

Quantitative Fluorescence *In Situ* Hybridization of Microbial Communities in the Rumens of Cattle Fed Different Diets[∇]

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At present there is little quantitative information on the identity and composition of bacterial populations in the rumen microbial community. Quantitative fluorescence *in situ* hybridization using newly designed oligonucleotide probes was applied to identify the microbial populations in liquid and solid fractions of rumen digesta from cows fed barley silage or grass hay diets with or without flaxseed. *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were abundant in both fractions, constituting 31.8 to 87.3% of the total cell numbers. They belong mainly to the order *Bacteroidales* (0.1 to 19.2%), hybridizing with probe BAC1080; the families *Lachnospiraceae* (9.3 to 25.5%) and *Ruminococcaceae* (5.5 to 23.8%), hybridizing with LAC435 and RUM831, respectively; and the classes *Deltaproteobacteria* (5.8 to 28.3%) and *Gammaproteobacteria* (1.2 to 8.2%). All were more abundant in the rumen communities of cows fed diets containing silage (75.2 to 87.3%) than in those of cows fed diets containing hay (31.8 to 49.5%). The addition of flaxseed reduced their abundance in the rumens of cows fed silage-based diets (to 45.2 to 58.7%) but did not change markedly their abundance in the rumens of cows fed hay-based diets (31.8 to 49.5%). Fibrolytic species, including *Fibrobacter succinogenes* and *Ruminococcus* spp., and archaeal methanogens accounted for only a small proportion (0.4 to 2.1% and 0.2 to 0.6%, respectively) of total cell numbers. Depending on diet, between 37.0 and 91.6% of microbial cells specifically hybridized with the probes used in this study, allowing them to be identified *in situ*. The identities of other microbial populations (8.4 to 63.0%) remain unknown.

The rumen is an anaerobic ecosystem used by herbivores to convert fibrous plant material into fermentation products that are in turn used as energy by the host. Fibrolytic degradation is accomplished by a complex microbial community which includes specialized fungi, protozoa, and bacteria (14). More than 200 bacterial species (5) have been isolated from rumen, and many of these have been phylogenetically and physiologically characterized. Several of these, including *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*, have the ability to hydrolyze cellulose in axenic culture (24). Despite the presence of these fibrolytic populations, a large portion of the fiber in low-quality forage diets passes through the rumen undigested. In the rumen, fibrolytic bacteria do not digest plant cell walls in isolation but rather interact with a consortium of bacteria (18). Although culture-dependent studies have improved our understanding of rumen microbiology, the importance of the isolates to the structure and function of the rumen microbial community, with the possible exception of the fibrolytic strains, is still unknown. Expanding our knowledge of the structure and function of the rumen microbial community may provide insights into approaches to improve the efficiency of fiber digestion and biofuel production (14).

To provide a high-resolution view of the population structure of the rumen bacterial community, we used quantitative

fluorescence *in situ* hybridization (qFISH) to investigate the composition and distribution of bacterial populations associated with the liquid and solid rumen contents from 12 ruminally cannulated Holstein dairy cows (3 cows were used for each diet) fed (for at least 21 days) grass hay or barley silage diets with or without flaxseed (Table 1). Six new 16S rRNA-targeted FISH probes (Table 2) for not only the fibrolytic groups but also other unclassified bacterial groups in the rumen were designed, using ARB software (17), against the rumen 16S rRNA gene sequences (data not shown) retrieved from the Ribosomal Database Project (RDP) database (6). The new probes target *Bacteroidales*-related clones (probe BAC1080) (phylum *Bacteroidetes*), *Lachnospiraceae*- and *Ruminococcaceae*-related clones (probes LAC435 and RUM831, respectively) (phylum *Firmicutes*), *Butyrivibrio fibrisolvens*-related clones (probe BFI826), and *R. albus*- and *R. flavefaciens*-related clones (probes RAL1436 and RFL155, respectively).

The optimal formamide concentrations (OFC) of the new probes used in FISH were assessed in different ways. Probes RUM831 and BAC1080 were assessed by using pure cultures of *Ruminococcus* and *Prevotella* strains with zero and one mismatch (Fig. 1) to the probes. The OFC of probes LAC435 and BFI826 were assessed using Clone-FISH (21) with zero and one mismatch 16S rRNA clone (Fig. 1) by following the procedure described previously (9, 10). The highest formamide concentration (tested in 5% stepwise increases) at which a clear fluorescent signal was observed with the reference bacterium or competent cells with zero mismatches after FISH probing, but not with bacteria or competent cells with one mismatch, was selected. The OFC of probes FIB225 (designed

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TABLE 1. Composition of diets used in this study

Ingredient	Diet composition (% dry weight)			
	Hay-based diet	Hay and flaxseed diet	Silage-based diet	Silage and flaxseed diet
Alfalfa grass hay (chopped)	47.5	47.5	0	0
Barley silage	0	0	47.5	47.5
Steamed rolled barley grain	47.5	32.5	47.5	32.5
Ground flaxseeds	0	15	0	15
Other	5	5	5	5

by Stahl et al. [23]), RFL155, and RAL1436 were assessed using only pure cultures of *F. succinogenes*, *R. flavefaciens*, and *R. albus*, respectively, all having perfect matches to each probe (Fig. 1). The highest formamide concentration (tested in 5% stepwise increases) at which a clear fluorescent signal was observed with the reference bacterium after FISH probing was selected. These probes were employed with other available probes (Table 2) chosen from probeBase (16) based on the alignment and classification of the 16S rRNA gene sequences retrieved from rumen communities.

The digest samples from the top, bottom, and middle of the rumen were collected through a cannula, thoroughly mixed, and fractionated as liquid fraction (LiqF) and solid fraction (SolF). On-site, about 100 ml was transferred to a heavy-wall 250-ml beaker and squeezed using a Bodum coffee maker plunger (Bodum Inc., Triengen, Switzerland). The extruded liquid samples (containing the planktonic cells) were fixed in ethanol and paraformaldehyde (PFA) for FISH probing (3).

The remaining liquid was discarded, and the squeezed particulate samples (used to collect particulate-attached cells) were washed with 100 ml phosphate buffer (5.23 g/liter K_2HPO_4 , 2.27 g/liter KH_2PO_4 , 3.00 g/liter $NaHCO_3$, and 20 ml/liter 2.5% cysteine HCl) by stirring gently with a spatula, followed by squeezing again and decanting. Washed particulate samples (5 g) were then fixed for FISH as described above.

After fixation, the particulate samples plus the fixation solution were transferred into a stomacher bag and "stomached" (Stomacher 400 Circulator, Seaward England) at 230 rpm for 6 min. Treated samples were then transferred into a clean 250-ml beaker and squeezed again. Microscopic examination of the squeezed residues after DAPI (4',6-diamidino-2-phenylindole) staining (100 μ l [0.003 mg/ml] for 10 min) showed only a few bacterial cells attached on the plant fibers, indicating that most bacterial cells had been "stomached" into the liquid (data not shown). To recover cells, filtrates were centrifuged (5,000 \times g), and the cell pellet was washed three times with phosphate buffer before being used for FISH probing. On the day of sampling, each cow was sampled twice, at 1100 h and 1600 h. The liquid FISH samples obtained from the 3 cows fed with the same diet (at two different sampling times) were mixed, as were the particulate FISH samples, and used in qFISH analysis. FISH was carried out according to Amann (3). FISH was carried out on glass coverslips (24 by 60 mm) coated with gelatin (9). DAPI staining of biomass samples was carried out after FISH probing. FISH and DAPI images were captured with a Zeiss epifluorescence microscope (Zeiss PM III) equipped with a Canon 5D Mark II camera. Raw images captured randomly were transferred into gray TIF images and sharpened in Adobe Photoshop CS3. Cells stained with DAPI

TABLE 2. Oligonucleotide probes and their target populations used in this study for FISH analyses

Probe name ^a	Target rRNA	Designed target(s)	% FA ^b	Reference
EUB338 (00159)	16S	Domain <i>Bacteria</i>	0–50	16
EUB338II (00160)	16S	Phylum <i>Planctomycetes</i>	0–50	16
EUB338III (00161)	16S	Phylum <i>Verrucomicrobia</i>	0–50	16
NONEUB (00243)	16S	Control probe complementary to EUB338	0–50	16
ALF968 (00021)	16S	Class <i>Alphaproteobacteria</i> , phylum <i>Proteobacteria</i>	20	16
BET42a (00034)	23S	Class <i>Betaproteobacteria</i> , phylum <i>Proteobacteria</i>	35	16
GAM42a (00174)	23S	Class <i>Gammaproteobacteria</i> , phylum <i>Proteobacteria</i>	35	16
SRB385 (00300)	16S	Class <i>Deltaproteobacteria</i> , phylum <i>Proteobacteria</i>	35	16
SRB385Db (00301)	16S	Class <i>Deltaproteobacteria</i> , phylum <i>Proteobacteria</i>	35	16
HGC69a (00182)	23S	Phylum <i>Actinobacteria</i>	25	16
GNSB941 (00718)	16S	Phylum <i>Chloroflexi</i>	35	16
CFX1223 (00719)	16S	Phylum <i>Chloroflexi</i>	35	16
SPIRO1400 (01004)	16S	Subgroup of family <i>Spirochaetaceae</i>	20	16
TM7-905 (00600)	16S	Candidate phylum TM7	20	16
LGC354A (00195)	16S	Phylum <i>Firmicutes</i>	35	16
LGC354B (00196)	16S	Phylum <i>Firmicutes</i>	35	16
LGC354C (00197)	16S	Phylum <i>Firmicutes</i>	35	16
RUM831	16S	Rumen clones in family <i>Ruminococcaceae</i> , phylum <i>Firmicutes</i>	35	This study
RAL1436	16S	<i>Ruminococcus albus</i> -related clones, phylum <i>Firmicutes</i>	20	This study
RFL155	16S	<i>Ruminococcus flavefaciens</i> -related clones, phylum <i>Firmicutes</i>	45	This study
LAC435	16S	Clones in family <i>Lachnospiraceae</i> , phylum <i>Firmicutes</i>	35	This study
BFI826	16S	<i>Butyrivibrio fibrisolvans</i> -related clones, phylum <i>Firmicutes</i>	35	This study
BAC1080	16S	Clones in order <i>Bacteroidales</i> , phylum <i>Bacteroidetes</i>	20	This study
Fibr225 (00005)	16S	<i>Fibrobacter succinogenes</i> -related clones, phylum <i>Fibrobacteres</i>	20 ^c	16
ARCH915 (00027)	16S	Domain <i>Archaea</i>	20	16

^a The numbers in parentheses after the probe names represent the probe accession numbers in probeBase (16).

^b FA, formamide concentration used in the FISH buffer.

^c The optimum formamide concentration for the probe was determined in this study.

RUM831 (S-S-Rum-0831-a-16)	5' GGTCAGTCCCCCACA3'	BAC1080 (S-S-Bac-1080-a-18)	5' GCACTTAAGCCGACACCT3'
Target	3' CCAGUCAGGGGGUGU5'	Target	3' CGUGAAUUCGGCUGUGGA5'
<i>R. albus</i> (ATCC27210)	3'-----5'	<i>P. brevis</i> (ATCC19188)	3'-----5'
<i>R. albus</i> (LRC054)	3'----A-----5'	<i>P. ruminicola</i> (ATCC19189)	3'-----E-----5'
LAC435 (S-S-Lac-0435-a-16)	5' TCTTCCTGTGATAGA3'	BFI826 (S-S-But-0826-a-18)	5' ATGGCACCCAACACCTAG3'
Target	3' AGAAGGGACGACUAUCU5'	Target	3' GACCGUGGGUUGUGGAUC5'
clone CAL2F04 (GQ327004)	3'-----5'	clone CAL2D06 (GQ326993)	3'-----5'
clone CARS1E09 (GQ327255)	3'-----G-----5'	clone CTRS1H12 (GQ327799)	3'---E-----5'
FIB225 (S-S-Fib-0225-a-21)	5' AATCGGACGCAAGCTCATCCC3'	RFL155 (S-S-Rum-0155-a-15)	5' TACCATCCGTTTCCAGA5'
Target	3' UUAGCCUGCGUUCGAGUAGGG5'	Target	3' AUGGUAGGCAAAGGUCU5'
<i>F. succinogenes</i> (ATCC19169)	3'-----5'	<i>R. flavefaciens</i> (ATCC19208)	3'-----5'
RAL1436 (S-S-Rum-1436-15)	5' CGGTTAGAACACAGG3'		
Target	3' GCCAAUCUUGUGUCC5'		
<i>R. albus</i> (ATCC27210)	3'-----5'		

FIG. 1. Alignments of the probe sequences and their target sites and sequences of corresponding sites in reference bacteria or clones. The probe names in parentheses after the abbreviated names are according to Oligonucleotide Probe Database nomenclature (2). Only the nucleotides that are different from target sequences are shown. E, empty space; *R.*, *Ruminococcus*; *P.*, *Prevotella*; *F.*, *Fibrobacter*.

and hybridized to the probes were enumerated using the function provided in ImageJ (1). The percent compositions of these probe-defined groups (against all DAPI-stained cells in the same microscopic field) in the different fractions of rumen contents from cows fed different diets are presented in Table 3.

We provided quantitative data by using qFISH to show that *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were abundant in both the LiqF and the SolF, constituting 31.8 to 87.3% of the total cell numbers. These FISH data add weight to the view that *Firmicutes* and *Bacteroidetes* might be dominant in rumens, as suggested previously from their high ratios retrieved from 16S rRNA clone libraries (e.g., see references 12, 26, and 27). However, information emerging from 16S rRNA gene clone library data cannot be used to reach conclusions on the quantitative composition of the rumen bacterial community.

Bacteria may have 1 to 14 copies of rRNA genes, and several biases are known to be associated with their PCR amplification (8).

These 3 dominant bacterial groups have been identified at a high-resolution level. They belong mainly to the order *Bacteroidales* (0.1 to 19.2%), hybridizing with probe BAC1080 (Fig. 2A); the families *Lachnospiraceae* (9.3 to 25.5%) and *Ruminococcaceae* (5.5 to 23.8%), hybridizing with LAC435 (Fig. 2E) and RUM831 (Fig. 2D), respectively; and the classes *Deltaproteobacteria* (5.8 to 28.3%) and *Gammaproteobacteria* (1.2 to 8.2%), hybridizing with SRBmix (equal moles of SRB385 and SRB385Db) (Fig. 2C) and GAM42a (Fig. 2B), respectively. All were more abundant in the microbial communities in the rumens of cows fed diets containing silage (75.2 to 87.3%) than in those in the rumens of cows fed diets containing hay (31.8 to

TABLE 3. Distribution and composition of FISH probe-defined groups in rumen microbial communities in cows fed with different diets

Probe-defined microbial group	Composition (mean value [%] ± SD) ^a							
	Hay-based diet		Hay and flaxseed diet		Silage-based diet		Silage and flaxseed diet	
	LiqF	SolF	LiqF	SolF	LiqF	SolF	LiqF	SolF
BAC1080	9.6 ± 1.33	0.1 ± 0.02	19.2 ± 3.71	4.2 ± 0.72	14.2 ± 3.11	18.8 ± 3.88	14.4 ± 2.89	16.7 ± 4.33
ALF968	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.03	0.2 ± 0.04	0.7 ± 0.14	1.5 ± 0.41	0.1 ± 0.01	0.1 ± 0.01
BET42a	0	0	0.6 ± 0.01	1.2 ± 0.27	0.1 ± 0.01	<0.1	0.4 ± 0.06	0.2 ± 0.04
GAM42a	3.2 ± 0.53	4.4 ± 0.57	4.2 ± 0.76	4.5 ± 0.67	2.0 ± 0.32	1.2 ± 0.23	8.2 ± 1.23	5.3 ± 0.95
SRBmix	5.8 ± 0.88	11.6 ± 2.43	9.0 ± 1.52	10.1 ± 2.56	28.3 ± 4.43	23.3 ± 4.54	7.7 ± 0.78	13.2 ± 2.22
CHLmix	1.7 ± 0.27	0	0.5 ± 0.01	0 ± 0	0.2 ± 0.02	0.4 ± 0.07	0.1 ± 0.01	0.1 ± 0.02
SPIRO1400	0.5 ± 0.09	1.9 ± 0.32	1.7 ± 0.33	2.0 ± 0.21	1.4 ± 0.31	1.9 ± 0.33	0.4 ± 0.03	0.4 ± 0.07
TM7-905	0.6 ± 0.08	0.8 ± 0.07	0.5 ± 0.01	0.1 ± 0.03	1.5 ± 0.23	0.2 ± 0.02	0.6 ± 0.02	0.3 ± 0.08
HGC69a	1.3 ± 0.28	2.1 ± 0.31	0.3 ± 0.06	0.3 ± 0.05	0.4 ± 0.03	0.1 ± 0.02	0.5 ± 0.09	0.2 ± 0.02
RUM831	5.5 ± 0.13	5.7 ± 0.89	5.8 ± 0.73	8.9 ± 1.32	18.0 ± 4.13	23.8 ± 3.11	5.6 ± 1.14	7.4 ± 1.32
RAL1436	0.4 ± 0.06	0.3 ± 0.03	0.2 ± 0.06	0.2 ± 0.03	0.3 ± 0.05	0.6 ± 0.09	0.7 ± 0.13	0.6 ± 0.12
RFL155	0.7 ± 0.11	0.2 ± 0.03	0.3 ± 0.07	0.7 ± 0.19	0.1 ± 0.01	0.8 ± 0.11	0.5 ± 0.06	1.2 ± 0.34
LAC435	25.5 ± 3.98	10.0 ± 1.51	9.6 ± 1.31	11.7 ± 1.67	12.6 ± 2.56	20.2 ± 3.23	9.3 ± 1.51	16.1 ± 3.31
BFI826	0.3 ± 0.06	0.4 ± 0.05	0.4 ± 0.06	0.7 ± 0.12	0.5 ± 0.05	0.3 ± 0.08	2.4 ± 0.37	0.2 ± 0.02
Fibr225	0	0	0.2 ± 0.04	0.1 ± 0.02	0.8 ± 0.14	0.7 ± 0.14	0.4 ± 0.11	0.1 ± 0.04
ARCH915	0.3 ± 0.08	0.2 ± 0.07	0.6 ± 0.01	0.3 ± 0.07	0.6 ± 0.09	0.1 ± 0.02	0.4 ± 0.05	0.4 ± 0.06
Total hybridized ^b	54.1	37	52.4	43.7	80.9	91.6	48	60.7
Other ^c	45.9	63	47.6	56.3	19.1	8.4	52	39.3

^a The two numbers represent the mean value (%) and the standard deviation of individual probe-defined microbial groups in a specified rumen digest fraction, which were calculated based on 3 mean values, each consisting of 20 enumerations.

^b The numbers represent the sum of percentages of all individual probe-defined microbial groups in a specified rumen digest fraction. The percentages obtained with FISH probes RAL1436, RFL155, and BFI826 were not counted in the sum because the bacterial cells hybridizing with the former two probes also hybridized with RUM831, and the bacterial cells hybridizing with the last probe also hybridized with probe LAC435.

^c The numbers represent the percentages of microorganisms which were not identified by FISH in a specified rumen digest fraction.

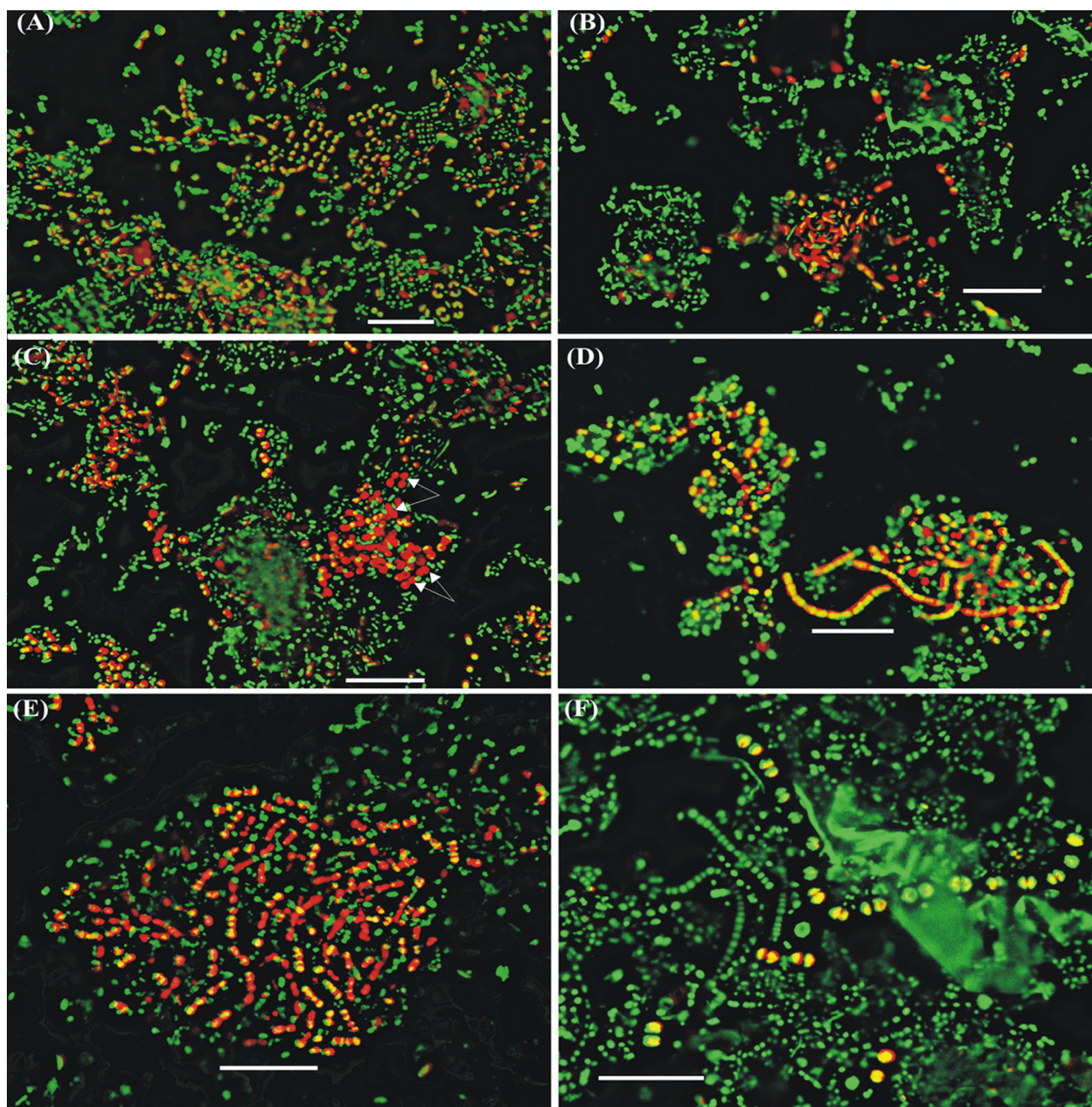


FIG. 2. Images of digest samples from the rumens of cows fed hay- or silage-based diets with and without flaxseed after color combination. Images from probes are labeled in red, and those from DAPI staining are in green. The yellow (combination of red and green), including those partly colored cells in panels A to F, hybridized with probes BAC1080, GAM42a, SRBmix, RUM831, LAC435, and ARCH915, respectively. A few cells (arrows) hybridizing with SRBmix (C) were not stained by DAPI. Bars, 10 μ m.

49.5%). These results show how diets containing different forages (hay or silage) may influence the distribution of the microbial populations, which is in line with data by Tajima et al. (25). We also found in this study that the addition of flaxseed (to inhibit methane emission) reduced their abundance in the rumens of cows fed silage-based diets (to 45.2 to 58.7%) but did not change markedly their abundance in the rumens of cows fed hay-based diets (31.8 to 49.5%), suggesting that adding flaxseed to these diets also affected rumen microbial community composition, although the extent of its influence

reflected the forage used, being more profound with a silage-based diet than when hay was used.

We also present evidence here to suggest that *Proteobacteria* are common members of the microbial community, with sulfur-reducing bacteria (SRB) belonging to *Deltaproteobacteria* in particular being readily detected (up to 28% of the total cells) in both the LiqF and the SolF of rumen contents from cows fed the four different diets examined here. SRB have seldom been retrieved in clone libraries obtained from rumen samples. Lin et al. (15) have estimated SRB abundance in the

rumen using DNA hybridization and concluded that they were of minor importance (0.7 to 0.8% of the total rRNA). Our estimates are much higher than those for every diet regime examined, possibly reflecting the coverage of the probes used in the two different studies. The probe mixture SRBmix used here targets most members of the *Deltaproteobacteria*, while those of Lin et al. (15) covered mainly members of the *Desulfobacteraceae*, *Desulfovibrionaceae*, and *Desulfobulbaceae*. We also recognized that the probe mixture SRBmix perfectly matched with the 16S rRNA genes of some bacteria other than SRB in *Deltaproteobacteria*. The possibility of overestimation of SRB cannot be ruled out. Interestingly, our data suggest that *Gammaproteobacteria* were abundant in some of the rumen communities we examined by FISH, comprising 1.2 to 8.2% of total cells.

The other unexpected finding was that the fibrolytic bacteria and archaeal methanogens accounted for only a minor fraction of the communities. Of the three characterized fibrolytic bacterial species, *F. succinogenes* was not detected in the rumen digesta from cattle fed the hay-based diet but was present in the remainder of the diets. In contrast, *R. albus* and *R. flavefaciens* were present in both the LiqF and the SolF of the rumen digesta from cows fed all four diets. Although the importance of these bacteria within the rumen microbial community cannot be denied, these three populations accounted for only 0.7 to 2.1% of the total microbial cells. This numerical range compares well with that determined previously for *F. succinogenes* (0.1 to 6.9% of total rRNA) (4, 23) and *Ruminococcus* spp. (1.5 to 2.9% of total rRNA) (11), considering that different animals and diets were used in those studies and that different specificities of the probes and different detection methods were used. However, this is much lower than the 9% (of total rRNA) detected by Michalet-Doreau et al. (19) in their work. The abundance of fibrolytic *B. fibrisolvens*-related species was also low, being present at <1% in all fractions, except in the LiqF in cows fed the mixture of silage and flaxseed, where they contributed 2.4% of total cells.

Methanogens hybridized to ARCH915 (Fig. 2F) were present (0.1 to 0.6%) in all rumen samples examined by FISH, which is close to or within the range (0.3 to 3.3%) estimated in other studies (15, 22). Interestingly, no marked difference in abundance of the methanogens could be seen between the samples from the rumens of cows fed diets with flaxseed and those from the rumens of cows fed diets without flaxseed, although it has been reported (7) that the addition of fatty acids could decrease methane production in the rumen. This may be due to the presence of methanogens with different activities in different rumen samples or the inability of probe ARCH915 to hybridize to all methanogens in the rumen samples examined here.

Bacteria belonging to *Chloroflexi*, TM7, *Spirochetes*, and *Actinobacteria* hybridizing with CHLmix, TM7-905, SPRO1400, and HGC69a, respectively, accounted for only a minor fraction of the total cell numbers observed. In most cases, their abundances in each fraction did not change markedly with diet, always being present in small numbers (0 to 1%), suggesting that they have a minor role there. This conclusion, however, has to be confirmed since many (8.4 to 63.0%, depending on diet) of the bacteria could not be identified in the rumens of cows fed with all diets except the silage-based diet (Table 3).

FISH with the probes designed in this study failed to identify all of the bacterial cells. This is because the probes do not target all rumen 16S rRNA gene sequences and/or the true extent of rumen biodiversity has not been revealed from cloning analyses. This indicates that our current understanding of the quantitative composition of the rumen microbial community is far from complete. Moreover, no physiological data were generated in this study to suggest what the role(s) of most of the dominant populations (except the SRB hybridized with probe SRBmix) identified by FISH might be, meaning that it is still not possible to link their abundance to their *in situ* function. Furthermore, each FISH-probed population probably includes bacteria with different phenotypes. Clearly, much needs to be done before the structure and function of the rumen microbial community are fully understood.

FISH is a useful tool in the investigation of microbial composition in complex ecosystems (3). However, FISH probes targeting rumen bacterial populations are limited. By comparison with other culture-independent methods, e.g., quantitative PCR, FISH has several advantages (8). In particular, in combination with histochemical staining methods (20) and microautoradiography (MAR-FISH) (13), the *in situ* ecophysiology of a targeted population can be determined under specified electron acceptor conditions. These techniques may provide important clues as to the functional role of microbial populations within complex communities, like that of the rumen. The possession of the FISH probes described in this paper could allow such studies to be undertaken in herbivore rumens.

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