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Accumulation of *trans* $C_{18:1}$ Fatty Acids in the Rumen after Dietary Algal Supplementation Is Associated with Changes in the *Butyrivibrio* Community▽

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Optimization of the fatty acid composition of ruminant milk and meat is desirable. Dietary supplementation of algae was previously shown to inhibit rumen biohydrogenation, resulting in an altered milk fatty acid profile. Bacteria involved in biohydrogenation belong to the *Butyrivibrio* group. This study was aimed at relating accumulation of biohydrogenation intermediates with shifts in *Butyrivibrio* spp. in the rumen of dairy cows. Therefore, an experiment was performed with three rumen-fistulated dairy cows receiving a concentrate containing algae (9.35 g/kg total dry matter [DM] intake) for 20 days. Supplementation of the diet with algae inhibited biohydrogenation of $C_{18:2}$ omega 6 (*n*-6) and $C_{18:3}$ *n*-3, resulting in increased concentrations of biohydrogenation intermediates, whereas $C_{18:0}$ decreased. Addition of algae increased ruminal $C_{18:1}$ *trans* fatty acid concentrations, mainly due to 6- and 20-fold increases in $C_{18:1}$ *trans* 11 (*t*11) and $C_{18:1}$ *t*10. The number of ciliates (5.37 log copies/g rumen digesta) and the composition of the ciliate community were unaffected by dietary algae. In contrast, supplementation of the diet with algae changed the composition of the bacterial community. Primers for the *Butyrivibrio* group, including the genera *Butyrivibrio* and *Pseudobutyrivibrio*, were specifically designed. Denaturing gradient gel electrophoresis showed community changes upon addition of algae without affecting the total amount of *Butyrivibrio* bacteria (7.06 log copies/g rumen DM). Clone libraries showed that algae affected noncultivated species, which cluster taxonomically between the genera *Butyrivibrio* and *Pseudobutyrivibrio* and might play a role in biohydrogenation. In addition, 20% of the clones from a randomly selected rumen sample were related to the $C_{18:0}$-producing branch, although the associated $C_{18:0}$ concentration decreased through supplementation of the diet with algae.

Rumen biohydrogenation is the microbial saturation of dietary unsaturated fatty acids, which limits the availability of health-associated polyunsaturated fatty acids (PUFA) in ruminant meat and milk. Therefore, understanding the ruminal biohydrogenation process is important to generate healthier ruminant products. Interest in research on microorganisms involved in rumen biohydrogenation of linoleic ($C_{18:2}$ omega 6 [*n*-6]) or linolenic ($C_{18:3}$ *n*-3) acid is currently growing. Polan et al. (26) associated rumen biohydrogenation activity with a *Butyrivibrio fibrisolvens* strain. Since then, several researchers have confirmed the active role of *Butyrivibrio* species in the partial or complete biohydrogenation of unsaturated C_{18} fatty acids (19). Bacteria involved in C_{18} biohydrogenation are grouped in the "*Butyrivibrio* group," which includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* and the species *Clostridium proteoclasticum* (24). The last was recently proposed for reclassification as *Butyrivibrio proteoclasticus* (21), and hereafter, we refer to *C. proteoclasticum* as *B. proteoclasticus*. Although isolation and pure-culture studies have provided fundamental insight into the bacteria involved in rumen biohydrogenation, the relative importance of individual strains in the in vivo ruminal lipid metabolism remains largely unclear (25). Indeed, complete in vivo biohydrogenation is thought to be a synergistic process involving a consortium of bacteria, each having a share in the conversion of unsaturated to more saturated fatty acids (15).

Marine products, such as fish oil and algae, proved to possess high effectiveness in the inhibition of rumen biohydrogenation of unsaturated fatty acids (5, 30). The long-chain PUFA eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) was found to be the active compounds in this process (1, 7). Their supplementation reduced $C_{18:0}$ production, resulting in the accumulation of various hydrogenation intermediates, predominantly $C_{18:1}$ *trans* 11 (*t*11) and $C_{18:1}$ *t*10. Incomplete biohydrogenation, induced by dietary algae, was found to be associated with the disappearance of some ciliates (5). Based on this study, we hypothesized that ciliates and/or their associated bacteria could play a role in rumen biohydrogenation (5). However, further research on rumen biohydrogenation of $C_{18:2}$ *n*-6 by pure *Isotricha prostoma* and its associated bacteria showed only minor biohydrogenation by the protozoal species, its bacterial symbionts, or a mixture of both (6).

Since the majority of bacteria are as yet unculturable and in vitro findings do not always reflect in vivo mechanisms, the

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current in vivo research with DHA-enriched microalgae is aimed at studying mutual changes in the rumen C_{18} biohydrogenation and the composition of the rumen microbial community through cultivation-independent techniques. More specifically, a PCR-denaturing gradient gel electrophoresis (DGGE) and real-time quantitative PCR (qPCR) method was optimized for the *Butyrivibrio* group to examine the time-dependent shifts in rumen *Butyrivibrio* species and rumen $C_{18:1}$ *trans* accumulation following supplementation of the diet with algae.

MATERIALS AND METHODS

Animals and diets. Three rumen-cannulated (10-cm inside diameter; Bar-Diamond Inc., Parma, ID) Holstein-Friesian cows (612 ± 32 kg body weight) in mid-lactation (172 ± 45 days in milk) were used to evaluate the effect of feeding a concentrate containing algae on both the rumen fatty acid composition and the rumen microbial population. The experimental setup involved a period without supplementation of the diet with algae, with rumen sampling 2 days before the start of the supplementation (day −2) and 20 days of dietary algal supplementation with samplings on days 6, 13, and 20. The basal diet consisted of a mixed ration based on grass silage, corn silage, wheat, straw, rapeseed meal, soybean meal, and cane molasses. The basal diet was supplemented with 2 kg of concentrate incorporating no or 11% (on a concentrate-product basis) DHA-enriched microalgae (*Schizochytrium* sp.; DHA Gold; Martek Biosciences Corp., Columbia, MD). The concentrate was offered in two equal portions, after the morning (0900 h) and after the evening (2100 h) milking. Following 15 min of feeding with the concentrate containing algae, refusals were placed into the rumen via the fistula to ensure each cow had its allotment. This was required only on the first day of the algal-concentrate feeding. After concentrate feeding, the basal diet was offered ad libitum (30 kg fresh matter after both morning and evening concentrate feedings). Grass silage, corn silage, and concentrate were sampled four times during the experimental period and stored frozen. Samples were freeze-dried and pooled by feed component prior to fatty acid analysis. The ingredient, chemical, and fatty acid compositions of both diets are shown in Table 1.

Sampling. Ruminal digesta were collected from each animal on days −2, 6, 13, and 20 and sampled just before (0 h) and 1, 2, 4, and 6 h after the morning feeding. Equal amounts of ruminal digesta were collected by hand from the front and middle of the ventral sac and from the cranial sac. After thorough mixing, an aliquot of 10 ml was stored at −80°C until DNA extraction was performed. Another aliquot of approximately 250 g was freeze-dried prior to fatty acid analysis.

Rumen fatty acid analysis. Fatty acids in freeze-dried ruminal digesta (2.5 g) were extracted with chloroform/methanol (2/1 [vol/vol]) as described by Chow et al. (11). Tridecanoic acid (10 mg; Sigma, Bornem, Belgium) was added as an internal standard. The extracted fatty acids were methylated as described by Raes et al. (27) with NaOH/MeOH (0.5 mol/liter; 3 ml), followed by HCl/MeOH (1/1 [vol/vol]; 2 ml). The FAME were extracted with 3 and 2 ml hexane, pooled, and evaporated to dryness under N_2. The residue was dissolved in 1 ml hexane and analyzed by gas chromatography (HP 6890; Agilent Technologies, Brussels, Belgium) on a CP-Sil88 column for FAME (100 m by 250 μm by 0.2 μm; Chrompack, Middelburg, The Netherlands) (27). FAME were identified using external standards (S37 [Supelco, Poole, Dorset, United Kingdom]; conjugated linoleic acid [CLA] *cis* 9 [*c*9] *t*11, CLA *t*10*c*12, and odd- and branched-chain fatty acids [Larodan Fine Chemicals AB, Malmö, Sweden]) and quantified using the internal standard. Some C_{18} fatty acids were identified according to the elution sequence reported by Ratnayake (28) and Shingfield et al. (31).

Bacterial strains, growth conditions, and DNA extraction. *B. fibrisolvens* DSM 3071 (10) was purchased from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). *B. fibrisolvens* MDT-5 (13) was kindly donated by T. Hino (Meiji University, Kawasaki, Japan). The bacteria were anaerobically cultured for 64 h at 39°C in a rumen medium containing glucose, cellobiose, maltose, and starch as carbon sources (medium 330; DSMZ, Braunschweig, Germany). Bacterial DNA (2 ml of growth medium) and total DNA of ruminal digesta (0.5 g), sampled before the morning feeding (0 h), were extracted following the method of Boon et al. (9). DNA extracts of *Escherichia coli* DH5α (Invitrogen, Merelbeke, Belgium), *Lactobacillus brevis* LMG 7761 (38), and *Bifidobacterium animalis* LMG 11580 (4) were used as negative controls. The *Lactobacillus* and *Bifidobacterium* strains were purchased from the Belgian culture collection (BCCM/LMG, Gent, Belgium).

TABLE 1. Ingredient, chemical, and fatty acid composition of the diet before (day −2) and during algal-concentrate feeding

Parameter	Value	
	Day −2	ALG[a]
Ingredients (g/kg DM)		
Grass silage	412	401
Corn silage	281	273
Wheat	83.7	81.3
Extracted rapeseed meal	62.7	61.0
Soybean meal	62.7	61.0
Straw	21.6	21.0
Standard concentrate	75.7	16.2
Algal concentrate	−[b]	85.0
Chemical composition (g/kg DM)[c]		
OM	930	933
Crude protein	158	160
Crude fat	30.9	34.4
Crude fiber	197	189
NDF	398	383
ADF	223	213
Fatty acid composition (g/kg DM)		
$C_{12:0}$	1.15	0.82
$C_{14:0}$	0.43	0.90
$C_{16:0}$	3.14	4.54
$C_{18:0}$	0.45	0.45
$C_{18:1}$ *c*9	3.88	3.56
$C_{18:2}$ *n*-6	6.32	6.58
$C_{18:3}$ *n*-3	4.14	4.30
$C_{22:6}$ *n*-3	−	2.09
Total fatty acids	21.4	26.4

[a] ALG, algal-concentrate feeding.
[b] −, not present.
[c] OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber.

Ciliate, bacterial, and *Butyrivibrio* PCRs. A nested-PCR approach was used to amplify a fragment of the 18S rRNA genes of ciliates for DGGE according to the method of Boeckaert et al. (5). General bacterial PCR for DGGE was performed as described by Boon et al. (8) using the bacterial primers P338F-GC and P518r. It should be noted that research performed by Huws et al. (17) indicated that these primer pairs also amplify nonspecific protozoal 18S rRNA, fungal 18S rRNA, and archaeal 16S rRNA. A third PCR was aimed at amplifying a fragment of the 16S rRNA gene of the *Butyrivibrio* group. *Butyrivibrio*-specific primers were designed using PRIMROSE software (3) based on sequences from the genera *Butyrivibrio* and *Pseudobutyrivibrio*. After primer design, the primer pair candidates were tested in silico using PRIMROSE (3) and the Ribosomal Database Project (34). The sequences (5′-3′) of the forward and reverse primer were GYG AAG AAG TAT TTC GGT AT (B395f) and CCA ACA CCT AGT ATT CAT C (B812r), respectively. These primers also allowed annealing for other genera within the family *Lachnospiraceae*. A 40-bp GC clamp (9) was attached to the forward primer for DGGE. The amplification conditions were as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and final extension at 72°C for 10 min. The PCR mixture was prepared according to the manufacturer's instructions (Fermentas, St. Leon-Rot, Germany) and contained 1 μl DNA extract, 0.5 μl of each primer (10 μM stock), 0.5 μl deoxynucleotide triphosphate mixture (10 mM each), 2.5 μl 10× *Taq* buffer with KCl (500 mM), 1.5 μl $MgCl_2$ (25 mM), 0.125 μl *Taq* DNA polymerase (0.6 U), 0.06 μl bovine serum albumin, and DNase-RNase-free filter-sterilized water (Sigma, Bornem, Belgium) to a final volume of 25 μl. The amplicons were visualized by gel electrophoresis with 1% agarose and ethidium bromide.

DGGE analysis. Ciliate and bacterial DGGE were performed as described by Boeckaert et al. (5) and Boon et al. (8), respectively. For the *Butyrivibrio*-specific DGGE, *Butyrivibrio*-specific PCR fragments were loaded onto a 7% (wt/vol) polyacrylamide gel (40% acrylamide, 77.8%; 2% bis-acrylamide, 22.2%) in 1× TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA, pH 8.5) with denaturing

gradients ranging from 45% to 60%. The electrophoresis was run for 16 h at 60°C and 45 V. DGGE patterns were visualized by staining with Sybr green I nucleic acid gel stain (Molecular Probes, Eugene, OR). The DGGE patterns obtained were analyzed with BioNumerics software version 3.5 (Applied Maths, Kortrijk, Belgium). Similarities were calculated by the Pearson correlation, taking into account band intensity and band position. The clustering algorithm of Ward (35) was used to calculate dendrograms.

Cloning and identification of *Butyrivibrio* species. The *Butyrivibrio* sp. PCR product of cow 1 on day 6 of algal feeding was cloned using a Topo-TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions in order to create a clone library. For each clone, an aliquot of 800 μl was stored in 40% (vol/vol) glycerol, while plasmid DNA was isolated from the remaining liquid using the High Pure plasmid isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Subsequent DGGE analysis excluded identical clones. The resulting 23 exclusive clones were identified by sequencing the partial 16S rRNA gene fragments (ITT Biotech, Bielefeld, Germany). Additionally, *Butyrivibrio* DGGE bands, which specifically modified upon algal feeding, were excised. After *Butyrivibrio*-specific PCR and purification of the PCR product (Qiaquick PCR Purification Kit; Qiagen Benelux B.V., Venlo, The Netherlands), these bands were cloned with a Topo-TA cloning kit as described above. Close relatives of the 16S rRNA sequences were identified with the sequence match server of Ribosomal Database Project II (34). Additionally, sequences were aligned with the NAST software (12). The alignment was manually checked using the ARB aligner tool (ARB Software) (37), after which the sequences were added to the original phylogenetic tree (Greengenes database, January 2008 [12]) using Parsimony (ARB Software) (37) without changing the tree topology.

qPCR. Ciliate and total bacterial rRNA gene copies present in the DNA extract of each ruminal-digesta sample were quantified as described by Boeckaert et al. (5) and Boon et al. (9), respectively. *Butyrivibrio* rRNA gene copies present in the DNA extract of each sample were quantified using an ABI Prism SDS 7000 instrument (Applied Biosystems, Lennik, Belgium) following the principle of Heid et al. (16). Dilutions (1:20) of DNA from all samples were added to amplification reaction mixtures (25 μl) containing 12.5 μl Sybr green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom), 6 μl RNA-free water, 0.75 μl B395f primer (10 μM stock), 0.75 μl B812r primer (10 μM stock), and 5 μl DNA. The cycling conditions were 1 cycle of 50°C for 2 min and 95°C for 10 min and 40 cycles of 95°C for 1 min, 54°C for 30 s, and 60°C for 1 min. Measurements were done in triplicate for each run. A standard curve for qPCR was constructed using six different DNA concentrations ($n = 3$) ranging from 2.67 copies to 2.67×10^8 copies of DNA per μl. A *Butyrivibrio* 417-bp PCR fragment inserted in a Topo vector (see above) was used as a template for the standard curve. The slope of the standard curve was −3.42 ($R^2 = 0.99$).

Statistical analysis. Rumen fatty acid and qPCR data were analyzed using the Mixed procedure of the SAS Institute (29). The model for rumen fatty acid data included the fixed effects of day and time of sampling and their interaction and the random effect of cow, assuming an autoregressive order one covariance structure fitted on the basis of Akaike information and Schwarz Bayesian model fit criteria. The time of sampling was treated as a repeated measure. The statistical model for qPCR data included the fixed effect of day and the random effect of cow, assuming the covariance structure as described above. Least squares means were reported, and significance was declared at a P value of <0.05.

Nucleotide sequence accession numbers. The nucleotide sequences for clones NC1 through NC23 and B1-1 through B4-3 have been deposited in the GenBank database under accession numbers EU839861 to EU839892.

RESULTS

Rumen fatty acid composition. Supplementation of the diet with algae reduced saturated fatty acids ($P < 0.001$), while monounsaturated fatty acid ($P < 0.001$) increased ($P < 0.001$) (Table 2). PUFA ($P = 0.001$) and odd- and branched-chain fatty acids ($P < 0.001$) also increased upon algal feeding, whereas the amounts of $C_{18:0}$ strongly decreased ($P < 0.001$). All $C_{18:1}$ fatty acids increased ($P < 0.001$) after algal feeding, except for $C_{18:1}$ *c*12. Dietary algae resulted in 6- and 20-fold increases in $C_{18:1}$ *t*11 and $C_{18:1}$ *t*10, respectively. $C_{18:1}$ *t*4 ($P < 0.001$), $C_{18:1}$ *t*5 ($P < 0.001$), and $C_{18:1}$ *t*11 ($P < 0.001$) showed maximum concentrations on day 6, after which they decreased, whereas the greatest concentration of $C_{18:1}$ *t*10 ($P < 0.001$) was observed on day 13. $C_{18:1}$ *c*14 + *t*16 ($P < 0.001$), $C_{18:1}$ *c*15 ($P < 0.001$), and $C_{18:1}$ *t*12 ($P < 0.001$) progressively increased with time of algal feeding. Besides increased monounsaturated fatty acid proportions, dietary algae increased PUFA proportions due to an increase in $C_{18:3}$ *c*9*t*11*c*15 ($P = 0.008$), $C_{18:2}$ *t*11*c*15 ($P < 0.001$), CLA isomers ($P < 0.001$), and $C_{22:6}$ *n*-3 ($P < 0.001$). CLA *c*9*t*11 reached its maximum concentration on day 6 after algal feeding and significantly decreased thereafter. Concentrations of CLA *c*9*t*11 on day 20 did not differ from the values before algal feeding, whereas the increased concentrations of CLA *t*10*c*12 and CLA *c*9*c*11 + *t*11*c*13 after dietary algal supplementation were stable until at least day 20 after algal feeding. Bacterial synthesized odd- and branched-chain fatty acids increased through algal feeding. This was mainly due to higher proportions of *iso* $C_{15:0}$ ($P < 0.001$), *iso* $C_{17:0}$ ($P < 0.001$), *anteiso* $C_{15:0}$ ($P < 0.001$), and *anteiso* $C_{17:0}$ ($P < 0.001$).

Ciliate community analysis. DGGE profiles of rumen ciliates before and during algal feeding were similar for all cows (data not shown). Cluster analysis according to Pearson correlation grouped 9 of the 12 DGGE profiles in one cluster independent of cow or days on algal feeding. This group showed four to six bands. Ciliate DGGE profiles of cow 1 on day 6 and day 20 and of cow 2 on day −2 showed greater diversity, with the number of bands varying between 7 and 10. Therefore, these DGGE patterns clustered separately.

The number of ciliates present in the bovine rumen content before and during algal feeding was estimated by amplifying the 18S rRNA gene fragment of the ciliates and measuring the increasing amounts of amplification products. Before algal feeding, ciliates numbered 5.68 ± 0.52 log copies per g ruminal digesta (mean ± standard deviation). The numbers of ciliates were unaffected ($P = 0.359$) by algal feeding (5.14 ± 0.10, 5.17 ± 0.66, and 5.48 ± 0.07 log copies per g ruminal digesta on day 6, day 13, and day 20, respectively).

Bacterial community analysis. Rumen bacterial DGGE profiles showed high complexity, with more than 30 bands both before and during algal feeding. Cluster analysis (Pearson correlation) resulted in two major clusters corresponding to the dietary treatments (Fig. 1). Nevertheless, these shifts in the bacterial community were difficult to link with the biohydrogenation activity, since no (dis)appearance of specific bands could be identified.

Algal feeding had no influence on total bacterial numbers ($P = 0.091$), and the numbers were on average 10.5 ± 0.15 log copies per g dry ruminal digesta (mean ± standard deviation).

***Butyrivibrio* community analysis.** The primers designed to detect *Butyrivibrio* spp. and relatives were tested for their specificity with *B. fibrisolvens* DSM 3071 and *B. fibrisolvens* MDT-5 as positive controls, whereas *E. coli* DH5α, *L. brevis* LMG 7761, and *B. animalis* LMG 11580 were used as negative controls. The primer set produced PCR products for both *B. fibrisolvens* strains of the expected size of 417 bp, whereas no amplification was observed for the negative control strains.

Butyrivibrio-specific DGGE generated around 15 bands, which clearly changed with time on dietary algae. Cluster analysis separated the DGGE profiles of rumen contents taken on day 13 and day 20 from cows 1 and 2 from the other DGGE profiles (Fig. 2). For these two cows, a further distinction was possible between DGGE profiles of rumen contents prior to and after the 6 days of algal feeding. For the third cow, algal

TABLE 2. Effect of time of algal feeding on the ruminal fatty acid composition[a]

Fatty acid	Sampling day				SEM[b]	Significance		
	−2	6	13	20		D[c]	T[d]	D*T[e]
$C_{12:0}$	0.50a	0.10b	0.11b	0.11b	0.014	<0.001	0.043	0.007
$C_{14:0}$	0.44b	0.54ab	0.54ab	0.64a	0.039	0.003	0.021	0.298
$C_{16:0}$	4.81b	6.28a	6.21a	6.73a	0.217	<0.001	0.598	0.652
$C_{14:1}$ *c*9	0.01ab	0.01ab	0.01b	0.01a	0.001	0.051	0.468	0.624
$C_{16:1}$ *c*9	0.03c	0.07b	0.07a	0.07ab	0.006	<0.001	0.036	0.796
$C_{15:0}$	0.35	0.37	0.33	0.37	0.030	0.684	0.572	0.701
$C_{17:0}$	0.14	0.16	0.16	0.17	0.011	0.144	0.064	0.568
iso $C_{13:0}$	0.01b	0.02a	0.02a	0.02a	0.001	<0.001	0.016	0.440
iso $C_{14:0}$	0.03	0.03	0.03	0.03	0.003	0.101	0.003	0.417
iso $C_{15:0}$	0.11b	0.18a	0.15a	0.17a	0.010	<0.001	0.004	0.372
iso $C_{16:0}$	0.04c	0.08a	0.06b	0.08a	0.009	<0.001	0.027	0.769
iso $C_{17:0}$	0.07b	0.14a	0.15a	0.16a	0.008	<0.001	0.003	0.128
anteiso $C_{13:0}$	0.03a	0.03b	0.03b	0.03ab	0.003	0.014	0.001	0.431
anteiso $C_{15:0}$	0.22c	0.30a	0.27b	0.30ab	0.011	<0.001	0.001	0.258
anteiso $C_{17:0}$	0.06c	0.10b	0.10b	0.11a	0.003	<0.001	<0.001	0.310
$C_{18:3}$ *n*-3	1.41ab	1.29b	1.65a	1.47ab	0.085	0.019	<0.001	0.201
$C_{18:2}$ *n*-6	2.37	2.21	2.39	2.08	0.131	0.254	<0.001	0.497
$C_{18:3}$ *c*9*t*11*c*15	0.08b	0.16a	0.15a	0.15a	0.020	0.008	0.788	0.527
$C_{18:2}$ *t*11*c*15	0.22b	0.65a	0.63a	0.67a	0.043	<0.001	<0.001	0.142
CLA *c*9*t*11	0.05c	0.13a	0.09b	0.08bc	0.013	<0.001	0.616	0.380
CLA *t*10*c*12	0.04b	0.06a	0.07a	0.07a	0.005	<0.001	0.033	0.265
CLA *c*9*c*11 + t11*c*13	0.02c	0.03bc	0.03b	0.04a	0.002	<0.001	0.009	0.740
$C_{18:1}$ *c*9	1.78b	2.59a	2.45a	2.32a	0.123	<0.001	<0.001	0.233
$C_{18:1}$ *c*11	0.35b	0.69a	0.67a	0.68a	0.024	<0.001	0.319	0.698
$C_{18:1}$ *c*12	0.21a	0.12b	0.12b	0.14b	0.013	<0.001	0.384	0.390
$C_{18:1}$ *c*13	0.02c	0.09b	0.09ab	0.10a	0.006	<0.001	0.124	0.257
$C_{18:1}$ *c*14 + *t*16	0.26c	0.40b	0.43ab	0.52a	0.055	<0.001	0.044	0.634
$C_{18:1}$ *c*15	0.08c	0.25b	0.28a	0.31a	0.014	<0.001	0.002	0.134
$C_{18:1}$ *t*4	0.02c	0.05a	0.04b	0.04b	0.003	<0.001	0.013	0.534
$C_{18:1}$ *t*5	0.02c	0.07a	0.06b	0.05b	0.002	<0.001	0.005	0.388
$C_{18:1}$ *t*6 to *t*8	0.13b	0.38a	0.37a	0.34a	0.023	<0.001	0.013	0.506
$C_{18:1}$ *t*9	0.10b	0.57a	0.56a	0.63a	0.054	<0.001	0.009	0.621
$C_{18:1}$ *t*10	0.23c	3.60b	5.19a	5.08a	0.368	<0.001	0.129	0.392
$C_{18:1}$ *t*11	0.92[d]	6.38a	4.32c	5.42b	0.201	<0.001	0.001	0.117
$C_{18:1}$ *t*12	0.23c	1.17ab	1.05b	1.21a	0.056	<0.001	0.002	0.227
$C_{18:1}$ *t*13 to *t*14	0.44b	1.45a	1.41a	1.69a	0.118	<0.001	<0.001	0.090
$C_{18:1}$ *t*15	0.27b	0.67a	0.69a	0.81a	0.060	<0.001	0.007	0.384
$C_{18:0}$	16.0a	1.27b	1.39b	1.22b	0.354	<0.001	0.534	0.762
$C_{22:6}$ *n*-3[f]	ND[l]	0.72a	0.71a	0.91a	0.088	0.225	<0.001	0.904
SFA[g]	22.8a	9.61b	9.54b	10.2b	0.536	<0.001	0.553	0.542
MUFA[h]	5.10b	18.5a	17.8a	19.4a	0.460	<0.001	0.042	0.399
PUFA[i]	4.19b	5.25a	5.72a	5.48a	0.248	0.001	<0.001	0.405
OBCFA[j]	1.08b	1.42a	1.29a	1.45a	0.051	<0.001	0.007	0.242
n-3[k]	1.41b	2.01a	2.36a	2.38a	0.107	<0.001	<0.001	0.178
n-3/*n*-6	0.59c	0.93b	0.99b	1.14a	0.033	<0.001	0.261	0.444

[a] In mg/g ruminal digesta DM ($n = 60$). The data represent the means of fatty acids present in ruminal digesta collected at five different time points from three different cows. Means within a row with different lowercase letters following (a to c) differ ($P \leq 0.05$).
[b] SEM, standard error of the mean.
[c] D, effect of days on algal-concentrate feeding.
[d] T, effect of time of rumen sampling.
[e] D*T, interaction between days and sampling time.
[f] $Y_{ijk} = \mu + A_i + B_j + C_k + AB_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the response, A_i the effect of day (day 6, day 13, and day 20), B_j the effect of time of sampling (0, 1, 2, 4, and 6 h), C_k the random effect of cow, AB_{ij} the interaction between day and time of sampling, and ε_{ijk} the residual error. Means with letters following differ from 0.
[g] SFA, saturated fatty acids. Σ ($C_{12:0}$; $C_{14:0}$; $C_{15:0}$; $C_{16:0}$; $C_{17:0}$; $C_{18:0}$; *iso* $C_{13:0}$; *iso* $C_{14:0}$; *iso* $C_{15:0}$; *iso* $C_{16:0}$; *iso* $C_{17:0}$; *anteiso* $C_{13:0}$; *anteiso* $C_{15:0}$; *anteiso* $C_{17:0}$).
[h] MUFA, monounsaturated fatty acids. Σ ($C_{14:1}$ *c*9; $C_{16:1}$ *c*9; $C_{18:1}$ *c*9; $C_{18:1}$ *c*11; $C_{18:1}$ *c*12; $C_{18:1}$ *c*13; $C_{18:1}$ *c*14 + *t*16; $C_{18:1}$ *c*15; $C_{18:1}$ t4; $C_{18:1}$ *t*5; $C_{18:1}$ *t*6 to *t*8; $C_{18:1}$ *t*9; $C_{18:1}$ *t*10; $C1_{8:1}$ *t*11; $C_{18:1}$ *t*12; $C_{18:1}$ *t*13 to *t*14; $C_{18:1}$ *t*15).
[i] PUFA, Σ ($C_{18:3}$ *n*-3; $C_{18:2}$ *n*-6; $C_{18:3}$ *c*9*t*11*c*15; CLA *c*9*c*11 + *t*11*c*13; $C_{18:2}$ *t*11*c*13; CLA c9*t*11; CLA *t*10*c*12; $C_{22:6}$ *n*-3).
[j] OBCFA, odd- and branched-chain fatty acids. Σ ($C_{15:0}$; $C_{17:0}$; *iso* $C_{13:0}$; *iso* $C_{14:0}$; *iso* $C_{15:0}$; *iso* $C_{16:0}$; *iso* $C_{17:0}$; *anteiso* $C_{13:0}$; *anteiso* $C_{15:0}$; *anteiso* $C_{17:0}$).
[k] *n*-3, Σ ($C_{18:3}$ *n*-3; $C_{22:6}$ *n*-3).
[l] ND, not detected.

feeding did not result in major shifts in the *Butyrivibrio* population, as DGGE profiles clustered together. Some bands disappeared from day 6 onward, whereas other bands became more pronounced on day 13 and day 20 (Fig. 2).

The number of *Butyrivibrio* species in the rumen content was determined before and during algal feeding by qPCR. Before algal feeding, *Butyrivibrio* species numbered 7.21 ± 0.05 log copies per g ruminal digesta (mean ± standard deviation). The

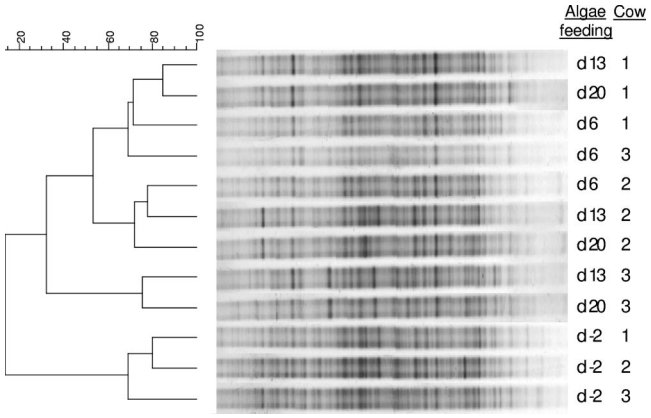

FIG. 1. Cluster analysis of the DGGE profile of total bacteria present in the rumen of cows fed algae. d, day.

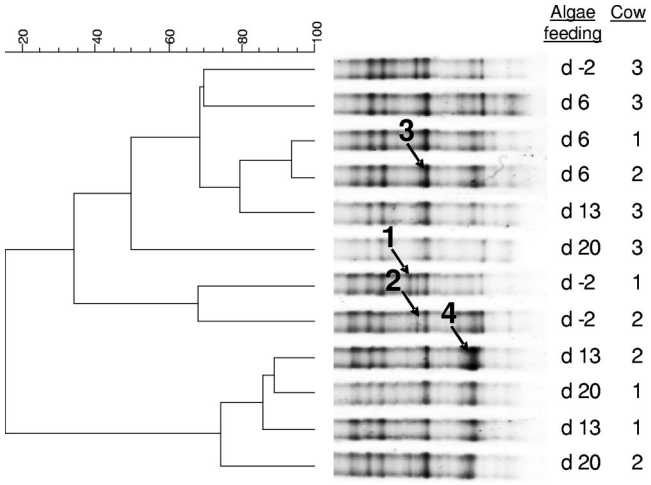

FIG. 2. Cluster analysis of the DGGE profile of *Butyrivibrio* spp. present in the rumen of cows fed algae. The numbers in the profile indicate disappearing (1 and 2) and more pronounced (3 and 4) bands following algal-concentrate feeding. d, day.

numbers decreased numerically ($P = 0.246$) during algal feeding (6.96 ± 0.18, 7.04 ± 0.02, and 7.02 ± 0.28 log copies per g ruminal digesta on day 6, day 13, and day 20, respectively).

Cloning and sequence analysis of the *Butyrivibrio* community. A clone library was constructed with the PCR product of cow 1 on day 6 in order to identify the bacterial species detected with the *Butyrivibrio*-specific primers. Phylogenetic clustering (Fig. 3) based on 16S rRNA sequence (417 bp) analysis indicated that the 23 clones were related to bacterial species within the genus *Butyrivibrio*, the genus *Lachnospiraceae* incertae sedis, and other, so far unknown genera within the family *Lachnospiraceae* and were closely related to the genera *Butyrivibrio* and *Pseudobutyrivibrio*.

The nine clones originating from four specific, changing DGGE bands upon supplementation of the diet with algae were phylogenetically classified (Fig. 3). The majority of these clones (eight) clustered on branches between the genus *Butyrivibrio* and the genus *Pseudobutyrivibrio*. They include the four clones (B1-1, B1-2, B2-1, and B2-2) originating from the two disappearing bands (bands 1 and 2 in Fig. 2) and four of the five clones (B3-1, B3-2, B4-1, and B4-3) originating from the more pronounced bands (bands 3 and 4 in Fig. 2). Disappearing bands did not cluster apart from more pronounced bands and did not cluster in the *B. proteoclasticus* branch. Clone B4-2 clustered distinctly and showed genetic relationship with butyrate-producing bacteria.

DISCUSSION

The aim of the current study was to relate inhibition of rumen biohydrogenation with changes in ciliates, total bacteria, and *Butyrivibrio* species. In order to induce accumulation of biohydrogenation intermediates in the rumen, DHA-enriched algae were used (5). A unique feature of this study was the identification of altering *Butyrivibrio* DGGE bands by means of cloning and their possible relationship with decreased $C_{18:0}$ and increased $C_{18:1}$ *trans* fatty acid concentrations.

Ciliate DGGE and qPCR (on average 5.37 log copies/g ruminal digesta) analysis showed no major changes in the ciliate community structure after supplementation of the diet with algae. This is in contrast with a previous experiment in which some ciliates, especially *I. prostoma*, disappeared after algal feeding (5). However, the daily amount of DHA supplied in the latter experiment (76.1 g DHA/day) was almost twice as high as in the current experiment (43.7 g DHA/day), probably explaining these results. In addition, further research on the specific role of *I. prostoma* in rumen biohydrogenation indicated that this ciliate and its associated bacteria are not directly involved (6).

The change in the total bacterial community structure with dietary supplementation of marine products was observed before (5, 20). Cluster analysis of the DGGE profile showed two clusters based on the absence or presence of algae in the diet. However, due to the complexity of the DGGE pattern of the total bacterial community, we examined in more detail bacterial groups of importance in rumen biohydrogenation. As C_{18} biohydrogenation is thought to be mainly performed by strains of the *Butyrivibrio* group, including the genera *Butyrivibrio* and *Pseudobutyrivibrio* (24), a primer set to study bacteria within this *Butyrivibrio* group was developed. In spite of major shifts in rumen biohydrogenation, supplementation of the diet with algae did not affect the total amount of *Butyrivibrio* spp. However, *Butyrivibrio*-specific DGGE profiles showed a shift in this group with time on a diet supplemented with algae. Some DGGE bands disappeared upon supplementation of the diet with algae, which was associated with a decrease in rumen $C_{18:0}$ concentrations (Fig. 2, bands 1 and 2). We hypothesize that bacteria associated with these disappearing bands might play a role in the conversion of $C_{18:1}$ *trans* to $C_{18:0}$. Until now, $C_{18:0}$ production was linked with isolates belonging to the *B. proteoclasticus* group (19) and the *Butyrivibrio* sp. branch (21), which all possess a close phylogenetic relationship. However, none of the species present in the disappearing bands belonged to this *B. proteoclasticus* group. On the other hand, 20% of the clones from the clone library of cow 1 on day 6 were classified in the *B. proteoclasticus* branch despite the reduced ruminal $C_{18:0}$ concentration (17.0 versus 1.65 mg/g ruminal digesta DM before and on day 6 of dietary algal supplementation, respectively). The presence of these bacteria in the *B. proteoclasticus*

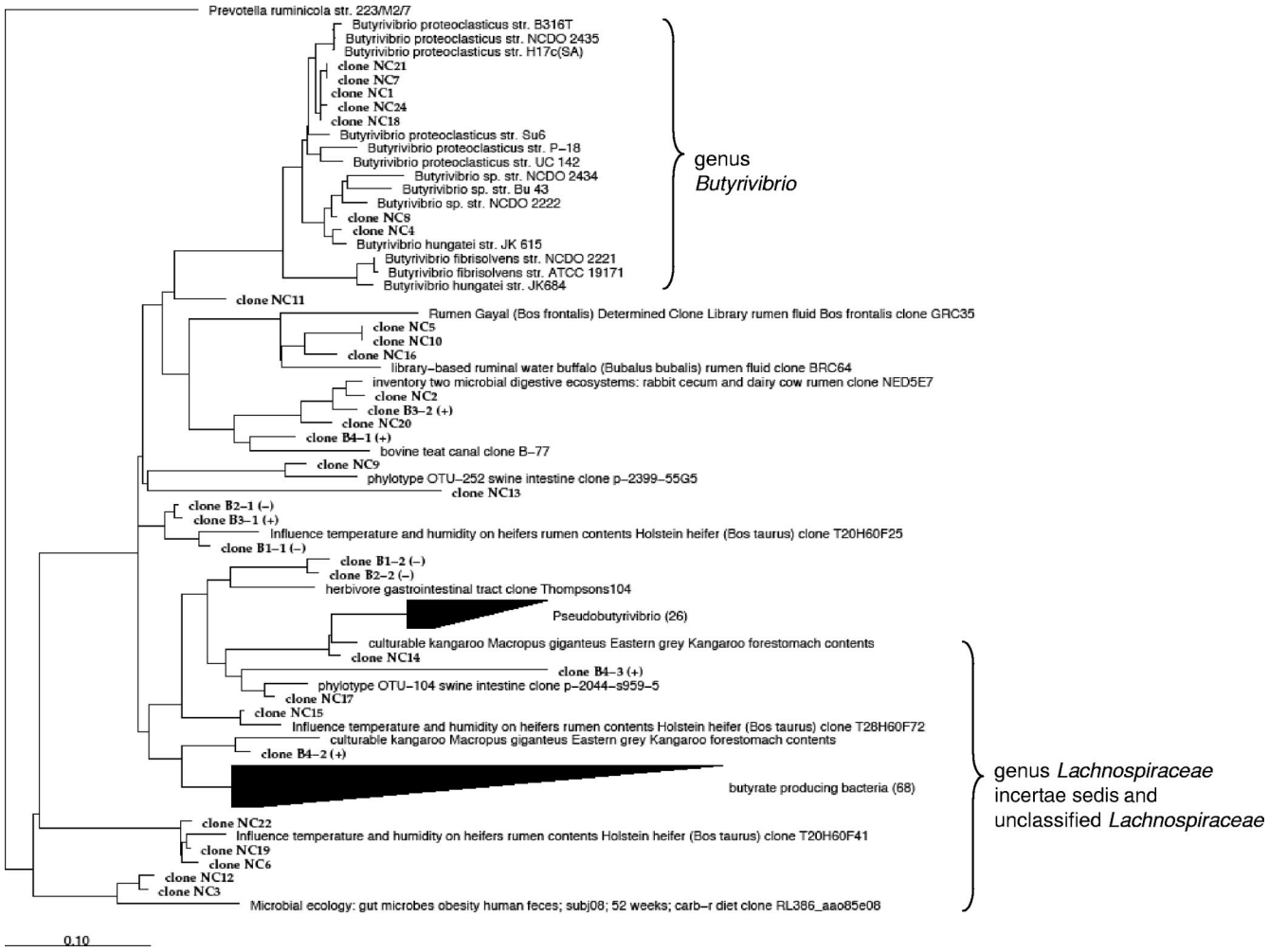

FIG. 3. Phylogenetic tree representing the classification of 23 clones (denoted as clone NCi; 417 bp), obtained from rumen fluid of a cow fed a diet supplemented with algae (cow 1 on day 6), within the genus *Butyrivibrio*, the genus *Lachnospiraceae* incertae sedis, and other (unknown) genera within the family *Lachnospiraceae*. Clones Bi to Bj are derived from changing DGGE bands upon algal-concentrate feeding (i, band excised and cloned from *Butyrivibrio* DGGE [Fig. 1]; j, clone number).

branch was unexpected, as the bacteria are highly sensitive to PUFA (19) and $C_{18:0}$ production was significantly decreased. This might indicate that the presence of DHA decreases the capacity of these bacteria to hydrogenate $C_{18:1}$ *trans* fatty acids rather than the bacteria as such. Competitive inhibition of bacterial isomerases and reductases and/or competition for hydrogen used in the simultaneous biohydrogenation of DHA and unsaturated C_{18} fatty acids (1, 36) might also explain the limited conversion of $C_{18:1}$ *trans* fatty acids to $C_{18:0}$ by bacteria belonging to the *B. proteoclasticus* group. Alternatively, these results could indicate that bacteria within the *B. proteoclasticus* branch have a limited contribution to in vivo $C_{18:0}$ formation. Kim et al. (20) found that the decreased duodenal $C_{18:0}$ flow in steers fed fish oil was not associated with *C. proteoclasticum* 16S rRNA gene concentrations in strained rumen fluid. Similarly, Huws et al. (18) reported that DNA concentrations from the *Butyrivibrio* $C_{18:0}$-producing group did not correlate with the $C_{18:0}$ concentrations of rumen planktonic and biofilm samples. This suggests that other, yet-uncultivated microbial species might be involved in $C_{18:0}$ production and might fulfill a more important role in the final step of the biohydrogenation process. Wallace et al. (33) stated that isolation of more $C_{18:0}$ producers might have been hampered by the fact that these bacteria must be growing in order to carry out biohydrogenation and that PUFA themselves have a strong tendency to inhibit this growth. It is generally recognized that only a minor part of the diversity of microorganisms in nature is presently known (2). Hence, it is not unlikely that reduced $C_{18:0}$ production is associated with the disappearance of uncultivated species. Identification of the disappearing bands through cloning and subsequent phylogenetic classification indicated that these noncultivated species were located on a separate branch between the genera *Butyrivibrio* and *Pseudobutyrivibrio*, belonging to unknown *Lachnospiraceae* strains genetically more distant from the *B. proteoclasticus* group. The concomitant disappearance of these species and decrease in the rumen $C_{18:0}$ concentration might suggest that they play a role in in vivo $C_{18:0}$ production. Further research is under way to evaluate the disappearance of these unknown *Lachnospiraceae* strains and reduced $C_{18:0}$ production under various rumen conditions.

Besides reduced $C_{18:0}$ concentrations, increased $C_{18:1}$ *t*11 and $C_{18:1}$ *t*10 concentrations were observed after feeding with the algal concentrate. This was associated with some more pronounced bands in the *Butyrivibrio* DGGE profile (Fig. 2, bands 3 and 4). Identification through cloning and subsequent phylogenetic classification indicated that these bands also represented noncultivated species located on a separate branch between the genera *Butyrivibrio* and *Pseudobutyrivibrio*. This might indicate that some *Butyrivibrio*-like bacteria are associated with changes in the rumen resulting in $C_{18:1}$ *t*11 and $C_{18:1}$ *t*10 accumulation. It remains to be determined whether the more pronounced appearance of these bands and accumulation of $C_{18:1}$ *t*10 or $C_{18:1}$ *t*11 is a causal relationship. In addition, the origin of these increased amounts of $C_{18:1}$ *t*10, either isomerization of $C_{18:1}$ *c*9 (23) or $C_{18:1}$ *t*11 (22) or biohydrogenation of CLA *t*10*c*12 (14), needs to be clarified.

Although this research mainly focused on molecular techniques to describe shifts within the rumen microbial community, other bacterial markers, such as odd- and branched-chain fatty acids (32), also showed shifts upon algal feeding. In the present study, supplementation of the diet with algae increased rumen *iso* $C_{17:0}$, *anteiso* $C_{15:0}$, and *anteiso* $C_{17:0}$ concentrations. In milk, these fatty acids showed a negative correlation with $C_{18:0}$ ($r_{pearson}$ = −0.807, −0.207, and −0.446, respectively), whereas milk *iso* $C_{17:0}$ was positively correlated with milk $C_{18:1}$ *t*11 ($r_{pearson}$ = 0.983) and, to a lesser extent, with $C_{18:1}$ *t*10 ($r_{pearson}$ = 0.522) (32).

Conclusion. Supplementation of the diet with algae inhibited rumen C_{18} biohydrogenation, resulting in decreased $C_{18:0}$ concentrations, whereas $C_{18:1}$ *t*11 and $C_{18:1}$ *t*10 concentrations increased. Changes in the rumen fatty acid profile were associated with changes in the structure of the bacterial community and, more specifically, with changes in the *Butyrivibrio* group. Clones associated with altered DGGE bands indicated that dietary algae affected noncultivated species, which cluster between the genus *Butyrivibrio* and the genus *Pseudobutyrivibrio*. Additionally, 20% of the clone library, from a randomly selected rumen sample after the start of supplementation of the diet with algae, clustered within the $C_{18:0}$-producing *B. proteoclasticus* branch, although $C_{18:0}$ production was reduced. This suggests that other, as-yet-uncultivated bacteria are involved in $C_{18:0}$ production, possibly being more important than *B. proteoclasticus*.

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