JULIANA DIAS

CHARACTERIZING THE GASTROINTESTINAL TRACT MICROBIOTA OF DAIRY CALVES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de *Doctor Scientiae*.

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DEDICATION

For my family: parents (Sebastião Carneiro Dias and Maria da Penha Dias), brothers (Francisco José Dias and Anelise Dias), nephews (Taís Dias de Assis, Pedro Dias de Assis, Valentina Lourenço Dias and Hugo Baldez) and brothers in law (Márcio Ferreira de Assis and Selma Lourenço) for all love, friendship and support.

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ABSTRACT

DIAS, Juliana, D.Sc., Universidade Federal de Viçosa, February, 2017. **Characterizing the gastrointestinal tract microbiota of dairy calves.** Adviser: Marcos Inácio Marcondes. Co-adviser: Fernanda Samarini Machado.

At birth, calves display an underdeveloped gastrointestinal tract (GIT) whose maturation is strictly related to microbiota colonization. However, little is known about the factors that affect the establishment of archaeal, bacterial and fungal communities in the GIT of calves, as well as the changes in their structure and abundance during calf development into a functional ruminant. To address these gaps in knowledge, this work employed next-generation sequencing to characterize the GIT microbiota of Holstein-Gyr crossbred dairy calves across pre-weaning development. The first study aimed to assess changes on the rumen archaeal, bacterial and fungal communities of crossbred dairy calves (n=45) across pre-weaning development (7, 28, 49, 63 days) on two different diets (M: only raw milk at 10% of body weight at birth (BW) and MC: raw milk (10% BW) plus starter concentrate *ad libitum*). In the second study, we characterized changes in the bacterial communities across GIT regions (rumen, jejunum, cecum and colon) of MC-fed calves (n=17) at 7, 28, 49, 63 days of age. The results of first study revealed that archaeal, bacterial and fungal communities co-occur in the rumen since early calf development but are impacted differently by pre-weaning diet and age. The inclusion of starter concentrate in the calf diet significantly affected rumen bacterial community by promoting increases of genera, direct and indirectly, related to degradation of readily fermentable carbohydrates (i.e. *Megasphaera, Sharpea* and *Succinivribrio*) and depressing those reliant on milk nutrients like lactose (i.e. *Lactobacillus*, *Bacteroides* and *Parabacteroides*). These bacterial changes resulted in apparent diet-driven archaeal differences due to altered fermentation patterns and availability of hydrogen in the rumen that favoured the colonization of members from genus *Methanosphaera* instead of *Methanobrevibacter*. No such differences were found for fungi community represented by members from genus *Caecomyces* and family Neocallimastigaceae, likely due to high inter-animal variation and low fibre content of concentrate used our study. Altogether, this study showed that manipulation of the microbiota in the developing rumen is possible through dietary intervention. Our results may be useful in designing strategies to promote colonization of target communities (i.e. butyrate- producers and lactate-utilizing) linked to functional development of the calf. In regards to second study, bacterial communities in the calf GIT differ qualitatively and quantitatively among compartments and respond differently to age advance that

encompass the GIT development (i.e. rumen) and progressive replacement of milk-based to grain-diet (i.e. increase of starter concentrate intake). In the rumen, bacterial community was composed majority by members from genera *Prevotella, Butyrivibrio and Ruminococcus* whose abundance increased proportionally with age possibly due greater availability of readily fermentable carbohydrates in the rumen. Members from genus *Lactobacillus* were overrepresented in the jejunum but their predominance was replaced by members from Clostridiaceae family in older calves. The cecum and colon displayed similar abundance at taxa level and the abundance of genera *Blautia*, *Paraprevotella, Prevotella, Phascolarctobacterium* and *Succiniclasticum* increased significantly with age. In summary, our results showed that although there are bacterial communities "common" to distinct regions, a closer look at their structure, abundance and dynamic reveals marked segregation and ecological succession in the calf GIT. Our study adds new insights into bacterial colonization across GIT of pre-ruminant that may be considered in formulating strategies to promote the colonization of target communities aiming improve health (i.e. bacteria with probiotic capability) and performance of dairy calves in the pre-weaning period.

RESUMO

DIAS, Juliana, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Caracterização da microbiota do trato gastrointestinal de bezerros leiteiros.** Orientador: Marcos Inácio Marcondes. Coorientadora: Fernanda Samarini Machado.

Ao nascimento, os bezerros exibem um trato gastrointestinal subdesenvolvido (TGI) cuja maturação é estritamente relacionada à colonização da microbiota. No entanto, pouco se sabe sobre os fatores que afetam o estabelecimento de comunidades de archaeas, bacterias e de fungos anaeróbicos no TGI dos bezerros, bem como as mudanças na estrutura e abundância desses grupos microbianos durante o período de transição da fase de pré-ruminante para verdadeiro ruminante. Para abordar essas lacunas no conhecimento, este trabalho empregou sequenciamento de próxima geração para caracterizar a microbiota do TGI de bezerros leiteiros mestiços (Holandês-Gir) durante o período pré-desmame. O primeiro estudo avaliou mudanças nas comunidades de archaeas metanogênicas, bacterias e fungos anaeróbicos no rúmen de bezerros leiteiros (n = 45) alimentados com duas dietas diferentes (M: somente leite cru (10% do peso vivo (PV)) e MC: leite cru (10% PV e concentrado *ad libitum*) e que foram abatidos aos 7, 28, 49, 63 dias de idade. No segundo estudo, caracterizamos as alterações nas comunidades bacterianas entre regiões GIT (rúmen, jejuno, ceco e cólon) de bezerros alimentados com MC (n = 17) que foram abatidos aos 7, 28, 49, 63 dias de idade. Os resultados do primeiro estudo revelaram que as comunidades de archaeas metanogênicas, bacterias e fungos coexistem no rúmen desde a primeira semana de vida, mas são afetadas diferentemente pela dieta e idade. A inclusão de concentrado na dieta de bezerros afetou significativamente a comunidade bacteriana do rúmen: observou-se um aumento na abundância de gêneros relacionados, direta e indiretamente, à degradação de amido (i.e. *Megasphaera, Sharpea* e *Succinivribrio*) e um decréscimo acentuado na abundancia de gêneros (i.e. *Lactobacillus*, *Bacteroides* e *Parabacteroides*) relacionados com a degradação de nutrientes do leite. Alterações na comunidade bacteriana, indiretamente afetaram a comunidade de metanogênicas: fermentação de carboidratos não fibrosos alterou padrões de fermentação (acetato:propionato) e disponibilidade de hidrogênio que por sua vez, favoreceu a colonização de *Methanosphaera* em vez de *Methanobrevibacter*. Na comunidade de fungos anaeróbicos, a abundância do genêro *Caecomyces* e família Neocallimastigaceae não variou significativamente com a dieta ou idade, provavelmente devido à alta variação inter-animal e baixo teor de fibra de concentrado usado em nosso estudo. In suma, este estudo mostrou que a manipulação da microbiota no rúmen em desenvolvimento é

possível através da intervenção dietética. Nossos resultados podem ser úteis na elaboração de estratégias para promover a colonização de comunidades-alvo (isto é, produtores de butirato e utilizadoras de lactato) que estão ligadas ao desenvolvimento de papilas e equilíbrio do pH ruminal. Em relação ao segundo estudo, as comunidades bacterianas diferem qualitativa e quantitativamente entre os compartimentos (rúmen, jejuno, cécum e colón) do trato gastrointestinal e também respondem diferentemente ao avanço da idade que inclui a substituição progressiva da dieta líquida para a dieta sólida (i.e. aumento do consumo de concentrado). No rúmen, a comunidade bacteriana foi composta em sua maioria pelos gêneros *Prevotella*, *Butyrivibrio* e *Ruminococcus* cuja abundância aumentou proporcionalmente com a idade devido a maior disponibilidade de carboidratos não fibrosos no rúmen. No jejuno, o gênero *Lactobacillus* foi abundante desde a primeira semana de vida, mas sua dominância foi substituída por membros da família Clostridiaceae em bezerros mais velhos. As comunidades do ceco e do cólon foram compostas pelos gêneros *Blautia*, *Paraprevotella, Prevotella Phascolarctobacterium* and *Succiniclasticum* cuja abundância aumentou com a idade. Em resumo, nossos resultados mostraram que, embora comunidades bacterianas coexistam em regiões distintas do TGI, uma análise mais detalhada da estrutura, abundância e dinâmica dessas comunidades revela uma marcante segregação e sucessão ecológica no TGI de bezerros. Nosso estudo acrescenta novos insights sobre a colonização bacteriana no TGI de pré-ruminantes que podem servir como base para formulação de estratégias para promover a colonização de comunidades-alvo (i.e. bactérias probióticas) para melhorar a saúde e desempenho de bezerros leiteiros no período pré-desmame.

GENERAL INTRODUCTION

The microbiota of the gastrointestinal tract (GIT) is closely related to immunological system development (Bauer et al., 2006) as well as to metabolic and physiological changes in digestive tracts of the calves during the critical period of transition from pre-ruminant to ruminant stage (Warner et al., 1956; Davis and Drackley, 1998; Guilloteau et al., 2009). However, there are many gaps in knowledge related to the GIT microbiota of dairy calves, as well as to their correlation to biological responses of economic importance (i.e. diahrea incidence and performance) during pre-weaning period.

Overall, our understanding of the GIT ruminant microbiome is very limited. The current knowledge is based on classical culture-based techniques responsible by identification up to 20% of the rumen microbial population (McSweeney et al., 2009). Nevertheless, the advent of molecular techniques, that overcomes some limitations inherent to classical methods, has stimulated new studies related to microbiota colonization in the calf GIT.

Studies performed using next-generation sequencing reported that the main proteolytic/cellulolytic bacterial species responsible for the degradation of feedstuffs were present in rumen of newborn calves (Jami et al., 2013; Rey et al., 2014; Guzman et al., 2015) and that microbial fermentative-and-enzymatic activities were rapidly established in the rumen of calves from second day of life (Rey et al., 2012). In a parallel study, several protein families related to carbohydrate degradation were identified in the rumen of calves at 14 days of age (Li et al., 2011), suggesting that rumen microbiota of calves possesses sufficient metabolic potential and should not be considered rudimentary (Li et al., 2011).

In addition, it was observed that rumen bacterial community changes as the calf develops (Rey et al., 2014; Meale et al., 2016; Dill-McFarland et. al, 2017). However, the most effective window of time for microbial interventions remains to be determined (Yáñez-Ruiz et al., 2015). Lastly, it was observed that bacterial community varies markedly between GIT regions (rumen vs. colon) and according sample (mucosal vs. ingesta) within each region (Malmuthuge et al., 2014; Guzman et al., 2016). Therefore, fecal samples are not a useful proxy of the GIT microbiota, and rumen digesta samples fail to adequately describe the diversity of mucosa-associated bacterial communities.

These findings represent new insights about the dynamic and colonization of microbiota in the GIT of dairy calves. However, the majority of sequencing-based studies has focused on the bacterial communities (Li et al., 2011, Wu et al., 2012; Jami

et al., 2013; Rey et al., 2014; Meale et al., 2016), while diversity of methanogenic archaea and anaerobic fungi in the calf GIT remain poorly characterized. Current information related to archaeal and fungal community in the calf GIT is limited to few works (Fonty et al., 1987; Zhou et al., 2014; Guzman et al., 2015; Dill-McFarland et. al, 2017) that highlight the need of further studies to address several gaps in knowledge.

Recent study identified archaea species (i.e. *Methanobrevibacter mobile* and *M. votae*) in the rumen of calves 20 minutes after birth (Guzman et al., 2015). This result suggests that methanogens colonization occurs prior to calving and raises questions about the current assumption that calf GIT is sterile at birth. Further, the presence of archaea species in the abomasum and jejunum of dairy calves suggest that methanogens may play other function beyond methanogenesis (Zhou et al., 2014) or have syntrophic partnership with bacteria (i.e. *Geobacter* spp.) to support (hydrogen transfer) their energy metabolism (Guzman et al., 2015, 2016).

The methanogenesis is a process inherent to energy metabolism of archaea species, which in turn, contributes to maintenance a low hydrogen partial pressure in the rumen, which is crucial to functioning of microbial enzymes (Janssen and Kirs, 2008). On the other hand, methanogenesis negatively impacts the environment and energy utilization efficiency of the diet. Thus, comprehensive analysis of hydrogen-utilizing communities such as methanogenic archaea and acetogenic bacteria in the calf GIT may facilitate efforts in formulation of strategies to decrease methane emissions.

In regards to anaerobic fungi, there are only two studies that characterized fungal community in the young ruminants. Fonty et al. (1987) identified *Neocallimastix frontalis* and *Caecomyces communis* in the rumen of lambs at 8 days of age, but these species disappeared in almost all the lambs after the concentrate and hay was offered. Dill-McFarland et al. (2017) tracked changes in the rumen and fecal microbiota of dairy calves (from 2 weeks until 2 years of age) and reported that anaerobic fungi were below detection until after weaning.

Like the anaerobic fungi, ciliate protozoa have a close association with rumen archaeal and bacterial communities and can impact nutrient digestibility, fermentation and methanogenesis (Newbold et al., 2015). However, unlike other microbial groups, the establishment of ciliate protozoa in the rumen of young ruminants is reliant on direct or indirect contact with the saliva of adult animals (Coleman, 1979). In contrast to beef cattle production system where calves experience maximum maternal care, dairy calves are separated from their dams right after the birth and kept isolated until weaning. Under these conditions, dairy calves are naturally protozoa-free (Sahoo et al., 2005).

In general, research is warranted to characterize the microbiota and to identify the factors that affect the establishment, distribution and survival of archaeal, bacterial, fungal and protozoa communities in the developing GIT. Further, advances in culture-based techniques coupled with metagenomics and metatranscriptomic tools are crucial to improve the current understanding about microbial ecology in the developing GIT. Lastly, the comprehensive analysis of GIT microbiome of ruminants may guide the elaboration of strategies to promote colonization of microbial groups linked to health and performance of dairy calves, especially bred in challenging systems.

To address these gaps in knowledge, we utilized next-generation sequencing to characterize changes in the rumen archaeal, bacterial and fungal communities of Holstein-Gyr crossbred dairy calves across pre-weaning development on two different diets. Further, we investigate bacterial segregation in the GIT (rumen, jejunum, cecum and colon) of calves at four developmental stages. We hypothesized that diet composition, GIT compartment and age drive changes in the microbial communities in the GIT of pre-ruminants.

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CHAPTER 1

Effect of pre-weaning diet on the rumen archaeal, bacterial and fungal diversity of dairy calves

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Abstract

At birth, calves display an underdeveloped rumen whose maturation is closely related to beginning solid feed intake and microbial activity. However, little is known regarding the impacts of pre-weaning diet on the establishment of the rumen microbiota. Here, we employed sequencing technology to investigate the effects of inclusion of starter concentrate (M: only milk-fed and MC: milk plus starter concentrate) on archaeal, bacterial and fungal communities in the rumen of 45 crossbred dairy calves across pre-weaning development (7, 28, 49 and 63 days). Our results showed that archaeal, bacterial and fungal taxa commonly found in the mature rumen were already established in the rumen of calves at 7 days old regardless of diet. This confirms that microbiota colonization occurs in the absence of solid substrate. However, diet did significantly impact some taxa. In the bacterial community, feeding starter concentrate promoted greater diversity of bacterial taxa (i.e. *Megasphaera*, *Sharpea* and *Succinivribrio*) related to degradation of readily fermentable carbohydrates in the rumen of MC calves. In contrast, M calves displayed a bacterial community dominated by taxa (i.e. *Lactobacillus*, *Bacteroides and Parabacteroides*) able to utilize milk nutrients to support their growth. However, in both diet groups, the dominance of these taxa decreased across developmental stages of calves, showing that diet and age simultaneously drive changes in the structure and abundance of bacterial communities in the developing rumen. Changes in the composition and abundance of archaeal communities were attributed exclusively to diet. The bacterial community in the rumen of MC calves shifted the fermentation pattern and availability of hydrogen favouring the colonization of members from genus *Methanosphaera* instead of *Methanobrevibacter*. The fungal community was dominated by members from genus *Caecomyces* and family Neocallimastigaceae whose abundance did not change in response to diet or age, probably due to high inter-animal variation and low fibre content of starter concentrate. This study adds new insights about colonization of methanogenic archaea, bacteria and anaerobic fungi communities in pre-ruminants that may be useful in designing strategies to promote colonization of target communities linked, to functional development of the calf rumen.

Introduction

In adult ruminants, the rumen harbours a diverse microbiota composed of bacterial, fungal, protozoal and archaeal species that act synergistically to degrade feedstuffs providing nutrients such as volatile fatty acids (VFAs), protein, minerals and vitamins that are metabolized and absorbed by the host (Hungate, 1966). However, at birth, ruminants display a sterile and immature gastrointestinal tract (GIT) whose development is closely related to the microbial colonization that occurs rapidly after birth (Taschuk and Griebe, 2012).

Until approximately the third week of life, calves are considered non-ruminants, because their rumen is anatomically and physiologically underdeveloped (Davis and Drackley, 1998; Baldwin et al. 2004). In this stage, the diet is predominantly milk, which bypasses the rumen and is carried by the esophagical groove to the fourth stomach, termed the abomasum (Davis and Drackley, 1998). Therefore, the rumen's contribution to nutrient degradation and the energetic needs of the animal are minimal compared to advanced developmental stages.

The beginning of solid feed intake (*i.e.* starter concentrate) triggers a critical process of transition from a functional non-ruminant to a true ruminant strictly reliant on the establishment and activity of the rumen microbiota. During the transition stage (3-8 weeks), solid intake increases with age and, consequently, an ascending supply of substrates become available for microbial degradation resulting in increasing VFA concentrations within the rumen. These events promote a cascade of morpho-physiological shifts (*i.e.* rumen papillation and volume) in the digestive system that result in a functional rumen at weaning (Warner et al., 1956; Davis and Drackley, 1998).

There are many knowledge gaps related to the establishment of the rumen microbiota and changes in its composition during the pre-weaning period. Because bacteria constitute a predominant and diverse GIT microbial domain that plays several roles, including modulation of the immune system (Bauer et al., 2006) and metabolism of nutrients (Hungate, 1966), most research has focused on bacterial community (Li et al., 2011, Wu et al., 2012; Jami et al., 2013; Rey et al., 2014), providing a restricted picture of microbial diversity existing in the calf rumen. Information related to archaea and fungi communities in the developing rumen is limited to a few studies (Fonty et al., 1987; Gagen et al., 2012; Zhou et al., 2014; Guzman et al., 2015) with only one study investigating bacteria, archaea, and fungi in calves concurrently (Dill-McFarland et. al, 2017).

Methanogenic archaea colonize the GIT right after birth (Gagen et al., 2012; Guzman et al., 2015) and play an important ecological role in the mature rumen by continuous removal of hydrogen gas (H_2) which in high levels may constrain the microbial growth and carbohydrate degradation (Wolin et al., 1997). However, ruminal archaea also synthesize methane (CH_4), which is a potent greenhouse gas released during eructation and a relevant source of dietary energy loss by the host (Liu and Whitman, 2008; Knapp et al., 2014). Given that manipulation of archaeal community is desirable from both environmental and nutritional aspects, the identification of the factors that affect its establishment in the developing rumen may guide efforts in the formulation of strategies to mitigate enteric methane emissions with long-term effects and economic viability.

Anaerobic fungi are known to play a prominent role in fibrous plant material degradation due their ability to disrupt vegetal tissues and facilitate the colonization of fibre particles by fibrolytic bacteria. Fungi also produce a wide range of polysaccharide-hydrolyzing enzymes to ferment complex carbohydrates releasing great amounts of H_2 that favours the archaeal community (Bauchop, 1983; McAllister et al., 1993). Therefore, anaerobic fungi activity may shape the structure of bacterial and archaeal communities in the mature rumen and consequently influence fibre utilization efficiency and methanogenesis (Tripathi et al., 2007; Kittelmann et al., 2012). Some anaerobic fungi communities are able to establish in the rumen even in the absence of dietary fibre such as observed in lambs (Fonty et al., 1987) but the factors that affect their establishment, distribution and survival in the developing rumen remain poorly explored.

Previous works reported that bacterial communities change with age (Jami et al., 2013; Rey et al., 2014; Dill-McFarland) but no sequencing-based analysis has been done to concurrently assess archaeal, bacterial and fungal communities in response to pre-weaning diets. Here, we hypothesized that diet composition impacts the establishment of rumen microbiota by selecting taxa adapted to new substrates and that microbial communities also change with age that encompass the increase of solid feed intake, microbial fermentation and rumen metabolic development. As such, the objective of this study was to characterize changes on the rumen archaeal, bacterial and fungal communities of Holstein-Gyr crossbred dairy calves across pre-weaning development on two different diets.

Material and Methods

Animals

All animal procedures were conducted according to the protocols approved by the Universidade Federal de Viçosa, MG-BR Animal Care and Use Committee, protocol number 27/2013. The study was carried out at the Experimental Field of Embrapa Dairy Cattle, located in Coronel Pacheco/MG, during Brazilian spring (October to February, 2014). A total of 45 newborn male crossbreed (3/4 to 15/16 Holstein × Gyr) dairy calves from a fixed-time insemination protocol were removed from their dam after birth (within 24 h), weighed (35±3.6 kg), identified and housed in individual shelters to avoid direct contact between calves and mature animals. Colostrum from their dams was offered to 10% of body weight at birth (BW), fractionated into two daily meals (morning and afternoon) until the 3rd day of life. Calves were randomly assigned to one of two diets (M: only whole milk to 10% of BW and MC: whole milk to 10% of BW plus starter concentrate *ad libitum*) and four slaughter age groups (7, 28, 49 and 63 days). Thus, 7 groups were formed: M-7d (n=6), M-28d (n=6), M-49d (n=7), M-63d (n=6), MC-28d (n=6), MC-49d (n=8) and MC-63d (n=6). Regardless diet group, water was available to all animals *ad libitum*. Calves slaughtered at 7 days of age were fed exclusively with colostrum and milk because the starter concentrate intake it is negligible in the first week of age. The starter concentrate was formulated to provide NRC (2001) dietary recommendations for dairy calves, and it was made available in buckets, which were refilled daily after evaluating intake (Supplementary Table 1).

Sample Collection

Calves at respective diet and age group were euthanized with an injection of Acepromazine (0,013mg/kg), Thiopental (0,125 mg/kg), and Potassium chloride (80 to 120 mL). Immediately after euthanasia, the body cavity was opened and the rumen was isolated with polyethylene seal (zip locks) to avoid reflux of ingesta between compartments. Then, ruminal aliquots (50 and 25 mL) were collected and stored at -80°C until DNA extraction and VFA analysis.

VFA analysis

The VFA concentration at rumen samples was determined using high-performance liquid chromatography (HPLC). In brief, samples were treated following methods described by Siegfried et al. (1984) and analyzed in Dionex Ultimate 3000 Dual with a refractive index detector Shodex RI-101 using an ion-exclusion column

Phenomenex Rezex ROA (300 x 7.8 mm) at 45ºC. The mobile phase was composed by H_2SO_4 (5 mM) at flow of 0.7 mL/min. Samples were compared to standards containing increasing concentrations (up to 20 mM) of acetate, butyrate, propionate, formate, isobutyrate, isovalerate, succinate, valerate and lactate.

DNA extraction and sequencing

Total genomic DNA was extracted from rumen fluid following methods described by Stevenson and Weimer (2007). Briefly, microbial cells were collected by centrifugation and lysed by heating and mechanical disruption. DNA was purified by phenol and phenol:chloroform:isoamyl alcohol extraction and resuspended in TE buffer. DNA was quantified by Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and shipped on dry ice to the University of Wisconsin-Madison for sequencing. The V3-V4 hypervariable regions of the bacterial 16S rRNA (Klindworth et al., 2013), V6-V8 of the archaeal 16S rRNA (Kittelmann et al., 2013), and fungal internal transcribed spacer (ITS1) (Kittelmann et al., 2013) were amplified along with Illumina sequencing primers. For bacteria, PCR reactions consisted of 50 ng template DNA, 0.4 μM of each primer, 1X Kapa Hifi HotStart ReadyMix (KAPA Biosystems), and water to 25 μL. For archaea and fungi, DNA was increased to 100 ng and primers to 1.6 μM each. PCR was performed at 95°C for 3 minutes, 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds (25 cycles for bacteria; 35 cycles for archaea and fungi) and a final extension step at 72°C for 5 minutes. PCR products were purified by PureLink™ Pro 96 PCR Purification Kit (Invitrogen) and a second PCR was performed on products to attach Illumina sequencing adapters and unique dual indices. PCR reactions were similar to those for V3-V4 (bacteria) except 5 μL of unquantified PCR product was used as template DNA and 8 cycles were performed. PCR products were recovered by gel extraction in AquaPōr LM low-melt agarose (National Diagnostics, Atlanta, GA) and with Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Purified DNA was quantified by Qubit® Fluorometer (Invitrogen) and equimolar pooled to create a single sample at 1×10^9 ng per μL). Sequencing was performed using the 2x300 bp paired-end method on the Illumina MiSeq following manufacturer's guidelines (Illumina, Inc., San Diego, CA, USA).

Bioinformatics analysis

Archaea, bacteria and fungi sequences were processed separately using mothur (v1.35.0) with procedures modified from Schloss et al. (2009). Briefly, sequences with a

length shorter than 250bp or longer than 600bp containing ambiguous characters or exhibiting a homopolymer greater than 8 bp were removed. Archaeal and bacterial sequences were aligned using the SILVA 16S rRNA gene reference database (Pruesse et al., 2007) and pre-clustered to remove sequencing errors. Fungal sequences were *de novo* aligned and pre-clustered.

The Uchime algorithm was used to detect chimeric sequences (Edgar et al., 2011) and sequences that did not align to the correct region or were chimeric were removed. Archaeal and bacterial sequences were classified using the GreenGenes database (DeSantis et al., 2006) and fungal sequences were classified by the UNITE dynamic ITS database (Kõljalg et al., 2013). All sequences were grouped into operational taxonomic units (OTUs) by uncorrected pairwise distances clustered by the nearest neighbor method with a similarity cutoff of 97%.

Coverage was assessed by Good's coverage calculated in mothur and samples that displayed coverage less than 95% (two samples in the fungi data set) or low number of sequences (3 samples in the archaea data set) were discarded prior the normalization. Archaeal (103 seqs), bacterial (3012 seqs), fungal (107 seqs) communities were normalized to equal sequence counts, and these normalized OTU tables were used to determine the alpha diversity (Chao1, Shannon and inverse Simpson) indexes and the relative abundance (reads /total reads in a sample) of OTUs and higher taxa (phylum, family and genus level) in each rumen sample.

Statistical analysis

Previous analysis showed that calf genetic group presented non-significant effects ($P > 0.05$) and therefore, was not included in the models here. Differences in archaeal, bacterial and fungal alpha diversity represented by Chao1, inverse Simpson's and Shannon's diversity indexes in response to diet, age and interaction (diet*age) were assessed by a two-way ANOVA (type III error). The *P*-values were adjusted to False Discovery Rate (FDR) using the Benjamini-Hochberg method and values below 0.05 were considered significant. This analysis was performed in R (v.3.2.3) using functions available at car package (Fox and Weisberg, 2011).

Beta diversity of archaeal, bacterial and fungal communities was explored by two approaches: Venn diagram to visualize which unique OTUs are shared across developmental stages of M and MC fed calves, as well as, canonical analysis of principal coordinates (CAP) to assess dissimilarities in the microbial communities composition among calves grouped according diet and age. Venn diagrams were built

with OTUs that represented ≥0.1% of the total community within each of the microbial groups (bacteria, archaea or fungi) in at least one sample and that were detected in at least two calves in each diet*age group using functions available at packages VennDiagram (Chen, 2015) and venn diagram (Dusa, 2016).

The CAP analysis was performed with OTUs (at > 0.1% relative abundance in at least one sample) clustered by the Bray-Curtis dissimilarity index with correction of Legendre & Legendre (1998). The Monte Carlo permutation test (nperm=999) was performed to assess the significance of model factors (diet, age and interaction) and constrained axis (CAP1 and CAP2). The Bray-Curtis dissimilarities ordinated by CAP were plotted with ellipses defined by standard deviation with a 95% confidence limit. This analysis was performed in R using functions available at vegan (Oksanen et al., 2013) and ggplot2 (Wickham, 2009).

Differences in the relative abundance of archaeal, bacterial and fungal taxa in response to diet, age and diet*age were assessed by Poisson regression (Jonsson et al., 2016) followed by analysis of deviance (F-test, type III error) and Tukey's Honest Significant Difference test (HSD). The *P*-values were adjusted to False Discovery Rate (FDR) using the Benjamini-Hochberg method and values below 0.05 were considered significant. This analysis was performed using functions available at packages car and agricolae of R (De Mendiburu, 2015). Only genera that represented ≥0.1% of the total community within each of the microbial groups (bacteria, archaea or fungi) in at least one sample and that were detected in at least 50% of all samples were included.

The inter- and intra-genera association patterns of archaeal, bacterial and fungal communities were assessed by two approaches: the Dice index to measure the ecological distance among genera indicating their co-occurrence (Dice, 1945) and Spearman´s ranks, a correlation analysis that indicates the strength of association among genera. These analysis were performed using functions of packages arules (Hahsler et al., 2005), Hmisc (Harrell, 2016) and corrplot (Wei, 2016). Only genera that represented ≥0.5% of the total community within each of the microbial groups (bacteria, archaea or fungi) in at least one sample and that were detected in at least 50% of all samples were included. The Spearman´s rank also was employed to assess the relationship between molar proportions of VFAs (acetate, butyrate, propionate, acetate-to-propionate ratio, total VFA) and relative abundance of archaeal, bacterial and fungal taxa (represented at least 0.1% of the total community within each of the microbial groups in at least one sample and that were detected in at least 50% of all samples) across samples of calves grouped according diet and age.

Results

Sequencing

After sequence trimming, quality filtering and removal of chimeras, 84,219 (mean 2,005.22 ± SD 1,563.959 per sample) high-quality archaeal, 979,883 (21,775.18 ± 18,017.65) bacterial and 32,099 (746.48 ± 660.49) fungal sequences were obtained. The Good's coverage estimator ranged from 0.98 to 1.0, indicating that sequences sufficiently covered the diversity of archaeal, bacterial and fungal communities in all samples. A summary of sequencing and OTU numbers prior to and after normalization according to microbial domain, diet and age group is shown in Supplementary Table 2.

Archaea

For our archaeal alpha-diversity analysis, Chao1 richness, inverse Simpson's diversity and Shannon's diversity, respectively, did not differ by diet (ANOVA, P=0.693, 0.387, 0.509), age (P=0.707; 0.620, 0.693) or the interaction of these two factors (P=0.707; 0.693; 0.707; Supplementary Table 3).

Beta-diversity analysis showed that Bray-Curtis dissimilarities in the archaeal community were significantly different according to diet (Permutation test, P=0.019). This diet effect (*i.e.* inclusion of starter concentrate) was more evident among M and MC calves at 49 and 63 days, which clustered separately (Figure 1A). However, these shifts were not significantly ascribed to age group or interaction (P = 0.509; 0.293). Our Venn diagram analysis showed that out of 6 archaeal OTUs (at > 0.1% relative abundance in at least one sample) only 2 were shared across developmental stages (7, 28, 49 and 63 days old) of M or MC calves (Figure 2). However, in both diet groups, the number of shared OTUs (n=5) did not change among calves at 28, 49 and 63 days old, indicating that archaeal community composition of calves at 7 days old is distinct from older calves (Figure 2).

Taxonomic composition analysis of archaeal communities revealed 8 OTUs (mean 3.30 ± SD 0.84) annotated to the phylum Euryarchaeota (100%) including the families Methanobacteriaceae (94.02 ± 1.80%) and Methanomassiliicoccaceae (5.97 ± 1.80%), as well as the genera *Methanobrevibacter* (56.25 ± 6.45%), *Methanosphaera* (37.77± 6.20%) and *vadinCA11* (5.97 ± 1.80%). The distributions of archaeal taxa (OTUs at the phylum, family and genus levels) among individual calves and diet-age groups are displayed in Supplementary Figures 1 and 3, respectively.

Our analysis of deviance showed that the relative abundances of the genera *Methanobrevibacter* and *Methanosphaera* varied significantly as a function of diet

(Poisson regression, P = 0.039; 0.039). The genus *Methanobrevibacter* was more abundant (TukeyHSD, $P < 0.05$) in the rumen of M calves whereas *Methanosphaera* was more abundant in MC calves. Although the average abundance of *Methanobrevibacter* decreased over the developmental stages of MC calves, the effect of age (P = 0.833) or the interaction of diet*age (P = 0.420) were non-significant. Further, the relative abundance of *vadinCA11* did not change in response to diet, age or interaction (P = 0.466; 0.926; 0.833, respectively) (Tables 1, 2 and 3).

Bacteria

In our bacterial alpha-diversity analysis, Chao1 richness, inverse Simpson's diversity and Shannon's diversity did not vary significantly in response to diet (P = 0.490; 0.472; 0.496), age (ANOVA, P = 0.672; 0.490; 0.672) or the interaction of these factors (P = 0.490; 0.490; 0.496; Supplementary Table 3).

Our beta-diversity analysis showed that Bray-Curtis dissimilarities in the bacterial community were attributed to diet and age (Permutation test, P = 0.001). The dissimilarities in the bacterial communities among M and MC calves persisted across age groups (28, 49 and 63 days old), as shown by sample clustering (Figure 1). In the M calves, dissimilarities in the bacterial communities decreased markedly with age, given the decrease of distance between samples of older calves (Figure 1B). However, the increase in similarity with age was less apparent in MC calves (Figure 1B). Our Venn diagram analysis showed that, out of 519 OTUs (at > 0.1% relative abundance), only 37 and 42 of them were shared (by at least two calves in each group) across developmental stages (7, 28, 49 and 63 days) of both M and MC calves, respectively (Figure 2). In both diet groups, the number of shared OTUs increased with age, indicating that bacterial community composition tended be less heterogeneous among older calves (Figure 2). Lastly, bacterial communities of M and MC calves had only 26 and 42 OTUs in common between age groups 7 and 63 days, and 28 and 63 days, respectively, thereby confirming the dissimilarities observed in the CAP analysis (Figure 1).

Our taxonomic composition analysis of the bacterial communities revealed a total of 1,125 OTUs (mean 112.46 ± SD 32.91) assigned to 20 phyla, 106 families and 140 genera. A total of 12 phyla, 41 families and 38 genera had relative abundances ≥ 0.1%, and regardless of diet and age group, bacterial communities were dominated by the phyla Firmicutes (57.75 ± 3.44%), Bacteroidetes (28.19 ± 2.84%), Actinobacteria (5.15 ± 1.78%), Proteobacteria (3.94 ± 0.79%), Verrucomicrobia (1.67 ± 1.26%) and

Synergistetes (1.26 ± 0.56%). Abundant families included the Ruminococcaceae (15.15 ± 1.17%), Lachnospiraceae (15.16 ± 2.16%), Prevotellaceae (11.71 ± 2.34%), Bacteroidaceae (6.22 ± 1.97%), Veillonellaceae (3.70 ± 0.79%) and Coriobacteriaceae (2.89 ± 1.61%). Lastly, the genera *Prevotella* (12.85 ± 2.32%), *Ruminococcus* (6.45 ± 1.13%), *Bacteroides* (6.17 ± 1.97%), *Shuttleworthia* (3.02 ± 1.66%), *Lactobacillus* (2.02 ± 1.18%), *Butyrivibrio* (1.95 ± 0.50%) and *Bifidobacterium* (1.39 ± 0.63%) were abundant in our calf ruminal bacterial communities (Supplementary Figure 1; Figure 3).

Our analysis of deviance showed that the relative abundance of several bacterial genera varied according to diet (25 genera), age (27 genera) and the interaction of these factors (18 genera) (Tables 1, 2 and 3). The bacterial genera that were not significantly different between diet, age or interaction are presented in Supplementary Table 4. A total of 8 bacterial genera varied significantly and exclusively according to diet group. The genera *Bifidobacterium*, *Bulleidia* and *Succiniclasticum* (Poisson, $P < 0.001$; 0.001; 0.002, respectively) were more abundant (Tukey HSD, $P < 0.05$) in rumen samples of MC calves than in M calves (Table 1). Further, the relative abundance of 9 genera varied significantly and exclusively according to age group. The abundance of the genera *Pseudoramibacter Eubacterium* and *SHD.231* (Poisson, $P < 0.001$) increased significantly with age (TukeyHSD, $P < 0.05$) but did not reach at least 1% abundance in rumen samples from calves at 63 days old. In contrast, the genera *Bifidobacterium, Butyricimonas* and *Oscillospira* (Poisson, $P < 0.001$; 0.028; 0.001) displayed abundances $\geq 0.5\%$ in the rumen of calves at 7 days of age, but their proportion decreased (Tukey HSD, $P < 0.05$) in calves by 63 days old (Table 2).

The relative abundance of 18 genera varied simultaneously with diet and age (Table 3). This included the *Bacteroides*, *Parabacteroides*, *Lactobacillus* and *Streptococcus* (Poisson, $P < 0.001$), which displayed high abundance in younger M calves (7 and or 28 days old), but their proportion decreased markedly and significantly (Tukey HSD, $P < 0.05$) in older calves (63 days old). In contrast, the abundance of *Parabacteroides*, *Lactobacillus* and *Streptococcus* remained low and unchanged across the developmental stages of MC calves, while the *Bacteroides* increased significantly in MC calves at 63 days of age (Table 3). In addition, the abundance of the genera *Megasphaera*, *Sharpea* and *Succinivibrio* increased significantly (Poisson, $P < 0.001$; Tukey HSD, $P<0.05$) in the rumen of MC calves at 28 days (20 days after starter concentrate intake began) and decreased proportionally as age increased. Finally, the abundance of *Megasphaera* and *Sharpea* were significantly lower (TukeyHSD, $P < 0.05$) across the developmental stages of M calves, compared with MC calves (Table 3).

Fungi

In our fungal alpha-diversity analysis, Chao1 richness, inverse Simpson's diversity and Shannon's diversity did not differ by diet (ANOVA, P = 0.966; 0.966; 0.966), age (P = 0.966; 0.966; 0.966) or interaction of these factors (P = 0.966; 0.966; 0.379; Supplementary Table 4). Our beta-diversity analysis showed that Bray-Curtis dissimilarities in the fungal community were significantly ascribed to diet*age (Permutation test, P = 0.025), whereas variations observed with diet (P = 0.690) and age (P = 0.462) were not significant (Figure 1). The dissimilarities in the fungal communities were more evident among M and MC calves at 49 days old, where diet groups tended to cluster separately (Figure 1). However, this segregation was not observed among calves at 63 days old where community composition was more homogeneous. In addition, our Venn diagram analysis showed that, out of 41 OTUs (at > 0.1% relative abundance in at least one sample), only 3 and 4 of them were shared across all developmental stages of M and MC calves, respectively (Figure 2).

In our taxonomic composition analysis of the fungal community, a total of 84 OTUs (mean 6.86 ± SD2.04) were assigned to the phylum Chytridiomycota (88.759 ± 2.872%), including the family Neocallimastigaceae (88.759 ± 2.872%) and the genera *Caecomyces* (43.349 ± 4.538%), *Neocallimasti*x (7.210 ± 2.546%), *Orpinomyces* (3.062 ± 1.282%) and *Piromyces* (4.430 ± 2.428%). The OTUs assigned to the genera *Anaeromyces* (0.239 ± 0.103%) and *Candida* (0.086% ± 0.086%) were observed in only two samples and excluded from the analyses. The distribution of fungal taxa among individual calves and diet-age groups are shown in Supplementary Figures 1 and 3.

Our analysis of deviance showed that the relative abundances of the genera *Neocallimastix* (P = 0.043) and *Orpinomyces* (P = 0.021) varied simultaneously with diet and age (Table 3). Conversely, the abundance of members from the family Neocallimastigaceae and the genus *Caecomyces*, identified in all animals, did not vary with diet (P = 0.930; 0.603), age (P = 0.184; 0.778) or interaction (P = 0.184; 0.184). In the M calves, the abundance of *Orpinomyces* and *Neocallimastix* increased at 28 and 49 days, respectively, but decreased in older calves (Table 3). In contrast, the abundance of *Orpinomyces* did not change across developmental stages of MC calves, while *Neocallimastix* increased at 28 days old, but decreased in older calves (Tables 1, 2, 3).

Co-occurrence and correlation inter-and intra-microbial communities

The co-occurrence and correlation among the most abundant genera (3 archaea, 15 bacteria and 4 fungi) were assessed in each diet-age group using two approaches: the

Dice index, where co-occurrence among genera ranges from 0 to 1 (indicating no, to moderate, to high co-occurrence), and Spearman's rank correlation, which indicates the strength of association among genera in the range from -1 to 1 (perfect negative to perfect positive correlation). Several correlations were observed in the rumen of calves at 7 days, but the type and extent of these associations varied in response to diet and age (Figures 4 and 5; Supplementary Table 5).

For the archaeal community, the genera *Methanobrevibacter* and *Methanosphera* displayed high co-occurrence (Dice > 0.90) and negative correlation in MC calves at 49 (Spearman= -0.93, $P< 0.01$) and 63 days of age (Spearman= -0.83, $P= 0.041$) (Figures 4 and 5; Supplementary Table 5). Archaeal and bacterial genera showed high co-occurrence (Dice >0.8) in both diet groups, but a negative association was observed among the genera *Methanosphaera* and *Parabacteroides* in MC calves at 28 days (Spearman= -0.87; $P = 0.024$) and 63 days old (Spearman= -0.85; $P= 0.034$) (Figures 4, 5; Supplementary Table 5). The co-occurrence and correlation among archaeal and fungal genera oscillated across the developmental stages of both M and MC calves, making it difficult to identify an association pattern. In M calves, *Methanobrevibacter* and *Methanosphera* were positively (Spearman=0.85; $P=0.034$) and negatively correlated (Spearman=-0.85; $P=0.034$), respectively, with *Orpinomyces* at 28 days of age (Figure 4; Supplementary Table 5). In contrast, *Methanobrevibacter* and *Methanosphera* were positively (Spearman= 0.91; $P=0.005$) and negatively correlated (Spearman=-0.94; $P=0.005$), respectively, with members of the family Neocallimastigacea in the rumen of MC calves at 63 days of age (Figures 5; Supplementary Table 5).

In the bacterial community, several significant correlations were identified, but few showed a stable pattern across developmental stage. For example, the genera *Bulleidia* and *Oscillospira* displayed high co-occurrence (Dice ≥ 0.75), positive and significant correlations (Spearman ≥ 0.79, $P< 0.05$) in M calves at 28, 49 and 63 days old. Both *Bulleidia* (Dice= 0.75, 0.86; Spearman= 0.85, 0.83; $P= 0.016$, 0.041) and *Oscillospira* (Dice= 0.89, 0.89; Spearman= 0.86, 0.87; $P= 0.013$, 0.024) also displayed a positive association with the genus *Succiniclasticum* in M calves at 49 and 63 days old. Lastly, the abundance of the genus *Succiniclasticum* was positively correlated to *Succinivibrio* in M calves at 28 (Dice= 0.8; Spearman=0.83; $P=0.042$) and 49 days old (Dice = 0.67; Spearman=0.83; $P=0.020$). Similar association patterns were not observed across developmental stages for MC calves (Figures 4 and 5; Supplementary Table 5).

In the fungal communities, only members of the *Caecomyces* and Neocallimastigacea displayed moderate and high co-occurrence (Dice = 0.50 to 0.85) across developmental stages of M and MC calves, but significant correlations were not observed in any diet-age groups. For associations between fungal and bacterial genera, a negative association of the genera *Streptococcus* with both *Caecomyces* (Dice= 0.80; Spearman= -0.83; *P*= 0.042) and *Neocallimastix* (Dice= 0.50; Spearman= -0.93; *P*= 0.008) was observed in the rumen of neonate calves. Both *Caecomyces* and *Neocallimastix* also displayed a positive association with the genera *Acidaminococcus* (Dice= 0.57; Spearman=0.85, *P*=0.034) and *Megasphaera* (Dice= 0.80; Spearman= 0.82, *P*= 0.045) in M calves at 7 days of age (Figure 4; Supplementary Table 5). High co-occurrence and positive correlation between *Caecomyces* and *Prevotella* in the rumen of MC calves at 63 days of age (Dice = 1.00; Spearman= 0.89; *P*= 0.020) were observed. In contrast, inverse associations among *Caecomyces* and *Prevotella* (Dice=1.00; Spearman= -0.83; *P*= 0.042) were observed in M calves at 63 days old (Figures 4 and 5; Supplementary Table 5).

Correlation among VFA and microbial taxa

VFA concentrations (acetate, propionate, butyrate, total VFA) were higher in MC calves, relative to M calves, and increased significantly at 63 days of age (Supplementary Table 6). Several correlations between molar proportions of VFAs and archaeal, bacterial or fungal taxa were identified, but few were stable across developmental stages in either M or MC calves (Figure 6; Supplementary Table 7).

In MC calves, the bacterial genera *Bacteroides* and *Lactobacillus* were positively correlated with acetate molar proportions (Spearman = 0.88, 0.84; *P* = 0.004, 0.009) in calves at 49 days old, as well as to the acetate-to-propionate ratio in calves at 49 (Spearman = 0.97, 0.98; *P* < 0.001, < 0.001) and 63 days old (Spearman = 0.89, 0.88; *P* = 0.019, 0.020). The butyrate proportion was strongly correlated with the abundance of the family Paraprevotellaceae (Spearman = 0.87, *P* = 0.020) and the genus *Succinivibrio* (Spearman = 0.83, *P* = 0.040) in calves at 63 days old. The increase of propionate proportion was positively associated to members of the family Lachnospiraceae (Spearman = 0.83, *P* = 0.040), as well as the genera *Bulleidia* (Spearman = 0.84, *P*=0.040), *Caecomyces* (Spearman = 0.91, *P* = 0.010) and *Methanosphaera* (Spearman=0.92, *P*=0.010). In contrast, several members from genera *Bacteroides* (Spearman=-0.76, *P*=0.030), *Lactobacillus* (Spearman=-0.75, *P*=0.030), and *Methanobrevibacter* (Spearman=-0.83, *P*=0.040) as well as the families

Ruminococcaceae (Spearman= -0.82, *P*= 0.050) and Neocallimastigaceae (Spearman= -0.86, *P*=0.030) were negatively correlated to propionate proportion in the rumen of calves at 49 and 63 days of age (Figure 6; Supplementary Table 7).

For M calves, the genera *Streptococcus* and *Lactobacillus* were positively correlated with total VFAs (Spearman = 0.86, 0.94; *P* = 0.030, < 0.001) and acetate-to-propionate ratio (Spearman = 0.89, 0.79; *P* = 0.010, 0.030) in calves at 7 and 49 days of age. The acetate proportion was negatively correlated with the abundance of the genera *Butyrivibrio, Bulleidia, Pseudoramibacter, Eubacterium* and *Shuttleworthia,* which in turn were positively correlated to butyrate proportion in calves at 28 days old. Further, the genera *Butyrivibrio* was positively correlated (Spearman= 0.90, 0.81; *P*= 0.01, 0.03) to butyrate in calves at 28 and 49 days old. Similar to MC calves at 63 days old, the abundance of *Succinivibrio* was negatively correlated with acetate proportion (Spearman= -0.77, *P*= 0.04), but positively associated with butyrate proportion (Spearman= 0.89, *P*= 0.01) in the M calves at 49 days of age. In contrast to MC calves, the family Neocallimastigaceae was positively correlated to propionate proportion (Spearman= 0.84, *P*= 0.04) in M calves at 49 days (Figure 6; Supplementary Table 7).

Discussion

Here, we characterize archaeal, bacterial and fungal communities in the rumen of dairy calves fed two diets (M and MC) across four developmental stages (7, 28, 49 and 63 days old) in the pre-weaning period. In addition, we investigate the impact of the starter concentrate inclusion on the composition and abundance of these communities and determine the cross-domain associative patterns.

The results of the present study reveal that the main microbial groups commonly found in the mature rumen were already established in the rumen of dairy calves at one week of age. These included methanogenic archaea responsible for methane production (*Methanobrevibacter, Methanosphera*), bacteria that occupy different metabolic niches (*Bacteroides*, *Butyrivibrio*, *Lactobacillus*, *Megasphaera*, *Prevotella*, *Ruminococcus* and *Streptococcus*) and anaerobic fungi with a prominent role in degradation of fibre (*Caecomyces* and Neocallimastigaceae). In line with previous reports (Bryant et al., 1958; Fonty et al., 1987; Rey et al., 2014; Guzman et al., 2016), these results shows that colonization of archaeal, bacterial and fungal communities occurs prior to solid feed arrival and reinforces the microbiota-related interventions should be explored right after birth (Abecia et al., 2013).

Overall, the process by which the microbiota colonizes the rumen has not been fully elucidated. Studies performed with culture-based techniques suggested that microbiota colonization may be sequential because bacteria is the first microbial group found in the neonatal rumen (1-2 days old) followed by methanogenic archaea (calves 3-14 days of age), anaerobic fungi (lambs 8-10 days of age) and protozoa (calves >56 days of age) (Fonty et al., 1987; Anderson et al., 1987; Minato et al., 1992). However, studies performed with molecular techniques identified archaea and bacteria in rumen samples of calves and lambs 20 minutes, 17 and 24 hours after birth (Skillman et al., 2004; Gagen et al., 2012; Guzman et al., 2015) suggesting that colonization of these communities may occur before calving.

The main substrate source for rumen microorganisms in calves prior to solid feed intake is liquid feed (*i.e.* colostrum, milk or milk replacer) that fails to flow directly to the abomasum through the oesophageal groove (Wise and Anderson, 1939, Ørskov, 1972). Generally, little milk enters the rumen due to failures in the oesophageal groove reflex; however, the occurrence is more frequent in bucket vs. bottle-fed calves (Wise et al., 1942; Labussière et al., 2014).

Indeed, this feeding system was adopted in our study, and we identified VFAs in ruminal samples from 7-day-old calves fed exclusively colostrum and milk (Figure 6). These factors support the establishment of some bacteria (*i.e. Bacteroides, Lactobacillus, Prevotella, Ruminococcus, and Streptococcus*) and fungi *(i.e. Neocallimastix and Caecomyces*) capable of utilizing milk nutrients, such as lactose, as a carbon and energy source (Russell and Baldwin, 1978; Marounek et al., 1987; Phillips and Gordon, 1995; Rainey, 2009).

Further, the presence of members from genus *Methanobrevibacter* in the rumen of M calves may be favoured by lactose fermentation performed by bacteria and fungi species which releases among others, great amounts of hydrogen (H_2) and carbon dioxide (CO_2) that are substrates for methanogenesis (Bauchop and Mountfort, 1981; Wolin and Miller, 1983). However, this assumption fails to explain the presence of members from the genus *Methanosphera* whose species (*i.e. Methanosphaera stadtmanae*) obtain energy for growth exclusively by reduction of methanol, which can be produced in the rumen by hydrolysis of pectin, a carbohydrate absent in milk (Miller and Wolin, 1985; Pol and Demeyer, 1987; Fricke et al., 2006). Therefore, we speculate the existence of alternatives pathways of ruminal methanol production (Dorokhov et al., 2015) or that *Methanosphaera sp.* may utilize other substrates to produce methane and support its energy metabolism in the rumen of M calves.

Likewise, the spillage of milk into the rumen may not be directly related to the presence of members from genus *Succiniclasticum* whose specie (*i.e. Succiniclasticum ruminis*) convert succinate to propionate as the sole energy-yielding mechanism (Van Gylswyk, 1995). We suggest that establishment of *Succiniclasticum* may be supported by *Bulleidia* and *Succinivibrio* (succinate-producers) given the positive and strong association among these genera across the developmental stages of M calves (Figure 4). Interestingly, similar association among *Succiniclasticum* and *Succinivibrio* was not observed at rumen of MC-fed calves (Figure 5) probably due greater availability of succinate resulting from starch fermentation by others succinate-producers, suggesting the existence of opportunistic association in response to diet. In this sense, it is important to note that the relationship among leakage of milk into the rumen and establishment of microbiota remains to be explored and that correlations determined at genus-levels only provide clues to further studies designed, exclusively, to validate species-interactions in the developing rumen.

While the establishment of the microbiota occurs regardless of solid feed intake, diet was a strong determinant of abundance of bacterial and archaeal taxa in the rumen of calves. In the bacterial community, beginning of starter concentrate intake promoted an increase in the abundance of taxa related to degradation of readily fermentable carbohydrates (*i.e. Succinivibrio, Sharpea, Megasphaera*) in the rumen of MC calves at 28 days of age, but these shifts did not persist with increase of age. In contrast, we observed that bacterial community in the rumen of M calves was dominated by taxa from genera *Lactobacillus*, *Bacteroides* and *Parabacteroides* whose abundance decreased with age but remained highest compared to MC calves (Table 3). This result suggests that diet is the main factor that affected bacterial community in short time by the "selection" of taxa adapted to new substrates, however, the persistence of responses diet-related depends on other factors naturally encompassed in the age effect, such as, increase of solid feed intake, ruminal metabolic development and microbial-associations.

Consistent with our results, the genus *Succinivibrio* was identified in low proportion in the rumen of calves 2 days old and its abundance increased and subsequent decreased after the provision of solid feed (Rey et al., 2014) and post-weaning (Meale et al., 2016). The main *Succinivibrio* (*Succinivibrio dextrinosolvens*) found in the rumen ferments starch hydrolysis products (maltose, dextrin, glucose) and produces mainly succinate, acetate and formate (Bryant and Small, 1956) and

apparently has a negative association with ruminal pH (Rey et al., 2014) and acetate molar proportion (Figure 6).

Another specialist genus whose abundance increased in response to beginning of starter was *Megasphaera*. In similar study, the abundance of this genus was very low at rumen of neonate calves and displayed tendency to increase with age or solid feed provision (Jami et al., 2013). In contrast, *Megasphaera* was identified at low proportion (0.4-0.6%) in the rumen of calves pre- and post-weaning (Meale et al., 2016) and its presence was not reported in studies performed with calves fed with milk replacer (Li et al., 2011; Wu et al., 2012) or milk plus starter concentrate and hay (Rey et al., 2014; Malmuthuge et al., 2014). Taken together, these results suggest that abundance of *Megasphaera* in the developing rumen is low and dietary strategies that promote its colonization may be interesting because *Megasphaera elsdenii* is an efficient lactate-utilising bacterium whose activity results in butyrate production that stimulates the development and differentiation of rumen epithelial cells (Counotte et al., 1981; Malhi et al., 2013). Moreover, the activity of *Megasphaera elsdenii* may prevent the accumulation of lactic acid from lactate-producers and minimize ruminal acidosis risk in calves during adaptation to starter concentrate intake.

In this sense, during the transition from a milk-based to solid-based diet, calves often experience subacute ruminal acidosis (pH < 5.8 for extended periods) due to increased lactate and VFA concentrations within rumen (Anderson et al., 1987; Quigley et al., 1992; Kim et al., 2016). In the adult ruminant, the incidence of ruminal acidosis is closely related to proliferation of lactate-producing species from genera *Streptococcus* and *Lactobacillus* (Russell and Hino, 1985; Petri et al., 2013; Wang et al., 2015). In calves, this association remains to be explored, because despite the amylolytic and saccharolytic capabilities of *Streptococcus* and *Lactobacillus*, starter concentrate did not promote their colonization in the rumen of MC calves here (Table 3) or in other studies (Rey et al., 2014; Meale et al., 2016; Kim et al., 2016). These results suggest that *Streptococcus* and *Lactobacillus* are not directly responsive to the availability of readily fermentable carbohydrates in the calf rumen.

Other lactate-producing organisms like *Sharpea* may be more relevant in the developing rumen and linked to incidence of pre-weaning acidosis. *Sharpea* species produce mainly the D-lactate (Morita et al., 2008) isomer of lactic acid, which greatly decreases (<5.5) ruminal pH (Anderson et al., 1987) and when absorbed, is poorly metabolized causing metabolic acidosis in calves (Ewaschuk et al., 2004). Indeed, the genus *Sharpea* was identified in very low proportions in the rumen of 7-day-old calves

here and the beginning of starter concentrate intake promoted an increase in its abundance in MC calves at 28 days of age. However, as animals continued to age, *Sharpea* decreased in MC calves but remained higher compared to M calves (Table 3).

This is similar to other studies where *Sharpea* was identified in high proportions (6%) in the rumen of 3-week-old calves fed with whole milk and concentrate (Malmuthuge et al., 2014), and its abundance increased considerably (1.48 to 7.67%) post-weaning (Meale et al., 2016). Therefore, the *Sharpea* community is likely more responsive to the availability of readily fermentable carbohydrates in the calf rumen and perhaps, competes in this niche more efficiently than other lactate-producers such as *Streptococcus* and *Lactobacillus*. However, further studies are needed to characterize *Sharpea spp.* isolated from the calf GIT and to assess its relationship with ruminal acidosis in calves.

Interestingly, the genera *Prevotella*, *Ruminococcus* and *Butyrivibrio* were identified in high proportion (>5%) across development stages of M and MC calves, and their abundance did not change significantly in response to diet or age, corroborating previous reports (Li et al., 2011; Rey et al., 2014; Meale et al., 2016; Kim et al., 2016; Wang et al., 2016). A recent study reported that *Prevotella*, *Butyrivibrio* and *Ruminococcus* are part of the rumen microbiome from a wide range of ruminant species, diets and geographical locations (Henderson et al., 2015), and our results suggest that dominance of these communities begin in the pre-weaning phase.

Despite of inter-individual variability that is a hallmark of calves microbiome studies (Li et al., 2011; Wu et al., 2012; Rey et al., 2014) this lack of significance is not surprising because the genera *Prevotella* and *Butyrivibrio* includes species endowed with great nutritional versatility that allow them to fill different metabolic niches (Hazlewood et al., 1979; Cotta and Hespel, 1986; Purushe et al., 2010) and "justifies" their abundance in the rumen of calves fed with different diets. Likewise, despite of recognized cellulolytic capability, *Ruminococcus spp*. isolated from calf rumen possess alternative pathways beyond of cellulose degradation (i.e. ferment lactose, glucose, sucrose and maltose) (Bryant et al., 1958; Rainey, 2009) to support their growth at absence of dietary fibre (Jami et al., 2013; Rey et al., 2014; Guzman et al., 2016).

In regards to archaeal community, the abundance of methanogens was not influenced by age but responsive to inclusion of concentrate in the calf´s diet. Previous works reported that *Methanobrevibacter* was dominant at rumen of calves fed with milk plus starter concentrate (Zhou et al., 2014; Rey et al., 2012) while *Methanosphaera*

increased markedly after the beginning of solid feed intake (Rey et al., 2012), suggesting a strong diet effect.

Here, we observed higher relative abundances of *Methanosphaera* in the rumens of MC calves. This is likely directly attributed to diet composition, as pectin was present in the main ingredients (Malathi and Devegowda, 2001) of the starter concentrate used in our study (Supplementary Table 1). Thus, we posit that the methanol produced through the fermentation of pectin is made available to *Methanosphaera* sp. A4 as well as methanol-utilizing bacteria such as *Eubacterium*, whose abundance was also significantly higher in the rumen of MC calves (Table 1).

In addition, bacterial changes resulting from the inclusion of concentrate intake (*i.e.* increase in the abundance of amylolytic populations) can also lead to decreases in the acetate-to-propionate ratio and hydrogen availability within the rumen (Johnson and Johnson, 1995; Martin et al., 2010), which in turn affects the archaeal community (Table 1). These dynamics may reflect changes in the archaeal community by favoring *Methanosphaera* sp. that require only 1 mol of H_2 to produce 1 mol of CH_4, as opposed to *Methanobrevibacter* species that require 4 mols of H_2 in order to produce 1 mol of CH_4 (Miller and Wolin, 1985; Fricke et al., 2006; Sun et al., 2015). Moreover, the abundance of *Methanobrevibacter* in our study was negatively correlated with *Methanosphaera* across developmental stages of M and MC calves (Figures 3 and 4), possibly due to competition for H_2 within the rumen (Miller and Wolin, 1985; Janssen and Kirs, 2008).

In contrast to archaeal and bacterial communities, diet and age did not affect the rumen fungal communities in our study. Members from genus *Caecomyces* and family Neocallimastigaceae were identified in all calves at 7 days old, remained abundant, and were irresponsive to inclusion of starter concentrate. In addition, we observed that members from genus *Piromyces* (identified < 50% of samples), *Neocallimastix* and *Orpinomyces* (~55% of all samples) did not establish well in the developing rumen and were not identified as part of the core of M or MC calves.

This is comparable to calves raised on milk plus starter pellets, where rumen fungi were highly variable pre-weaning and not dramatically impacted by diet (milk-replacer plus calf starter) until the weaning transition (Dill-McFarland et al., 2017). In contrast to these results, Fonty et al. (1987) identified *Neocallimastix frontalis* and *Caecomyces communis* (previously aka *Sphaeromonas communis sensu Orpin*) in the rumen of lambs 8-10 days old, however, these species disappeared in almost all the lambs after the concentrate and hay was offered.

Although the fungal genera identified in our study (*Neocallimastix*, *Piromyces* and *Orpinomyces*) and previous reports (Fonty et al., 1987; Dill-McFarland et al., 2017) include species able to degrade several carbohydrates, such as starch and products of its breakdown, the extent to which this occurs depends on the fungal strains and carbohydrate source (McAllister et al., 1993; Phillips and Gordon, 1995). Moreover, starch fermentation constitutes a sought-after niche in the rumen and the slow growth rate of fungi species constitutes a limiting factor for competition with bacteria (McAllister et al., 1993). Lastly, the rapid release of acetate, formate and lactate from starch fermentation causes a drop in ruminal pH that may inhibit fungal growth (Srinivasan et al., 2001). These factors have been ascribed to decrease in the rumen fungi community of adult ruminants fed with grain-rich diets in comparison to animals fed forage-based diets (Denman et al., 2008; Boots et al., 2013; Kumar et al., 2015). Together, these results suggest that rumen fungal communities are not fully established until regular or substantial dietary fiber intake, which occurs from weaning period. Further researches are needed to assess the impact of others pre-weaning diets (i.e. fiber-rich concentrates with or without hay) on the fungal colonization in the calf rumen.

Our results provide new insights into colonization and associations within the microbiota in the developing rumen. In summary, archaeal, bacterial and fungal communities co-occur in the rumen since early calf development but are impacted differently by pre-weaning diet and age. We observed that inclusion of starter concentrate significantly affected rumen bacterial communities by promoting increases in bacteria capable of degrading readily fermentable carbohydrates and depressing those reliant on milk components like lactose. These bacterial changes resulted in apparent diet-driven archaeal differences due to altered fermentation patterns and availability of hydrogen in the rumen. No such differences were found for fungi, likely due to high inter-animal variation and low fibre content of concentrate used. These results indicate that pre-weaning manipulation of the rumen microbiota is possible through dietary intervention and may be useful in designing strategies to promote colonization of target communities linked to functional development of the calf.

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TABLE 1| Diet effect on the relative abundance of archaeal, bacterial and fungal taxa in the rumen of the dairy calves.

		Diet[1]			
	Taxa	**M**	**MC**	***P*-value**	**FDR[2]**
Archaea	*Methanobrevibacter*	71.803±8.031[a]	39.153±8.971[b]	0.009	0.039
	Methanosphaera	23.909±6.847[b]	53.020±9.710[a]	0.005	0.039
	vadinCA11	4.289±1.687	7.828±3.299	0.259	0.466
Bacteria	*Acidaminococcus*	0.533±0.316[b]	1.130±0.695[a]	<.001	<.001
	Bacteroides	10.015±3.346[a]	1.369±0.645[b]	<.001	<.001
	*Bifidobacterium***	0.239±0.153[b]	2.828±1.352[a]	<.001	<.001
	Blautia	1.026±0.488[a]	0.478±0.242[b]	0.031	0.050
	*Bulleidia***	0.303±0.162[b]	2.161±0.434[a]	<.001	0.001
	Campylobacter	0.226±0.115[a]	0.146±0.059[b]	<.001	<.001
	CF231	0.439±0.267[a]	0.040±0.020[b]	<.001	<.001
	Corynebacterium	0.935±0.590[a]	0.227±0.166[b]	<.001	<.001
	*Eubacterium***	0.011±0.108[b]	0.098±0.044[a]	<.001	<.001
	*Faecalibacterium***	0.043±0.040[b]	0.207±0.184[a]	<.001	<.001
	Lactobacillus	3.351±2.103[a]	0.364±0.206[b]	<.001	<.001
	Megasphaera	0.052±0.026[b]	1.109±0.482[a]	<.001	<.001
	Odoribacter	0.361±0.156[a]	0.036±0.013[b]	<.001	<.001
	Oribacterium	0.107±0.091[a]	0.028±0.017[b]	<.001	<.001
	p.75.a5	0.392±0.215[a]	0.020±0.014[b]	<.001	<.001
	Parabacteroides	1.208±0.591[a]	0.175±0.085[b]	<.001	<.001
	*Pseudoramibacter Eubacterium***	0.023±0.007[b]	0.239±0.076[a]	<.001	<.001
	Sharpea	0.109±0.050[b]	0.601±0.263[a]	<.001	<.001
	*SHD.231***	0.306±0.211[a]	0.000±0.000[b]	<.001	<.001
	Shuttleworthia	1.346±0.560[b]	5.100±3.673[a]	<.001	<.001
	Streptococcus	0.984±0.516[a]	0.053±0.021[b]	<.001	<.001
	*Succiniclasticum***	0.463±0.253[b]	2.370±0.644[a]	0.001	0.002
	Succinivibrio	0.461±0.312[b]	1.357±0.765[a]	<.001	<.001
	Synergistes	0.091±0.079[a]	0.058±0.022[a]	0.009	0.016
	*YRC22***	0.034±0.019[b]	0.093±0.039[a]	0.014	0.024
Fungi	*Caecomyces*	38.616±5.768	48.792±7.118	0.452	0.603
	Neocallimastix	6.159±2.836	8.418±4.467	0.903	0.930
	Orpinomyces	3.760±2.296[a]	2.259±0.854[b]	<.001	<.001
	f_Neocallimastigaceae	32.711±5.195	27.891±7.045	0.930	0.930

The values represent relative abundance average percentage and standard deviation.[1]Calves fed with whole milk (M) or with whole milk plus starter concentrate *ad libitum* (MC); [2]*P*-value adjusted by FDR method; FDR≤ 0.05 were considered significant; [**]Genera that varied regardless of diet-age interaction.

TABLE 2| Age effect on the relative abundance of archaeal, bacterial and fungal taxa in the rumen of the dairy calves.

	Taxa	Age				*P*-value	FDR[1]
		7d	28d	49d	63d		
Archaea	*Methanobrevibacter*	33.010±31.088	62.338±11.301	55.224±11.305	57.271±12.295	0.741	0.833
	Methanosphaera	59.531±29.950	30.846±10.534	38.358±11.440	38.522±11.297	0.739	0.833
	vadinCA11	7.459±2.581	6.815±2.814	6.418±4.120	4.207±2.575	0.926	0.926
Bacteria	*Acidaminococcus*	0.354±0.322^{b}	0.937±0.636^{a}	1.183±0.932^{a}	0.399±0.151^{b}	<.001	<.001
	Bacteroides	12.281±4.185^{a}	11.681±6.545^{b}	3.138±1.355^{c}	1.403±1.039^{d}	<.001	<.001
	*Bifidobacterium***	0.745±0.574^{b}	4.245±2.152^{a}	0.388±0.347^{bc}	0.108±0.093^{c}	<.001	<.001
	Blautia	2.930±1.858^{a}	0.257±0.076^{c}	0.673±0.325^{b}	0.372±0.255^{c}	<.001	<.001
	*Butyricimonas***	0.508±0.233^{a}	0.146±0.096^{b}	0.080±0.067^{b}	0.044±0.036^{b}	0.017	0.028
	Campylobacter	0.006±0.006^{c}	0.341±0.236^{a}	0.175±0.069^{b}	0.152±0.058^{bc}	<.001	<.001
	CF231	0.039±0.028^{b}	0.042±0.025^{b}	0.205±0.105^{b}	0.663±0.546^{a}	<.001	<.001
	Corynebacterium	3.576±2.245^{a}	0.223±0.148^{b}	0.029±0.018^{b}	0.278±0.278^{b}	<.001	<.001
	*Eubacterium***	0.485±0.445^{a}	0.030±0.017^{b}	0.069±0.057^{b}	0.064±0.025^{b}	<.001	<.001
	*Faecalibacterium***	0.000±0.000^{b}	0.017±0.006^{b}	0.321±0.251^{a}	0.017±0.014^{b}	<.001	<.001
	Lactobacillus	0.631±0.305^{c}	2.377±1.853^{b}	3.712±3.249^{a}	0.256±0.179^{c}	<.001	<.001
	Megasphaera	0.039±0.025^{c}	1.309±0.781^{a}	0.248±0.172^{bc}	0.318±0.175^{b}	<.001	<.001
	Odoribacter	0.259±0.149^{b}	0.455±0.303^{a}	0.161±0.093^{bc}	0.028±0.013^{c}	<.001	<.001
	Oribacterium	0.000±0.000^{b}	0.008±0.006^{b}	0.058±0.025^{b}	0.189±0.189^{a}	<.001	<.001
	*Oscillospira***	2.009±0.822^{a}	0.484±0.109^{b}	0.242±0.095^{c}	0.452±0.216^{b}	0.001	0.001
	p.75.a5	0.061±0.054^{c}	0.474±0.329^{a}	0.272±0.253^{b}	0.006±0.006^{c}	<.001	<.001
	Parabacteroides	2.670±2.344^{a}	0.698±0.407^{b}	0.470±0.193^{b}	0.190±0.140^{c}	<.001	<.001
	*Phascolarctobacterium***	0.503±0.218^{a}	0.039±0.013^{b}	0.060±0.032^{b}	0.085±0.085^{b}	0.014	0.024
	Porphyromonas	3.274±1.271^{a}	0.655±0.515^{b}	0.020±0.012^{c}	0.885±0.882^{b}	0.001	0.002
	*Pseudoramibacter***	0.022±0.011^{b}	0.050±0.018^{b}	0.166±0.091^{a}	0.177±0.076^{a}	<.001	<.001
	Sharpea	0.006±0.006^{c}	0.572±0.383^{a}	0.251±0.160^{b}	0.340±0.178^{b}	<.001	<.001
	*SHD.231***	0.000±0.000^{b}	0.000±0.000^{b}	0.302±0.293^{a}	0.261±0.258^{a}	<.001	<.001
	Shuttleworthia	1.905±1.905^{b}	0.549±0.383^{d}	6.916±4.859^{a}	1.157±0.497^{c}	<.001	<.001
	Streptococcus	3.208±1.943^{a}	0.111±0.032^{b}	0.276±0.186^{b}	0.077±0.033^{b}	<.001	<.001
	Succinivibrio	0.006±0.006^{c}	2.094±1.326^{a}	0.450±0.266^{b}	0.563±0.351^{b}	<.001	<.001
	Synergistes	0.039±0.028^{b}	0.214±0.163^{a}	0.018±0.011^{b}	0.030±0.019^{b}	0.025	0.041
	*Treponema***	0.000±0.000^{b}	0.473±0.237^{a}	0.055±0.032^{b}	0.119±0.083^{b}	<.001	<.001
Fungi	*Caecomyces*	28.986±8.639	48.086±8.881	45.087±8.548	42.82±8.857	0.649	0.778
	Neocallimastix	0.943±0.517	12.736±7.900	8.224±4.215	3.488±1.719	0.125	0.187
	Orpinomyces	0.187±0.187^{b}	6.414±4.625^{a}	2.398±1.054^{b}	2.016±1.222^{b}	<.001	<.001
	f_Neocallimastigaceae	46.132±9.293	15.388±4.806	33.942±8.432	33.425±8.50	0.107	0.184

The values represent relative abundance average percentage and standard deviation.[1]*P*-value adjusted by FDR method; FDR≤ 0.05 were considered significant;**Genera that varied regardless of diet-age interaction. Means between diet groups followed by the same letter are not significantly different ($P > 0.05$) by Tukey HSD test.

TABLE 3| Diet-age interaction effect on the relative abundance of archaeal, bacterial and fungal taxa in the rumen of the dairy calves.

		Diet*Age[1]								
	Taxa	**M_07d**	**M_28d**	**M_49d**	**M_63d**	**MC_28d**	**MC_49d**	**MC_63d**	***P*-value**	**FDR[2]**
Archaea	*Methanobrevibacter*	33.010±31.088	63.754±16.569	85.887±9.396	82.816±12.953	60.923±16.93	28.394±13.882	31.726±15.373	0.187	0.420
	Methanosphaera	59.531±29.953	31.715±13.473	11.875±7.965	12.33±8.516	29.977±17.506	61.53±16.697	64.714±14.651	0.130	0.390
	vadinCA11	7.459±2.581	4.531±4.155	2.238±1.493	4.854±4.477	9.100±3.936	10.075±7.609	3.560±2.994	0.583	0.833
Bacteria	*Acidaminococcus*	0.354±0.322^{c}	1.286±1.286^{b}	0.096±0.069^{c}	0.467±0.257^{c}	0.588±0.281^{c}	2.135±1.726^{a}	0.332±0.181^{c}	<.001	<.001
	Bacteroides	12.281±4.185^{b}	22.652±11.842^{a}	5.302±2.709^{c}	0.611±0.334^{e}	0.710±0.358^{e}	1.244±0.528^{e}	2.194±2.095^{d}	<.001	<.001
	Blautia	2.930±1.858^{a}	0.409±0.110^{bc}	0.718±0.460^{b}	0.100±0.043^{c}	0.105±0.062^{c}	0.634±0.487^{b}	0.644±0.506^{b}	<.001	<.001
	Campylobacter	0.006±0.006^{b}	0.610±0.464^{a}	0.176±0.055^{b}	0.122±0.038^{b}	0.072±0.026^{b}	0.174±0.124^{b}	0.182±0.115^{b}	<.001	<.001
	CF231	0.039±0.028^{c}	0.017±0.011^{c}	0.396±0.207^{b}	1.310±1.071^{a}	0.067±0.050^{c}	0.037±0.033^{c}	0.017±0.011^{c}	<.001	<.001
	Corynebacterium	3.576±2.245^{a}	0.308±0.294^{bc}	0.010±0.010^{c}	0.000±0.000^{c}	0.139±0.084^{bc}	0.046±0.033^{c}	0.555±0.555^{b}	<.001	<.001
	Lactobacillus	0.631±0.305^{c}	4.709±3.595^{b}	7.301±6.963^{a}	0.106±0.034^{c}	0.044±0.038^{c}	0.572±0.448^{c}	0.407±0.361^{c}	<.001	<.001
	Megasphaera	0.039±0.025^{d}	0.011±0.011^{d}	0.048±0.032^{d}	0.110±0.104^{cd}	2.606±1.418^{a}	0.424±0.317^{bc}	0.525±0.327^{b}	<.001	<.001
	Odoribacter	0.259±0.149^{b}	0.871±0.579^{a}	0.297±0.193^{b}	0.028±0.016^{c}	0.039±0.026^{c}	0.041±0.024^{c}	0.027±0.022^{c}	0.001	0.002
	Oribacterium	0.000±0.000^{b}	0.006±0.006^{b}	0.053±0.031^{b}	0.378±0.378^{a}	0.011±0.011^{b}	0.062±0.041^{b}	0.000±0.000^{b}	<.001	<.001
	p.75.a5	0.061±0.054^{c}	0.892±0.637^{a}	0.574±0.540^{b}	0.011±0.011^{c}	0.056±0.044^{c}	0.008±0.008^{c}	0.000±0.000^{c}	0.007	0.013
	Parabacteroides	2.670±2.344^{a}	1.280±0.768^{b}	0.878±0.364^{b}	0.061±0.023^{c}	0.116±0.067^{c}	0.112±0.036^{c}	0.319±0.281^{c}	<.001	<.001
	Porphyromonas	3.274±1.271^{a}	1.304±0.999^{c}	0.019±0.010^{d}	0.006±0.006^{d}	0.006±0.006^{d}	0.021±0.021^{d}	1.765±1.765^{b}	0.014	0.024
	Sharpea	0.006±0.006^{d}	0.116±0.069^{cd}	0.182±0.137^{cd}	0.121±0.121^{cd}	1.027±0.748^{a}	0.312±0.284^{bc}	0.559±0.325^{b}	<.001	<.001
	Shuttleworthia	1.905±1.905^{b}	0.954±0.748^{bc}	1.427±1.042^{b}	1.084±0.670^{bc}	0.145±0.138^{c}	11.719±8.982^{a}	1.230±0.797^{b}	<.001	<.001
	Streptococcus	3.208±1.943^{a}	0.149±0.056^{c}	0.578±0.380^{b}	0.067±0.029^{c}	0.072±0.028^{c}	0.012±0.009^{c}	0.088±0.064^{c}	<.001	<.001
	Succinivibrio	0.006±0.006^{d}	1.131±1.124^{b}	0.010±0.006^{d}	0.772±0.694^{bc}	3.058±2.470^{a}	0.835±0.469^{bc}	0.354±0.208^{cd}	<.001	<.001
	Synergistes	0.039±0.028^{b}	0.335±0.328^{a}	0.005±0.005^{b}	0.000±0.000^{b}	0.094±0.061^{b}	0.029±0.019^{b}	0.061±0.036^{b}	<.001	<.001
Fungi	*Caecomyces*	28.986±8.639	44.877±16.81	53.603±9.338	23.94±9.139	50.761±9.881	37.635±13.843	61.7±10.911	0.081	0.184
	Neocallimastix	0.943±0.517^{c}	1.705±0.760^{c}	13.848±8.571^{b}	5.247±3.231^{c}	21.928±13.831^{a}	3.303±2.033^{c}	1.730±1.153^{c}	0.014	0.043
	Orpinomyces	0.187±0.187^{b}	10.566±10.332^{a}	2.293±0.988^{b}	2.778±2.415^{b}	2.954±1.417^{b}	2.491±1.853^{b}	1.254±0.710^{b}	0.005	0.021
	f_Neocallimastigaceae	46.132±9.293	12.279±5.383	25.056±7.113	47.483±12.273	17.98±7.900	41.717±14.498	19.368±9.426	0.093	0.184

The values represent relative abundance average percentage and standard deviation. [1]Calves fed with only whole milk (M) or whole milk and starter concentrate (MC) that were slaughtered at 7, 28, 49 and 63 days old; [2]*P*-value adjusted by FDR method; FDR≤ 0.05 were considered significant;Means between diet and age groups followed by the same letter are not significantly different ($P > 0.05$) by Tukey HSD test.

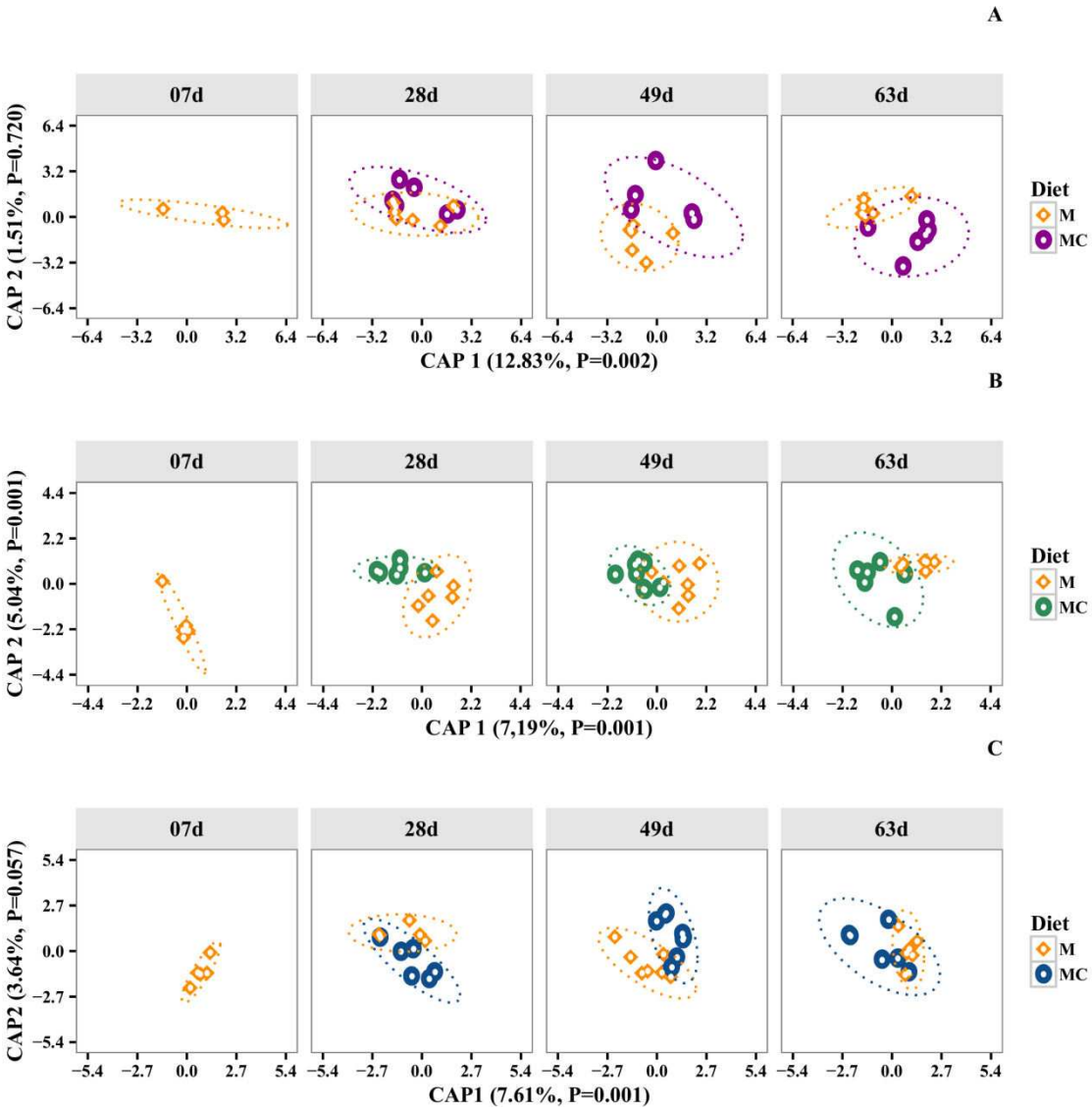

FIGURE 1| Canonical Analysis of Principal Coordinates (CAP) of the Bray-Curtis dissimilarity metric for archaeal (A), bacterial (B) and fungal (C) communities in the rumen of dairy calves. On the plots each point represents a rumen sample, different shapes represents the diet (M: only whole milk and MC: whole milk and starter concentrate) and each facet represents the age group (7, 28, 49 and 63 days old). Percentages and *P*-values showed along the axes represent, respectively, the proportion of dissimilarities captured by CAP and significance by Permutation test. The increasing distance between samples equates to more dissimilarity in the community composition.

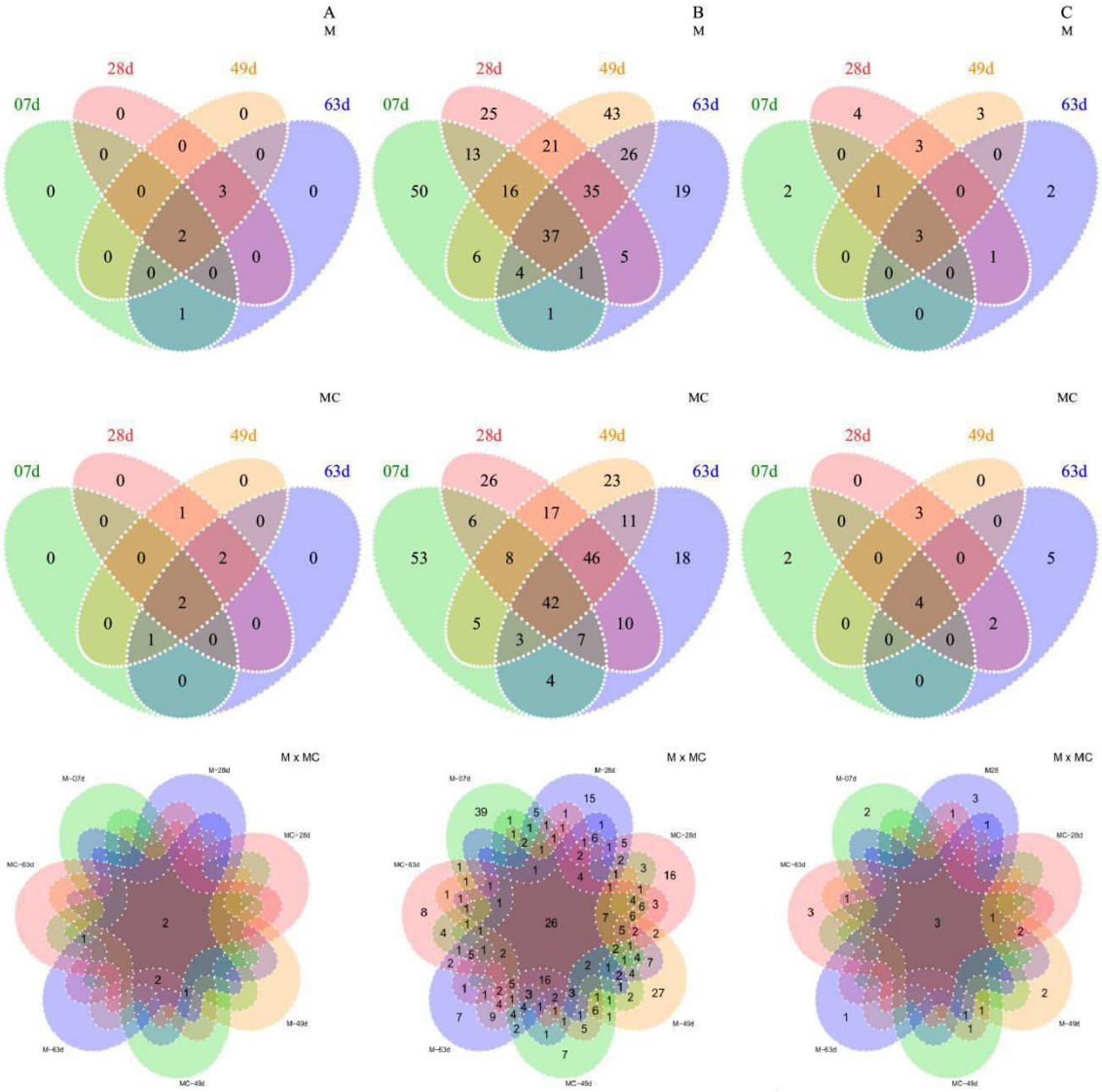

FIGURE 2| Venn diagrams of shared archaeal (A), bacterial (B), and fungal (C) OTUs among calves according to diet (M: milk-fed or MC: milk and starter concentrate fed) and age group (7, 28, 49 and 63 days old). In all plots of MC calves, the M-07d group was included as a reference. Only OTUs at > 0.1% relative abundance (archaea n=6; bacteria n=519; fungi n=48) and present in at least two rumen samples in each diet-age groups were included.

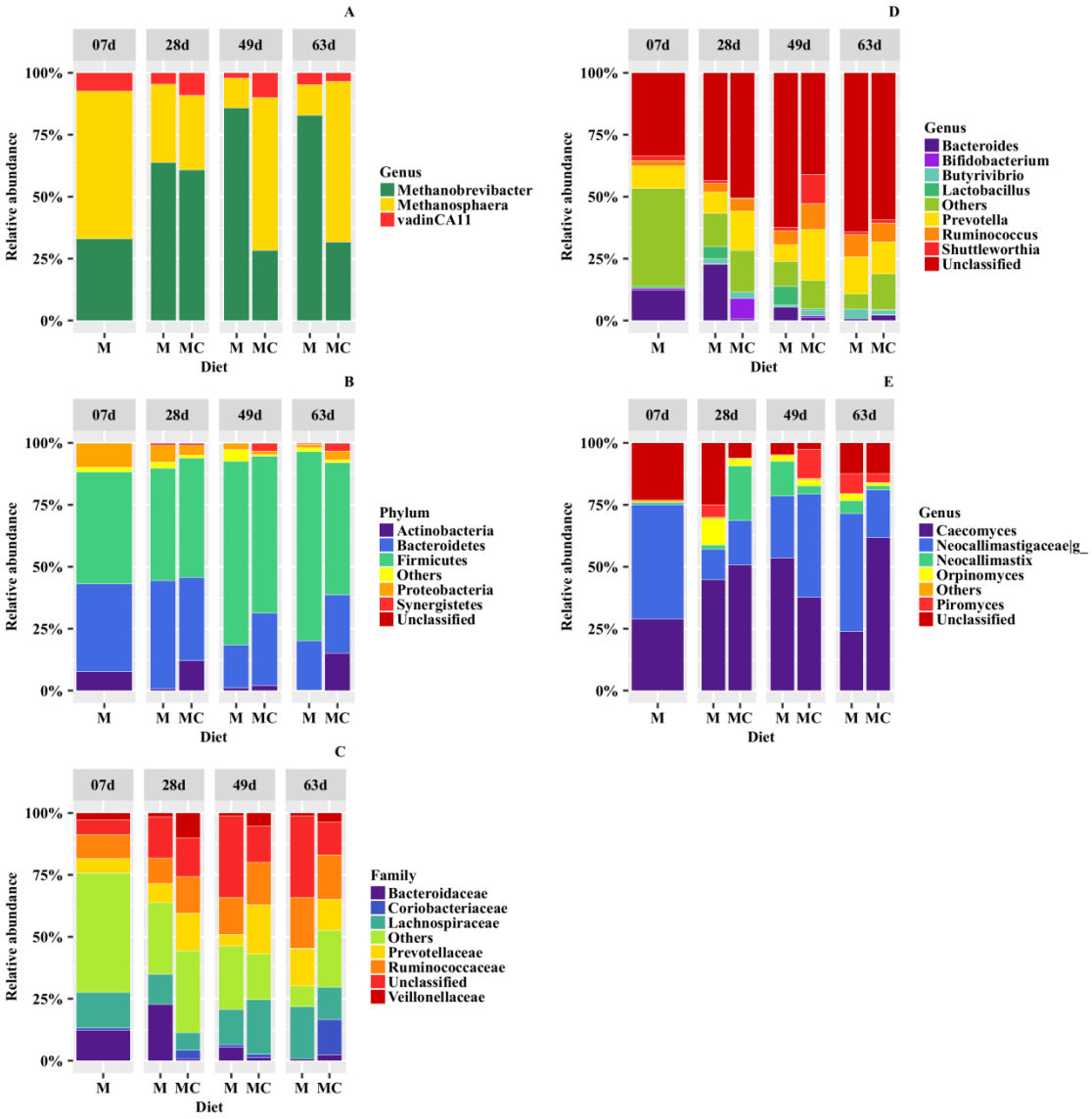

FIGURE 3| Distribution of the most abundant archaeal (A), bacterial (B, C, D) and fungal taxa (E) in the rumen samples from dairy calves grouped by diet (M: milk-fed or MC: milk and starter concentrate fed) and age (7, 28, 49 and 63 days old).

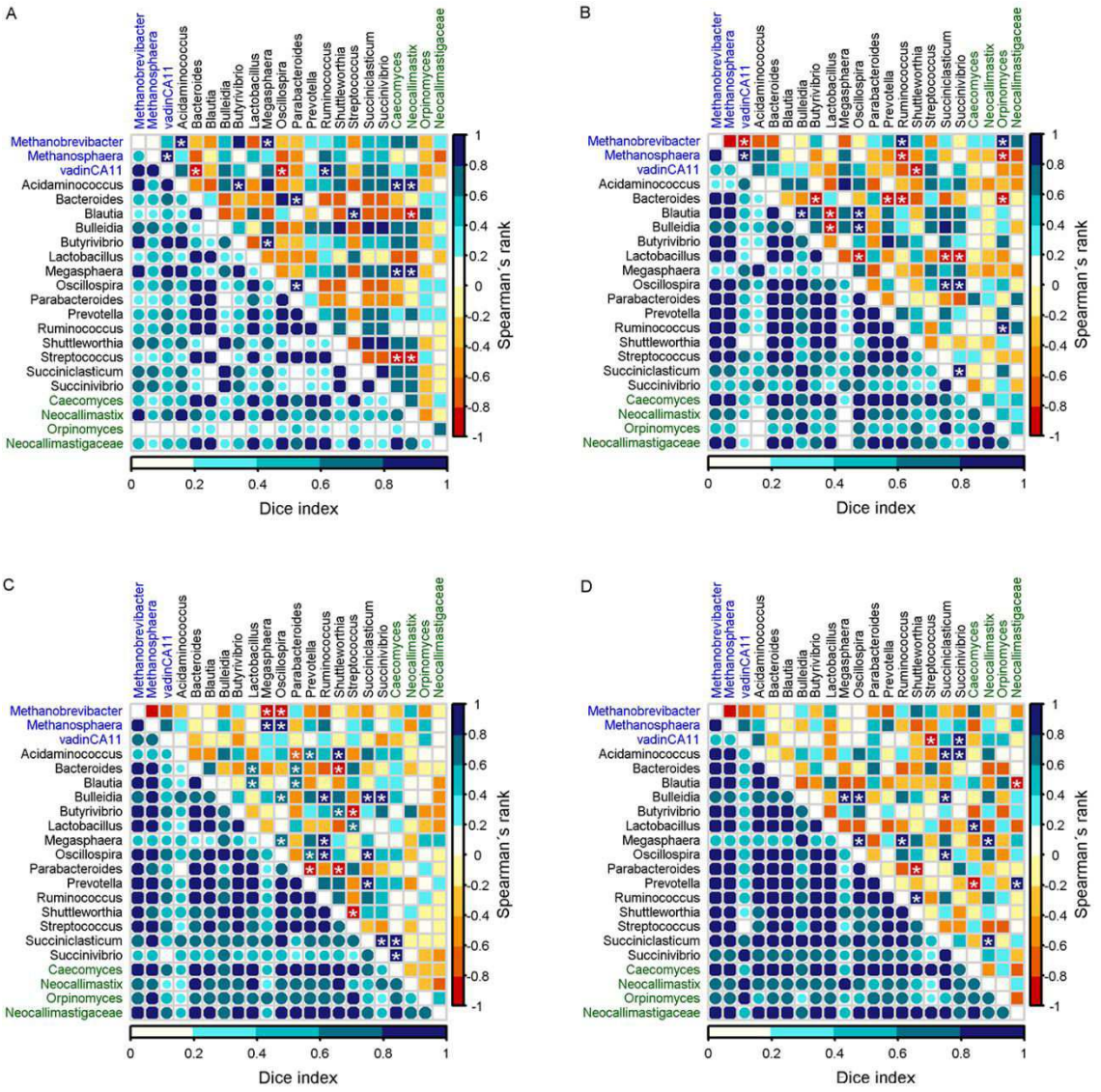

FIGURE 4 | The co-occurrence and correlation among archaeal (blue), bacterial (black) and fungal (green) genera in the rumen samples of milk-fed calves at four development stages: 7 (A), 28 (B), 49 (C) and 63 (D) days of age. The co-occurrence across genera is shown by Dice's index (lower matrix), ranging from 0 to 1, represented by color key (dark blue: high, cyan: moderate, light blue: low co-occurrence). The correlation across genera is shown by Spearman's rank correlation (upper matrix), ranging from -1 to 1 and represented by color key dark red (perfect negative correlation) to dark blue (perfect positive correlation). The correlations marked with asterisks were significant at a $P<0.05$ (Supplementary Table 5).

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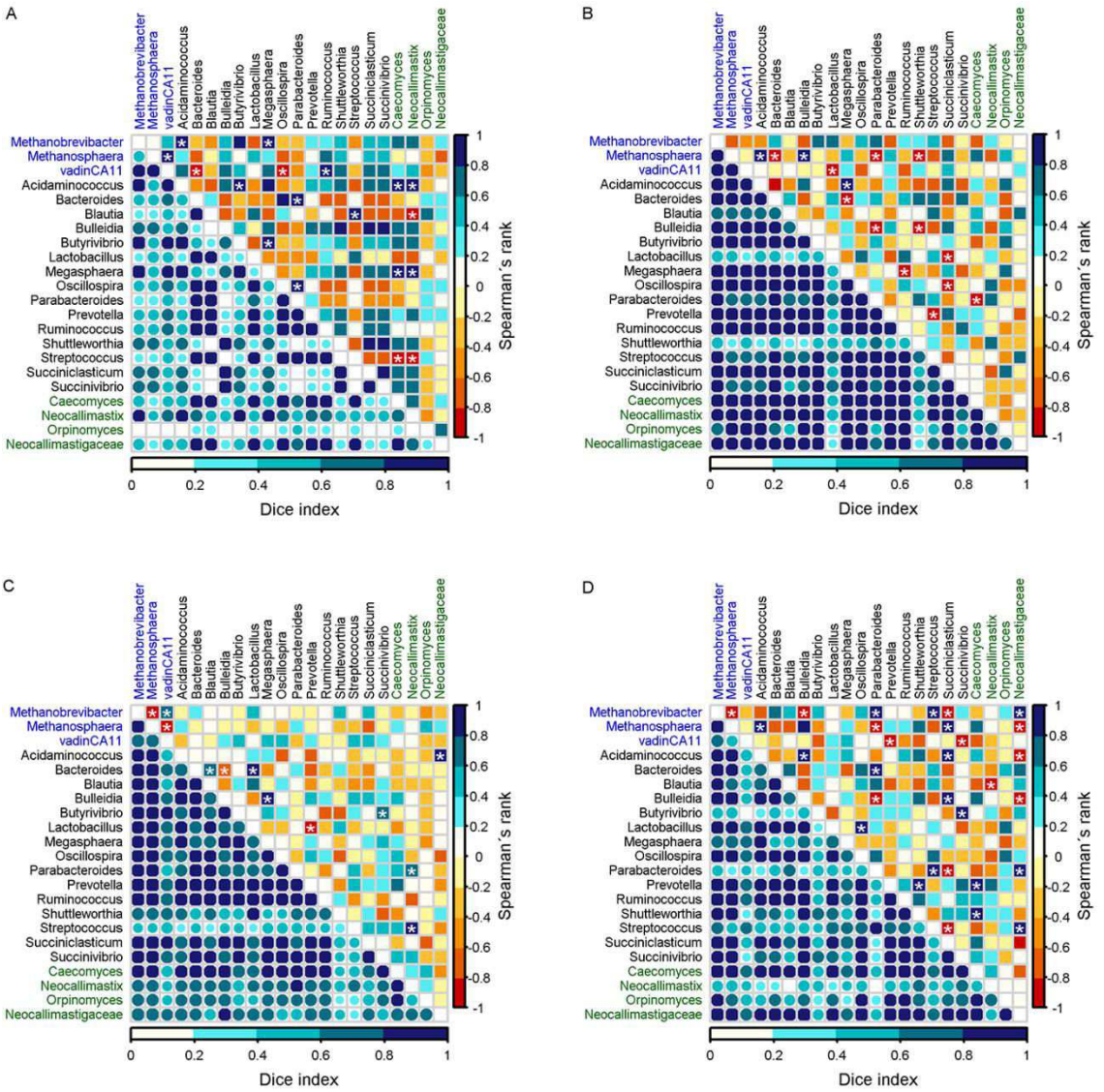

FIGURE 5 | The co-occurrence and correlation among archaeal (blue), bacterial (black) and fungal (green) genera in the rumen samples of the milk and starter concentrate fed calves at three development stages: 7 (A), 28 (B), 49 (C) and 63 (D) days of age. Plot (A) represents co-occurrence and correlation analysis for calves at 7 days old (Figure 4A) and is shown for comparative purposes. The co-occurrence across genera is shown by Dice's index (lower matrix) ranging from 0 to 1, represented by color key (dark blue: high, cyan: moderate, light blue: low co-occurrence). The correlation across genera is indicated by Spearman's rank correlation (upper matrix), ranging from -1 to 1 and represented by color key dark red (perfect negative correlation) to dark blue (perfect positive correlation).The correlations marked with asterisk were significant at a $P<0.05$ (Supplementary Table 5).

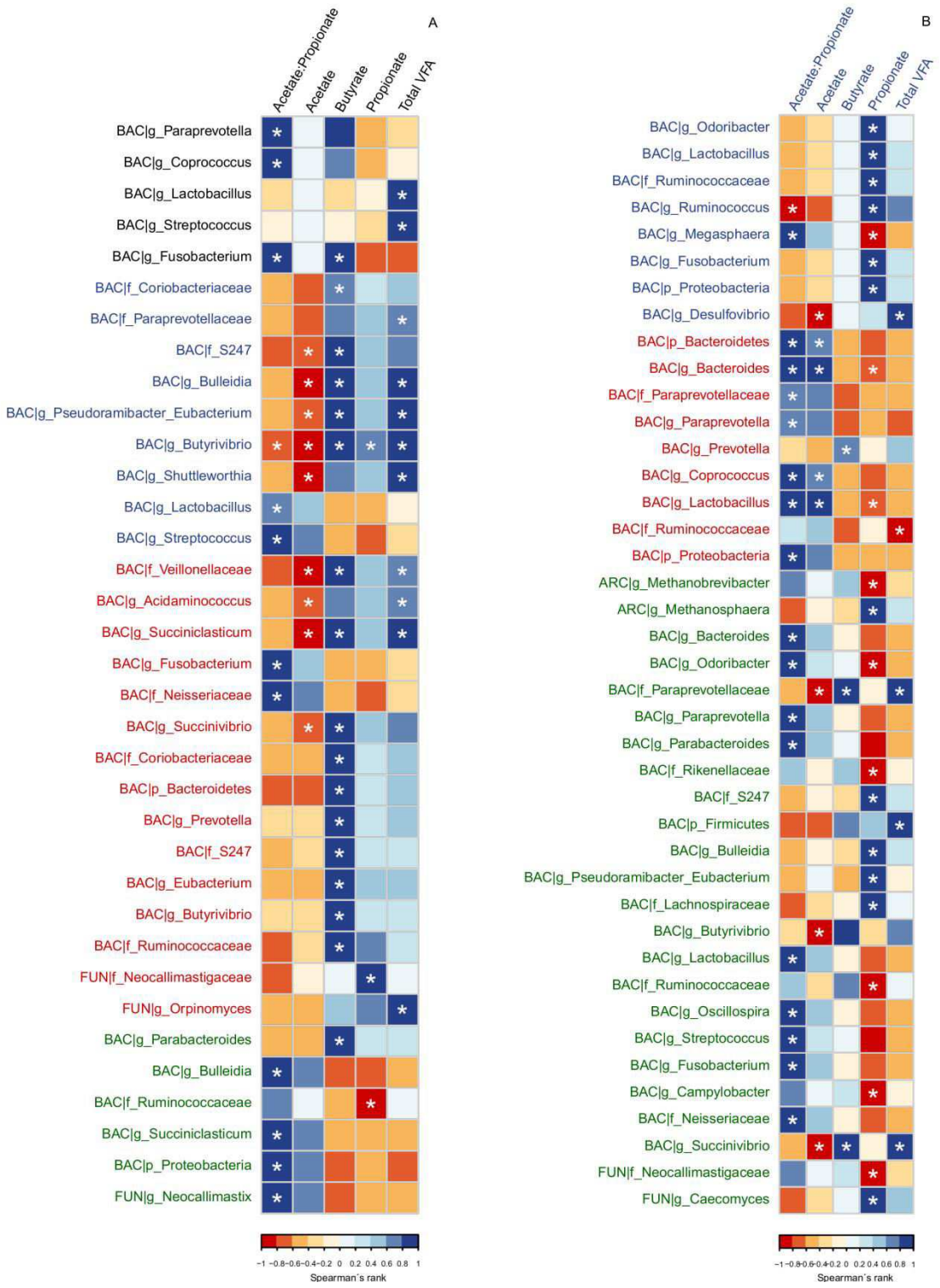

FIGURE 6 | Correlations between molar proportions of volatile fatty acids and relative abundance of archaeal, bacterial and fungal taxa in the rumen samples of milk-fed (A) and milk and starter concentrate fed (B) calves in their respective age groups: 7 (black), 28 (blue), 49 (red) and 63 (green) days old. The correlation is indicated by Spearman´s rank correlation, ranging from -1 to 1 and represented by color key dark red (perfect negative correlation) to dark blue (perfect positive correlation). The correlations marked with asterisks were significant at a $P<0.05$ (Supplementary Table 7).

Appendix

Table S1. Chemical composition of whole milk and starter concentrate fed to calves during experimental period (7 to 63 days).

Composition	Milk	Starter concentrate[1]
Dry matter (%)	12.32	89.33
Crude protein (DM basis %)	25.89	17.45
Ether extract (DM basis %)	37.64	1.940
Neutral Detergent Fiber (DM basis %)	-	12.99
Non-Fiber Carbohydrate (DM basis %)	31.23	62.62
Ashes (DM basis %)	5.240	5.01

[1]Contained 62.637% corn ground, 32.486% soybean meal, 3.094% wheat meal, 0.303% dicalcium phosphate, 1.123% limestone, 0.237% sodium chloride, 0.121% commercial mineral premix (180 g/kg Zinc sulfate , 150 g/kg Copper Sulfate, 10 g / kg Cobalt Sulfate, 10 g / kg sodium selenite and 10 g / kg Potassium Iodate. [*]In spite of distribution from day 4, the starter concentrate intake began from day 8 and calves slaughtered at 28, 49 and 63 days of age consumed mean 188.68 ± SEM 0.01, 265.57±0.01 and 459.99±0.01 g of DMI concentrate/day.

Table S2. Summary of sequencing of archaea, bacteria and fungi from rumen samples of dairy calves according to diet and age group

				After clean-up and prior to normalization								After normalization					
				Sequence			OTU			Coverage		Sequence			OTU		
	Diet[1]	Age[2]	Calves	Total	Mean	SEM[3]	Total	Mean	SEM	Mean	SEM	Total	Mean	SEM	Total	Mean	SEM
Archaea	M	07d	3	7493.000	2497.667	860.388	15.000	5.000	1.000	0.999	0.001	308.000	102.667	0.333	9.000	3.000	0.577
		28d	6	13045.000	2174.167	787.831	25.000	4.167	0.601	0.999	0.001	618.000	103.000	0.000	19.000	3.167	0.401
		49d	7	10099.000	1442.714	553.438	25.000	3.571	0.481	1.000	0.000	720.000	102.857	0.261	24.000	3.429	0.481
		63d	6	11489.000	1914.833	794.196	27.000	4.500	0.500	1.000	0.000	616.000	102.667	0.333	22.000	3.667	0.494
	MC	28d	6	13410.000	2235.000	919.420	25.000	4.167	0.307	1.000	0.000	616.000	102.667	0.333	21.000	3.500	0.428
		49d	8	9141.000	1142.625	535.473	28.000	3.500	0.423	1.000	0.000	823.000	102.875	0.125	25.000	3.125	0.398
		63d	6	19542.000	3257.000	1622.311	24.000	4.000	0.365	1.000	0.000	617.000	102.833	0.167	19.000	3.167	0.401
Bacteria	M	07d	6	41499.000	6916.500	994.973	687.000	114.500	8.625	0.998	0.000	18089.000	3014.833	3.135	642.000	107.000	10.040
		28d	6	217861.000	36310.167	11456.553	1008.000	168.000	3.077	0.998	0.001	18056.000	3009.333	5.463	670.000	111.667	16.808
		49d	7	282921.000	40417.286	9475.404	1880.000	268.571	48.987	0.999	0.000	20940.000	2991.429	5.830	901.000	128.714	16.643
		63d	6	240662.000	40110.333	6380.079	1063.000	177.167	7.993	0.999	0.000	18017.000	3002.833	3.371	596.000	99.333	9.566
	MC	28d	6	64378.000	10729.667	2710.992	896.000	149.333	28.393	0.997	0.001	18047.000	3007.833	4.430	712.000	118.667	22.921
		49d	8	72065.000	9008.125	2520.070	895.000	111.875	12.710	0.997	0.001	24095.000	3011.875	2.682	813.000	101.625	15.565
		63d	6	60497.000	10082.833	3478.488	969.000	161.500	33.798	0.997	0.001	18069.000	3011.500	8.702	727.000	121.167	21.642
Fungi	M	07d	5	7795.000	1559.000	556.267	60.000	12.000	1.265	0.999	0.001	529.000	105.800	0.490	33.000	6.600	0.980
		28d	5	2055.000	411.000	153.557	56.000	11.200	1.908	0.988	0.006	529.000	105.800	0.200	42.000	8.400	0.927
		49d	7	1502.000	214.571	68.444	63.000	9.000	1.431	0.989	0.004	743.000	106.143	0.459	50.000	7.143	0.986
		63d	6	2903.000	483.833	279.104	61.000	10.167	2.372	0.987	0.005	643.000	107.167	0.543	42.000	7.000	1.000
	MC	28d	6	3906.000	651.000	272.819	59.000	9.833	1.447	0.995	0.003	641.000	106.833	0.543	42.000	7.000	0.931
		49d	8	8108.000	1013.500	449.988	66.000	8.250	1.840	0.997	0.002	852.000	106.500	0.327	41.000	5.125	1.060
		63d	6	5830.000	971.667	442.993	95.000	15.833	3.439	0.994	0.002	635.000	105.833	0.477	45.000	7.500	0.764

[1]Calves fed only milk (M) or milk and starter concentrate (MC); [2] slaughter age 7, 28, 49 and 63 days old; [3]Standard error of the mean.

Table S3. Alpha-diversity of archaeal, bacterial and fungal communities in the rumen of calves according diet, age groups.

		Diet[1]								
	Index	**M**				**MC**			***P*-value**	**FDR***
Arc	Chao1	3.455±1.101				3.300±1.1290			0.460	0.693
	Invsimpson	1.641±0.623				1.485±0.524			0.129	0.387
	Shannon	0.577±0.354				0.495±0.317			0.246	0.590
Bac	Chao1	165.564±72.734				173.746±94.046			0.219	0.490
	Invsimpson	11.704±7.322				12.431±7.151			0.157	0.472
	Shannon	2.915±0.690				2.954±0.832			0.391	0.496
Fun	Chao1	9.036 ± 4.413				8.588 ± 5.444			0.741	0.966
	Invsimpson	2.953 ± 1.126				2.372 ± 1.190			0.709	0.966
	Shannon	1.227 ± 0.367				0.951 ± 0.509			0.767	0.966
		Age[2]								
		07d		**28d**		**49d**		**63d**		
Arc	Chao1	3.000±1.000		3.333±0.985		3.333±1.175		3.583±1.24	0.675	0.707
	Invsimpson	1.233±0.153		1.717±0.564		1.413±0.501		1.692±0.703	0.310	0.620
	Shannon	0.367±0.153		0.633±0.306		0.460±0.366		0.583±0.351	0.520	0.693
Bac	Chao1	143.93±28.079		166.602±91.008		176.645±87.304		175.128±89.866	0.641	0.672
	Invsimpson	12.193±6.712		14.238±7.492		11.839±7.214		9.97±7.279	0.322	0.490
	Shannon	2.975±0.686		3.138±0.666		2.88±0.885		2.772±0.709	0.672	0.672
Fun	Chao1	7.500 ± 3.202		8.909 ± 3.846		8.500 ± 5.441		9.715 ± 5.803	0.847	0.966
	Invsimpson	3.172 ± 0.917		2.666 ± 1.018		2.446 ± 1.347		2.789 ± 1.246	0.897	0.966
	Shannon	1.294 ± 0.342		1.185 ± 0.309		0.923 ± 0.610		1.158 ± 0.340	0.966	0.966
		Diet*Age[3]								
		M_07d	**M_28d**	**M_49d**	**M_63d**	**MC_28d**	**MC_49d**	**MC_63d**		
Arc	Chao1	3.000±1.000	3.167±0.983	3.571±1.272	3.833±1.169	3.500±1.049	3.125±1.126	3.333±1.366	0.611	0.707
	Invsimpson	1.233±0.153	1.700±0.452	1.500±0.548	1.950±0.901	1.733±0.703	1.337±0.481	1.433±0.339	0.500	0.693
	Shannon	0.367±0.153	0.633±0.308	0.514±0.38	0.700±0.443	0.633±0.333	0.413±0.372	0.467±0.207	0.707	0.707
Bac	Chao1	143.93±28.079	172.835±109.299	195.941±84.597	144.488±40.254	160.37±78.625	159.761±91.715	205.768±117.871	0.327	0.490
	Invsimpson	12.193±6.712	14.185±8.793	13.246±8.176	6.937±4.059	14.29±6.793	10.607±6.563	13.003±8.832	0.309	0.490
	Shannon	2.975±0.686	3.012±0.825	3.07±0.778	2.578±0.468	3.263±0.507	2.714±0.989	2.965±0.893	0.413	0.496
Fun	Chao1	7.500 ± 3.202	10.400 ± 4.602	9.000 ± 5.627	9.222 ± 4.277	7.667 ± 2.927	8.062 ± 5.622	10.208 ± 7.43	0.689	0.966
	Invsimpson	3.172 ± 0.917	2.630 ± 1.461	3.057 ± 1.192	2.918 ± 1.150	2.697 ± 0.604	1.911 ± 1.308	2.660 ± 1.433	0.408	0.966
	Shannon	1.294 ± 0.342	1.170 ± 0.451	1.249 ± 0.430	1.195 ± 0.321	1.198 ± 0.168	0.638 ± 0.621	1.122 ± 0.385	0.126	0.379

Values represent mean and standard deviation. [1]milk (M), milk and concentrate (MC); [2]slaughter age (7, 28, 49 and 63 days); [3]Calves fed only with milk (M) or milk and starter concentrate (MC) that were slaughtered at 7, 28, 49 and 63 days of age; *P-value adjusted by FDR method; FDR≤ 0.05 were considered significant.

Table S4. Bacterial taxa that present not significantly different between diet, age or interaction of these factors.

	Diet[1]			
Taxa	**M**	**MC**	***P*-value**	**FDR***
Butyricimonas	0.218±0.086	0.055±0.023	0.163	0.215
Butyrivibrio	1.793±0.708	2.153±0.696	0.651	0.720
Clostridium	0.083±0.039	0.058±0.023	0.098	0.140
Coprococcus	0.441±0.123	0.207±0.104	0.037	0.060
Desulfovibrio	0.286±0.090	0.289±0.056	0.626	0.700
Fusobacterium	0.232±0.080	0.089±0.057	0.291	0.353
Leuconostoc	0.101±0.064	0.075±0.061	0.196	0.250
Oscillospira	0.706±0.245	0.464±0.139	0.418	0.486
Phascolarctobacterium	0.158±0.065	0.073±0.053	0.823	0.860
Porphyromonas	1.105±0.452	0.540±0.529	0.688	0.740
Prevotella	9.677±2.424	16.817±4.144	0.223	0.276
Ruminococcus	5.136±0.949	8.081±2.238	0.889	0.897
Treponema	0.121±0.071	0.245±0.135	0.423	0.487
	Age[2]			
	07d	**28d**	**49d**	**63d**		
Bulleidia	0.327±0.327	1.127±0.387	1.32±0.432	1.292±0.668	0.207	0.259
Butyrivibrio	0.066±0.043	2.368±0.826	1.675±0.669	2.830±1.426	0.090	0.130
Clostridium	0.006±0.006	0.028±0.015	0.102±0.058	0.111±0.047	0.051	0.079
Coprococcus	0.072±0.054	0.307±0.189	0.36±0.136	0.471±0.182	0.319	0.379
Desulfovibrio	0.519±0.304	0.271±0.074	0.162±0.049	0.343±0.104	0.147	0.200
Fusobacterium	0.441±0.220	0.225±0.118	0.069±0.042	0.102±0.087	0.101	0.142
Leuconostoc	0.000±0.000	0.022±0.014	0.237±0.126	0.017±0.014	0.064	0.095
Prevotella	9.118±3.987	12.196±2.941	14.054±5.227	13.865±4.878	0.406	0.477
Ruminococcus	2.180±1.040	4.389±1.465	8.259±2.780	8.365±1.624	0.047	0.073
Succiniclasticum	0.986±0.986	1.829±0.801	1.310±0.682	0.956±0.387	0.847	0.870
YRC22	0.110±0.07	0.030±0.027	0.042±0.029	0.088±0.054	0.206	0.259

Continued Table S4

	Diet*Age[3]								
	M-07d	**M-28d**	**M-49d**	**M-63d**	**MC-28d**	**MC-49d**	**MC-63d**		
Bifidobacterium	0.745±0.574	0.250±0.250	0.000±0.000	0.000±0.000	8.239±3.732	0.728±0.645	0.216±0.183	0.038	0.061
Bulleidia	0.327±0.327	0.222±0.183	0.568±0.503	0.050±0.032	2.033±0.545	1.977±0.614	2.533±1.160	0.183	0.237
Butyricimonas	0.508±0.233	0.225±0.194	0.149±0.143	0.000±0.000	0.066±0.026	0.021±0.012	0.088±0.070	0.110	0.153
Butyrivibrio	0.066±0.043	2.167±1.232	1.082±0.370	3.977±2.574	2.569±1.210	2.193±1.223	1.683±1.342	0.479	0.541
Clostridium	0.006±0.006	0.022±0.022	0.181±0.12	0.106±0.073	0.033±0.023	0.033±0.025	0.116±0.065	0.137	0.188
Coprococcus	0.072±0.054	0.608±0.348	0.596±0.232	0.461±0.253	0.006±0.006	0.153±0.126	0.480±0.287	0.175	0.230
Desulfovibrio	0.519±0.304	0.194±0.107	0.100±0.054	0.361±0.178	0.349±0.102	0.216±0.078	0.326±0.127	0.663	0.726
Eubacterium	0.485±0.445	0.011±0.007	0.010±0.006	0.011±0.007	0.050±0.032	0.121±0.107	0.116±0.041	0.844	0.870
Faecalibacterium	0.000±0.000	0.006±0.006	0.149±0.143	0.000±0.000	0.028±0.010	0.472±0.462	0.033±0.027	0.066	0.096
Fusobacterium	0.441±0.220	0.427±0.211	0.067±0.056	0.022±0.007	0.022±0.016	0.070±0.066	0.182±0.175	0.064	0.095
Leuconostoc	0.000±0.000	0.017±0.011	0.324±0.215	0.028±0.028	0.028±0.028	0.162±0.152	0.006±0.006	0.856	0.871
Oscillospira	2.009±0.822	0.337±0.153	0.263±0.169	0.288±0.132	0.631±0.141	0.224±0.111	0.617±0.422	0.446	0.508
Phascolarctobacterium	0.503±0.218	0.061±0.020	0.081±0.053	0.000±0.000	0.017±0.011	0.041±0.041	0.170±0.170	0.161	0.215
Prevotella	9.118±3.987	8.490±3.281	6.676±2.876	14.923±8.447	15.903±4.668	20.509±9.138	12.808±5.737	0.304	0.365
Pseudoramibacter	0.022±0.011	0.028±0.018	0.024±0.019	0.017±0.011	0.072±0.029	0.290±0.162	0.338±0.123	0.058	0.089
Ruminococcus	2.180±1.040	3.676±1.815	5.631±2.136	8.974±1.435	5.101±2.438	10.558±4.892	7.757±3.064	0.755	0.797
SHD.231	0.000±0.000	0.000±0.000	0.647±0.625	0.522±0.515	0.000±0.000	0.000±0.000	0.000±0.000	1.000	1.000
Succiniclasticum	0.986±0.986	0.116±0.055	0.586±0.376	0.144±0.077	3.541±1.284	1.944±1.230	1.767±0.623	0.260	0.319
Treponema	0.000±0.000	0.454±0.265	0.019±0.019	0.028±0.010	0.491±0.421	0.087±0.058	0.209±0.163	0.669	0.726
YRC22	0.110±0.070	0.005±0.005	0.000±0.000	0.028±0.028	0.055±0.055	0.079±0.052	0.149±0.103	0.738	0.786

Values represent mean and standard deviation. [1]milk (M), milk plus concentrate (MC); [2]slaughter age (7, 28, 49 and 63 days); [3]Calves fed with only with milk (M) or milk plus starter concentrate (MC) that were slaughtered at 7, 28, 49 and 63 days of age; [*]*P*-value adjusted by FDR method; FDR≤ 0.05 were considered significant.

Table S5. Co-occurrence (Dice index) and correlation (Spearman´s rank) analysis inter-intra microbial genera in the rumen of dairy calves grouped according diet and age.

Group[1]	Association[2]	Dice	Spearman	*P*-value[3]
M_07d	ARC|g_vadinCA11:ARC|g_Methanosphaera	0.80	0.83	0.043
	BAC|g_Acidaminococcus:ARC|g_Methanobrevibacter	1.00	0.92	0.009
	BAC|g_Bacteroides:ARC|g_vadinCA11	0.67	-0.82	0.046
	BAC|g_Butyrivibrio:BAC|g_Acidaminococcus	1.00	0.92	0.009
	BAC|g_Megasphaera:ARC|g_Methanobrevibacter	1.00	0.92	0.009
	BAC|g_Megasphaera:BAC|g_Butyrivibrio	1.00	0.92	0.009
	BAC|g_Oscillospira:ARC|g_vadinCA11	0.67	-0.82	0.046
	BAC|g_Parabacteroides:BAC|g_Bacteroides	0.80	0.90	0.015
	BAC|g_Parabacteroides:BAC|g_Oscillospira	0.80	0.90	0.015
	BAC|g_Ruminococcus:ARC|g_vadinCA11	0.67	0.82	0.046
	BAC|g_Streptococcus:BAC|g_Blautia	1.00	0.94	0.005
	FUN|g_Caecomyces:BAC|g_Acidaminococcus	0.57	0.85	0.034
	FUN|g_Caecomyces:BAC|g_Megasphaera	0.57	0.85	0.034
	FUN|g_Caecomyces:BAC|g_Streptococcus	0.80	-0.83	0.042
	FUN|g_Neocallimastix:BAC|g_Acidaminococcus	0.80	0.82	0.045
	FUN|g_Neocallimastix:BAC|g_Blautia	0.50	-0.93	0.008
	FUN|g_Neocallimastix:BAC|g_Megasphaera	0.80	0.82	0.045
	FUN|g_Neocallimastix:BAC|g_Streptococcus	0.50	-0.93	0.008
M_28d	ARC|g_vadinCA11:ARC|g_Methanobrevibacter	0.50	-0.85	0.034
	ARC|g_vadinCA11:ARC|g_Methanosphaera	0.50	0.85	0.034
	BAC|g_Bulleidia:BAC|g_Blautia	0.67	0.94	0.005
	BAC|g_Butyrivibrio:BAC|g_Bacteroides	0.91	-0.89	0.019
	BAC|g_Lactobacillus:BAC|g_Blautia	0.91	-0.89	0.019
	BAC|g_Lactobacillus:BAC|g_Bulleidia	0.50	-0.88	0.021
	BAC|g_Oscillospira:BAC|g_Blautia	0.80	0.84	0.036
	BAC|g_Oscillospira:BAC|g_Bulleidia	0.86	0.89	0.016
	BAC|g_Oscillospira:BAC|g_Lactobacillus	0.67	-0.99	<.001
	BAC|g_Prevotella:BAC|g_Bacteroides	1.00	-0.89	0.019
	BAC|g_Ruminococcus:ARC|g_Methanobrevibacter	1.00	0.94	0.005
	BAC|g_Ruminococcus:ARC|g_Methanosphaera	1.00	-0.94	0.005
	BAC|g_Ruminococcus:BAC|g_Bacteroides	1.00	-0.83	0.042
	BAC|g_Shuttleworthia:ARC|g_vadinCA11	0.00	-0.82	0.044
	BAC|g_Succiniclasticum:BAC|g_Lactobacillus	0.50	-0.88	0.021
	BAC|g_Succiniclasticum:BAC|g_Oscillospira	0.86	0.89	0.016
	BAC|g_Succinivibrio:BAC|g_Lactobacillus	0.29	-0.85	0.034
	BAC|g_Succinivibrio:BAC|g_Oscillospira	0.67	0.86	0.029
	BAC|g_Succinivibrio:BAC|g_Succiniclasticum	0.80	0.83	0.043
	FUN|g_Orpinomyces:ARC|g_Methanobrevibacter	0.50	0.85	0.034
	FUN|g_Orpinomyces:ARC|g_Methanosphaera	0.50	-0.85	0.034
	FUN|g_Orpinomyces:BAC|g_Bacteroides	0.50	-0.85	0.034
	FUN|g_Orpinomyces:BAC|g_Ruminococcus	0.50	0.85	0.034
M_49d	BAC|g_Lactobacillus:BAC|g_Bacteroides	1.00	0.79	0.036
	BAC|g_Lactobacillus:BAC|g_Blautia	0.91	0.77	0.041
	BAC|g_Megasphaera:ARC|g_Methanobrevibacter	0.44	-0.80	0.030

	BAC|g_Megasphaera:ARC|g_Methanosphaera	0.50	0.80	0.030
	BAC|g_Oscillospira:ARC|g_Methanobrevibacter	0.83	-0.85	0.016
	BAC|g_Oscillospira:ARC|g_Methanosphaera	0.91	0.85	0.016
	BAC|g_Oscillospira:BAC|g_Bulleidia	0.89	0.79	0.036
	BAC|g_Oscillospira:BAC|g_Megasphaera	0.57	0.76	0.046
	BAC|g_Parabacteroides:BAC|g_Acidaminococcus	0.00	-0.79	0.036
	BAC|g_Parabacteroides:BAC|g_Bacteroides	0.91	0.79	0.033
	BAC|g_Parabacteroides:BAC|g_Blautia	0.80	0.77	0.042
	BAC|g_Prevotella:BAC|g_Acidaminococcus	0.44	0.76	0.049
	BAC|g_Prevotella:BAC|g_Oscillospira	0.83	0.77	0.041
	BAC|g_Prevotella:BAC|g_Parabacteroides	0.83	-0.81	0.027
	BAC|g_Ruminococcus:BAC|g_Bulleidia	0.73	0.82	0.025
	BAC|g_Ruminococcus:BAC|g_Megasphaera	0.44	0.80	0.030
	BAC|g_Ruminococcus:BAC|g_Oscillospira	0.83	0.83	0.021
	BAC|g_Shuttleworthia:BAC|g_Acidaminococcus	0.57	0.81	0.028
	BAC|g_Shuttleworthia:BAC|g_Bacteroides	0.73	-0.85	0.016
	BAC|g_Shuttleworthia:BAC|g_Butyrivibrio	0.83	0.77	0.041
	BAC|g_Shuttleworthia:BAC|g_Parabacteroides	0.60	-0.84	0.019
	BAC|g_Streptococcus:BAC|g_Butyrivibrio	0.83	-0.81	0.027
	BAC|g_Streptococcus:BAC|g_Lactobacillus	0.73	0.77	0.041
	BAC|g_Streptococcus:BAC|g_Shuttleworthia	0.60	-0.87	0.010
	BAC|g_Succiniclasticum:BAC|g_Bulleidia	0.75	0.85	0.016
	BAC|g_Succiniclasticum:BAC|g_Oscillospira	0.89	0.86	0.013
	BAC|g_Succiniclasticum:BAC|g_Prevotella	0.73	0.85	0.015
	BAC|g_Succinivibrio:BAC|g_Bulleidia	0.67	0.83	0.020
	BAC|g_Succinivibrio:BAC|g_Succiniclasticum	0.67	0.83	0.020
	FUN|g_Caecomyces:BAC|g_Succiniclasticum	0.73	0.82	0.025
	FUN|g_Caecomyces:BAC|g_Succinivibrio	0.44	0.80	0.030
M_63d	BAC|g_Megasphaera:BAC|g_Bulleidia	0.80	0.83	0.043
	BAC|g_Oscillospira:BAC|g_Bulleidia	0.75	0.88	0.021
	BAC|g_Oscillospira:BAC|g_Megasphaera	0.57	0.85	0.034
	BAC|g_Ruminococcus:BAC|g_Megasphaera	0.50	0.85	0.034
	BAC|g_Shuttleworthia:BAC|g_Parabacteroides	0.67	-0.90	0.015
	BAC|g_Shuttleworthia:BAC|g_Ruminococcus	0.80	0.84	0.036
	BAC|g_Streptococcus:ARC|g_vadinCA11	0.00	-0.82	0.044
	BAC|g_Succiniclasticum:BAC|g_Acidaminococcus	0.89	0.81	0.050
	BAC|g_Succiniclasticum:BAC|g_Bulleidia	0.86	0.83	0.040
	BAC|g_Succiniclasticum:BAC|g_Oscillospira	0.89	0.87	0.024
	BAC|g_Succinivibrio:ARC|g_vadinCA11	0.80	0.90	0.015
	BAC|g_Succinivibrio:BAC|g_Acidaminococcus	0.75	0.88	0.021
	FUN|g_Caecomyces:BAC|g_Lactobacillus	1.00	0.89	0.019
	FUN|g_Caecomyces:BAC|g_Prevotella	1.00	-0.83	0.042
	FUN|f_Neocallimastigaceae:BAC|g_Blautia	1.00	-0.84	0.036
	FUN|f_Neocallimastigaceae:BAC|g_Prevotella	1.00	0.89	0.019
	FUN|g_Neocallimastix:BAC|g_Megasphaera	0.80	0.83	0.043
	FUN|g_Neocallimastix:BAC|g_Succiniclasticum	0.86	0.95	0.003
MC_28d	BAC|g_Acidaminococcus:ARC|g_Methanosphaera	1.00	0.89	0.019
	BAC|g_Bacteroides:ARC|g_Methanosphaera	0.80	-0.89	0.019

	BAC|g_Bulleidia:ARC|g_Methanosphaera	1.00	0.94	0.005
	BAC|g_Lactobacillus:ARC|g_vadinCA11	0.29	-0.85	0.034
	BAC|g_Megasphaera:BAC|g_Acidaminococcus	0.91	0.83	0.042
	BAC|g_Megasphaera:BAC|g_Bacteroides	0.91	-0.83	0.042
	BAC|g_Parabacteroides:ARC|g_Methanosphaera	0.67	-0.87	0.024
	BAC|g_Parabacteroides:BAC|g_Bulleidia	0.67	-0.87	0.024
	BAC|g_Ruminococcus:BAC|g_Megasphaera	1.00	-0.83	0.042
	BAC|g_Shuttleworthia:ARC|g_Methanosphaera	0.29	-0.85	0.034
	BAC|g_Shuttleworthia:BAC|g_Bulleidia	0.29	-0.85	0.034
	BAC|g_Streptococcus:BAC|g_Prevotella	0.80	-0.81	0.050
	BAC|g_Succiniclasticum:BAC|g_Lactobacillus	0.50	-0.85	0.034
	BAC|g_Succiniclasticum:BAC|g_Oscillospira	1.00	-0.83	0.042
	FUN|g_Caecomyces:BAC|g_Parabacteroides	0.80	-0.84	0.036
MC_49d	ARC|g_Methanosphaera:ARC|g_Methanobrevibacter	1.00	-0.93	0.001
	ARC|g_vadinCA11:ARC|g_Methanobrevibacter	0.77	0.73	0.042
	ARC|g_vadinCA11:ARC|g_Methanosphaera	0.77	-0.86	0.006
	BAC|g_Blautia:BAC|g_Bacteroides	0.92	0.75	0.031
	BAC|g_Bulleidia:BAC|g_Bacteroides	0.86	-0.71	0.047
	BAC|g_Lactobacillus:BAC|g_Bacteroides	0.77	0.92	0.001
	BAC|g_Megasphaera:BAC|g_Bulleidia	0.92	0.85	0.007
	BAC|g_Prevotella:BAC|g_Lactobacillus	0.86	-0.84	0.009
	BAC|g_Succinivibrio:BAC|g_Butyrivibrio	0.92	0.77	0.027
	FUN|f_Neocallimastigaceae:BAC|g_Acidaminococcus	0.73	0.83	0.010
	FUN|g_Neocallimastix:BAC|g_Parabacteroides	0.80	0.75	0.031
	FUN|g_Neocallimastix:BAC|g_Streptococcus	0.67	0.81	0.014
MC_63d	ARC|g_Methanosphaera:ARC|g_Methanobrevibacter	0.91	-0.83	0.042
	BAC|g_Acidaminococcus:ARC|g_Methanosphaera	0.89	0.90	0.015
	BAC|g_Bulleidia:ARC|g_Methanobrevibacter	0.91	-0.83	0.042
	BAC|g_Bulleidia:BAC|g_Acidaminococcus	0.89	0.90	0.015
	BAC|g_Oscillospira:BAC|g_Lactobacillus	0.91	0.83	0.042
	BAC|g_Parabacteroides:ARC|g_Methanobrevibacter	0.50	0.85	0.034
	BAC|g_Parabacteroides:ARC|g_Methanosphaera	0.29	-0.85	0.034
	BAC|g_Parabacteroides:BAC|g_Bacteroides	0.57	0.85	0.034
	BAC|g_Parabacteroides:BAC|g_Bulleidia	0.29	-0.85	0.034
	BAC|g_Prevotella:ARC|g_vadinCA11	0.67	-0.82	0.046
	BAC|g_Shuttleworthia:BAC|g_Prevotella	0.80	0.81	0.050
	BAC|g_Streptococcus:ARC|g_Methanobrevibacter	0.67	0.82	0.046
	BAC|g_Streptococcus:BAC|g_Parabacteroides	0.80	0.90	0.015
	BAC|g_Succiniclasticum:ARC|g_Methanobrevibacter	0.91	-0.94	0.005
	BAC|g_Succiniclasticum:ARC|g_Methanosphaera	1.00	0.94	0.005
	BAC|g_Succiniclasticum:BAC|g_Acidaminococcus	0.89	0.84	0.036
	BAC|g_Succiniclasticum:BAC|g_Bulleidia	1.00	0.94	0.005
	BAC|g_Succiniclasticum:BAC|g_Parabacteroides	0.29	-0.85	0.034
	BAC|g_Succiniclasticum:BAC|g_Streptococcus	0.50	-0.82	0.046
	BAC|g_Succinivibrio:ARC|g_vadinCA11	0.29	-0.92	0.008
	BAC|g_Succinivibrio:BAC|g_Butyrivibrio	0.67	0.86	0.029
	FUN|g_Caecomyces:BAC|g_Prevotella	1.00	0.89	0.019
	FUN|g_Caecomyces:BAC|g_Shuttleworthia	0.80	0.93	0.008

FUN|f_Neocallimastigaceae:ARC|g_Methanobrevibacter	1.00	0.94	0.005
FUN|f_Neocallimastigaceae:ARC|g_Methanosphaera	0.91	-0.94	0.005
FUN|f_Neocallimastigaceae:BAC|g_Acidaminococcus	0.80	-0.84	0.036
FUN|f_Neocallimastigaceae:BAC|g_Bulleidia	0.91	-0.94	0.005
FUN|f_Neocallimastigaceae:BAC|g_Parabacteroides	0.50	0.85	0.034
FUN|f_Neocallimastigaceae:BAC|g_Streptococcus	0.67	0.82	0.046
FUN|g_Neocallimastix:BAC|g_Blautia	0.00	-0.82	0.044

[1]Calves fed with whole milk (M) or whole milk and starter concentrate (MC) slaughtered at 7, 28, 49 and 63 days old; [2]ARC (archaea); BAC (bacteria), FUN (fungi); [3]Significance of Spearman´s correlation.

Table S6. Volatile fatty acids (VFAs) concentration in the rumen samples of calves according diet and age group.

	Diet[1]			
VFA (mmol/L)	**M**	**MC**	***P*-value**	**FDR***
Acetate:Propionate	3.492 ± 1.554	3.288 ± 1.973	0.777	0.829
Acetate	12.523 ± 4.601[b]	35.443 ± 12.532[a]	<.001	<.001
Butyrate	2.200 ± 1.733[b]	5.829 ± 4.552[a]	<.001	<.001
Isobutyrate	0.931 ± 1.101[b]	1.917 ± 1.281[a]	0.021	0.049
Isovalerate	0.713 ± 0.576[b]	1.878 ± 1.267[a]	<.001	<.001
Propionate	4.427 ± 2.847[b]	13.252 ± 7.621[a]	<.001	<.001
Valerate	0.693 ± 0.978[b]	2.287 ± 1.943[a]	<.001	0.001
Total VFA	46.066 ± 21.06[b]	124.879 ± 57.095[a]	<.001	<.001

	Age[2]					
	07d	**28d**	**49d**	**63d**		
Acetate:Propionate	3.487 ± 0.813	3.136 ± 1.354	3.961 ± 2.511	2.925 ± 1.059	0.714	0.816
Acetate	6.168 ± 1.500[d]	17.172 ± 6.344[c]	24.103 ± 10.228[b]	34.776 ± 18.153[a]	<.001	<.001
Butyrate	0.938 ± 0.536	3.251 ± 2.108	3.514 ± 3.059	6.186 ± 5.295	0.682	0.808
Isobutyrate	0.212 ± 0.122	1.101 ± 0.473	1.666 ± 1.642	1.845 ± 1.274	0.341	0.474
Isovalerate	0.097 ± 0.15	1.257 ± 0.657	1.163 ± 0.706	1.857 ± 1.629	0.113	0.191
Propionate	1.875 ± 0.645	6.424 ± 3.259	8.102 ± 5.104	13.82 ± 9.646	0.312	0.453
Valerate	0.065 ± 0.159	1.347 ± 1.202	1.251 ± 1.161	2.312 ± 2.468	0.585	0.748
Total VFA	20.313 ± 5.329	65.717 ± 21.788	80.397 ± 36.644	127.733 ± 77.648	0.043	0.081

Continued Table S6

	Diet*Age[3]								
	M_07d	**M_28d**	**M_49d**	**M_63d**	**MC_28d**	**MC_49d**	**MC_63d**		
Acetate:Propionate	3.487±0.813	3.177±1.651	4.124±2.278	3.073±1.042	3.817±1.848	3.095±1.142	2.777±1.152	0.985	0.985
Acetate	6.168±1.500^{f}	11.213±0.516^{ef}	14.213±1.882^{de}	18.217±1.202^{cd}	23.132±1.749^{c}	32.758±4.771^{b}	51.335±8.088^{a}	<.001	<.001
Butyrate	0.938±0.536^{b}	2.703±2.098^{b}	2.506±2.108^{b}	2.602±1.363^{b}	3.798±2.158^{b}	4.396±3.606^{b}	9.77±5.385^{a}	0.027	0.035
Isobutyrate	0.212±0.122	1.040±0.539	1.347±1.863	1.057±0.671	1.162±0.439	1.945±1.492	2.633±1.275	0.290	0.441
Isovalerate	0.097±0.150^{c}	1.187±0.798^{bc}	0.877±0.180^{bc}	0.665±0.356^{bc}	1.327±0.547^{bc}	1.413±0.903^{b}	3.05±1.517^{a}	0.002	0.006
Propionate	1.875±0.645^{c}	4.613±2.913^{c}	4.700±3.273^{c}	6.475±2.038^{bc}	8.235±2.647^{bc}	13.079±4.607^{b}	21.165±8.429^{a}	0.007	0.016
Valerate	0.065±0.159^{b}	1.102±1.444^{b}	0.856±0.958^{b}	0.723±0.818^{b}	1.592±0.975^{b}	1.596±1.271^{b}	3.902±2.582^{a}	0.017	0.033
TotalVFA	20.313±5.329^{d}	50.865±17.63^{cd}	49.009±18.403^{cd}	63.587±13.389^{cd}	80.568±14.289^{bc}	107.861±23.408^{b}	191.88±56.654^{a}	<.001	<.001

Values represent mean and standard deviation. [1]milk (M), milk plus concentrate (MC); [2]slaughter age (7, 28, 49 and 63 days); [3]Calves fed with only with milk (M) or milk plus starter concentrate (MC) that were slaughtered at 7, 28, 49 and 63 days of age; [*]*P*-value adjusted by FDR method; FDR ≤ 0.05 were considered significant. Means between groups followed by the same letter are not significantly different ($P > 0.05$) by Tukey HSD test.

Table S7. Significant correlations (Spearman´s rank) between molar proportions of volatile fatty acids (acetate, propionate, butyrate, total VFA, acetate-to-propionate ratio) and relative abundance of archaeal, bacterial and fungal taxa in the rumen of dairy calves grouped according diet and age.

Group[1]	Association[2]	Spearman	*P*-value
M_07d	Acetate-Propionate:BAC|g_Coprococcus	0.83	0.042
	Acetate-Propionate:BAC|g_Fusobacterium	0.90	0.015
	Acetate-Propionate:BAC|g_Paraprevotella	0.90	0.016
	Butyrate:BAC|g_Fusobacterium	0.89	0.017
	Total VFA:BAC|g_Lactobacillus	0.94	0.004
	Total VFA:BAC|g_Streptococcus	0.86	0.030
M_28d	Butyrate:BAC|f_Coriobacteriaceae	0.92	0.010
	Butyrate:BAC|f_Ruminococcaceae	0.89	0.018
	Butyrate:BAC|f_S247	0.94	0.005
	Butyrate:BAC|g_Butyrivibrio	0.90	0.014
	Butyrate:BAC|g_Eubacterium	0.95	0.004
	Butyrate:BAC|g_Prevotella	0.87	0.024
	Butyrate:BAC|p_Bacteroidetes	0.88	0.021
	Propionate:FUN|f_Neocallimastigaceae	0.82	0.044
	Total VFA:FUN|g_Orpinomyces	0.82	0.045
M_49d	Acetate:BAC|f_S247	-0.76	0.047
	Acetate:BAC|f_Veillonellaceae	-0.85	0.014
	Acetate:BAC|g_Acidaminococcus	-0.77	0.042
	Acetate:BAC|g_Bulleidia	-0.86	0.014
	Acetate:BAC|g_Butyrivibrio	-0.91	0.005
	Acetate:BAC|g_Pseudoramibacter_Eubacterium	-0.78	0.037
	Acetate:BAC|g_Shuttleworthia	-0.85	0.015
	Acetate:BAC|g_Succiniclasticum	-0.87	0.011
	Acetate:BAC|g_Succinivibrio	-0.77	0.042
	Acetate-Propionate:BAC|f_Neisseriaceae	0.88	0.008
	Acetate-Propionate:BAC|g_Butyrivibrio	-0.77	0.041
	Acetate-Propionate:BAC|g_Fusobacterium	0.80	0.030
	Acetate-Propionate:BAC|g_Lactobacillus	0.79	0.034
	Acetate-Propionate:BAC|g_Streptococcus	0.89	0.007
	Butyrate:BAC|f_Coriobacteriaceae	0.77	0.041
	Butyrate:BAC|f_S247	0.81	0.028
	Butyrate:BAC|f_Veillonellaceae	0.92	0.004
	Butyrate:BAC|g_Bulleidia	0.82	0.024
	Butyrate:BAC|g_Butyrivibrio	0.81	0.029
	Butyrate:BAC|g_Pseudoramibacter_Eubacterium	0.81	0.029
	Butyrate:BAC|g_Succiniclasticum	0.83	0.020
	Butyrate:BAC|g_Succinivibrio	0.89	0.007
	Propionate:BAC|g_Butyrivibrio	0.80	0.032
	Total VFA:BAC|f_Paraprevotellaceae	0.76	0.045
	Total VFA:BAC|f_Veillonellaceae	0.80	0.032
	Total VFA:BAC|g_Acidaminococcus	0.80	0.031
	Total VFA:BAC|g_Bulleidia	0.93	0.002
	Total VFA:BAC|g_Butyrivibrio	0.87	0.010

	Total VFA:BAC|g_Pseudoramibacter_Eubacterium	0.87	0.012
	Total VFA:BAC|g_Shuttleworthia	0.91	0.004
	Total VFA:BAC|g_Succiniclasticum	0.89	0.008
M_63d	Acetate-Propionate:BAC|g_Bulleidia	0.97	0.002
	Acetate-Propionate:BAC|g_Succiniclasticum	0.85	0.030
	Acetate-Propionate:BAC|p_Proteobacteria	0.92	0.010
	Acetate-Propionate:FUN|g_Neocallimastix	0.85	0.032
	Butyrate:BAC|g_Parabacteroides	0.90	0.015
	Propionate:BAC|f_Ruminococcaceae	-0.86	0.030
MC_28d	Acetate:BAC|g_Desulfovibrio	-0.88	0.020
	Acetate-Propionate:BAC|g_Megasphaera	0.89	0.018
	Acetate-Propionate:BAC|g_Ruminococcus	-0.84	0.038
	Propionate:BAC|f_Ruminococcaceae	0.88	0.021
	Propionate:BAC|g_Fusobacterium	0.85	0.033
	Propionate:BAC|g_Lactobacillus	0.81	0.049
	Propionate:BAC|g_Megasphaera	-0.83	0.043
	Propionate:BAC|g_Odoribacter	0.85	0.031
	Propionate:BAC|g_Ruminococcus	0.85	0.031
	Propionate:BAC|p_Proteobacteria	0.85	0.034
	Total VFA:BAC|g_Desulfovibrio	0.89	0.019
MC_49d	Acetate:BAC|g_Bacteroides	0.88	0.004
	Acetate:BAC|g_Coprococcus	0.75	0.031
	Acetate:BAC|g_Lactobacillus	0.84	0.009
	Acetate:BAC|p_Bacteroidetes	0.77	0.024
	Acetate-Propionate:BAC|f_Paraprevotellaceae	0.74	0.035
	Acetate-Propionate:BAC|g_Bacteroides	0.97	<.001
	Acetate-Propionate:BAC|g_Coprococcus	0.94	0.001
	Acetate-Propionate:BAC|g_Lactobacillus	0.98	<.001
	Acetate-Propionate:BAC|g_Paraprevotella	0.77	0.026
	Acetate-Propionate:BAC|p_Bacteroidetes	0.92	0.001
	Acetate-Propionate:BAC|p_Proteobacteria	0.82	0.013
	Butyrate:BAC|g_Prevotella	0.71	0.047
	Propionate:BAC|g_Bacteroides	-0.76	0.030
	Propionate:BAC|g_Lactobacillus	-0.75	0.030
	Total VFA:BAC|f_Ruminococcaceae	-0.89	0.003
MC_63d	Acetate:BAC|f_Paraprevotellaceae	-0.92	0.010
	Acetate:BAC|g_Butyrivibrio	-0.84	0.036
	Acetate:BAC|g_Succinivibrio	-0.91	0.012
	Acetate-Propionate:BAC|f_Neisseriaceae	0.89	0.019
	Acetate-Propionate:BAC|g_Bacteroides	0.89	0.019
	Acetate-Propionate:BAC|g_Fusobacterium	0.89	0.019
	Acetate-Propionate:BAC|g_Lactobacillus	0.88	0.020
	Acetate-Propionate:BAC|g_Odoribacter	0.82	0.044
	Acetate-Propionate:BAC|g_Oscillospira	0.85	0.032
	Acetate-Propionate:BAC|g_Parabacteroides	0.87	0.025
	Acetate-Propionate:BAC|g_Paraprevotella	0.84	0.034
	Acetate-Propionate:BAC|g_Streptococcus	0.81	0.049
	Butyrate:BAC|f_Paraprevotellaceae	0.87	0.025
	Butyrate:BAC|g_Succinivibrio	0.83	0.043
	Propionate:ARC|g_Methanobrevibacter	-0.83	0.039

	Propionate:ARC|g_Methanosphaera	0.92	0.010
	Propionate:BAC|f_Lachnospiraceae	0.83	0.040
	Propionate:BAC|f_Rikenellaceae	-0.85	0.033
	Propionate:BAC|f_Ruminococcaceae	-0.82	0.046
	Propionate:BAC|f_S247	0.82	0.046
	Propionate:BAC|g_Bulleidia	0.84	0.038
	Propionate:BAC|g_Campylobacter	-0.87	0.026
	Propionate:BAC|g_Odoribacter	-0.84	0.037
	Propionate:BAC|g_Pseudoramibacter_Eubacterium	0.87	0.024
	Propionate:FUN|f_Neocallimastigaceae	-0.86	0.028
	Propionate:FUN|g_Caecomyces	0.91	0.013
	Total VFA:BAC|f_Paraprevotellaceae	0.83	0.043
	Total VFA:BAC|g_Succinivibrio	0.85	0.034
	Total VFA:BAC|p_Firmicutes	0.90	0.015

[1]Calves fed with whole milk (M) or whole milk and starter concentrate (MC) slaughtered at 7, 28, 49 and 63 days old; [2]ARC (archaea); BAC (bacteria), FUN (fungi);

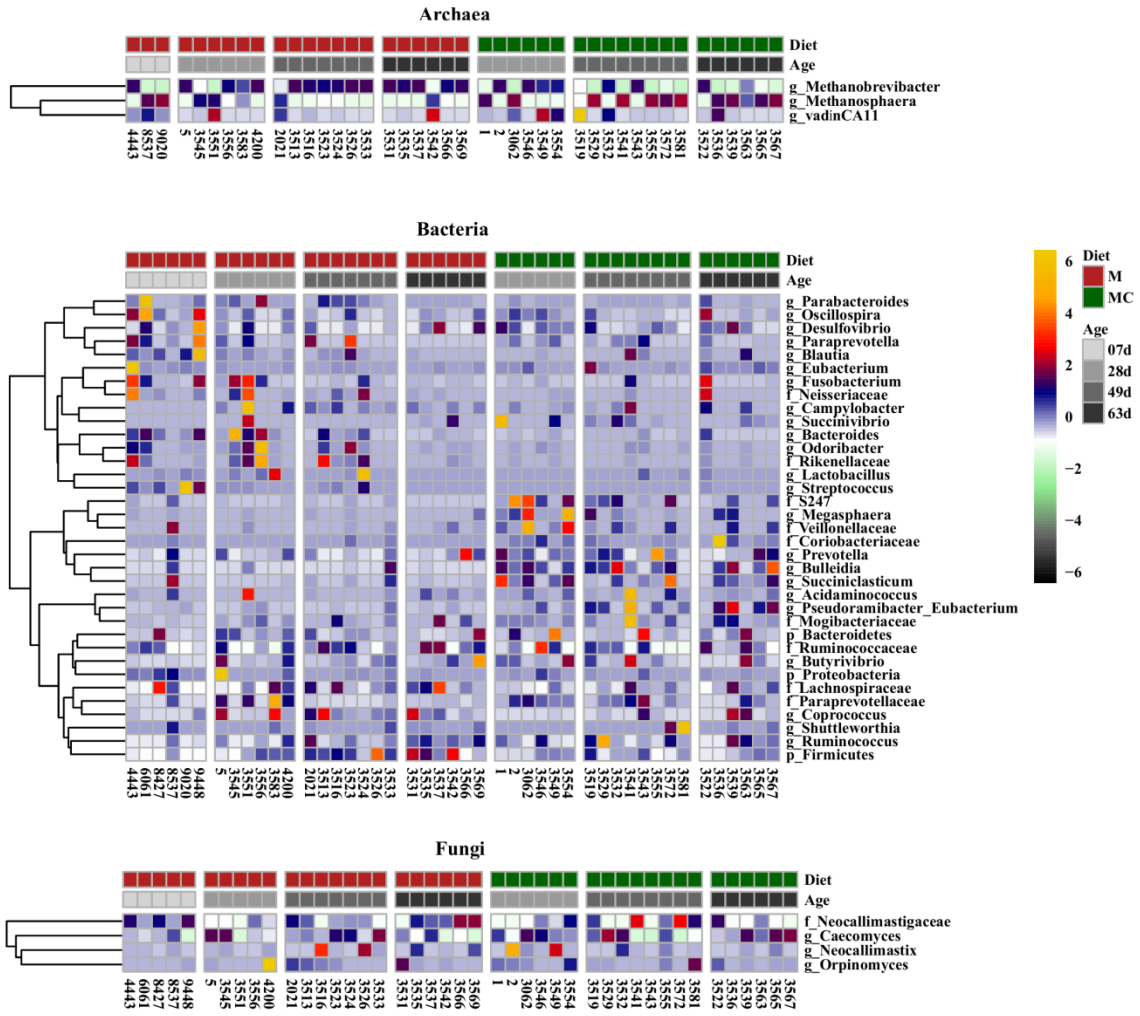

Figure S1. Distribution of the most abundant archaeal, bacterial and fungal taxa (at relative abundance ≥0.5% in at least one sample and present in at least 50% of all samples) among individual calves according diet and age group. Colors in the horizontal bars at the top of the plot represent diet (M: dark red, MC: dark green) and age (7 days: light grey, 28 days: grey medium, 49 days: grey medium-dark and 63 days old: dark grey). The scale (Z-score) represents the relative abundance at gradient of color from dark grey (low abundance) to gold (high abundance). The hierarchical dendrogram was established through Minkowski distances of the taxa along the y axis and Ward.D2 linkage clustering method.

CHAPTER 2

Changes in the bacterial community of gastrointestinal tract of dairy calves across pre-weaning development

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Abstract

The bacterial community play several roles in the gastrointestinal tract (GIT) influencing the performance and health of pre-ruminants. However, little is known about the establishment of the bacterial communities in the GIT of calves, as well as the changes in their structure and abundance during calf development into a functional ruminant. Here, next generation sequencing was used to characterize and assess changes in the bacterial communities in the rumen, jejunum, cecum and colon of 17 crossbred calves in four developmental stages (7, 28, 49 and 63 days old) that were fed with raw milk and starter concentrate. Alpha diversity analysis showed that richness and evenness of the bacterial community was lower in the jejunum compared to other GIT regions. Beta diversity analysis showed the existence of regional segregation and succession of species in the calf GIT evidenced by low number of OTUs shared inter-compartments within given age group and inter-age within given compartment. Therefore, each GIT region harbours a distinct bacterial community that become less heterogeneous as calf aged. Taxonomic composition analysis showed that several bacterial taxa commonly found in the mature GIT coexist in the rumen, jejunum, cecum and colon of milk-fed calves 7 days old, confirming that bacterial colonization in the GIT occur regardless presence of solid substrate. In addition we observed that region and age drive changes in the relative abundance of bacterial communities in the calf GIT. In the rumen, bacterial community was composed majority by members from genera *Prevotella, Butyrivibrio* and *Ruminococcus* whose abundance increased proportionally with age possibly due to greater availability of readily fermentable carbohydrates in the rumen. Members from genus *Lactobacillus* were overrepresented in the jejunum but their predominance was replaced by members from Clostridiaceae family in older calves. Similar shift in the abundance of *Lactobacillus* and Clostridiaceae was observed in the cecum and this result suggests an inverse relationship among *Lactobacillus* colonization in the calf gut with increase of starter concentrate intake. The bacterial communities in the cecum and colon were composed by members from genera *Blautia*, *Paraprevotella* and *Prevotella* whose abundance increased with age. The abundance of *Prevotella* (succinate-producer) in these sites may have favoured the colonization of *Phascolarctobacterium* and *Succiniclasticum* (succinate-utilizing) whose abundance also increased as the calf age. This work adds new insights about bacterial colonization in the developing GIT that may be useful in formulating strategies to improve health and performance of dairy calves in pre-weaning stage.

Introduction

At birth, calves display an immature gastrointestinal tract (GIT) that is quickly colonized by archaeal, bacterial, and fungal communities (Fonty et al., 1987; Rey et al., 2014; Guzman et al., 2015) acquired from maternal (i.e. vagina, skin and nursing) and environmental (i.e. colostrum, milk and housing) sources during and after delivery (Taschuk and Griebe, 2012). Bacterial communities constitute a predominant and diverse group that plays several roles in the ruminant GIT, including fiber degradation, modulation of the immune system (Bauer et al., 2006), rumen morphological development (Warner et al., 1956) and supply of nutrients like volatile fatty acids (VFAs) and protein (Armstron and Smithard, 1979).

The rumen of neonate calves is underdeveloped in terms of volume, absorptive capacity and metabolism (Davis and Drackley, 1998; Baldwin et al. 2004) and become functional during process of transition of pre-ruminant to true ruminant which is triggered by solid substrate arrival and microbial colonization within rumen. The rumen development constitutes a prerequisite to anticipate weaning and reduce feeding cost; thus, dairy calves raised in commercial herds are fed restricted amounts of liquid feed (milk or milk replacer) to stimulate the intake of solid feeds (starter concentrate).

As solid feed intake and bacterial activity increases (i.e. butyrate synthesis), the rumen increases in surface area through increases in volume and papillation. Additonally, the main site of digestion and absorption shifts from the intestine to the rumen, and the calf's metabolism switches from primarily glucose to VFA utilization. Non-rumen development also occurs with changes in intestinal enzyme activity from lactase to maltase as well as development of the salivary apparatus and rumination behavior (Warner et al., 1956; Baldwin et al., 2004; Guilloteau et al., 2008; Khan et al., 2015).

Previous studies reported changes in the rumen and fecal microbiota from birth until weaning (Jami et al., 2013; Rey et al., 2014; Uyeno et al., 2010; Oikonomou et al., 2013). However, bacterial changes in other GIT regions and their correlation with performance and disease incidence at pre-weaning period remain poorly explored. Apart from pioneer studies of Malmuthuge et al. (2014) and Guzman et al. (2016), no sequencing-based analysis has been performed to characterize bacterial communities throughout the GIT of pre-weaned calves. Although extremely important, these studies were performed with pooling of samples from 3 week-old calves (Malmuthuge et al., 2014) and with target species (Guzman et al., 2016), that respectively prevents

identification of inter-animal variation and provide a restricted picture of bacterial diversity existing in the calf GIT.

To address these gaps in knowledge, we utilized next-generation sequencing to assess changes in the bacterial community of gastrointestinal tract (rumen, jejunum, cecum and colon) of dairy calves across pre-weaning development (7, 28, 49 and 63 days old). We hypothesized that both diet composition (i.e. milk-based to solid diet) and GIT development (i.e. rumen development) drive changes in the bacterial communities during calf transition into a functional ruminant.

Material and Methods

Animals Experiment and Sampling

All animal procedures were conducted according to the protocols approved by the University of Viçosa, MG-BR Animal Care and Use Committee, process number 27/2013. The study was conducted at the Experimental Field of Embrapa Gado de Leite, located in Coronel Pacheco/MG, Brazil from October 2013 to April 2014. A total of 17 newborn male crossbreed (3/4 to7/8 Holstein-Gyr) dairy calves from a fixed-time insemination protocol were locally obtained shortly after birth (within 24 h). The calves were weighed (36.4±3.7 kg), identified, and housed in individual stalls with *ad libitum* access to water. Colostrum from their dams was offered at 10% of body weight at birth (BWB), fractionated into two daily meals (morning and afternoon) until the 3rd day of life. Calves were randomly distributed to one of the age group: 7 (n=4), 28 (n=4), 49 (n=4) and 63d (n=5) and fed with raw milk to 10% of BWB plus starter concentrate *ad libitum* until the slaughter (Table 1). Given that starter concentrate intake is negligible at first week of life, calves slaughtered at 7 days old were fed exclusively with colostrum (until 3rd day) and raw milk (from 4th until the 6th day of life). The calves at respective age group were euthanized with an injection of Acepromazine (0,013 mg/kg), Thiopental (0,125 mg/kg), and Potassium chloride (80 to 120 mL). After euthanasia, the body cavity was opened and the rumen, jejunum, cecum and colon were isolated with polyethylene seal (zip locks) to avoid reflux of ingesta among compartments. Then, 50 mL of ingesta from each GIT region was collected and immediately transported on wet ice and stored at -80°C prior to DNA extraction.

DNA Extraction and Sequencing

Total genomic DNA was extracted from GIT fluid samples following the procedures described by Stevenson and Weimer (2007). Briefly, microbial cells were

collected by centrifugation and lysed by heating and mechanical disruption. DNA was purified by phenol and phenol:chloroform:isoamyl alcohol extraction and resuspended in TE buffer. DNA was quantified by Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and shipped on dry ice to the University of Wisconsin-Madison to perform to sequencing. The V3-V4 hypervariable regions of the bacterial 16S rRNA (Klindworth et al., 2013) were amplified along with Illumina sequencing primers. PCR reaction consisted of 50 ng template DNA, 0.4 μM of each primer, 1X Kapa Hifi HotStart ReadyMix (KAPA Biosystems), and water to 25 μL. The PCR reaction was performed at 95°C for 3 minutes, 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds (25 cycles) and a final extension step at 72°C for 5 minutes. PCR products were purified by PureLink™ Pro 96 PCR Purification Kit (Invitrogen) and a second PCR was performed on products to attach Illumina sequencing adapters and unique dual indices. PCR reaction was composed by 5 μL of unquantified PCR product used as template DNA and performed with 8 cycles. PCR products were recovered by gel extraction in AquaPōr LM low-melt agarose (National Diagnostics, Atlanta, GA) and with Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Purified DNA was quantified by Qubit® Fluorometer (Invitrogen) and equimolar pooled to create a single sample at 1×10^9 ng per μL. Sequencing was performed using the 2x300 bp paired-end method on the Illumina MiSeq following manufacturer's guidelines (Illumina, Inc., San Diego, CA, USA).

Bioinformatics Analysis

The sequences were processed using mothur (v1.35.0) with procedures modified from Schloss et al. (2009). Briefly, sequences with a length shorter than 250 bp or longer than 600 bp containing ambiguous characters or exhibiting a homopolymer greater than 8 bp were removed. Sequences were aligned using the SILVA 16S rRNA gene reference database (Pruesse et al., 2007) and pre-clustered to remove sequencing error. The Uchime algorithm was used to detect chimeric sequences (Edgar et al., 2011) and sequences that did not align to the correct region or were chimeric were removed. The sequences were classified using the GreenGenes database (DeSantis et al., 2006) and grouped into operational taxonomic units (OTUs) by uncorrected pairwise distances clustered by the nearest neighbor method with a similarity cutoff of 97%. Coverage was assessed by Good's coverage calculated in mothur and due to different sequencing depth, OTUs table was normalized to equal sequence counts (1959 sequences per

sample) and used to determine the alpha diversity indexes (Chao1's richness, Shannon´s and inverse Simpson´s diversity) and relative abundance of OTUs and higher taxon (sequences of OTU or taxa/total sequences in the sample) in each sample.

Statistical Analysis

All statistical analyses and plots were carried out in R (v.3.2.3) using functions available in the packages: car (Fox and Weisberg, 2011), MASS (Venables and Brian, 2013), multcomp (Hothorn et al., 2016), vegan (Oksanen et al., 2013), VennDiagram (Chen, 2011), pheatmap (Kolde, 2015) and ggplot2 (Wickham, 2009).

Differences in the alpha diversity (Chao1, inverse Simpson's and Shannon's diversity indexes) and relative abundance of bacterial communities were analyzed using a repeated-measures mixed model (Anova, type III error), including GIT region, age group and their interaction (GIT*age) as fixed effects and calf as the random effect. The means were compared using Tukey's Honestly Significant Difference test. The *P*-values were adjusted to False Discovery Rate (FDR) using the Benjamini-Hochberg method and values below 0.05 were considered significant. Only taxa that represented ≥0.1% in at least one sample and that were detected in at least 50% of all samples were assessed.

Beta-diversity was assessed by Venn diagrams and Canonical Analysis of Principal Coordinates (CAP). Venn diagrams were built using OTUs at a relative abundance > 0.1% in at least one sample and that were detected in at least two samples in each group (GIT*Age). In the CAP analysis, OTUs were clustered by the Bray-Curtis dissimilarity index and a permutation test (nperm=999) was performed to assess the significance of model factors (GIT region, age group and GIT*age) and constrained axis (CAP1 and CAP2). The dissimilarities ordinated by CAP were plotted with ellipses defined by standard deviation with a 95% confidence limit.

Results

Sequencing

After sequence trimming, quality filtering and removal of chimeras, a total of 2,173.784 high-quality sequences (mean 32936.12±SE 3278.94 per sample) and 44,436 OTUs (mean 673.27±SE 52.53 per sample) clustered at 97% similarity were obtained in current study. After normalization, remained 129,294 (1,959 sequences per sample) and 3,655 OTUs (mean 178.67 ± SE 10.13 per sample). The Good's coverage estimator ranged from 95 to 100% indicating that the sequences sufficiently covered the diversity of bacterial community in the GIT of calves (Table 2).

Alpha diversity

Alpha diversity that refers to diversity of bacterial OTUs of each GIT region was represented by Chao1's richness, Shannon´s and inverse Simpson´s diversity indexes that varied significantly across GIT regions (P=0.003, <0.001, <0.001). Overall, jejunum bacterial community showed lower richness and evenness (Tukey HSD, P<0.05) among GIT regions. In contrast, alpha diversity indexes did not change significantly according to age (P=0.745, 0.116, 0.409) and only Shannon's diversity index varied according to GIT*Age (P=0.026) and increased significantly within jejunum and cecum (TukeyHSD, P<0.05) of calves at 63 days (Table 3).

Beta diversity

Beta diversity indicates the degree of differentiation among communities, and it was assessed by two approaches: Venn diagram and Canonical Analysis of Principal Coordinates (CAP). Out of 3655 OTUs, 1408 displayed abundance ≥0.1% in at least one sample and Venn diagrams revealed that only 39, 36, 49 and 55 OTUs (identified in at least two calves in each age group) were shared, respectively, by rumen, jejunum, cecum, and colon samples of calves at 7, 28, 49 and 63 days of age (Figure 1A). Regardless of the age, jejunum samples exhibited the lowest number of unique OTUs and shared the least number of OTUs with other GIT regions. In contrast, the number of OTUs shared by colon and cecum, and by colon and rumen tended to increase with age (Figure 1A). Further, Venn diagrams revealed that only 6 (rumen), 7 (jejunum), 20 (cecum) and 30 (colon) OTUs were shared across age groups (Figure 2A).

CAP analysis showed that Bray-Curtis dissimilarities in the bacterial composition were ascribed to GIT region and age (Monte Carlo permutation test, P<0.001, 0.002) and of calves (Figures 1B and 2B). The segregation of bacterial communities through the GIT of calves was evident since the first week of age given that rumen, jejunum and cecum samples were clustered separately (Figure 1B). However, the decreasing distance among rumen, jejunum and colon samples of calves 63 days old shows that bacterial community composition tend to be less heterogeneous with age (Figure 1B). The age effect was evident in each GIT region, mainly among calves at 7 and 63 days of age among whose samples clustered separately (Figure 2B).

Taxonomic composition

A total of 27 phyla, 119 families or 189 genera were identified at GIT samples of dairy calves. The most abundant phyla, families and genera (with average abundance

≥1% and present in at least 50% of all samples) among GIT region of calves in their respective age group are displayed at Figure 3. Additionally, the distribution of the most abundant bacterial taxa among individual calves is shown at heat map (Figure 2). At rumen, all sequences were assigned to some phylum, whereas 11.95±11.68% and 45.95±18.54% of sequences could not be assigned to any family and genus levels respectively. A total of 7 phyla, 15 families and 11 genera displayed abundance over 1% among them are highlighted the phyla Firmicutes (46.95±3.64), Bacteroidetes (29.85±3.37) and Actinobacteria (12.62±4.28) that include the families Ruminococcaceae (13.77±2.64) and Prevotellaceae (13.03±2.63) and Lachnospiraceae (8.41±1.68), as well as, the genera *Prevotella* (14.5±2.5), *Ruminococcus* (4.8±1.9) and *Bacteroides* (4.0±1.7) (Figure 3). At jejunum, 2.49% (±2.80) and 31.86% (±26.48) of sequences were not assigned to any family and genera respectively. Only 4 phyla, 10 families and 4 genera displayed abundance over 1% and the majority of sequences were assigned to phylo Firmicutes (87.47±2.85), families Lactobacillaceae (54.71±8.88) and Clostridiaceae (13.47±3.97) and genus *Lactobacillus* (54.7±8.9) (Figure 3).

Like observed at rumen, cecum and colon samples displayed high percentage of sequences unsigned to family (10.70±9.27%; 16.33±14.14%) and genus levels (44.68±11.86%; 54.85±14.75%), respectively. A total of 5 and 4 phyla, 18 and 13 families, 12 and 9 genera with relative abundance over 1% were identified at cecum and colon, respectively. The most abundant phyla at cecum and colon were, respectively, Firmicutes (65.15±4.28; 73.6±12.94), Bacteroidetes (23.75±4.24; 18.53±2.91) and Proteobacteria (5.92±3.00; 3.71±1.65) that includes families Lachnospiraceae (18.27±3.43; 18.16±2.95), Ruminococcaceae (17.01±3.37; 18.75±2.45) and Lactobacillaceae (9.14±2.33; 7.08 ±2.14). Finally, the genera *Lactobacillus* (9.14±2.33; 7.08±2.14) follow by *Prevotella* (8.16±2.55; 5.80±1.37), *Bacteroides* (7.68±2.24; 5.68±2.21) and *Blautia* (5.32±1.81; 5.12±1.05) were the most abundant genera in the cecum and colon, respectively (Figure 3).

Changes in the bacterial community

A total of 8, 6 and 32 bacterial taxa varied significantly according to GIT region, age and their interaction, respectively (Table 4). Regarding to GIT and age effects, the relative abundance of phyla Bacteroidetes and Firmicutes varied significantly according GIT region (P<.001;<.001) and age group (P=0.020; 0.004). Members from phylo Bacteroidetes and Firmicutes were significantly less abundant in the jejunum and

regardless the GIT region the abundance of these taxa increased as the calves aged (Table 4).

In addition, the relative abundance of Ruminococcaceae family varied exclusively according GIT ($P<.001$) while Enterobacteriaceae varied according to GIT ($P=0.002$) and age ($P=0.001$). Members from Ruminococcaceae family were more abundant in the cecum and colon compared to rumen and jejunum while members from Enterobacteriaceae family were more abundant in the jejunum and cecum compared to the rumen. Regardless of the GIT region, the abundance of members from Enterobacteriaceae family decresead significantly as the calves aged (49 and 63 days) (Table 4).

Lastly, the genera *Clostridium* and *Oscillospira* varied exclusively with GIT region ($P<.001$; $<.001$) and were more abundant in the cecum and colon. In addition, the abundance of genera *Butyrivibrio,* and *Ruminococcus* varied according GIT region ($P<.001$; 0.004) as well as according to age ($P=0.011$; 0.018). The genus *Butyrivibrio* was more abundant in the rumen while members from genus *Ruminococcus* were more abundant in the rumen and colon compared to jejunum and cecum. Regardless GIT region, the abundance of *Butyrivibrio* and *Ruminococcus* increased as the calves aged.

Regarding to interaction (GIT*age) effect, the genera *Bacteroides* ($P=0.019$), *Blautia* ($P<.001$), *Desulfovibrio* ($P=0.031$), *Dorea* ($P=0.021$), *Parabacteroides* ($P<.001$), *Paraprevotella* ($P<.001$), *Phascolarctobacterium* ($P<.001$) and *Streptococcus* ($P=0.003$) were abundant ($\geq$1%) in the rumen of calves at 7 days but decreased marked with increase of age. In addition, it was observed that relative abundance of members from families Coriobacteriaceae ($P<.001$), Lachnospiraceae ($P=0.014$) and Mogibacteriaceae ($P=0.019$) as well as genera *Bulleidia* ($P<.001$), *Succiniclasticum* ($P<.001$) and *Sharpea* ($P=0.016$) increased markedly in the rumen of older calves (49 and or 63 days). Lastly, the genera *Bifidobacterium* ($P<.001$), *Megasphaera* ($P<.001$) and *Succinivibrio* ($P<.001$) were present in low proportions in the rumen of calves at 7 days old and their abundance increased in the rumen of calves at 28 and 49 days and subsequently decreased at 63 days (Table 4).

Like observed in the rumen, the abundance of genera *Bacteroides*, *Blautia* and *Parabacteroides* decreased while the abundance of Coriobacteriaceae, Lachnospiraceae and *Bulleidia* increased in the jejunum of calves at 63 days. In addition, the abundance of genus *Lactobacillus* ($P=0.045$) decreased while members from families Clostridiaceae ($P=0.005$), Peptostreptococcaceae ($P <.001$) and Synergistaceae (P

<.001) and genera *Eubacterium* (*P*<.001) and *Turicibacter* (*P*<.001) increased markedly in the jejunum of calves at 63 days (Table 4).

In the cecum and colon, similar changes in the relative abundance of bacterial taxa were observed. The abundance of members from families Coriobacteriaceae and S24-7 (*P*=0.039) as well as genera *Coprococcus* (*P*<.001), *Paraprevotella*, *Prevotella* (*P*=0.036) and *Phascolarctobacterium* increased in the cecum and colon of older calves. Like in the jejunum, the abundance of Clostridiaceae increased while *Lactobacillus* decrased markedly in the cecum and colon of calves at 63 days. In contrast to the rumen and jejunum, the abundance of *Bacteroides* (*P*=0.019) and *Dorea* (*P*=0.021) in the cecum and colon remained high and unchanged across the developmental stages of calves (Table 4).

In the cecum, the abundance of *Parabacteroides* increased with age while the abundance of *Blautia* increased after beginning of starter concentrate intake (28 days) but decreased in older calves (63 days). Like observed in the rumen and jejunum, the abundance of members from Lachnospiraceae family (*P*=0.014) increased in the cecum of older calves. In contrast, the abundance of *Parabacteroides* increased and subsequent decreased in the colon of calves at 63 days while the abundance of *Blautia* in the colon remained high and unchanged across the developmental stages of calves (Table 4).

Lastly, the abundance of Erysipelotrichaceae family (*P*<.001) as well as genus *Collinsella* (*P*<.001) increased after beginning of starter concentrate intake (28 and 49 days) but decreased in the colon of older calves (63 days). Like observed in the rumen, the abundance of genus *Succiniclasticum* (*P*<.001) increased in the colon of older calves but remained low or unchanged in the jejunum and cecum. In contrast to the rumen, the genera *Megasphaera* (*P*<.001) and *Streptococcus* (*P*=0.003) were observed in low proportion in the colon of calves at 7 days but its abundance increased in calves at 63 days. Like observed in the jejunum, the abundance of genus *Turicibacter* (*P*<.001) increased markedly in the colon of calves at 63 days and remained low and unchanged in the rumen and cecum.

Discussion

Information related to microbiota composition in the calf GIT has been mostly restricted to the bacterial community in the rumen and feces of young ruminants (Li et al., 2011; Wu et al., 2012; Rey et al., 2014; Uyeno et al., 2010; Oikonomou et al., 2013; Dill-McFarland et al., 2017). This is due to the fact that the rumen is the major fermentation site in adult ruminants and because feces constitute a non-invasive

sampling technique and has been employed such as proxy for the GIT microbiota (Tapio et al., 2016). Overall, little is known about bacterial community in other compartments of the GIT, their potential roles in the host development and how the gradual replacement of calf's diet (from birth until weaning) affects their composition and abundance. To our knowledge, this is the first study performed in tropical environment that employed next-generation sequencing to analyze bacterial community in the GIT (rumen, jejunum, cecum and colon) of calves across pre-weaning development.

In our study, the structure and abundance of bacterial community varied markedly among individual calves under the same diet and age group (Figure 3). Indeed, the inter-individual variability is a hallmark of calf microbiota studies and suggests the host effect (Li et al., 2011; Wu et al., 2012; Rey et al., 2014; Zhou et al., 2014). For example, calves in our study had little maternal contact after birth, were individually housed and their microbial exposition was limited to surrounding environment whose influence is less apparent in the first days of life, thus the large inter-animal variation observed in calves 7 days of age suggests that the host plays a role on the selection of microbial community colonizing the GIT tract (Weimer, 2015).

Alpha diversity analysis showed that bacterial community in the jejunum was less diverse compared to other GIT region (Table 2). This result is consistent with previous studies performed with calves and steers (de Oliveira et al., 2013; Malmuthuge et al., 2014; Myer et al., 2016) and may be related to shorter retention time of digesta in the jejunum of calves (2 to 8hr) compared to other compartment such as the rumen (11-17hr) (Smith, 1964; Oura et al., 1986). Indeed, retention time of digesta may determine indigenous bacteria colonization as well as the degree of microbial fermentation (Stevens and Hume, 1998).

Beta-diversity analysis showed that few OTUs were shared among rumen, jejunum, cecum and colon (Figure 1A), confirming the existence of bacterial segregation throughout the calf GIT (Malmuthuge et al., 2014). Indeed, each GIT region shows peculiar characteristics (i.e. morphology, pH, secretions, rate of passage of digesta, physical structure and size of particle) and harbours distinct microbial communities that co-evolved symbiotically with the host to occupy different niches in these micro-environments (Stevens and Hume, 1998; Shapira, 2016).

The regional segregation observed in our study suggest that bacterial community at distal region (i.e. colon) represent a small part of the community at proximal GIT (i.e. rumen and jejunum) and reinforce that faecal samples may not be useful as proxy of gut

microbiota (Malmuthuge et al., 2014; Guzman et al., 2016; Tapio et al., 2016). Besides regional segregation, we observed that bacterial populations within each GIT region display fluctuations in terms of composition (i.e. emergence and vanishing of species) along of developmental stages of the young ruminant. However, bacterial community composition became less heterogeneous with increase of age due to greater number of OTUs shared among calves 49 and 63 days old (Figure 2A), corroborating previous reports (Uyeno et al., 2010; Malmuthuge et al., 2012).

In young ruminants, the age effect encompasses sub-effects such as replacement of calf´s diet (i.e. milk-origin vs. vegetable-origin protein), increase of solid feed intake, modifications in the digestive system (i.e. rumen development) and cross-domain interactions that, collectively, "selects" microbial communities adapted to new substrates and becomes an intrinsic characteristic of each compartment (Le Huerou et al., 1992; Bauer et al., 2006; Guilloteau et al., 2008; Comolli, 2014). These factors apparently "explain" the decreased of dissimilarities in the bacterial communities among older calves.

The taxonomic composition analysis showed that several bacteria (i.e. genera *Bacteroides*, *Prevotella, Ruminococcus* and *Streptococcus*) commonly found in the mature GIT (de Oliveira, 2013; Mao et al., 2015; Myer et al., 2016) and playing prominent roles in the degradation of fibre and starch (Purushe et al., 2010; Rainey, 2009), were identified in the rumen, jejunum, cecum and colon of calves 7 days old (Figure 4), reinforcing that bacterial colonization in the calf GIT occurs prior to solid feed intake (Rey et al., 2014; Guzman et al., 2016). Although a phylogenetically similar bacterial community coexist in distinct sites such as the rumen, jejunum and colon of neonate calves, their abundance vary markedly among compartments and developmental stages of the animal.

In the rumen, bacterial community was dominated by taxa (i.e. *Butyrivibrio, Prevotella and Ruminococcus*) endowed of specific and diverse fermentative capabilities related, direct and indirectly, to degradation of rapidly fermented carbohydrates that are commonly abundant in the calf starters (Table 4). In particular, the abundance of *Butyrivibrio* populations is especially important for morphological development of rumen due to production of butyrate that stimulates the differentiation of rumen epithelial increasing the absorptive surface (Cotta et al., 1986; Malhi et al., 2013).

In addition, we observed that rumen bacterial community changed as the calf develops. Members from genera *Bacteroides*, *Streptococcus* and *Parabacteroides* were

overrepresented in the rumen of neonate calves but their dominance decresead markedly in older calves (Table 4). Lastly, we observed the “expansion” of taxa related to production (*Bifidobacterium* and *Sharpea*) and utilization of lactate (*Megasphaera*) in the rumen of calves at 28 and 49 days old, possibly due to starter concentrate intake. Interestingly, the abundance of those genera was synchronized and decreased proportionally with increase of the age (Table 4). This result suggest that other factors (e.g. antagonistic interactions), beyond the availability of rapidly fermented carbohydrates, determine the abundance of *Bifidobacterium* and *Sharpea* populations in the developing rumen and highlight the importance of *Megasphaera* to prevent accumulation of lactic acid (Counotte et al., 1981) and minimize ruminal acidosis risk in calves during adaptation to starter concentrate intake (Quigley et al., 1992).

In contrast to rumen, bacterial community in the jejunum was dominated by members from genus *Lactobacillus* in calves 7 to 49 days old, but their abundance was replaced by members belonging to family Clostridiaceae and *Turicibacter* genus in calves 63 days old (Table 4). Similar results were observed in previous studies performed with calves and steers (Oikonomou et al., 2013; Meale et al., 2016; de Oliveira et al., 2013; Myer et al., 2016). This result suggests that gradual adaptation of calf GIT to increase of starter concentrate intake, specifically, the starch flow to small intestine, favouring the proliferation of Clostridiaceae-related groups other than *Lactobacillus*.

Members from genus *Lactobacillus* ferment simple sugars (i.e. lactose) and produce mainly lactate, an intermediate of several pathways of VFAs production. However, its ability to ferment starch is limited and strain-dependent (Satter and Esdale, 1968; Marounek et al., 1988; Gänzle and Follador, 2012). This fact apparently “explains" the decrease in the prevalence of *Lactobacillus* in the calf GIT as animals consumed more starter concentrate relative to milk. On the other hand, the increase of *Turicibacter* (lactate producer) in the jejunum of older calves may be direct and indirectly related to concentrate intake because *Turicibacter sanguinis* is specialized in the fermentation of maltose (produced from starch breakdown by amylase) and 5-ketogluconate (produced from glucose by some bacterial species) (Bosshard, 2002). Likewise, members from the Clostridiaceae family constitutes a large and heterogeneous group commonly found in the GIT of mammals with diverse capabilities related to degradation of complex carbohydrates, such as starch, which was abundant in the starter concentrate used in our study (Biddle et al., 2013).

Given the enzymatic and fermentative capabilities of members from Clostridiaceae family, we speculate that abundance of these populations at jejunum of older calves may be related to starch degradation at small intestine. In adult ruminant, starch that escape rumen fermentation reaches the small intestine and undergoes enzymatic hydrolysis by α-amylase and maltase produced by pancreas and intestinal mucosa. However, the activity of these enzymes in the small intestine of calves is very low and age-dependent (Le Huerou et al., 1992; Gilbert et al., 2015). Moreover, recent study verified that starch disappearance in the small intestine of calves is due to bacterial fermentation rather than enzymatic hydrolysis (Gilbert et al., 2015), but it remains to be determined which microbial species may be involved in this process.

In regards to cecum and colon, our results showed that bacterial communities in these compartments were similar in terms of composition and abundance (Figure 4). The cecum and colon are important fermentation sites of starch, fibre and protein from microbial/vegetal origin that escape the digestion in the proximal GIT (Armstron and Smithard, 1979). Bacterial communities in these sites were dominated by members from Ruminococcaceae family and genera *Bacteroides, Blautia, Lactobacillus* and *Prevotella* (Table 4). This results are consistent with previous studies performed with calves and cows (Oikonomou et al., 2013; Malmuthuge et al., 2014; Meale et al., 2016) and suggest that these taxa are part of the gut microbiota of ruminant (Rainey, 2009; Krieg et al., 2011).

The Ruminococcaceae family includes several genera whose specie are endowed of cellulolytic capability and play prominent role in the fibre degradation in rumen of adult ruminants (Rainey, 2009; Biddle et al., 2013). Further, species Ruminococcaceae-related are able to ferment simple and complex carbohydrates (i.e. lactose and starch) (Rainey, 2009) and such ability apparently “explain" their establishment in the calf GIT at absent of dietary fibre (Jami et al., 2013; Guzman et al., 2016).

Moreover, the presence and abundance of the genus *Blautia* in the cecum and colon (Table 4) may be important due to its acetogenic capability. Indeed, *Blautia* spp. (*B. producta, B. coccoides* and *B.schinkii*) isolated from rumen and feces of calves and lambs may ferment a variety of substrate such as H_2 and CO_2 to produce acetate in a pathway to compete with methanogens (Bryant et al., 1958; Rieu-Lesme et al., 1996; Fonty et al., 2007). Although, the methanogenesis is a process energetically more efficient than acetogenesis, a mixotrophic capability (grow sustained by simultaneous utilization of hydrogen and organic substrates) of some *Blautia* spp constitutes a competitive advantage over H_2-consuming methanogens (Cord-Ruwisch et al., 1988;

Breznak et al., 1991). Therefore, comprehensive characterization of *Blautia* spp endowed with mixotrophic capability isolated from calf GIT may drive efforts in formulating optimal strategies to mitigate enteric methanogenesis.

In addition, we observed the "expansion" of amylolytic and saccharolytic taxa such as *Paraprevotella* and *Prevotella* as well as propionate-producers like *Phascolarctobacterium* and *Succiniclasticum* in the cecum and colon of older calves (Table 4). These results suggest that these populations are responsive to increase of starter concentrate intake and highlight their importance to VFA production in calf gut. Under this model, increase of starter concentrate intake promoted the colonization of succinate-producers like *Prevotella* and *Paraprevotella* which in turn favoured the establishment of *Phascolarctobacterium* and *Succiniclasticum* species that are specialized in convert succinate to propionate (van Gylswyk, 1995, Watanabe et al., 2012), the most important precursor of glucose synthesis in ruminants (Reynolds, 2006).

Lastly, similar shifts in the abundance of *Lactobacillus* and Clostridiaceae observed in jejunum were also identified in the cecum and colon. This result suggests an inverse relationship between *Lactobacillus* colonization in the calf gut and increases in starter concentrate intake. Besides saccharolytic activity, some *Lactobacillus spp.* (i.e. *L. salivarius*) isolated from faeces of calves play important roles related to pathogen inhibition (i.e. *Salmonella* and *Escherichia* which cause diarrhea in calves) and modulation of immune responses (i.e. increases the calf's ability to respond to the *Salmonella* challenge) (Schneider et al., 2004; Yu et al., 2011; Frizzo et al., 2012; Soto et al., 2015). The probiotic capability of some *Lactobacillus* specie highlights the importance of their establishment in the cecum and colon, sites that can be colonized by bacterial pathogens causing diarrhea in calves. Further studies designed to characterize *Lactobacillus* isolated from calf gut are required to identify species with probiotic capability, as well as to assess factors (i.e. inverse relationship with starter concentrate intake) that affect their colonization in the developing GIT.

In summary, our results showed that although there are bacterial communities "common" to distinct regions, a closer look at their structure, abundance and dynamic reveals marked segregation and ecological succession in the calf GIT. Overall, bacterial communities in the calf GIT differ qualitatively and quantitatively among compartments and respond differently to age advance that encompass the progressive replacement of milk-based to grain-diet and GIT development. In addition, given the degree of instability in the bacterial community structure of younger calves (7 and 28 days old),

our results suggest that microbial-related interventions explored straight from birth (Abecia et al., 2013) may be not effective and economically viable. In this sense, considering that once established, the microbiota is resilient to changes in any direction (Weimer, 2015) and that dissimilarity in the bacterial community decrease while the intake of solid feed increases with age, we speculate that weaning transition (8-9 weeks of life) constitutes a potential window for GIT microbiota manipulation in dairy calves. This work adds new insights about bacterial colonization in the GIT of pre-ruminant that may be considered in formulating optimal strategies to promote the colonization of target communities aiming improve health and performance of dairy calves in pre-weaning stage.

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TABLE 1| Chemical composition of whole milk and starter concentrate fed to calves during experimental period.

Composition	Milk	Starter concentrate[1]
Dry matter (%)	12.32	89.33
Crude protein (DM basis %)	25.89	17.45
Ether extract (DM basis %)	37.64	1.940
Neutral Detergent Fiber (DM basis %)	-	12.99
Non-Fiber Carbohydrate (DM basis %)	31.23	62.62
Ashes (DM basis %)	5.240	5.01

[1]Contained 62.637% corn ground, 32.486% soybean meal, 3.094% wheat meal, 0.303% dicalcium phosphate, 1.123% limestone, 0.237% sodium chloride, 0.121% commercial mineral premix (180 g/kg Zinc sulfate , 150 g/kg Copper Sulfate, 10 g / kg Cobalt Sulfate, 10 g / kg sodium selenite and 10 g / kg Potassium Iodate. In spite of distribution from day 4, the starter concentrate intake began from day 8 and calves slaughtered at 28, 49 and 63 days of age consumed mean 188.684±0.013, 265.574±0.012 and 459.996±0.019 g of DMI concentrate/day.

TABLE 2| Summary of sequencing according to gastrointestinal tract (GIT) region and age group.

GIT	Age	Calves (n)	After filtering and clean-up			After normalization
			Sequence	OTUs	Coverage	OTUs
Rumen	07d	4	12170.75±2820.25	380.25±85.89	0.99±0.00	164.50±41.96
	28d	4	21025.00±10380.63	508.75±280.20	0.98±0.02	190.00±105.31
	49d	4	9330.75±2595.51	501.50±219.53	0.97±0.02	234.50±83.55
	63d	5	19442.80±14224.23	568.60±265.04	0.99±0.00	184.60±55.73
Jejunum	07d	4	40772.00±41728.19	406.50±254.45	0.99±0.00	131.25±82.68
	28d	4	27455.25±15222.08	261.75±160.55	1.00±0.01	68.75±23.92
	49d	3	50664.67±18067.01	417.00±187.01	0.99±0.01	74.00±44.17
	63d	5	18088.20±12125.40	417.60±210.31	0.99±0.01	142.00±33.64
Cecum	07d	4	25389.25±10499.34	502.00±359.86	0.99±0.00	109.00±43.66
	28d	4	39074.75±31911.19	783.50±271.10	0.99±0.01	211.25±71.49
	49d	4	40326.25±35467.62	776.00±304.73	0.99±0.01	197.25±80.51
	63d	4	27886.00±10354.28	928.50±425.17	0.98±0.01	236.25±88.54
Colon	07d	4	49031.25±26942.71	1000.50±613.98	0.99±0.01	197.75±121.48
	28d	4	46396.00±24321.12	886.25±363.25	0.99±0.00	192.25±48.70
	49d	4	47205.00±33308.12	1215.75±429.97	0.98±0.01	262.75±75.51
	63d	5	57977.20±46833.40	1129.8±655.07	0.98±0.01	231.00±55.08

Values refers to mean and standard deviation

TABLE 3| Changes in alpha diversity represented by Chao1's richness, Shannon´s and inverse Simpson´s diversity indexes according to gastrointestinal tract (GIT) region, age and their interaction (GIT*age).

Factor		Chao1	Shannon	inverse Simpson´s
GIT	Rumen	370.706±35.768[b]	5.207±0.235[a]	19.308±2.628[a]
	Jejunum	238.950±34.306[c]	2.635±0.339[b]	4.067±0.730[b]
	Cecum	409.581±52.129[ab]	5.081±0.263[a]	19.982±3.375[a]
	Colon	558.053±87.076[a]	5.351±0.175[a]	18.490±2.284[a]
	P-value	0.001	<.001	<.001
	FDR[1]	0.003	<.001	<.001
Age	07d	390.350±92.032	4.072±0.337	10.922±2.343
	28d	327.725±40.907	4.434±0.439	15.449±2.933
	49d	441.067±65.664	4.673±0.476	17.725±3.823
	63d	424.221±46.495	5.092±0.230	17.870±2.420
	P-value	0.714	0.068	0.273
	FDR	0.745	0.116	0.409
GIT*Age	Rumen_07d	317.050±24.936	4.968±0.625[a]	20.388±7.499
	Rumen_28d	349.050±95.675	5.245±0.498[a]	20.391±6.262
	Rumen_49d	461.425±102.250	5.772±0.327[a]	23.352±4.910
	Rumen_63d	358.380±53.921	4.916±0.457[a]	14.342±3.502
	Jejunum_07d	275.750±106.908	2.572±0.754[ab]	3.518±1.247
	Jejunum_28d	159.950±30.031	1.675±0.239[b]	2.046±0.380
	Jejunum_49d	177.467±63.086	1.680±0.716[b]	1.791±0.379
	Jejunum_63d	309.600±46.861	4.026±0.215[a]	7.489±0.865
	Cecum_07d	257.575±71.902	3.982±0.189[b]	8.745±1.266
	Cecum_28d	423.650±75.524	5.442±0.178[ab]	19.784±4.557
	Cecum_49d	399.675±92.920	5.110±0.727[ab]	24.079±10.569
	Cecum_63d	557.425±140.547	5.787±0.418[a]	27.321±5.300
	Colon_07d	711.025±325.354	4.768±0.318[a]	11.036±0.839
	Colon_28d	378.250±65.240	5.372±0.369[a]	19.576±5.650
	Colon_49d	659.800±142.646	5.380±0.445[a]	17.696±5.692
	Colon_63d	498.120±103.580	5.776±0.203[a]	24.219±3.501
	P-value	0.373	0.013	0.298
	FDR	0.454	0.026	0.421

Values refer to mean and standard deviation; [1]*P*-value corrected to FDR; FDR≤ 0.05 were considered significant; Means between groups (GIT, Age and Age within GIT (GIT*Age)) followed by the same letter are not significantly different ($P > 0.05$) by Tukey HSD test.

TABLE 4| Relative abundance of the bacterial taxa that exhibited significant (FDR≤0.05) changes according to gastrointestinal tract region (GIT), age and interaction (GIT*age).

	GIT					
Taxa[1]	**Rumen**	**Jejunum**	**Cecum**	**Colon**	***P*-value**	**FDR[2]**
p_Bacteroidetes	3.057±1.659[a]	0.134±0.047[b]	1.739±0.481[a]	1.582±0.392[a]	<.001	<.001
p_Firmicutes	8.726±1.733[b]	2.256±0.699[c]	8.847±2.394[b]	14.569±3.536[a]	<.001	<.001
f_Enterobacteriaceae	0.288±0.230[b]	4.521±2.083[a]	3.200±1.722[a]	2.303±1.283[ab]	0.002	0.002
f_Ruminococcaceae	8.119±1.389[b]	0.778±0.227[c]	14.577±2.934[a]	14.650±2.222[a]	<.001	<.001
g_*Butyrivibrio*	2.681±0.990[a]	0.737±0.606[b]	0.204±0.161[c]	0.279±0.122[c]	<.001	<.001
g_*Clostridium*	0.354±0.323[b]	0.195±0.079[c]	0.973±0.361[a]	0.679±0.330[a]	<.001	<.001
g_*Oscillospira*	0.820±0.268[b]	0.048±0.014[c]	1.104±0.278[a]	1.450±0.411[a]	<.001	<.001
g_*Ruminococcus*	4.795±1.936[a]	1.180±0.590[b]	0.865±0.247[c]	2.264±0.594[ab]	0.003	0.004
	Age					
	07d	**28d**	**49d**	**63d**		
p_Bacteroidetes	0.469±0.219[b]	2.045±1.326[ab]	2.569±1.354[a]	1.582±0.424[ab]	0.017	0.020
p_Firmicutes	3.803±2.040[b]	13.613±3.584[a]	10.849±2.414[a]	6.961±1.491[a]	0.003	0.004
f_Enterobacteriaceae	5.838±2.344[a]	2.406±1.342[ab]	1.405±1.212[b]	0.768±0.414[b]	0.001	0.001
g_*Butyrivibrio*	0.191±0.142[b]	0.616±0.433[ab]	0.660±0.341[ab]	2.241±0.960[a]	0.010	0.011
g_*Ruminococcus*	1.145±0.506[b]	1.136±0.590[b]	3.852±2.033[ab]	3.076±0.887[a]	0.015	0.018
	GIT*age					
	Rumen_07d	**Rumen_28d**	**Rumen_49d**	**Rumen_63d**		
f_Christensenellaceae	0.472±0.325[a]	0.715±0.558[a]	0.166±0.057[a]	2.032±2.019[a]	<.001	<.001
f_Clostridiaceae	0.370±0.353[a]	0.128±0.128[a]	0.255±0.223[a]	0.041±0.041[b]	0.004	0.005
f_Coriobacteriaceae	0.944±0.944[c]	5.704±2.526[b]	2.093±1.077[b]	17.723±13.291[a]	<.001	<.001
f_Erysipelotrichaceae	0.064±0.048[a]	0.013±0.013[a]	0.038±0.038[a]	0.020±0.013[a]	<.001	<.001
f_Lachnospiraceae	3.548±3.111[b]	3.637±1.945[b]	2.897±0.872[b]	5.758±2.444[a]	0.012	0.014
f_Mogibacteriaceae	0.115±0.115[c]	1.085±0.340[b]	0.651±0.303[b]	1.899±0.777[a]	0.017	0.019
f_Paraprevotellaceae	0.000±0.000[b]	0.740±0.428[a]	0.370±0.155[a]	0.368±0.355[a]	<.001	<.001
f_Peptostreptococcaceae	0.102±0.102[a]	0.000±0.000[b]	0.000±0.000[b]	0.010±0.010[a]	<.001	<.001
f_S24-7	1.314±1.314[b]	10.120±4.232[a]	5.309±1.833[a]	2.348±1.338[b]	0.035	0.039
f_Synergistaceae	0.013±0.013[c]	0.128±0.085[b]	4.581±3.418[a]	3.093±2.940[a]	<.001	<.001
f_Veillonellaceae	1.659±1.659[b]	5.015±2.574[a]	1.302±0.586[b]	1.501±0.815[b]	<.001	<.001
g_*Bacteroides*	11.753±5.488[a]	0.804±0.572[b]	1.646±0.980[b]	2.389±2.250[b]	0.016	0.019
g_*Bifidobacterium*	0.242±0.242[c]	11.434±5.102[a]	1.914±1.618[b]	0.306±0.257[c]	<.001	<.001

g_*Blautia*	2.489±1.950[a]	0.115±0.057[b]	0.115±0.057[b]	0.092±0.067[c]	<.001	<.001
g_*Bulleidia*	0.510±0.510[b]	1.582±0.540[b]	2.578±1.100[a]	2.950±1.090[a]	<.001	<.001
g_*Butyricimonas*	0.804±0.479[a]	0.217±0.156[a]	0.191±0.053[a]	0.265±0.136[a]	<.001	<.001
g_*Collinsella*	0.051±0.036[a]	0.000±0.000[a]	0.064±0.064[a]	0.000±0.000[a]	<.001	<.001
g_*Coprococcus*	0.140±0.109[a]	0.038±0.024[b]	0.306±0.290[a]	0.041±0.019[b]	<.001	<.001
g_*Desulfovibrio*	1.161±0.557[a]	0.255±0.116[b]	0.332±0.148[b]	0.449±0.187[b]	0.027	0.031
g_*Dorea*	1.314±0.567[a]	0.485±0.327[b]	0.842±0.774[b]	0.010±0.010[c]	0.018	0.021
g_*Eubacterium*	0.140±0.038[a]	0.089±0.073[b]	0.370±0.320[a]	0.245±0.103[a]	<.001	<.001
g_*Faecalibacterium*	0.000±0.000[b]	0.000±0.000[b]	0.102±0.102[a]	0.000±0.000[b]	<.001	<.001
g_*Lactobacillus*	0.944±0.531[a]	0.077±0.061[a]	0.932±0.932[a]	0.490±0.427[a]	0.001	0.045
g_*Megasphaera*	0.026±0.026[c]	4.250±2.511[a]	1.289±0.965[ab]	1.041±0.628[b]	<.001	<.001
g_*Parabacteroides*	3.280±3.145[a]	0.102±0.063[b]	0.102±0.063[b]	0.225±0.200[b]	<.001	<.001
g_*Phascolarctobacterium*	1.225±0.547[a]	0.013±0.013[c]	0.128±0.128[b]	0.276±0.276[b]	<.001	<.001
g_*Prevotella* (f_Paraprevotellaceae)	3.573±2.308[a]	0.855±0.518[b]	1.327±0.616[a]	0.510±0.263[b]	<.001	<.001
g_*Prevotella* (f_Prevotellaceae)	9.252±6.527[a]	11.626±3.821[a]	17.522±4.014[a]	13.558±6.546[a]	0.032	0.036
g_*Pseudoramibacter Eubacterium*	0.013±0.013[b]	0.102±0.036[ab]	0.153±0.108[ab]	0.408±0.118[a]	<.001	<.001
g_*Sharpea*	0.128±0.128[c]	0.613±0.190[b]	1.161±1.010[a]	0.858±0.525[b]	<.001	<.001
g_*Streptococcus*	5.653±3.693[a]	0.115±0.048[b]	0.077±0.033[c]	0.184±0.134[c]	0.002	0.003
g_*Succiniclasticum*	2.527±2.510[b]	3.165±1.166[a]	1.672±0.663[a]	2.573±0.938[a]	<.001	<.001
g_*Succinivibrio*	0.115±0.115[b]	0.510±0.510[ab]	1.021±0.769[a]	0.123±0.073[b]	<.001	<.001
g_*Treponema*	0.000±0.000[b]	0.230±0.197[a]	0.230±0.107[a]	0.153±0.067[a]	<.001	<.001
g_*Turicibacter*	0.064±0.064[b]	0.026±0.026[a]	0.000±0.000[c]	0.010±0.010[a]	<.001	<.001
	Jejunum_07d	**Jejunum_28d**	**Jejunum_49d**	**Jejunum_63d**		
f_Christensenellaceae	0.089±0.060[a]	0.000±0.000[b]	0.051±0.051[a]	0.000±0.000[b]	<.001	<.001
f_Clostridiaceae	8.295±8.109[b]	5.513±5.394[b]	1.055±0.495[b]	30.454±3.339[a]	0.004	0.005
f_Coriobacteriaceae	0.153±0.088[b]	0.026±0.026[b]	0.391±0.391[b]	11.853±4.626[a]	<.001	<.001
f_Erysipelotrichaceae	0.013±0.013[a]	0.013±0.013[a]	0.000±0.000[b]	0.020±0.020[a]	<.001	<.001
f_Lachnospiraceae	0.766±0.277[b]	8.946±3.557[a]	0.596±0.417[b]	6.115±2.081[a]	0.012	0.014
f_Mogibacteriaceae	0.115±0.067[a]	0.013±0.013[a]	0.034±0.034[a]	0.919±0.350[a]	0.017	0.019
f_Paraprevotellaceae	0.026±0.015[a]	0.013±0.013[a]	0.017±0.017[a]	0.000±0.000[b]	<.001	<.001
f_Peptostreptococcaceae	1.608±1.591[b]	0.523±0.490[b]	0.051±0.029[c]	2.072±0.426[a]	<.001	<.001
f_S24-7	0.179±0.111[a]	0.026±0.026[a]	0.119±0.119[a]	0.133±0.133[a]	0.035	0.039
f_Synergistaceae	0.026±0.015[b]	0.000±0.000[c]	0.153±0.128[b]	1.011±0.631[a]	<.001	<.001
f_Veillonellaceae	0.000±0.000[c]	0.013±0.013[b]	0.204±0.204[a]	0.051±0.032[b]	<.001	<.001

g_*Bacteroides*	0.689±0.211[a]	0.740±0.374[a]	0.204±0.059[b]	0.051±0.016[c]	0.016	0.019
g_*Bifidobacterium*	0.396±0.362[a]	0.038±0.024[a]	1.089±1.089[a]	0.184±0.095[a]	<.001	<.001
g_*Blautia*	0.268±0.127[a]	0.000±0.000[b]	0.000±0.000[b]	0.061±0.038[b]	<.001	<.001
g_*Bulleidia*	0.013±0.013[b]	0.064±0.024[b]	0.153±0.153[b]	1.552±0.809[a]	<.001	<.001
g_*Butyricimonas*	0.013±0.013[a]	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	<.001	<.001
g_*Collinsella*	0.447±0.430[a]	0.026±0.026[a]	0.000±0.000[b]	0.000±0.000[b]	<.001	<.001
g_*Coprococcus*	0.026±0.026[a]	0.026±0.015[a]	0.000±0.000[b]	0.010±0.010[a]	<.001	<.001
g_*Desulfovibrio*	0.013±0.013[a]	0.051±0.029[a]	0.017±0.017[a]	0.061±0.049[a]	0.027	0.031
g_*Dorea*	0.102±0.069[a]	0.038±0.024[a]	0.051±0.051[a]	0.071±0.060[a]	0.018	0.021
g_*Eubacterium*	0.038±0.024[b]	0.013±0.013[b]	0.000±0.000[c]	1.348±0.886[a]	<.001	<.001
g_*Faecalibacterium*	0.102±0.055[a]	0.026±0.015[a]	0.017±0.017[a]	0.010±0.010[a]	<.001	<.001
g_*Lactobacillus*	71.095±12.915[a]	71.082±9.082[a]	84.193±7.915[a]	10.812±7.599[b]	0.001	0.045
g_*Megasphaera*	0.281±0.264[a]	0.000±0.000[b]	0.221±0.221[a]	0.092±0.057[a]	<.001	<.001
g_*Parabacteroides*	0.153±0.075[a]	0.217±0.184[a]	0.034±0.034[b]	0.010±0.010[b]	<.001	<.001
g_*Phascolarctobacterium*	0.077±0.049[a]	0.013±0.013[a]	0.000±0.000[b]	0.051±0.040[a]	<.001	<.001
g_*Prevotella* (f_Paraprevotellaceae)	0.332±0.218[a]	0.166±0.149[a]	0.119±0.119[a]	0.082±0.035[a]	<.001	<.001
g_*Prevotella* (f_Prevotellaceae)	0.651±0.328[a]	0.217±0.096[a]	0.238±0.148[a]	0.419±0.169[a]	0.032	0.036
g_*Pseudoramibacter Eubacterium*	0.000±0.000[c]	0.013±0.013[b]	0.000±0.000[c]	0.368±0.153[a]	<.001	<.001
g_*Sharpea*	0.128±0.111[ab]	0.013±0.013[b]	0.698±0.598[ab]	0.521±0.208[a]	<.001	<.001
g_*Streptococcus*	0.281±0.179[b]	0.026±0.026[c]	0.204±0.179[b]	0.664±0.295[a]	0.002	0.003
g_*Succiniclasticum*	0.038±0.024[b]	0.000±0.000[c]	0.034±0.034[b]	0.102±0.067[a]	<.001	<.001
g_*Succinivibrio*	0.013±0.010[a]	0.018±0.013[a]	0.136±0.112[a]	0.051±0.023[a]	<.001	<.001
g_*Treponema*	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]	0.010±0.010[a]	<.001	<.001
g_*Turicibacter*	0.191±0.105[b]	0.153±0.137[b]	0.068±0.068[b]	13.813±9.128[a]	<.001	<.001
	Cecum_07d	**Cecum_28d**	**Cecum_49d**	**Cecum_63d**		
f_Christensenellaceae	0.000±0.000[b]	0.026±0.026[a]	0.000±0.000[b]	0.038±0.013[a]	<.001	<.001
f_Clostridiaceae	3.943±2.930[b]	1.544±0.592[b]	0.613±0.280[c]	3.956±1.471[a]	0.004	0.005
f_Coriobacteriaceae	0.000±0.000[b]	0.408±0.277[a]	0.944±0.927[a]	0.995±0.569[a]	<.001	<.001
f_Erysipelotrichaceae	0.102±0.069[a]	0.179±0.162[a]	0.830±0.612[a]	0.242±0.053[a]	<.001	<.001
f_Lachnospiraceae	1.493±0.766[b]	3.790±0.378[ab]	18.415±11.169[a]	8.104±1.927[ab]	0.012	0.014
f_Mogibacteriaceae	0.026±0.026[a]	0.089±0.053[a]	0.498±0.249[a]	0.051±0.036[a]	0.017	0.019
f_Paraprevotellaceae	0.013±0.013[a]	0.051±0.051[a]	0.306±0.243[a]	0.140±0.070[a]	<.001	<.001
f_Peptostreptococcaceae	0.026±0.026[a]	0.842±0.742[a]	0.038±0.024[a]	0.077±0.033[a]	<.001	<.001
f_S24-7	0.077±0.049[b]	3.918±2.954[ab]	3.701±3.434[ab]	4.275±2.766[a]	0.035	0.039

f_Synergistaceae	0.000±0.000[a]	0.000±0.000[a]	0.000±0.000[a]	0.000±0.000[a]	<.001	<.001
f_Veillonellaceae	0.013±0.013[a]	0.038±0.024[a]	0.153±0.122[a]	0.038±0.038[a]	<.001	<.001
g_*Bacteroides*	11.588±7.875[a]	4.109±2.077[a]	5.909±2.770[a]	9.099±3.879[a]	0.016	0.019
g_*Bifidobacterium*	0.000±0.000[b]	0.447±0.274[a]	0.026±0.015[b]	0.051±0.051[b]	<.001	<.001
g_*Blautia*	2.935±2.585[b]	11.830±6.113[a]	2.591±0.628[ab]	3.931±1.277[ab]	<.001	<.001
g_*Bulleidia*	0.013±0.013[b]	0.000±0.000[c]	0.204±0.204[a]	0.089±0.057[ab]	<.001	<.001
g_*Butyricimonas*	0.000±0.000[b]	0.153±0.153[a]	0.013±0.013[a]	0.026±0.026[a]	<.001	<.001
g_*Collinsella*	1.314±1.280[a]	1.761±1.548[a]	0.166±0.101[a]	0.447±0.397[a]	<.001	<.001
g_*Coprococcus*	0.000±0.000[b]	0.102±0.069[a]	0.242±0.089[a]	0.447±0.296[a]	<.001	<.001
g_*Desulfovibrio*	0.013±0.013[b]	0.089±0.089[ab]	0.038±0.038[ab]	0.179±0.129[a]	0.027	0.031
g_*Dorea*	2.782±0.546[a]	2.246±0.945[a]	4.479±2.855[a]	7.874±5.735[a]	0.018	0.021
g_*Eubacterium*	0.064±0.024[a]	0.089±0.044[a]	0.102±0.069[a]	0.089±0.089[a]	<.001	<.001
g_*Faecalibacterium*	0.983±0.915[a]	0.102±0.036[a]	0.574±0.359[a]	0.204±0.021[a]	<.001	<.001
g_*Lactobacillus*	15.901±5.52[a]	6.343±3.550[a]	13.221±4.525[a]	1.097±0.590[c]	0.001	0.045
g_*Megasphaera*	0.000±0.000[b]	0.013±0.013[a]	0.051±0.051[a]	0.013±0.013[a]	<.001	<.001
g_*Parabacteroides*	0.128±0.128[b]	3.522±2.296[a]	0.944±0.557[a]	1.748±0.927[a]	<.001	<.001
g_*Phascolarctobacterium*	0.306±0.169[b]	1.021±0.936[a]	2.476±1.432[a]	1.621±0.264[a]	<.001	<.001
g_*Prevotella* (f_Paraprevotellaceae)	0.204±0.123[b]	1.901±1.042[a]	3.560±2.178[a]	12.072±4.177[a]	<.001	<.001
g_*Prevotella* (f_Prevotellaceae)	0.319±0.229[b]	1.072±0.307[ab]	4.058±1.935[a]	9.444±2.207[a]	0.032	0.036
g_*Pseudoramibacter Eubacterium*	0.000±0.000[b]	0.038±0.013[a]	0.089±0.053[a]	0.000±0.000[b]	<.001	<.001
g_*Sharpea*	0.000±0.000[b]	0.064±0.064[a]	0.128±0.128[a]	0.026±0.026[a]	<.001	<.001
g_*Streptococcus*	0.294±0.228[a]	2.067±2.050[a]	0.051±0.029[a]	0.038±0.024[a]	0.002	0.003
g_*Succiniclasticum*	0.038±0.038[ab]	0.013±0.013[b]	0.115±0.115[ab]	0.166±0.092[a]	<.001	<.001
g_*Succinivibrio*	0.000±0.000[b]	0.064±0.064[a]	0.064±0.032[a]	0.447±0.231[a]	<.001	<.001
g_*Treponema*	0.013±0.013[c]	1.123±0.585[a]	0.128±0.111[b]	0.459±0.376[ab]	<.001	<.001
g_*Turicibacter*	0.000±0.000[c]	4.020±3.782[a]	0.217±0.067[b]	0.651±0.278[b]	<.001	<.001
	Colon_07d	**Colon_28d**	**Colon_49d**	**Colon_63d**		
f_Christensenellaceae	0.038±0.038[a]	0.026±0.026[a]	0.013±0.013[a]	0.633±0.633[a]	<.001	<.001
f_Clostridiaceae	6.075±4.864[b]	1.442±0.727[b]	2.412±1.254[b]	8.127±6.548[a]	0.004	0.005
f_Coriobacteriaceae	0.000±0.000[c]	0.217±0.136[b]	1.225±0.955[ab]	4.717±2.451[a]	<.001	<.001
f_Erysipelotrichaceae	0.140±0.124[b]	0.268±0.064[ab]	1.251±0.579[a]	0.276±0.136[ab]	<.001	<.001
f_Lachnospiraceae	11.320±8.156[a]	4.747±1.231[a]	17.905±10.471[a]	5.738±2.466[a]	0.012	0.014
f_Mogibacteriaceae	0.191±0.099[a]	0.026±0.015[a]	0.217±0.167[a]	0.276±0.099[a]	0.017	0.019
f_Paraprevotellaceae	0.153±0.120[a]	0.357±0.276[a]	0.294±0.146[a]	0.143±0.069[a]	<.001	<.001

f_Peptostreptococcaceae	0.115±0.038[a]	0.077±0.061[a]	0.191±0.099[a]	0.306±0.224[a]	<.001	<.001
f_S24-7	0.140±0.140[b]	2.935±1.921[ab]	4.377±3.030[ab]	5.278±3.683[a]	0.035	0.039
f_Synergistaceae	0.013±0.013[a]	0.000±0.000[b]	0.000±0.000[b]	0.010±0.010[a]	<.001	<.001
f_Veillonellaceae	0.013±0.013[b]	0.038±0.038[ab]	0.026±0.026[b]	0.480±0.294[a]	<.001	<.001
g_*Bacteroides*	13.872±7.811[a]	1.863±0.611[a]	2.476±0.982[a]	4.737±3.217[a]	0.016	0.019
g_*Bifidobacterium*	0.013±0.013[b]	0.574±0.449[a]	0.664±0.630[a]	0.112±0.076[b]	<.001	<.001
g_*Blautia*	1.927±0.950[a]	8.231±2.546[a]	6.406±2.068[a]	4.145±1.871[a]	<.001	<.001
g_*Bulleidia*	0.115±0.115[b]	0.000±0.000[c]	0.013±0.013[b]	0.184±0.117[a]	<.001	<.001
g_*Butyricimonas*	0.000±0.000[c]	0.294±0.277[a]	0.013±0.013[b]	0.041±0.019[b]	<.001	<.001
g_*Collinsella*	0.472±0.455[b]	0.715±0.398[a]	1.621±0.640[a]	0.378±0.201[b]	<.001	<.001
g_*Coprococcus*	0.064±0.024[b]	0.064±0.048[b]	0.179±0.099[ab]	0.500±0.312[a]	<.001	<.001
g_*Desulfovibrio*	0.038±0.024[a]	0.179±0.147[a]	0.013±0.013[a]	0.061±0.038[a]	0.027	0.031
g_*Dorea*	3.216±1.481[a]	2.808±1.238[a]	3.280±2.071[a]	1.776±0.749[a]	0.018	0.021
g_*Eubacterium*	0.026±0.015[a]	0.064±0.038[a]	0.230±0.213[a]	0.184±0.105[a]	<.001	<.001
g_*Faecalibacterium*	0.587±0.416[a]	0.268±0.111[a]	0.459±0.165[a]	0.245±0.173[a]	<.001	<.001
g_*Lactobacillus*	9.342±3.300[b]	6.611±3.872[a]	13.183±6.837[a]	0.755±0.508[c]	0.001	0.045
g_*Megasphaera*	0.000±0.000[c]	0.064±0.064[b]	0.026±0.026[b]	0.939±0.801[a]	<.001	<.001
g_*Parabacteroides*	0.268±0.163[b]	1.940±0.465[a]	0.498±0.302[ab]	0.970±0.694[ab]	<.001	<.001
g_*Phascolarctobacterium*	0.625±0.307[b]	11.307±9.271[a]	0.868±0.240[a]	1.827±1.158[a]	<.001	<.001
g_*Prevotella* (f_Paraprevotellaceae)	0.536±0.440[b]	2.016±0.597[ab]	1.506±0.717[ab]	4.247±1.777[a]	<.001	<.001
g_*Prevotella* (f_Prevotellaceae)	1.774±1.219[a]	2.118±0.477[a]	3.612±1.298[a]	6.207±3.492[a]	0.032	0.036
g_*Pseudoramibacter Eubacterium*	0.115±0.115[a]	0.051±0.021[a]	0.179±0.105[a]	0.071±0.047[a]	<.001	<.001
g_*Sharpea*	0.000±0.000[b]	0.026±0.026[a]	0.281±0.094[a]	0.643±0.468[a]	<.001	<.001
g_*Streptococcus*	0.664±0.380[b]	0.013±0.013[c]	0.396±0.176[b]	1.776±1.638[a]	0.002	0.003
g_*Succiniclasticum*	0.255±0.153[ab]	0.077±0.033[b]	0.128±0.085[ab]	1.235±0.848[a]	<.001	<.001
g_*Succinivibrio*	0.051±0.029[a]	0.140±0.082[a]	0.013±0.013[a]	0.500±0.437[a]	<.001	<.001
g_*Treponema*	0.166±0.057[a]	0.804±0.334[a]	0.204±0.029[a]	0.133±0.047[a]	<.001	<.001
g_*Turicibacter*	1.238±1.238[a]	0.217±0.101[a]	0.153±0.081[a]	3.226±2.834[a]	<.001	<.001

Values are relative abundance average percentage and standard deviation; [1]Sequences summarised to phylum (p_), family (f_) or genus (g_) levels; [2]*P*-value corrected to FDR; FDR≤ 0.05 were considered significant; Means between groups (GIT, Age and Age within GIT (GIT*Age)) followed by the same letter are not significantly different ($P > 0.05$) by Tukey HSD test.

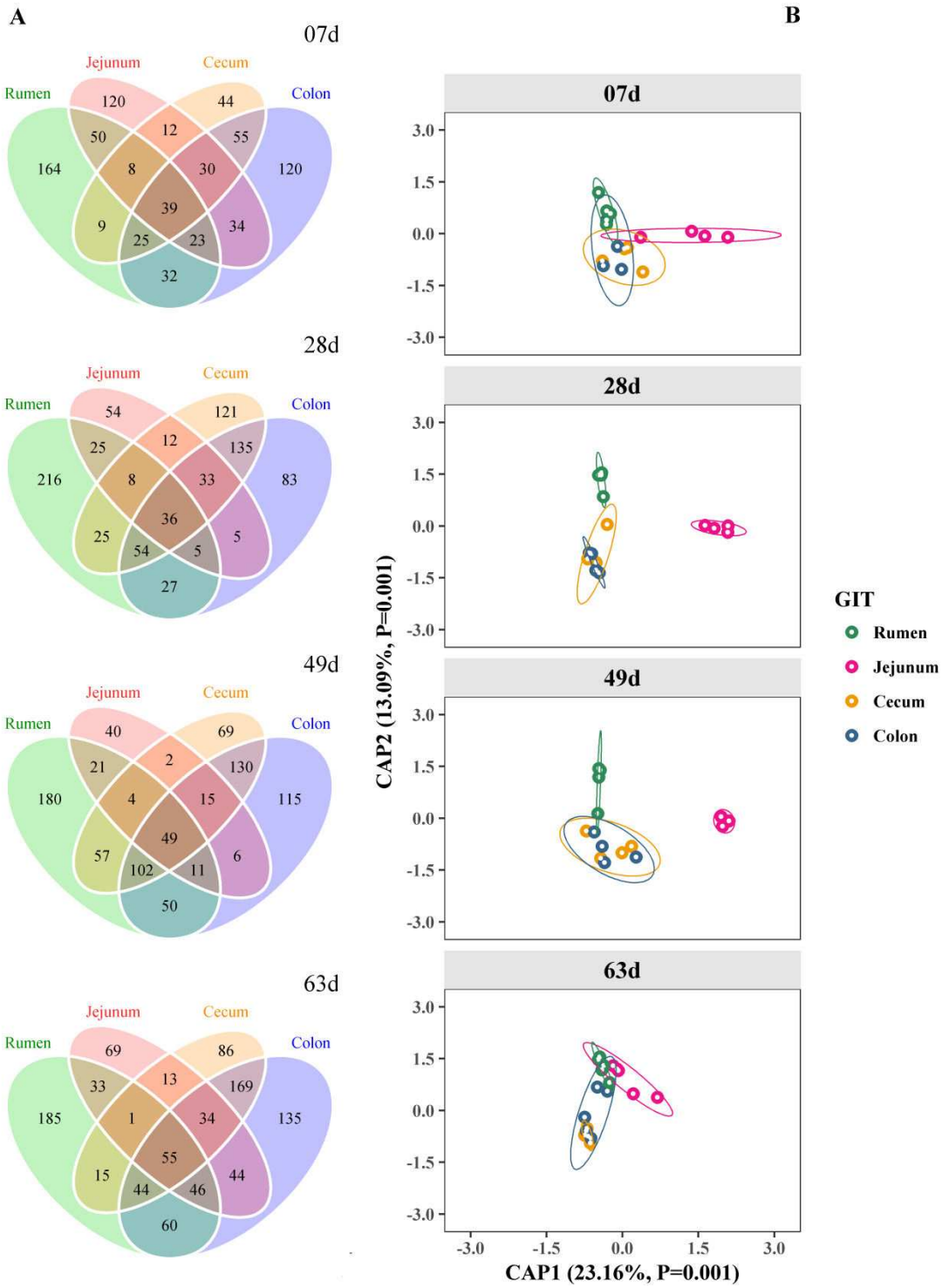

FIGURE 1| A. Venn diagram of OTUs shared among rumen, jejunum, cecum and colon samples of dairy calves at 7, 28, 49 and 63 days of age. B. Canonical Analysis of Principal Coordinates (CAP) of the Bray-Curtis dissimilarity metric for bacterial community ordinated by Canonical Analysis of Principal Coordinates (CAP). On the CAP plots each point represents a sample, colors represent the GIT region and facets represent the age group. The increasing distance between samples equates to more dissimilarity in bacterial community.

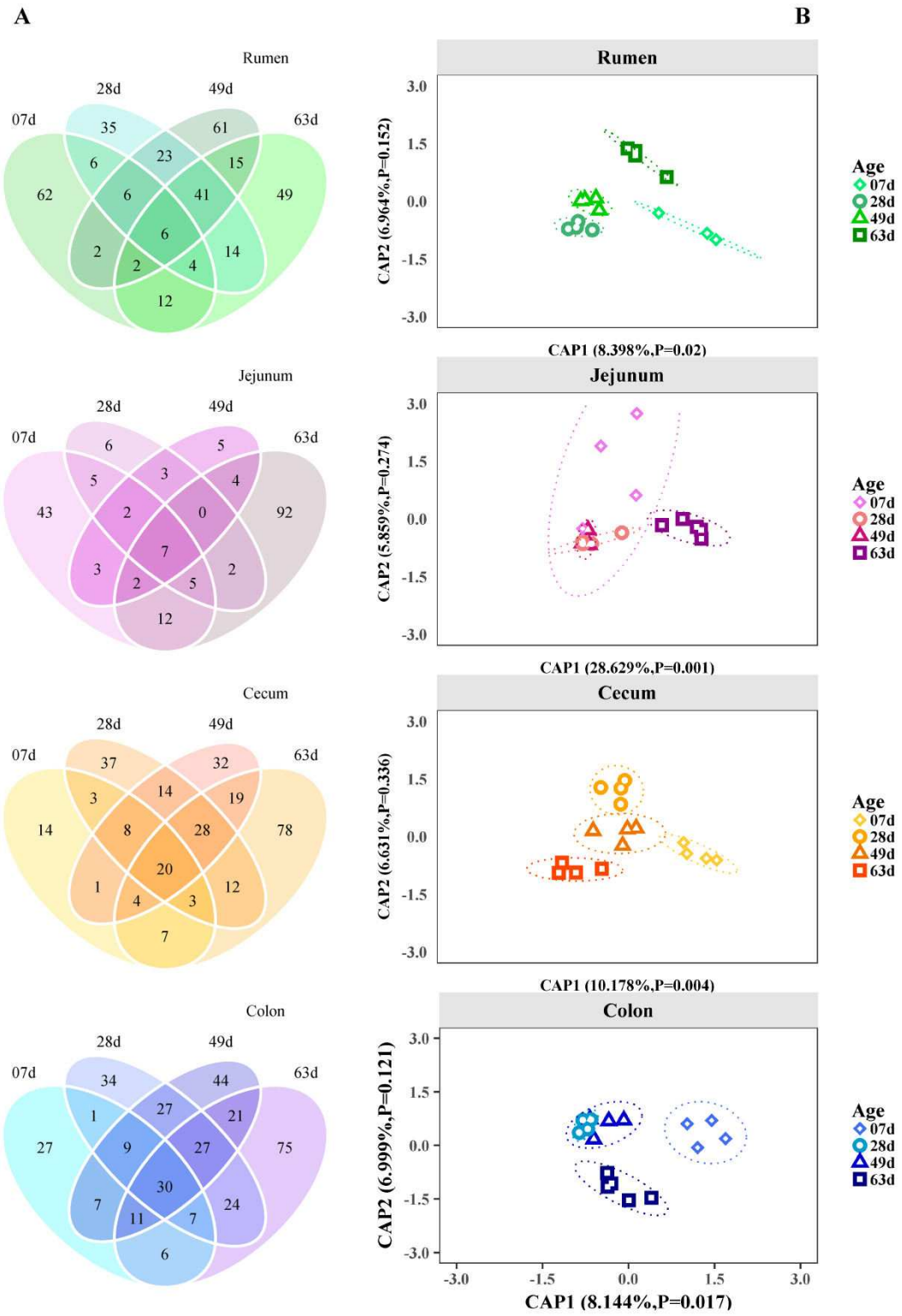

FIGURE 2| A. Venn diagram of shared OTUs among calves 7, 28, 49, 63 days according GIT region (rumen, jejunum, cecum and colon). B. Canonical Analysis of Principal Coordinates (CAP) of the Bray-Curtis dissimilarity metric for bacterial community among calves according age in each GIT region.

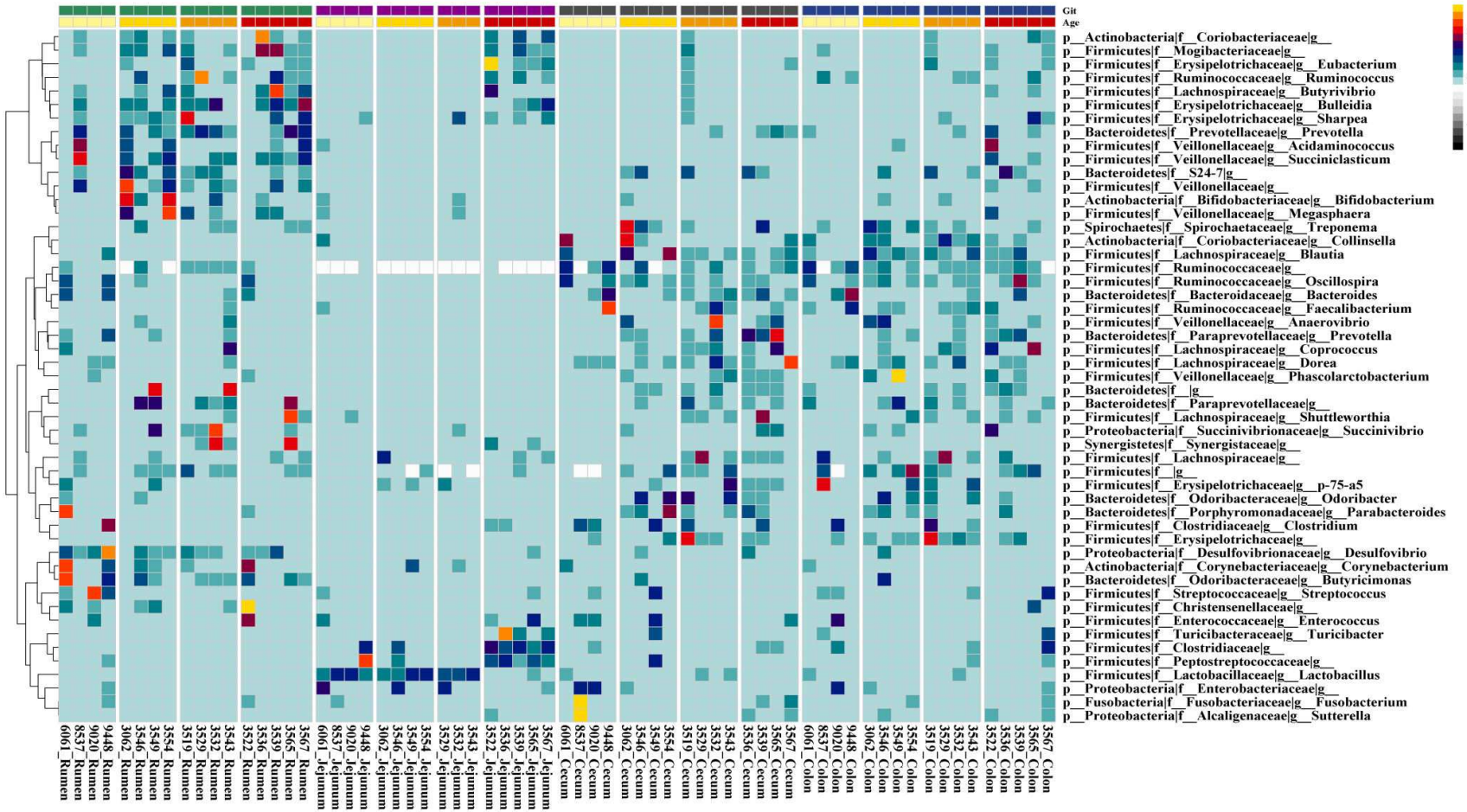

FIGURE 3| Distribution of the most abundant bacterial taxa (sequences summarized at phylo, family and genus levels at average abundance ≥1% and present at least 50% of all samples) among individual calves. Colors in the horizontal bars at the top of the plot represent GIT region (Rumen: dark green, Jejunum: magenta, Cecum: black, Colon: blue) and age (7d: light yellow, 28d: gold, 49d: orange and 63d: red). The scale (Z-score) represents the relative abundance of taxa in a given sample at gradient of color from black (low abundance) to gold (high abundance). The hierarchical dendrogram was established through Minkowski distances of the taxa along the y axis and Ward.D2 linkage clustering method.

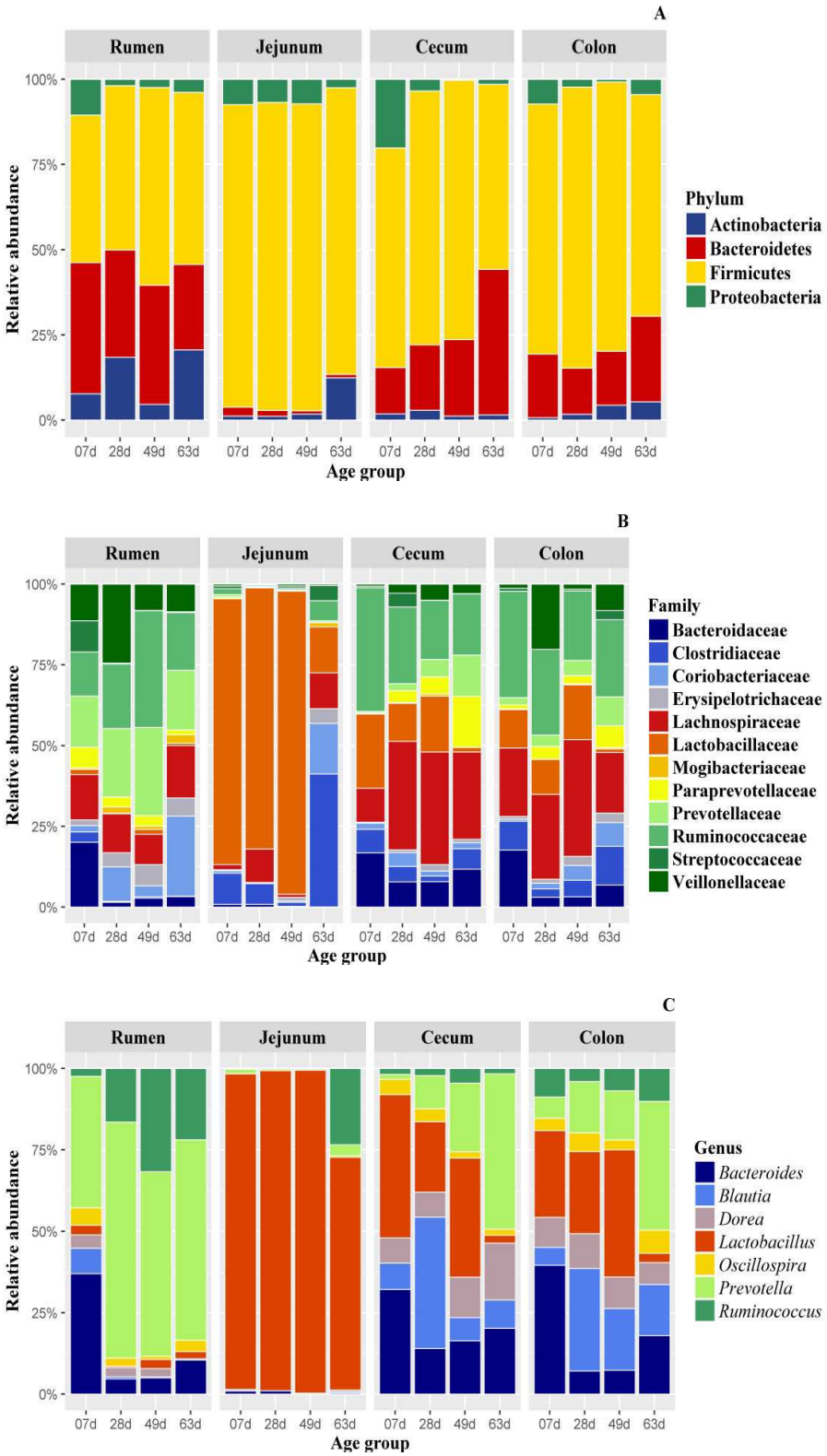

FIGURE 4| Distribution of the most abundant bacterial taxa (average abundance ≥1% and present at least 50% of all samples) assigned to phylum (A), family (B) and genus (C) among rumen, jejunum, cecum and colon of calves in their respective age group (7, 28, 49 and 63 days old).

GENERAL CONCLUSIONS

Our results confirmed the hypothesis that diet, age and GIT region drives changes in the structure and abundance of microbial communities in the GIT of pre-ruminants. This study provides new insights into the microbial colonization within GIT of pre-ruminants as well as targets for improving health and performance of dairy calves through manipulation of the microbiota along the GIT, not just in the rumen.