Characterization of The Microbiome That Colonizes The Digestive Tract of Calves From 0 To 42 Days of Age, Raised In The Mexican Tropics.

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Abstract

In the first few weeks of a calf's life, the early colonization of microbes throughout the gastrointestinal tract (GIT) is critical in its digestive system and immunity development. Analyses of data generated from next-generation sequencing platforms have revolutionized the understanding of host-associated microbial communities; these analyses can be done through a variety of bioinformatics pipelines. This study aimed to describe the diversity and evolution of gastrointestinal tract microbial communities in the first days of life of calves; for which we used the USEARCH and KRAKEN2 algorithms, in terms of alpha and beta diversity analysis in the rumen and jejunum contents of calves from 0, 7, 28, 42 days-born in the Mexican Tropic and its changes. The two pipelines detected similar abundance distributions in the three most abundant taxa *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. The present study revealed the changes occurring in the rumen and jejunum ecosystem in the first week of life, reflected by a decline in the phylum *Proteobacteria* and an increase in phylum *Bacteroidetes* in rumen and *Firmicutes* in the jejunum. We observed an increase in the similarity in the phylum taxa with age, suggesting a homogeneous and specific mature community, compared with the primary community.

Introduction

Ruminants and the microorganisms in their digestive tract have evolved to create a symbiosis that has allowed them to adapt their diet to the consumption of forages, obtaining the necessary energy for all metabolic processes. Relationships between digestive tract bacterial communities and their mammalian hosts have been shown to have an important role in the host's well-being and proper function.

Colonization by the initial microbiota is influenced by many factors such as diet, feeding methods (breastfeeding or artificial rearing), birth canal microbiota, and the environment where the animal grows up. This is a dynamic, stable process, but changes in the proportions of microorganism taxa are dependent on the surrounding metacommunity, which is important to achieve a correct function that can influence the health, immunity, and productivity of animals when reaching adulthood (Jiao et al. 2005).

The microbiome of the digestive tract of ruminants was first investigated using bacterial culture techniques, however, with this methodology, it was only possible to identify 11% of the species (Pitta et al. 2009) since it was not possible to grow a large proportion of bacteria in laboratory conditions. The emergence of the new generation of sequencing techniques has allowed us to expand our knowledge about the bacterial species of the digestive tract and to understand the interactions between the host and the microbiota, and their impact on the development of immunity and health. In this study, we set out to study the changes in the microbiota of calves of the Mexican Tropic, to dissect the possible microbial colonization pattern in the gastrointestinal tract of calves.

Materials And Methods

Animals and sampling

The experimental protocols (CICUA.E.DC-2019/4 – 2) were approved by the internal committee for the experimental animal care and use (CICUA.E, UNAM) according to the NOM-062-ZOO-1999. The animals were obtained from the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical (CEIEGT), Tlapacoyan, Veracruz.

A total of 12 newborn calves ($n = 3$) of ages 0, 7, 28, and 42 days were used. The three animals of 0 days were sacrificed immediately after birth, without having ingested colostrum (Zhong et al. 2017). The calves of ages 7, 28, and 42 days had the same management in the livestock unit (up to sacrifice), which consists of colostrum ingestion and staying with the dam for 4 days in a maternity pen. After this period, the calves were separated from the cow and moved to the rearing area with the rest of the calves where they were fed a milk substitute (22% protein and 12% fat), roughage ad libitum, and from the second week, a concentrate.

Ruminal and intestinal (jejunum) contents were collected from all animals. The samples were immediately frozen in liquid nitrogen for further analysis (Liang et al. 2014 and 2016).

DNA extraction

To determine bacterial diversity, genomic DNA was extracted from samples previously collected. DNA extraction was performed according to the RBB + C method (Yu and Morrison 2004); zirconium beads and lysis buffer were used for cell disruption. 10M ammonium acetate, isopropanol, and 70% ethanol were used for the precipitation of nucleic acids. Finally, for protein removal and DNA purification, the columns of the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) were used.

DNA concentration was quantified using a Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Product integrity was observed on 1% agarose gel. Subsequently, the samples were sent to RTL genomics to perform the sequencing of the DNA samples, using primers covering the V4 region (357wF:3'-CCTACGGGNGGCWGCAG-5'; 785R:3'-GACTACHVGGGTATCTAATCC-5'). The Illumina MiSeq platform was used with paired readings (2 x 250).

Data quality control and analyses were performed using the USEARCH V.11 pipeline. The quality control was applied to the fastq files, which included removing adapters and cutting the sequences to length-based filtering of 400 bp (< 200 bp was excluded from the analysis). The next step was to align the obtained sequences to define operational taxonomic units (OTUs) for eventual taxonomy assignment; the OTUs table was generated at 97% identity. The UCLUST method was used to cluster the reads into OTUs (Edgar, 2010).

For KRAKEN2 (Wood et al. 2019), fastq files were firstly uploaded into FastQC for quality control and removal of ambiguous sequences, and then the software Trimmomatic (version 0.32; Bolger et al. 2014) was used to trim residual artificial sequences, cut bases with quality scores below 20, and filtering of 400 bp (< 200 bp was excluded from the analysis). After pre-processing, the protocol proposed by Lu et al. (2020) was used.

Taxonomy was assigned using RDP V.16 against the 16S reference database with 97% identity for both pipelines (Edgar, 2010). The data were analyzed on the R platform with the help of the pyloseq (McMurdie et al. 2013) library. Alpha diversity values for bacterial communities with ruminal and jejunal content were obtained using various diversity indexes (observed OTUs, Chao's estimate, Shannon's diversity index).

Statistical analysis

Additional statistical analyses were performed using a completely random model using the GLM function of the SAS statistical program (SAS 9.3):

$$y_{jk} = \mu + D_j + \varepsilon_{(j)k}$$

Y_{jk} = response variable of j^{th} day

μ = general mean

D_j = effect of day j

$\varepsilon_{(j)k}$ = associated error of the k^{th} sample of the j^{th} day.

Results

Rumen bacterial composition across different age groups

Alpha diversity metrics summarize the structure of an ecological community concerning its richness (number of taxonomic groups), uniformity (distribution of group abundances), or both.

Alpha-diversity was measured using Shannon, Chao estimate, and observed OTUs (Fig. 1). Observed OTUs were greater in the 28-day sample in the rumen (418.3 ± 42.19) compared to the other days in both rumen and jejunum. Concerning metrics related to the richness, the Chao estimate for rumen samples increased from 0-day to 42-day; samples of day 28 having the greater richness followed by 42-day samples (476.23 ± 52.23 ; 368.60 ± 52.23 respectively; $P < 0.05$), we did not find a difference in the jejunum ($P > 0.05$). Diversity did not fluctuate greatly ($P > 0.05$) and was similar for all the trials in both regions (rumen and jejunum).

Analyzing the abundance of the regions, we found that the *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* phyla represent almost 90% of all species found in both rumen and jejunum.

In the case of the rumen (Fig. 2), a greater proportion of the *Proteobacteria* phylum was observed on day 0 (decreasing from 34% on 0-day to 18.7% on 42-day), which was replaced by an increasing proportion of the *Bacteroidetes* phylum which remains as the more abundant phylum throughout all later measurements (increasing from 16.6% on 0-day to 70% on 42-day; $P \leq 0.05$, Fig. 2). *Firmicutes* and

Actinobacteria phyla on day 0 represent 24.7% and 13.7%, respectively of the abundance in the rumen ($P \leq 0.05$), this diminished on days 7 and 28, by day 42 it represents 20% for *Firmicutes* and 8% for *Actinobacteria*.

These findings were supported by both programs, with small differences found between them. KRAKEN2 detected an increase in the proportion of the phylum *Firmicutes* on day 0 (decreasing from 45–18% by day 42 $P \leq 0.05$; similar to USEARCH), and the two programs agree on the proportions of the phylum on days 7, 28, 42.

Jejunum samples were dominated by *Firmicutes* phylum in older ages, being the most abundant phylum in 7, 28, and 42-days samples, representing 39%; 87%; 74% of the abundance respectively, according to both programs (Fig. 3). On the other hand, there is a decrease in the age of the *Proteobacteria* phylum (decreasing from 70% on 0-day to 5% on 42-day), similar to what is observed in the rumen. *Actinobacteria* phylum did not show a difference between the days under study ($P > 0.05$). *Bacteroidetes* phylum has an increase in 7-day representing 24% of the abundance for this day and a decrease on day 42 representing less than 10% of the phylum for this day.

We also analyzed the microbiome shared in each region (microbial core) among the four ages, the jejunum microbiota had the most (OTUs) in common with and those of the rumen sample had the least.

This similarity between the newborn and older calves microbiota was evident when comparing the jejunum sample's microbial composition (Fig. 4). As a group, newborns shared more phylum-level taxa with older ages (114 of OTUs were shared by all jejunum samples in USEARCH analysis (Fig. 4b), and 107 of OTUs were shared by all jejunum samples in KRAKEN2 analyses (Fig. 4d). In rumen only, 11 of the OTUs analyzed with USEARCH were exclusively shared between all ages, and only 8 of the OTUs analyzed with KRAKEN2 (Fig. 4a and 4c) were shared by all ages; rumen 0-day had the greatest amount of individual OTUs (204 OTUs detected with USEARCH, and 355 OTUs detected with KRAKEN2 were not shared with other ages).

Discussion

A diverse microbial population colonizes the sterile mammalian gastrointestinal tract during and after birth. There is increasing evidence that this complex microbiome plays a crucial role in the development of the mucosal immune system and influences newborn health. Microbial colonization is a complex process influenced by a two-way interaction between host and microbes and a variety of external factors, including maternal microbiota, the birth process, diet, and antibiotics.

The objective of this study was to evaluate the composition of the total bacteria in the rumen and jejunum of newborn calves, and determine how the composition changes during normal development. Our results suggest that each sampled age group has its distinct microbiota, as reflected by the clustering of the samples by age group, with two different pipelines.

In this study, the α -diversity of the bacterial community was greatest at the middle-end of the trial, richness was following the adaptation period to the growing diet, and diversity was invariable throughout much of the trial. Bacterial community diversity was lower by the beginning of the trial, increasing in the next weeks following the adaptation period.

Oikonomou et al. (2014) and Klein- Jöbstl et al. (2014) in their studies have described an increase in species richness and diversity with the animals increasing age. Our data agree with Austin et al. (2018) in that the 28-day samples had increased richness compared with samples at weaning, suggesting a stabilization of the microbiome in more mature animals. In contrast, in the study by Klein et al. (2019), richness, diversity, and the number of observed OTUs, decreased significantly between 6 and 24 hours after birth. In the same way, a decrease in mean Chao estimate, Shannon and observed OTUs, during the first day after birth was seen in calves in the studies of Alipour et al. (2018) and Yeoman et al. (2018). Possible explanations given by these authors are selective effects from the environment, especially the diets given to the animals, and a higher diversity caused by prenatal colonization.

The ruminal ecosystem is a complex consortium of different bacterial species living in a symbiotic relationship with the host. Zhou et al. (2015) in their review on rumen bacteria found that in ruminal populations the most abundant phylum always falls on *Firmicutes* and *Bacteroidetes*. Similarly, Tapio et al. (2017), report that the main phylum is represented by *Firmicutes* (55.9–86.8%) followed by *Bacteroidetes* (8- 24.4%) and *Proteobacteria* (0.9–13.4%), with other phyla such as *Actinobacteria*, *Cyanobacteria* representing less than 0.5%.

Next-generation sequencing and bioinformatics tools have greatly advanced our knowledge about the phylogenetic diversity and ecological role of microbes inhabiting the mammalian gut. However, there is a lack of information on the evaluation of these computational tools in the context of the gastrointestinal ruminants microbiome as these programs have mostly been benchmarked on real or simulated datasets generated from human studies. In this study, we also compared the outcomes from two different methods, 1) KRAKEN2, and 2) a pipeline based in USEARCH, to assess the taxonomic profiles (bacteria) of rumen and jejunum microbial communities using DNA sequencing. Our study finds that these programs perform similarly and achieve the same conclusions.

Similarly, Neves et al. (2017), in their study, compared the outcomes of KRAKEN2 and a pipeline developed in-house based on MOTHUR (Schloss 2009; 2020), both approaches revealed a similar phylum distribution of the most abundant taxa, with *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* accounting for approximately 80% of total bacterial abundance. Similar results were described in the study of Glendinning et al. (2020); they performed a metagenomic analysis of samples taken from the ruminal contents of cattle, sheep, reindeer, and red deer, with the taxonomies assigned with KRAKEN2; in all ruminant species, *Bacteroidetes* was the most abundant phylum, with *Firmicutes* being the second. Similarly, Palomba et al. (2017), using USEARCH with rumen and reticulum samples, detected high levels of phylum *Planctomycetes*; phylum *Firmicutes*; phylum *Bacteroidetes*; and phylum *Synergistetes*), plus

several other families with a moderate relative abundance (> 3%), belonging to the usual components of the ruminal microbiota of sheep, cattle and dairy cows.

The main identified phylum's and their changes over time in this work were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. These findings agree with the proportions reported by Jiao et al. (2015), and Malmuthuge et al. (2015), and Sbardellati et al. (2020), using 16S rRNA, observed dominance of *Proteobacteria* phylum during the first week, but after that were replaced by *Firmicutes* and *Bacteroidetes* phyla. Also, O'Hara et al. (2020) in their study using USEARCH commands, found that the proportion of bacteria were mainly *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, also *Actinobacteria*, *Fibrobacteres*, *Spirochaete*, and *Cyanobacteria*, same as our finding with both programs USEARCH and KRAKEN2.

Gut microbial colonization during the preweaning period has primarily been studied using fecal samples because it is a non-invasive way to collect samples from the same individual over time (Klein et al. 2019; Alipour et al. 2018 and Yeoman et al. 2018). However, Malmuthuge et al. (2014) reported that bacterial composition could vary depending on the GIT region and sample type used (content versus mucosal tissue). Palomba et al. (2017), and Zeng et al. (2017) using USEARCH commands, found an important difference in the taxonomy across the GIT of the pre-weaned lambs' understudy, being *Firmicutes* and *Bacteroidetes* the most abundant phylum in all GIT, as in our data wherefrom 7-day the proportion of *Firmicutes* and *Bacteroidetes* increase in jejunum; also Taschuk and Griebel (2012) and Klein et al. (2019) reported *Firmicutes* and *Bacteroidetes* dominated the bacterial communities along with the GIT and the relative abundance of these 2 phyla varied greatly among local gut regions and between the tissue and content within each region (Malmuthuge and Guan, 2017).

In animals raised on calf starter, GIT microbial communities in those fed silage or mixed diets became more alike and more diverse with age. Animals had a succession of microorganisms representing similar taxa but different specific OTUs present at different ages. Therefore, age is one of the major driving forces in the establishment of microbial communities in developing calves (Jami et al. 2013; Dill-McFarland et al. 2017, 2019). Ma et al. (2020) detected changes in gut microbial diversity in healthy calves that were fed only milk replacer (same amount under the same age), and therefore, speculate that host (age and growth) was the main factor driving the development of microbial diversity in the early life of calves. Li et al. (2012), reported that calves fed with a milk substitute and subsequently with a starter feed showed a predominance of the phylum *Bacteroidetes* during the first 6 weeks of age; this is similar to the results obtained in this work ($P \leq 0.05$, Fig. 3c).

As we can see throughout many studies, the microbial populations maintain some stability through time, and for that reason, many authors speak of a microbial core. A microbial core is defined as a set of microbial species present in individuals within a given species (Taschuk et al. 2012).

Henderson et al. (2015) carried out a study of the composition of the microbial community of the rumen in different species, including cows, goats, camels, buffaloes, sheep, and how this microbiome could vary by factors like diet and the host, however, thanks to the fact that a central microbiome (a core) is

hypothesized to be present, variations are small even in a wide geographical range. They collected samples in 35 different countries, where the dominant bacterial phylum was mainly *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Fibrobacter* (representing about 90%). The seven most abundant bacterial groups comprised 67.1% of all bacterial sequence data, were detected in all samples, and can be considered the "dominant" ruminal bacteria. These were *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales*.

Xue et al. (2018), analyzed the microbiome of 334 dairy cows, finding that there was a bacterial core that represented 46% of all the bacteria found in the samples, these phyla were *Firmicutes* (21.67%), *Bacteroidetes* (20.68%), *Proteobacteria* (0.52%).

There is also an intestinal core; Fraune et al. (2007) studied this nucleus over three decades and he defined it as a stable population across samples that is relatively constant, with an endosymbiotic relationship between the host and its microbiota. Taschuk et al. (2012) mention that the central microbiome (core) must be redefined at a functional level, with greater emphasis on the processes carried out by the resident biota, and less emphasis on the absolute or relative abundance of individual organisms.

Therefore, maintaining the tolerance and stability of populations may require the presence and interaction of a relatively consistent and specific microbiota, for example, many diseases, such as inflammatory bowel disease, or ruminal acidosis have been associated with changes in the microbial population, so it is not uncommon to find a species-bacterial core that helps to maintain homeostasis (Fraune et al. 2007; Taschuk et al. 2012).

Research continues to show that small changes in ruminal microbial taxa or the abundance of specific microbes impact livestock productivity. Factors such as age, diet, feeding system, host species, and even geography, can alter the proportions of ruminal microorganisms. Several studies have analyzed different factors that affect the colonization of the digestive tract, such as age, diet, geographic location, feeding system, etc. Most studies concluded that the common factor that affects how populations of the digestive tract microbiota move are diet. We can observe this in works such as those of Malmuthuge et al. (2011), Petri et al. (2013), Iqbal et al. (2018), Clemmons et al. (2019), Guo et al. (2020), and many other authors that focus on the relationship between the type of diet or the diet changes and microbiome and how this impacts production.

The microbiota plays a fundamental role in the development of immunity and gastrointestinal metabolism, so knowing these populations allows not only to prevent diseases but also to improve productive parameters. This work used the USEARCH and KRAKEN2 programs, and demonstrated that they both are good tools in the analysis of the GIT microbiota in ruminants; an important point to mention is that we found USEARCH to be user-friendly because it has good technical support, its user manuals remain updated, and they were complete, with explanations of the function and use of each of its commands. Therefore, we recommend USEARCH for those who are beginning in microbiome analysis. Even so, we think that using two pipelines supported by a collection of tools could help control the

sources of variation present in these kinds of analyses (e.g. analytical pipelines, related databases, and software parameters), which will lead to more reliable biological interpretations and improved taxonomic evaluation at the species level (minimizing unclassified sequences).

Our results showed the profile of microbial colonization of the gastrointestinal tract of 0 to 42-day-old calves from a tropical region of Mexico. However, it is necessary to analyze other aspects of this colonization and how it can influence factors such as the pattern of microbial succession along with the GIT in pre-ruminant and ruminant animals. This knowledge is essential since it influences the development and maturation of the host's GIT, as well as the development of the immune system and therefore, the health of the animals. In addition, information on the viability, genetic sequences (metagenomics), or even gene expression (metatranscriptomics and metaproteomics) of the described microbial core is required. Therefore, much remains to be understood regarding the underlying mechanisms of possible interactions between the ruminal and intestinal microbial communities and their host. In addition, more emphasis should be placed on dysbiosis caused by antimicrobials in feed and the possibility of using the gut microbiome as prebiotics and probiotics as antimicrobial substitutes. In the future, it will be possible with the collection of microbiota data to create bioinformatics tools, such as algorithms based on the establishment of digestive tract populations to predict patterns with which we can prevent diseases and help design preventive treatment strategies.

Declarations

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Conflict of interest The authors declare no competing interests.

Availability of data and material The authors assure that the data and materials support the published claims and comply with field standards. The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Data were analyzed using SAS (9.3).

Author contributions Ofelia Mora, Armando Shimada, and Carla Daniela Robles-Espinoza contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Carolina Robles and Laura González-Dávalos. The first draft of the manuscript was written by Carolina Robles-Rodríguez and all the authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

Ethics approval All the procedures used in this study have been approved by the internal committee for the experimental animal care and use (CICUAE.DC-2019/4-2, UNAM) according to the NOM-062-ZOO-1999.

Consent to participate Not applicable.

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Figures

Figure 1

Alpha diversity. Temporal changes of the rumen and jejunum bacterial diversity, richness, and observed otus in calves 0, 7, 28, 42 days old from the Mexican tropics. Data are visualized as box-and-whisker plots showing the median and the interquartile (midspread) range (boxes containing 50% of all values), the whiskers (representing the 25 and 75 percentiles), and the extreme data points. Letters above boxes indicate significant differences (n = 3, P ≤ 0.05)

Figure 2

Temporal variations in the rumen. a) Relative abundance of rumen phyla in calves 0, 7, 28, 42 days old from the Mexican tropics (detected by USEARCH). b) Changes over time of the relative abundance of the most representative phyla that inhabit the rumen (detected by USEARCH) c) Relative abundance of rumen phyla in calves 0, 7, 28, 42 days old from the Mexican tropics (detected by KRAKEN2). d) Changes over time of the relative abundance of the most representative phyla that inhabit the rumen (detected by KRAKEN2). Different literals between columns indicate statistical difference ($n = 3$, $P \leq 0.05$)

Figure 3

Temporal variations in the jejunum. a) Relative abundance of jejunum phyla in calves 0, 7, 28, 42 days old from the Mexican tropics (detected by USEARCH). b) Changes over time of the relative abundance of the most representative phyla that inhabit the jejunum (detected by USEARCH). c) Relative abundance of jejunum phyla in calves 0, 7, 28, 42 days old from the Mexican tropics (detected by KRAKEN2). d) Changes over time of the relative abundance of the most representative phyla that inhabit the jejunum (detected by KRAKEN2). Different literals between columns indicate statistical difference ($n = 3$, $P \leq 0.05$)

Figure 4

Shared OTUs across different groups. Venn plot showing the shared and unique OTUs found in each plotted group. a) Shared OTUs across rumen sample (USEARCH). b) Shared OTUs across the jejunum sample (USEARCH). c) Shared OTUs across rumen sample (KRAKEN2). d) Shared OTUs across jejunum sample (KRAKEN2)