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Role of the protozoan *Isotricha prostoma*, liquid-, and solid-associated bacteria in rumen biohydrogenation of linoleic acid

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From the simultaneous accumulation of hydrogenation intermediates and the disappearance of Isotricha prostoma *after algae supplementation, we suggested a role of this ciliate and/or its associated bacteria in rumen biohydrogenation of unsaturated fatty acids. The experiments described here evaluated the role of* I. prostoma *and/or its associated endogenous and exogenous bacteria in rumen biohydrogenation of C18:2n-6 and its main intermediates CLA c9t11 and C18:1t11. Fractions of* I. prostoma *and associated bacteria, obtained by sedimentation of rumen fluid sampled from a monofaunated sheep, were used untreated, treated with antibiotics or sonicated to discriminate between the activity of* I. prostoma *and its associated bacteria, the protozoan or the bacteria, respectively. Incubations were performed in triplicate during 6 h with unesterified C18:2n-6, CLA c9t11 or C18:1t11 (400 μg/ml) and 0.1 g glucose/cellobiose (1/1, w/w).* I. prostoma *did not hydrogenate C18:2n-6 or its intermediates whereas bacteria associated with* I. prostoma *converted a limited amount of C18:2n-6 and CLA c9t11 to* trans *monoenes. C18:1t11 was not hydrogenated by either* I. prostoma *or its associated bacteria but was isomerized to C18:1c9. A phylogenetic analysis of clones originating from* Butyrivibrio*-specific PCR product was performed. This indicated that 71% of the clones from the endogenous and exogenous community clustered in close relationship with* Lachnospira pectinoschiza*. Additionally, the biohydrogenation activity of solid-associated bacteria (SAB) and liquid-associated bacteria (LAB) was examined and compared with the activity of the non-fractioned* I. prostoma *monofaunated rumen fluid (LAB + SAB). Both SAB and LAB were involved in rumen biohydrogenation of C18:2n-6. SAB fractions performed the full hydrogenation reaction to C18:0 while C18:1 fatty acids, predominantly C18:1t10 and C18:1t11, accumulated in the LAB fractions. SAB and LAB sequence analyses were mainly related to the genera* Butyrivibrio *and* Pseudobutyrivibrio *with 12% of the SAB clones closely related to the C18:0 producing* B. proteoclasticus *branch. In conclusion, this work suggests that* I. prostoma *and its associated bacteria play no role in C18:2n-6 biohydrogenation, while LAB convert C18:2n-6 to a wide range of C18:1 fatty acids and SAB produce C18:0, the end product of rumen lipid metabolism.*

Keywords: protozoa, *Butyrivibrio*, *Lachnospira*, conjugated linoleic acids, *trans* fatty acids

Implications

Saturated fatty acids in animal products are undesirable. A better understanding of the microbial biohydrogenation is necessary to limit this process in the rumen and increase the proportion of health-associated polyunsaturated fatty acids in ruminants' meat and milk. The protozoan *Isotricha prostoma* is not involved in the rumen lipid metabolism whereas its associated bacteria convert a limited amount of C18:2n-6 to C18:1t11. Solid-associated bacteria perform the full hydrogenation reaction to C18:0 while liquid-associated bacteria accumulate C18:1 fatty acids, predominantly C18:1t10 and C18:1t11.

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Introduction

In a recent *in vivo* trial, incomplete rumen biohydrogenation after algae supplementation was associated with the disappearance of ciliate protozoa (Boeckaert *et al.*, 2007). Some protozoal species were less abundant in the rumen of algae-fed cows such as *Isotricha intestinalis*, *Epidinium caudatum*, *Eudiplodinium maggii* and *Diplodinium dentatum* but the holotrich *Isotricha prostoma* was particularly affected. Previously, *I. prostoma* (Gutierrez *et al.*, 1962) and

I. intestinalis (Williams *et al*., 1963) have been shown to hydrogenate oleic acid (C18:1c9) to stearic acid (C18:0), whereas Wright (1959) reported *in vitro* hydrogenation of linoleic (C18:2n-6) to C18:0 by mixtures of ruminal ciliates comprising mainly holotrichs and *Epidinium* sp. However, the unequivocal role of protozoa in the hydrogenation of unsaturated fatty acids cannot be assessed from these *in vitro* studies since bacterial contamination cannot be excluded from protozoal fractions (Dawson and Kemp, 1969). It is well known that protozoa contain bacteria in vesicles in their endoplasm (endosymbionts) and/or have bacteria attached to the outside of the pellicle (ectosymbionts) (Williams and Coleman, 1992). The above-mentioned results are therefore inconclusive. Later studies concluded that *Isotricha* species possess little or no ability to hydrogenate unsaturated fatty acids in the absence of bacteria (Chalupa and Kutches, 1968; Abaza *et al*., 1975) while co-incubations with bacteria harvested from the rumen liquid phase resulted in biohydrogenation of C18:2n-6 to C18:0 (Girard and Hawke, 1978). These findings illustrate that protozoa require bacteria for rumen biohydrogenation. In contrast, it remains unclear whether bacterial biohydrogenation is enhanced by protozoa. The main bacteria involved in the biohydrogenation process have been identified. So far known, biohydrogenation is performed by bacteria belonging to the *Butyrivibrio* group, including the genera *Butyrivibrio* and *Pseudobutyrivibrio* (Paillard *et al*., 2007a). Kemp *et al*. (1975) identified C18:0-producing bacteria as *Fusocillus* spp., which actually corresponds to *Clostridium proteoclasticum* isolated by Wallace *et al*. (2006) and was recently reclassified as *Butyrivibrio proteoclasticus* (Moon *et al*., 2008). However, it is uncertain whether or not bacteria within the *Butyrivibrio* group are associated with protozoa, in particular *I. prostoma*. Following on our previous results regarding the disappearance of *I. prostoma* when biohydrogenation is obstructed by algae, this study aimed at evaluating the hydrogenating capacity of *I. prostoma* and its endogenous and exogenous associated bacteria. Therefore, *in vitro* incubations with rumen fluid from a monofaunated sheep were performed. The latter houses only one genus or species of protozoa in its rumen, i.e. *I. prostoma* in this study. Fractions of *I. prostoma* were prepared and incubated to discriminate between the biohydrogenating activity of *I. prostoma* together with its endogenous and exogenous associated bacteria, *I. prostoma* alone or the associated bacteria of *I. prostoma* alone. Biohydrogenation of C18:2n-6 and its main intermediates CLA c9t11 and C18:1t11 has been studied. Additionally, the biohydrogenation capacity of this protozoal fraction was compared with that of non-fractioned rumen fluid inoculum and its liquid-associated bacteria (LAB) and the solid-associated bacteria (SAB), respectively.

Material and methods

Rumen fluid and fatty acid preparations

Animal care was in accordance with the guidelines for animal research of the French Ministry of Agriculture. A wether monofaunated with *I. prostoma* (6.6×10^3 *I. prostoma*/ml rumen fluid) was used as donor (Jouany *et al*., 1981). The wether was fitted with a rumen cannula and fed once a day 600 g alfalfa pellets, 400 g barley grain and 150 g meadow hay. Rumen contents were sampled before morning feeding (0800 h) and were strained through a nylon monofilament membrane (800 μm) under an O_2-free CO_2 stream to remove large particulate matter.

Unesterified C18:2n-6 and CLA c9t11 (Larodan Fine Chemicals AB, Malmö, Sweden) were dissolved in hexane (200 μg/ml) after which 50 μl of the hexane solution (10 mg fatty acid) was added to the incubation flask and evaporated by CO_2 before incubation. C18:1t11 (Larodan Fine Chemicals AB), dissolved in hexane, crystallized when evaporated by CO_2 before incubation. This hampered a homogeneous mixing of the fatty acid with the incubation fluid. Therefore C18:1t11 was dissolved in ethanol (100 mg/ml) and 100 μl of the ethanol solution was added directly to the buffered rumen fluid in the incubation flask. This volume of ethanol does not affect fermentations (Morgavi *et al*., 2004) and was not considered toxic for the rumen microorganisms. The average amounts of C18:2n-6, CLA c9t11 and C18:1t11 in the serum bottles with 25 ml incubation fluid were 9.21 ± 0.78, 10.6 ± 0.35 and 11.4 ± 0.17 mg in the first experiment (protozoan fractions) and 10.7 ± 0.57, 11.9 ± 0.11 and 12.1 ± 1.81 mg in the second experiment (LAB, SAB and LAB + SAB fractions).

In vitro *incubations*

In vitro incubations were performed during 6 h in 120-ml serum bottles containing fractionated or monofaunated rumen fluid (8 ml), Goering–Van Soest buffer (17 ml) (Goering and Van Soest, 1970), either unesterified C18:2n-6, CLA c9t11 or C18:1t11 (10 mg) and 0.1 g glucose/cellobiose (1/1, w/w) (Sigma, Saint-Quentin Fallavier, France) as substrate. Additionally, incubations without the addition of a particular fatty acid were performed for each fraction and acted as control incubations in terms of rumen fermentation. Flasks that had been previously filled with pure CO_2 once more were gassed with CO_2 after rumen fluid addition to eliminate trace amounts of oxygen. Finally, they were closed by a crimped butyl septum and were maintained at 39°C in a water bath. The medium in each incubation flask was regularly agitated by a magnetic rod (300 r.p.m. for 30 s followed by a resting period of 3 min). All incubations were performed in three runs on separate days.

Experiment 1: Role of I. prostoma *and its associated bacteria in rumen biohydrogenation*

Protozoa were obtained using a sedimentation procedure as described by Williams and Coleman (1992) with some modifications. Briefly, 200 ml of *I. prostoma* monofaunated rumen fluid was mixed with 400 ml of Simplex type Coleman buffer (Coleman, 1978), prepared as recommended by Broudiscou and Lassalas (2000), and 0.6 g of glucose. The mixture was transferred into a separating funnel and incubated at 39°C for 30 min to allow *I. prostoma* to settle.

Protozoa accumulated in a clear white zone at the bottom of the separating funnel. The remaining liquid above was removed by suction. The sediment was collected in a jar and diluted with autoclaved *I. prostoma* monofaunated rumen fluid to the initial volume of 200 ml (=protozoal fraction). To discriminate between the biohydrogenation capacity of *I. prostoma* and its associated bacteria (IP + B), *I. prostoma* alone (IP) or the associated bacteria alone (B), incubations with this protozoal fraction were further subdivided. Batch incubators containing the complete protozoal fraction (IP + B), including endogenous and exogenous associated bacteria, were supplemented with an antibiotic mixture (0.3 mg/ml of chloramphenicol crystalline/streptomycin/ampicillin sodium salt, 1/1/1, w/w/w) to halt bacterial metabolism (IP) or were sonicated (40 kHz, 240 V) (Deltasonic, Meaux, France) during 15 min to lyse *I. prostoma* cells (B). Preliminary tests indicated that these sonication parameters were efficient to lyse protozoal cells without negative effects on the associated bacteria (Onodera *et al*., 1977). Each fraction was incubated with unesterified C18:2n-6, CLA c9t11 or C18:1t11. The latter two incubations starting with biohydrogenation intermediates were performed since *I. prostoma* and/or its associated bacteria are possibly involved in only part of the rumen biohydrogenation of C18:2n-6.

Experiment 2: Role of LAB and SAB in rumen biohydrogenation

LAB and SAB were obtained following a centrifugation procedure of Legay-Carmier and Bauchart (1989) with some modifications. Briefly, 2 × 200 ml of *I. prostoma* monofaunated rumen fluid was centrifuged during 3 min at 500 × **g**. From one aliquot, the supernatant was collected by suction and diluted with autoclaved *I. prostoma* monofaunated rumen fluid to the initial volume of 200 ml. This fraction represented the LAB. The remaining white interface layer and the pellet were also diluted with autoclaved *I. prostoma* monofaunated rumen fluid to the initial volume of 200 ml and represented the SAB fraction containing bacteria as well as *I. prostoma* with its endosymbionts and ectosymbionts. Another aliquot was mixed again by means of a magnetic stirrer and represented the *I. prostoma* monofaunated rumen fluid, further referred to as LAB + SAB. The last aliquot was also subjected to centrifugation to ensure similar manipulations of the rumen microorganisms. Each fraction (LAB, SAB and LAB + SAB) was incubated with unesterified C18:2n-6, CLA c9t11 or C18:1t11.

Sampling and analysis

Gas production was recorded after 6 h of incubation using a pressure transducer. A headspace sample was collected and fermentation gases were analyzed by gas chromatography (DI-700, Rungis, France) as described by Broudiscou *et al*. (1999). The pH was measured immediately after opening the incubation flasks. Aliquots of incubation fluid were sampled for short chain fatty acid (SCFA) analysis and DNA extraction. For SCFA determination, gas chromatography was applied as described by Broudiscou and Lassalas (2000). The net amount of SCFA produced during 6 h of incubation was calculated by deducting the amount of SCFA in the corresponding 0 h incubation sample. For DNA extraction, 2 ml of incubation fluid was stored at −80°C until further analysis. The remaining incubation fluid (21.2 ml) was transferred to a recipient after which the incubation flask was rinsed with 7 ml of chloroform/methanol (C/M, 2/1, v/v) and added to the recipient to stop further reactions. Recipients were stored at −20 °C until fatty acid analysis.

Lipid extraction followed the method of Folch *et al*. (1957) with some adaptations as described by Chow *et al*. (2004). Methylation of fatty acids and subsequent identification of fatty acid methyl esters were performed as described in Boeckaert *et al*. (2008).

Microbial community analysis

Total DNA of 0.5 g incubation fluid from LAB, SAB and B fractions was extracted as described by Boon *et al*. (2003). A PCR to amplify a fragment of the 16S rRNA gene of the *Butyrivibrio* group was performed following Boeckaert *et al*. (2008). The amplified PCR product of one LAB, SAB and B sample was cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) 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% glycerol (v/v) while plasmid DNA was isolated from the remaining liquid using the high pure plasmid isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). The resulting 41 clones for LAB, 52 clones for SAB and 51 clones for B were identified by sequencing the partial 16S rRNA gene fragments (Genetic Service Unit, Gent, Belgium). Close relatives of the 16S rRNA sequences were identified with the sequence match server of the Ribosomal Database Project II (Wang *et al*., 2007). Nucleotide sequences for clones 1 through 144 have been deposited in the GenBank database under accession numbers FJ345536–FJ345679. Additionally, sequences were aligned with the NAST software (DeSantis *et al*., 2006). The alignment was manually checked using the ARB aligner tool (ARB software, Ludwig *et al*., 2004), after which sequences were added to the original phylogenetic tree (Greengenes database January 2008) using Parsimony (ARB software, Ludwig *et al*., 2004) without changing the tree topology. Clones from the same original samples (SAB, LAB or B), showing 97% or more sequence similarity (ClustalW2), were considered the same species and were grouped in the phylogenetic tree. For example, samples LAB-146 and LAB-147 showed 99% similarity and are represented by Lab-cl1 (2), denoting cluster 1 contains two clones.

Statistical analysis

Rumen fatty acid were statistically analyzed using the MIXED procedure of SAS Institute (SAS Institute Inc., 2004). The statistical model for rumen fatty acid data included the fixed effect of fraction, incubation time and their interaction and the random effect of repetition assuming an autoregressive order one covariance structure fitted on the basis of Akaike information and Schwarz Bayesian model fit

criteria. Incubation time was treated as a repeated measure. Least squares means are reported and significance was declared at $P < 0.05$. Differences among fractions were evaluated using a multiple comparison test following the Tukey–Kramer method.

Results

Experiment 1: Role of I. prostoma and its associated bacteria in rumen biohydrogenation

Incubations with C18:2n-6. C18:2n-6 concentrations decreased during 6 h of incubation for all fractions (Table 1). However, the decrease was more pronounced for the IP + B and B fractions as compared to the IP fraction. Decreases in C18:2n-6 were associated with increased CLA c9t11 concentrations being substantially higher for the IP + B and B fractions. Additionally, CLA t10c12, CLA c9c11 and unidentified non-conjugated C18:2 fatty acids were formed by all fractions. The high increase in CLA t10c12 concentrations for the B fraction is noteworthy. Also C18:1c12 and C18:1t12 concentrations increased whereas C18:1t11 only showed a trend to increase. None of the fractions significantly produced C18:0 during 6 h of incubation with C18:2n-6.

Incubations with CLA c9t11. Similar decreases in the concentration of CLA c9t11 were observed for all fractions and were associated with accumulation of CLA t10c12, CLA c9c11, a CLA t,t and an unidentified non-conjugated C18:2 fatty acid (Table 2). C18:1c9, C18:1c11, C18:1t11 and C18:1t12 concentrations increased for all fractions, although the C18:1t11 increase was only prominent for the IP + B and B fractions. As with the C18:2n-6 substrate, C18:0 production did not take place.

Incubations with C18:1t11. A non-significant decrease ($P = 0.110$) in C18:1t11 concentrations occurred whereas C18:1c9 was produced by all fractions, i.e. +4.5, +10.9 and +4.2 μg/ml for the IP + B, IP and B fractions, respectively (full tables not shown). C18:1t9 concentrations decreased in IP + B and IP and increased in B while other C18:1 fatty acids did not change. As with C18:2n-6 and CLA c9t11, C18:0 concentrations did not increase.

SCFA, CH_4 and H_2. Rumen fermentation parameters were measured to further evaluate *in vitro* incubations. The IP fraction produced minimal amounts of SCFA (1.01 ± 0.44 mmol/l, $n = 9$), mainly as butyrate. No CH_4 was produced for the IP fraction while H_2 accumulation was on average 2.23 ± 0.15 μmol/flask. Incubation of IP without the addition of a fatty acid generated a net total amount SCFA of 3.91 mmol/l ($n = 3$) with acetate, propionate and butyrate (56 : 12 : 31, mol : mol : mol) as the main SCFA. The IP + B and B fraction produced low net total amounts of SCFA, which was independent of the addition of fatty acids (5.83 ± 1.02 ($n = 24$) and 7.80 ± 0.96 ($n = 24$) mmol/l, with or without fatty acid addition, respectively). Acetate, propionate and butyrate (on average 79 : 10 : 11, mol : mol : mol) were again the main SCFA. CH_4 production was low, on average 0.96 ± 0.002 and 0.94 ± 0.11 μmol/flask for the

Table 1 *Concentration of C18 fatty acids (μg/ml) before (0 h) and after (6 h) incubation of* Isotricha prostoma *and its associated bacteria (IP + B),* I. prostoma *alone (IP) and the associated bacteria alone (B) with C18:2n-6 (*n *= 3)*

	IP + B		IP		B					
	0 h	6 h	0 h	6 h	0 h	6 h	s.e.	$F^{\dagger}$	$T^{\ddagger}$	$F * T^{\S}$
C18:2n-6	381[a]	324[ab]	381[a]	368[a]	381[a]	299[b]	17.06	**	**	0.118
CLA c9t11	0.10[b]	8.38[a]	0.10[b]	0.52[b]	0.10[b]	7.48[a]	1.226	*	***	*
CLA c9c11	0.40	n.d.	0.40	0.367	0.40	0.13	0.103	0.103	*	0.352
CLA t10c12	n.d.[b]	0.10[b]	n.d.[b]	0.40[b]	n.d.[b]	6.87[a]	1.218	*	*	0.032
CLA t,t	0.61	n.d.	0.61	n.d.	0.61	0.72	0.368	0.402	0.193	0.476
C18:1c9	17.5	18.8	17.5	18.6	17.5	17.1	0.977	0.569	0.493	0.690
C18:1c11	4.42	4.88	4.42	4.75	4.42	3.68	0.350	0.156	0.973	0.277
C18:1c12	1.32	2.01	1.32	3.03	1.32	2.20	0.508	0.579	*	0.583
C18:1c14 + t16	4.98	4.34	4.98	4.41	4.98	3.51	0.385	0.146	0.051	0.608
C18:1t5	n.d.	0.10	n.d.	0.09	n.d.	n.d.	0.054	0.402	0.188	0.620
C18:1t6–t8	1.81	1.68	1.81	1.58	1.81	1.39	0.190	0.227	0.231	0.843
C18:1t9	1.28	1.17	1.28	1.16	1.28	1.14	0.149	0.987	0.457	0.998
C18:1t10	2.64	10.1	2.64	3.25	2.64	11.9	3.328	0.123	0.062	0.430
C18:1t11	33.2	42.4	33.2	32.6	33.2	43.9	3.753	0.200	0.090	0.379
C18:1t12	2.66	3.63	2.66	2.89	2.66	3.00	0.261	0.135	0.050	0.398
C18:0	247	282	247	294	247	228	33.40	0.413	0.392	0.506

n.d.: not detected.
s.e.: standard error of mean.
[a,b]Means within a row with a different superscript differ ($P < 0.05$).
[†]Effect of fraction (*F*).
[‡]Effect of time (*T*).
[§]Interaction between fraction and time (F * T).

Table 2 *Concentration of C18 fatty acids (μg/ml) before (0 h) and after (6 h) incubation of* Isotricha prostoma *and its associated bacteria (IP + B),* I. prostoma *alone (IP) and the associated bacteria alone (B) with CLA c9t11 (*n = *3)*

	IP + B		IP		B					
	0 h	6 h	0 h	6 h	0 h	6 h	s.e.	$F^{\dagger}$	$T^{\ddagger}$	$F^{*}T^{\S}$
CLA c9t11	426[a]	363[b]	426[a]	378[b]	426[a]	371[b]	14.49	0.752	***	0.735
CLA c9c11	0.40[b]	2.73[a]	0.40[b]	1.84[ab]	0.40[b]	2.11[ab]	0.424	0.590	***	0.563
CLA t10c12	n.d.[b]	11.0[a]	n.d.[b]	11.3[a]	n.d.[b]	11.4[a]	0.486	0.856	***	0.901
CLA t,t	0.61[b]	18.0[a]	0.61[b]	12.6[ab]	0.61[b]	17.5[a]	2.634	0.578	***	0.527
C18:1c9	17.5[b]	31.4[a]	17.5[b]	32.6[a]	17.5[b]	33.9[a]	1.460	0.600	***	0.727
C18:1c11	4.42	5.50	4.42	4.84	4.42	4.91	0.447	0.589	*	0.545
C18:1c12	1.32	1.46	1.32	1.66	1.32	1.49	0.220	0.806	0.216	0.868
C18:1c14 + t16	4.98	4.38	4.98	4.17	4.98	4.35	0.459	0.673	0.189	0.981
C18:1t6–t8	1.81	1.55	1.81	1.55	1.81	1.62	0.186	0.828	0.194	0.984
C18:1t9	1.28	1.33	1.28	1.21	1.28	1.12	0.153	0.143	0.675	0.828
C18:1t10	2.64	3.06	2.64	2.60	2.64	3.04	0.273	0.497	0.315	0.702
C18:1t11	33.2[b]	58.5[ab]	33.2[b]	34.3[b]	33.2[b]	76.3[a]	6.984	**	**	0.051
C18:1t12	2.66[b]	3.97[a]	2.66[b]	3.28[b]	2.66[b]	4.05[a]	0.385	0.055	***	0.063
C18:0	247	297	247	230	247	242	23.96	0.375	0.585	0.271

n.d.: not detected.
s.e.: standard error of mean.
[a,b]Means within a row with a different superscript differ ($P < 0.05$).
[†]Effect of fraction (*F*).
[‡]Effect of time (*T*).
[§]Interaction between fraction and time (*F* * *T*).

IP + B and B fraction, respectively, whereas H_2 production was on average 3.35 ± 0.40 and 2.43 ± 0.14 μmol/flask for all incubations (with and without the addition of a fatty acid).

Experiment 2: Role of LAB and SAB in rumen biohydrogenation

Incubations with C18:2n-6. Concentrations of C18:2n-6 decreased in all fractions (Table 3) but was most pronounced for the SAB fraction and was considerably higher than that observed with the protozoal fractions. Disappearance of C18:2n-6 from the incubation media was associated with increased amounts of all C18:2 isomers, except for CLA c9c11. Likewise, most C18:1 fatty acids, in particular C18:1t10 and C18:1t11, increased in concentration. The increase in C18:1 isomers was substantially higher for the LAB fraction, with the exception of C18:1c11 and C18:1c14 + t16. This higher concentration of C18:1 fatty acids for the LAB fraction was associated with lower C18:0 concentrations as compared to the SAB and LAB + SAB fractions. Overall, the LAB, SAB and LAB + SAB fractions converted 25%, 69% and 87%, respectively, of the C18:2n-6 substrate into C18:0.

Incubations with CLA c9t11. C18:2 isomers increased at the expense of CLA c9t11 with some unidentified non-conjugated C18:2 fatty acids typically higher for the SAB fraction (Table 4). There was a particularly large increase in the production of CLA t,t. Furthermore, concentrations of all C18:1 fatty acids increased, except for C18:1c13. However, the increase in C18:1t10 was less important than in incubations with C18:2n-6. The LAB fraction again showed substantially high increases in most C18:1 fatty acid concentrations. Accordingly, C18:0 concentrations increased less for the LAB fraction as compared to the SAB and LAB + SAB fractions.

Incubations with C18:1t11. C18:1t11 concentrations only decreased numerically ($P < 0.1$, full tables not shown). Nevertheless, C18:1c9 concentrations increased while C18:1t10 and C18:1t12 concentrations decreased in all fractions. Changes in the other C18:1 *trans* fatty acids were dependent upon the fraction. C18:1t4 and C18:1t5 concentrations increased for the LAB and SAB fractions and were unchanged for the LAB + SAB fraction. C18:1t6–t8 and C18:1t9 increased for the LAB + SAB and LAB fractions but decreased for the SAB fraction. C18:0 production was low in all fractions, increasing by 42, 85 and 35 μg/ml incubation fluid for the LAB, SAB and LAB + SAB fractions, respectively.

SCFA, CH_4 and H_2. SCFA produced during 6 h of incubation with C18:2n-6, CLA c9t11 or no additional fatty acids did not differ between fractions (47.3 ± 3.22 mmol/l, $n = 27$). However, for incubations with C18:1t11, net total amounts of SCFA were lower for the LAB fraction (40.2 mmol/l, $n = 3$) as compared to the SAB (55.9 mmol/l, $n = 3$) and LAB + SAB (52.0 mmol/l, $n = 3$) fractions. The proportion of main SCFA produced was 57 : 23 : 20 (mol : mol : mol) for acetate, propionate and butyrate. For all incubations, CH_4 production was lower for the LAB fraction (51.2 ± 2.58 μmol/flask, $n = 12$) as compared to the SAB (74.0 ± 8.41 μmol/flask, $n = 12$) and LAB + SAB (72.2 ± 9.80 μmol/flask, $n = 12$) fractions while H_2 production remained very low for all fractions (0.56 ± 0.17 μmol/flask, $n = 36$).

Table 3 *Concentration of C18 fatty acids (μg/ml) before (0 h) and after (6 h) incubation of* Isotricha prostoma *monofaunated rumen fluid (LAB + SAB), liquid-associated bacteria (LAB) and solid-associated bacteria (SAB) with C18:2n-6 (*n = *3)*

	LAB + SAB		LAB		SAB					
	0 h	6 h	0 h	6 h	0 h	6 h	s.e.	$F^{\dagger}$	$T^{\ddagger}$	$F^{*}T^{\S}$
C18:2n-6	421^{a}	276^{b}	414^{a}	215^{b}	424^{a}	203^{b}	24.99	0.067	***	0.054
CLA c9t11	3.22	4.80	3.42	6.68	2.78	6.48	1.244	0.299	*	0.681
CLA c9c11	0.13	0.33	n.d.	n.d.	0.25	0.12	0.120	0.313	0.722	0.175
CLA t10c12	n.d.^{b}	6.92^{a}	n.d.^{b}	5.82^{a}	n.d.^{b}	4.80^{a}	0.512	0.160	***	0.160
CLA t,t	n.d.^{b}	4.77^{a}	n.d.^{b}	5.62^{a}	n.d.^{b}	4.66^{a}	0.413	0.503	***	0.506
C18:1c9	15.2^{bc}	26.3^{ab}	6.23^{c}	38.8^{a}	22.0^{cb}	46.3^{a}	4.029	**	***	*
C18:1c11	3.39^{d}	7.85^{cd}	4.98^{cd}	16.0^{ac}	5.72^{bd}	17.1^{ab}	2.027	***	**	0.341
C18:1c12	0.94^{c}	4.04^{bc}	1.25^{c}	15.7^{a}	1.40^{c}	6.35^{b}	1.005	***	***	***
C18:1c13	1.09^{ab}	1.08^{ab}	1.22^{a}	0.91^{ab}	0.98^{ab}	0.73^{b}	0.078	**	*	0.324
C18:1c14 + t16	2.92^{bc}	6.97^{b}	1.52^{c}	7.68^{b}	4.95^{bc}	15.6^{a}	0.960	***	***	*
C18:1t4	n.d.^{bc}	0.76^{abc}	n.d.^{bc}	3.25^{a}	n.d.^{b}	2.11^{a}	0.278	**	***	**
C18:1t5	n.d.^{c}	0.69^{c}	n.d.^{c}	4.10^{a}	n.d.^{c}	1.88^{b}	0.221	***	***	***
C18:1t6–t8	1.75^{c}	4.76^{c}	1.29^{c}	27.4^{a}	2.87^{c}	14.7^{b}	1.602	***	***	***
C18:1t9	1.55^{c}	3.20^{c}	0.93^{c}	16.6^{a}	2.07^{c}	8.85^{b}	0.999	***	***	***
C18:1t10	2.77^{c}	11.0^{c}	1.84^{c}	63.1^{a}	3.68^{c}	28.7^{b}	3.047	***	***	***
C18:1t11	38.6^{cd}	47.2^{bc}	19.0^{d}	69.7^{ab}	53.8^{ac}	79.3^{a}	5.111	***	***	*
C18:1t12	2.75^{b}	7.57^{b}	1.94^{b}	25.1^{a}	4.34^{b}	18.7^{a}	1.951	**	***	**
C18:1t15	n.d.^{c}	n.d.^{c}	n.d.^{c}	5.13^{a}	n.d.^{c}	2.92^{b}	0.258	***	***	***
C18:0	270^{c}	475^{b}	151^{d}	223^{cd}	435^{b}	651^{a}	12.41	***	***	**

n.d.: not detected.
s.e.: standard error of mean.
^{a,b,c,d}Means within a row with a different superscript differ ($P < 0.05$).
†Effect of fraction (F).
‡Effect of time (T).
§Interaction between fraction and time ($F * T$).

Cloning and sequence analysis of the LAB, SAB and B fractions
Based on the results of the different fractions (LAB, SAB, IP + B, IP and B) in rumen biohydrogenation of C18:2n-6, CLA c9t11 or C18:1t11, clone libraries were constructed with the *Butyrivibrio*-specific PCR products of one LAB, SAB and B sample. Sequence match analysis indicated that all 144 clones were related to bacterial species within the family of the *Lachnospiraceae*. Clones matched as follows: 39 clones within the genus *Butyrivibrio* (29 SAB, 4 LAB and 6 B), 14 clones within the genus *Pseudobutyrivibrio* (5 SAB, 6 LAB and 3 B), two clones within the genus *Oribacterium* (2 B), three clones within the genus *Lachnobacterium* (3 B), 56 clones within the genus *Lachnospiraceae Incertae Sedis* (8 SAB, 11 LAB and 37 B) and 30 clones were unclassified *Lachnospiraceae* (10 SAB and 20 LAB). The phylogenetic clustering of the clones, based on 16S rRNA sequence (417 bp) analysis, is presented in Figure 1. The branch with bacteria known so far to convert C18:2n-6 into C18:0 (i.e. *B. proteoclasticus* and *Butyrivibrio* species) contains nine clones among which six SAB, one LAB and two B. Seventy clones were in close phylogenetic relationship with bacteria converting C18:2n-6 into C18:1 fatty acids (i.e. the genera *Butyrivibrio* and *Pseudobutyrivibrio*) including 32 SAB, 28 LAB and 10 B. The remaining 65 clones (14 SAB, 12 LAB and 39 B) clustered further away from the genera *Butyrivibrio* and *Pseudobutyrivibrio* with 36 B clones closely related to *Lachnospira pectinoschiza* species.

Discussion

Role of I. prostoma *and its associated bacteria in rumen biohydrogenation*
In vitro incubations with fractions of *I. prostoma* monofaunated rumen fluid were performed to investigate the activity of *I. prostoma* and its associated bacteria in rumen biohydrogenation of C18:2n-6 and its main intermediates CLA c9t11 and C18:1t11.

The minor disappearance of the added fatty acids and the lack of a substantial formation of intermediates or end products observed for the IP fraction (protozoan alone) indicate that *I. prostoma* is not directly involved in rumen biohydrogenation of C18:2n-6, CLA c9t11 and C18:1t11. Similarly, Devillard *et al*. (2006) reported little C18:2n-6 biohydrogenation activity by a mixed protozoal fraction. These results confirm previously published information that attributed the minor hydrogenating capacity of this ciliate to ingested or associated bacteria (Abaza *et al*., 1975; Girard and Hawke, 1978). Nonetheless, two particular features are worth mentioning for the IP incubations. Firstly, the slight decrease in C18:2n-6 and CLA c9t11 concentrations with a concomitant increase in CLA isomers, which may indicate some isomerase activity (Bauman *et al*., 2000). Or-Rashid *et al*. (2008) earlier demonstrated CLA production from C18:2n-6 by mixed rumen protozoa, whereas Paillard *et al*. (2007b) associated the capacity to

Table 4 *Concentration of C18 fatty acids (μg/ml) before (0 h) and after (6 h) incubation of* Isotricha prostoma *monofaunated rumen fluid (LAB + SAB), liquid-associated bacteria (LAB) and solid-associated bacteria (SAB) with CLAc9t11 (n = 3)*

	LAB + SAB		LAB		SAB					
	0 h	6 h	0 h	6 h	0 h	6 h	s.e.	$F^{\dagger}$	$T^{\ddagger}$	$F^{*}T^{\S}$
CLA c9t11	479^{a}	133^{b}	479^{a}	123^{b}	478^{a}	131^{b}	25.68	0.915	***	0.978
CLA c9c11	0.14^{b}	10.5^{a}	n.d.^{b}	9.32^{a}	0.25^{b}	7.46^{a}	1.086	0.433	***	0.366
CLA t9c11	n.d.^{b}	13.1^{a}	n.d.^{b}	9.54^{ab}	n.d.^{b}	10.1^{ab}	2.126	0.676	***	0.679
CLA t10c12	n.d.^{b}	4.85^{a}	n.d.^{b}	4.45^{a}	n.d.^{b}	4.52^{a}	0.802	0.862	***	0.964
CLA t,t	n.d.^{b}	127^{a}	n.d.^{b}	98.4^{ab}	n.d.^{b}	123^{ab}	25.73	0.836	***	0.836
C18:1c9	15.2^{dc}	40.2^{b}	6.23^{d}	40.9^{b}	22.0^{c}	55.3^{a}	2.586	***	***	0.121
C18:1c11	3.38^{c}	8.07^{bc}	4.98^{c}	12.6^{ac}	5.72^{bc}	17.2^{a}	1.562	*	***	0.110
C18:1c12	0.94^{b}	1.54^{b}	1.25^{b}	2.57^{a}	1.40^{b}	3.18^{a}	0.224	**	***	*
C18:1c13	1.09	0.91	1.22	1.24	0.98	0.51	0.155	*	0.104	0.274
C18:1c14 + t16	2.92^{bc}	7.52^{b}	1.52^{c}	5.72^{bc}	4.95^{bc}	14.4^{a}	1.001	***	***	0.059
C18:1t4	n.d.	0.48	n.d.	0.58	n.d.	0.77	0.185	0.733	**	0.733
C18:1t5	n.d.	0.42	n.d.	0.65	n.d.	0.76	0.193	0.669	**	0.669
C18:1t6–t8	1.75^{b}	4.54^{ab}	1.29^{b}	9.60^{a}	2.87^{b}	8.66^{a}	0.859	*	***	*
C18:1t9	1.55^{b}	4.47^{ab}	0.93^{b}	13.3^{a}	2.07^{b}	9.29^{a}	1.166	**	***	**
C18:1t10	2.77^{b}	6.03^{b}	1.84^{b}	15.3^{a}	3.68^{b}	11.6^{a}	1.247	**	***	**
C18:1t11	38.6^{b}	52.9^{b}	19.0^{b}	158^{a}	53.8^{b}	90.8^{b}	21.22	0.164	**	**
C18:1t12	2.75^{b}	7.59^{b}	1.94^{b}	18.2^{a}	4.34^{b}	16.2^{a}	1.522	***	***	**
C18:1t15	n.d.^{c}	n.d.^{c}	n.d.^{c}	4.29^{a}	n.d.^{c}	3.14^{b}	0.185	***	***	***
C18:0	270^{c}	396^{b}	151^{d}	186^{d}	435^{b}	607^{a}	13.78	***	***	**

n.d.: not detected.
s.e.: standard error of mean.
^{a,b,c,d}Means within a row with a different superscript differ ($P < 0.05$).
†Effect of fraction (*F*).
‡Effect of time (*T*).
§Interaction between fraction and time (*F* * *T*).

isomerize C18:2n-6 to CLA c9t11 entirely with the bacterial *Butyrivibrio* group. The isomerase activity observed suggests that either *I. prostoma* can isomerize C18:2n-6 or that either the associated bacteria or their enzymes were not entirely or immediately inactivated by the antibiotic treatment. Ultimately, isomerization might also be the result of an abiotic process in the rumen. Secondly, C18:1c9 concentrations considerably increased upon incubation with C18:1t11. Research by Mosley and McGuire (2008) demonstrated that the biohydrogenation of ^{13}C-labeled C18:1t11 by ruminal microbes *in vitro* involves the formation of positional isomers of *cis* and *trans* C18:1 and C18:0. However, in the present study, only C18:1c9 concentrations increased upon C18:1t11 incubations. Moreover, the amount of C18:1c9 formed was similar for the IP + B, IP as well as the B fraction. These equal amounts of C18:1c9 in all fractions, despite the lower bacterial activity for the IP fraction, sustain the hypothesis of the abiotic isomerization of C18:1t11 at high *in vitro* concentrations.

In contrast to the IP fraction, substantial disappearance of C18:2n-6 and CLA c9t11 occurred for both the IP + B and B fraction. Both CLA c9t11 and CLA t10c12 were produced by the B fraction upon C18:2n-6 incubation. However, it is not clear why the IP + B fraction produced similar amounts of CLA c9t11 but not of CLA t10c12. Biohydrogenation proceeded until C18:1t11 but no further reduction to C18:0 was observed. The incubation of a protozoal suspension in the absence of antibacterial agents by Or-Rashid *et al.* (2008) also resulted in the production of C18:1t11 and C18:1t10. The absence of differences between IP and IP + B fractions regarding the extent of biohydrogenation suggest the hydrogenating activity originated from the endogenous and exogenous associated bacteria. The fact that C18:1t11 was not converted to C18:0 by these endosymbionts and ectosymbionts indicates that they do not belong to the group of bacteria currently known to transform C18:2n-6 into C18:0 and/or that they lack the reductase enzyme necessary for this final biohydrogenation step. On the other hand, incubation length might have been insufficient to enable C18:0 production since Or-Rashid *et al.* (2008) observed increased C18:0 concentrations when protozoal suspensions were incubated without antibiotics for 12 h or longer.

The fatty acid results exclude, besides a direct role of *I. prostoma* in rumen biohydrogenation, also the indirect role (e.g. H transfer) of *I. prostoma* in rumen lipid metabolism. The latter disproves the hypothesis of a required interaction between ciliate and bacterial communities for rumen biohydrogenation (Boeckaert *et al.*, 2007). Moreover, overall biohydrogenation by the IP + B and B fractions remained very limited as compared to hydrogenation performed by monofaunated rumen fluid. On average only 18% of C18:2n-6 and 14% of CLA c9t11 disappeared when the IP + B or B fraction was incubated while 46% of C18:2n-6 and 72% of CLA c9t11 disappeared with non-fractioned monofaunated rumen fluid.

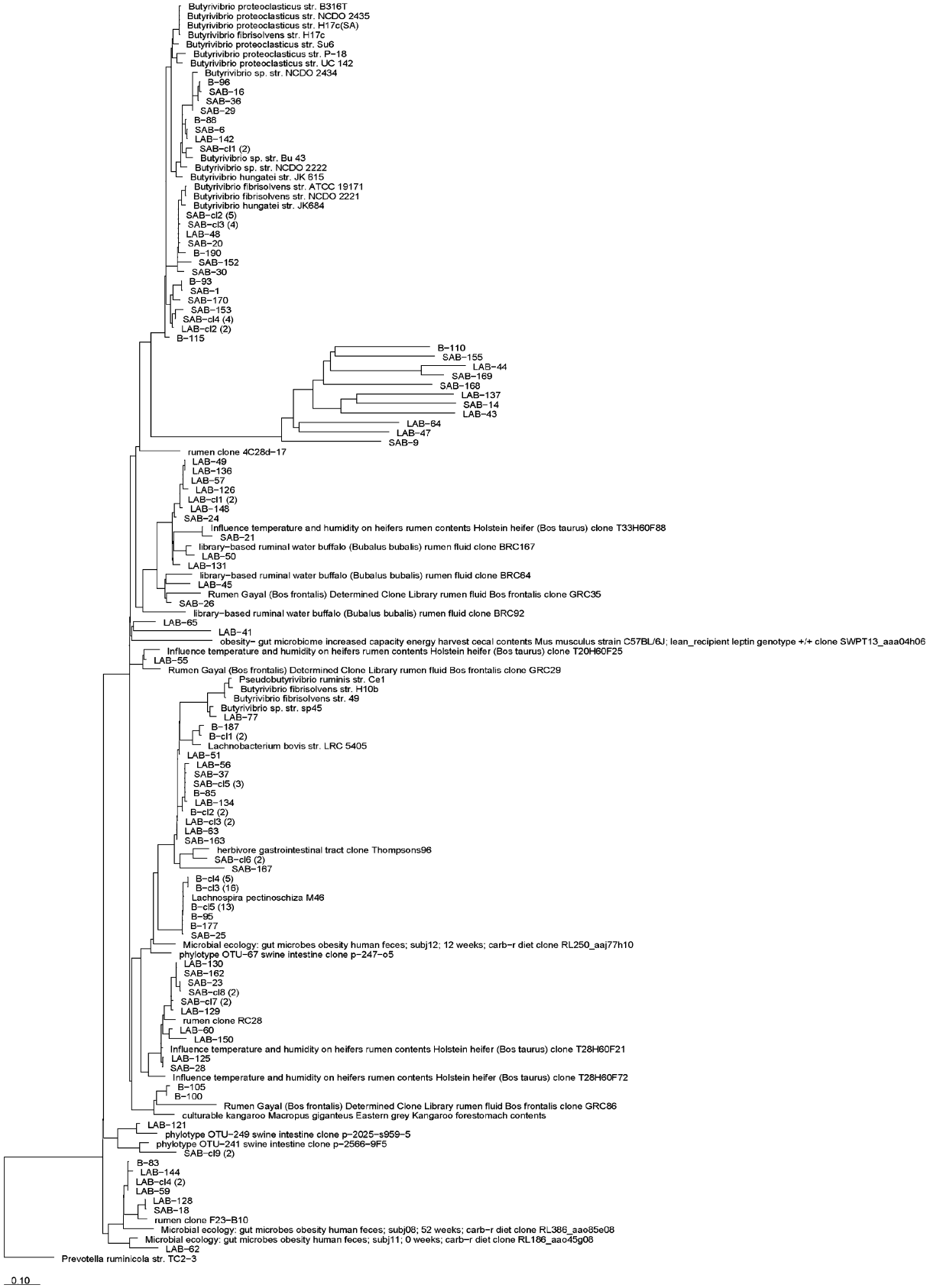

Figure 1 Phylogenetic tree representing the classification of 144 clones obtained from a liquid-associated bacteria (LAB) sample (42), a solid-associated bacteria (SAB) sample (52) and a sample containing the endogenous and exogenous associated bacteria (B) of *Isotricha protoma* (51) within the *Lachnospiraceae* family. Sequences were added to the original phylogenetic tree (Greengenes database January 2008) using Parsimony (ARB software, Ludwig *et al.*, 2004) without changing the tree topology.

Role of LAB and SAB in rumen biohydrogenation

A second aim of the current study was to assess the biohydrogenation capacity of SAB *v.* LAB. In this study, SAB represented the bacteria attached to the particulate solid matter (pellet) obtained after filtration (800 μm mesh) and centrifugation (500 × **g**, 3 min) of the monofaunated rumen fluid. Hence, SAB were not obtained from bulk, coarser solids retained by a wire gauze (1 mm mesh) as in other studies, e.g. Legay-Carmier and Bauchart (1989). It should be also noted that LAB and SAB fractions obtained in the current study could be atypical since the source of microorganisms was a single wether monofaunated with *I. prostoma*.

Incubations with the different fractions showed that decreased C18:2n-6 and CLA c9t11 concentrations were associated with increased amounts of hydrogenation intermediates and C18:0 in all fractions. Since biohydrogenation took place in both LAB and SAB fractions, biohydrogenating bacteria are not exclusive to any of the two phases. Previous research showed that hydrogenation of C18:2n-6 to C18:1t11 and subsequently to C18:0 was mainly associated with rumen solids (Harfoot *et al*., 1973; Singh and Hawke, 1979). However, these authors used a different methodology to obtain their bacteria, cell-free supernatant and food particle fractions as they were collected after incubation with lipid supplements. It was postulated that the preferential adhesion of lipids to the particulate matter resulted in an uneven distribution of biohydrogenating bacteria in the rumen (Ward *et al*., 1964) rather than an intrinsic difference in hydrogenating capacity between SAB and LAB (Harfoot *et al*., 1973). Our results indicate that the addition of equal amounts of fatty acids to the LAB and SAB fraction indeed results in a similar disappearance of C18:2n-6, CLA c9t11 and C18:1t11. However, differences between SAB and LAB did occur in terms of intermediate biohydrogenation steps with LAB fractions containing substantially higher concentrations of C18:1 fatty acids and lower concentrations of C18:0, suggesting that bacteria involved in the conversion of C18:1 fatty acids to C18:0 are largely absent in the LAB fraction. Probably, the LAB fraction mainly contains bacteria that have been categorized as group A bacteria by Kemp and Lander (1984) and which hydrogenate C18:2n-6 to C18:1t11 whereas the SAB fraction possibly contains the group B bacteria, which convert C18:1c9, C18:1t11 and C18:2n-6 to C18:0.

In general, the production of C18:0 (μg/ml) is lower for incubations with CLA c9t11 followed by C18:1t11, as compared to incubations with C18:2n-6. Most probably, this is related to the different biohydrogenation pathways (e.g. CLA c9t11 and CLA t10c12) that may arise from incubations with C18:2n-6 and that all contribute to C18:0 production. As an example, the LAB fraction contained similar concentrations of C18:1t10 and C18:1t11 when incubated with C18:2n-6 while incubations with CLA c9t11 were mainly associated with increased C18:1t11 concentrations. This suggests that C18:1t10-producing bacteria can proliferate only when the substrate CLA t10c12 is present. Moreover, it is not surprising that the formation of CLA t10c12 proceeds more smoothly from C18:2n-6 (one isomerization) than from CLA c9t11 (two isomerizations). Indeed, incubations with C18:2n-6 resulted in similar amounts of CLA c9t11, CLA t10c12 and CLA t,t, which all represented only a minor proportion of the fatty acids being formed.

It should be noted that the C18:0 production relative to the disappearance of C18:2n-6 is higher for the LAB + SAB fraction as compared to the SAB fraction. This might indicate that the presence of both bacterial groups increases the overall hydrogenation efficiency. In this respect, Kemp and Lander (1984) pointed out that high yields of C18:0 require a balance between the number of group A and group B bacteria. A striking feature is the high production of CLA t,t upon incubation with CLA c9t11. This reveals that the disappearance of added CLA c9t11 for all fractions is predominantly attributed to isomerization to CLA t,t.

Phylogenetic analysis of I. prostoma*-associated, LAB and SAB bacteria*

Bacteria that are currently known to be involved in rumen biohydrogenation belong to the *Butyrivibrio* group (Paillard *et al*., 2007a). Therefore, amplified *Butyrivibrio* PCR products were cloned in this study. A phylogenetic analysis of 42 LAB, 52 SAB and 51 B clones was performed to relate lipid metabolism with the clones' phylogenetic position (Figure 1). The majority (71%) of the B clones clustered in close relationship with *L. pectinoschiza*. This species was isolated from the pig intestine (Cornick *et al*., 1994) and is related to *Lachnospira multiparus*. Both bacteria are pectinolytic, decomposing pectin into oligogalacturonides in the rumen, which are further metabolized intracellularly with a high yield of acetic acid (Dušková and Marounek, 2001). Protozoa themselves also degrade pectin to oligouronides and methanol, but these products are not further metabolized (Abou Akkada and Howard, 1961). *L. pectinoschiza* might be associated with *I. prostoma* to further degrade the oligogalacturonides produced by *I. prostoma* into acetic acid. This might explain the high acetate proportions relative to propionate and butyrate proportions (79 : 10 : 11, mol : mol : mol) measured for the fractions containing active associated bacteria. An other 12 B clones were closely related with the genera *Butyrivibrio* and *Pseudobutyrivibrio*, both of which contain species previously shown to be involved in rumen biohydrogenation of C18 fatty acids (Paillard *et al*., 2007b). Among these, 2 B clones were situated on the branch involving the C18:0-producing *B. proteoclasticus* (Moon *et al*., 2008), although no C18:0 was produced (Figure 1).

The LAB fraction also had a minor role in C18:0 production. Nonetheless, the 1 LAB clone clustered within the C18:0-producing branch (Figure 1). The SAB fraction produced considerable amounts of C18:0 and 6 SAB clones were closely associated with the C18:0-producing branch whereas 32 SAB clones were positioned on different sub-clusters between both genera (Figure 1). Similar results were obtained in a previous experiment with dairy cows supplemented with docosahexaenoic acid-enriched algae, which dramatically decreased ruminal C18:0 production, although 20% of the clones remained clustered in close relationship with the C18:0-producing branch (Boeckaert *et al*., 2008).

Conclusion

I. prostoma cannot biohydrogenate C18:2n-6 whereas its associated bacteria play a minor role in the conversion of C18:2n-6 to C18:1t11. Both SAB and LAB possess hydrogenating capacity. SAB perform the full hydrogenation reaction to C18:0 while LAB accumulate C18:1 fatty acids, predominantly C18:1t10 and C18:1t11. Phylogenetic analysis of clones originating from *Butyrivibrio*-specific PCR products indicated that bacteria associated with *I. prostoma* show a close relationship with *L. pectinoschiza*. SAB and LAB clones clustered mainly within the genera *Butyrivibrio* and *Pseudobutyrivibrio*. SAB clones predominantly clustered with unknown and yet-uncultivated microbial species between the genera *Butyrivibrio* and *Pseudobutyrivibrio* and to a lesser extent within the known C18:0-producing *B. proteoclasticus* branch.

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References

Abaza MA, Abou Akkada AR and El-Shazly K 1975. Effect of rumen protozoa on dietary lipid in sheep. Journal of Agricultural Science 85, 135–143.

Abou Akkada A and Howard BH 1961. The biochemistry of rumen protozoa. 4. Decomposition of pectic substances. Biochemical Journal 78, 512–517.

Bauman DE, Baumgard LH, Corl BA and Griinari JM 2000. Biosynthesis of conjugated linoleic acid in ruminants. Journal of Animal Science 77, 1ae–15ae.

Boeckaert C, Fievez V, Van Hecke D, Verstraete W and Boon N 2007. Changes in rumen biohydrogenation intermediates and ciliate protozoa diversity after algae supplementation to dairy cattle. European Journal of Lipid Science and Technology 109, 767–777.

Boeckaert C, Vlaeminck B, Fievez V, Maignien L, Dijkstra J and Boon N 2008. Accumulation of *trans* C18:1 fatty acids in the rumen after algae supplementation is associated with changes in the *Butyrivibrio* community. Applied and Environmental Microbiology 74, 6923–6930.

Boon N, Top EM, Verstraete W and Siciliano SD 2003. Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. Applied and Environmental Microbiology 69, 1511–1520.

Broudiscou LP and Lassalas B 2000. Effects of *Lavandula officinalis* and *Equisetum arvense* dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. Reproduction Nutrition Development 40, 431–440.

Broudiscou LP, Papon Y and Broudiscou A 1999. Effects of inorganic nitrogen and amino acids on the degradation of ammonia-treated barley straw and proteosynthesis in a continuous culture of rumen microbes. Animal Feed Science and Technology 77, 149–162.

Chalupa W and Kutches AJ 1968. Biohydrogenation of linoleic-1-14C acid by rumen protozoa. Journal of Animal Science 27, 1502–1508.

Chow TT, Fievez V, Moloney AP, Raes K, Demeyer D and De Smet S 2004. Effect of fish oil on *in vitro* rumen lipolysis, apparent biohydrogenation of linoleic and linolenic acid and accumulation of biohydrogenation intermediates. Animal Feed Science and Technology 117, 1–12.

Coleman GS 1978. Rumen entodiniomorphid protozoa. In Methods of cultivating parasites *in vitro* (ed. JR Baker and AER Taylor), pp. 39–54. Academic Press, London.

Cornick NA, Jensen NS, Stahl DA, Hartman PA and Allison MJ 1994. *Lachnospira pectinoschiza* sp. nov., an anaerobic pectinophile from the pig intestine. International Journal of Systematic Bacteriology 44, 87–93.

Dawson RMC and Kemp P 1969. The effect of defaunation on the phospholipids and on the hydrogenation of unsaturated fatty acids in the rumen. Biochemical Journal 115, 351–352.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P and Andersen GL 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72, 5069–5072.

Devillard E, McIntosh FM, Newbold CJ and Wallace RJ 2006. Rumen ciliate protozoa contain high concentrations of conjugated linoleic acids and vaccenic acid, yet do not hydrogenate linoleic acid or desaturate stearic acid. British Journal of Nutrition 96, 697–704.

Dušková D and Marounek M 2001. Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rumen bacterium *Lachnospira multiparus*. Letters in Applied Microbiology 33, 159–163.

Folch J, Lees M and Sloane Stanley SGH 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226, 497–509.

Girard V and Hawke JC 1978. The role of holotrichs in the metabolism of dietary linoleic acid in the rumen. Biochimica et Biophysica Acta 528, 17–27.

Goering HK and Van Soest PJ 1970. Forage fiber analysis (apparatus, reagents, procedures, and some applications). Agriculture handbook 379. USDA-ARS, US Government Printing Office, Washington, DC, USA.

Gutierrez J, Williams PP, Davis RE and Warwick EJ 1962. Lipid metabolism of rumen ciliates and bacteria. 1. Uptake of fatty acids by *Isotricha prostoma* and *Entodinium simplex*. Applied Microbiology 10, 548–551.

Harfoot CG, Noble RC and Moore JH 1973. Food particles as a site for biohydrogenation of unsaturated fatty acids in the rumen. Biochemical Journal 132, 829–832.

Jouany J-P, Zainab B, Senaud J, Groliere CA, Grain J and Thivend P 1981. Role of the rumen ciliate protozoa *Polyplastron multivesiculatum*, *Entodinium* sp., and *Isotricha prostoma* in the digestion of a mixed diet in sheep. Reproduction Nutrition Development 21, 871–884.

Kemp P and Lander DJ 1984. Hydrogenation *in vitro* of alpha-linolenic acid to stearic acid by mixed cultures of pure strains of rumen bacteria. Journal of General Microbiology 130, 527–533.

Kemp P, White RW and Lander DJ 1975. The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. Journal of General Microbiology 90, 100–114.

Legay-Carmier F and Bauchart D 1989. Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. British Journal of Nutrition 61, 725–740.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar , Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A and Schleifer K-H 2004. ARB: a software environment for sequence data. Nucleic Acids Research 32, 1363–1371.

Moon CD, Pacheco DM, Kelly WJ, Leahy SC, Li D, Kopecny J and Attwood GT 2008. Reclassification of *Clostridium proteoclasticum* as *Butyrivibrio proteoclasticus* comb. nov., a butyrate-producing ruminal bacterium. International Journal of Systematic and Evolutionary Microbiology 58, 2041–2045.

Morgavi DP, Boudra H, Jouany JP and Michalet-Doreau B 2004. Effect and stability of gliotoxin, an *Aspergillus fumigatus* toxin, on *in vitro* rumen fermentation. Food Additives and Contaminants 21, 871–878.

Mosley EE and McGuire MA 2008. Biohydrogenation of vaccenic-1-13C acid by ruminal microbes *in vitro*. Journal of Animal Science 86(E-suppl. 2), 88.

Onodera R, Yamaguchi H, Eguchi C and Kandatsu M 1977. Limits of survival of mingled rumen bacteria in washed cell suspension of rumen ciliate protozoa. Agricultural and Biological Chemistry 41, 2465–2466.

Or-Rashid MM, AlZahal O and McBride BW 2008. Studies on the production of conjugated linoleid acid from linoleic and vaccenic acids by mixed rumen protozoa. Applied Microbiology and Biotechnology 81, 533–541.

Paillard D, McKain N, Rincon MT, Shingfield KJ, Givens DI and Wallace RJ 2007a. Quantification of ruminal *Clostridium proteoclasticum* by real-time PCR using a molecular beacon approach. Journal of Applied Microbiology 103, 1251–1261.

Paillard D, McKain N, Chaudhary LC, Walker ND, Pizette F, Koppova I, McEwan NR, Kopecny J, Vercoe PE, Louis P and Wallace RJ 2007b. Relation between phylogenetic position, lipid metabolism and butyrate production by different *Butyrivibrio*-like bacteria from the rumen. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 91, 417–422.

SAS Institute Inc. 2004. Output delivery system: user's guide. SAS Institute Inc., Cary, NC, USA.

Singh S and Hawke JC 1979. The *in vitro* lipolysis and biohydrogenation of monogalactosyldiglyceride by whole rumen contents and its fractions. Journal of the Science of Food and Agriculture 30, 603–612.

Wallace RJ, Chaudhary LC, McKain N, McEwan NR, Richardson AJ, Vercoe PE, Walker ND and Paillard D 2006. *Clostridium proteoclasticum*: a ruminal bacterium that forms stearic acid from linoleic acid. FEMS Microbiology Letters 265, 195–201.

Wang Q, Garrity GM, Tiedje JM and Cole JR 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied Environmental Microbiology 73, 5261–5267.

Ward PFV, Scott TW and Dawson MRC 1964. The hydrogenation of unsaturated fatty acids in the ovine digestive tract. Biochemical Journal 92, 60–68.

Williams AG and Coleman GS 1992. The rumen protozoa. Springer, New York, USA.

Williams PP, Gutierrez J and Davis RE 1963. Lipid metabolism of rumen ciliates and bacteria. 2. Uptake of fatty acids and lipid analysis of *Isotricha intestinalis* and rumen bacteria with further information on *Entodinium simplex*. Applied Microbiology 11, 260–264.

Wright DE 1959. Hydrogenation of lipids by rumen protozoa. Nature 184, 875–876.