Effect of Feeding Cows Genetically Modified Maize on the Bacterial Community in the Bovine Rumen[▽]

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Rumen-cannulated cows ($n = 4$) were fed successively silage made from either conventional or genetically modified (GM) maize. Results revealed no effects of GM maize on the dynamics of six ruminal bacterial strains (investigated by real-time PCR) compared to the conventional maize silage.

The composition of the ruminal microbial ecosystem in the forestomach of ruminants is known to be affected by type and quantity of the ration, feeding intervals, specific additives (e.g., antibiotics), and the host animal itself (2, 9). Maize silage represents a major diet component of beef and dairy cows. Currently, potential risks of feeding genetically modified (GM) Bt maize containing the *cry1Ab* gene from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 to ruminants are publicly discussed. In several studies no adverse health or production effects in cattle, pigs, or broiler chicken consuming GM forages were found (reviewed by Flachowsky et al. [7]). Furthermore, during digestion of GM maize in the bovine rumen, recombinant DNA and protein were degraded (3, 11, 14, 18). However, little is known about the effect of feeding GM crops on the rumen microbiota (4).

Therefore, the objectives of this study were (i) to examine the dietary influence of Bt176 maize compared to the non-Bt isoline and an additional conventional non-Bt maize variety on the ruminal microbial community and (ii) to assess the dynamics of six different bacterial strains for 33 days in order to characterize sampling day and/or animal variability.

Three different maize varieties (A, B, and C) were planted on experimental fields of the Bavarian State Research Centre for Agriculture (Poing-Grub, Germany). After harvest, whole plants were chopped and ensiled according to standard schedules. Maize silage A represented a conventional non-Bt maize variety. Silage B was produced from the isogenic non-Bt maize hybrid (Antares), and silage C originated from the transgenic maize hybrid Bt176 (Navares; both Syngenta International AG, Basel, Switzerland). The contents of Cry1Ab DNA and protein fragments of maize silage C were shown previously (10, 18). The experiments were carried out using four mature, rumen-cannulated, nonlactating Holstein-Friesian cows (body weight, 650 to 750 kg) fed twice daily (0700 and 1600 h) in equal meals according to Fig. 1. Prior to the 0700 h feeding, samples of rumen contents were collected from the ventral rumen sac using a 50-ml Falcon tube, immediately frozen, and stored at −20°C. During the first experimental period (maize

silage A; Fig. 1), samples were taken on three consecutive days. During the second (silage B) and third (silage C) experimental periods, six samples were taken (days 4, 7, 8, 9, 10, and 11). During the fourth experimental period (silage A), 15 samples were collected in intervals of 2 to 3 days. Prior to the fourth experimental period one animal was replaced due to technical reasons. Total DNA was extracted using the bead-beating Fast-Prep technique (BIO101, Carlsbad, CA). Along with 0.06 g Matrix Green ceramic beads (BIO101), 300 μl of rumen content was shaken twice at 5.5 m/s for 30 s. In between those homogenization intervals, samples were placed on ice for 3 min. After 400 μl lysis buffer (C1) and 10 μl RNase A (both from the NucleoSpin plant kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany) were added, the tube contents were mixed thoroughly and 100 μl chloroform was applied. The FastPrep homogenization step was repeated, and the mixture was incubated for 45 min at 60°C and centrifuged for 5 min at $11,000 \times g$. The supernatant (300 μl) was transferred into a new tube. All succeeding DNA purification steps were performed using a silica spin column according to the manufacturer's protocol, with the addition of a CW buffer washing step and elution of DNA in 50 μl CE buffer (both from the NucleoSpin plant kit). DNA concentrations were determined by UV absorption at 260 nm; DNA integrity was estimated from the 260/280-nm UV absorption ratio.

Potential quantitative changes in ruminal bacterial community were analyzed using the LightCycler instrument (Roche, Mannheim, Germany). Tajima et al. (16) described primers and real-time PCR conditions for the detection and quantification of 12 ruminal bacteria. Melting curve analyses, standard curve accuracy, and additional verification of agarose gel electrophoresis patterns of the real-time PCR products yielded specific results for the quantification of six bacterial strains (*Anaerovibrio lipolyticus*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Ruminococcus flavefaciens*, *Selenomonas ruminantium-Mitsuokella multiacida*, and *Treponema bryantii*). The reaction mixtures (10-μl final volume) for those six bacterial strains contained 10 to 40 ng extracted DNA, 1 μl LightCycler DNA Master SYBR Green I (10×), 3 mM $MgCl_2$ (Roche), and 0.4 μM forward and reverse primers. The amplification of a universal 530-bp 16S rRNA gene fragment (www.arb-home .de) involved 1 cycle at 95°C for 10 min and 40 cycles of 95°C for 15 s, 57°C for 10 s, and 72°C for 25 s. To specify the integrity

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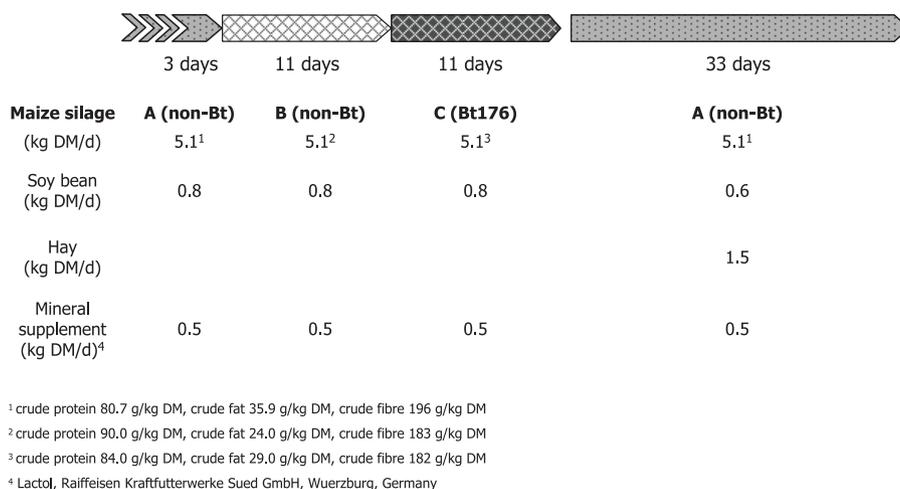
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Maize silage	A (non-Bt)	B (non-Bt)	C (Bt176)	A (non-Bt)
(kg DM/d)	5.1[1]	5.1[2]	5.1[3]	5.1[1]
Soy bean (kg DM/d)	0.8	0.8	0.8	0.6
Hay (kg DM/d)				1.5
Mineral supplement (kg DM/d)[4]	0.5	0.5	0.5	0.5

(Top bar: 3 days | 11 days | 11 days | 33 days)

[1] crude protein 80.7 g/kg DM, crude fat 35.9 g/kg DM, crude fibre 196 g/kg DM

[2] crude protein 90.0 g/kg DM, crude fat 24.0 g/kg DM, crude fibre 183 g/kg DM

[3] crude protein 84.0 g/kg DM, crude fat 29.0 g/kg DM, crude fibre 182 g/kg DM

[4] Lactol, Raiffeisen Kraftfutterwerke Sued GmbH, Wuerzburg, Germany

FIG. 1. Experimental setup and feeding scheme including ingredients and compositions of the diets derived from non-Bt maize silage A, isogenic control maize silage B, and transgenic Bt176 maize silage C. DM, dry matter.

of amplification, all products underwent melting curve analyses by slow heating with a 0.1°C/s increment from 60°C to 99°C and fluorescence collection at 0.1°C intervals after the last cycle. The product sizes of amplicons were verified by electrophoresis after PCR. Dilutions of one representative DNA sample were used to construct gene-specific calibration curves. Data sets of at least two measurements were averaged for each sample, and the results were expressed as ratios of specific bacterial RNA gene concentration to universal 16S rRNA gene concentration.

SigmaStat 3.0 software (SPSS Inc., Chicago, IL) was used for statistical analyses. Comparison of effects of maize silage variety, sampling day, and individual animal in the same subjects were conducted using repeated-measures analysis of variance. When applicable, data were expressed as logarithms ($\log_{10}$) to achieve normal distribution. If an overall significant effect of treatment was found, subsequent Holm-Sidak post hoc analysis was performed. A P value of <0.05 was considered significant.

Figure 2 shows typical patterns of representation for three bacterial species for each of four cows. During the experimental periods considerable population fluctuations occurred within animals and there was substantial animal-to-animal variation. However, each animal showed pronounced and distinctive individual peaks and nadirs throughout the trial that appeared to be independent of the maize silage fed. Figure 3 shows the averaged concentrations of three other bacterial species measured (means for four animals ± standard deviations [SD]). The animal variability in bacterial populations resulted in large SD for each data point. Real-time PCR results are displayed in Fig. 4. In addition to isogenic (maize silage B) and transgenic (maize silage C) maize silages, the effect of another non-Bt maize variety (maize silage A) on the representation of the six bacterial species was also evaluated. The patterns revealed no significant effects of feeding GM Bt176 maize compared to the non-Bt control hybrid on any of the six bacterial species tested. The representations of two species (*F. succinogenes* and *P. ruminicola*) differed during the silage A feeding compared with silages B and C. The relative proportion of *S. ruminantium-M. multiacida* for transgenic silage C

was different from that for silage A ($P = 0.003$). These findings supplement several feeding studies on the influence of GM plants that found no effects on physiological conditions and milk production (summarized in reference 7). Investigations performed in our laboratories also revealed similar degradation patterns of endogenous plant DNA during digestion of Bt176 and the non-Bt isoline (18). Furthermore, a comparable distribution of bacterial clones after feeding Bt silage was reported (4). The present studies, however, showed that feeding another variety of non-Bt silage had an effect on three bacterial strains compared to the non-Bt silage B. Besides a possible relationship to slight variations in nutritional values of silage A (Fig. 1), this might be explained by the additional hay feeding during the fourth experimental period. In contrast to previous periods where the maximal possible amount of maize silage was fed, this period was carried out to determine sampling day and animal variability. Therefore, one specific diet was given over a longer time scale (33 days). In this diet, 1.5 kg hay was fed at 0900 h to decrease the risk of animals developing an acidosis due to high concentrate along with low fiber content. This early time of hay feeding was chosen to have a minimum effect on the sampling procedure, which started at 0700 h. Taking into account the substantial variation between the animals, more animals should be included in further experiments to reduce type II statistical error rates.

The analysis of sampling day variability showed a significant influence on the relative representation levels of *A. lipolyticus* and *S. ruminantium-M. multiacida* (Table 1). To the best of our knowledge, there are no data available on the influence of sampling day on ruminal bacteria. However, Weimer et al. (17) showed that sampling at several time points spanning 99 h had an impact on various analyzed parameters, like dry matter intake, milk production, and acetate, but not on the relative proportions of *Ruminococcus albus*, *R. flavefaciens*, and *F. succinogenes*. The underlying reasons for the significant influence of the sampling period on *S. ruminantium-M. multiacida* remain to be elucidated.

Analyzing the animal variability revealed statistically significant differences in rRNA gene proportion in four bacterial

Fibrobacter succinogenes

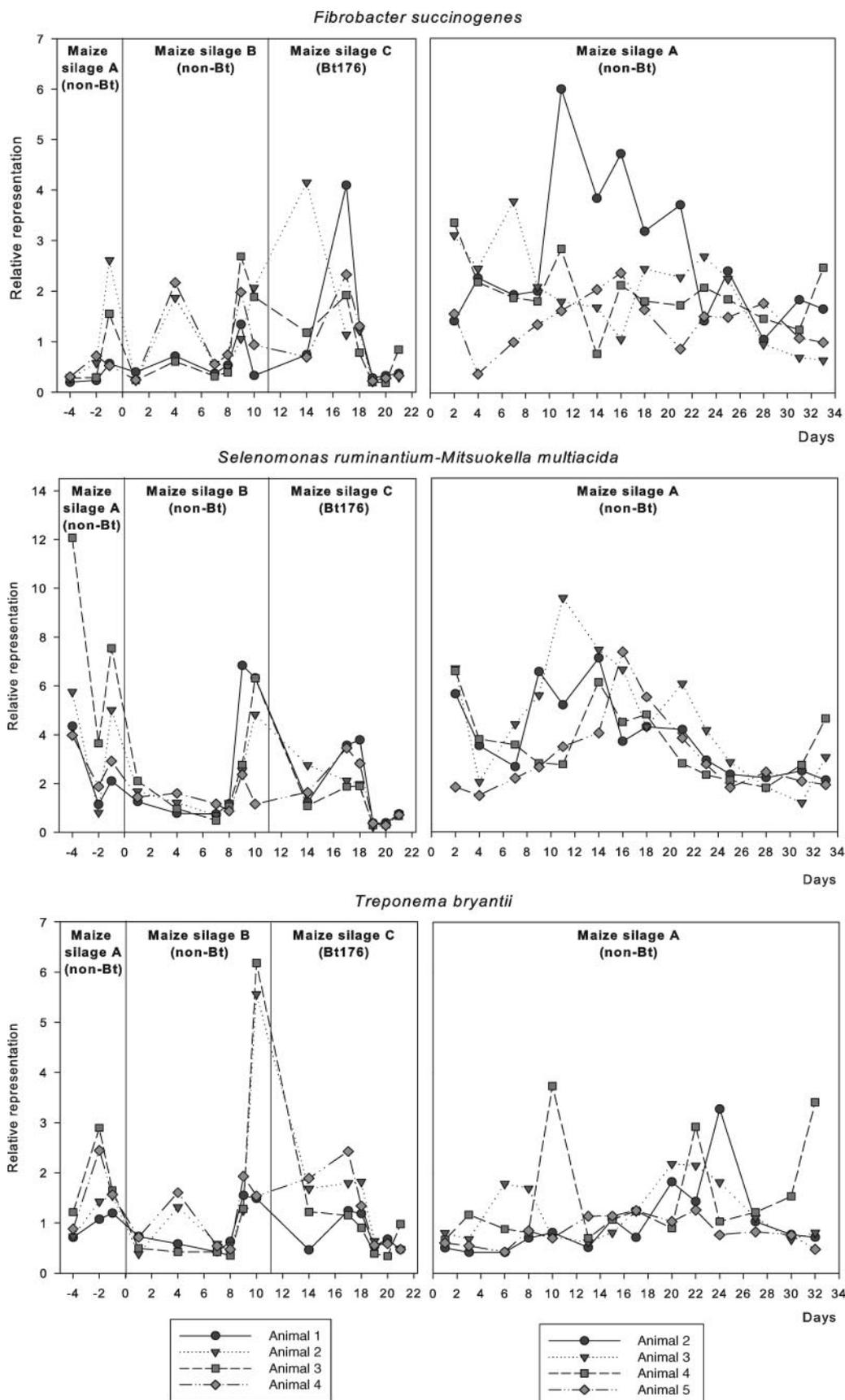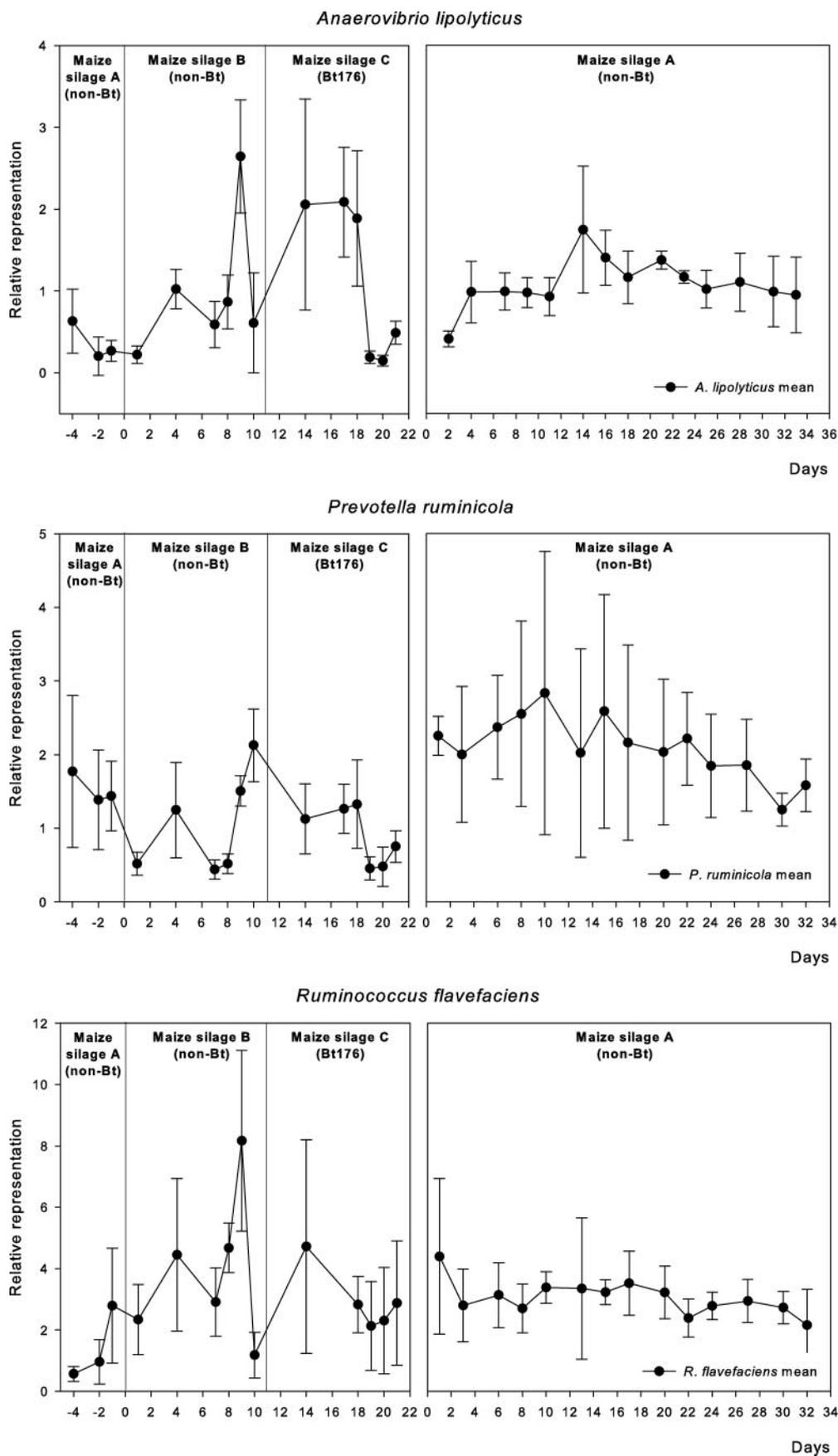## Selenomonas ruminantium-Mitsuokella multiacida

Treponema bryantii

FIG. 2. Relative representation of the ruminal bacteria *Fibrobacter succinogenes*, *Selenomonas ruminantium-Mitsuokella multiacida*, and *Treponema bryantii* in four individual cows after feeding non-Bt maize silages A and B and Bt176 maize silage C. Results are expressed as ratios of specific bacterial rRNA gene concentrations to the universal 16S rRNA gene concentration.
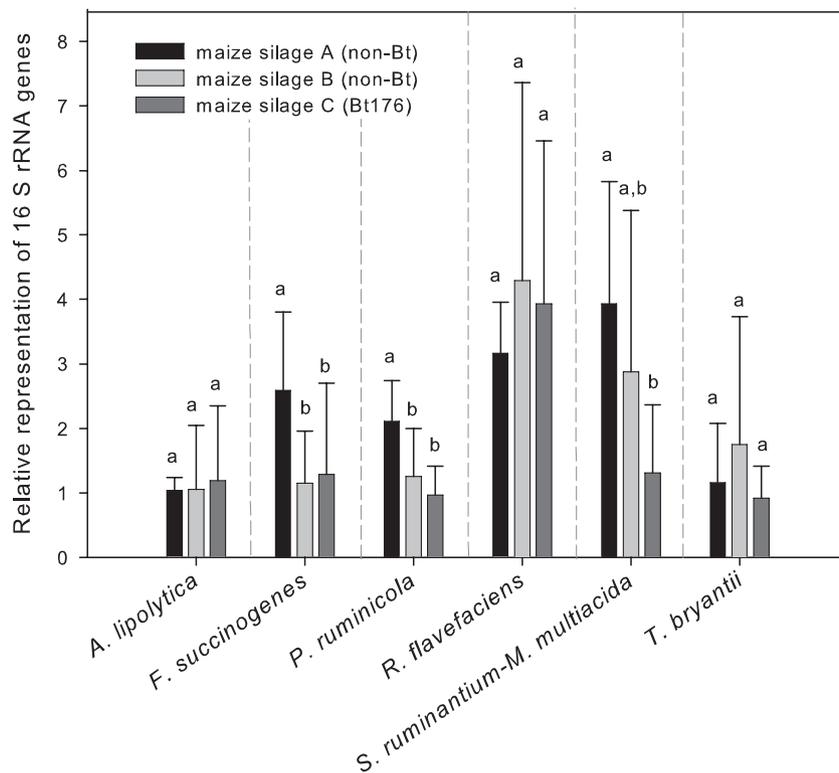
FIG. 3. Relative representation of the ruminal bacteria *Anaerovibrio lipolyticus*, *Prevotella ruminicola*, and *Ruminococcus flavefaciens* (mean ± SD, $n = 4$) after feeding non-Bt maize silages A and B and Bt176 maize silage C. Results are expressed as ratios of specific bacterial rRNA gene concentrations to the universal 16S rRNA gene concentration.

FIG. 4. Effect of feeding non-Bt maize silage A-, isogenic non-Bt maize silage B-, and transgenic Bt176 maize silage C-based diets on the representation of normalized 16S rRNA genes of *Anaerovibrio lipolyticus*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Ruminococcus flavefaciens*, *Selenomonas ruminantium-Mitsuokella multiacida*, and *Treponema bryantii*. Results are expressed as ratios of specific bacterial concentrations to the universal 16S rRNA gene concentration. Each bar represents the mean ± SD for three cows at 4, 7, 9, and 11 days after introduction of the diet; means without common letters are significantly different ($P < 0.05$).

species (Table 1). In the remaining two bacterial species, namely, the saccharolytic spirochetes *T. bryantii* and *R. flavefaciens*, a trend towards an impact of the individual animal was found ($P = 0.076$ and 0.069, respectively).

Taken together, data obtained in our studies are in agreement with those of Weimer et al. (17), who reported no statistical effect of diets containing various amounts of alfalfa hay and barley concentrate on three cellulolytic bacteria (*F. succinogenes*, *R. flavefaciens*, and *R. albus*). Thus, the community of ruminal microbiota seems to be more influenced by the microbial composition of each individual animal than by changes induced by different diets. This may be due to the impact of distinct rumen motility, rumen repletion, rumen outflow, and

the physical condition of cows. Individual and characteristic dynamics and turnover of bacteria were found in each experimental animal. Taking into account the intensive fluid turnover in the rumen, an influence of slightly different sampling sites is unlikely. In order to rule out the effect of nonrepresentative rRNA gene isolation or measurements on estimates of bacterial populations, we repeated DNA extraction procedures until the DNA quality and integrity reached a high standard.

Real-time PCR techniques using 16S rRNA/DNA have been applied to the quantification of ruminal microbes to overcome the inconveniences of conventional techniques like noncultivability, media and/or substrate requirements, and phenotype-based classification of important active bacterial species (15). Measuring the concentration of a specific bacterium in an ecosystem containing many bacterial members using this technique is complex. It is influenced by small-scale variation in the number of rRNA operons, even among strains of a given species (1, 6). No pattern of high or low operon numbers among more distantly related organisms was reported, so correction factors can be applied only to overall estimates of microbial diversity, not to individual phylogenetic groups. The number of replication forks and the location of the rRNA operon relative to the origin of replication can further increase the number of rRNA operons (8, 13). However, it should be considered that in quantifying bacterial numbers in environmental samples real-time PCR has been shown to be more reliable than other methods such as single-strand conformation polymorphism

TABLE 1. Probability values showing the effect of the sampling day and the individual animal on six bacterial strains in the rumen of cows fed non-Bt maize silage A for 33 days

Bacterial species	P^a for effect of:	
	Sampling day	Individual animal
A. lipolyticus	<0.001*	0.003*
F. succinogenes	0.364	0.010*
P. ruminicola	0.592	0.001*
R. flavefaciens	0.523	0.069
S. ruminantium-M. multiacida	<0.001*	0.032*
T. bryantii	0.264	0.076

^{a} *, significant difference.

analysis, temperature gradient gel electrophoresis, and fluorescence in situ hybridization (5, 12). Reproducibility and accuracy of our real-time PCR measurements were confirmed by the low intra- and interday coefficients of variance (0.67 to 1.71%).

In conclusion, the microbial population is the result of complex interactions and seems to depend more on the individual animal and sampling day than on the variety of maize used for the production of the respective maize silage. To further evaluate the animal variability during one specific feeding regimen, it is advisable to include more animals on different sampling days. Additionally, the feeding rotation may be changed to completely exclude rotation effects.

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