First insights into the fecal bacterial microbiota of the black–tailed prairie dog (*Cynomys ludovicianus*) in Janos, Mexico

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Abstract

*First insights into the fecal bacterial microbiota of the black–tailed prairie dog (*Cynomys ludovicianus*) in Janos, Mexico*. Intestinal bacteria are an important indicator of the health of their host. Incorporating periodic assessment of the taxonomic composition of these microorganisms into management and conservation plans can be a valuable tool to detect changes that may jeopardize the survival of threatened populations. Here we describe the diversity and abundance of fecal bacteria for the black–tailed prairie dog (*Cynomys ludovicianus*), a threatened species, in the Janos Biosphere Reserve, Chihuahua, Mexico. We analyzed fecal samples through next generation massive sequencing and amplified the V3–V4 region of the 16S rRNA gene using Illumina technology. The results were analyzed with QIIME based on the EzBioCloud reference. We identified 12 phyla, 22 classes, 33 orders, 54 families and 263 genera. The phyla Firmicutes and Bacteroidetes were the most abundant groups and are associated with healthy intestinal communities and high efficiency in the energy diet. Most of the bacterial genera reported here for *C. ludovicianus* are not pathogenic and are normally found in mammalian feces. Some of the other bacteria are associated with soil, water and plants, possibly in relation to the habitat of the black–tailed prairie dog. This is the first study to report the fecal bacteria of *C. ludovicianus* in Mexico and it provides a baseline for determining this species' health for use in long–term conservation strategies.

Key words*:* 16s rRNA, Bacteria, Diversity, Metagenomics, Rodent

Resumen

*Primeros datos sobre la microbiota bacteriana fecal del perrito de las praderas de cola negra (*Cynomys ludovicianus*) en Janos, México*. Las bacterias intestinales son un indicador importante de la salud de su hospedero y la incorporación de una evaluación periódica de la composición taxonómica de estos microorganismos en los planes de gestión y conservación puede ser una herramienta valiosa para detectar cambios que puedan poner en peligro la supervivencia de las poblaciones amenazadas. En este estudio describimos la diversidad y abundancia de las bacterias fecales de una especie amenazada, el perrito de la pradera de cola negra (*Cynomys ludovicianus*), en la Reserva de la Biosfera de Janos, en Chihuahua, México. Se analizaron muestras fecales mediante secuenciación masiva de siguiente generación y se amplificó la región V3–V4 del gen que codifica el ARNr 16S utilizando la tecnología Illumina. Los resultados se analizaron con QIIME a partir de la referencia EzBioCloud. Se identificaron 12 filos, 22 clases, 33 órdenes, 54 familias y 263 géneros. Los filos Firmicutes y Bacteroidetes, que fueron los grupos más abundantes, se asocian con comunidades intestinales saludables y una alta eficiencia en la dieta energética. La mayoría de los géneros bacterianos detectados en este estudio para *C. ludovicianus* no son patógenos y se encuentran habitualmente en las heces de mamíferos. Algunas de las otras bacterias están asociadas al suelo, el agua y las plantas, posiblemente en relación con el hábitat del perrito de las praderas de cola negra. Este es el primer estudio que reporta las bacterias fecales de *C. ludovicianus* en México y que proporciona un punto de referencia para determinar la salud de esta especie con vistas a utilizar esta información en estrategias de conservación a largo plazo.

Palabras clave: ARNr 16s, Bacteria, Diversidad, Metagenómica, Roedor

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Introduction

The black–tailed prairie dog (*Cynomys ludovicianus*) occurs in the region of Casas Grandes and Janos, Chihuahua, Mexico (CONABIO, 2011). It is a keystone species since, together with the bison (*Bison bison*), it controls the proliferation of shrubby plants, maintaining the structure of a grassland ecosystem (Cid et al., 1991). The 'Norma Oficial Mexicana 059' (SEMARNAT, 2010) has listed this species as threatened since 1994. In 2005, it was estimated that populations of black–tailed prairie dog had decreased by 98%, with 97% of its original range being lost to urban, agricultural and livestock development (CONABIO, 2011). Currently, the largest surviving colonies occur within the Janos Biosphere Reserve, considered the primary area for this species' conservation in Mexico and the United States of America (Ceballos et al., 1993). These rodents are well suited for measuring the conservation status of a habitat. Because of their keystone function, they are environmental indicators to assess short and medium term changes in their habitat. Environmental indicator species are sensitive to variations in the environment due to their dependence on certain physical and climatic characteristics that affect their physiology, survival and reproduction (Wilson and Reeder, 2005; Aragón et al., 2012; Tzab–Hernández and Macswiney–Gónzalez, 2014).

Intestinal bacteria play an important role in the health of the host by participating in the synthesis of essential vitamins, renewing the intestinal epithelium and facilitating the digestion of food (Guarner and Malagelada, 2003). However, the microbiota within the host shows a generalized variation in the composition of the intestinal bacterial community depending on the modifications in the diet over time and space (Caporaso et al., 2011). Variation occurs as a response to seasonal changes in feeding patterns, which can affect the ecology of the host itself by altering its nutritional status and overall health (Lee and Mazmania, 2010; Amato et al., 2015; Aivelo et al., 2016, Amato et al., 2016).

Due to the influence of intestinal bacteria on the health of their hosts (Lee and Mazmania, 2010), it is important to document the taxonomic composition of these microorganisms. This is especially important in threatened animal species as this information will inform management and conservation plans, and allow the detection of future changes in the fecal microbiota that could risk the survival of this population.

Here we report on the diversity and abundance of fecal bacteria of *C. ludovicianus*, determined by the 16S rRNA metagenomic technique in the Janos Biosphere Reserve, Chihuahua, Mexico. This information constitutes a first approach to knowledge of the fecal microbiota of this species, serving as a baseline and contributing to future approaches to management and conservation strategies of this threatened species in Mexico.

Material and methods

Study area

The Janos Biosphere Reserve is located in the northeastern part of the state of Chihuahua, Mexico, south of the border with the United States of America (fig. 1). It forms part of the Chihuahuan Desert ecoregion and limits the southwest of the area with the Sierra Madre Occidental and the east with meadows and mountains.

Sample collection

In October 2015, we identified a colony with the highest activity of the black–tailed prairie dog in the Janos reserve. During the first hours of the day, observations were made using binoculars at a distance of approximately 50 m. After 15 to 20 minutes of recording the activity of the prairie dogs outside their burrows, we collected ten samples of fresh fecal scat using sterile tweezers. We sprayed each sample with an antiseptic solution composed of super oxidized water (H_2O_2), sodium chloride (NaCl), hypochlorous acid (HClO) and sodium hypochlorite (NaClO), Microdacyn®, to remove soil bacteria that had adhered to the scat. The collected samples were deposited in BashingBead™ Zymo Research™ cell lysis tubes, and 750 µl of lysing/stabilizing solution and 25 g of fecal material were added. Each tube was processed in a cellular disruptor (TerraLyzer™) for 20 seconds. The vibration of the processor (3600 beats/minute) breaks down the bacterial cells with the help of the silica beads contained in the tube, allowing the DNA to come into contact with the stabilizing buffer. This process conserves the genetic material at room temperature, making it viable for up to two weeks according to the manufacturer's specifications. All the sampling procedures followed the guidelines approved by the American Society of Mammalogists (Sikes et al., 2011).

Laboratory work

We extracted DNA from the samples using the Xpedition™ Soil/Fecal DNA MiniPrep kit in a laminar UV flow hood in sterile conditions. The extracted DNA was then combined in a pool and run on a 1.2% agarose gel at 80V for 45 minutes in the BIO–RAD electrophoresis chamber to visualize the presence of high molecular weight DNA. The visualization was carried out in a GelMax™ photodocumenter (UVP®).

The amount of DNA obtained was measured in a Qubit® fluorometer. The amplification of the V3 and V4 regions of the 16S rRNA gene was carried out using the following primers (Klindworth et al., 2013): 5'–CCTACGGGNGGCWGCAG–3' and 5'–GACTACHVGGGTATCTAATCC–3'; which produces an amplicon of about ~460 bp. By joining these sequences to the 'overhang' adapters of the Illumina protocol (2017a), they were as follows:

5'–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3'and 5'–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3´ (amplicon of ~550 bp). The Illumina PCR protocol (2017a) was performed by using 12.5 µl of MyTaqTM Ready Mix 1X (Bioline®), 1 µl of each primer (10 uM), 5 µl of DNA (50 ng total) and 5.5 µl of molecular grade H_2O; the following cycle was used: 95ºC for 3 minutes; 25 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 sec-

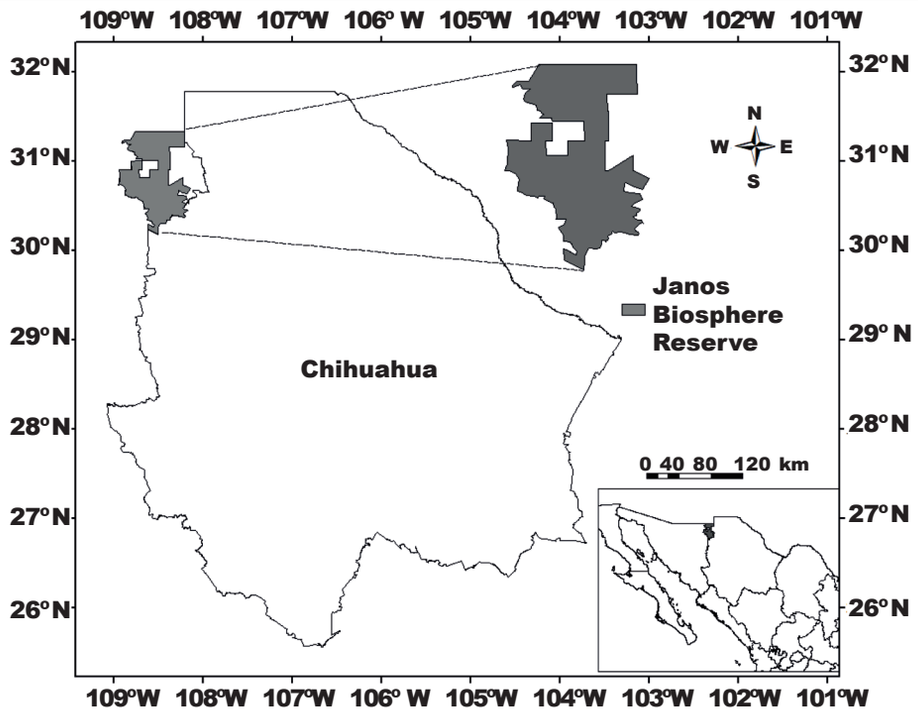

Fig. 1. Geographical location of the Janos Biosphere Reserve, Chihuahua, Mexico.

Fig 1. Ubicación geográfica de la Reserva de la Biosfera Janos, en Chihuahua, México.

onds; 72 ºC for 5 minutes in a Labnet Multigene™ Gradient PCR thermal cycler. One µl of the PCR products were placed on a Bioanalyzer DNA 1000 chip to verify the size of the amplicon (~550 bp). Purification of the amplicons was performed with Agentcourt® AMPure® XP 0.8 % beads. Subsequently, Nextera XT Index Kit™ was used to create the library, following the Illumina protocol (2017b), using 25 µl of MyTaq™ Ready Mix 1X (Bioline®), 5 µl of each primer (N7xx and S5xx), 5 µl of DNA and 10 µl of molecular grade H_2O; the following cycle was used: 95 ºC for 3 minutes; 10 cycles of 95 ºC for 30 seconds, 55 ºC for 30 seconds, 72 ºC for 30 seconds; 72 ºC for 5 minutes. Purification of the libraries was carried out with Agencourt® AMPure® XP 1.2 % beads. One µl of the final library of some randomly selected PCR products was placed on a Bioanalyzer DNA 1,000 chip to verify amplicon size of ~630 bp. Finally, quantification, normalization (equimolarity) and next generation massive sequencing (MiSeq Illumina® of 2 x 250 paired final readings) were performed following the 16S metagenomic protocol (Illumina, 2017a).

Bioinformatic analysis

The sequence was analysed in VM Oracle VirtualBox 5.1.14 on the MGLinux platform using the Quantitative Insights into Microbial Ecology bioinformatics software (QIIME) v.1.9.0 (Caporaso et al., 2010). The process was started by assembling the forward and reverse sequences of the samples using the PEAR program (Zhang et al., 2014) with an overlap of 50 bp, a minimum reading length of 430 bp and a maximum of 470 bp, a quality criterion Q30 (one false base for every 1,000 bases) and a value of $P < 0.0001$. The files were then converted to FASTA format and chimeric sequences of the samples with VSEARCH were eliminated (Edgar, 2010). The operational taxonomic units (OTUs) were selected with the VCLUST method (Edgar, 2010) at 97 % similarity; a representative sequence was obtained for each OTU and the taxonomy was assigned, taking the EzBioCloud database as reference (Yoon et al., 2017). The OTUs tables were built in Biom format (Biological observation matrix; McDonald et al., 2012) and the domains were separated. We calculated Simpson and Shannon alpha diversity indices and also Faith's phylogenetic index. The relative abundance of the taxonomic levels of phylum, class, order, family, genus and species was then obtained using Krona (Ondov et al., 2011).

Results

We obtained 131,828 non–chimeric bacterial sequences. The OTUs resulted in 12 phyla, 22 classes, 33 orders, 54 families, 263 genera and 333 species. The most abundant phyla were Firmicutes (83.14 %), Bacteroide-

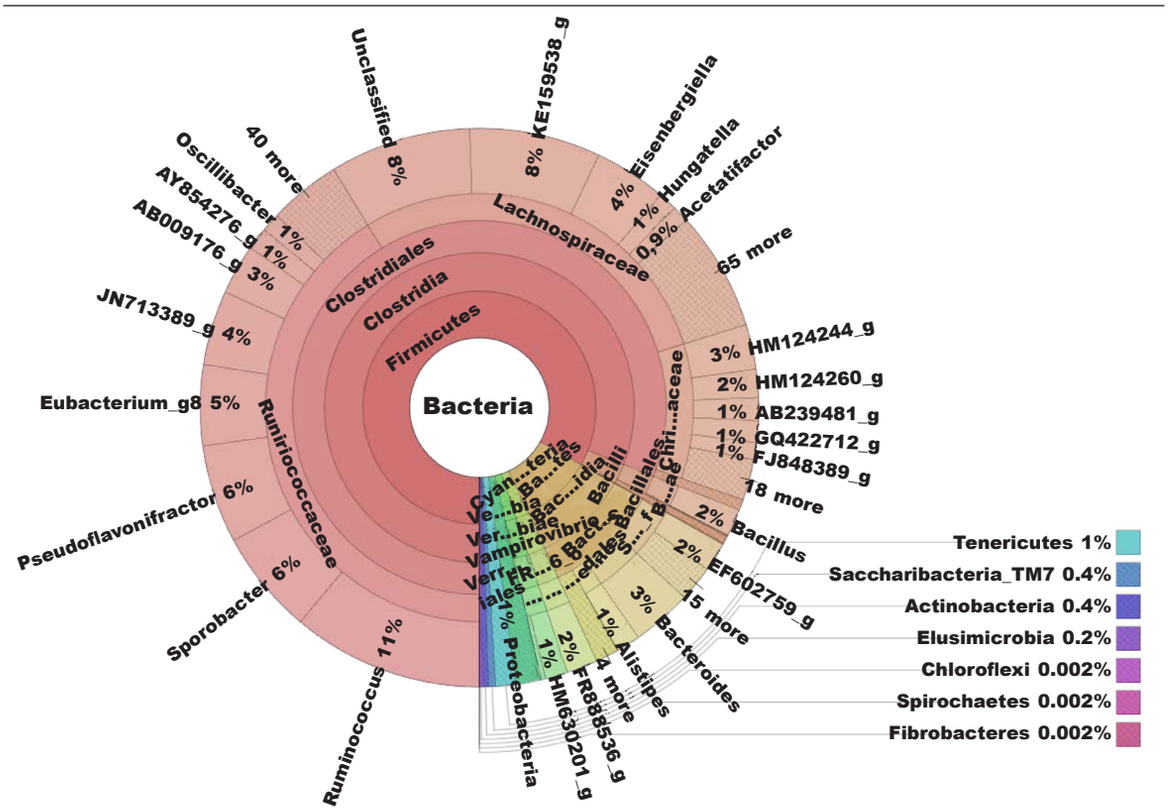

Fig. 2. Relative abundance of bacterial taxa found in the fecal pool of *Cynomys ludovicianus* in Janos, Mexico.

Fig. 2. Abundancia relativa de taxones bacterianos encontrados en las heces de Cynomys ludovicianus *en Janos, México.*

tes (9.94 %), Cyanobacteria (1.79 %), Verrucomicrobia (1.56 %), Proteobacteria (1.45 %) and Tenericutes (1.16 %) (fig. 2). The class Clostridia was the most abundant in the fecal pool of *C. ludovicianus* with 80.97 %, followed by the classes Bacteroidia (9.94 %), Bacilli (2.10%), Vampirovibrio_c (1.79 %), and Verrucomicrobiae (1.55 %) (fig. 2). The order Clostridiales (80.97 %) was dominant in the pool, while Bacteroidales (9.9 %), FR888536_o (1.79 %) and Bacilliales (1.6 %) followed in abundance (fig. 2).

The three most abundant families were Ruminococcaceae (41.26 %), Lachnospiraceae (28.93 %) and Christensenellaceae (10.16 %) (fig. 2). At the gender level, 30 % of the OTUs in the faecal pool was taxonomically classified (fig. 2), with Ruminoccocus being the most abundant (11.08 %); 65 % were bacteria that currently only have an identification key and the remaining 5 % were unknown OTUs. Finally, 333 species were determined, of which 75 % are not known, 23.7 % have a key nomenclature and only 0.9 % have a known name (*Acetatifactor muris*, *Bacteroides rodentium*, and *Streptococcus gallolyticus*). The alpha diversity of the sample was 0.99 with the Simpson index, 9.65 with the Shannon index, and 179.46 with the Faith's phylogenetic index.

Discussion

We determined that the fecal microbiota of *C. ludovicianus* at the phylum level is similar to that of wild rodents and other species of the order Rodentia (Lu et al., 2012; Maurice et al., 2015) with two main groups, Firmicutes and Bacteroidetes. These two groups represented about 90 % of the total readings in the sequencing of the 16S rRNA gene. This type of bacteria have been found in healthy intestinal communities, associated with a high efficiency in the energetic diet and with high probabilities that the host individual develops obesity (Duncan et al., 2008; Ley et al., 2008a; Mai and Draganov, 2009). In areas of extreme climates such as the Chihuahuan desert, precipitation and food availability vary from one year to the next (González–Romero et al., 2005). The high proportion of Firmicutes and Bacteroidetes can therefore be attributed to the need to extract and store energy from food sources occasionally limited within the area.

Within the phylum Firmicutes, the order Clostridia was identified as the most abundant. This group represents a crucial factor in the modulation of physiological, metabolic and immune processes within the intestine (Lopetuso et al., 2013). Within this class,

the families Lachnospiraceae and Ruminococcaceae were the most abundant; this was also reported in a fecal metagenomic analysis in field mice (*Microtus agrestis, M. oeconomus*, and *Myodes glareolus*) where more than 50 % of the classified sequences belonged to those two families (Koskela et al., 2017). There are precedents that indicate that the families Lachnospiraceae and Rumiococcaceae are common in the intestinal microbiota of animals that metabolize complex carbohydrates such as cellulose (Rainey, 2009). It should be noted that *C. ludovicianus* feeds mainly on grasses and some species of cactus (Sánchez–Cordero, 2003). These results confirm that the composition of the intestinal and fecal microbiota of mammals is similar regardless of the host species (Ley et al., 2008a; Muegge et al., 2011), showing the limited set of microorganisms that have adapted to life in the gastrointestinal tract (Ley et al., 2008b). Genera within Lachnospiracea, such as *Eubacterium*, *Coprococcus*, and *Roseburia* have been associated with the production of butyrate, playing a fundamental protective role, necessary for the health of intestinal epithelial tissue in mice and other hosts (Stanton and Savage, 1983). Genera that are included in the Ruminococcaceae have been found as part of the intestinal flora in cattle, sheep and goats degrading cellulose and colonizing the rumen (Rainey, 2009).

Most bacterial genera reported in the present study for *C. ludovicianus* are not pathogenic and are common in mammalian feces. Others are associated with soil, water and plants, which could be related to the habitat of the black–tailed prairie dog (Cai and Dong, 2010; Pindi et al., 2016). However, the possible existence of pathogenic bacteria due to climatic changes in the distribution zone of this species was not ruled out. These changes can affect bacterial diversity due to the influence of ecological and environmental factors such as temperature (Muegge et al., 2011).

Although only one colony of *C. ludovicianus* was studied in the present study, it is important to report the alpha diversity registered for this population, since comparisons with other colonies may be carried out in future research. The diversity and abundance of microorganisms in diverse vertebrate body regions can be important to understand the symbiotic associations with their hosts; nevertheless they are currently rarely used for conservation applications. In the case of *C. ludovicianus,* we documented the basic composition of intestinal bacteria represented in their feces, which provides a baseline of microbiological knowledge for this species. Future research should focus on determining the factors that affect this bacterial diversity and abundance, in addition to the spatiotemporal dynamics of its intestinal microbiota (Bobbie et al., 2017). This information can extend the effectiveness of conservation strategies for species at risk by incorporating aspects of health and nutrition of the population that is being protected. Fortunately, as 16S rRNA sequencing is now an economically feasible approach, microbial analyses can be integrated into conservation strategies for the benefit of vulnerable species (Stumpf et al., 2016), as is the case with the black–tailed prairie dog in Mexico.

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