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ORIGINAL ARTICLE

Exploring the bovine rumen bacterial community from birth to adulthood

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The mammalian gut microbiota is essential in shaping many of its host's functional attributes. One such microbiota resides in the bovine digestive tract in a compartment termed as the rumen. The rumen microbiota is necessary for the proper physiological development of the rumen and for the animal's ability to digest and convert plant mass into food products, making it highly significant to humans. The establishment of this microbial population and the changes occurring with the host's age are important for understanding this key microbial community. Despite its importance, little information about colonization of the microbial populations in newborn animals, and the gradual changes occurring thereafter, exists. Here, we characterized the overall bovine ruminal bacterial populations of five age groups, from 1-day-old calves to 2-year-old cows. We describe the changes occurring in the rumen ecosystem after birth, reflected by a decline in aerobic and facultative anaerobic taxa and an increase in anaerobic ones. Some rumen bacteria that are essential for mature rumen function could be detected as early as 1 day after birth, long before the rumen is active or even before ingestion of plant material occurs. The diversity and within-group similarity increased with age, suggesting a more diverse but homogeneous and specific mature community, compared with the more heterogeneous and less diverse primary community. In addition, a convergence toward a mature bacterial arrangement with age was observed. These findings have also been reported for human gut microbiota, suggesting that similar forces drive the establishment of gut microbiotas in these two distinct mammalian digestive systems.
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Introduction

Relationships between gut bacterial communities and their mammalian hosts have been shown in recent years to have an important role in the host's well being and proper function (Ley *et al.,* 2008; Zilber-Rosenberg and Rosenberg, 2008). A classic example of these relationships is found in the first compartment of the digestive tract of ruminants—the rumen, where plant digestion enables the conversion of plant fibers into chemical compounds, which are subsequently absorbed and digested by the animal (Mackie 2002). This process is of extreme importance to mankind as it enables utilization of the solar energy stored in plant fibers via their conversion into food products, such as milk and meat. The digestive strategy, architecture and physiology of this system have evolved for over millions of years to allow efficient digestion of plant materials (Mackie, 2002). This system's effectiveness is conferred by its design, which prolongs and maximizes plant biomass exposure to specialized microorganisms that degrade the plant fibers, while providing stable and favorable conditions for their growth. During the first weeks of life, when the animals are still suckling milk, the rumen is not functional: the suckled milk does not pass through it due to closure of the esophageal groove by reflex action (Van Soest, 1994). Its relative proportions are considerably smaller than in the adult and some of its functional components, such as the rumen wall villi, which serve for absorption of nutritional components, have not yet developed (Van Soest, 1994). The changes in the structural and physiological properties of the rumen with age are linked to the development of the rumen microorganisms, as their fermentation products are important for the development of the rumen wall villi (Klein *et al.,* 1987; Beharka *et al.,* 1998). Early research into the emergence of microbial communities in the rumen of newborn animals revealed rapid colonization of the rumen by aerobic and facultative anaerobic microbial taxa close to birth, which decreased

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gradually to a constant level at between 6 and 8 weeks of age, being gradually replaced by exclusively anaerobic taxa (Bryant *et al.,* 1958; Fonty *et al.,* 1987; Minato *et al.,* 1992). Interestingly, bacteria with cellulolytic capabilities appeared in 3–5-day-old animals, becoming abundant in 2-3-week-olds (Fonty *et al.,* 1987; Minato *et al.,* 1992). However, those studies used classical culture-based microbiology methods, which describe only a small fraction of the total bacterial populations (Janssen, 2006). Thus, knowledge of the composition and establishment of primary bacterial communities in newborn animals, and rumen composition in general, was limited to only a few cultured species (Dehority, 2003; Hungate *et al.,* 1964). Recently, a study examining ruminal microbial communities of pre-ruminant calves—three 14-day-olds and three 42-day-olds—reported the existence of bacteria and functions found in mature animals (Li *et al.,* 2011). Nevertheless, the identity and composition of the primary rumen bacterial communities acquired shortly after birth, and the changes occurring in these communities at different growth stages of the animal remain largely unknown, despite their importance for understanding the forces governing this microbial ecosystem and its similarities to others. Therefore, we sought to characterize the composition of rumen bacterial communities in newborn calves and their changes with growth stages throughout the animal's life. We used a robust pyrosequencing approach that provides vast knowledge on the bacterial communities, and quantitative real-time PCR for accurate monitoring of selected taxa.

Materials and methods

Animal handling and sampling

The experimental procedures used in this study were approved by the Faculty Animal Policy and Welfare Committee of the Agricultural Research Organization Volcani Research Center, and were in accordance with the guidelines of the Israel Council for Animal Care.

One month prior to sample collection, 2-year-old ($n=5$) and 6-month-old ($n=5$) Israeli Holstein cows were fed a diet *ad libitum* consisting of 70% concentrated food, mineral and vitamin mix and 30% roughage (Supplementary Table S1); 2-month-old calves ($n=5$), just before weaning, were fed milk and a specifically designed solid starter diet (Supplementary Table S1); newborn calves ($n=6$) were fed exclusively colostrum. One hour after the morning feeding, rumen fluid was extracted via the mouth using a stomach tube with a rumen vacuum sampler. Samples were transferred to CO_2-containing centrifuge bottles to maintain anaerobic conditions and kept on ice for no more than 20 min before processing. Immediately after collection, the ruminal samples were processed as previously described (Stevenson and Weimer, 2007). Sample handling by cooling on ice has been reported not to affect the samples or their subsequent analyses (Wu *et al.,* 2010). The samples were centrifuged at 10 000 *g* and the pellet was dissolved in extraction buffer (100 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.15 M NaCl; pH 8.0); 1 g of pellet was dissolved in 4 ml of buffer and incubated at 4 °C for 1 h, as chilling has been shown to maximize the release of particle-associated bacteria from ruminal contents (Dehority and Grubb, 1980). The suspension was then centrifuged at 500 *g* for 15 min at 4 °C to remove ruptured plant particles while keeping the bacterial cells in suspension. The supernatant was then passed through four layers of cheesecloth, centrifuged (10 000 *g*, 25 min, 4 °C) and the pellets were kept at −20 °C until DNA extraction.

DNA extraction

DNA extraction was performed as previously described (Stevenson and Weimer, 2007). In brief, cells were lysed by bead disruption with phenol followed by phenol/chloroform DNA extraction. The final supernatant was precipitated with 0.6 volume of isopropanol and resuspended overnight in 50–100 µl TE (10 mM Tris-HCl, 1 mM EDTA), then stored at 4 °C for short-term use, or archived at −80 °C.

454 bacterial tag-encoded amplicon pyrosequencing and data analyses

The Research and Testing Laboratory (Lubbock, TX, USA) performed 454 amplicon pyrosequencing of the ruminal DNA samples, using primers covering the V2 and V3 regions (Gray28F 5′-TTTGATC NTGGCTCAG-3′ and Gray519r 5′-GTNTTACNGC GGCKGCTG-3′). The tagging and sequencing protocol was performed as previously described by (Dowd *et al.,* 2008). PCR was performed under the following conditions: 94 °C for 3 min followed by 32 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. A secondary PCR was performed for FLX (Roche, Nutley, NJ, USA) amplicon sequencing under the same conditions by using specially designed fusion primers with different tag sequences (Supplementary Table S3). Data quality control and analyses were mostly performed using the QIIME pipeline (Caporaso *et al.,* 2011). First, reads were assigned to their designated rumen sample, then length-based filtering (<200 bp was excluded from the analysis) and read-quality filtering was performed. The next step was to align the obtained sequences to define operational taxonomic units (OTUs) for eventual taxonomy assignment. The Uclust method was used to cluster the reads into OTUs (Edgar, 2010). Chimeric OTUs were removed from the analysis using ChimeraSlayer (Haas *et al.,* 2011), as were clusters of only one or two reads.

Taxonomy was assigned using the BLAST algorithm against the 16S reference database found at: http://blog.qiime.org, designated as 'most recent Greengenes OTUs'. For similarity measurement between the bacterial communities in the samples, the Bray–Curtis similarity index was used to compare samples according to both presence and absence of OTUs and abundance of OTUs between samples. Additional statistical analysis were performed using the Paleontological Statistics software (Hammer et al., 2001). Sequencing data and information were deposited in the MG-RAST server under IDs 4514864.3 to 4514868.3.

Real-time PCR

Quantitative real-time PCR analysis was performed on 11 bacterial species through amplification of their copy of the 16S rRNA gene as previously described (Jami and Mizrahi, 2012a), using the primers shown in Supplementary Table S2. Briefly, a standard curve was generated for each individual bacterial strain selected and for the total bacterial rRNA gene, using universal primers. Real-time PCR was performed in a 10-μl reaction mixture containing 5 μl of Absolute Blue SYBR Green Master Mix (Thermo Scientific, Waltham, MA, USA), 0.5 μl of each primer (10-μM working concentration), 3 μl of nuclease-free water and 2 μl of 10 ng DNA templates. Amplification involved one holding cycle at 95 °C for 15 min for initial denaturation and activation of the hot-start polymerase system, and then 40 cycles at 95 °C for 10 s followed by annealing at 60 °C for 15 s and extension at 72 °C for 20 s. Relative quantification was achieved by comparing the total bacterial 16S count using 16S universal primers (HDA) to the 16S count for each bacterial species tested.

Results

Rumen bacterial composition across different age groups

We selected animals from four different age groups: 1–3-day-old calves (three 1-day-olds and three 3-day-olds, $n=6$), 2-month-old calves ($n=5$), 6-month-old heifers ($n=5$) and 2-year-old lactating dairy cows ($n=5$). These groups were fed with three different diets according to standard husbandry feeding programs (Supplementary Table S1). We collected the rumen content of the 21 animals from the different groups, the microbial cells were separated and metagenomic DNA was extracted as previously described (Stevenson and Weimer, 2007). We then used bacterial tag-encoded amplicon pyrosequencing generated from the V2 and V3 regions of the 16S rRNA gene to identify and characterize the overall ruminal bacterial composition of each of our samples. In total, after size filtering, quality control and chimera removal using the QIIME pipeline (Caporaso *et al.,* 2011), 229 897 quality reads were generated with an average of 10 938 ± 2860 reads per sample. The overall number of OTUs detected by the analysis reached 6594 based on ⩾97% nucleotide sequence identity between reads. To assess whether our sampling effort provided sufficient OTU coverage to accurately describe the bacterial composition of each group, sample- and individual-based rarefaction curves were generated for each group. These implied that our sampling effort was sufficient for the samples from younger animals but remained incomplete for the 6-month- and 2-year-old animals because of the high complexity in these samples (Figure 1 and Supplementary Figure S1a). The number of OTUs and the taxon diversity differed significantly ($P<0.05$ using *t*-test analysis) between the age groups (Figure 1 and Supplementary Figure S1b). This was also apparent with the Shannon–Weaver diversity index, which was significantly different between the groups ($P<0.05$ using *t*-test analysis) (Supplementary Figure S1b).

Overall, 15 phyla were detected in the samples. Among them, the Firmicutes, Bacteroidetes and Proteobacteria were detected as the dominant phyla regardless of age group (Figure 2a), but their ratio and composition among the groups varied considerably (Figure 2, Supplementary Figures S2, S3, S4 and Supplementary Table S4). The phylum Firmicutes found in the samples taken from the 1–3-day group was abundant compared with the other groups, with the vast majority of the reads belonging to the genus *Streptococcus* (Supplementary Figure S2); in one sample, as much as 75% of the reads belonged to this genus. In samples from all other

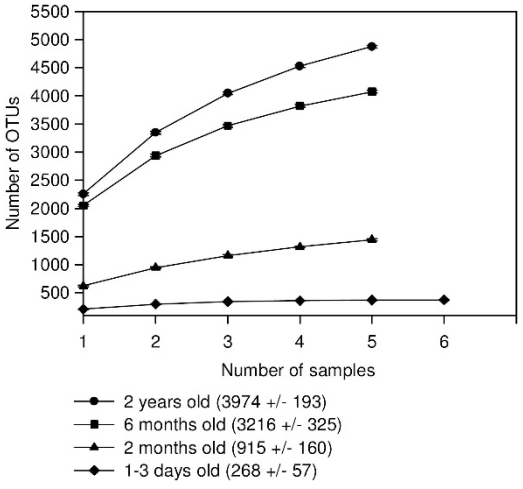

Figure 1 Sample-based rarefaction analysis. Species-accumulation curves showing the increase in OTU numbers as a function of the number of individuals sampled. Each curve represents one individual age group and its corresponding rarefaction ($n=21$); 1–3-day-old calves ($n=6$), 2-month-old calves ($n=5$), 6-month-old heifers ($n=5$) and 2-year-old dairy cows ($n=5$). The values in brackets represent the average Chao1 species richness estimation for each age group.

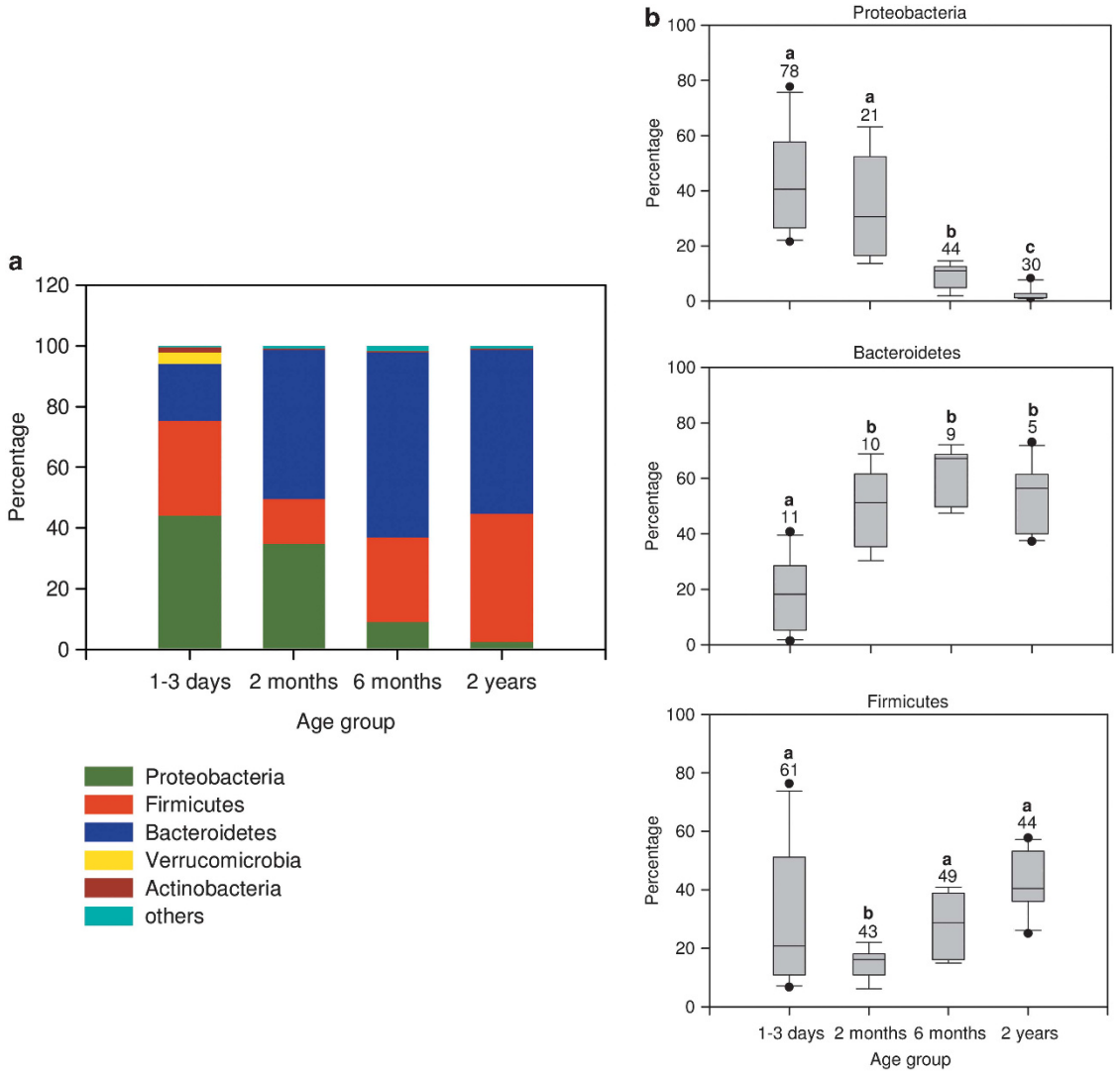

Figure 2 Phylum level composition. (**a**) Color-coded bar plot showing the average bacterial phylum distribution across the different age groups sampled. (**b**) Box plot showing the relative abundance of the three main bacterial phyla found in every age group: Proteobacteria, Bacteroidetes and Firmicutes, represented as log percentage on the Y-axis. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively. The numbers above the boxes denote the number of genera composing each phylum. Boxes with a different letter above their whiskers are significantly different at $P<0.05$ using *t*-test analysis.

groups, only a small proportion of the reads could be associated to the *Streptococci* (Supplementary Figure S2). The Firmicutes decreased in the 2-month-old group and significantly increased again ($P<0.05$ using *t*-test analysis) in the two older age groups (Figure 2). The phylum Bacteroidetes was significantly lower ($P<0.05$ using *t*-test analysis) in samples taken from the 1–3-day group compared with all other groups, but became the most abundant phylum in samples from older animals (Figure 2b). The genus composition of this phylum also changed considerably between the age groups: in the newborn animals (1- and 3-day old) it was mainly composed of the genus *Bacteroides,* whereas in the older age groups, it was almost exclusively composed of *Prevotella* (Supplementary Figure S3). Other minor phyla present in the sampled animals, such as Actinobacteria and Fusobacteria, could be found in all age groups, but were more prominent in newborn calves. The Actinobacteria taxon composition was markedly different between newborn calves (1- and 3-day old) and older animals, changing from being made up mainly of Actinomycetales order in newborns to Coriobacteriales order in 2-year-olds (Supplementary Figure S4). Other phyla, such as Tenericutes, Cyanobacteria and TM7, were more abundant in older animals ($P<0.05$ using *t*-test) (Figure 2a and Supplementary Table S4). The phylum TM7 was previously found to be part of the core community in lactating dairy cows (Jami and Mizrahi, 2012b).

OTU diversity and similarity analysis

Community OTU comparisons by non-metric multidimentional scaling (NMDS; OTU $\geqslant$97% identity, species level similarity) of each group using the Bray–Curtis similarity metric revealed that the samples clustered together according to their particular age group, suggesting that each group hosts its own distinct bacterial community (Figure 3a). This was also apparent when analysis of similarity (ANOSIM) was performed for the different groups, showing significantly high R values ($P<0.05$) between the groups (Supplementary Table S5). The difference between the 6-month-old and 2-year-old animals is also evidenced by the degree of similarity between the two groups (Supplementary Figure S5). The average within-group similarity analysis showed a significant difference between the groups and increased in an age-dependent manner, with the exception of the 6-month- and 2-year-old groups that only exhibited an increasing trend (Figure 3b). Our results also revealed a subclustering within the 1–3-day group, with the 1- and 3-day-old calves clustering separately from each other. Despite its high R values, however, this clustering was not significant, possibly due to the low number of samples when the 1–3-day group was divided into two groups of three animals each ($P=0.1$, using ANOSIM).

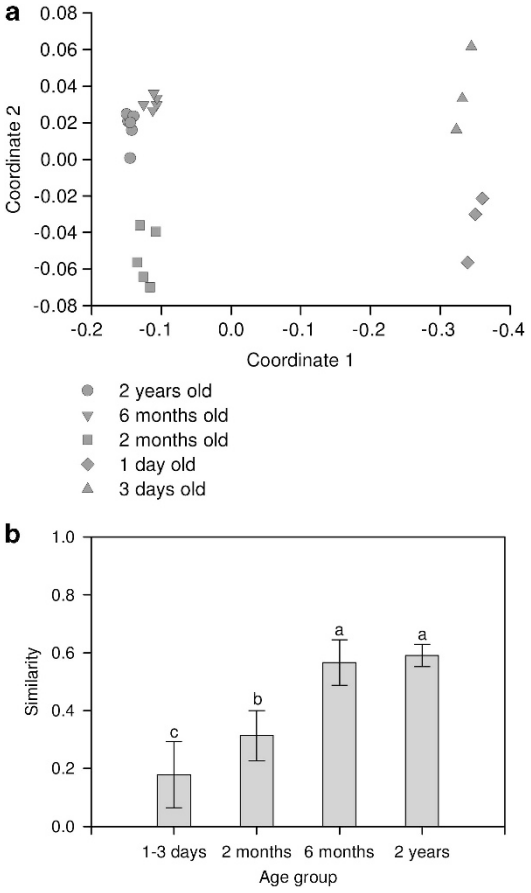

Figure 3 Similarity of the bacterial communities between and within age groups. (**a**) Distance between the samples, based on similarity in OTU composition (OTU similarity $\geqslant$0.97%) of each sample calculated using the Bray–Curtis similarity index and plotted using non-metric multidimensional scaling (NMDS). Each point represents a different sample plotted according to their OTU composition and abundance (stress value = 0.12). A greater distance between two points infers a lower similarity between them, whereas samples with a more similar bacterial composition and abundance cluster closer together. (**b**) Bar plot showing within-group similarity, calculated as the average of the pairwise similarity between each paired sample within each group using the Bray–Curtis metric. The X-axis denotes the age group of the animals from which the rumen samples were taken, and the Y-axis represents the degree of similarity: the closer the similarity is to 1, the higher the average similarity within a group. Letters above the bars indicate significance between groups; groups with different letters have significantly different similarity values at $P<0.05$ using t-test analysis.

Analysis of bacterial composition in 1- and 3-day-old animals

The observed differences between the 1- and 3-day-old animals and their low similarity when compared with all other groups (Figure 3a), along with the observation that only a few genera are shared between the bacterial community during primary stages of colonization and the communities found in mature animals (Figure 4), suggested different and rapidly changing bacterial communities during the first days of life. Therefore, we examined the 1-day-old ($n=3$) and 3-day-old ($n=3$) animals separately. At the phylum level, a substantial decrease in the phylum Firmicutes and a rise in the phylum Verrucomicrobia were observed (Figure 5a). This latter increase was caused by one specific 3-day-old calf with a high proportion of Verrucomicrobia (23%), all belonging to the genus *Akkermansia*. In other newborn samples, Verrucomicrobia could only be found in trace amounts or not at all, and could not be detected in any of the samples from older animals. At the genus level, the two groups shared 23 genera that were present in all animals in both

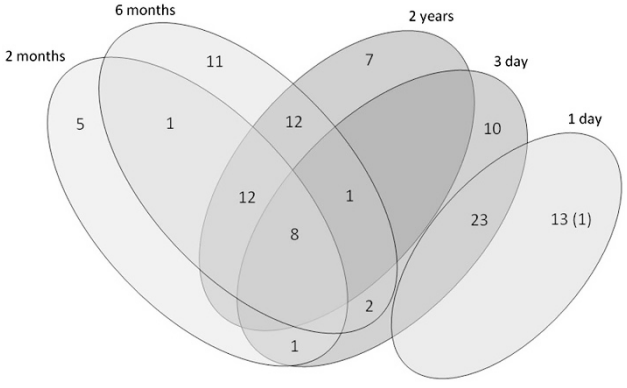

Figure 4 Shared genera across different groups. Venn plot showing the shared and unique genera found in each plotted group. Only taxa that are shared by all of the animals (core) within each group are plotted. The parenthesis in the 1-day-old group denotes the one genus it shares with all other animals (not seen in the plot, see Table 1).

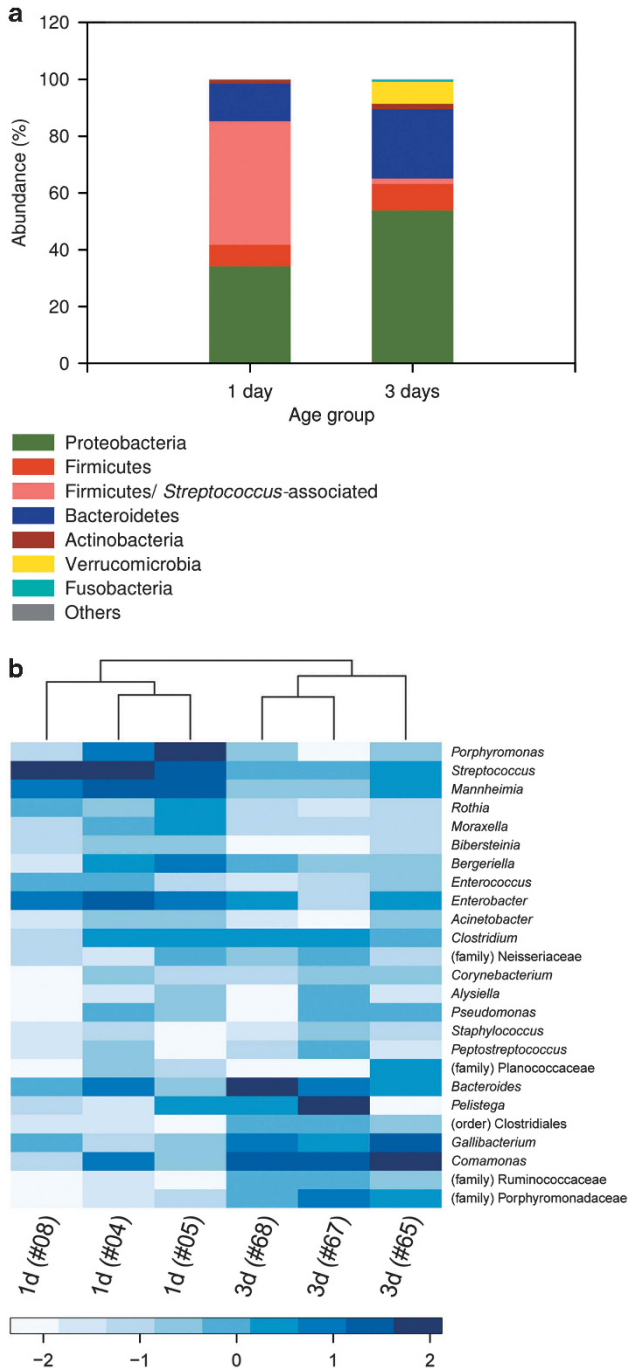

Figure 5 Taxonomic comparison of 1-day-old and 3-day-old animals. (**a**) Bar plot comparing the phylum composition of 1-day-old calves ($n=3$) and 3-day-old calves ($n=3$). The light red bar segment represents the proportion of Firmicutes associated with the genus *Streptococcus,* whereas the dark red segment represents Firmicutes associated with all other genera. (**b**) Log-scaled percentage heat map of genus-level rumen composition; the abundance of the 23 genera shared by all of the 1-day-old and 3-day-old calves (core genera) are displayed. Taxa that could not be assigned a genus but were still present in all of the samples were displayed using the highest taxonomic level that could be assigned to them and the level is shown in parentheses.

groups, but their levels were radically different between the two groups (Figures 4 and 5b). We examined the genera exhibiting a five-fold or more change between the 1- and 3-day-old groups, taking into account only those that were present in all samples in at least one of the groups. Thus, we examined genera such as *Streptococcus*, *Enterococcus* or *Enterobacter*, which were substantially represented in one of the age groups, but were at potentially undetectable levels in all animals from the other group.

Interestingly, most of the genera that showed a substantial decrease were associated with aerobic or facultative anaerobic functions, whereas most of the genera exhibiting a similar increase were associated with strictly anaerobic functions according to the Bergey's Manual of Systematic Bacteriology (Garrity, 2005; Krieg, 2010; Vos, 2010; Whitman, 2012) (Figure 6). This compositional change in functional associations (aerobic vs anaerobic) was found to be highly significant (using χ^2 at $P<0.001$).

Monitoring of specific functional bacterial species

Some of the genera that emerged in 3-day-old animals were present in the adult functional rumen and are considered important to its proper function. These genera included the *Coprococcus* (0.13%), *Prevotella* (3.9%), which in later samples became the most abundant genus, and *Ruminococcus* (0.2%), a genus containing two main cellulolytic bacterial species—*Ruminococcus albus* and *Ruminococcus flavefaciens* (Table 1 and Supplementary Table S4). Therefore, by using quantitative real-time PCR, we investigated whether these genera are composed of species known to be involved in fundamental functions in the mature rumen. Indeed, from the quantification of 11 typically rumen-associated and extensively studied bacterial species, it became evident that bacterial species that occupy the adult rumen could already be found in the first days of life in the pre-functioning rumen (Figure 7). The cellulolytic bacteria *R. flavefaciens* was detected in samples taken from 1-day-old calves and increased significantly in abundance in samples taken from 3-day-old calves, whereas *R. albus* first emerged in the 3-day-old animals. *Fibrobacter succinogenes*, another major cellulolytic bacterium, could only be detected in samples from animals that were at least 2-months old. Three ruminal species of the pivotal *Prevotella* genus that can account for as much as 70% of the rumen bacterial population (Jami and Mizrahi, 2012b) were also analyzed; these species are capable of utilizing starches, other non-cellulosic polysaccharides and simple sugars as energy sources with succinate being produced as the major fermentation end product (Purushe *et al.,* 2010). *Prevotella ruminicola* was found in 1-day-old animals, albeit in minute amounts, increasing by day 3. The other *Prevotella* species tested appeared only in the 2-month and older age groups, exhibiting little change between groups. *Streptococcus bovis*, which is considered to be a starch utilizer and lactose fermenter (Dehority, 2003), was found in

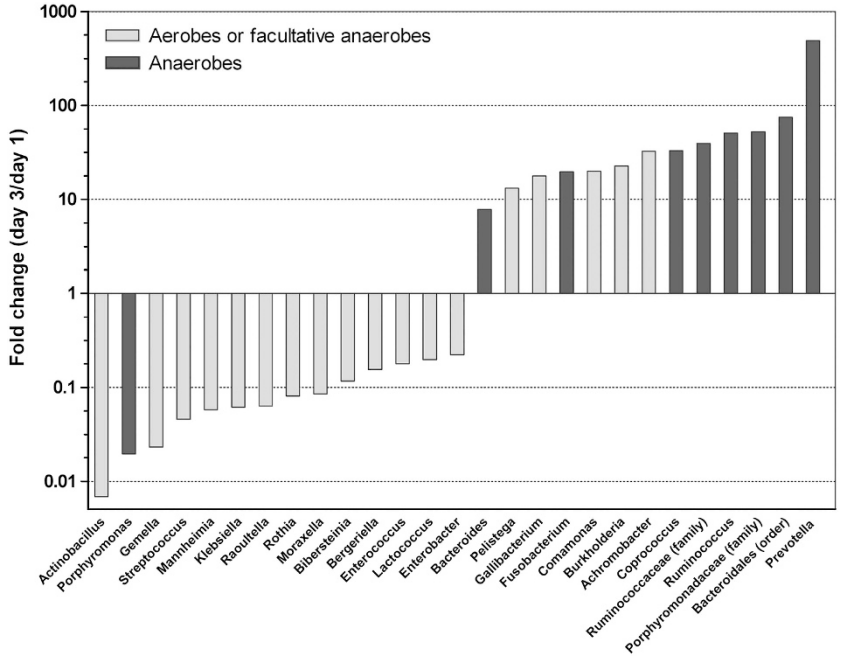

Figure 6 Fold changes of taxa between 1-day-old and 3-day-old calves (3 day/1 day). The Y-axis denotes the log-scale fold change occurring within the taxon specified on the X-axis. Only taxa that were shared by all animals within at least one of the two groups are displayed. The light gray bars represent taxa associated with aerobic or facultative anaerobic functions, and the dark gray bars represent taxa associated with strictly anaerobic traits, according to the Bergey's Manual of Systematic Bacteriology (Garrity, 2005, Krieg, 2010, Vos, 2010, Whitman, 2012).

Table 1 Shared genera between groups

Phylum	*Genus*	*1-day old (%)*	*s.e.m. (%)*	*3-days old (%)*	*s.e.m. (%)*	*2-months old (%)*	*s.e.m. (%)*	*6-months old (%)*	*s.e.m. (%)*	*2-years old (%)*	*s.e.m. (%)*
Bacteroidetes	*Prevotella*			3.85	3.35	48.88	6.98	59.68	5.15	56.66	5.28
	*Bacteroidales			1.48	0.82	0.34	0.16	0.52	0.14	0.27	0.05
	Clostridium	2.16	1.02	1.81	0.35	0.86	0.15	5.24	1.19	4.60	0.67
	*Lachnospiraceae			0.08	0.02	3.58	0.73	5.02	0.73	7.02	1.03
	Succiniclasticum			0.22	0.13	0.76	0.08	1.35	0.17	2.01	0.36
	Coprococcus			0.13	0.11	1.24	0.38	1.68	0.23	1.88	0.20
	Ruminococcus			0.20	0.13	1.30	0.44	1.50	0.20	1.60	0.11
	*Ruminococcaceae			0.67	0.28	0.80	0.18	5.30	1.34	5.56	0.82
	Butyrivibrio					1.41	0.86	2.44	0.61	9.17	1.66
	*Clostridiales FamilyXIII.IncertaeSedis					0.15	0.06	0.99	0.21	1.38	0.25
Firmicutes	*Selenomonas*					0.13	0.02	0.27	0.07	1.03	0.22
	Mitsuokella					0.08	0.01	0.14	0.02	0.58	0.20
	Eubacterium					0.50	0.05	0.56	0.07	0.71	0.04
	Roseburia					0.06	0.01	0.22	0.03	0.41	0.05
	Moryella					0.15	0.01	0.10	9.03E-05	0.38	0.10
	Bulleidia					0.06	0.02	0.10	0.02	0.21	0.05
	Lachnobacterium					0.08	0.04	0.11	0.02	0.16	0.04
	Oscillospira					0.55	0.16	0.13	0.02	0.11	0.02
	Lachnospira					0.15	0.06	0.05	5.31E-05	0.08	0.02
Proteobacteria	*Succinivibrio*					34.45	9.47	8.45	2.29	1.41	0.32
	Sum	2.16		8.44		95.53		93.85		95.23	

Identity of the genera shared by all animals from the different age groups and their average abundance (and s.e.m.) within each group are displayed. Taxa that could not be assigned a genus but were still present in all samples are displayed using the highest taxonomic level that could be assigned to them (marked with an asterisk, *). The sum of all taxa present in the table within each group is shown in the last row of the table.

relatively high abundance in samples from 1- and 3-day-old calves, correlating with the high proportion of *Streptococcus* reads obtained from the pyrosequencing results. In samples of older age groups, its abundance decreased and was relatively similar among those groups (Figure 7). The lactate

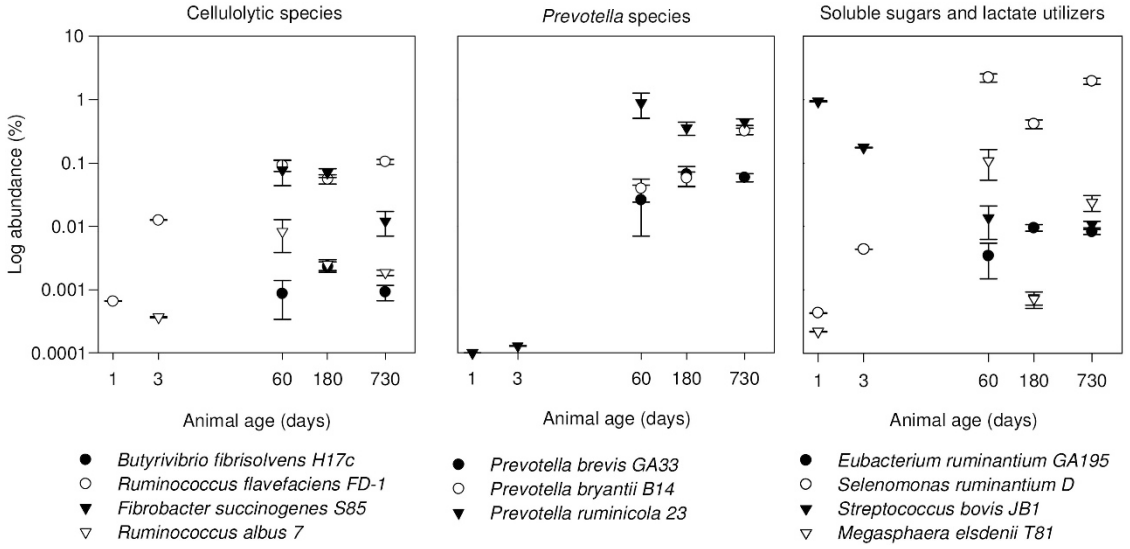

Figure 7 Quantification of known functional bacterial species in the samples using real-time PCR. Each graph displays bacteria that are either taxonomically or functionally related. The Y-axis denotes the abundance in log scale of the species quantified and the X-axis the age group. Whiskers represent s.e.m. for the 1-day-old (group 1, $n=3$), 3-day-old (group 3, $n=3$), 2-month-old (group 60, $n=5$), 6-month-old (group 180, $n=5$) and 2-year-old (group 730, $n=5$) animals.

utilizers *Selenomonas ruminantium* and *Megasphaera elsdenii* could also be detected on the first day of life.

Discussion

The objective of this study was to evaluate the composition of the total ruminal bacteria in newborn calves, and to determine how the composition changes during normal development. Our results suggest that each sampled age group has its own distinct microbiota, as reflected by the clustering of the samples by age group using NMDS and confirmed by ANOSIM ($P<0.01$) (Figure 3 and Supplementary Table S5). Even though animals from the 6-month and 2-year groups received the same diet, their microbiota were still significantly different (Supplementary Table S5), which might indicate that at 6 months of age, the rumen microbiota undergoes developmental changes that are independent of diet.

There were few shared genera between the bacterial community during the primary stages of colonization and the communities found in mature animals (Figure 4 and Table 1). This could be because in the first days of life, the rumen is still inactive and not involved in the digestion of plant material (Van Soest, 1994). The diversity index and OTU number increased with age, as did the within-group similarity in bacterial communities (Pearson $r=0.76$; Figure 3b and Supplementary Figure S1b). This suggests that the mature rumen environment, although more diverse, is a more restricted niche, housing a more specific and homogeneous bacterial community, in comparison to the primary community that allows for more heterogeneity between different animals. In addition, the between-group similarity of the animals to the 2-year group increased with age (Supplementary Figure S5), suggesting a convergence toward a mature bacterial arrangement. Interestingly, similar observations have been reported for human infants: in one study, the pairwise similarity of six infants increased with age (Palmer *et al.,* 2007); in another study, a gradual increase in gut bacterial diversity from birth to 2.5 years of age was related to a gradual change in community diversity (Koenig *et al.,* 2011). These remarkable similarities in (a) the age-dependent increase in bacterial diversity, (b) the increase in within-age-group similarity and (c) the convergence toward a mature bacterial arrangement with age, were also recently reported in a study of the gut microbiomes of human populations from different geographical locations (Yatsunenko *et al.,* 2012) across ages.

The dominant phyla found in all age groups were Firmicutes, Bacteroidetes and Proteobacteria: these varied considerably between the groups in abundance and in the number of genera composing them (Figure 2).

The phylum Bacteroidetes, which was significantly less abundant in newborns, was composed mainly of the genus *Bacteroides*, whereas in the older animals it was prominent and composed almost exclusively of the genus *Prevotella* (Supplementary Figure S3), reaching up to 72% of the total reads in some samples (Table 1). A similar compositional change in the Bacteroidetes phylum has been reported in a study comparing the gut microbiota of children from Europe and rural Africa (De Filippo *et al.*, 2010). In that study, the genus

Prevotella dominated the Bacteroidetes phylum, comprising 53% of the total gut bacteria, in the African children; on the other hand, in the European children, this genus was not present and instead *Bacteroides* was dominant. Moreover, a much higher representation of the Bacteroidetes phylum was observed in the African children, along with a lower representation of Firmicutes. These differences were attributed to the different diets consumed by these children: the African diet is composed mainly of plant fiber, whereas the European diet is high in animal protein, sugar, starch and fat, but low in fiber (De Filippo *et al.,* 2010). These dietary differences resemble those in our study, with the newborn diet, the colostrum, being of high caloric value, rich in protein, fat and sugar (Kehoe *et al.,* 2007), whereas the older animal's diet is composed mainly, of plant fiber. The similarities in the changing trends of phylum distribution and composition as a function of diet reinforce the general and global functions of these bacterial taxa in the digestive tract of living beings as related to dietary content. More specifically, the Bacteroidetes phylum is less abundant when high-calorie diets are consumed, and its composition changes to being predominantly composed of the genus *Prevotella* when high-fiber diets are introduced. Other phyla could be found in a higher proportion in calves fed exclusively milk, such as the Actinobacteria and Verrucomicrobia. Verrucomicrobia, represented exclusively by the genus *Akkermansia*, was found in a relatively high proportion in only one 3-day-old calf. This was also observed in another study in one 42-day-old calf (Li *et al.*, 2011). This observation may reflect an opportunistic trend for this genus in young calves. The Actinobacteria phylum decreased with age along with a compositional change in newborn calves vs older animals. This was most apparent at the order level where the Actinomycetales that was the most dominant order of this phylum in 1–3-day-old animals significantly decreased in older groups ($P<0.05$ using *t*-test), whereas the Coriobacteriales order significantly increased in the 2-year- and 6-month-old animals (Supplementary Figure S4).

Twenty genera were shared between the 2-month, 6-month and 2-year groups with relatively similar abundance, accounting for between 95% and 96% of the community for each group; in contrast, the 3-day-old animals shared eight genera with the older animals, accounting for only ~8.5% of the former's total bacterial community, and only one taxon was shared between the 1-day-old and older animals (Figure 4, Table 1). The 1- and 3-day animals shared 23 genera. However, unlike the taxa shared by the older groups, the abundance of each genus from this shared community differed greatly between the two groups but was similar within them (Figure 5b). This might indicate that the change is a structured and ordered process occurring during the first days of life. A similarly non-random assembly of human gut bacterial communities was suggested in a study that monitored the colonization of gut bacterial communities in one human infant over a 2.5-year period (Koenig *et al.,* 2011). The changes observed here between the 1- and 3-day-old animals and their individual clustering in the NMDS indicate a rapidly changing environmental niche in the rumen in the first days after birth. We therefore used these groups to examine the composition and dynamics of the primary bacterial populations in the first days of life and their impact on the adult rumen bacterial communities.

The most noticeable and statistically significant change occurring between the first and third day of life was the decrease in taxa associated with aerobic or facultative anaerobic function and the increase in those associated with obligatory anaerobic function ($P<0.0001$ by χ^2 analysis) (Figure 6). Almost all genera that showed a sharp decrease on day 3 were either aerobic or facultative anaerobic. Interestingly, a similar observation was made in newborn lambs using classical culturing methods (Fonty *et al.,* 1987).

Some of the anaerobic genera that showed a substantial increase in samples taken from 3-day-old calves are known to be permanent residents of the bacterial community in the mature rumen. Chief among these were the *Prevotella*, exhibiting an almost 500-fold increase on average: this genus is the most abundant in the adult rumen (Stevenson and Weimer, 2007; Jami and Mizrahi, 2012b) and is assumed to comprise a large part of the rumen microbial genetic and metabolic diversity (Purushe *et al.,* 2010). It was detected in only a single 1-day-old calf (<0.03%) and increased in abundance to an average of 3.85% in 3-day-old animals, where it could already be found in all of the samples.

Also exhibiting an increase in samples from 3-day-old calves was *Ruminococcus*, a genus that includes two of the major cellulolytic bacteria commonly found in the adult rumen, *R. flavefaciens* and *R. albus* (Flint and Bayer, 2008). Following these observations, we examined whether cellulolytic bacteria and other functional rumen bacterial species exist in the rumen of newborn calves using quantitative real-time PCR. Indeed, we found cellulolytic bacterial species as early as 1 day after birth. *R. flavefaciens* could already be found in the rumen of 1-day-old animals and at increasing abundance on the third day (Figure 7). The increase in abundance might suggest pathways other than cellulose degradation in *R. flavefaciens*, or its possible reliance on other bacterial species for its nutritional needs (Fonty *et al.,* 1983). To the best of our knowledge, this is the earliest detection of these bacterial species in the pre-functioning rumen, and it is reinforced by the observations of Fonty *et al.* (1987) and Minato *et al.* (1992) who reported the isolation of cellulolytic bacteria from the rumen in the first week after birth, albeit without taxonomic identification (Fonty *et al.,* 1987; Minato *et al.,* 1992). *R. albus* appeared in low abundance in

samples taken from 3-day-old calves and the third major cellulolytic bacterium, *F. succinogenes*, could only be detected in samples from 2-month and older animals, in a quantity similar to that of *R. flavefaciens*. *S. bovis* was the most abundant species quantified on the first day after birth, and a ten-fold decrease could already be observed in the samples from day 3, although this decrease was not significant. This species was also detected in samples from older animals in lower abundance. Consequently, establishment in the rumen of bacterial species that are important for its proper function in adult animals begins on the first day of life, when the animals are still being fed exclusively colostrum, that is, before the intake of plant material. This notion has also been advanced for microbial communities in the developing human infant's gut microbiome (Koenig *et al.,* 2011).

Until now, the composition of ruminal bacterial communities in the first days after birth had only been studied using culture-dependent methods, which allowed examination of only a small portion of the bacteria (Janssen, 2006; Stevenson and Weimer, 2007). By describing the overall ruminal bacterial communities in newborn animals and other age groups, we were able to capture the changes occurring in these communities on the first days after birth and their remnants at more mature growth stages. Our findings led us to conclude that these populations undergo dramatic and rapid changes involving an increase in the number of anaerobic genera by day 3 of age, indicating the emergence of a new anaerobic environmental niche as early as 3 days after birth. Furthermore, we detected cellulolytic bacteria and other bacterial species important to the proper functioning of the adult rumen on the first day of age, long before weaning or exposure to plant material. These bacteria increased in number as the animals matured. Our results suggest that the rumen bacterial communities are not only influenced by diet, but also by the age of the animal. This is especially evident in the separate clustering of the 6-month and 2-year groups, and in the distinct and considerably different bacterial compositions in the 1-day-old and 3-day-old animals. An impact of age on gut bacterial communities was also observed by Koenig *et al.,* (2011) and Yatsunenko *et al* (2012) in their aforementioned studies on changes in human gut microbiota with age. Overall, the remarkable similarities observed throughout this study between the development of the rumen and human gut bacterial communities, as well as their changes with diet, suggest that intestinal bacterial development and function are common to different living beings. This in turn raises intriguing questions regarding the evolution of these bacteria with various hosts, and may also suggest the bovine rumen as a model for the study of gastrointestinal tract bacteria. The current findings also call for further investigation regarding the changes in the rumen population with age, the origin of these bacteria, and the way in which they are transferred to the newborn animals. This primary bacterial community might be transmitted from the mother via skin, birth canal, or saliva, as was recently demonstrated in humans, where skin and birth canal were the main modes of acquisition (Dominguez-Bello *et al.,* 2010). The correlations and similarities of these processes to the acquisition and structure of the human gut microbiome raise an attractive topic for further investigation.

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