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RESEARCH ARTICLE

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Asma Zened[1,2,3], Sylvie Combes[1,2,3], Laurent Cauquil[1,2,3], Jérôme Mariette[4], Christophe Klopp[4], Olivier Bouchez[5,6], Annabelle Troegeler-Meynadier[1,2,3] & Francis Enjalbert[1,2,3]

[1]Université de Toulouse INPT ENVT, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, Toulouse, France; [2]INRA, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, Castanet-Tolosan, France; [3]Université de Toulouse INPT ENSAT, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, Castanet-Tolosan, France; [4]INRA Plateforme bio-informatique Genotoul, Biométrie et Intelligence Artificielle, Castanet-Tolosan, France; [5]INRA, UMR444 Laboratoire de Génétique Cellulaire, INRA Auzeville, Castanet-Tolosan, France; and [6]GeT-PlaGe, Genotoul, INRA Auzeville, Castanet-Tolosan, France

**Keywords**
rumen bacteria; diversity; dietary starch; dietary oil; 454 pyrosequencing; taxonomy.

## Abstract

To provide a comprehensive examination of the bacterial diversity in the rumen content of cows fed different diets, high-throughput 16S rRNA gene-based pyrosequencing was used. Four rumen fistulated nonlactating Holstein cows received 12 kg of dry matter per day of four diets based on maize silage during four periods: the low-starch diet (22% starch, 3% fat); the high-starch diet, supplemented with wheat plus barley (35% starch, 3% fat); the low-starch plus oil diet, supplemented with 5% of sunflower oil (20% starch, 7.6% fat) and the high-starch plus oil diet (33% starch, 7.3% fat). Samples were taken after 12 days of adaptation, 5 h postfeeding. Whatever the diet, bacterial community of sieved rumen contents was dominated by *Firmicutes* and *Bacteroidetes*. *Lachnospiraceae*, *Ruminococcaceae*, *Prevotellaceae*, and *Rikenellaceae* families were highly present and were clearly affected by cow diet. The highest abundance of *Prevotellaceae* and the lowest abundance of *Ruminococcaceae* and *Rikenellaceae* were found with the high-starch plus oil diet. Dietary starch increased the relative abundance of only three genera: *Barnesiella*, *Oribacterium* and *Olsenella*, but decreased the relative abundances of several genera, with very significant effects for *Rikenellaceae_RC9* and *Butyrivibrio-Pseudobutyrivibrio*. Oil alone had a limited effect, but interestingly, starch plus oil addition differently affected the bacterial populations compared to starch addition without oil.

## Introduction

The rumen is the major site of digestion in ruminants, due to a complex microbial community including bacteria, archaea, protozoa and fungi. The main functions of this microbiota are degradation of dietary polysaccharides to volatile fatty acids, which are the main energetic fuels of ruminants, degradation of a major part of dietary proteins, synthesis of microbial protein, lipolysis of glycerides and hydrogenation of dietary unsaturated fatty acids. Because of the complexity of the microbial community in the rumen, changing the diet has a cascading effect on rumen metabolism, which can impact both quantity and quality of animal production.

The equilibrium of ruminal microbiota is dependent on the diet, which carries fermentation substrates, and the efficiency of ruminal microbiota can be strongly affected by dietary changes. High-starch diets serve as an energy source for the animal and are often offered to dairy cows, whose energy requirements are very high. However, they lead to a decreased efficiency of fiber digestion, associated with a decreased number of some fibrolytic bacteria, i.e. *Ruminococcus flavefasciens* (Tajima *et al.*, 2001), *Fibrobacter succinogenes* (Tajima *et al.*, 2001;

Brown *et al.*, 2006; Fernando *et al.*, 2010) and *Butyrivibrio fibrisolvens* (Fernando *et al.*, 2010), and a shift toward more lactate producers like lactobacilli (Brown *et al.*, 2006). Similarly, enrichment of diets with unsaturated fat is interesting in dairy cows both for increasing dietary energy supply and to improve the profile of milk fatty acids. However, it is also known to negatively affect the fibrolytic activity of ruminal microbiota (Brooks *et al.*, 1954). *B. fibrisolvens*, a fibrolytic bacterium, is usually assumed as being the main biohydrogenating bacterium in the rumen (Enjalbert & Troegeler-Meynadier, 2009), so that a reduction in the relative abundance of this species could reduce ruminal biohydrogenation and enhance the transfer of unsaturated fatty acids to tissues and milk. Recent data indicate that this biohydrogenation is a detoxification process necessary to prevent the bacteriostatic effect of polyunsaturated fatty acids (Maia *et al.*, 2010). Dietary unsaturated fatty acids have been shown to inhibit the growth of numerous bacteria in *in vitro* pure cultures (Maczulak *et al.*, 1981), including *B. fibrisolvens* (Maia *et al.*, 2007), but *in vivo* experiments showed more variable and limited effects (Yang *et al.*, 2009; Belenguer *et al.*, 2010; Huws *et al.*, 2010). Most of these studies regarding the effects of dietary changes that can disturb the microbiota have focused on bacterial species with known metabolic functions, as assessed by *in vitro* studies. However, as outlined by Kelly *et al.* (2010), direct amplification and sequencing of 16S ribosomal RNA gene indicate that, in the rumen, the majority of sequences are derived from organisms that are phylogenetically distinct from currently cultivated species. Such molecular methods have already been used to approach the effects of changes of dietary forage (Pitta *et al.*, 2010) or concentrates (Khafipour *et al.*, 2009; Callaway *et al.*, 2010). The objective of this study was to assess the effects of starch and/or oil addition to the diet on the rumen bacterial community of dairy cows, using FLX amplicon pyrosequencing.

## Materials and methods

### Experimental design

Four dry ruminally fistulated Holstein cows were assigned to a 4 × 4 Latin square design with four different diets (Table 1) based on maize silage and soybean meal during four periods. Chemical composition of feed was determined according to the Association of Official Analytical Chemists (Association of Official Analytical Chemists, 1998) procedures; Neutral Detergent Fibre was determined according to the procedure of Van Soest *et al.* (1991). To ensure the same quality of dietary components, we used the same batch of concentrate over the duration of the experiment during which analysis of the silage occurred showing no variation of nutrient composition. Cows were housed in individual stalls and received 12.5 kg of dry matter daily, in two equal meals at 08:00 and 17:00 hours and were allowed *ad libitum* access to water. Periods were divided into weeks of washout subperiod during which all cows received the control diet, and 2 weeks of treatment subperiod, during which each cow received one of the four experimental diets. To avoid carry-over effects, treatment orders were different among cows: [high-starch + oil (HSO), low-starch + oil (LSO), high starch (HS) and low starch (LS) for cow 1; LSO, LS, HSO, HS for cow 2; LS, HS, LSO, HSO for cow 3; HS, HSO, LS, LSO for cow 4]. One liter of rumen fluid was taken from the ventral sac of each cow 5 h postfeeding on day 12 of the treatment subperiod, strained through a metal sieve (1.6-mm mesh) and then 200 μL of filtrate were precisely weighed and stored in a 2-mL sterile Eppendorf tube at −80 °C.

**Table 1.** Ingredients and chemical composition of donor cow diets

| Diets* | LS | LSO | HS | HSO |
|---|---|---|---|---|
| Ingredients (% of dry matter) | | | | |
| Maize silage | 68.7 | 63.2 | 37.7 | 32.3 |
| Alfalfa hay | 14.3 | 14.3 | 0.0 | 0.0 |
| Wheat and barley mix | 0.0 | 0.0 | 48.5 | 48.5 |
| Soybean meal | 15.5 | 16.2 | 12.4 | 13.1 |
| Sunflower oil | 0.0 | 4.8 | 0.0 | 4.7 |
| Minerals and vitamins | 1.5 | 1.5 | 1.5 | 1.5 |
| Composition (% of dry matter) | | | | |
| Crude protein | 14.6 | 14.6 | 14.2 | 14.2 |
| Neutral detergent fiber | 39.7 | 37.3 | 30.9 | 28.6 |
| Starch | 21.5 | 19.8 | 34.8 | 33.1 |
| Crude fat | 2.9 | 7.6 | 2.7 | 7.3 |

*LS = low-starch diet, LSO = low-starch + oil diet, HS = high-starch diet, HSO = high-starch + oil diet.

### DNA extraction and 16S rRNA gene amplicon pyrosequencing

Total DNA was extracted from 0.2 g of ruminal sample, combining a mechanic lysis with a FastPrep instrument (MP Biomedicals, Illkirch, France) and the QIAamp DNA stool minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer (Nano- Drop Technologies, Wilmington, DE) was used to quantify DNA.

Amplicons from the V3 to V4 regions of 16S rRNA genes (460 bp on *E. coli*, GenBank number J01695) of day 12 samples were amplified with bacterial forward 343F (TACGGRAGGCAGCAG; Liu *et al.*, 2007) and reverse 784R (TACCAGGGTATCTAATCCT; Andersson *et al.*, 2008) primers. Each primer had a barcode sequence of ten nucleotides on 5′ which was unique for

each sample. PCRs were performed in a total volume of 100 μL containing 1X PCR buffer, 200 μM of dNTP, 1U Isis$^{TM}$ DNA polymerase (MP Biomedicals), 0.5 μM of forward and reverse primers, and 0.5–3 ng of DNA template. The amplification program consisted of an initial denaturation step at 94 °C for 2 min; 32 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s; and a final extension step at 72 °C for 7 min. PCR of products were purified with the QIAquick PCR Purification kit (Qiagen) followed by DNA yield quantification and quality estimation using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies). The size of the PCR products was confirmed by gel electrophoresis. The purified PCR products were quantified using Quant-iT PicoGreen ds DNA Assay kit (Invitogen, Saint Aubin, France) on a ABI Prism 7900HT sequence detection system (Life technologies-Invitrogen-A-BIOSYSTEM, Villebon-sur-Yvette, France) and then mixed in equimolar amounts to a final DNA concentration of 1000 ng $\mu L^{-1}$ for each library. The pooled amplicons were pyrosequenced using an on 454 FLX Titanium (454 Life Sciences – a Roche Company, Branford, CT) sequencer at the GeT (Genomic and Transcriptomic) platform of Toulouse. Sequencing occurred on a picotiter plate, of which a half space was available for samples included in this study. To facilitate pyrosequencing using titanium chemistry, each forward primer was appended with the titanium sequencing adaptor A and the reverse primer carried the titanium adaptor B at the 5′ end.

### Pyrotag handling and analysis

A total of 138 764 16S rRNA gene sequences (also referred to as 16S pyrotags) were obtained from 454 Titanium pyrosequencing run for the 16 samples. The mean quality score for the base between position 1 and 500 pb was 32.8 ± 4.5 (mean ± SD, max = 38.9; min = 18.0). The 16S pyrotags were sorted based on their respective barcodes to form a total of 16 pyrotag library representing the 16 collected ruminal samples. Sequences were sequentially filtered using a Python script developed by the bioinformatic platform of Toulouse, removing first sequences with a short sequencing length (less than 150 nt; 7768 sequences removed); those with at least one ambiguous base (12 345 sequences removed) or with a long homopolymer (> 9; four sequences); those that did not match the proximal PCR primer sequences (with two mismatches allowed; 2241 sequences removed) and finally those having both primers but with a length shorter than 350 pb (227 sequences removed). A total of 116 183 sequences were retained corresponding to 7088 ± 1258 sequences per samples. The average read length was of 393 ± 81 nucleotides after trimming the barcodes.

### Taxonomical classification and statistical analysis

After cleaning, sequences were analyzed using MOTHUR software (v 1.24; Schloss *et al.*, 2009). Reads were aligned over SILVA alignment database provided by MOTHUR software (14 956 sequences corresponding to the unique sequences in the SSU REF database v102) (Pruesse *et al.*, 2007) and an alignment quality was calculated using the SILVA secondary structure map file (1072 bad aligned sequences were removed). After calculating a pairwise distance between aligned sequences, they were clustered into Operational Taxonomic Unit (OTU, cutoff 0.05 using a furthest neighbor clustering). Taxonomic assignment was performed with a bootstrap of 60%. Finally, all unclassified bacteria at the phylum level were considered as chimera and were removed from statistical analysis (1692 sequences). Rarefaction curves, Abundance-based coverage estimator (ACE) and Chao1 richness were calculated using the relevant Ribosomal Database Project modules (Cole *et al.*, 2008), including Rarefaction and Chao1 Estimator. Shannon α-diversity index was calculated according to Hayek & Buzas (1996).

Sequences corresponding to *Butyrivibrio* genus which were not identified at species level in SILVA database were compared with deposited sequences in NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This procedure did not allow discrimination between *Butyrivibrio proteoclasticus* and *Butyrivibrio hungatei*.

### Statistical analysis

The relative abundances of bacteria in the rumen fluids were analyzed by ANOVA, using the General Linear Model of SYSTAT (version 9, SPSS Inc., Chicago, IL), according to the following model:

$$Y = \mu + C + P + S + O + S \times O + \varepsilon,$$

where μ is the mean, $C$, $P$, $S$ and $O$ are the effects of cow, period, dietary starch level and sunflower oil addition, respectively, and ε the residual error. Significant differences among treatments were declared at $P < 0.05$ and trends at $P < 0.10$. Genera under 0.1% of relative abundance for the four studied diets were excluded from the analysis.

## Results

### Microbial community in the rumen of dairy cows

Diversity within the different samples was estimated by the calculation of the number of OTUs and the measure

of richness estimators. Of the overall 16 samples, the number of OTUs with a cutoff of 0.05 was 2297 ± 665 with coverage on average of 81.1 ± 6.3%. The Chao1 and ACE estimators were on 4412 ± 1551; 6369 ± 2494 and 6.7 ± 0.7, respectively.

Within the bacterial population, 13 phyla were found across all samples. *Firmicutes* and *Bacteroidetes* together represented more than 80% of total sequences: 49.6% and 40.2% of total sequences on average, respectively, but with high variations (residual SEM = 4.5 and 4.7 respectively for mean values presented on Fig. 1a). Rumen bacterial populations had *Firmicutes* and *Bacteroidetes* in varying relative abundances, ranging from 24.1% to 67.2% and from 11.5% to 62.9% of the total microbial community, respectively. The remaining rumen microbiota was composed of low relative abundance phyla (< 20% of total microbial community). *Proteobacteria* and *Actinobacteria* represented around 4.3% and 5.1% of total sequences on average, respectively. Other phyla (< 0.5% of total microbial community), such as *Fibrobacteres*, *Spirochaetes*, *Lentisphaerae* or *Fusobacteria*, were not consistently present in all the different host populations.

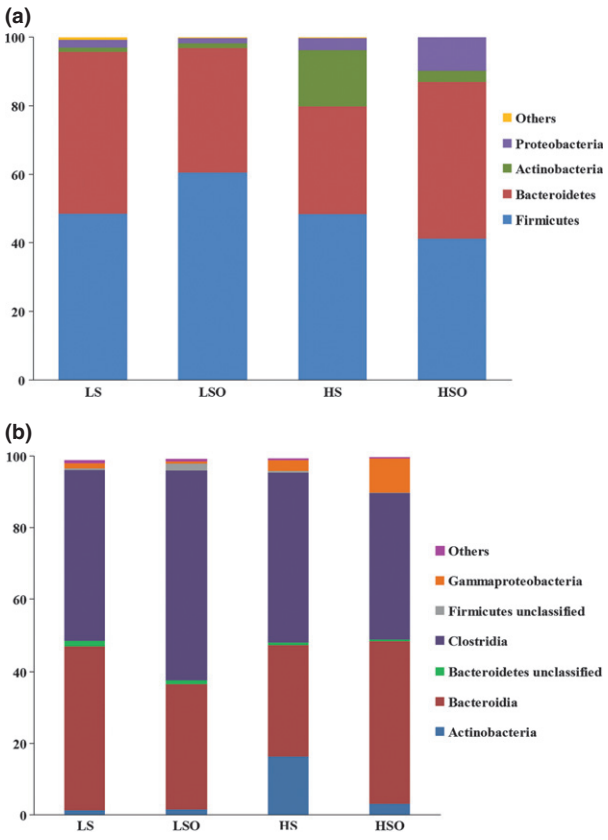


**Fig. 1.** Percentage contribution of sequences (%) evaluated at the phylum (a) and class (b) levels to the total number of sequences in the database (LS = low-starch diet, LSO = low-starch + oil diet, HS diet = high-starch diet, HSO = high-starch + oil diet).

Evaluation at finer taxonomical levels was carried out to determine the distribution of the different bacteria. When sequences were analyzed at the class level (Fig. 1b), *Firmicutes* were dominated by *Clostridia* (48.6% on average of the total sequences) and *Bacteroidia* class made a significant contribution (39.2% on average of the total sequences) in the *Bacteroidetes* phylum. Using a minimal bootstrap of 60%, 83.3% and 52.6% of sequences were assigned to family and genus levels, respectively. On the totality of samples, 45 orders and 98 families were detected. Further evaluation of the two major classes showed that *Clostridia* were mainly composed of the families *Lachnospiraceae* and *Ruminococcaceae* (26.1% and 17.2% on average of the total sequences, respectively), belonging to the *Clostridiales* order. Predominant bacterial families in the *Bacteroidia* class were *Prevotellaceae* and *Rikenellaceae* (17.1% and 5.9% on average of the total sequences, respectively), belonging to the *Bacteroidales* order. Regarding *Actinobacteria* phylum, the major class was *Actinobacteria* represented mainly by *Bifidobacteriaceae* family (4.0% of total sequences on average).

Over the totality of samples, 219 genera were detected (Supporting information, Table S1a indicates the relative abundances of genera representing more than 0.3% of total sequences). Among these genera, 11.3% of the sequences belonged to the genus *Prevotella* followed by more than 5% of the genera *Ruminococcaceae* Incertae_Sedis (5.7%) and *RC9* (5.6%). The most common uncultured and unclassified bacteria are presented in Table S1b: 11.9% of total sequences belonged to the *Lachnospiraceae* family, 9.2% to unclassified families of the *Bacteroidales* order and 7.9% to the *Ruminococcaceae* family. Uncultured genera were also found such as *Lachnospiraceae* at 5% of total sequences.

## Clustering differences in bacterial community and their relative abundances

After 12 days of experimental diet, the comparison of the bacterial communities by PCA (Fig. 2) showed that cows fed HS and HSO diets were separated from those receiving low-starch diets, but LS and LSO diets were very close in the plot. Importantly, the efficiency of the washout carried out in this study was verified: the structure of bacterial communities (tested by Capillary Electrophoresis – Single Strand Conformation Polymorphism) of ruminal samples taken on the last day of the washout did not differ according to the diet of the previous period (data not shown).

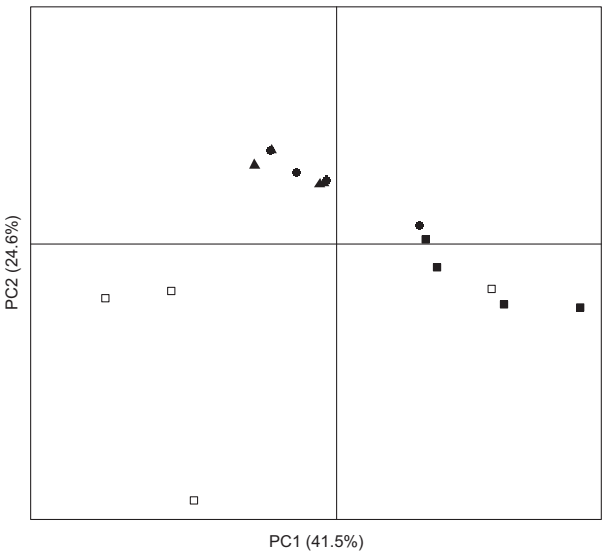


**Fig. 2.** Principal component analysis (PCA) of bacterial 16S rRNA gene sequence tags generated from 454 pyrosequencing run of 5 h postfeeding ruminal contents taken on day 12, corresponding to the last day of the treatment subperiod. The marks relate to the diets of donor cows: low-starch diet (●), low-starch + oil diet (▲), high-starch diet (□) and high-starch + oil diet (■).

## Effect of diet on the relative abundance and diversity of bacterial groups

Starch addition reduced Chao1 richness, ACE and Shannon diversity indexes (Table 2). At the phylum level, both starch and oil additions negatively affected ($P < 0.05$) *Fibrobacteres* and *Spirochaetes* phyla, and starch addition resulted ($P = 0.05$) in high *Actinobacteria* relative abundance, but the major phyla were not significantly affected (data not shown).

The dietary starch level and/or the addition of sunflower oil had a number of effects on bacteria encompassing *Clostridia* and *Bacteroidia*. For example, among *Clostridia*, in rumen fluids of cows fed HSO diet, the relative abundance of *Ruminococcaceae* was 2.4 times lower than with the other diets due to an interaction between starch and oil additions (Table 3). Oil addition had a positive effect on the abundance of *Clostridiales Family_XIII Incertae_Sedis* (Table 3). In the *Bacteroidia* class, the relative abundances of *BS11* and *Rikenellaceae* were lower and that of *Porphyromonadaceae* was higher in ruminal microbiota of cows fed high-starch diets compared to cows fed low-starch diets (Table 3). In addition to the effect of starch, the abundances of *Rikenellaceae* and *RF16* families were negatively affected by the addition of sunflower oil (Table 3), resulting in a very low abundance (0.74% and 0.07% of total sequences, respectively) with HSO diet compared to the other diets (7.57% and 0.65% on average of total sequences). *Prevotellaceae* relative abundance was numerically higher with HSO diet due to a trend ($P = 0.06$) toward an interaction between starch level and oil addition (Table 3). The relative abundance of *Clostridiales* unclassified was significantly lowered with high-starch diets (Table 3). Among Actinobacteria, *Bifidobacteriaceae* relative abundance tended ($P = 0.08$) to be affected by an interaction between starch level and oil addition leading to the highest value with HS diet.

Taxonomic evaluation of bacterial genus of the ruminal fluids (LS, LSO, HS and HSO) showed that the relative abundances of some genera were affected by the addition of starch and oil to the diet (Table 4). Single effect of starch addition increased the relative abundance of only three genera: *Barnesiella*, *Oribacterium* and *Olsenella*, but decreased the relative abundance of several genera, with very significant effects ($P < 0.001$) for *Rikenellaceae_RC9* and *Butyrivibrio-Pseudobutyrivibrio*. Oil addition decreased the relative abundance of *Rikenellaceae_RC9* and *Acetitomaculum* (Table 4). As a consequence of an additive effect of oil and starch additions, *Rikenellaceae_RC9* abundance was lowest with HSO diet.

Significant interactions between starch level and oil addition were observed on some genera. Starch addition

**Table 2.** Estimators of diversity within cow's ruminal fluids on day 12

| Diets[†] | LS | LSO | HS | HSO | SEM[‡] | Effects[§] |
|---|---|---|---|---|---|---|
| Number of operational taxonomic units | 2939 | 2467 | 2055 | 1729 | 166.28 | |
| Chao1 estimated richness | 5886 | 4826 | 3876 | 3060 | 387.87 | *S** |
| ACE | 8713 | 6976 | 5599 | 4188 | 623.50 | *S** |
| Shannon diversity index | 7.33 | 7.14 | 6.37 | 6.16 | 0.15 | *S*** |

*$P < 0.05$.

**$P < 0.01$.

[†]LS, low-starch diet; LSO, low-starch + oil diet; HS, high-starch diet; HSO, high-starch + oil diet.

[‡]Residual standard error of the mean.

[§]Effects: *S* = high-starch vs. low-starch diet.

**Table 3.** Bacterial families affected by dietary changes on day 12, ranked by class and average relative abundance order (% of total sequences)

| Diets[†] | LS | LSO | HS | HSO | SEM[‡] | Significance of effects[§] |
|---|---|---|---|---|---|---|
| *Clostridia* class | | | | | | |
| *Lachnospiraceae* | 26.02 | 29.92 | 20.51 | 27.81 | 2.69 | *O*° |
| *Ruminococcaceae* | 17.10 | 21.31 | 23.05 | 7.28 | 3.67 | *O* × *S** |
| *Family_XIII_Incertae_Sedis* | 1.55 | 2.24 | 0.86 | 1.74 | 0.30 | *O**, *S*° |
| *Veillonellaceae* | 0.08 | 0.23 | 0.56 | 1.36 | 0.35 | *S*° |
| *Clostridiales* unclassified | 2.81 | 4.60 | 2.36 | 2.46 | 0.46 | *O*°, *S** |
| *Bacteroidia* class | | | | | | |
| *Prevotellaceae* | 17.17 | 14.38 | 12.22 | 24.73 | 3.33 | *O* × *S*° |
| *Rikenellaceae* | 11.78 | 7.39 | 3.54 | 0.74 | 0.96 | *O**, *S**** |
| *BS11* | 4.40 | 4.10 | 1.85 | 0.16 | 0.57 | *S*** |
| *Porphyromonadaceae* | 1.36 | 1.77 | 3.93 | 4.63 | 0.98 | *S** |
| *RF16* | 1.18 | 0.34 | 0.42 | 0.07 | 0.17 | *O**, *S** |
| *Actinobacteria* class | | | | | | |
| *Bifidobacteriaceae* | 0.45 | 0.33 | 15.03 | 0.38 | 3.47 | *O* × *S*° |
| *Corionobacteraceae* | 0.73 | 0.77 | 1.13 | 2.64 | 0.54 | *S*° |

°$P < 0.10$.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

[†]LS, low-starch diet; LSO, low-starch + oil diet; HS, high-starch diet; HSO, high-starch + oil diet.

[‡]Residual standard error of the mean.

[§]Effects: *O* = effect of oil addition, *S* = high-starch vs. low-starch diets, *O* × *S* = effect of interaction of starch by oil additions.

lowered the relative abundances of *Prevotella* and *Lachnospiraceae_Incertae_Sedis* when diets did not contain oil but increased these abundances with oil supplemented diets, so that the highest abundances were observed with the HSO diet. The slope of the effect of starch addition on relative abundance was positive without oil addition and negative with oil addition for *Ruminococcaceae_Incertae_Sedis*, *Acetitomaculum* and *Bifidobacterium*, which exhibited very high percentages only with the HS diet. *Butyrivibrio* genus relative abundance tended to be decreased by starch addition: in particular *B. proteoclasticus* or *B. hungatei* was significantly decreased by starch; *B. fibrisolvens* was present at low level and a trend toward an interaction between starch and oil addition led to the lowest value with HSO diet. Many of the affected genera were unclassified or uncultured (Table S2). In particular, unclassified and uncultured *Ruminoccaceae* and uncultured *Lachnospiraceae* presented lowest abundances with HSO diet.

## Discussion

### Average microbial community

In this study, high-throughput sequencing approach proved to be a powerful tool to reveal bacterial diversity of sieved rumen contents, and effects of diet on that diversity. It also gave much greater coverage of bacterial diversity than could be provided by other molecular diversity techniques (fingerprints, cloning…). Our 16S rRNA gene pyrosequencing revealed that, at the phylum level, the microbial community of the cows, regardless of individual and diet, was dominated by *Firmicutes* and *Bacteroidetes*. This microbial distribution of major phyla is similar to those found with culture-based or molecular approaches (Stewart *et al.*, 1997; Kim *et al.*, 2011) suggesting that *Firmicutes* and *Bacteroidetes* play important roles in the ruminal metabolism (Turnbaugh *et al.*, 2006, 2008). Similarly, the most abundant classes observed in this study were *Clostridia* and *Bacteroidia*. At the family level, common features between this study and other rumen 16S rRNA gene studies (Koike *et al.*, 2003; Edwards *et al.*, 2004; Kong *et al.*, 2010) included the dominance of *Lachnospiraceae*, *Ruminococcaceae* and *Prevotellaceae*. The fourth prevalent family was Bacteroidales unclassified, which fits with the results of Kong *et al.* (2010).

Our study, using short-length pyrosequencing, was not able to identify the bacterial groups at species level, but allowed identification of numerous genera, part of them being uncultured or unclassified (Table S2). Our observed predominance of *Prevotella* (Table S2) is in accordance with literature showing that *Prevotella* was the major ruminal genus, representing up to 52% of total bacteria of rumen content (Pitta *et al.*, 2010). High abundance of *Rikenella-like* bacteria was noticed, in which *Rikenellaceae_RC9* was

**Table 4.** Bacterial genera and selected species (#) affected by dietary changes on day 12, ranked by family and genus or species average relative abundance order (% of total sequences)

| Diets† | LS | LSO | HS | HSO | SEM‡ | Effects§ |
|---|---|---|---|---|---|---|
| ***Clostridia* class** | | | | | | |
| *Lachnospiraceae* | | | | | | |
| *Acetitomaculum* | 1.36 | 2.08 | 3.09 | 1.19 | 0.19 | *O**, *S*°, *O* × *S**** |
| *Incertae_Sedis* | 1.84 | 1.50 | 0.53 | 2.89 | 0.51 | *O*°, *O* × *S** |
| *Butyrivibrio* | 2.45 | 2.68 | 1.60 | 1.85 | 0.33 | *S*° |
| *B. proteoclasticus* or *B. hungatei*# | 0.86 | 0.67 | 0.45 | 0.24 | 0.17 | *S** |
| *B. fibrisolvens*# | 0.07 | 0.12 | 0.09 | 0.05 | 0.01 | *O* × *S*° |
| *Butyrivibrio-Pseudobutyrivibrio* | 0.72 | 0.61 | 0.15 | 0.17 | 0.07 | *S**** |
| *Blautia* | 0.29 | 0.64 | 0.11 | 0.16 | 0.10 | *O*°, *S** |
| *Lachnospira* | 0.58 | 0.12 | 0.03 | 0.49 | 0.19 | *O* × *S*° |
| *Catabacter* | 0.11 | 0.15 | 0.02 | 0.01 | 0.03 | *S*** |
| *Oribacterium* | 0.01 | 0.03 | 0.06 | 0.15 | 0.03 | *S** |
| *Ruminococcaceae* | | | | | | |
| *Incertae_Sedis* | 4.12 | 5.22 | 11.95 | 1.45 | 2.43 | *O* × *S*° |
| *Ruminococcus* | 0.93 | 0.59 | 0.44 | 0.41 | 0.14 | *S*° |
| *Fastidiosipila* | 0.62 | 0.67 | 0.34 | 0.03 | 0.14 | *S** |
| *Oscillibacter* | 0.47 | 0.48 | 0.24 | 0.02 | 0.09 | *S** |
| *Papillibacter* | 0.16 | 0.13 | 0.07 | 0.02 | 0.03 | *S** |
| *Veillonellaceae* | | | | | | |
| *Selenomonas* | 0.01 | 0.04 | 0.16 | 0.10 | 0.04 | *S*° |
| ***Bacteroidia* class** | | | | | | |
| *Prevotellaceae* | | | | | | |
| *Prevotella* | 10.99 | 9.12 | 7.85 | 17.22 | 2.77 | *O* × *S*° |
| *Rikenellaceae* | | | | | | |
| *RC9* | 11.26 | 7.03 | 3.40 | 0.56 | 0.98 | *O**, *S**** |
| *Porphyromonadaceae* | | | | | | |
| *Barnesiella* | 0.98 | 1.35 | 3.17 | 4.10 | 0.95 | *S** |
| ***Actinobacteria* class** | | | | | | |
| *Bifidobacteraceae* | | | | | | |
| *Bifidobacterium* | 0.37 | 0.24 | 14.48 | 0.38 | 3.40 | *O*°, *S*°, *O* × *S*° |
| *Corionobacteraceae* | | | | | | |
| *Olsenella* | 0.18 | 0.13 | 0.70 | 2.48 | 0.56 | *S** |
| *Atopobium* | 0.15 | 0.18 | 0.08 | 0.05 | 0.03 | *S*** |
| ***Spirochaetes* class** | | | | | | |
| *Spirochaetaceae* | | | | | | |
| *Termite_Treponema_cluster* | 0.18 | 0.06 | 0.07 | 0.05 | 0.02 | *O*° |

°$P < 0.10$.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.
†LS, low-starch diet; LSO, low-starch + oil diet; HS, high-starch diet; HSO, high-starch + oil diet.
‡Residual standard error of the mean.
§Effects: *O* = effect of oil addition, *S* = high-starch vs. low-starch diets, *O* × *S* = effect of interaction of starch by oil additions.

among the most abundant ruminal bacteria genera. Similar results were found by Pitta *et al.* (2010) but minor abundance of *Rikenella* was observed in the study of de Menezes *et al.* (2011). We also found high relative abundances of *Ruminocaccaceae* genera and *Lachnospiraceae* genera, including *Butyrivibrio*, *Acetitomaculum*, *Incertae-Sedis*, *Moriella* and *Syntrophococcus*.

*Fibrobacter* and *Ruminoccocus* species are major cellulolytic bacteria in the rumen. In our study, the *Ruminococcus* genus represented 0.92% of total bacteria in ruminal content of cows receiving LS diet. These results contrast with those of Palmonari *et al.* (2010), who found that *Ruminococcus albus* represented more than 10% of total 16S rRNA gene. However, in our study, a *Ruminococcaceae Incertae-sedis* represented about 6% of total bacteria. Similarly, Kong *et al.* (2010) found a much higher abundance of *Ruminococcaceae unclassified* than *Ruminococcus*.

In our study, the relative abundance of *Fibrobacteraceae* was only 0.34% on average of total bacterial community in LS diet but it accounted for almost 10% of the sequences in the study of de Menezes *et al.* (2011). These authors showed that *Fibrobacteres* were considerably more prevalent in the solid phase than in the liquid phase which could partly explain the discrepancy between their results and ours. However, in our study, the ruminal samples were strained and sieved through a 1.6 mm mesh so that particles were not completely removed. Similar to our results, Pitta *et al.* (2010) found a low abundance of *Fibrobacter* in the rumen of cows. As the four diets tested in our study contained starch, the growth or the activity of fibrolytic bacteria could have been negatively affected (Klieve *et al.*, 2003). Moreover, *Fibrobacter* DNA is known to be difficult to amplify (Tajima *et al.*, 2001), so that the difference between results of the different experiments could reflect PCR bias. Besides, comparison between results must be done with caution because studies targeting specific bacteria are different from pyrosequencing approach which poses no such restrictions.

## Effects of diets

In our study, the washout subperiod was successful in restoring a microbiota that did not depend on the experimental diet given during the previous period (data not shown). Sequencing analysis revealed that diets affected bacterial communities, which could relate to diet being used as a growth substrate for ruminal bacteria, and/or from diet potentially resulting in toxic or inhibiting effects on the ruminal bacteria, due to dietary components or results of diet fermentation. Our deep sequencing enabled quantitative study of the effects of diet on gut microbiota.

As outlined in Fig. 2, dietary starch affected the ruminal bacterial community, which was clearly separated from that of cows fed low-starch diets. Interestingly, the combination of starch and oil supplementations modified the composition of the bacterial community, which was

different from that induced by starch addition alone. Quantitative methods evaluating richness and diversity such as the Shannon index measure the evenness of the α-diversity. In our study, Shannon index was negatively affected by the addition of starch, which suggested a reduced ruminal bacterial diversity.

Most effects of starch addition were observed at the genus level. Starch addition increased the relative abundance of one genus belonging to *Firmicutes* (Table 4), *Oribacterium* which has recently been identified in the rumen of cows fed forage based diets (Kong *et al.*, 2010; Kenters *et al.*, 2011), and whose functions in the rumen are not known. To our knowledge, effects of diet on the abundance of this genus have not been published. Starch addition tended ($P = 0.066$) to increase the abundance of *Selenomonas*, a starch utilizing bacteria, but in a much more narrow range than observed by Tajima *et al.* (2001), whose dietary changes from a low-grain to a high-grain diet were much greater than ours. Previous studies, investigating the effects of dietary starch addition on ruminal bacteria, showed variable effects on *Streptococcus bovis* (Tajima *et al.*, 2001; Fernando *et al.*, 2010; Palmonari *et al.*, 2010), another amylolytic bacteria, involved in ruminal acidosis. The relative abundance of *Streptococcus* exhibited a wide variability in our data set, so that the effect of starch addition was not statistically significant ($P = 0.28$). Similarly, the relative abundance of *Lactobacillus* was 0.04%, and the diet did not affect this genus, consistent with Brown *et al.* (2006), who reported increases of this genus only when the diets contained 71% of concentrates.

Starch addition to the diet also decreased the relative abundance of *Ruminococcaceae* family, including *Ruminococcus*, a major cellulolytic genus (Bryant, 1959). *R. flavefaciens* has already been shown to be highly depressed when switching from hay to grain diet (Tajima *et al.*, 2001). In our study, the genera most affected by starch addition were *Rikenellaceae_RC9* and *Butyrivibrio-Pseudobutyrivibrio*. *Rikenellaceae_RC9* has not been extensively studied in the rumen, but Pitta *et al.* (2010) already reported effects of diet on a *Rikenella*-like genus, whose abundance was much lower in both solid and liquid fractions of rumen contents in cows receiving bermudagrass hay than in cows receiving wheat pasture diets. They underlined that, in the liquid fraction of hay fed cows, this genus clustered with other genera including *Fibrobacter*, and suggested that this bacterium is involved in structural carbohydrates degradation. The genus *Butyrivibrio-Pseudobutyrivibrio* also contains fibrolytic bacteria, including *P. ruminis* and *P. xylanivorans* (Kopečný *et al.*, 2003), which could explain the negative effect of starch addition to the diet. Other *Lachnospiraceae* were also negatively affected by the high-starch diets, including *B. proteoclasticus* or *B. hungatei*. In our study, *B. fibrisolvens* was not affected by the single effect of starch addition, as opposed to the results of Fernando *et al.* (2010). However, in this latter study, the reference values were obtained when animals received a diet containing only hay, progressively changed to a diet containing 80% grain, which represents a much greater dietary change that our switch from 20% to 35% starch.

Besides these observed differences in the *Firmicutes* phylum, we also observed differences due to dietary starch level in the relative abundances of the quantitatively minor phyla *Fibrobacteres* and *Spirochaetes*, whose relative abundances were decreased by more than two times when the dietary starch level increased. A decreased abundance of *Spirochaetes* has already been described by de Menezes *et al.* (2011) when switching from 100% to 36% forage in the diet, and increasing dietary starch resulted in a strong decrease of *F. succinogenes* in the experiment of Tajima *et al.* (2001). On the contrary, de Menezes *et al.* (2011) observed that a concentrate diet resulted in a higher abundance of *Fibrobacteraceae* than a forage diet, and explained this paradoxical effect by the presence of straw in their concentrate diet. In our experiment, the high-starch diets had less fibrous material than the low-starch diets, so this paradoxical effect was not observed. In the *Bacteroidetes* phylum, *Barnesiella*, which has recently been identified in the ruminal content (Kim *et al.*, 2011), was strongly and positively affected by starch addition. In the *Actinobacteria* phylum, *Bifidobacteriaceae* were strongly represented (15.0% relative abundance in average) in ruminal samples of cows receiving HS diet. This bacterial family was not found by Pitta *et al.* (2010) in cattle receiving forage diets or by Callaway *et al.* (2010) in cattle receiving very high concentrate diets, but was found in the study of Boguhn *et al.* (2010) in the rumen of dairy cows with diets that were similar to ours. The addition of starch increased the abundance of *Bifidobacterium* and *Olsenella* genera but decreased that of *Atopobium* genus. Information about these genera is not commonly found in the literature except *Bifidobacterium* genus which seems to be implicated in the production of conjugated linoleic and conjugated linolenic acids isomers (Gorissen *et al.*, 2010; Park *et al.*, 2011). *Bifidobacterium* spp. have been identified in animals receiving diets rich in starch, which implies a role for these microorganisms in the hydrolysis of this carbohydrate; hence, they can ferment starch to produce acetic and lactic acids (Stewart *et al.*,1997). The genera *Olsenella*, *Atopobium* and *Bifidobacterium* constitute a group of bacteria that ferments carbohydrates to lactic acid (Kraatz *et al.*, 2011). Uncultured or unclassified bacteria were also negatively affected by starch addition (Table S2), the three most abundant affected genera being *Clostridiales*.

Oil addition had only a positive effect on two unclassified taxa (*Firmicutes*;unclassified and *Clostridiales*;Family_XIII_Incertae_Sedis;unclassified). Oil addition negatively affected some minor or poorly known genera, but had no significant effect on *B. proteoclasticus* or *B. hungatei*, and interacted with the effects of starch addition for *B. fibrisolvens*, whose lowest relative abundance was observed with the HSO diet. These three *Butyrivibrio* species are known to be able to biohydrogenate unsaturated fatty acids. Contrary to our results, Maia *et al.* (2007) showed in pure cultures that many bacteria, including *B. hungatei*, *B. proteoclasticus*, together with cellulolytic bacteria belonging to the *Ruminococcus* and *Fibrobacter* genera, have increased lag phases during growth when the medium contains linoleic acid. In our experiment, the limited effect of sunflower oil on these bacteria could be due to linoleic acid being in an esterified form, or to differences between *in vivo* and *in vitro* studies, for example due to interactions with other bacterial species or substrates. *In vivo*, Belenguer *et al.* (2010) did not find any effect of 2% sunflower oil in the diet of ewes on the abundance of *Butyrivibrio* involved in ruminal biohydrogenation. Similarly, in the bovine rumen, Yang *et al.* (2009) did not determine any effect of dietary addition of 4% of soybean oil, another high linoleic acid fat, on the amount of *B. fibrisolvens*, and showed a negative effect on *F. succinogenes* and *R. albus* but not *R. flavefaciens*. In the rumen, *R. flavefaciens* abundance is 100 times that of *R. albus* (Yang *et al.*, 2009), which explains the lack of effect of dietary oil on the *Ruminococcus* genus in our study.

The *Prevotella* genus comprises a wide range of species, the best known in the rumen being *P. ruminicola* and *P. bryantii*, who have important roles in the utilization of polysaccharides of plant origin, including xylans, pectins, and starch (Avguštin *et al.*, 1997). However, most rumen *Prevotella* do not belong to these two main species (Bekele *et al.*, 2010). In the present experiment, the relative abundance of this genus was not affected by single effects of starch or oil addition. Literature data on the effects of starch diets on this genus are not consistent: switching from a hay to a starch diet, Tajima *et al.* (2001) showed a decrease of *P. ruminicola* but an increase of *P. bryantii* whereas Bekele *et al.* (2010) found an increase of both genera. As far as we are aware, limited knowledge is available regarding the relationship between *Prevotella* and dietary fat: these bacteria have a low *in vitro* sensitivity to linoleic acid (Maia *et al.*, 2007), are not affected *in vivo* by fish oil (Huws *et al.*, 2010), and a strain of *Prevotella* is linked to rumen biohydrogenation of fatty acids (Huws *et al.*, 2011). In our study, *Prevotella* relative abundance was affected by an interaction between starch and oil additions: switching from LS to HS diet slightly decreased their relative abundance whereas switching from LSO to HSO diet resulted in a twofold increase of relative abundance. This high relative abundance of *Prevotella* with the HSO diet could be related to their ability to degrade a wide variety of substrates and their resistance to unsaturated fatty acids. However, due to the limited knowledge on the relationship between this genus and dietary lipids, understanding this interaction will require further research on the metabolic characteristics of this genus. Moreover, an accurate study of rumen *Prevotellaceae* should take into account that liquid and solid rumen fractions have abundances that are different and are differently affected by dietary changes (de Menezes *et al.*, 2011).

Such interaction, resulting in highest relative abundances with the HSO diet, was also observed on *Lachnospiraceae Incertae_Sedis*. An opposite interaction between starch and oil, with the lowest relative abundance being found with the HSO diet, was observed for *B. fibrisolvens*, *Acetitomaculum* (Table 4), *Ruminococcaceae Incertae_Sedis*, *Ruminococcaceae* unclassified, *Ruminococcaceae* uncultured, *Lachnospiraceae* uncultured and Firmicutes unclassified (Table S2). Interestingly, bacteria involved in interactions between starch and oil in our experiment in part fit with the results of Huws *et al.* (2011). These authors found that putative taxonomic identification of many terminal restriction fragments associated with *trans*-11 18 : 1 and *cis*-9,*trans*-11 CLA, intermediates of ruminal biohydrogenation of dietary unsaturated fatty acids, revealed that more than 60% of the predicted phylotypes belonged to the genera *Prevotella*, *Lachnospiraceae Incertae-Sedis* and unclassified *Bacteroidales, Clostridia*, Ruminococcaceae and *Lachnospiraceae*.

In conclusion, this study, based on 16S rRNA gene pyrosequencing of ruminal microbiota sampled from cows fed different diets, provides a detailed account of the microbial community inhabiting the rumen and the effects of four diets on the relative abundances of some bacterial groups. The diets had significant effects in shaping the microbial community in the rumen of dairy cows, in particular starch and starch plus oil addition. Bacterial profile was primarily predominated by *Firmicutes* and *Bacteroidetes*, and a large portion of the bacteria genera were not identified because they are as yet uncultured. With a better understanding of the bacterial diversity in dairy cows, future studies would aim to investigate their relationship with digestion products and efficiency to elucidate their functional roles. Such a study should be of great interest for researchers investigating rumen microbial ecology and bacteria involved in rumen digestion.

## Acknowledgements

The authors thank the staff at the experimental station (UMR1289 Tandem). We would also like to thank Lluch

J (INRA, Plateforme Génomique, Génopole Toulouse/ Midi-pyrénées, Castanet-Tolosan, France).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1a.** Most common bacteria genera on day 12 ranked by average relative abundance order (% of total sequences).
**Table S1b.** Most common uncultured or unclassified bacteria genera on day 12 ranked by average relative abundance order (% of total sequences).
**Table S2.** Unclassified or uncultured bacterial genera affected by diet at day 12, ranked by average relative abundance order (% of total sequences).

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