	-0.53*	0.88**	0.76**	0.74**					
A:P	-0.06	-0.41	-0.16	0.85**	-0.62*	0.58*	0.82**	0.05	-0.87**	-0.23	-0.41				
<i>R. albus</i>	0.44	0.53*	0.56*	-0.16	0.06	-0.38	-0.05	0.39	0.29	0.55*	0.43	-0.18			
<i>R. flavefaciens</i>	0.05	0.48	0.50	-0.44	0.40	-0.68**	-0.44	0.46	0.54*	0.62*	0.61*	-0.45	0.77**		
<i>F. succinogenes</i>	0.23	0.11	-0.06	-0.11	0.44	0.01	-0.15	-0.03	0.22	0.11	0.09	-0.32	0.59*	0.47	
Sum ⁴	0.39	0.49	0.48	-0.20	0.19	-0.38	-0.13	0.35	0.39	0.53*	0.43	-0.26	0.98**	0.81**	0.71**

¹Correlations involving production variables (DMI, milk production, FCM, fat, or protein) were from 15 combinations of cow and diet. Correlations not involving these variables were from 16 combinations of cow and diet.

²pH prefeeding.

³pH at 3 h postfeeding.

⁴Sum of the relative populations of the three cellulolytic species.

* $P < 0.05$.

** $P < 0.01$.

Despite the potential bias that might have resulted from sampling at a single postfeeding time point or from intraspecific differences in probe response, the relative proportions of the three cellulolytic species determined in this study by RNA probes were in general agreement with several studies in which hundreds of ruminal isolates were classified on the basis of classical determinative (morphological and physiological) methods. In both heifers (24) and sheep (39), *R. albus* has been reported to represent a greater fraction of isolates than *R. flavefaciens*. Moreover, Van Gylswyk (39) has reported that coccoid rods, a morphological characteristic of *F. succinogenes*, accounted for a mean of only 4% of the cellulolytic isolates from the rumens of four sheep fed different diets. At a reported cellulolytic bacterial count of ~3% of the viable cell count (39), this value translates to a cellulolytic coccoid rod population that averaged ~0.1% of the viable cell count. In our study, *F. succinogenes* ranged from 0.1 to 0.8% of the total

bacterial RNA, and Stahl et al. (36) reported similar values in the original RNA probe study for this species using a single cow examined over a period of several weeks in the presence or absence of the ionophore monensin. However, these values are considerably lower than the 6% of bacterial RNA reported by Briesacher et al. (4) for three steers fed orchardgrass. Although the same probe sequence was used for *F. succinogenes* in these last three studies, variations in the hybridization of individual strains within this species could account for some or all of the discrepancies among the reported data.

The pH is a strong determinant of many microbial processes, particularly ruminal fiber digestion (15, 16, 38). Cows in this trial showed a wide range in ruminal pH, both prefeeding (5.4 to 6.5) and 3 h postfeeding (5.0 to 5.9; Figure 1). Correlations between the relative cellulolytic populations with ruminal pH were not affected by the range in the ruminal pH values reported here (Table 5). This result is

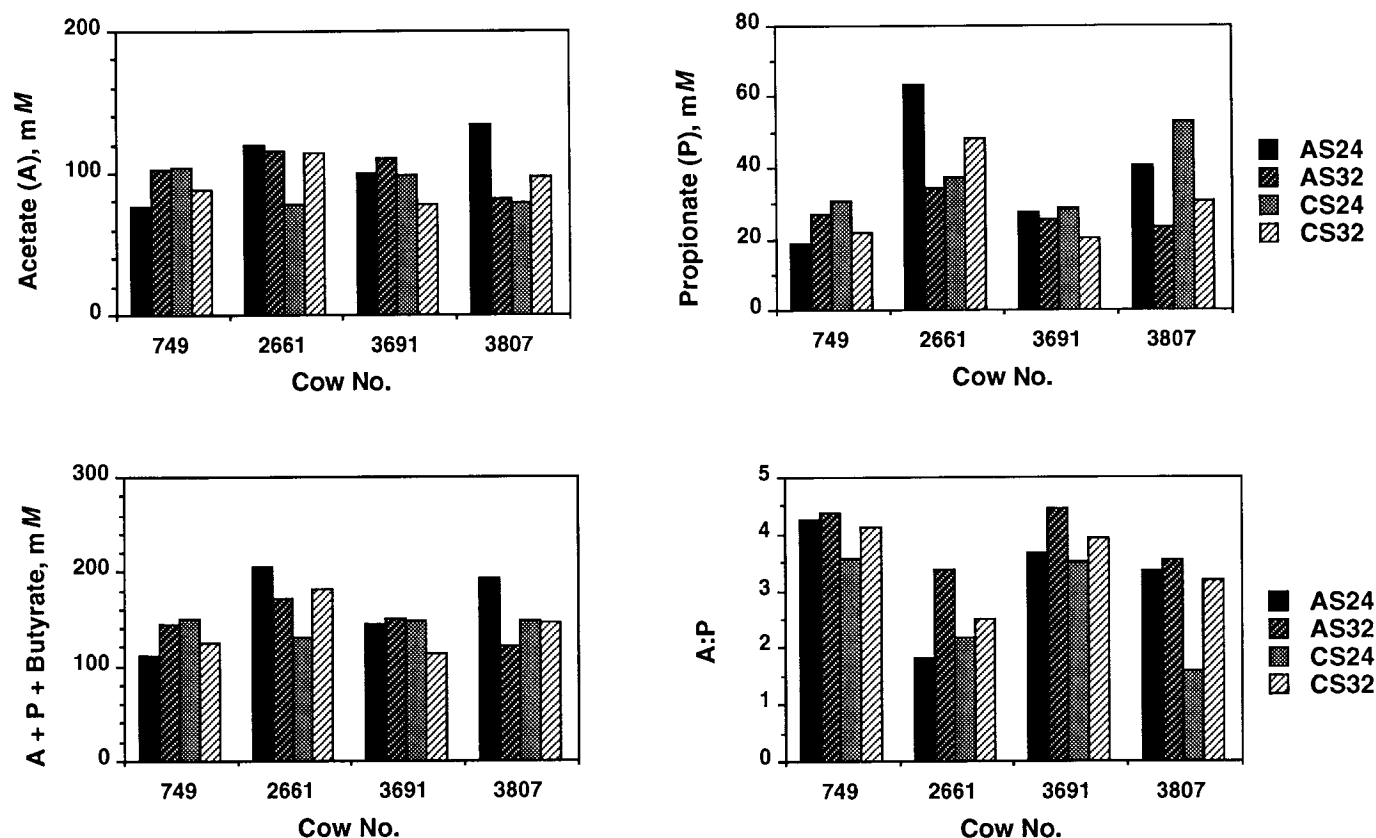


Figure 2. Mean VFA concentrations and ratios of acetate to propionate (A:P) for all combinations of cow and diet; each value was calculated from 14 samples collected during the sampling period. Diets contained alfalfa silage (AS) or corn silage (CS) as the principal fiber source with 24 or 32% aNDF (NDF determined after α -amylase treatment). Diets: AS24, AS at 24% aNDF; AS32, AS at 32% aNDF; CS24, CS at 24% aNDF; and CS32, CS at 32% aNDF.

surprising, given that pH values at 3 h postfeeding were <6.0 in many instances and, therefore, were below the lower limit for the growth of these species in culture (27, 29, 43). An adaptation of a distinct population of cellulolytic bacteria that are able to grow at a low but fluctuating pH is unlikely. Attempts to establish enrichment cultures of cellulolytic microbes at pH 5.5 by inoculation of SRF from these rumen samples into media with cellulose as sole energy source (45) were consistently unsuccessful, indicating that the indigenous strains, similar to their counterparts in laboratory culture, could not initiate growth at this pH. The low relative populations of the three cellulolytic species in samples collected 3 h postfeeding may reflect a temporary suppression of growth of these bacteria relative to the noncellulolytic species during the relatively acidic conditions that developed immediately after feeding.

Hiltner and Dehority (16) have reported that pure cultures of cellulolytic bacteria subjected to prolonged (48 h) exposure to low pH, upon return to more neutral pH, resumed cellulose digestion at rates comparable with those of the same strains that were not exposed to low pH. These cellulolytic species may maintain themselves in the rumen by rapid recovery of growth and cellulose digestion during periods of moderate pH (> 6.0) that offsets dilution from the rumen during periods of nongrowth (pH < 6.0) when cellulolytic activity continues at a reduced rate.

The impact of pH on microbial end products and milk composition also requires careful interpretation. For example, in this trial, milk fat percentage and A:P were more strongly ($P < 0.01$) related to the pH at 3 h postfeeding ($r = 0.80$ and 0.82 , respectively) than to the pH prefeeding [$r = 0.47$ (NS at $P = 0.05$) and 0.58 ($P < 0.05$)] despite a generally nonsignificant

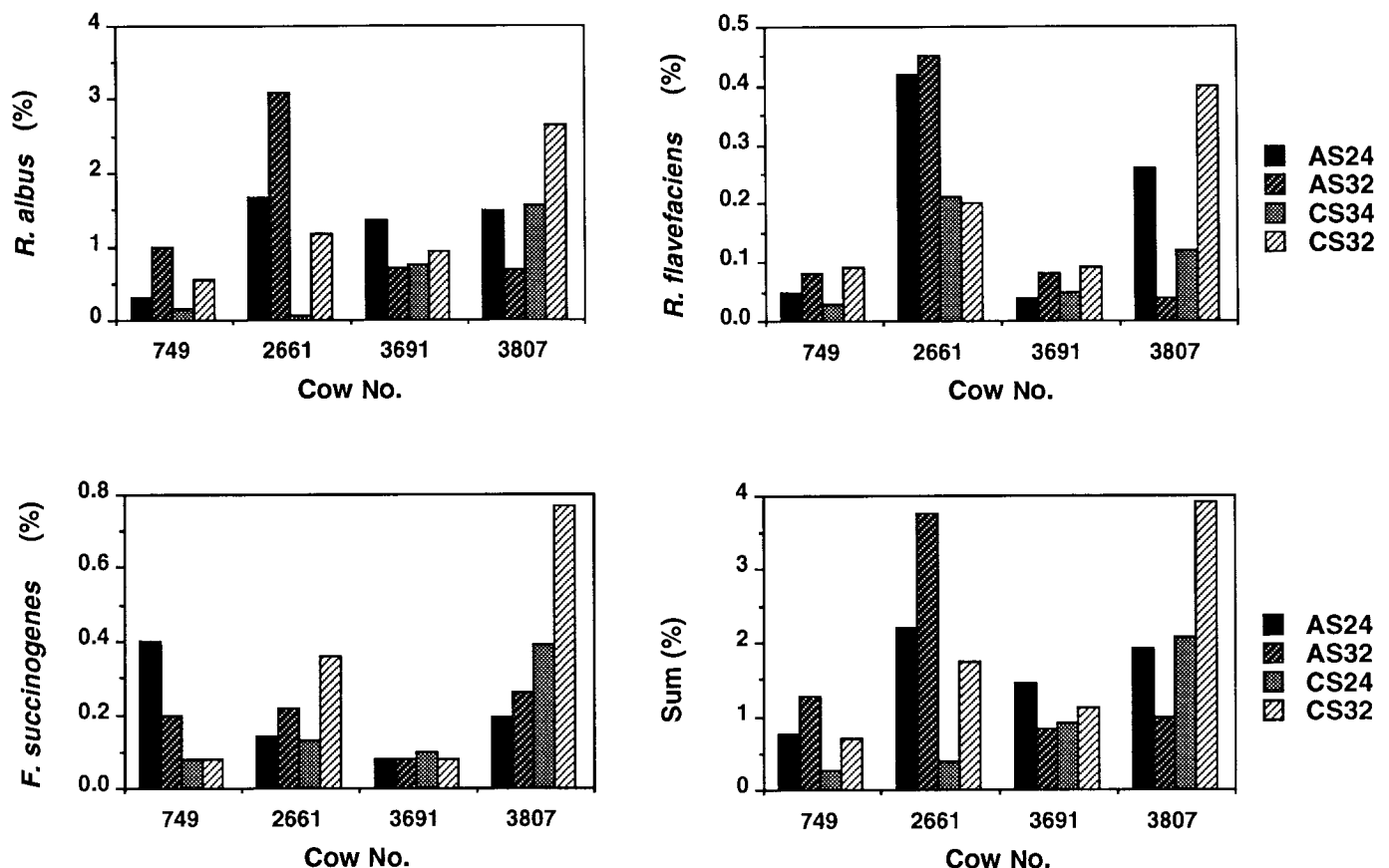


Figure 3. Relative populations of the ruminal cellulolytic bacteria *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* and their sums for all combinations of cow and diet, expressed as a fraction of total bacterial RNA. Results are mean values of three to five samples collected 3 h postfeeding during the sampling period. Diets contained alfalfa silage (AS) or corn silage (CS) as the principal fiber source with 24 or 32% aNDF (NDF determined after α -amylase treatment). Diets: AS24, AS at 24% aNDF; AS32, AS at 32% aNDF; CS24, CS at 24% aNDF; and CS32, CS at 32% aNDF. Note differences in the scale of each ordinate.

relationship between ruminal pH and milk fat percentage in studies reviewed by Erdman (11). Results reported here show that the time of sampling relative to feeding can affect the interpretation of pH data.

When effects of both cow and diet were taken into account, this trial achieved a 2.7-fold range in A:P and a 1.8-fold range in milk fat percentage. These parameters were strongly correlated ($r = 0.85$) across the broad range of treatments, and the correlation was even stronger ($r = 0.94$) at acetate concentrations of >100 mM. This fact, combined with the negative correlation between milk fat and ruminal propionate concentration and the lack of effect of acetate concentration on milk fat (Table 3), reinforced the suggestion that propionate itself has a strong negative influence on milk fat production (40). However, neither ruminal A:P nor milk fat showed a significant relationship with any of the cellulolytic bacterial populations (Table 5), probably because of their small contribution to the ruminal bacterial population. The relative population of *R. flavefaciens*, which produces primarily acetate and lesser amounts of succinate (a propionate precursor), displayed some positive correlations with ruminal acetate ($r = 0.46$; NS at $P = 0.05$) and propionate ($r = 0.54$; $P < 0.05$) (Table 5). By contrast, the relative population sizes of *F. succinogenes*, which produces primarily succinate and lesser amounts of acetate, did not display significant correlations with either acetate or succinate concentration. The cellulolytic bacteria measured here are apparently important in digesting fiber, but their total population is too small to have a major impact on the proportions of ruminal VFA or the production parameters that derive from ruminal chemistry.

Diets used in this study differed slightly in protein concentration (Table 1), which was reflected in the

differences in ruminal concentrations of ammonia and total amino acids (Table 4). The higher ammonia concentrations and the lower amino acid concentrations of the AS diets were in accord with the lower efficiency of alfalfa protein utilization (10). The ammonia concentrations always exceeded the concentration (3 mM) typically regarded as sufficient for ammonia-utilizing ruminal bacteria (31). The poor correlation of ammonia or amino acid concentrations with the relative populations of the three cellulolytic species indicated that slight differences in dietary protein do not affect the quantitative distribution of the three cellulolytic species, which utilize ammonia as a primary or sole nitrogen source.

The three cellulolytic species did represent a slightly greater fraction of the total bacterial population in the high fiber diets than in the low fiber diets (Table 6), which supported the notion (41, 42, 44) that the rate of cellulose digestion in the rumen is limited by substrate (i.e., an increase in cellulose concentration increases the cellulolytic population). The relative populations of both *Ruminococcus* species displayed positive ($P < 0.05$) correlations with ruminal butyrate concentration, which was surprising because neither species is known to produce butyrate (17); this result suggests that the ruminococci might have positively affected the populations or activities of cohabitant species that produce butyrate. This type of observation highlights the complex synergy that affects microbial populations and the need to identify populations in vivo to maximize the efficiency of feed utilization for production.

Individual cows were responsible for larger differences in the proportions of these cellulolytic species than were changes brought about by contrasting diets (Table 3). In this regard, our data support the results

TABLE 6. Mean values for cellulolytic microbial species (as a percentage of the total bacterial population) in ruminal samples collected at 3 h postfeeding from four cows fed diets that varied in source of forage and concentration of fiber.

	Diet ¹				Forage		aNDF ²		Cow no.				Pooled SE
	AS24	AS32	CS24	CS32	AS	CS	24%	32%	749	2661	3691	3807	
<i>Ruminococcus albus</i>	1.19 ^a	1.37 ^a	0.63 ^a	1.33 ^a	1.28 ^a	0.98 ^a	0.91 ^a	1.35 ^a	0.59 ^a	1.49 ^a	0.94 ^a	1.59 ^a	0.64
<i>Ruminococcus flavefaciens</i>	0.19 ^a	0.16 ^a	0.10 ^a	0.20 ^a	0.18 ^a	0.15 ^a	0.15 ^a	0.18 ^a	0.06 ^b	0.32 ^a	0.07 ^b	0.21 ^{ab}	0.03
<i>Fibrobacter succinogenes</i>	0.20 ^a	0.19 ^a	0.18 ^a	0.32 ^a	0.20 ^a	0.25 ^a	0.19 ^a	0.26 ^a	0.19 ^a	0.21 ^a	0.09 ^a	0.40 ^a	0.19
Sum ³	1.59 ^a	1.71 ^a	0.91 ^a	1.87 ^a	1.65 ^a	1.39 ^a	1.25 ^a	1.79 ^a	0.75 ^b	2.02 ^{ab}	1.08 ^{ab}	2.22 ^a	0.84

^{a,b}Means within the same group (diet, forage, aNDF, or cow) with different superscripts differ ($P < 0.05$).

¹AS24 = Alfalfa silage (AS) at 24% aNDF, AS32 = AS at 32% aNDF, CS24 = corn silage (CS) at 24% aNDF, and CS32 = CS at 32% aNDF.

²NDF determined after α -amylase treatment.

³Sum of the relative populations of the three cellulolytic species.

of the study by Van Gylswyk (39), who observed considerable differences in the species composition of cellulolytic isolates from four sheep fed the same diets. Moreover, we observed larger differences in ruminal pH at 3 h postfeeding among cows (mean difference = 0.46 pH units) than among diets (mean difference = 0.41 pH units), and even greater differences in favor of cow effects were observed for the A:P (1.61 vs. 1.23) and VFA concentrations (Table 3). Although diet undoubtedly affects the populations of some ruminal microbial species, the failure of the three cellulolytic species measured to respond to differences in source or concentration of fiber in a consistent manner across cows suggests that each cow maintains a unique assemblage of ruminal microbial strains. The mechanisms by which these cellulolytic populations remain in the rumen over time are unknown. Nevertheless, the stability of these populations, combined with the well-known inherent resistance of native populations to displacement by invading species (1), suggests that improvement of ruminal cellulolysis through the introduction of engineered microbial strains (12, 25) may be more problematic than was originally suspected, even if these strains are engineered from the predominant culturable ruminal cellulolytic species.

In our study, substantial shifts in the source and amount of fiber in the diet resulted in shifts in production, ruminal chemistry, and microbial populations, but these shifts were not consistent among cows. This result reinforces the notion that the ruminal microbial population is the result of complex interactions with the host (of which diet is only one component) and suggests that a more thorough understanding of these interactions will require that each animal be considered as an individual having a unique assemblage of microbial symbionts.

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