Long-term defaunation increases the abundance of cellulolytic ruminococci and methanogens but does not affect the bacterial and methanogen diversity in the rumen of sheep

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ABSTRACT: Protozoa are commensal eukaryotes in the rumen of herbivores. Protozoa are large producers of hydrogen, which is utilized by methanogenic archaea to produce methane, a greenhouse gas. The removal of protozoa from the rumen (defaunation) decreases methanogenesis, but also negatively affects fiber digestion, which is the main function of the rumen. The aim of this study was to examine the effect of long-term defaunation on the structure of the microbiota and particularly methanogenic archaea and fibrolytic bacteria to better understand the microbial mechanisms responsible for the decrease in methanogenesis and fibrolysis. The trial was conducted in 5 adult sheep subjected successively to long-term defaunation (2 yr), refaunation (12 wk), and short-term defaunation (10 wk). Methanogens were enumerated by quantitative PCR targeting the rrs (16S ribosomal RNA subunit) and mcrA (methyl coenzyme-M reductase) genes. The rrs gene was used to quantify the 3 major culturable rumen cellulolytic bacterial species (i.e., Fibrobacter succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens) and total bacteria. Bacterial and methanogen diversity was also examined by PCR-DGGE (PCR-denaturing gradient gel electrophoresis) analysis targeting the rrs and mcrA genes, respectively. Total rumen bacterial density estimated as rrs copies per gram of DM of rumen content increased in response to long- and short-term defaunation (+1 log, P < 0.001), but without noticeable shifts in diversity. Defaunation increased the rrs copies per gram of DM of rumen content of R. albus and R. flavefaciens (+2 log, P < 0.001), but did not affect that of F. succinogenes. Despite a 20% reduction in methane emission in the 2 defaunated periods, the mcrA and rrs copies of methanogens per gram of DM of rumen content increased (+1 log, P < 0.001) in the absence of protozoa, whereas the diversity of the dominant methanogenic community was not modified. This study shows no major difference between long- and short-term defaunation in abundance and diversity of bacteria and archaea. It also provides evidence that monitoring the abundance and diversity of methanogens is not sufficient to comprehend the microbial mechanisms leading to a reduction in methane emissions by ruminants. This study also reports for the first time in sheep a selective effect of defaunation on the abundance of cellulolytic bacterial species.

Key words: cellulolytic bacteria, defaunation, methanogen, polymerase chain reaction-denaturing gradient gel electrophoresis, quantitative polymerase chain reaction, rumen

INTRODUCTION

Ruminants depend for their growth and maintenance on the activity of a dense and diverse microbial community present in the rumen. However, rumen micro-
methanogenesis by reducing the density of methanogens. However, the effect of defaunation on the abundance of methanogens is still controversial (Machmüller et al., 2003; Ohene-Adjei et al., 2007; McAllister and Newbold, 2008). In parallel, it is necessary to consider the decrease in fiber digestion and investigate why defaunation induces such an effect. To our knowledge, there is only 1 report of a differential effect of defaunation on the abundance of cellulolytic bacterial species in cattle (Ozutsumi et al., 2006).

We have examined the effect of defaunation on methanogens and fibrolytic bacteria using molecular approaches, in sheep submitted successively to long-term defaunation, refaunation, and short-term defaunation. This experimental design was chosen because, unlike others, it presented the opportunity to monitor functional communities within the same animals in response to defaunation and refaunation, and because there is little information on the persistence of the effect of defaunation on rumen microbiota over long periods.

**MATERIALS AND METHODS**

The animal experimental procedure was reviewed and approved by the Local Ethics Committee before beginning the trial.

**Animals and Sampling**

The animals and treatments were described previously (Morgavi et al., 2008). Briefly, 5 adult, rumen-cannulated Texel wethers (*Ovis aries*) were used in a trial consisting of 3 periods. The animals had been kept defaunated (i.e., without protozoa) for a long period (2 yr, Def P1). They were successively refaunated (12 wk, Ref P2) and defaunated for a short period (10 wk, Def P3). Defaunation was carried out by rumen emptying and washing (Jouany and Senaud, 1979). Strained, pooled rumen fluid obtained from 3 conventional (protozoa-harboring) wethers that received a maintenance diet (see below) was used as protozoal inoculum in Ref P2. The inoculum was administered intraruminally and contained 1,000 mixed protozoal cells. The protozoa-free status of the animals was verified during the 2 defaunated periods (Def P1 and Def P3), whereas protozoa were enumerated (7.6 ± 3.4 × 10³ cells/mL) at the end of the refaunated period (Ref P2; Morgavi et al., 2008). Protozoa were mainly represented by small entodiniomorphs (88% of total), but large entodiniomorphs (>80 μm; 9%) and holotrichs (3%) were also found. The protozoal populations of refaunated animals, in terms of number and composition, were similar to those of donor animals.

Animals were housed in individual pens and were fed a maintenance diet consisting of 900 g of forage (75% alfalfa pellets, 25% hay) and 300 g of cracked corn grain. Feed was given once daily at 0800 h and was eaten within 1 h. No orts were observed during the trial.

Access to water and mineral salt block supplement was unrestricted. Methane production was measured 6 times during the trial over a 4-d period each time using the sulfur hexafluoride tracer technique (Morgavi et al., 2008).

Rumen contents were taken through the cannula from different rumen locations using an adapted collecting device that opens and closes at the sampling site. Sampling was done on the last day of each period, just before feeding, and was fractionated as follows. A first aliquot (about 40 g) was stored without any preservation (g of DM per 100 g of wet weight of rumen content). A second aliquot (about 100 g) was filtered through a polyester monofilament fabric (250-μm mesh aperture) to obtain rumen liquid and solid phase fractions. One-milliliter aliquots of the filtrate (liquid phase) were dispensed in microtubes, centrifuged at 1,000 × g, 10 min, 4°C, the supernatant was removed, and the pellet was stored at −80°C. The retentate (solid phase) was washed with 50 mL of sterile PBS, filtered as above, and 2 g was stored at −80°C. Whole rumen content samples were used for quantitative PCR (qPCR) analysis, and solid and liquid rumen samples were used for PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis.

**Bacterial and Archaeal Strains**

* Fibrobacter succinogenes* S85 (ATCC19169), *Ruminococcus flavefaciens* c94 (ATCC19208), *Ruminococcus albus* 7 (ATCC27210), *Butyrivibrio fibrisolvens* (DSM3071), *Eubacterium limosum* (ATCC80489), *Prevotella bryantii* B14 (DSM11371), *Peptostreptococcus* sp. D1 (isolated in our laboratory), *Megaphaera elsdenii* (DSM20460), *Selenomonas ruminantium* (DSM2872), and *Streptococcus bovis* (DSM20480) were routinely cultivated in a rumen fluid medium (Leedle and Hespell, 1980). *Escherichia coli* DH5α was cultivated on Luria-Bertani medium. *Methanobrevibacter smithii* (DSM861) was cultivated under 2 bar pressure of H2/CO2 (80/20) as already described (Balch et al., 1979).

**DNA Extraction**

Total DNA was extracted in duplicate from 0.25 g of rumen content or from solid and liquid rumen samples as described previously (Yu and Morrison, 2004). This method has been reported to extract PCR-quality microbial community DNA (DNA free of inhibitory substances) from rumen samples and was shown to produce the best representation of bacterial diversity in comparison with other DNA extraction methods. It was also used to assess archaeal diversity (Yu et al., 2008). The yield of total extracted DNA (μg of DNA/g of DM of rumen content) was expressed as the mean of 2 extractions per animal.
Genomic DNA from pure bacterial species was obtained as described before (Mosoni et al., 2007). Isolation of genomic DNA from *Mbb. smithii* was performed as follows. After 5 d of growth, cells (40 mL culture) were harvested by centrifugation (8,000 × g, 15 min, 4°C), washed in 2 mL of sodium phosphate buffer (50 mM, pH 7), centrifuged again (8,000 × g, 15 min, 4°C), and resuspended in 200 μL of the same buffer. Enzymatic cell lysis was accomplished by adding 20 μL of mutanolysin (1 mg/mL; Sigma-Aldrich, Saint-Quentin Fallavier, France), 10 kU/mg) and 12 μL of lysozyme (50 mg/mL; Sigma-Aldrich, 93 kU/mg) and incubating the mixture for 15 min at 37°C. An additional incubation for 5 min at 37°C with 1 μL of proteinase K (20 mg/mL; Sigma-Aldrich, 47 U/mg) was also performed. Cells were then disrupted by bead beating for 3 min with 0.2 g of zirconia beads (0.1 mm diameter, Sigma-Aldrich). Subsequent genomic DNA extraction was performed using the easy DNA Kit (Invitrogen, Cergy Pontoise, France) according to the manufacturer’s recommendations. Nucleic acid (RNase-treated DNA) concentration was estimated by absorbance at 260 nm.

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**qPCR**

Quantitative PCR (SYBR green chemistry) was carried out using a Mastercycler ep realpha 2S (Eppendorf, Le Pecq, France), 96-well plates (Twin.tec plate 96 skirted, Eppendorf), and realpha version 2.0 software (Eppendorf). Primers targeting the *rrs* gene were used to quantify total bacteria (Edwards et al., 2007, 2008), *R. flavefaciens* and *F. succinogenes* (Denman and McSweeney, 2006), and *R. albus* (Koike and Kobayashi, 2001). Methanogenic archaea were quantified by targeting the methyl coenzyme-M reductase (*mcr*) gene (Denman et al., 2007) and the *rrs* gene (Ohene-Adjei et al., 2007). The archaeal *rrs* primers are specific to methanogenic archaea (i.e., *Methanobrevibacter*, *Methanosphaera*, and *Methanobacterium*) present in the rumen ecosystem.

The qPCR reaction mixture (final volume of 20 μL) contained forward and reverse primers (0.3 μM for all primer sets except for total bacteria: 0.4 μM, 0.75x SYBR Premix Ex Taq (Perfect Real Time, Lonza, Levallois-Perret, France), and 40 to 100 ng of DNA template. The qPCR program was adapted to each target gene according to the instructions provided for the SYBR Premix Ex Taq (Perfect Real Time, Lonza), taking into account the already described qPCR conditions (Koike and Kobayashi, 2001; Denman and McSweeney, 2006; Denman et al., 2007; Edwards et al., 2007; Ohene-Adjei et al., 2007) and the PCR efficiencies (>95%) obtained with the standard curves (see below). For each rumen content sample, the results were expressed as the mean of 2 determinations (obtained with the 2 DNA extracts per rumen content sample) in *rrs* or *mcrA* copies per gram of DM of rumen content.

The standard curves (10^4 to 10^5 *rrs* copies) targeting *F. succinogenes* S85, *R. flavefaciens* c94, *R. albus* 7, and total bacteria were established as already described (Mosoni et al., 2007), except that for total bacteria we used equal amounts of the *rrs* DNA fragment (about 1,485 bp) amplified from genomic DNA of the 11 bacterial species cited above.

The standard curve (10^4 to 10^5 *mcrA* or *rrs* copies) targeting methanogenic archaea was determined with the *mcrA* DNA fragment (about 470 bp) or the *rrs* DNA fragment (about 1,100 bp) amplified from genomic DNA of *Mbb. smithii* DSM861 using PCR conditions and primers already described: *mcrA*-F: GGT GGT GTM GGA TTC ACA CAR TAY GCW GC and *mcrA*-R: TTC ATT GCR TAG TTW GGR TAG TT (Luton et al., 2002) or 344F: ACG GGG YGC AGC AGG CGC GA and 1406 to 1389R: ACG GGC GGT GTG TGC AAG (Ohene-Adjei et al., 2007).

Three dilutions of each DNA sample extracted from the rumen contents allowed us to obtain the slope of the sample curve and hence calculate the PCR efficiency for each DNA sample. In this way, PCR efficiency with DNA samples was confirmed to be similar to that obtained with the standard curves (>95%).

**PCR-DGGE**

Using DNA extracted from liquid and solid rumen samples as template, the V3 variable region of the *rrs* gene of bacteria was amplified by PCR with universal primers 341f and 532r as described before (Sadet et al., 2007). For methanogens, a fragment of the *mcrA* gene of about 470 bp was amplified according to Luton et al. (2002), except that a GC clamp was added to the 5’ end of the forward primer (Muyzer et al., 1993). The PCR run also had an initial denaturation step of 95°C for 15 min to activate the HotStartTaq DNA Polymerase (Qiagen, Hilden, Germany), a “slow-ramp” temperature increase to allow extension of mismatched primers in the first 5 cycles (Luton et al., 2002), and a final elongation step of 72°C for 30 min to eliminate artifactual double bands (Janse et al., 2004). All PCR products were analyzed by electrophoresis on 2% agarose gels containing ethidium bromide to check their size and estimate their concentration using a Low DNA Mass Ladder (Invitrogen, Carlsbad, CA) and an imaging system (Cheminager, Alpha Innotech, San Leandro, CA).

Denaturing gradient gel electrophoresis was performed as described before for total bacteria (Sadet et al., 2007) using a denaturing gradient between 40 and 60% for bacterial *rrs* fragments and between 20 and 40% for *mcrA* fragments. Samples of DNA from rumen liquid and solid phases from each animal and for the 3 periods were analyzed. In addition, amplified DNA fragments from each wether were mixed at equal con-
centrations to obtain pooled liquid and solid samples for each period. Approximately 100 ng of PCR product was loaded per well, and the electrophoresis was run for 5 h at 60°C using a fixed voltage of 200 mV in a DGGE-2001 (CBS Scientific Co., Solana Beach, CA). Gels were then silver stained using a commercial kit (Bio-Rad Laboratories, Hercules, CA). A known PCR product from a rumen sample was used as internal control for subsequent gel analysis (Sadet et al., 2007). Gel images were acquired using an optical density calibrated scanner (ImageScanner, GE Healthcare, Piscataway, NJ) at a spatial resolution of 400 dpi. The percent-age similarity above which samples were considered to be the same was 95.8 and 91.4% for bacterial rrs and methanogens mcrA, respectively, and was calculated using the internal controls.

**Statistics and DGGE Image Processing**

In this study 5 adult sheep were submitted successively to long-term defaunation, refaunation, and short-term defaunation, and microbial communities within the same animals were monitored in response to treatment (defaunation and refaunation). With such an experimental design, the treatment effect was confounded with the period effect. Because wethers were adult, nonproductive, fed a restricted diet, and maintained in the same controlled environment throughout the trial, the period effect was considered to be negligible. The qPCR data, converted into \( \log_{10} \) values, were statistically evaluated by ANOVA with the Tukey adjustment for multiple pairwise comparisons using the mixed procedure (SAS Inst. Inc., Cary, NC). The animal was considered as the random effect. Images of DGGE gels were analyzed with GelCompar II version 4.0 package (Applied Maths, Kortrijk, Belgium). Profiles were compared by hierarchical clustering to join similar patterns into groups (Fromin et al., 2002). To this end, all the images of DGGE gels were matched using the internal control sample and the bands were quantified after local background subtraction. A tolerance in the band position of 1% was applied. The similarity among profiles was calculated with the Pearson product-moment correlation coefficient, recommended for the analysis of complex profiles (Savelkoul et al., 1999), and the clustering was done with the unweighted pair-group method using arithmetic averages. A significance test based on pairwise similarity measures was used to compare the community profiles of different groups of samples (Kropf et al., 2004). This permutation test was done using the PROC IML procedure of SAS version 8 with \( 10^5 \) random permutations and based on the model of Kropf et al. (2004). For all statistical analysis, effects were declared significant at \( P < 0.05 \).

**RESULTS AND DISCUSSION**

An absolute quantification by qPCR targeting the mcrA and rrs genes of methanogens or the rrs gene of total bacteria and of the 3 cellulolytic bacteria F. succinogenes, R. albus, and R. flavefaciens was performed using already described qPCR conditions and primers. Results of quantification of the 5 microbial targets are given as target gene copies per gram of DM of rumen content (Table 1).

The DNA extraction yields, expressed as micrograms of DNA per gram of DM of rumen content, were greater \( (P < 0.001) \) in the defaunated periods \((858 \pm 102 \text{ in Def P1 and } 1,307 \pm 281 \text{ in Def P3})\) than in the faunated period \((202 \pm 131 \text{ in Ref P2})\). As already suggested in our previous study, DNA extraction may be affected by fiber content or microbiota composition or both in rumen samples (Mosoni et al., 2007). The DNA extraction method used in this study was shown to be very efficient with bacterial and archaeal communities \((Yu \text{ and Morrison, 2004; Yu et al., 2008}, \) whereas it is ineffective for dietary DNA recovery \((P. \text{ Mosoni, unpublished data})\). To our knowledge, no studies have reported DNA yields from rumen fungi using this method. In the faunated period, we assumed that the extraction
yields in protozoal DNA were low because protozoal N (used as biomass marker) represents less than 13% of the total rumen microbial N (Sylvester et al., 2005) and protozoal DNA recovery is less than 40% when using a DNA extraction method with a mechanical cell lysis step (J. L. Firkins, The Ohio State University, Department of Animal Sciences, Columbus, personal communication). In consequence, the much greater DNA yield per gram DM of rumen content from protozoa-free animals could reflect a denser bacterial population, as already shown by several authors (reviewed by Eugène et al., 2004b). It could also reflect a denser archaeal and fungal population. The latter assumption is unlikely though because defaunation had no effect on (Williams and Withers, 1993) or slightly increased fungal numbers (Newbold and Hillman, 1990).

**Effect of Long- and Short-Term Defaunation on Total Bacteria**

Long- and short-term defaunation increased (approximately 1 log, \( P < 0.001 \)) the bacterial \( rrs \) copies per gram of DM of rumen content (Table 1). Bacterial density tended to be greater in Def P3 than in Def P1 (\( P = 0.06 \)). An increase in viable bacteria as a result of defaunation was also measured in sheep by cultural enumeration (Demeyer and Van Nevel, 1979; Rowe et al., 1985; Newbold and Hillman, 1990; Williams and Withers, 1991).

In our work, the variations in total bacterial numbers observed at different periods were not accompanied by a change in the structure of the community as analyzed by DGGE. Indeed, the DGGE profiles of the amplified bacterial \( rrs \) fragments showed marked individual differences, a feature that masked any possible treatment effect. To circumvent this problem, DGGE profiles from every single wether were analyzed separately. For each wether, the rumen solid and liquid phase samples separated into 2 distinct clusters, but within each cluster periods with and without protozoa were not further separated (data not shown). Similarly, with pooled samples, the solid and liquid phases were separated at the first node (permutation test, \( P = 0.002 \)), but no period effect was found (Figure 1A). Yáñez-Ruiz et al. (2007), also using DGGE, reported differences in rumen bacterial communities between fauna-free and faunated lambs. These contradictory results could be explained by differences in experimental design between our study and theirs, such as the use of 2 groups of animals by Yáñez-Ruiz et al. (2007), with the fauna-free group never exposed to protozoa. In addition, the absence of changes in the bacterial community observed in the present work may be explained by the experimental periods of defaunation and refaunation, which were significantly longer than those usually reported (Demeyer and Van Nevel, 1979; Rowe et al., 1985; Santra and Karim, 2002; Yáñez-Ruiz et al., 2007) and could have allowed the community to stabilize.
**Effect of Long- and Short-Term Defaunation on Methanogens**

The main objective of the present study was to examine the effect of long- and short-term defaunation on methanogens. In the absence of protozoa, a 20% decrease in methane emissions was measured, and this decrease was stable for a period of up to 2 yr (Morgavi et al., 2008). Production of methane was $33 \pm 8$, $42 \pm 5$, and $35 \pm 7$ L per animal per day ($P < 0.05$; means ± SD, n = 5) in Def P1, Ref P2, and Def P3, respectively. Conversely, the amount of rumen methanogens increased by approximately 1 log in both defaunated periods whatever the target gene used for qPCR determination (Table 1). No significant difference was observed between the 2 defaunated periods. It is unlikely that the decrease in methane emissions was the result of a reduction in rumen volume considering that defaunation tends to increase retention time of feeds and rumen volume (Eugène et al., 2004b).

This uncoupling between methanogenesis and methanogen abundance has already been suggested (reviewed by Hegarty, 1999; Firkins and Yu, 2006), but never shown on a long-term basis. Recent studies on defaunation (Machmiller et al., 2003; Ohene-Adjei et al., 2007) or on treatments (i.e., addition of tea saponins or monensin; Guo et al., 2008; Hook et al., 2009) that decreased protozoa numbers and methanogenesis support the data obtained in the present study, suggesting that it is not the number of methanogens which is affected. Our results are in contradiction with 1 study showing that protozoa-free lambs produced 26% less methane per kilogram of DMI than faunated lambs and that the proportions of methanogens (measured by qPCR relative to total bacteria) were less in protozoa-free animals (McAllister and Newbold, 2008). Estimating methanogens by qPCR relative to total bacteria to investigate a defaunation effect on methanogen abundance may not be adequate considering that bacterial density is also affected by defaunation.

Our qPCR data obtained with mcrA primers were 1 log greater than those obtained with rrs primers targeting Methanobrevibacter, Methanosphaera, and Methanobacterium, 3 rumen dominant methanogenic genera in sheep (Ohene-Adjei et al., 2007). This difference might be due to the fact that the mcrA primers cover a broader range of methanogens than the rrs primers. Quantifying methanogens using 2 target genes, as done in the present study, increases the reliability of the qPCR for detecting fluctuation in the number of methanogens in response to treatments. If the decrease in methanogenesis cannot be explained by a decrease in methanogen abundance, it could be speculated that the methanogenic community has shifted in composition to a community less active or less efficient at producing methane. However, the mcrA DGGE profiles reflecting the composition of the dominant methanogens did not confirm this hypothesis either. Indeed, no differences between faunated and defaunated periods were observed in the mcrA DGGE profiles determined with pooled mcrA samples (Figure 1B). As observed for bacteria, only the mcrA DGGE profiles from rumen solid and liquid phases could be distinguished (permutation test, $P = 0.004$). Again, these DGGE results are in agreement with the recent studies using tea saponins and monensin (Guo et al., 2008; Hook et al., 2009). Based on phylogenetic analysis, Ohene-Adjei et al. (2007) observed that different archaeal phylotypes were associated with specific groups of protozoa, but that an uncultured archaeal community seemed dominant and ubiquitous in the ovine rumen ecosystem. The latter observation is in agreement with our conclusion from the mcrA DGGE profiles. Finally, only 1 study using bromochloromethane showed that this compound, which is a direct inhibitor of the methanogenesis pathway, induced a 30% decrease in methanogenic populations and a shift in their diversity (Denman et al., 2007).

Reduced methanogenesis with an increase in numbers of methanogens has been associated with reduced metabolic activity of methanogens (using FISH and order-specific oligonucleotide probes) in sheep after coconut oil and defaunation treatment (Machmüller et al., 2003). Reduced metabolic activity could be due to reduced production of H$_2$ in the rumen. Machmüller et al. (2003) hypothesized that the rate of methane production per single methanogenic cell was significantly reduced. Another explanation could be that hydrogen transfer from H$_2$-producing microorganisms to methanogens is less efficient in defaunated animals. Indeed, H$_2$ transfer from protozoa to their methanogenic symbionts is probably more direct than in other associations such as H$_2$-producing bacteria or fungi with methanogens. This is in agreement with the production of propionate measured in the rumen of the 5 defaunated wethers, which increased with decreased methanogenesis, indicating that the propionate pathway was used as an alternative electron sink consuming hydrogen (Morgavi et al., 2008). In consequence, monitoring the metabolic activity of the methanogens and studying their interactions with H$_2$ producers (protozoa, bacteria, fungi) as well as the impact of non-H$_2$ producers on these interactions are key factors in further investigating the effect of anti-methanogenic treatments in the rumen.

**Effect of Long- and Short-Term Defaunation on Cellulolytic Bacteria**

Despite numerous studies over the past 50 yr, the mechanisms by which the absence of protozoa negatively affect rumen fiber degradation are still unclear. As shown and discussed above, defaunation increases total bacterial numbers, and some authors have also reported shifts in numbers of functional bacterial groups, including cellulolytic bacteria (Williams and Withers, 1993). In addition, total polysaccharidase activities...
have been shown to be greater in the rumen of faunated animals compared with defaunated ones (Williams and Withers, 1991; Santra and Karim, 2002; Eugène et al., 2004a). Hence, another objective of this study was to examine if defaunation has an effect on the abundance of bacterial species that are considered the major rumen fibrolytic bacteria: *F. succinogenes*, *R. albus*, and *R. flavefaciens* (Morrison et al., 2009). However, it is possible that other very active cellulolytic bacteria not yet cultivated exist in the rumen. Recently, a new species was identified in the rumen of yak (Cai et al., 2010). The prevalence of this species in domesticated ruminants will have to be evaluated in future studies.

The re-establishment of protozoa after long-term defaunation did not modify the population of *F. succinogenes* (i.e., *F. succinogenes* rrs gene copies/g of DM of rumen content), whereas the subsequent short-term defaunation positively influenced (+1.6 log) its level ($P < 0.001$; Table 1). However, when *F. succinogenes* rrs gene copies were expressed as a percentage of bacterial rrs gene copies, no significant effect of period was observed, even though it tended to be greater in Def P3 than in Def P1 ($P = 0.06$) and Ref P2 ($P = 0.12$). In contrast to *F. succinogenes*, *R. flavefaciens*, and *R. albus* populations decreased by almost 2 log in the presence of protozoa ($P < 0.001$), whereas their concentrations (estimated as rrs gene copies per g of DM of rumen content) were similar in both long- and short-term defaunated periods. This effect of the presence of protozoa was also observed when the rrs gene copies of both *Ruminococcus* species were expressed as a percentage of those of total bacteria (Table 1). The *R. flavefaciens* and *R. albus* percentages decreased 6-fold ($P = 0.020$ and $P = 0.032$, respectively) from Def P1 to Ref P2 and increased ($P = 0.045$ for *R. albus* only) from Ref P2 to Def P3. Our results obtained in sheep are partly in agreement with those of Ozutsumi et al. (2006) who found by qPCR that defaunated cattle harbored a greater number of *R. flavefaciens* ($\times 2$) and *R. albus* ($\times 3.5$) and a smaller number of *F. succinogenes* ($\times 2.4$) than faunated cattle. Protozoa have been reported to ingest cellulolytic bacteria preferentially (Williams and Withers, 1993). The fact that both *Ruminococcus* species decreased in number in the presence of protozoa, whereas this was not observed for *F. succinogenes*, may be due to the greater susceptibility of gram-positive bacteria (i.e., ruminococci) to predation (Coleman and Hall, 1972). However, an indirect effect, such as competition for substrate between cellulolytic ruminococci and protozoa may not be ruled out. The quantitative shift in the cellulolytic community was not underlined by bacterial PCR-DGGE analysis, probably because this method only detects dominant bacterial species and is not quantitative.

Independently of period, *F. succinogenes* was the most abundant cellulolytic species, followed by *R. flavefaciens* and *R. albus*. This hierarchy has been observed in most studies in which these 3 cellulolytic bacterial species were quantified (Firkins and Yu, 2006; Koike and Kobayashi, 2009).

Finally, the increase in cellulolytic ruminococci, which are large producers of hydrogen, does not correlate with the observed decrease in methane emission in protozoa-free animals. Either this increase did not compensate for the loss of hydrogen produced by protozoa or, as suggested earlier, methanogenic activity is decreased because of less efficient hydrogen transfer between cellulolytic ruminococci and methanogens or both.

Overall, long-term defaunation was shown to enhance the population of *R. albus* and of *R. flavefaciens* as estimated by qPCR, but not that of *F. succinogenes*, which is, among the 3 species, the most efficient in degrading low-digestible plant cell walls (Matulova et al., 2005). Hence, protozoa might have a hydrolytic action on plant cell walls that cannot be compensated for in vivo by the increase in number of cellulolytic *Ruminococci*. In the literature, it has also been suggested that protozoa may have a more indirect effect. For instance, their competition for starch with the amylolytic bacterial community associated with the predation of these bacteria when engulfing starch granules may have a buffering effect on the rumen environment and hence favor the development or activity of fibrolytic bacteria and fungi or both (Jouany, 1994).

In conclusion, this study shows that short-term defaunation of 10 wk affects the abundance and diversity of bacteria and archaea in the same way as long-term defaunation of 2 yr, but the bacterial community might not be totally stabilized after short-term defaunation, as observed for *F. succinogenes*. This work also provides evidence that, under the conditions of the study, reduction in methane emissions in the absence of protozoa cannot be explained by a decrease in numbers of methanogens, or a modification in composition of the dominant methanogens. In this respect, estimating the activity of methanogens, by quantifying mcrA transcripts for instance, would provide useful information in studies aiming to reduce methane emission by ruminants. Finally, this study demonstrates for the first time in sheep a negative effect of protozoa on the population of 2 rumen cellulolytic bacterial species, *R. flavefaciens* and *R. albus* (gram-positive bacteria). In contrast, *F. succinogenes* (gram-negative bacterium) was not affected by the presence of protozoa. In future studies, it would be interesting to monitor the abundance of *Cellulosilyticum ruminicola*, a newly identified cellulolytic species (Cai et al., 2010), and also other functional bacterial species in response to defaunation and refaunation and thereby better assess the effect of protozoa on the different functional bacterial communities.

**LITERATURE CITED**


