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Influence of the Composition of the Cellulolytic Flora on the Development of Hydrogenotrophic Microorganisms, Hydrogen Utilization, and Methane Production in the Rumens of Gnotobiotically Reared Lambs▽

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We investigated the influence of the composition of the fibrolytic microbial community on the development and activities of hydrogen-utilizing microorganisms in the rumens of gnotobiotically reared lambs. Two groups of lambs were reared. The first group was inoculated with *Fibrobacter succinogenes*, a non-H_2-producing species, as the main cellulolytic organism, and the second group was inoculated with *Ruminococcus albus*, *Ruminococcus flavefaciens*, and anaerobic fungi that produce hydrogen. The development of hydrogenotrophic bacterial communities, i.e., acetogens, fumarate and sulfate reducers, was monitored in the absence of methanogens and after inoculation of methanogens. Hydrogen production and utilization and methane production were measured in rumen content samples incubated *in vitro* in the presence of exogenous hydrogen (supplemented with fumarate or not supplemented with fumarate) or in the presence of ground alfalfa hay as a degradable substrate. Our results show that methane production was clearly reduced when the dominant fibrolytic species was a non-H_2-producing species, such as *Fibrobacter succinogenes*, without significantly impairing fiber degradation and fermentations in the rumen. The addition of fumarate to the rumen contents stimulated H_2 utilization only by the ruminal microbiota inoculated with *F. succinogenes*, suggesting that these communities could play an important role in fumarate reduction *in vivo*.

Hydrogen is a major intermediary metabolite in anaerobic degradation of organic matter. In ruminants, hydrogen is produced by hydrolytic and fermentative microorganisms and is mainly used by methanogenic *Archaea* to reduce carbon dioxide into methane. These microorganisms represent the main ruminal microbial community implicated in this pathway (33). Hydrogen transfer through methanogenesis is beneficial to the degradation of plant cell wall carbohydrates in the rumen (19, 43, 46). However, as a result of this process, methane is eructated by ruminants (400 to 500 liters per day per adult animal) and represents a loss of carbon and energy, accounting for 8 to 12% of the gross energy content of the diet (27, 37, 44). The amount of methane produced varies according to the diet (forage or concentrate) and the production system (intensive or extensive) (27, 40). The contribution of livestock agriculture to greenhouse gas (GHG) emissions has been estimated to range between 9% and 18% of anthropogenic emissions, with methane representing between 30% and 50% of the total GHG emitted from the livestock sector (34). In that context, various strategies have been suggested in order to mitigate ruminant methane production (27, 28, 34). Reducing hydrogen production should be achieved without impairing efficacy of feed digestion and fermentation. Inhibition of activity and/or number of methanogens should be done in association with a stimulation of hydrogen-consuming pathways in order to avoid the negative effect of an increased partial pressure of this gas (28).

The aim of the present work was to investigate the effect of cellulolytic microbial populations on the development and activity of hydrogen-consuming microorganisms and methane production in the rumen. We used gnotobiotically reared lambs harboring cellulolytic species that differed in their capacity to produce hydrogen. Among the major cellulolytic communities, anaerobic fungi and ruminococci are major contributers to H_2 production, whereas *Fibrobacter succinogenes* does not produce any hydrogen (17). *F. succinogenes* has been found to be the predominant cellulolytic species in the rumen of bison, and the presence of methanogens has been reported to be less important in this animal than in other ruminants, where the abundance of ruminococci and methanogens was strongly correlated (35).

MATERIALS AND METHODS

Animals and diet. Four naturally born lambs (INRA Romane breed) from the INRA sheep flock were utilized in this study. They had been left with their dams for the first 15 to 20 h after birth before they were transferred to sterile incubators (La Calhène, Vendôme, France) where they were reared gnotobiotically (14) until they were 5 months old (end of the experiment). Two pairs of animals

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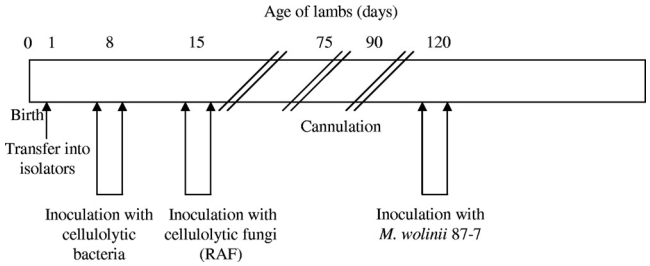

FIG. 1. Timeline showing interventions according to the age of the lambs.

were studied. (i) Group FS contained two lambs inoculated with *Fibrobacter succinogenes* (strains S85, U, and HM2) as the sole cellulolytic organism. (ii) Group RAF contained two lambs inoculated with cellulolytic microflora composed of *Ruminococcus albus* 7, *Ruminococcus flavefaciens* FD1 and two strains of anaerobic fungi (one monocentric and one polycentric).

Until the lambs were 40 days old, they were exclusively fed cow's milk sterilized by ultrahigh temperature (UHT) processing. Once they were 40 days old, they received at libitum a diet of dehydrated alfalfa hay in 7-mm pellets (SAFE, Augy, France) that had been sterilized by gamma irradiation (4 megarads; Ionizos, Dagneux, France). Lambs were weaned after 50 days. When the animals were between 2.5 and 3 months of age, they were fitted with a permanent ruminal cannula (external diameter, 27 mm; internal diameter, 20 mm).

The experimental protocol had been reviewed and validated by the local Ethics Committee before the beginning of the experiment.

Inoculation of the different microbial communities (Fig. 1). (i) Origin of cellulolytic strains. *Fibrobacter succinogenes* S85 and HM2 were obtained from the American Type Culture Collection (ATCC) (ATCC 19169 and ATCC 43856, respectively), and *F. succinogenes* U was provided by the Agriculture and Agri-Food Canada Lethbridge Research Centre, Lethbridge, Alberta, Canada. *Ruminococcus albus* 7 and *Ruminococcus flavefaciens* FD1 were obtained from the culture collection of the University of Illinois at Urbana-Champaign.

Ruminal fungal strains were isolated in our laboratory from a hay-fed sheep with a cannula in its rumen. On the basis of microscopic observation, a monocentric strain (presumptively close to *Neocallimastix frontalis*) and a polycentric strain (morphologically close to *Orpinomyces joyonii*) were selected from bacterium-free culture enrichments in the medium of Orpin (38) supplemented with a mixture of antibiotics (8) and a strip of filter paper (Whatman no. 1).

All microbes were cultured under strictly anaerobic conditions (23).

(ii) Inoculation with cellulolytic strains. The absence of cellulolytic bacteria and fungi in each lamb rumen before inoculation with pure cultures was carefully checked by inoculating triplicate Hungate tubes containing culture medium and filter paper cellulose strips with 10^{-1}, 10^{-2}, and 10^{-3} dilutions of fresh rumen contents, obtained by stomach tubing, from the 2-day-old and 5-day-old lambs. The tubes were incubated at 39°C for at least 2 weeks, and we checked that the filter paper strips remained totally undegraded.

The cellulolytic bacterial inocula were prepared in a ruminal-fluid-based anaerobic medium (20). After 24 h of incubation at 39°C, one 10-ml tube containing a mixture of *F. succinogenes* strains or one 10-ml tube containing a mixture (50:50) of both *R. albus* and *R. flavefaciens* were inoculated into the 8-day-old lambs for three consecutive days. The RAF lambs were inoculated with fungal cultures over a period of three consecutive days 1 week later. One 10-ml culture tube containing a mixture (50:50) of each fungal isolate grown for 24 to 48 h in a ruminal-fluid- and cellobiose-based medium was given to each lamb per day using a stomach polyvinyl chloride (PVC) tube (external diameter, 6 mm; internal diameter, 3 mm).

(iii) Inoculation of the methanogenic strain. When the animals were 120 days old, they all received an inoculum of strain 87-7, a methanogenic strain isolated from lamb rumen in our laboratory (F. Chaucheyras-Durand, unpublished data). On the basis of morphological and metabolic criteria, this strain was related to a *Methanobrevibacter* species. Sequencing of the 16S rRNA gene (EMBL accession number AM269413) indicated that this strain was phylogenetically close to *Methanobrevibacter wolinii* (98% identity). The contents of one culture tube containing the 87-7 strain, grown on a complex medium (3) under a pressure of 202 kPa in a mixture of H_2 and CO_2 (80:20), was administered to the animals during three consecutive days through the cannula in the rumen.

Microbiological analyses. Samples from the rumen were collected before the morning feeding by aspiration with a stomach tube in lambs less than 3 months old and through the cannula afterwards. Serial dilutions of each sample were prepared in the anaerobic mineral solution (6). These dilutions were then utilized for enumeration of different microbial functional groups, i.e., total viable anaerobic bacteria, cellulolytic bacteria, anaerobic fungi, and methanogenic *Archaea*, according to previously described methods (15).

Acetogenic bacteria, methanogenic *Archaea*, and sulfate-reducing bacteria (SRB) were enumerated in specific liquid media by the methods of Doré et al. (11) and Morvan et al. (35, 36) after incubation at 39°C in a H_2-CO_2 (80:20) atmosphere (202 kPa) for 2 to 3 weeks. Fumarate-reducing bacteria (FRB) were enumerated in the *Wolinella succinogenes* medium of Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (DSMZ medium number 157) modified by the method of Fonty et al. (16). Cultures were incubated for 3 weeks at 39°C in a H_2-N_2 (50:50) atmosphere (202 kPa). Growth of FRB was considered to occur when a decrease in gas pressure (compared to uninoculated pressurized tubes) together with an increase in succinate and/or propionate were observed. Bacterial numbers were estimated according to the most-probable-number method (9).

Degradation of alfalfa hay in bags in the rumen. Fiber degradation in the rumens of all lambs was determined before and after inoculation of methanogens (at 100 and 140 days of age, respectively) by the nylon bag method (31). Each bag (pore size, 50 μm) contained 0.8 g of ground alfalfa pellets. The bags were incubated for periods of 24 and 48 h in the rumen, and the incubations were repeated four times for each lamb. After incubation, the bags were removed from the rumen, washed with tap water, dried at 70°C, and weighed. The amount of alfalfa hay digested was then estimated by the percentage loss of dry matter (DM).

Hydrogen utilization by hydrogenotrophic communities. We estimated the hydrogen-utilizing capacities of the ruminal hydrogenotrophic populations harbored by the different animal models. To achieve this goal, we performed *in vitro* incubations of the contents of the rumen in the presence of hydrogen either produced from alfalfa hay degradation and fermentation or added as an exogenous substrate as described previously (13, 16). Rumen content samples (approximately 120 g) were obtained at the following times: 2 h after the morning feeding, from each lamb when they were 98 to 100 days old, i.e., before inoculation of methanogens, and when they were 138 to 140 days old, i.e., after inoculation of methanogens. The samples were filtered through two layers of cheesecloth, and 25-ml portions of filtered rumen samples were diluted in 15 ml of anaerobic mineral solution (6) and incubated *in vitro* for 0, 24, and 48 h in 125-ml flasks under the following gaseous atmospheres and conditions: 100% CO_2 plus 200 mg ground alfalfa pellets, 202 kPa of H_2-CO_2 (80:20), 202 kPa of H_2-N_2 (50:50), or 202 kPa of H_2-N_2 (50:50) plus 30 mM sodium fumarate.

At the beginning and end of incubation, total gas volume in each flask was measured by displacement using a glass syringe, and gas composition was determined by gas chromatography. Five milliliters of each sample was also centrifuged (1,000 × *g*, 15 min, 4°C), and supernatants were used for determination of the short-chain fatty acid (SCFA) concentration by one-dimensional (1D) ^{1}H nuclear magnetic resonance (NMR) analysis (29).

Statistical analyses. In the figures and tables, results are given as means with their standard deviations (SDs). Data were analyzed with one-way analysis of variance, and differences between groups were considered to be significant at $P < 0.10$ using Student's *t* test.

RESULTS

Establishment of microbial communities in the rumens of gnotobiotically reared lambs. Total anaerobic bacteria rapidly established at high levels in all lambs; 5 days after birth, this community already ranged between 1.6×10^9 and 7.4×10^9 CFU · ml^{-1} and slightly increased during the first weeks. No significant differences were observed for total bacterial counts within the two groups of animals.

Cellulolytic bacteria were not detected in the rumen of any lamb before inoculation of the different species. In both groups, they began to be detected a few days after their inoculation with pure cultures. The respective population mean sizes were 2.5×10^7 and 5.0×10^6 cells · ml^{-1} for FS and RAF lambs before inoculation of methanogens and 5.0×10^7 and 1.6×10^6 cells · ml^{-1} after inoculation of methanogens.

Cellulolytic fungi were never detected in the rumens of FS

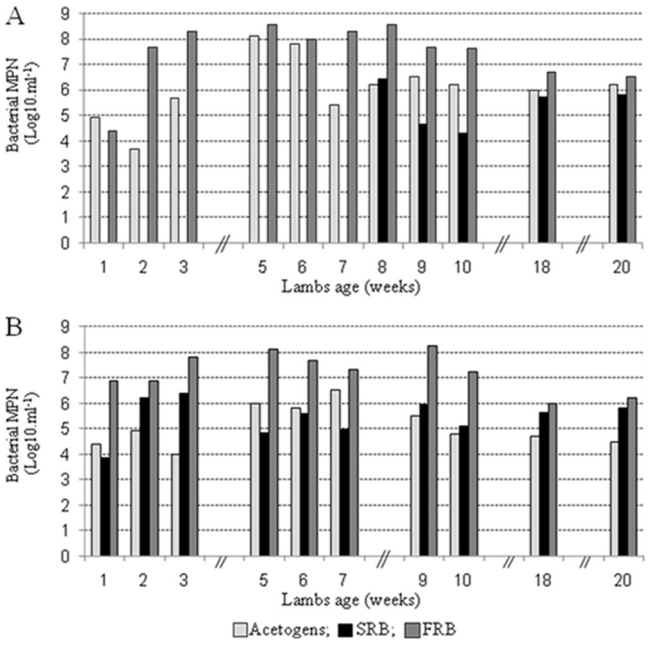

FIG. 2. Ruminal concentrations of acetogens, sulfate-reducing bacteria (SRB), and fumarate-reducing bacteria (FRB) according to the age of the lambs. (A) FS lambs; (B) RAF lambs. For each time point, the results are the means of two most-probable-number (MPN) determinations in each group.

lambs and not detected in the rumens of RAF lambs before their inoculation with pure cultures. After their inoculation, they were detected consistently in the rumen of only one lamb from the RAF group, whereas they were found only sporadically in the rumen of the other RAF lamb; zoospore concentrations ranged between 10^2 and $10^3 \cdot ml^{-1}$ of rumen contents.

Methanobrevibacter wolinii-related strain 87-7 established rapidly in the rumens of all lambs. The mean number of strain 87-7 bacteria was close to 10^6 cells $\cdot ml^{-1}$ 1 week after inoculation and increased thereafter to reach mean values above 5×10^7 cells $\cdot ml^{-1}$.

In FS lambs, in the absence of methanogens, acetogenic bacteria were detected in the rumen at low levels just after *Fibrobacter succinogenes* inoculation, then the size of the population increased to $\sim 10^8$ bacteria $\cdot ml^{-1}$ (Fig. 2A). After 6 weeks, it fell to lower levels. In the presence of methanogens, only one sample per lamb was analyzed, and the population was close to 10^6 cells $\cdot ml^{-1}$. Sulfate-reducing bacteria (SRB) were detected only after 50 days in the rumens of FS lambs; the numbers of bacteria were rarely above 10^6 cells $\cdot ml^{-1}$.

In RAF lambs, acetogenic bacteria were detected soon after birth, but their population size rarely reached 10^6 bacteria $\cdot ml^{-1}$ (Fig. 2B). After inoculation of methanogens, acetogen concentrations decreased in the rumens of RAF lambs and were detected only at low levels (3.0×10^4 to $4.9 \times 10^4 \cdot ml^{-1}$). SRB were present very soon after birth. Their population size stabilized between 10^5 and 10^6 bacteria $\cdot ml^{-1}$ even after establishment of methanogens.

In both FS and RAF lambs, fumarate-reducing bacteria (FRB) were detected soon after birth at a concentration close to 10^7 cells $\cdot ml^{-1}$. This concentration remained stable, between 10^7 and 10^8 bacteria $\cdot ml^{-1}$ in the absence of methanogens. Although only one ruminal fluid sample from each animal could be analyzed after inoculation of *M. wolinii*, we noticed that the FRB population tended to decrease (1-log-unit decrease) when methanogens were present in the ecosystem.

TABLE 1. Disappearance of alfalfa hay dry matter in bags in the rumens of gnotobiotically reared lambs before and after establishment of methanogens

Animal model	Methanogenic status[a]	DM disappearance[b] (%) in samples incubated for the following time:	
		24 h	48 h
FS lambs	Absent	49.9 ± 2.1	50.3 ± 3.6
	Present	51.9 ± 3.6	53.6 ± 3.3
RAF lambs	Absent	53.5 ± 4.4	53.9 ± 2.5
	Present	57.4 ± 5.5	57.8 ± 6.4

[a] Absent, methanogens absent; Present, methanogens present.
[b] The disappearance of dry matter (DM) is shown. The values are means ± standard deviations (SDs) for at least 4 values.

Fiber degradation in bags in the rumen. The percentages of alfalfa hay dry matter (DM) degradation were close to 50% after 24 h of incubation (Table 1). DM degradation was not statistically significantly different between the two groups.

***In vitro* hydrogen production and consumption and methane production. (i) Short-chain fatty acid (SCFA) concentrations in lamb rumen contents.** Before incubation of the flasks, SCFA concentrations were determined in the ruminal fluid samples obtained 2 h after feeding. The concentrations of the main SCFAs (acetate, propionate, and butyrate) were not significantly different in the two groups of animals (Table 2). However, the composition of the SCFA mixture varied. Before inoculation of strain 87-7, we observed a high percentage of butyrate in the samples from FS lambs and a high percentage of acetate and propionate in the samples from RAF lambs. After inoculation with methanogens, acetate proportion increased in FS lambs and propionate and butyrate proportions sharply decreased, whereas no great changes in the SCFA proportions were observed in RAF lambs.

TABLE 2. Main short-chain fatty acid concentrations and proportions in ruminal fluid samples of gnotobiotically reared lambs[a]

Animal model	Methanogenic status[b]	Concn of the main SCFAs[c] (mmol $\cdot$ liter^{-1})	Proportion (%) of the following SCFA in ruminal fluid samples		
			Acetate	Propionate	Butyrate
FS lambs	Absent	95.5 ± 18.3	71.2	17.9	10.9
	Present	102.1 ± 14.2	84.0	9.2	6.5
RAF lambs	Absent	87.6 ± 11.4	77.7	19.8	2.6
	Present	95.0 ± 16.4	75.6	21.3	3.1

[a] The samples were obtained 2 h after the morning feeding in the absence or presence of methanogens.
[b] Absent, methanogens absent; Present, methanogens present.
[c] The main short-chain fatty acids (SCFAs) were acetate, propionate, and butyrate. The values are means ± standard deviations (SDs) for at least 4 values.

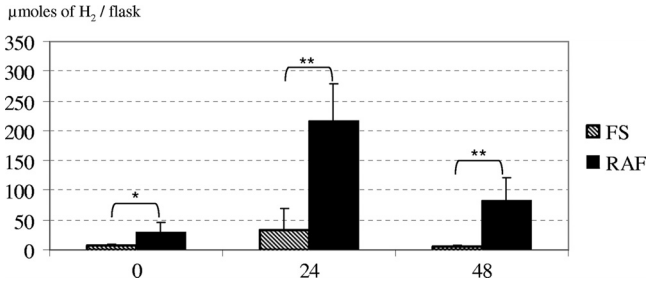

FIG. 3. Hydrogen production from *in vitro* incubations of ruminal fluid samples obtained from the two groups of lambs (FS and RAF lambs). The samples were incubated in the presence of ground alfalfa. Values represent means plus standard deviations (SDs) (error bars) from two values for each lamb within each group. Within the same incubation time, values for samples from the two groups were significantly different as follows: *, $P < 0.05$; **, $P < 0.01$.

(ii) Incubations in the presence of ground alfalfa hay and 100% CO_2 in the gas phase. When alfalfa was added to the flasks containing rumen contents taken before establishment of methanogens, H_2 production peaked after 24 h of incubation and decreased thereafter (Fig. 3). Methane was never detected in the gas phase. Significantly higher H_2 quantities were produced from alfalfa fermentation by ruminal fluid samples from RAF lambs than by samples from FS lambs. After inoculation of methanogens, almost no hydrogen was recovered, and methane was detected in the flasks (Fig. 4A). The amount of methane produced from RAF lamb rumen contents was significantly greater than that produced from FS lamb rumen contents after 48 h of incubation, whereas no significant difference in major SCFA production was measured between the two lamb groups (Fig. 4B). Moreover, the amount of methane produced during incubation of samples from RAF lambs exceeded the amount expected to be generated from H_2 produced from alfalfa, based on H_2 quantities measured before inoculation of strain 87-7 and on stoichiometry of methanogenesis pathway (4 mol H_2 necessary to synthesize 1 mol CH_4).

(iii) Incubations with H_2-CO_2. In FS lambs, in the absence of methanogens, and when exogenous hydrogen was introduced in the headspace using a H_2-CO_2 mixture, approximately 78% of the initial H_2 was consumed within 48 h (Table 3). Methane was never detected. Ninety-eight percent of H_2 was consumed after 48 h of incubation of ruminal fluid obtained from the FS lambs 12 days after inoculation with the methanogenic strain, but based on the stoichiometry of methanogenesis, the calculated percentage of H_2 actually required for methane formation was only 47% (data not shown). However, when the ruminal fluid samples of FS lambs were obtained 27 days after inoculation of methanogens, all H_2 was accounted for by methane formation (Table 4).

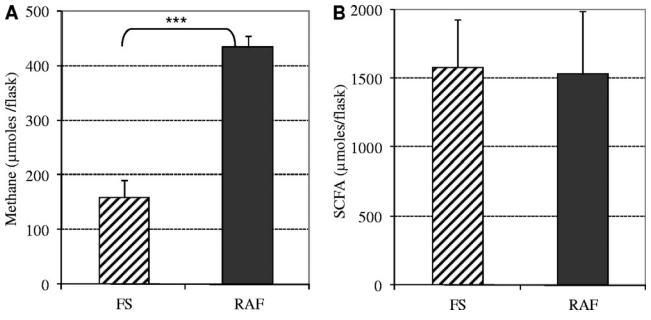

FIG. 4. *In vitro* methane (A) and major short-chain fatty acid (SCFA) (B) production after 48 h of incubation of rumen contents from both groups of lambs in the presence of ground alfalfa after establishment of *M. wolinii* 87-7. The values are the means of two values per lamb with their SDs. The values for the two groups were significantly different ($P < 0.001$) as indicated by the three asterisks.

TABLE 3. Utilization of H_2 after 48 h of incubation of mixed ruminal bacteria of the two groups of lambs with different gas mixtures before and after inoculation of methanogens

Animal model	Methanogenic status[a]	H_2 utilization (%)[b] by mixed ruminal bacteria under the following conditions:		
		H_2-CO_2	H_2-N_2	H_2-N_2 + fumarate
FS lambs	Absent	77.9	37.0	60.6
	Present	98.0	87.9	94.1
RAF lambs	Absent	48.7	41.9	42.6
	Present	96.8	86.5	99.6

[a] Absent, methanogens absent; Present, methanogens present.
[b] Utilization of H_2 is shown as a percentage of the initial concentration at the beginning of incubation of two flasks per animal.

(iv) Incubations in an atmosphere of H_2-N_2 with or without fumarate. When the contents of the rumens of FS lambs were incubated under a H_2-N_2 atmosphere, in the absence of *M. wolinii* 87-7, hydrogen consumption was lower than when H_2-CO_2 was used as the gas mixture (Table 3). Indeed, only 37% of the H_2 was used within 48 h. When fumarate was added to the incubation medium, the percentage of H_2 utilization by the flora increased to 60.6%. Methane was never detected in the gas phase.

Almost 42% of the H_2 was consumed within 48 h by the microflora of RAF lambs in the absence of methanogens; however, this percentage was not increased by the addition of fumarate, in contrast to what was observed with FS rumen samples (Table 3).

After establishment of methanogens, in the absence of fumarate, a sharp increase in H_2 utilization was observed in samples from FS and RAF lambs; in the presence of fumarate, almost 100% H_2 was consumed after 48 h of incubation of samples from both groups of lambs, but the recovery of methane produced related to the amount of hydrogen consumed was only 66% in FS samples, whereas it was a little higher than 100% in RAF samples (Table 4).

DISCUSSION

Establishment of microbial populations in the rumens of gnotobiotically reared lambs. We used animal models with

TABLE 4. Hydrogen consumed and methane produced after incubation of the contents of rumens from the two groups of lambs[a]

Incubation condition(s)	Animal model	Amt of H_2 consumed (μmol/flask)	Amt of CH_4 produced (μmol/flask)		
			Expected[b]	Measured	% Recovery
H_2-CO_2	FS lambs	4,960	1,240	1,234	99.5
	RAF lambs	5,290	1,323	1,459	110.3
H_2-N_2, without fumarate	FS lambs	6,561	1,640	1,686	103.0
	RAF lambs	5,860	1,465	1,455	99.3
H_2-N_2, with fumarate	FS lambs	6,030	1,508	999	66.2
	RAF lambs	6,148	1,537	1,881	122.4

[a] Ruminal fluid samples from the lambs were incubated for 48 h in H_2-CO_2 or H_2-N_2 atmosphere in the presence or absence of fumarate after establishment of methanogens.

[b] The amount of methane expected has been calculated from stoechiometry of the reaction $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$.

different fibrolytic species to better understand the relationships between hydrogen-producing and -consuming communities in the absence of methanogens and after inoculation of methanogens. Considering the poor knowledge of the physiology and metabolism of these communities, these animal models appeared particularly relevant to study these kinds of interactions. In these lambs, rumen bacterial and fungal population densities were found to be close to those reported for conventionally reared animals (15, 18).

The detection of acetogens very soon after birth was in accordance with previous findings from conventionally reared animals (16, 36). Indeed, it is very likely that colonization of the rumen by these communities occurs thanks to maternal transmission. Because of their metabolic versatility, acetogenic bacteria can use a wide range of substrates for growth in addition to H_2-CO_2 (12). Our enumeration method being based only on the capacity of H_2-CO_2 uptake, we could have underestimated the population density of acetogens as has been discussed elsewhere (16). A quantitative real-time PCR approach targeting a functional *fhs* gene encoding the formyltetrahydrofolate synthetase (FTHFS), a key enzyme implicated in reductive acetogenesis pathway, has been proposed (47) but has not been yet applied and validated for digestive ecosystems.

SRB were not detected in the rumens of FS lambs before 50 days (weaning period). This may be due to a low availability of hydrogen in the rumens inoculated with *F. succinogenes*. Although FRB population densities were found in accordance with other studies (16), they were quite variable, and consequently, it is not possible to know whether the nature of fibrolytic communities influences its development.

After establishment of *M. wolinii* 87-7, only a few samples were analyzed for bacterial enumeration, which made it difficult to reach a conclusion regarding its impact on other bacterial communities. We did not observe a significant modification of the cellulolytic bacterial community size, but we noticed fluctuations of acetogenic bacterial numbers, which declined in the rumens of RAF lambs. FRB also tended to decrease slightly in all groups after methanogen inoculation. These data confirm that methanogenic *Archaea* impact negatively on other hydrogen-consuming populations, even those established for a long time, particularly acetogenic bacterial communities which are known to have a higher H_2 utilization threshold than methanogens do (10).

Fiber degradation. In our animal models, the relatively high percentages of DM degradation illustrate an efficacious fibrolytic function in the rumens of both groups of lambs. The slight improvement of fiber degradation observed after establishment of methanogens is in accordance with previous findings in a similar animal model (19). Indeed, through interspecies hydrogen transfer, methanogens contribute to a low H_2 partial pressure, which is essential for optimal functioning of hydrolytic microorganisms. Considering the same methanogenic status, no significant differences were obtained between the two groups of lambs. However, the slightly better degradation noticed in the rumens of RAF lambs may be explained by the effect of efficient enzymatic equipment of both ruminococci and fungi and the mechanical activity of fungal rhizoids (45), although competitive interactions between ruminococci and fungi have been described *in vitro* (5).

Hydrogen production and utilization in the absence of methanogens. When ruminal fluid samples were incubated with alfalfa hay as the substrate, no exogenous gas was added to the flasks, and we measured only small amounts of hydrogen as a result of simultaneous production-uptake processes. However, accumulation of H_2 was observed only in the samples from RAF lambs after 24 h of incubation, suggesting that promoting non-H_2-producing fibrolytic bacteria such as *F. succinogenes* limits hydrogen disposal for hydrogenotrophic populations. The fact that some H_2 remained in flasks containing ruminal fluid samples from RAF lambs suggested that establishing H_2-producing microorganisms as the dominant fibrolytic microflora does not induce a greater capacity of H_2 utilization by hydrogenotrophic communities.

Addition of different gaseous mixtures to incubated flasks allowed us to focus on hydrogen-consuming activities of the different hydrogenotrophic communities present in the rumens of the two animal models. In the presence of H_2-CO_2, an efficient hydrogen-consuming bacterial population in the rumens of FS lambs used H_2, possibly through the reductive acetogenesis pathway; however, acetate constituted only 60% of the major SCFA mixture (data not shown), which suggests that this activity might be limited; indeed, the presence of *F. succinogenes* as the sole fibrolytic bacterial species could limit the activity of these hydrogenotrophic populations. Hydrogen could also be utilized through other routes. Among the bacterial consortia preferentially associated with *F. succinogenes*, *Butyrivibrio fibrisolvens* representatives have been identified

(41). In ruminal fluid samples from FS lambs obtained 2 h after feeding and in 48-h incubations in a H_2-CO_2 atmosphere, butyrate represented a large proportion of the SCFA mixture (10.9 and 20%, respectively), which would support the hypothesis of a high prevalence of *B. fibrisolvens*. This species is involved in the fatty acid biohydrogenation pathway, and it may contribute to hydrogen uptake. However, this reaction is likely to play a rather minor role in hydrogen consumption *in vivo* (25). Acetate might also be reutilized for butyrogenesis by *B. fibrisolvens* or other species, which could explain the low levels of acetate.

In RAF lambs, the presence of hydrogen producers such as ruminococci and fungi as cellulose-degrading organisms was likely to favor the H_2-consuming activity, but it is noteworthy that the rate of H_2 consumed was lower than that measured in incubated flasks with ruminal fluid samples from FS lambs, which was surprising. One possible explanation might be the production of reduced components by the ruminococci and fungi, such as formate, which was detected at the beginning of incubation (5.3 ± 1.4 mM) but which was not found after 48 h of incubation. Formate might have been used, instead of H_2, as an electron donor for hydrogen-consuming bacteria. However, the capacity of hydrogen utilization by acetogens appeared to be higher than that of fumarate-reducing bacteria, because the percentage of H_2 utilization in a H_2-N_2 atmosphere in both animal models was only around 40%. This observation suggests that reductive acetogenesis would represent an important hydrogen sink in the rumen and that it would be interesting to take strategies aiming at favoring this pathway into consideration (16, 24).

The fact that adding fumarate to incubated flasks containing ruminal fluid samples from lambs inoculated with *F. succinogenes* induced a significant increase in hydrogen utilization indicates that the microbiota containing *F. succinogenes* can use fumarate efficiently and increase the removal of H_2. This was supported by NMR data showing a 48% increase in propionate proportion in flasks supplemented with fumarate, which suggested that the fumarate reduction pathway was activated, generating succinate being decarboxylated into propionate by several bacterial species (17). The results of *in vitro* studies have suggested that *F. succinogenes* is able to reduce fumarate to succinate by using H_2 as an electron donor (1, 2), but earlier papers reported that fumarate reduction by *F. succinogenes* was achieved only in the presence of NADH or the reduced form of flavin mononucleotide ($FMNH_2$) (30, 32). Therefore, it cannot be concluded that the increase of H_2 utilization was due to the activity of *F. succinogenes* alone. However, other bacterial species such as *Wolinella succinogenes*, *Anaerovibrio lipolytica*, and *Selenomonas ruminantium* have been reported to use hydrogen as an electron donor to reduce fumarate (22, 26, 42). Clones related to several clusters within the *Proteobacteria* phylum, possessing the *frdA* gene encoding the α-subunit of fumarate reductase, have also been characterized (21). These species, potentially present in the rumen contents of our lambs, could also be implicated in hydrogen removal from the incubated flasks. Recent findings (41) indicate that different bacterial consortia may be associated with different subgroups of *F. succinogenes* strains. In this context, further studies are needed to better characterize the composition of the microbial communities harbored by the FS lambs.

Hydrogen utilization in the presence of methanogens. In most of our *in vitro* experiments and regardless of the composition of fibrolytic communities, the proportion of hydrogen required for methanogenesis was not very important just after inoculation with *M. wolinii* 87-7, indicating that non-CH_4-producing hydrogenotrophs were able to compete with methanogens for H_2 utilization at least during the period of establishment of *M. wolinii* in the rumen. When ruminal fluid samples obtained once the methanogens had stabilized (1 week to 10 days after inoculation) were incubated, almost all the hydrogen was involved in methane production, which confirms the dominance of methanogens in the ecological niche for H_2 utilization (16), because of higher substrate affinity and lower thresholds for H_2 uptake (12). These results also suggest that to express their complete potential of H_2 utilization, methanogens need some time to adapt to the ecosystem.

When ruminal fluid samples were incubated with alfalfa hay as the substrate, methane production was significantly higher when the microflora contained ruminococci and fungi instead of *F. succinogenes*, but at the same time, there was no significant difference in SCFA content in the rumens of the two groups. The hydrogen production status of the fibrolytic community could influence methane synthesis in the rumen. These data corroborate with earlier results from Morvan et al. (35), who observed that in the rumens of bison, *F. succinogenes* was largely dominant among the cellulolytic bacterial populations and that methanogenic *Archaea* densities were lower than in the rumens of other domestic ruminants. Similarly, in the feces of methane-excreting human subjects (carrying methanogens in their colon), the cellulolytic bacterial isolates were hydrogen-producing *Ruminococcus* and *Enterococcus* species (39), whereas in the feces of non-methane excretors, the cellulolytic isolates were related to non-H_2-producing *Bacteroides* species (7).

After inoculation of methanogens and in the presence of fumarate in the flasks containing ruminal fluid samples from FS lambs, the recovery of methane produced/allowed by hydrogen disposal was only 66%, suggesting an active contribution of the microbiota containing *F. succinogenes* in the fumarate reduction process.

Conclusion. Using an original animal model requiring gnotobiology techniques, we show here that the composition of the cellulolytic community (hydrogen producers versus nonhydrogen producers) may have an impact on hydrogen accumulation and subsequent methane production in the rumen ecosystem. When the animal is young, the promotion of fibrolytic organisms which do not produce any hydrogen such as *Fibrobacter succinogenes*, with some control of methanogen activity, would represent an interesting ecological means to help limit methane emissions in the rumen. Feed additives such as selected direct-fed microbials able to enhance growth or/and activity of this bacterial species, together with fumarate supplementation, would be worth further study.

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