UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE ECOLOGÍA

BIOLOGÍA EVOLUTIVA

DINÁMICA DEL MICROBIOMA EN LOS DIFERENTES ESTADÍOS REPRODUCTIVOS DE DOS POBLACIONES DE (*Leptonycteris yerbabuenae*)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

OSIRIS GAONA PINEDA

TUTORA PRINCIPAL DE TESIS: DRA. LUISA ISAURA FALCÓN ALVÁREZ
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CIUDAD DE MÉXICO. **ENERO 2020.**

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ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que, en la reunión ordinaria del Subcomité de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, celebrada el día 11 de noviembre de 2019, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **GAONA PINEDA OSIRIS,** con número de cuenta: **84335807** con la tesis titulada **"DINÁMICA DEL MICROBIOMA EN LOS DIFERENTES ESTADÍOS REPRODUCTIVOS DE DOS POBLACIONES DE *(Leptonycteris yerbabuenae)*",** bajo la dirección de la DRA. LUISA ISAURA FALCÓN ÁLVAREZ, quedando integrado de la siguiente manera:

Presidente:	DRA. ELLA GLORIA VÁZQUEZ DOMÍNGUEZ
Vocal:	DRA. LIVIA SOCORRO LEÓN PANIAGUA
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Suplente:	DR. ISAAC GONZÁLEZ SANTOYO
Suplente:	DR, LUIS EDUARDO SERVÍN GARCIDUEÑAS

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 07 de enero de 2020

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

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Dedicatoria

Alza tus palabras
no tu voz.
Es la lluvia la que hace
crecer las flores,
no los truenos.
(Anónimo)

Dedico esta tesis a todas las mujeres que siguen sueños, que a pesar de sentir miedo que a veces paraliza y entume los huesos, deciden seguir siempre adelante, aquellas que con el ejemplo y una sonrisa nos tienden la mano. A aquellas que han dejado su vida para dejarnos un mundo mejor.

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Resumen

En este trabajo hemos dilucidado la importancia que tiene la microbiota para el murciélago magueyero menor *Leptonycteris yerbabuenae*. En el primer capítulo se analizó la composición microbiana en muestras de heces de 33 individuos en diferentes estadíos reproductivos (juveniles y adultos c zxvfde ambos sexos y hembras preñadas y lactando) utilizando la region V4 del gen 16S rDNA y aplicando estadísticos de diversidad alfa y beta. Se encontró que la diversidad microbiana expresada en *Amplicon Sequence Variants* (ASV) es mayor en las hembras preñadas y lactando que en el resto de los estadíos reproductivos. La microbiota de adultos y juveniles está respresentada principalmente por Gammaproteobacteria y Firmicutes. Las hembras preñadas tienen una microbiota más diversa, con un incremento en los fila Bacteroidetes y Alfaproteobacteria. No se encontró diferencia significativa entre las hembras preñadas y lactando, ni entre los juveniles y adultos no reproductivos. Nuestros resultados sugieren que existe una diferencia en la microbiota relacionada a los estadíos reproductivos. En el segundo capítulo exploramos la composición microbiana del parche dorsal de *L. yerbabuenae*, que es exclusivo de los machos de esta especie, se desarrolla durante la temporada de apareamiento y desempeña un papel crucial en el reconocimiento parental y de apareamiento selectivo. Utilizando la secuenciación de la región V4 del gen 16S rDNA, identificamos 2,847 filotipos en los parches dorsales de 11 individuos. Veintiseis filotipos se compartieron entre todos los parches, los cuales representan del 30 al 75% de su abundancia relativa. Estas bacterias se especializan en la producción de ácidos grasos volátiles a través de procesos fermentativos. En el tercer capítulo se analizó como dos poblaciones discretas y geográficamente separadas del murciélago magueyero menor (*L. yerbabuenae*), una en el centro y otra en la región del Pacífico de México, difieren en su composición de microbiota fecal. Analizamos la región V4 del gen 16S rDNA de 68 individuos utilizando métricas de diversidad alfa y beta. Obtuvimos un total de 11,566 *Amplicon Sequence Variants* (ASV). Las comunidades bacterianas en las poblaciones del centro y el Pacífico presentan una diversidad de 6,939 y 4,088 ASV respectivamente, compartiendo una microbiota de 539 ASV. Nuestro estudio sugiere diferencias entre las dos poblaciones de *L. yerbabuenae*, lo cual no se había evaluado anteriormente, y aporta al conocimiento de su biología. Los resultados proporcionan una línea base para futuros estudios del microbioma de poblaciones silvestres.

Abstract

In this work we have elucidated the importance of the microbiota of the lesser long-nosed bat *Leptonycteris yerbabuenae*. In the first chapter, the microbial composition in fecal samples was analyzed among different life stages (both sexes juveniles and adults, and pregnant and lactating females). A total of 33 individuals were analysed focusing on the V4 region of the 16S rDNA gene using alpha and beta diversity metrics. We found that the microbial diversity expressed in Amplicon Sequence Variants (ASV) is higher in pregnant and lactating females than in the rest of the reproductive stages. The microbiota of adults and juveniles is mainly represented by Gammaproteobacteria and Firmicutes. The microbiota of pregnant females is more diverse, with an increased presence of Bacteroidetes and Alphaprotobacteria. No significant difference was found between pregnant and lactating females, or between non-reproductive adults and juveniles. These results suggest a difference in microbiota associated to reproductive stages. In the second chapter, we explored the microbial composition of *L. yerbabuenae* dorsal patch, which is exclusively found in reproductive males during mating season, and performs a crucial role in parental recognition and selective mating. Using sequences of the V4 region of the 16S rDNA gene, 2,847 phylotypes were identified in the dorsal patches of eleven individuals. Twenty-six phylotypes were shared among all patches, representing between the 30 to 75% of the relative abundance. These bacteria specialize in the production of volatile fatty acids through fermentation. In the third chapter, we explored how two discrete and geographically distanced populations of the lesser long-nosed bat (*L. yerbabuenae*), one in Central and the other in the Pacific region of Mexico, differ on their fecal microbiota composition. The V4 region of the 16S rDNA gene was assessed from 68 individual samples using alpha and beta diversity indexes. A total of 11,566 Amplicont Sequence Variants (ASV) were obtained. The microbiota of the Pacific and Central populations have 6,939 and 4,088 ASV respectively, sharing 539 ASV. This study identifies changes in the fecal microbiota of both Mexican populations, suggesting that these are in fact separated and different. This aspect of the biology of *L. yerbabuenae* had not been explored before, and the results provide a base line for future microbiome studies in two wild populations.

Introducción

¿Qué son la microbiota y el microbioma?

Después de estar en guerra con los microoganismos por más de dos siglos, las bacterias, arqueas, virus y hongos son vistos de una manera conciliadora. Leeuwenhoek en 1673 demuestra que existen universos fantásticos en una gota de lluvia, en el sarro que se deposita entre los dientes, en el líquido acuoso del ojo de buey; gracias a sus innovadoras lentes apiladas una sobre otra, nace la microbiología con su extraordinario invento y sus observaciones que maravillaron a la Real Sociedad (Kruif, 2018). Los padres de esta disciplina, Leeuwenhoek, Pasteur, Koch, entre otros, quizá nunca imaginaron que en los albores del siglo XXI seguiría esta cacería de microbios. A la fecha esta historia continúa, sólo cambia el escenario con las técnicas de secuenciación de nueva generación y la enorme cantidad de información con la que se dispone hoy en día. Se han acuñado nuevos términos para tratar de explicar ese mundo que sólo intuímos y que nuestros ojos son incapaces de ver, pero sin el cual nuestra existencia no sería la misma. Así nace el estudio de la "microbiota", descrita como los millones de microorganismos que viven en el cuerpo de un individuo animal o vegetal, y el "microbioma", que representa al conjunto de genomas que interactúan en la microbiota (Grice y Segre, 2014).

El microbioma es considerado, dada la función que desempeña, como un órgano esencial, ubicuo y simbiótico no sólo para los seres humanos (Moya y Ferrer, 2016), sino para todos los animales y plantas que habitan el planeta. Una parte significativa de éste órgano se encuentra en el intestino, en donde la asociación está generalmente enriquecida con especies de arqueas, bacterias, virus y eucariontes. La mayoría de estos microbios son simbiontes mutualistas, y su labor es promover la salud de los hospederos, facilitando los procesos de asimilación de nutrientes, resistiendo colonizaciones de bacterias antagónicas a la salud, colonizando el sistema inmune y estimulando una amplia variedad de funciones en el hospedero (Moya y Ferrer, 2016).

Existe un vínculo indirecto entre la composición microbiana y las respuestas metabólicas, influenciado fuertemente por la plasticidad metábolica y la redundancia funcional de las bacterias (Moya y Ferrer, 2016). El microbioma es un ejemplo de sucesión ecológica y dinámica poblacional, toda vez que existen cambios cíclicos en la microbiota

debido a los cambios en la disponibilidad de alimento del hospedero (Smits, 2017). La comunidad de microorganismos que residen en el intestino de los vertebrados realiza una variedad de funciones que afectan el fenotipo del huésped, la nutrición, la desintoxicación de los xenobióticos, la estimulación intestinal, el desarrollo inmune y el comportamiento (Ezenwa et al., 2012; Malmuthuge et al., 2015; Thaiss et al., 2016).

La simbiosis microbiana, considerada como una sub-disciplina de la rama del conocimiento de las Ciencias de la vida, y las interacciones cobran sentido en el haber científico al darnos cuenta que sin la presencia de los microbios muchas de las funciones fisiológicas que realizan los organismos serían imposibles. Un ejemplo de ello se refiere a los biofilms bacterianos de *Enterococcus casseliflavus* encontrados en dos insectos *Hyles ephorbiae* y *Brithys crini* que ayudan a degradar los terpenos tóxicos de las plantas que consumen (Vilanova et al., 2016). Es inevitable el cambio en los paradigmas filosóficos del significado del *Homo sapiens*, si consideramos que los humanos tenemos cientos de miles de millones de microbios en el intestino, por lo que las bacterias comensales, arqueas, virus y hongos que habitan en el cuerpo superan por 10 veces más al número de células que constituyen al ser humano (Hickman, 2005; Hird, 2014).

Los animales evolucionaron en un mundo microbiano: los procariontes preceden a los eucariontes en alrededor de 3 mil millones de años (Hickman, 2005). No es una sorpresa que cada animal sea el anfitrión de una comunidad microbiana compleja (microbioma), que contiene miles de millones de microorganismos, que pertenecen a cientos de miles de especies (Bäckhed et al., 2005; Qin et al., 2010; Consorcio del Proyecto Microbioma Humano, 2012).

La coevolución entre el hospedero y su microbiota es considerado un proceso de adaptación mutualista que dirige la especiación, jugando un papel crucial en la diversidad biológica (Moeller et al., 2009; Sharon et al., 2010; Grice y Segre 2011; Ley et al., 2008; Lee y Mazmanian, 2010), debido a que las bacterias pueden mejorar la asimilación de energía de diferentes fuentes de alimento a través de la síntesis de vitaminas necesarias para las funciones fisiológicas (Nicholson et al., 2005). Los genomas de los mamíferos no codifican la mayoría de las enzimas necesarias para degradar los polisacáridos estructurales presentes en el material vegetal (Van Soest, 2004), convirtiéndolos en dependientes de microorganismos intestinales simbióticos que son capaces de acceder a diferentes fuentes de

energía. Los herbívoros y omnívoros se benefician de la energía adicional de la fermentación microbiana de carbohidratos en el intestino (Flint et al., 2012).

Si sostenemos la hipótesis de que el microbioma endógeno refleja las firmas evolutivas de sus huéspedes y que las fuerzas evolutivas y ecológicas actúan tanto en el huésped como en su microbiota residente, entonces los hospederos y su microbiota constituirían nichos ecológicos complejos donde el equilibrio está también determinado por interacciones inter-específicas (microbios/microbios y microbios/hospederos) y la suma de los factores ambientales (Romano-Bertrand et al., 2015).

Lo anterior ha llevado a cambios importantes en la anatomía y fisiología digestiva. Un ejemplo se refiere a las formas y el tamaño de los aparatos digestivos determinados por el tipo de alimentación de los organismos.Esto es fácil de observar en el intestino de los murciélagos filostómidos que presentan diferentes gremios alimentarios que van desde insectívoros hasta hematófagos (Carrillo et al., 2015). et al

Factores que afectan el microbioma

El microbioma está relacionado a los cambios de crecimiento y metabolismo del hospedero (De Winter et al., 2015), dieta (Carrillo et al., 2015), la filogenia del hospedero (Anderson et al., 2012; Colman et al., 2012; Phillips et al., 2012), la ecología e historia natural del hospedero (Wong y Rawls 2012; Coon et al., 2014; Dill-McFarland et al., 2014) y la geografía (Hird et al., 2014).

El microbioma sin duda influyó en la evolución del huésped durante millones de años, contribuyendo potencialmente a las trayectorias evolutivas de comunidades enteras de vertebrados, ya que las bacterias dan como resultado comunidades microbianas convergentes entre los huéspedes con hábitos de alimentación similares (et alFujimura et al., 2010; Muegge et al., 2011). Asimismo, están involucradas en el reconocimiento entre especies, selección de pareja y selección intraparental que son procesos importantes para el mantenimiento y estabilidad de las poblaciones (Zilber-Rosenberg y Rosenberg 2008; Markov et al., 2009; Ley et al., 2009; Fraune y Bosch 2010). Tanto el microbioma intestinal como el cutáneo alteran el olfato en los mamíferos (Archie y Theis, 2011; Grice y Segre, 2011) y podría jugar un papel crucial en los procesos de reconocimiento parental (Lizé et al., 2013), así como en

otros procesos de interacción social entre los individuos regulados por el olfato, la visión y las claves acústicas (Bee, 2006). et al

Los cambios en la microbiota se deben a una variedad de factores que se pueden modificar en las diferentes etapas de la vida, incluidas las condiciones ambientales, la dieta, el peso y las hormonas (Koren et al., 2012).

Dieta

Se ha considerado a la dieta como el factor principal que determina la funcionalidad y la diversidad de la microbiota intestinal (Muegge et al., 2011). Existe evidencia de que la dieta da forma a la abundancia relativa de los filos dominantes de la microbiota, y las poblaciones de grupos bacterianos específicos están influenciadas por la composición de los macronutrientes consumidos. La comida en sí puede servir como sustrato para nuevas colonizaciones microbianas (Wu et al., 2011; Voreades et al., 2014), mientras que la microbiota responde rápidamente a los cambios en la dieta del huésped (Amato, 2013; Oriach et al. 2016). Asimismo las necesidades alimentarias de los animales cambian según su estadío reproductivo e historia de vida (Gaona et al., 2019). Un excelente ejemplo de ello es el de las abejas parasitoides del género *Nasonia*, en donde la microbiota difiere entre las tres etapas de desarrollo presentes, particularmente entre las etapas larvaria y adulta (Brucker et al., 2012). et al

La dieta del huésped tiene un efecto determinante en el microbioma del intestino, por ejemplo, se ha detectado alta biodiversidad en la composición de los fila de bacterias en carnívoros y omnívoros y menos en herbívoros (Muegge et al., 2008). La microbiota de los vertebrados está dominada por miembros de los fila Firmicutes, Bacteroidetes y Proteobacteria, independientemente de si el huésped es herbívoro u omnívoro, aunque la proporción de cada uno de estos varía sustancialmente dependiendo del gremio alimentario al que pertenece su huésped (Ley et al., 2009; Hird et al., 2014; Colston et al., 2015; Carrillo et al., 2015).

Además de los patrones generales observados para los mamíferos entre dietas carnívoras y herbívoras (Ley et al., 2008), se ha encontrado que los mamíferos filogenéticamente distantes que han convergido en dietas altamente especializadas (por ejemplo, hormigas) tienen microbios intestinales muy similares (Delsuc et al., 2014). Aún no

es posible dilucidar si esta convergencia ocurre en un rango filogenético más amplio de vertebrados en poblaciones en vida silvestre.

Los estudios en seres humanos sugieren que los miembros de Bacteroidetes que están presentes en el tracto gastrointestinal son a menudo responsables de la fermentación de carbohidratos, la degradación de material derivado de plantas y de potencialmente producir ácidos grasos de cadena corta que pueden ser absorbidos por el huésped e incluso contribuir a su nutrición (Walter y Ley, 2011). Sin embargo, esto no es tan claro en aves, y se ha encontrado que dos especies herbívoras que llevan a cabo la fermentación en el intestino anterior, como el hoatzin sudamericano, *Opisthocomus hoazin* y el kakapo, *Strigops habroptilus* de Nueva Zelanda, tienen microbiomas endógenos significativamente diferentes a pesar de tener estrategias alimentarias similares (Godoy-Vitorino et al., 2012; Waite et al., 2012; David et al., 2014).

Los microbiomas cutáneos y mucosos desempeñan un papel importante en la resistencia a la enfermedad del huésped (The Human Microbiome Consortium, 2012), y los cambios en el microbioma del intestino de los seres humanos pueden correlacionarse con los cambios en el microbioma de otras regiones del cuerpo.

Durante sus largos períodos de ayuno, las ballenas jorobadas (*Megaptera novaeangliae*) muestran cambios significativos en sus microbiomas cutáneos con respecto a aquellos periodos con alimentación continua, posiblemente reflejando el estrés o una menor salud durante los períodos de ayuno (Apprill et al., 2014). et al

Los patrones temporales en el microbioma de los animales que se alimentan de forma intermitente es otra área poco conocida. Muchos vertebrados tienen ciclos de alimentación y ayuno, un patrón de alimentación que es común en los reptiles, pero también se observa en anfibios y peces. Se sabe por ejemplo que el microbioma intestinal de las pitones birmanas cambia durante los períodos de ayuno y alimentación (Costello et al., 2010), lo que sugiere que existen cambios en otras regiones del microbioma del huésped. Los estudios en mamíferos han mostrado cambios en la estructura de la comunidad microbiana endógena después del ayuno (Morishita y Miyaki, 1979; Sonoyama et al., 2009) y lo mismo se ha sugerido para los peces (Xia et al., 2014) y reptiles (Costello et al. 2013; Colston et al., 2015). Es probable que los períodos prolongados de ayuno lleven a reducciones sustanciales en la disponibilidad de nutrientes para el microbioma endógeno, lo que podría conducir a los

cambios tanto en la diversidad general como en la composición filogenética. En una comparación entre diferentes clases de vertebrados, Kohl y colaboradores (2014) demostraron que el ayuno aumentó la diversidad en el microbioma del colon de los peces tilapia (*Oreochromis niloticus*) y anfibios sapos del sur (*Anaxyrus terrestris*), pero disminuyó la diversidad en el microbioma del colon de las aves (*Coturnix coturnix*) y en el reptil gecko leopardo (*Eublepharis macularius*). Este estudio sugirió algunas respuestas comunes de la comunidad microbiana del intestino vertebrado a la disponibilidad de alimentos (disminuciones en la abundancia relativa de géneros como *Ruminococcus* y *Coprobacillus*).

Por otra parte, en la actualidad se explora de manera activa el microbioma relacionado al sistema endócrino relacionándolo con el comportamiento del huésped (Foster y McVey Neufeld, 2013; Lyte, 2013). Las señales hormonales en el comportamiento de muchos taxones de vertebrados puedem tener cambios inducidos por la dieta en el microbioma intestinal y esto podría estar sucediendo en especies no mamíferas.

Animales en cautiverio

El microbioma no es estático, está en cambio continúo regulado por diversos factores, entre los que se encuentran la nutrición, tratamientos médicos, medio ambiente, condiciones sociales y estrés (Cheng et al., 2015). Aunque los zoológicos tratan de imitar las condiciones ambientales de los animales en vida silvestre, el cautiverio provoca cambios en su conducta y fisiológicos (et alDinan et al., 2012; Cheng et al., 2015). Por ejemplo, se registra mayor estabilidad en el microbioma en los vertebrados herbívoros de vida libre que en cautiverio (Alfano et al., 2015); otro ejemplo es el caso particular del demonio de Tasmania *Sarcophilus harrisii*, que presenta cambios del microbioma mayores en cautiverio que en vida silvestre, además de que el microbioma es más semejante entre los grupos con la condición de cautiverio que los que se encuentran en vida libre (Cheng et al., 2015).

Edad

Las etapas del ciclo de vida del hospedero son propias de la edad del mismo y están ligadas directamente a la composición del microbioma. Ello debido a que cada etapa presenta cambios a nivel intestinal como pH, potencial redox, la secreción biliar y enzimática, estas últimas ligadas a cambios hormonales (et alOchman et al*.,* 2000; Qin et al., 2010; Zhu et al.,

2011; Godoy-Vitorino et al*.,* 2012).

Geografía

Los estudios realizados sobre microbioma en humanos con base en la geografía demuestran que la vida moderna, alto consumo de medicamentos y una dieta pobre influye en el decremento de la biodiversidad del microbioma, así como en su transmisión. Ello es opuesto en comunidades humanas que han estado aisladas y con mínimo contacto con otros individuos, sin contacto con antibióticos, donde la biodiversidad de microbioma es mayor (Clemente et al., 2015). En anfibios de la misma especie se ha encontrado diferencia en la biodiversidad de microbioma en diferentes posiciones geográficas de una rivera (Clemente et al., 2015).

Los organismos voladores no se ajustan con tanta precisión a los patrones filogeográficos de los organismos no voladores u otras especies con poca dispersión (Ditchfield, 2000). La capacidad de volar confiere a los organismos mayor movilidad y por ende dispersión, de manera que las barreras geográficas son más fáciles de evadir para estos organismos, confiriéndoles mayor flujo génico (Ditchfield, 2000; Guevara-Chumacero et al., 2010; Turmelle et al*.,* 2010).et al

Principales grupos de bacterias encontrados en las asociaciones microbióticas en diferentes órganos de los vertebrados.

Actinobacteria

Las actinobacterias (se conocían como bacterias Gram-positivas), son consideradas como bacterias del suelo, aunque se encuentran en la mayoría de los ambientes, incluso asociadas con animales. Todos los miembros del filo son heterótrofos, e incluyen especies aerobias y anaerobias, así como algunos géneros patógenos (*Corynebacterium, Mycobacterium, Propionibacterium*). Consituyen el 50% de la comunidad microbiana de la piel humana y menos del 5% del microbioma intestinal. Este filo también se ha detectado en el intestino de peces y aves, así como en la mucosa y la piel de los peces. La mayoría de las actinobacterias que habitan en el intestino son especies de *Bifidobacterium*, que ayudan a mantener la homeostasis del huésped, la inhibición de los patógenos Gram-negativos y la fermentación

del ácido láctico. En humanos, *Bifidobacterium* son miembros dominantes del microbioma del intestino durante la infancia, donde probablemente tengan un papel metabolizando los azúcares de la leche.

Bacteroidetes

Los miembros de Bacteroidetes son bacterias heterotróficas que llevan a cabo una serie de metabolismos que van desde la respiración aeróbica hasta la fermentación. El filo se llamaba grupo de Cytophaga-Flavobacterium-Bacteroides, que representan géneros en sus tres clases dominantes (Cytophagia, Flavobacteriia, Bacteroidia). Bacteroidetes es uno de los fila bacterianos más abundantes en el tracto gastrointestinal de vertebrados y el género *Bacteroides* es el más común. Estos organismos son estrictamente anaeróbicos y tienen la capacidad de degradar polisacáridos y proteínas en el intestino, siendo de gran relevancia en las dietas herbívoras y carnívoras. El aumento de *Bacteroides* se ha relacionado con la obesidad en los mamíferos, potencialmente por su capacidad para liberar energía adicional de alimentos de otro modo indigeribles (Turnbaugh et al., 2006). Bacteroidetes contribuye en el desarrollo de la mucosa del huésped y la inmunidad sistémica. Flavobacterium y Cytophaga son patógenos en peces, causando enfermedades en piel y branquias, pero no son comunes en el tracto intestinal.

Firmicutes

Es el fila bacteriano más abundante en el tracto gastrointestinal de los vertebrados herbívoros y también son dominantes en piel. Los Firmicutes presentes en el tracto gastrointestinal pertenecen a los miembros de la clase Clostridia, anaerobios obligados que utilizan la fermentación como su único metabolismo, aunque también son importantes en la descomposición de los hidratos de carbono y la absorción de nutrientes. *Clostidium difficile* puede llegar a ser patógena en seres humanos después de utilizar antibióticos, debido a la alteración del microbioma del intestino. La mayoría son comensales y son importantes en el mantenimiento de la homeostasis intestinal y el desarrollo de inmunidad (Lopetuso et al., 2013). Otros Firmicutes incluyen las bacterias del ácido láctico, y aunque se encuentran en el tubo digestivo son más comunes en la piel. Lactobacillales también se encuentra con frecuencia en el tracto vaginal humano, donde desempeña un papel importante en la

reducción de patógenos a través de la producción de ácido láctico. Dichas bacterias también se han detectado en la cloaca de los anfibios.

Fusobacteria

Las Fusobacterias son uno de los fila menos abundantes en el tracto gastrointestinal de vertebrados y representan alrededor del 5% de las bacterias encontradas en la cavidad oral humana (Cho y Blaser, 2012). El papel de estos organismos en el tracto gastrointestinal es desconocido, la mayoría de las especies son anaeróbicas y metabolizan los aminoácidos en lugar de los azúcares, lo que sugiere un papel potencial en la degradación de proteínas. El aumento de Fusobacteria en el colon humano se relaciona con la presencia de células cancerosas (Gao et al., 2015), aunque no es claro si están involucradas en la formación de tumores o simplemente hacen uso de tumores como sitios de unión para el crecimiento. En los vertebrados que se alimentan de carroña como caimanes y buitres, se ha detectado un aumento de Fusobacteria indicando su capacidad de degradación de las proteínas.

Proteobacteria

Proteobacteria, junto con Firmicutes y Bacteroidetes, son los tres fila más abundantes en el tracto gastrointestinal de la mayoría de vertebrados. Aunque este filo ocupa el tercer lugar de abundancia en el tracto intestinal de mamíferos, es dominante en el tracto gastrointestinal de algunos peces, reptiles y aves. Proteobacteria es el filo bacteriano más grande en términos del número de bacterias cultivables y ha sido el más estudiado. Aunque todos sus miembros son Gram-negativos, metabólicamente son diversos e incluyen heterótrofos y autótrofos, con actividades que incluyen respiración, fermentación, fotosíntesis anoxigénica y quimioautotrofía. Las Proteobacterias se clasifican típicamente en Alfa, Beta, Gamma, Delta y Epsilon, de las cuales las Gammaproteobacterias son las más comunes en el tracto gastrointestinal vertebrado. Estas bacterias usualmente descomponen y fermentan los azúcares complejos e incluyen a *Salmonella* y *Escherichia* y estas últimas son importantes en la producción de vitaminas para el huésped. Los miembros de las Betaproteobacteria y Epsilonproteobacteria habitan el tracto gastrointestinal de los vertebrados, y las segundas incluyen al género *Helicobacter* que habita de manera natural en el estómago de los mamíferos. Estos y otros Epsilonproteobacteria también se han encontrado en el tracto

intestinal de aves y reptiles.

Tenericutes

Los Tenericutes se han agrupado típicamente con los Mollicutes, un grupo inusual de Firmicutes, pero se identifican más correctamente como su propio filo. Se caracterizan por la falta de pared celular y son muy pequeños, al igual que su genoma. Muchos son parásitos (de plantas y vertebrados) y requieren un hospedero. Son muy difíciles de cultivar y por ello han sido poco estudiados. Tenericutes se ha encontrado en el tracto intestinal de vertebrados como peces y anfibios juveniles, donde pueden estar involucrados en el procesamiento de nutrientes, particularmente para huéspedes detritívoros. Son miembros dominantes de los microbiomas de corales (Kellogg et al., 2009; Gray et al., 2011), lo que sugiere que pueden ser importantes para los organismos acuáticos.

Diversidad y función del microbioma en los vertebrados

Peces

Los peces cartilaginosos son el grupo de vertebrados más diverso, con 34,000 especies descritas (Nelson, 2006) y aquellos con aletas rayadas representan más del 50% de las especies conocidas actualmente. Los peces son ectotérmicos amnióticos y acuáticos obligados; representan uno de los grupos de vertebrados más exitosos, viven en aguas marinas y dulces en todo el mundo, y también están adaptados para vivir en ambientes extremos, incluso existen especies que toleran corrientes de sulfuro de hidrógeno. Tienen diferentes gremios alimentarios: herbívoros, omnívoros o carnívoros (Nelson 2006). Dependiendo de la filogenia del huésped y las condiciones ambientales, se pueden encontrar microbiomas con alta biodiversidad en los peces.

et alLos análisis filogenéticos y estadísticos de 25 librerías de diferentes taxones de peces usando 16S rDNA demuestran que la salinidad, el nivel trófico y la filogenia del hospedero modelan la composición anatómica del microbioma intestinal de peces, siendo estas comunidades bacterianas muy semejantes a las que se encuentran en mamíferos e insectos. El microbioma de peces es más especializado de lo que se creía y se relaciona más al de los mamíferos, lo que podría explicarse como una convergencia, y sugiere la posibilidad

de que los peces sean el primer grupo de vertebrados surgido de simbiosis y se haya extendido hasta el sistema de fermentación de los mamíferos (Colston et al., 2016).

En el microbioma de peces se encontró un ácido graso que opera como agente anti-microbiano, con actividad antagonista contra las siguientes bacterias: *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecium* y *Vibrio mimicus* (Sánchez et al., 2012).

Anfibios

Los anfibios (ranas, salamandras y caecilianos) pertenecen a los vertebrados ectotérmicos, anamnióticos. Sus hábitats son variados, desde acuáticos terrestres, arbóreos y de agua dulce en regiones templadas como tropicales (Vitt y Caldwell, 2013). El ciclo de vida de los anfibios incluye una larva acuática, con cambios drásticos en su fisiología durante la metamorfosis. Actualmente se conocen 7,517 especies de anfibios (amphibiaweb.org). Durante la etapa juvenil son herbívoros/detritívoros y en estadíos adultos, carnívoros u omnívoros.

La diversidad de sus comunidades microbianas asociadas puede estar relacionada con sus historia de vida y a las similitudes que presenta este grupo, tales como: hábitos alimentarios de carnívoros/omnívoros, estadíos larvales acuáticos y a las fluctuaciones ambientales como la temperatura (Colston et al*.,* 2016).

Los anfibios larvales carecen de estómago de manera general, pero la boca conduce inmediatamente a un intestino recubierto de mucosidad con un pH bajo y sin regiones distintas. El tracto gastrointestinal de los anfibios experimenta una reestructuración durante la metamorfosis y los anfibios adultos tienen un estómago recubierto de mucosa, intestino acortado y el intestino posterior definido. Los estudios de microbioma con hisopos cloacales, en las diferentes regiones intestinales (tejido intestinal, tracto gastrointestinal completo y heces fecales), muestran una variación baja en la región del gen 16S rDNA con estudios que utilizan típicamente la región V4.

El microbioma en piel de anfibios muestra diferencias en la biodiversidad dependiendo de la región donde se encuentre y dependiendo de la humedad, pH de la piel y otros factores endógenos y exógenos incluyendo los ambientales.

En individuos de la misma especie también se han detectado cambios en la biodiversidad del microbioma dependiendo del cuerpo acuático donde se encuentren, lo que

sugiere que el microbioma se ve influenciado por la geografía, siendo el cuerpo un simil de topografía de los anfibios y también en la situación geográfica (Colston et al.. 2016).

Reptiles

Se conocen 10,272 especies de reptiles (reptile-database.org), el segundo grupo más diverso de vertebrados amnióticos después de las aves (Pincheira-Donoso et al., 2013), y se encuentran en todos los continentes excepto la Antártida, en hábitats terrestres, de agua dulce y marinos (Vitt y Caldwell, 2013). Son vertebrados ectotérmicos. Se refieren a anfisbenios, lagartijas, serpientes, cocodrilos, tortugas y los tuátara. Los reptiles presentan una gran diversidad de historias de vida y estrategias reproductivas. Su reproducción puede ser sexual o asexual y en algunos casos tienen la capacidad de cambiar el modo de paridad, un rasgo bastante plástico en este grupo. Asimismo, tienen una variedad de estrategias de alimentación, incluyendo la herbivoría y la omnivoría, pero la gran mayoría de las especies son carnívoras. La mayoría de los reptiles, como las aves y los mamíferos, tienen glándulas salivales que ayudan en la deglución de los alimentos, que recorre el esófago desde la boca. El estómago en los reptiles es tubular y carece de un píloro separado, con la excepción de los cocodrilos. La superficie mucosa del estómago se divide en regiones gástricas, pilóricas y ocasionalmente cardiacas. El intestino medio de los reptiles carnívoros tiende a ser más largo que el de los herbívoros. Los reptiles herbívoros tienen generalmente un ciego y un colón proximal que se definen por los pliegues de la mucosa. Los microbios del intestino del reptil se caracterizan a menudo por muestras fecales que han sido expuestas al medio ambiente, aunque se han utilizado secciones del tejido del tracto gastrointestinal, hisopos y frotis cloacales. La porción del gen 16S rDNA secuenciado ha sido típicamente la región V1-V4, con estudios más recientes centrados en la región V4 (Colston et al., 2016).

Aves

Las aves son el grupo más diverso de vertebrados amnióticos con 10,425 especies y más de 20,000 variedades de subespecies (avibase.org), son endotérmicas, con una distribución general de plumas de los amniotes. Muchas especies experimentan migraciones estacionales a través de grandes distancias. Las aves presentan diferentes gremios alimentarios que están generalmente relacionados con el tamaño del cuerpo; especies más pequeñas (por ejemplo,

colibríes) tienden a ser herbívoras y las especies más grandes (ej. águilas) carnívoras, con excepción de las aves no voladoras. Los pájaros exhiben cuidado parental en un grado mayor que otros vertebrados con la excepción de los mamíferos, factores que se podría presumir tienen importancia respecto a sus relaciones con las comunidades microbianas asociadas (Colston et al., 2016).

El ventrículo, el proventrículo y la molleja en las aves llevan a cabo las funciones que realiza el estómago en otros vertebrados, tales como almacenamiento de alimento, secreción ácida y trituración. El tamaño relativo y las propiedades mucosas de estos órganos varían con la dieta, donde herbívoros suelen tener tejidos más grandes y mollejas musculares. El intestino medio de la mayoría de los pájaros es corto, y el intestino posterior consiste en un colon corto y recto y cloaca típicamente emparejado. Dentro de las especies de aves herbívoras, se sabe que el sitio de fermentación microbiana varía sustancialmente y puede ser el ciego, el intestino medio o el colon. Típicamente, el sitio de fermentación se agranda con respecto a otros órganos (por ejemplo, el emú tiene un colon corto, pero un intestino medio largo). El microbioma del intestino de las aves se ha determinado utilizando heces fecales, tejidos intestinales e hisopos cloacales. Se ha estudiado más el microbioma de las aves domésticas usando el tracto gastrointestinal. La variación en la región del gen 16S rDNA secuenciado ha sido sustancial, y la mayoría de los estudios utilizan la región V3-V4 (Colston et al., 2016).

Mamíferos

Existen alrededor de 5,487 especies, cinco son monotremas, 272 marsupiales y 5,209 placentarios. Son amniotas homeotermos, poseen glándulas mamarias y pelo en alguna etapa de su vida. Son vivíparos, excepto los monotremas (ornitorrinco y equidnas) que representan un taxón monofilético. El aparato digestivo de los mamíferos es un tubo visceral en el que los alimentos se someten a un intenso tratamiento para obtener el máximo aprovechamiento de los nutrientes. Durante el tránsito digestivo desde que se ingiere hasta que se excreta, el alimento pasa por un proceso de degradación mecánica y química en donde intervienen una serie de órganos y tejidos encadenados estratégicamente (Colston et al., 2016). Los mamíferos reciben beneficios de sus bacterias mutualistas no patógenas. Se le considera el tercer genoma principal en los mamíferos junto con los genomas nuclear y mitocondrial. En

general, los estudios se centran en dilucidar la complejidad de los organismos que comprenden el ecosistema único del tracto gastrointestinal y los asociados a superficies epidérmicas. Existen multiples estudios enfocados en el microbioma gastrointestinal, su relación con la salud y la enfermedad humanas con un enfoque particular en la fisiología de los mamíferos y los esfuerzos para modelar su composición como un enfoque terapéutico (Carroll et al., 2009).

Microbioma de murciélagos

El orden Chiroptera es un grupo evolutivo y ecológicamente diverso, representa al 20% de todos los mamíferos y es el segundo grupo con mayor riqueza después de los roedores (Wilson y Reeder, 2005). La familia Phyllostomidae incluye 6 subfamilias: Phyllostominae, Phyllonycterinae, Glossophaginae, Carolliinae, Stenodermatinae y Desmodontinae (Rojas et al*.,* 2011) e incluye alrededor de 123 especies (Fenton et al., 1992).

Para el caso particular de murciélagos, los fila bacterianos más abundantes en la piel de poblaciones sanas de especies de filostómidos como *Artibeus lituratus* y *Carollia brevicauda*, incluyen a *Actinobacteria, Proteobacteria, Firmicutes, Cyanobacteria, Bacteroidetes y Fusobacteria*, los cuales son reguladores del microbioma en la piel (Lemieux-Labonté et al., 2016). Las abundancias relativas de los fila bacterianos en el intestino están relacionados con la filogenia del hospedero (Carrillo et al., 2015). Los frugívoros y nectarívoros presentan una mayor diversidad bacteriana que los carnívoros (Carrilo-Araujo et al., 2015), las hembras activas tienen una mayor diversidad en la microbiota que las no reproductivas (Phillips et al., 2013; Gaona et al., 2019).

Las señales olfativas son muy comunes en los murciélagos, donde los componentes químicos están asociados al reconocimiento, la comunicación y la selección de pareja Estos componentes químicos se producen en glándulas cutáneas, anales y otras estructuras, en ocasiones heridas provocadas que producen olores, orina y heces fecales. Algunos murciélagos que presentan señales olfativas son los murciélagos pescadores *Noctilio leporinus* (Noctilonidae) que tienen un fuerte olor que se relaciona a las bacterias denominadas *Staphylococcus aureus* (Voigt et al., 2005; Ware y Gosden, 1980). Los machos de los murciélagos con sacos en las alas, *Saccopeteryx bilineata* (Embalonuridae) poseen una bolsa en las alas en donde guardan un líquido oloroso que usan para cortejar (Voigt et al.,

2005). El murciélago café, *Eptesicus fuscus* (Vespertilionidae) comparte un olor característico con sus compañeros de cueva, el cual difiere totalmente de los otros individuos que no son de su área de percha (Bloss et al., 2002). En el caso de *Leptonycteris yerbabuenae*, los machos suelen desarrollar una estructura denominada parche dorsal en la que ponen saliva y líquidos urogenitales.

Los murciélagos representan un buen modelo para estudiar microbioma debido a su gran diversificación evolutiva asociada con su alimentación, incluyendo insectivoría (rasgo ancestral) (Monteiro y Nogueira, 2011; Carrillo-Araujo et al., 2015) así como la alimentación de sangre, pequeños vertebrados, néctar, fruta y complejas dietas omnívoras (Gardner, 1979), lo que les confiere una gran plasticidad y abundancia, así como la facilidad en el manejo y obtención de heces fecales con métodos no invasivos (Phillip et al., 2012; Carrillo et al., 2015et al).

Historia de vida de Leptonycteris yerbabuenae

Es un murciélago de tamaño mediano, se le conoce como murciélago magueyero menor. *Leptonycteris yerbabuenae* es nectarívoro y frugívoro, pertenece a la familia Phyllostomidae y a la subfamilia Glossophaginae (Simmons, 2005). Los individuos de esta especie pesan entre 23-26 g y el antebrazo mide 51-56 mm (Ceballos et al., 1997). La subfamilia Glossophaginae se describe como una familia del Nuevo Mundo, con hoja nasal y con dieta altamente especializada de polen y néctar. Los murciélagos de esta subfamilia se caracterizan por tener el rostro elongado, lengua extensible y larga, y los dientes son reducidos comparados con otras subfamilias (Fleming, 2004). Otros miembros de la subfamilia Glossophaginae y del género *Leptonycteris* son *L. nivalis* y *L. curasoae* (Simmons, 2005), que son especies simpátricas desde Nuevo México hasta la mitad Este y la parte central de México, a lo largo de diversas latitudes (Arita, 1991). *Leptonycteris curasoae* habita la parte norte de Sudamérica y las islas del Caribe (Fleming y Nassar, 2002). Anteriormente *L. yerbauenae* se consideraba una sola especie, conocida como *L. curasoae* (Arita y Humphrey, 1988). Se ha determinado que *L. yerbabuenae* y *L. curasoae* son dos especies distintas (Simmons, 2005). Los nombres que se han utilizado para referirnos a esta especies son: *L. yerbabuenae*, *L. sanborni*, *L. curasoae* y *L. curasoae yerbabuenae*.

Figura 1. *Leptonycteris yerbabuenae*. Ilustración elaborada por Ximena Neri.

México tiene dos poblaciones diferenciadas de *L. yerbabuenae*: una a lo largo de la costa del Pacífico, incluidos los estados de Baja California, Sonora y Jalisco, y la otra en la región centro-sur, en los estados de Oaxaca y Morelos (Morales-Garza et al., 2007). Las dos poblaciones están separadas geográficamente, con bajo flujo genético, como lo demostró Morales-Garza et al. (2007) usando análisis de ADN polimórfico amplificado al azar (RAPD).

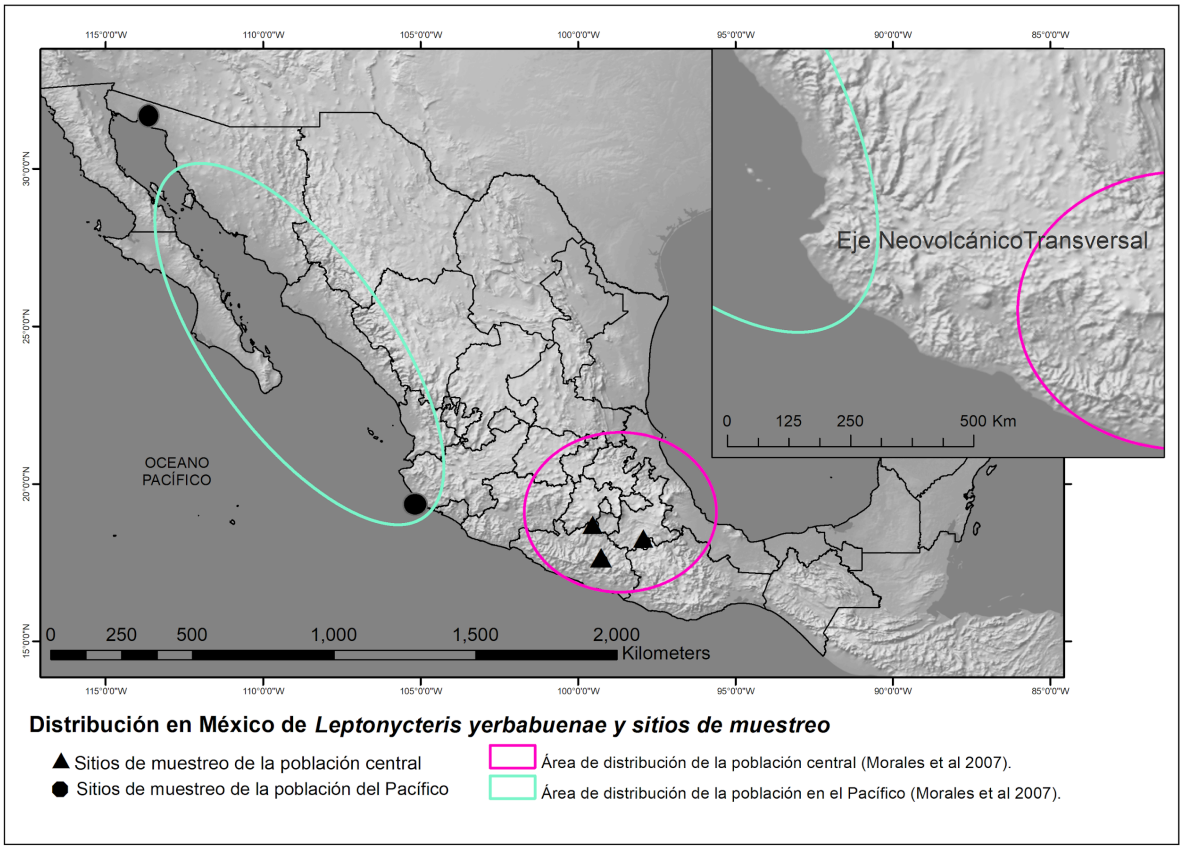

Figura 2. Distribución de *Leptonycteris yerbabuenae* en México. Mapa elaborado por Ximena Neri.

En ambas poblaciones, los refugios se diferencian por su etapa reproductiva (et alCockrum, 1991; Stoner et al., 2003; Ceballos et al., 2006;), con refugios de apareamiento y maternidad separados geográficamente, machos en estado reproductivo y no reproductivo (Hayward y Cockrum, 1971; Fleming y Nassar, 2002; Stoner et al., 2003; Sánchez y Medellín, 2007). Las hembras preñadas se congregan en colonias de maternidad para parir a sus crías, amamantar y cuidarlas (Ceballos et al., 2006), y generalmente regresan al mismo refugio año tras año en diferentes etapas del embarazo durante toda su vida (Hayward y Cockrum, 1971). Los machos adultos y las hembras no reproductivas a menudo se segregan en grupos llamados "colonias de solteros" (Ceballos et al., 2006). Antes de alimentarse por la noche, ambos sexos descansan en perchas nocturnas temporales (Ceballos et al., 2006; Cole y Wilson, 2006).

El murciélago magueyero menor se distribuye en el matorral y desierto semiárido y en la selva seca en diversos rangos. En Estados Unidos *L. yerbabuenae* se encuentra en

Arizona (Condado Pima, Condado Santa Cruz y Condado Cochise) y el suroeste de Nuevo México (Cockrum, 1991). En México, se encuentra en Nuevo León, centro de México hasta Chiapas, y su distribución se extiende hasta Sonora, Sinaloa y Baja California Sur (Woolozyn y Woolozyn, 1982; Arita, 1991), continuando hasta Guatemala, Honduras y El Salvador (Simmons, 2005).

De igual manera, *L. yerbabuenae* se ha encontrado desde el bosque seco hasta la selva baja, donde consume néctar, polen y frutos pertenecientes a una gran variedad de plantas (Fleming et al., 1993; Valiente-Banuet et al., 1996; Stoner et al., 2003et al). Se ha registrado que en el centro de México consumen plantas de la familia Cactaceae (Valiente-Banuet et al., 1996), y en la selva baja consumen además plantas de las familias Capparaceae, Malvaceae y Bombacaceae en la estación seca y en la época de lluvia consume Agavaceae, Bignoniaceae, Cactaceae y Eupharbiaceae (Stoner et al. 2003; Sperr et al., 2011).

Además existe una población residente de *L. yerbabuenae* en Baja California Sur (Woolozyn y Woolozyn, 1982; Fleming et al., 1993). Fleming y colaboradores (1993) han propuesto que es residente anual (no migra) debido a que existen recursos suficientes de cactáceas de las que se alimenta casi todo el año y en el invierno se alimenta de plantas de Agavaceae; la población del centro del país presenta el mismo patrón.

L. yerbabuenae es monoéstrico, es decir, tiene una cría por año (Stoner et al., 2003; Galindo et al.; 2004). Hacia finales del verano y principios de otoño, los jóvenes son destetados, y los murciélagos comienzan su migración hacia el sur hasta el suroeste de México (et alCockrum, 1991; Fleming et al., 1993; Wilkinson y Fleming, 1996; Ceballos et al., 1997). Las hembras nacidas en el invierno (octubre-diciembre) se quedan aparentemente todo un año en este refugio (Galindo et al., 2004), y salen de él para migrar al cumplir un año (machos y hembras de la misma manera) (Ceballos et al., 1997; Stoner et al., 2003).

Se ha comprobado la alta movilidad de *L. yerbabuenae* donde los movimientos migratorios exceden los 1,500 km y se han registrado movimientos nocturnos de hasta 100 km (Horner et al., 1998; Ramírez, 2011).

Este trabajo de tesis doctoral se presenta en tres capítulos: en el primer capítulo se analizó la composición microbiana en muestras de heces en los diferentes estadíos reproductivos de *L. yerbabuenae* (juveniles y adultos de ambos sexos y hembras preñadas y

lactando). Se analizó la region V4 del gen 16S rDNA de 33 individuos usando estadísticos de diversidad alfa y beta. Se encontró que la diversidad microbiana expresada en *Amplicon Sequence Variants* (ASV) es más alta en en las hembras preñadas y lactando que en el resto de los estadíos reproductivos. La microbiota de adultos y juveniles está representada principalmente por Gammaproteobacteria y Firmicutes. Las hembras preñadas tienen una microbiota más diversa, con un incremento en los fila Bacteroidetes y Alfaproteobacteria. No se encontró diferencia significativa entre las hembras preñadas y lactando, ni entre los juveniles y adultos no reproductivos. Nuestros resultados sugieren que existe una diferencia en la microbiota relacionada a los estadíos reproductivos. Se ha inferido que los machos mantienen una microbiota estable porque no tienen cambios fisiológicos imortantes como las hembras durante la preñez y lactancia, manteniendo una dieta más especializada que las hembras durante su ciclo de vida.

En el segundo capítulo exploramos la composición microbiana del parche dorsal de *L. yerbabuenae*, que es exclusivo de los machos y se desarrolla durante la temporada de apareamiento. Este parche desempeña un papel crucial en el reconocimiento parental y de apareamiento selectivo. Nuestros resultados sugieren que las bacterias que se encontraron en el parche dorsal podrían desencadenar procesos fermentativos y existen bacterias no compartidas que determinan la individualidad del huésped. Utilizando la secuenciación de nueva generación de la región V4 del gen 16S rDNA, identificamos 2,847 filotipos en los parches dorsales de 11 individuos. Veintiséis filotipos se compartieron entre todos los parches, los cuales representan del 30 al 75% de su abundancia relativa. Estas bacterias compartidas se distribuyen en 13 Familias, 10 Ordenes, 6 Clases y 3 Fila. Dos de estos componentes bacterianos comunes del parche dorsal son *Lactococcus* y *Streptococcus*. Siete de ellos *Helcococcus*, *Aggregatibacter*, *Enterococcus* y *Corynebacteriaceae*, incluyen bacterias con potencial patogénico. La mitad de los filotipos compartidos pertenecen a *Gallicola*, *Anaerococcus*, *Peptoniphilus*, *Proteus*, *Staphylococcus*, *Clostridium* y *Peptostreptococcus* y se especializan en la producción de ácidos grasos a través de procesos fermentativos. Este trabajo sienta las bases para futuros estudios de microbios simbióticos centrados en la hipótesis fermentativa involucrada en las estrategias de comunicación y reproducción en la vida silvestre.

En el tercer capítulo exploramos como dos poblaciones discretas y geográficamente separadas del murciélago de nariz larga menor (*L. yerbabuenae*), una en el centro y otra en la región del Pacífico de México, difieren en su composición de microbioma fecal. La región V4 del gen 16s rDNA de 68 individuos se analizó utilizando métricas de diversidad alfa y beta. Obtuvimos un total de 11,566 *Amplicon Sequence Variants* (ASV). Las comunidades bacterianas en las poblaciones central y pacífica tenían una diversidad de 6,939 y 4,088 ASV respectivamente, compartiendo una microbiota de 539 ASV. Las hembras lactantes y preñadas en las dos poblaciones tuvieron diferencias significativas en los patrones de diversidad beta con respecto a los adultos no reproductivos. La microbiota fecal es diferente entre poblaciones. Esto podría ser una consecuencia de la diversificación de la dieta entre poblaciones asociada a la separación geográfica. Nuestro estudio evalúa los cambios de la microbiota en dos poblaciones de L. yerbabuenae con poco flujo génico y separación geográfica. Estos resultados proporcionan una línea de base para futuros estudios del microbioma en poblaciones silvestres de *L. yerbabuenae*, polinizador de los agaves de los que se elaboran bebidas como el tequila y el mezcal.

Capítulo 1. Microbiota fecal en los diferentes estadíos reproductivos de la población central del murciélago de nariz larga menor *L. yerbabuenae*.

Gaona O, Gómez-Acata ES, Cerqueda- García D, Neri-Barrios CX, Falcón LI (2019) Fecal microbiota of different reproductive stages of the central population of the lesser-long nosed bat, *Leptonycteris yerbabuenae*. PLoS ONE 14(7): e0219982. https://doi.org/10.1371/journal. pone.0219982

PLOS ONE

RESEARCH ARTICLE

Fecal microbiota of different reproductive stages of the central population of the lesser-long nosed bat, *Leptonycteris yerbabuenae*

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Abstract

In this study we analyzed the microbiota composition of fecal samples from the lesser-long nosed bat *Leptonycteris yerbabuenae* in different reproductive stages (juveniles and adult bats of both sexes as well as pregnant and lactating females). The V4 region of the 16s rRNA gene from 33 individuals was analyzed using alpha and beta diversity metrics. We found that microbiota diversity (expressed in Amplicon Sequence Variants) is higher in pregnant and lactating females. The microbiota of the juveniles and non-reproductive adults was dominated by Gammaproteobacteria and Firmicutes. Reproductive females had a much more diverse microbiota, with a significant increase in phyla such as Bacteroidetes and Alphaproteobacteria. There was no difference in fecal microbiota diversity between pregnant and lactating females and juveniles and non-reproductive adults. Results suggest that differences in microbiota diversity are related to reproduction. We infer that males maintain stable microbiota composition because they do not undergo the large physiological changes that females do during reproduction and maintain a more specialized diet throughout all life stages.

Introduction

The community of microorganisms that reside in the vertebrate gut executes a variety of functions that impact host phenotype, nutrition, detoxification of xenobiotics, gut stimulation, immune development and behavior [1,2,3]. Thus, bacteria are directly involved in their hosts' fitness. In particular, bacteria can improve energy assimilation from different food sources through the synthesis of vitamins necessary for physiological functions [4]. For instance, mammalian genomes do not encode most of the enzymes needed to degrade the structural polysaccharides present in plant material [5], leaving them dependent on symbiotic gut

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microorganisms that are capable of accessing different sources of energy. Herbivores and omnivores benefit from additional energy from microbial fermentation of carbohydrates in the gut [6]. This has led to major changes in digestive anatomy and physiology that allow efficient microbial fermentation to take place alongside the recovery of dietary energy by the host [6]. In some vertebrates, such as ruminants, gut microbes are so essential that hosts coevolved specialized organs to enhance gut microbial functionality [7].

Studying the microbiotas of wildlife is difficult, since there are many variables that are likely to impact microbiota that cannot be controlled. For example, diet has been demonstrated to be a main factor shaping the functionality and diversity of the gut microbiota, resulting in convergent microbial communities among hosts with similar feeding habits [8,9]. There is evidence that diet shapes the relative abundance of dominant phyla, and populations of specific bacterial groups are influenced by the composition of macronutrients consumed; in addition, food itself can serve as a reservoir for new microbial introductions [10,11]. Empirical studies show that microbiota respond rapidly to changes in host diet [12,13].

Animals' dietary needs change depending on their life-history stage. In the genus *Nasonia*, the microbiota differs among the three developmental stages present in this genus, particularly between the pupal and adult stages [14]. As mentioned above, changes in the microbiota can be due to a variety of factors that may change among life stages, including environmental conditions, diet, weight, and hormones [15].

During pregnancy, there are various hormonal, immune, and metabolic changes that are associated with increases in the bacterial load in several organs, including the vagina, oral cavity, and intestine [15]. In the case of *L. yerbabuenae*, there is a significant change in females' feeding habits during reproduction, with pregnant and lactating females consuming an increased diversity of plants [16]. These changes may be temporary, related to the preferences and nutritional requirements of the individual during different reproductive stages [16]. Reproductive females of *L. yerbabuenae* and other females of nectarivorous bats (*Glossophaga soricina*) have been reported as active feeders on flowers in the driest season of the Mexican central highlands when flowers are the only food resource [17].

Microbes that reside in the gastrointestinal tract respond dynamically as a community to those changes over an individual's lifespan [10]. In small mammals, the direct costs of pregnancy and lactation include increased energy, protein and calcium demands. Organ re-modelling is necessary to achieve the high demands of lactation and involves growth of the alimentary tract and associated organs such as the liver and pancreas [18]. The pre-natal period is shaped by immunological and inflammatory changes that modify the functionality of the gut and bacterial composition in females as much as pregnancy [19,20], while in non-pregnant healthy females microbiota composition has been reported to be relatively stable [21]. Another factor affecting the functionality of the gut during the pregnancy and pre-natal stages are the hormones estrogen and progesterone [19]. Geography and behavior can also shape microbial composition. For example, the microbiota of an isolated human population of hunter-gatherers in Hadza, Tanzania presents a cyclical succession of bacterial species that correspond to the richness of functions associated with the season, and which differs from that of urbanized communities [22]. This population has limited access to plant-based foods and a carbohydrate-rich diet [23,24]. In amphibians, the biodiversity of the microbiota has been shown to vary within species depending on the geographic position along a river [25]. Some authors describe the host as a topographic map, since physical and chemical variables change along different parts of the host's body, including pH, texture, salinity, and sebaceous content; these variables are determined by factors that are intrinsic to the host (e.g. genotype, age, sex), as well as factors that are extrinsic but depend on the individual (e.g. in humans, occupation, lifestyle, geographic location, and use of antibiotics or cosmetics) [26, 27]. The host microbiota

can also be shaped by individual behavior [28]; social animals acquire much of their resident bacterial population directly through social grooming of their group members or indirectly from the environment [28].

Leptonycteris yerbabuenae is a migratory nectar specialist bat that is a pollinator of columnar cacti and Agave in North America [29]. Unlike other bat species such as *Phyllostomus hastatus* [30] or *Artibeus jamaicensis* [31] which carry out all of their activities and spend their whole life cycles in a single roost, *L. yerbabuenae* has a more complex life cycle in that it uses geographically separate roosts for copulation, giving birth, and rearing young, with roosts occupied by adult males and females [32–37]. This species is migratory, and has different roosts where they will complete their life cycles, which are limited by food availability [17].

L. yerbabuenae has two differentiated populations in Mexico: one along the Pacific coast including Baja California, Sonora and Jalisco, and a central population that occurs in south-central Mexico, in the states of Oaxaca, Morelos, and Guerrero [38–41]. This central population has local migration patterns, but is considered a resident population since there is constant food availability due to the large diversity of cacti in the region [38,39] (Fig 1). In this study we collected fecal samples from the central population of the lesser-long nosed bat to explore the microbiota composition in different reproductive stages (juvenile and adult males and females, and pregnant and lactating females), in order to describe how the microbiota differs among different reproductive stages over the complex life history of this species. Our hypothesis is that pregnant females will have the highest microbiota diversity due to the dietary and physiological changes that occur during pregnancy.

Methodology

Study site

Bat fecal microbiota samples were collected at three caves previously known to host specific reproductive stages of the lesser long-nosed bat from the Central population, between January and November, 2015 (as reproduction progressed in *L. yerbabuenae* reproductive stages). Reproductive adult males were sampled in San Juan Noxchitlan, Oaxaca (97° 40' N and 18° 03' W), a colony of 100,000 resident bats [42] in June, 2015. Pregnant and lactating females were captured in Juxtlahuaca Cave, Guerrero (17° 23" 3′ N and 99° 16" 1′W) in November, 2015, and non-reproductive adult females were sampled in Salitre Cave, Morelos (18° 45" 0.05′N and 99° 11" 23.17′) in April, 2015 (Table 1). As mentioned above, because caves are segregated by life history stage, it is not possible to find all of the different life stages in a single cave, and roosts can change from year to year.

The colony in "San Juan Nochixtlan" cave where adult males were sampled, has a mean annual rainfall of ca. 400 mm with an average temperature of 21° C in a semi-arid region [43]. The region has a high number of columnar cacti species, containing 19 of the 45 reported for south-central Mexico [29]. The landscape surrounding the "Juxtlahuaca" cave is characterized by deciduous forest vegetation and seasonal maize cultivation [44]. The characteristic climate region is dry sub-humid warm weather with rainy season in summer, with mean temperatures between 20 and 29° C [44,45]. Pregnant and lactating females were sampled here.

The "El Salitre" cave, where non-reproductive adult females were sampled, is located at an altitude of 1140 masl, in a warm sub-humid climate region with an annual total of 800–1000 mm of rain concentrated in the summer, dry winters and a mean temperature of 22°C [44,46] in a deciduous forest environment, with fragments of secondary vegetation and sugarcane and maize fields [44,46,47].

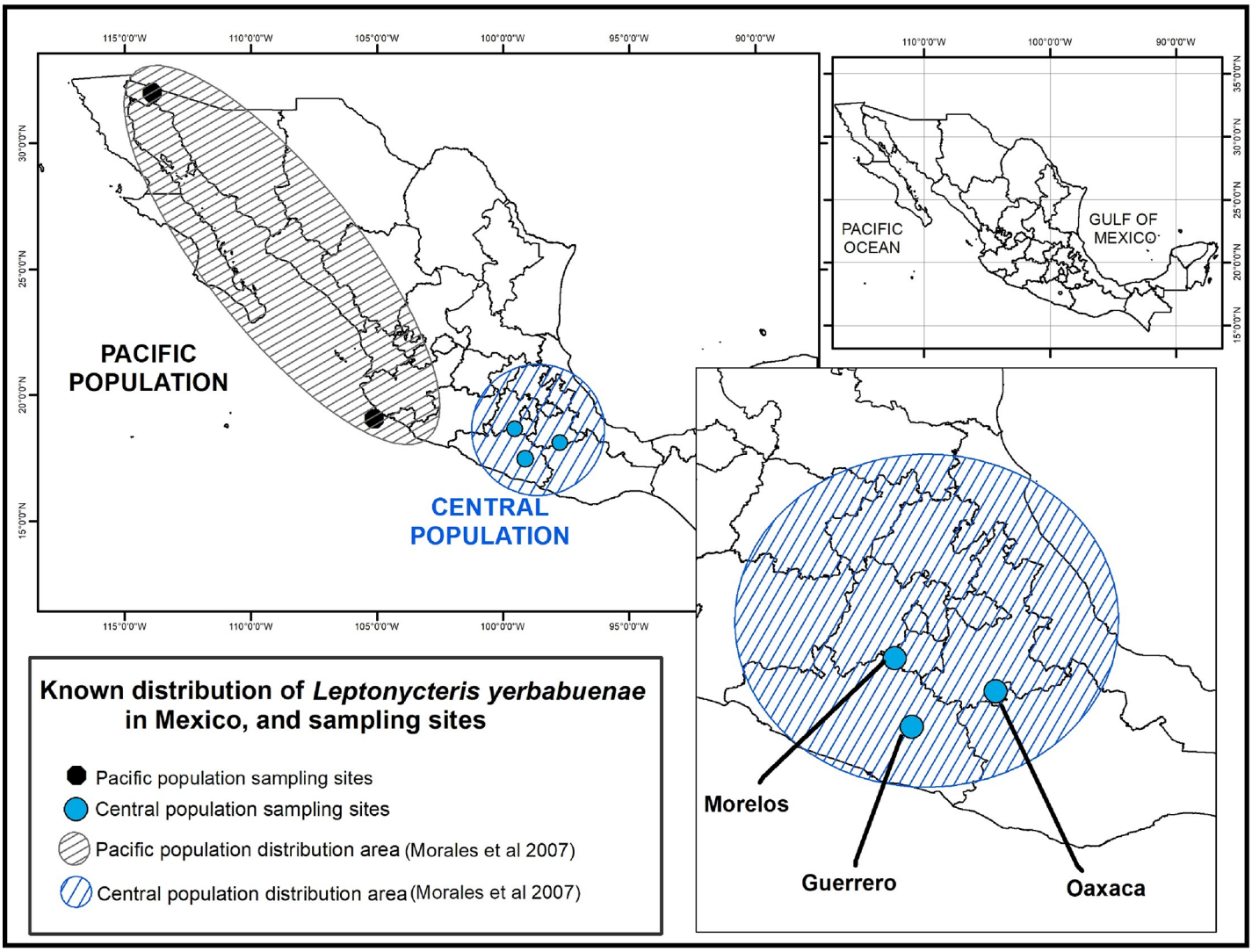

Fig 1. Distribution of the two differentiated *L. yerbabuenae* populations in Mexico. The Pacific population is found in the states of Baja California, Sonora and Jalisco. The Central population (from which our samples were collected) is found in Morelos, Guerrero and Oaxaca.

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Bat fecal microbiota sampling

Bats were captured with 12m-long mist nets (Avinet, Dryden, New York, USA) at the entrance of the caves using Kunz's technique [48], between 18:30 and 7:00 h. Each captured bat was individually placed into a clean sterile plastic bag, leaving a gap to ensure ventilation and avoid

Table 1. Sampling data from different reproductive stages of L. yerbabuenae.

Reproductive stage	Feeding	Study Site	Sex	Number of bats
Lactating	Nectar	Guerrero	Female	6
Pregnant	Nectar	Guerrero	Female	6
Adult	Nectar	Morelos	Female	6
Adult	Nectar	Oaxaca	Male	11
Juvenile	Nectar	Oaxaca	Male	4

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suffocation. Individuals defecated in a matter of seconds, and no chemical immobilizers, analgesics or sedatives, were needed. The fecal samples were collected using sterile 1.5 ml Eppendorf tubes and frozen in liquid nitrogen until they arrived at the laboratory, where they were stored at -80º C until processing. The bats were taken out of the bag to take standard body measurements and released *in-situ*. Total samples processed per reproductive stage are shown in Table 1.

Standard measurements of each individual included forearm length (measured using a manual caliper with a precision of 0.01 mm) and body mass (measured with a 100 g spring balance). Individuals' age category (juvenile or adult) was estimated based on the ossification of wing bones (metacarpals and phalanges) [49]. The condition of testes (scrotal or inguinal) was recorded in males to determine whether they were reproductively active. Pregnancy and lactation was confirmed in females by palpation of the belly and mammary glands, respectively.

Ethics statement

Samples were taken from wild bats that were released in the same area as capture immediately after fecal samples a body measurements were taken, causing no apparent harm to any of the individuals captured. *Leptonycteris yerbabuenae* is not under federal protection by Mexican law (NOM-059-SEMARNAT-2010). Scientific collection activities were carried out under a scientific collection permit number granted by the Mexican Secretary of the Environment and Natural Resources (SEMARNAT), number FAUT-0231, SGPA/DGVS/05780/15. SEMARNAT approved and authorized the tissue sampling methods under this collection permit. Laboratory activities were carried in the Ecology Institute of the Universidad Nacional Autónoma de Mexico (UNAM); no specific permit was needed because only tissue and skin samples were used (no *in-vivo* studies were included). All Biosecurity standard requirements from the Ecology Institute were satisfied.

Extraction of DNA from feces

Metagenomic DNA was extracted from the fecal samples using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, feces collected into 1.5 ml sterile tubes were diluted with 180 μl of ATL extraction buffer with 20μl of proteinase K (10 mg ml^{-1}), were mixed thoroughly by vortexing and incubated at 56˚C at 1500 rpm for 50 min. 200μl of AL Buffer with 200 μl ethanol (96–100%) were added and mixed thoroughly by vortexing. The mixture was transferred into the DNeasy Mini spin column, washed with Buffer AW1 and then with AW2. The DNA was eluted with 200 μl of Buffer AE and precipitated with absolute ethanol, 0.1 volume 3 M sodium acetate and 2 μL glycoblue. DNA was resuspended in 30 μL of molecular grade water and stored at -20˚C until PCR amplification.

16S rRNA gene amplification and sequencing

DNA samples were PCR amplified using the hypervariable region V4 of the 16S rRNA gene with universal bacteria/archaeal primers 515F/806R following the procedures reported by Caporaso et al. [50] and Carrillo et al. [51]. PCR reactions (25 μL) contained 2–6 ng of total DNA, 2.5 μL Takara ExTaq PCR buffer 10X, 2 μL Takara dNTP mix (2.5 mM), 0.7 μL bovine serum albumin (BSA, 20 mg ml^{-1}), 1 μL primers (10 μM), 0.125 μL Takara Ex Taq DNA Polymerase (5 U μl^{-1}) (TaKaRa, Shiga, Japan) and nuclease-free water. Samples were amplified in triplicate using a PCR protocol including an initial denaturation step at 95˚C (3 min), followed by 35 cycles of 95˚C (30 s), 52˚C (40 s) and 72˚C (90 s), followed by a final extension (72˚C, 12 min). Triplicates were then pooled and purified using the SPRI magnetic bead, AgencourtAMPure XP PCR purification system (Beckman Coulter, Brea, CA, USA). The purified 16S rRNA

fragments (~20 ng per sample) were sequenced on an Illumina MiSeq platform (Yale Center for Genome Analysis, CT, USA), generating ~250 bp paired-end reads. The sequence data are available from the NCBI Bioproject number PRJNA508738 with accession numbers SRR8303327 to SRR8303359.

Analysis of sequence data

Sequences were analyzed using the QIIME2 pipeline (v.2018.6) [50] (https://qiime2.org). Paired-end reads were demultiplexed with the Qiime plugin *demuxemp-paired*, then processed with the DADA2 plugin in the *denoise-paired* mode [52], trimmed at position 14 from the 5' end, and truncated at position 200 from the 3' end in both forward and reverse after manually verifying quality. Sequences were then denoised, and the amplicon sequence variants (ASVs) were resolved. Chimeric sequences were removed using the consensus method. Representative sequences of each ASV were taxonomically assigned using the QIIME plugin *feature-classifier classify-consensus-vsearch* (v 2.9.0) [53] searching in the SILVA database (release 132–99% OTUs, 515–806 region, L7 taxonomy) which was used to analyze the microbiota composition for each reproductive stage. The representative ASVs were aligned with the MAFFT algorithm [54]. After the subsequent masking of the positional conservation and gap filtering, a tree was built with the FastTree algorithm [55]. The feature table was rarified according to the same level of surveying effort of 12,569 reads per sample (S1 Fig). Mitochondrial and plastid sequences were filtered from all samples before rarefaction. The data was exported to the R environment (http://www.R-project.org/). Alpha diversity indices (Observed species, Shannon and Simpson index) were calculated with the phyloseq package [56] (Fig 2, S1 Table).

A Principal Coordinate Analysis was calculated with the weighted unifrac distance (Fig 3). To assess whether the differences between stages were statistically significant, a PERMANOVA was carried out using the vegan package [57] with the weighted unifrac distance matrix and 1000 permutations. A permutation test for homogeneity of variance (with betadisper and permutest functions) was carried out to assess the reliability of the beta diversity results.

A linear discriminant analysis (LDA) effect size (LEfSec) [58] was performed at the ASV level to find the microbial taxa whose abundances differed amongst reproductive stages, using an LDA cut-off of 2 and a Kruskal-Wallis alpha value of 0.01 (Fig 2).

Results

Microbiota composition during different reproductive stages

A total of 33 fecal samples were obtained and classified according to the sex, age and reproductive stage of the individual: Juvenile male, Adult male, Juvenile female, Adult (non-reproductive) female, Pregnant female, and Lactating female. Sample sizes per group are given in Table 1.

A total of 5,980,446 16S rRNA gene reads were obtained. After quality filtering, this was reduced to 4,120,896 reads. We found a total of 41 bacterial phyla in the bats' fecal microbiota. The dominant phyla in the samples as a whole were Firmicutes, Proteobacteria (principally class Alphaproteobacteria, Gammaproteobacteria and Deltaproteobacteria), followed by Bacteroidetes, Chloroflexi, Planctomycetes, Verrumicrobia, Acidobacteria, Tenericutes and Cyanobacteria in reproductive (pregnant and lactating) females (Fig 4).

The most abundant phylum in lactating females was Proteobacteria (35%) followed by Bacteroidetes (21%) and Cyanobacteria (20%). Pregnant females' gut microbiota was dominated by the phylum Cyanobacteria (27%) followed by Proteobacteria (21%) and Bacteroidetes (17%). The microbiota of non-reproductive adult females, adult males, and juveniles was

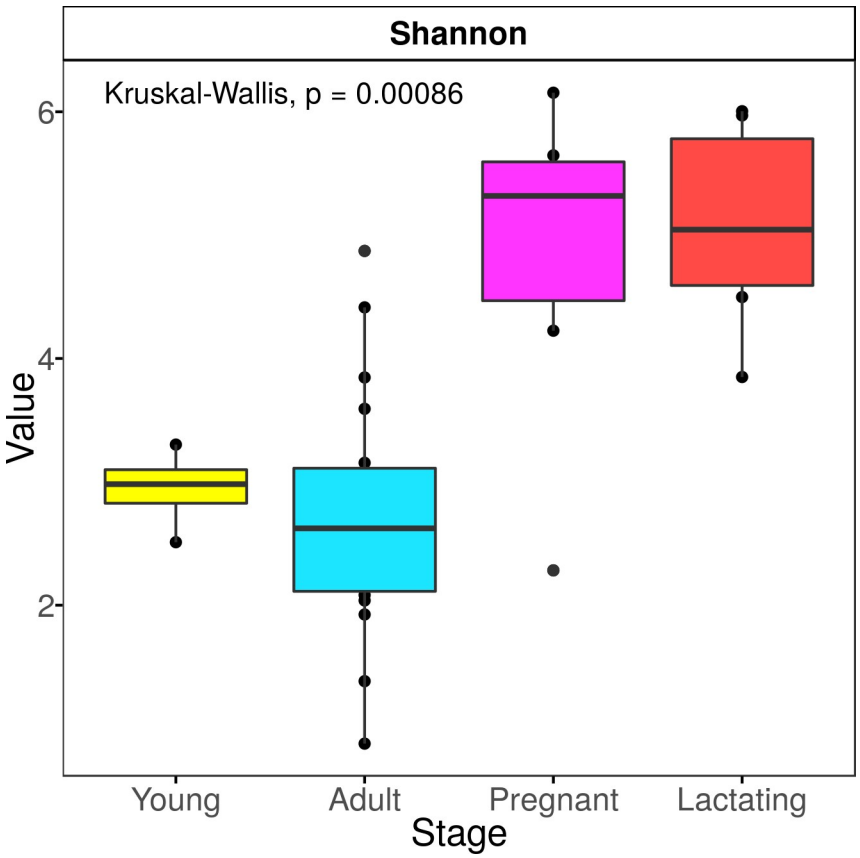

Fig 2. Alpha diversity index by reproductive stage.

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dominated by the phylum Proteobacteria (54%, 62% and 48% respectively), followed by Firmicutes (34%, 30% and 47% respectively) (Fig 4).

Lactating females' gut microbiota composition was dominated by the class Bacteroidia (20%), Oxyphotobacteria (19%), and Alphaproteobacteria (15%), while in pregnant females Oxyphotobacteria (27%) was the most abundant class followed by Bacteroidia (16%), and Alphaproteobacteria (11%). Non-reproductive adult females, adult males, and young males' gut bacteria were dominated by Gammaproteobacteria (51%, 60% and 46% respectively) and Bacilli (30%, 26% and 41% respectively) (Fig 5). The genus *Escherichia-Shigella* was the most abundant among juveniles and adults, followed by *Lactococcus* (Figs 6 and 7)

The PERMANOVA analysis with 1000 permutations and the weighted unifrac distance showed no significant difference between the fecal microbiota composition of pregnant females and lactating females or between juveniles and adults; the strongest differences were between pregnant and lactating females and adult males and females followed by pregnant and lactating females and juveniles (Table 2) (Fig 2). The PCoA showed that the bacterial community structure in pregnant and lactating females is different from that found in non-reproductive adult females, adult males and juvenile males. The PCoA grouped the fecal microbiota compositions of pregnant and lactating females together, differentiating them from the rest of the population (non-reproductive adult males and females and juvenile males) (Fig 3).

Bacteroidetes, Cyanobacteria, Alphaproteobacteria, Gammaproteobacteria, and Firmicutes had high abundances in pregnant females. In juveniles, Firmicutes was the most abundant, followed by Gammaproteobacteria. In adults of both sexes Gammaproteobacteria were more abundant than Firmicutes (Figs 4 and 5).

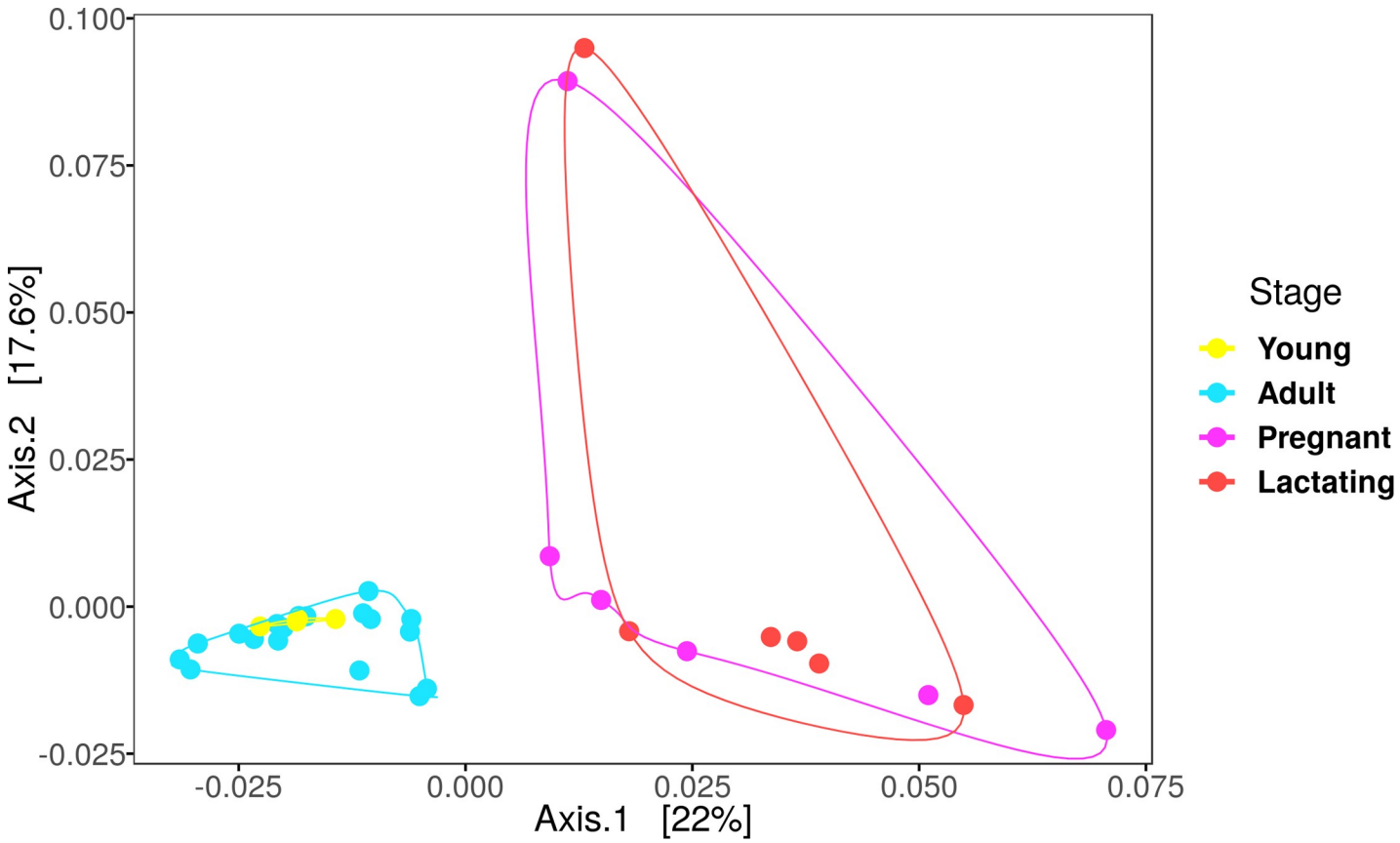

Fig 3. PCoA with the weighted unifrac distances by reproductive stage.

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The microbiota of the non-reproductive adult population was dominated by Gammaproteobacteria and Firmicutes. Reproductive females had a much more diverse microbiota, with a clear increase in phyla such as Cyanobacteria, Bacteroidetes and Alphaproteobacteria. Escherichia-Shigella bacteria were one of the main contributors to the microbiota of non-reproductive adults (Fig 6). The pregnant and lactating females shared 54 ASV at the Family level, juveniles and adults (of both sexes) shared 5 (Figs 7 and 8), and no families were shared by all four reproductive stages.

A Venn diagram (Fig 8) of microbiota composition shows more clearly how pregnant and lactating females differed from the rest of the population, with only 10% of their microbiota composition shared with the other stages. Interestingly, juveniles had 79% similarity in microbial composition to non-reproductive adults. Pregnant and lactating females shared 226 ASV; there were 125 representative ASV for pregnancy and 122 representative ASV of lactation. Juveniles and adults shared 52 ASV, and there were 2 ASV representative of adults and 3 representative of juveniles (Fig 8).

Discussion

Pregnant and lactating females

The analysis of the fecal microbiota of *Leptonycteris yerbabuenae* individuals in different reproductive stages shows a higher microbial diversity in pregnant and lactating females compared to non-reproductive females, males, and juvenile bats. This could be associated with

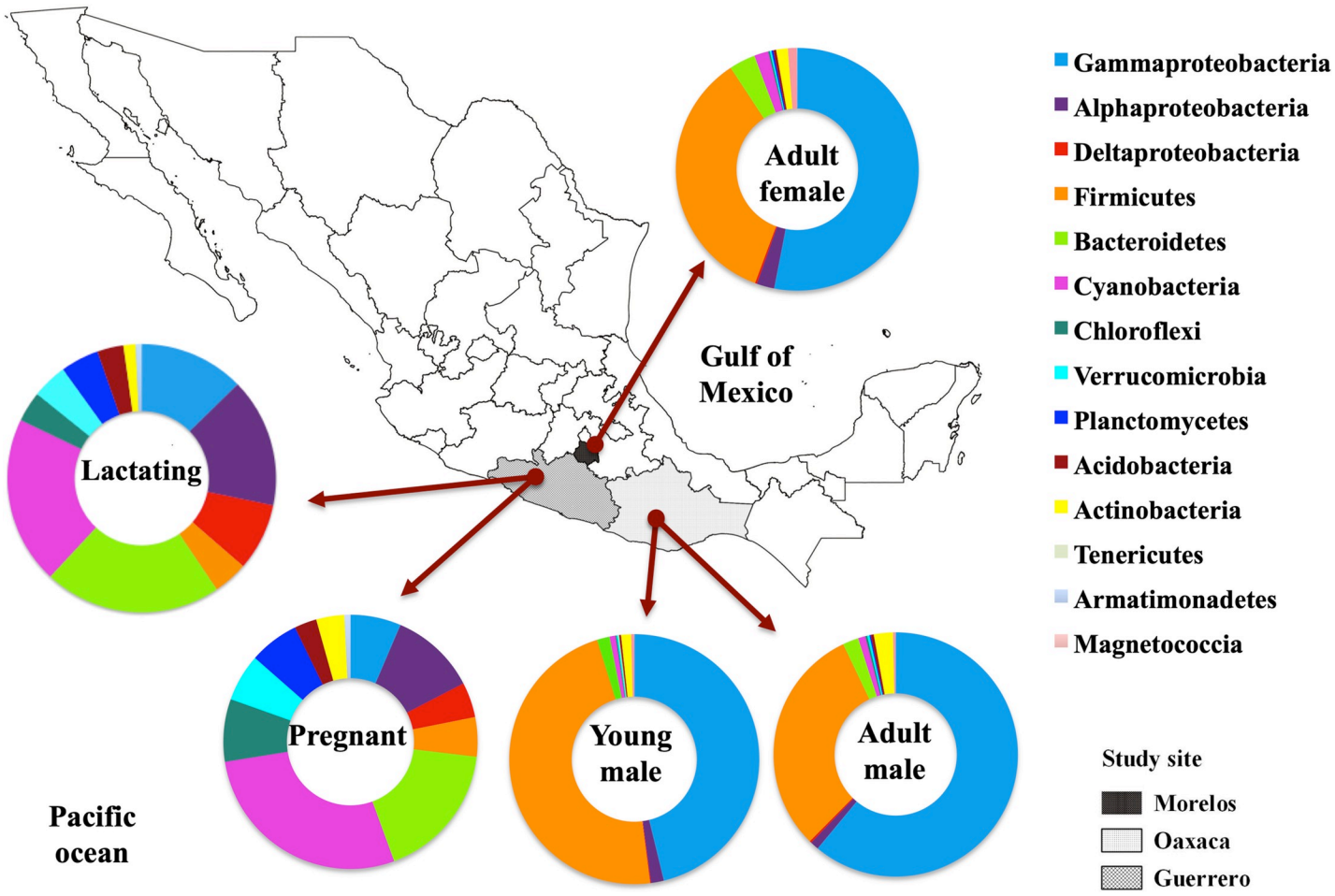

Fig 4. Distribution of bacterial phyla, class and order composition of fecal microbiota of *L. yerbabuenae* in different reproductive stages.

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their increased energy, protein and calcium requirements [18]. Pregnancy (in small mammals) results in the rearrangement of several organs including the stomach and the growth of the intestine [18]. This leads to an increased surface area for colonization by bacteria, which has been reported as an increase in the bacterial carrying capacity during pregnancy in humans and mice [15] and could explain the increase in microbial diversity among pregnant and lactating females. In *L. yerbabuenae*, there is a 2.4-fold increase in the diversity of plants consumed by pregnant and lactating females compared to non-reproductive females and males [16]. When there is little nectar available, reproductive females can consume fruits and insects, and females (but not males) of this species [17]. The diet of *L. yerbabuenae* has been widely studied, and while it is considered a strictly nectarivorous species [59], the microbiota data reported here suggest a dietary shift during pregnancy and lactation [15]. Bats are adapted to seasonal changes, and can adapt to food resources depending on the food available in the environment [59].

These changes in the gastrointestinal tract in reproductive female bats could suggest that an increase in the diversity of foods consumed and the assimilation of new food sources are the main physiological and morphological responses to the higher energetic demands during pregnancy and lactation [60]. Furthermore, the energy requirements of migration to the pregnancy and lactation caves to which females are phylopatric, imposes additional stress. These high

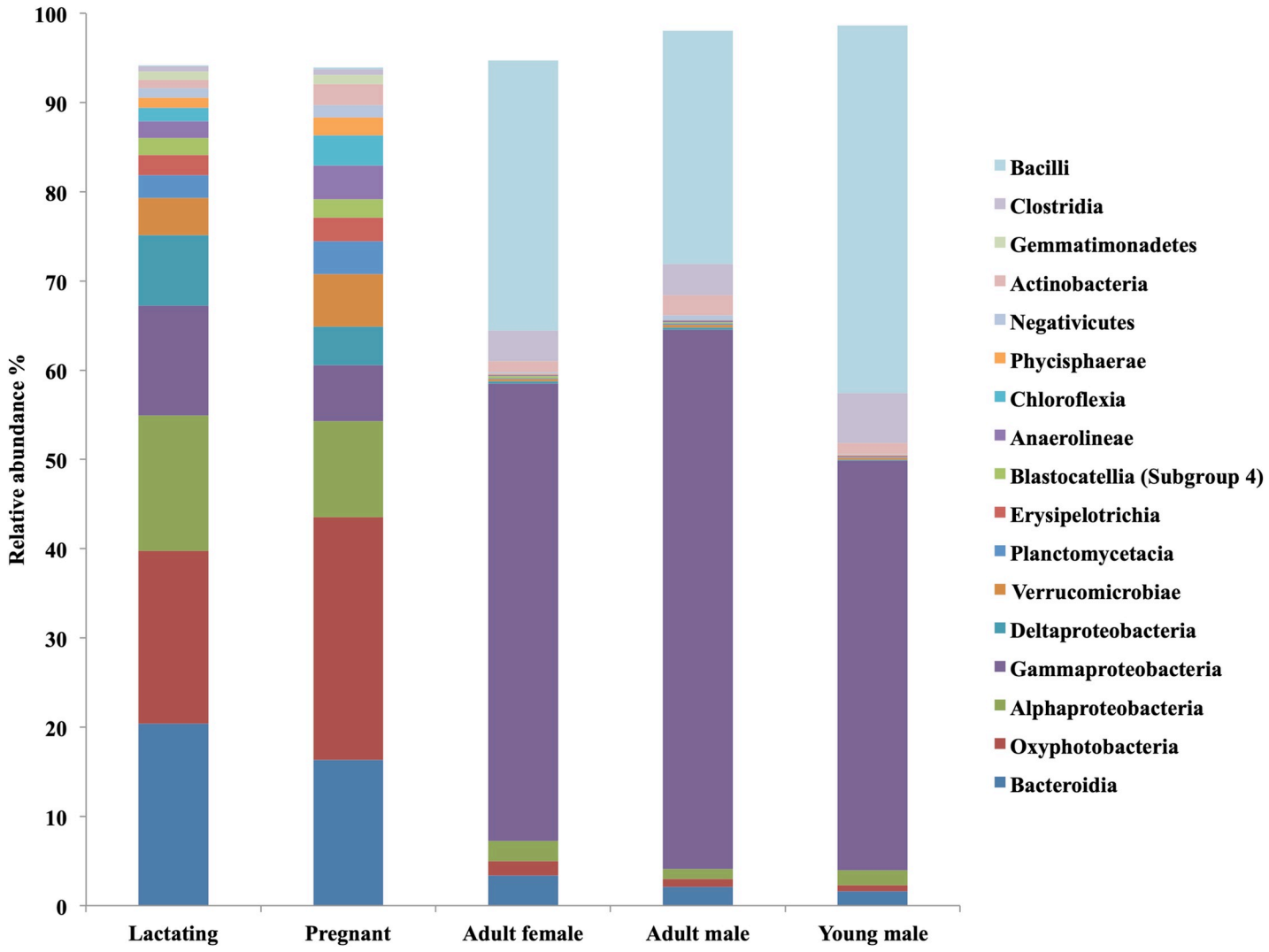

Fig 5. Distribution of bacterial Class composition of fecal microbiota of *L. yerbabuenae* in different reproductive stages.

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energy requirements may be offset by shifting from a nectar-specialist diet to a more generalist one. During pregnancy, levels of progesterone and estrogen increase, influencing the growth of some bacteria, and some of the changes during pregnancy are similar to changes that occur with illnesses such as obesity and diabetes, which can lead to changes in the microbiota [15].

L. yerbabuenae's physiological restrictions on hydrolyzing sucrose imposes physiological limitations due to the lack or inefficiency of sacarase to use this sugar as an energy source [61]. Our results show an increased abundance of Bacteroidetes in pregnant and lactating females. This change may be due to the increased requirements of the host to hydrolyze polysaccharides [62], since intestinal Bacteroidetes are specialized in the degradation of plant-derived polymers, such as plant cell wall compounds (e.g., cellulose, pectin, and xylan) [63].

There is a clear increase in the abundance of Bacteroidetes in both pregnant and lactating females compared to the rest of the population, this change in composition may be considered a host-microbiota adaptation determined by physiological changes during these reproductive stages that result in significant benefits to the host, increasing their nutrient adsorption

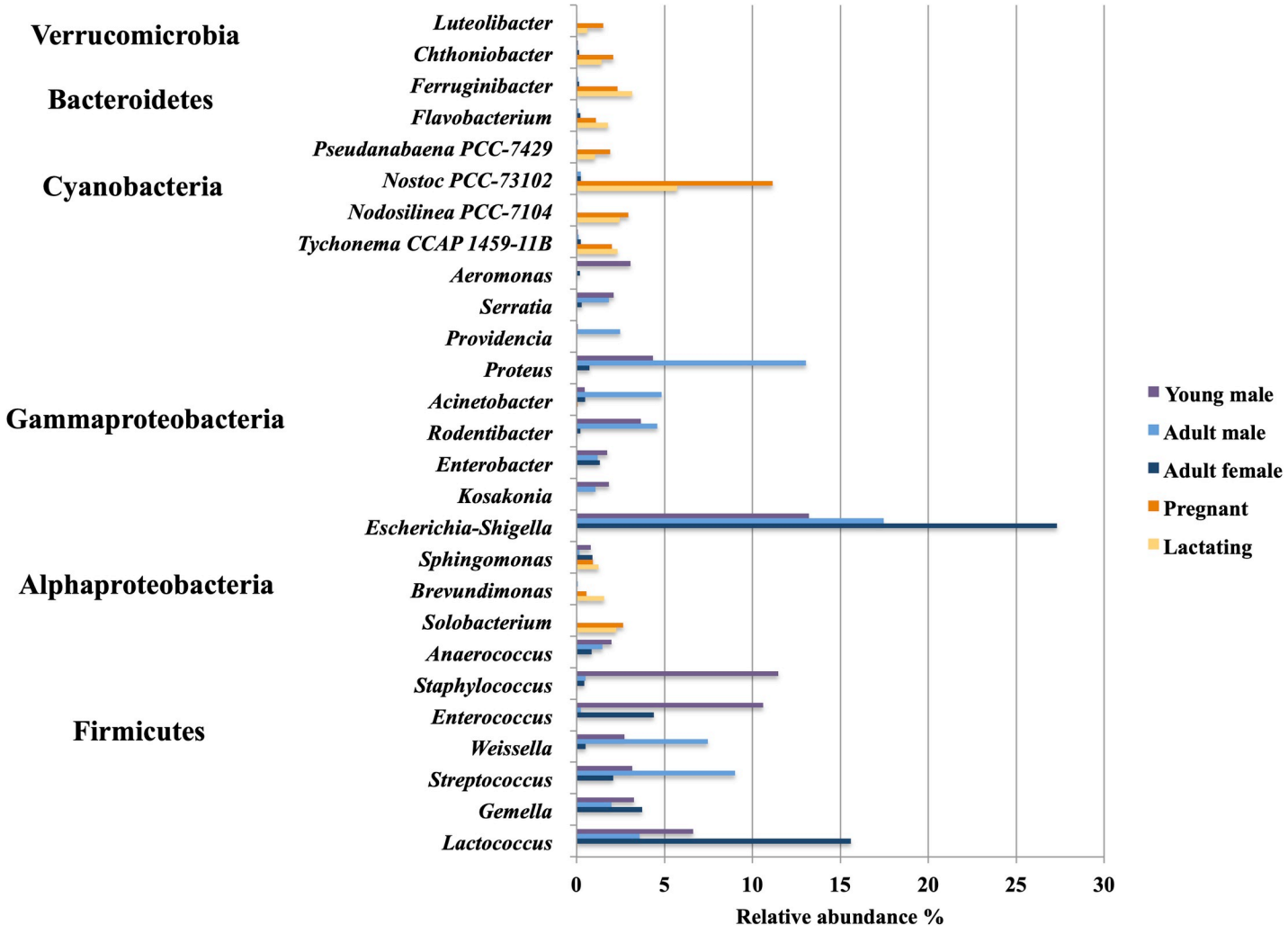

Fig 6. Distribution of bacterial Genus composition of fecal microbiota of *L. yerbabuenae* in different reproductive stages.

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capacity and energy intake. The phylum Bacteroidetes is a very diverse bacterial phylum, but generally the interactions between Bacteroidetes and their animal hosts are considered mutualistic rather than commensal, since both the bacterium and host receive fitness benefits from the association [63]. The bacteria-mediated fermentation of these foods and host-derived polysaccharides in the colon lead to the release of volatile short-chain fatty acids (mainly acetate,

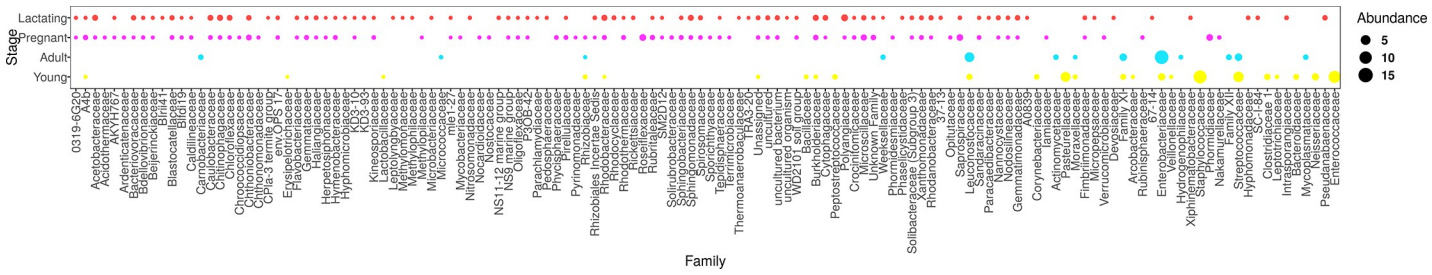

Fig 7. Linear discriminant analysis (LDA) effect size (LEfSec) showing similarities in the microbial communities of *L. yerbabuenae* at the family level.

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Table 2. PERMANOVA analysis with 1000 permutations and the weighted unifrac distance. In the pairwise mode, the p-value was adjusted using the false discovery rate (FDR) method. The significant comparisons at a p.value 0.05 are marked with asterisk. The permutest analysis was not significant (p.value = 0.149), thus the PERMANOVA result is reliable because the dispersion of the groups is homogeneous.

Pairwise	Model *F*	R^2	FDR-adjusted *P* value
Adult vs Lactating	4.8059527	0.17928677	0.006*
Adult vs Pregnant	4.3205027	0.1641497	0.006*
Adult vs Young	0.5715419	0.02778313	1
Lactating vs Pregnant	0.6055095	0.05709386	1
Lactating vs Juvenile	2.6686511	0.2501395	0.024*
Pregnant vs Juvenile	2.4378676	0.23355992	0.042*
Single	**Model *F***	**R^2**	**p.value**
Stage	2.8305	0.22061	0.001*

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propionate, and butyrate). The assimilation of short-chain fatty acids (SCFA) produced by microbial fermentation of polysaccharides can contribute more than 50% of the total caloric supply [64]. Hence, the presence of Bacteroidetes optimizes the extraction of energy from the diet, likely helping to provide an additional source of energy to pregnant and lactating females. Some members of this phylum can have strong pathogenic behavior, but the emergence of an infection seems to be linked to the assemblage of pathogens in bacterial consortia more than to the individual action of a particular species [65].

The change in the microbiota composition in pregnant and lactating females can be considered a short-term microbiota-host adaptation driven by the physiological changes during these reproductive stages, and which is beneficial because it increases their nutrient adsorption capacity and energy uptake. At the end of this cycle, the organs that were modified during pregnancy return to their non-reproductive state, reducing the size of the gut and the associated bacterial diversity [18]. Cyclical changes in the microbiota have been observed previously, such as seasonal changes marked by food availability [22].

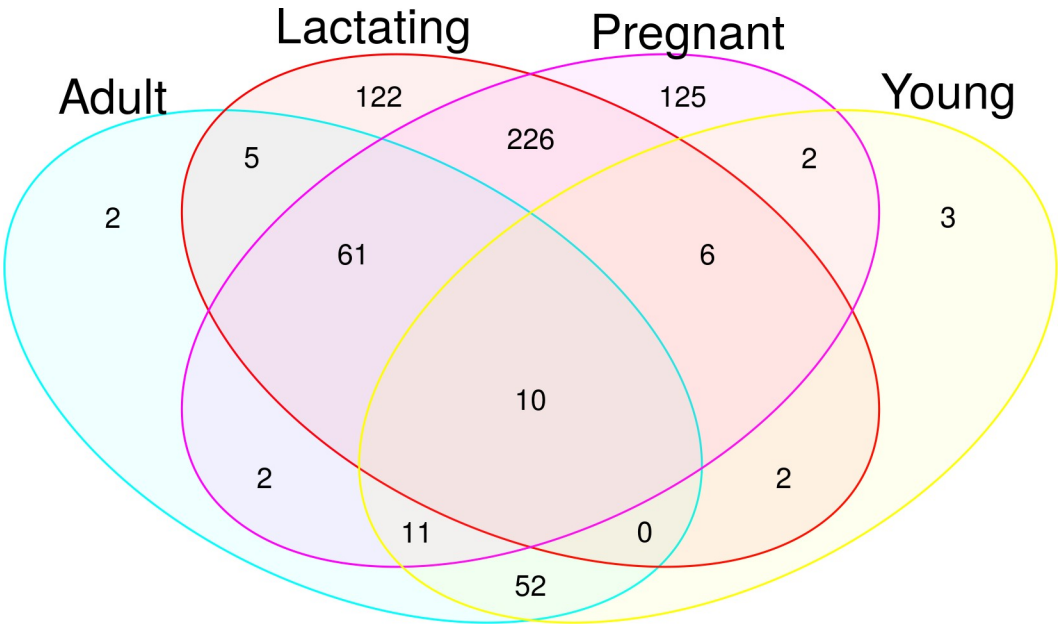

Fig 8. Venn diagram of different reproductive stages of *L. yerbabuenae*.

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There is no evidence to suggest that the microbiota varies between sexes of non-reproductive adults. Males do not present the changes to the intestine that females do; this suggests that after weaning, when females' energetic demands are reduced, their foraging behavior reverts to that of a nectar specialist. More studies are needed to test this hypothesis and more thorough studies on food availability and diet would be very informative in this context. On the other hand, while males from this species do spend energy on the development of the dorsal patch, a secondary sexual organ developed during mating season [66], without the excessive energy requirements imposed by giving birth they are likely consistently nectar-feeding specialists throughout their life-history and thus more effective pollinators of agaves and cacti. This hypothesis is consistent with reports by Ibarra-López [67] of Central-population males feeding mainly on two Cactaceae and Agavaceae families. In contrast, a maternity and lactation colony of *L. yerbabuenae* in Chiapas, Mexico was found to feed from seven different families and 19 species of plants; it was also reported that the diversity of plant species consumed was lower in lactating females than in pregnant females [16]. These findings are of relevance to conservation management planning of this species, including ecological restoration for mezcal and tequila production, alcoholic beverages of significant economic importance in Mexico.

Microbiota composition among non-reproductive *L. yerbabuenae*

Juvenile bats share 79% of their microbiota with non-reproductive adults and have a less diverse microbiota. In both juvenile and non-reproductive adult males and females, the phylum Firmicutes has a high relative abundance. Eight out of nine genera within this phylum recovered from this microbiota study are SCFA fermenters (genera *Solobacterium*, *Lactococcus*, *Enterococcus*, *Gemella*, *Streptococcus*, *Weissella*, *Anaerococcus* and *Staphylococcus*) [68]. A high proportion of the SCFA produced by microbial fermentation of indigestible carbohydrates in the large intestine is absorbed by the host. Thus, microbial activity contributes energy to the host (estimated to be around 10% of the calories obtained from the diet) that would otherwise be lost through excretion of undegraded substrate in the feces [6]. Increased SCFA concentration may also increase the solubility of certain minerals such as calcium and enhance absorption and expression of calcium-binding proteins. Bacteria of the phylum Firmicutes have a lower ability for polysaccharide degradation [69] and are better known for their production of butyric acid. Butyrate-producing bacteria play an important role in the human colon, supplying energy to the gut epithelium and regulating host cell responses [70]. Butyrate-producing gut bacteria are an important component of the microbiota, in terms of both abundance and functionality [70].

The change in diet and foraging behavior among the different reproductive stages determine the microbiota composition, shaping their physiological requirements. Adults and young could require the production of SCFA to aid calcium absorption [6]. Changes in the intestinal microbial metabolism following the consumption of inulin fructans have also been shown to benefit bone health by increasing calcium absorption, while B-glucans may lower total cholesterol levels [6]. There might be a higher intake of mono-saccharides in non-reproductive females and males due to differences in foraging. Inulin fructans affect gastrointestinal functions not because of their physico-chemical properties, but rather because of their biochemical and physiological attributes. In the colon, they are rapidly fermented to produce SCFA that are good candidates to explain some of the systemic effects of the inulin-type fructans.

Importance of the microbiota for *L. yerbabuenae* reproduction

Bacteria that colonize the mammalian intestine collectively possess a far wider diversity of genes and a larger repertoire of degradative enzymes and metabolic capabilities than their

hosts. Fermentation of complex carbohydrates in the intestine involves interactions between community members that include both nutritionally specialized and widely adapted species. Certain dominant species allow them to switch readily between different energy sources in the gut depending on availability, using sophisticated sensing and regulatory mechanisms to control gene expression [6].

The gut microbiota may also influence the expression of host peptides and hormones by producing SCFA via their interactions with free fatty acid receptors, influencing host energy metabolism and appetite regulation [6]. Another potential route linking microbial activity with the host is via the gut-brain axis, a bi-directional communication system based on neural, endocrine and immunological mechanisms [6]. The immune system is influenced by microbial metabolic products, leading to complex interactions between the species composition of the microbiota and the host's innate and adaptive immune systems that are thought to underlie many probiotic effects [71].

The relevance of the microbiota in *L. yerbabuenae* reproduction is not limited to females. In males, the skin microbiota plays an important role in pheromone production during the reproductive season. There is a synchrony between testicle growth and the development of a structure known as the sebaceous patch; a wound-like structure in the interscapular area where fermentative bacteria produce SCFAs, commonly known as pheromones [72]. In light of this, it is worthwhile to further examine the role of the microbiota in reproduction and mating behavior in wildlife.

Conclusion

This study focused on the diversity of the fecal microbiota in different reproductive stages of the central population of *Leptonycteris yerbabuenae*. Results suggest that the microbiotas of pregnant and lactating females are similar to each other and have higher abundance than juveniles and non-reproductive adults. There was no significant difference in microbiota between juveniles and non-reproductive adults in this population, regardless of the roost in which the adults were captured. Diet is considered to be the main factor shaping the diversity and function of the microbiota [8,9]. The reproductive stage of *L. yerbabuenae*, a strictly nectarivorous bat, is important in shaping the microbiota due to physiological changes in the energy requirements during pregnancy and lactation which are consistent with data from the literature that show increased dietary diversity during female reproduction. A relationship exists between the abundances of fecal microbial communities and the different reproductive stages of this nectar-feeding bat. This host-microbiota relationship is more evident in pregnant and lactating females than in other reproductive stages, due to the physiological, anatomical and energy-requirements changes associated with maternity. These requirements trigger a need to consume different foods, and the microbiota is strongly shaped by diet [9,13,51]. Pregnant and lactating females become more generalist feeders to cope with the increased energy requirements, feeding on resources other than nectar. This dietary modification suggests that non-reproductive individuals, which retain a specialist feeding strategy, are more efficient pollinators than reproductive females.

Migration and the segregation between females and males in maternity and bachelor caves might have evolved as a strategy to guarantee resource availability during the high-energy demanding stages of pregnancy and lactation. It may be speculated that the adaptation of gut microbiota could have been important to these evolutionary adaptations of populations. The flexibility of the gut microbiota to shift from a specialist to a generalist diet could have coevolved in reproductive females to increase their fitness and guarantee yearly reproductive success. There are more questions than answers in our understanding of the host-microbiota

relationship. Is there a signal that directs the change of abundances in microbiota composition? What is the ecological succession of these communities from one reproductive stage to the other? More research is needed to unravel the patterns of bat-microbiota association, to understand its implications in this species' ecology, evolution, and life-history.

Supporting information

S1 Fig. Rarefaction curve.
(TIF)

S1 Table. Alpha diversity indexes per sample.
(DOCX)

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Capítulo 2. Composición de la microbiota del parche dorsal de machos reproductivos de *Leptonycteris yerbabuenae.*

Gaona O, Cerqueda-García D, Falcón LI, Vázquez-Domínguez G, Valdespino-Castillo PM, Neri-Barrios C-X (2019) Microbiota composition of the dorsal patch of reproductive male *Leptonycteris yerbabuenae*. PLoS ONE 14(12): e0226239. https://doi.org/10.1371/journal.pone.0226239

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RESEARCH ARTICLE

Microbiota composition of the dorsal patch of reproductive male *Leptonycteris yerbabuenae*

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Abstract

Bacteria and other types of microbes interact with their hosts in several ways, including metabolic pathways, development, and complex behavioral processes such as mate recognition. During the mating season, adult males of the lesser long-nosed agave pollinator bat *Leptonycteris yerbabuenae* (Phyllostomidae: Glossophaginae) develop a structure called the dorsal patch, which is located in the interscapular region and may play a role in kin recognition and mate selection. Using high-throughput sequencing of the V4 region of the 16S rRNA gene, we identified a total of 2,847 microbial phylotypes in the dorsal patches of eleven specimens. Twenty-six phylotypes were shared among all the patches, accounting for 30 to 75% of their relative abundance. These shared bacteria are distributed among 13 families, 10 orders, 6 classes and 3 phyla. Two of these common bacterial components of the dorsal patch are *Lactococcus* and *Streptococcus*. Some of them—*Helcococcus*, *Aggregatibacter*, *Enterococcus*, and Corynebacteriaceae—include bacteria with pathogenic potential. Half of the shared phylotypes belong to *Gallicola*, *Anaerococcus*, *Peptoniphilus*, *Proteus*, *Staphylococcus*, *Clostridium*, and *Peptostreptococcus* and specialize in fatty acid production through fermentative processes. This work lays the basis for future symbiotic microbe studies focused on communication and reproduction strategies in wildlife.

Introduction

The microbiome is the assemblage of archaea, bacteria, viruses and other microorganisms associated with any multicellular organism (the host) in a particular environment [1,2]. The microbiome is a vital component in the evolution of the host, and in vertebrates it plays essential roles in almost all of the organism's functions [3–7]. The relationships between the animal host and its microbiome are, for the most part, mutually beneficial [8]. Gut and skin

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microbiota have coevolved with their hosts, and this mutualism has led to the evolution of intra- and interspecific interactions [9,10]. Microbiota associated with skin and glands in mammals are and diverse, and host species is an important predictor of the microbial community assemblage [11]. It has been demonstrated that microbial communities play a role in the production of odors and chemical signals, which are important in species recognition, kin recognition, and mate choice [12–15]. The interaction between host and microbiome can thus alter the host's social behavior at various points during its life cycle [14,15].

The role of bacteria in the processes of fermentation and production of volatile fatty acids has been previously reported in studies of canids and bats [16,17]. These short chain fatty acids, produced by bacteria, are the precursors of chemical signals, known as scents, which influence the intraspecific interactions of many mammals. Bacteria associated with the skin contribute to the host's scent through the direct production of odors and the fermentation of organic compounds produced endogenously by the host, which is known as the "fermentative hypothesis" [18,19]. The fermentative hypothesis is based on two main assumptions: first, that volatile odorants are produced by bacteria that colonize mammal scent glands and ephitelial tissues and second, that individual "odor prints" are generated by differences among individuals in the composition of their bacterial communities [20].

Scent production as a consequence of bacterial fermentation has been reported in several bat species [17,21,22], most often among reproductive males [22–24]. Some examples of bats that possess odor-producing structures colonized by bacteria are the piscivorous *Noctilio leporinus* (Noctilionidae), which have a typical strong odor associated with *Staphylococcus aureus* [17], male sac-winged bats, *Saccopteryx bilineata* (Emballonuridae), which have a scent pouch that contains an odoriferous liquid used in courtship [17,25], and the big brown bat *Eptesicus fuscus* (Vespertilionidae), in which roost-mates have a shared odor signature [21]. Adult males of the nectar-feeding bats *Leptonycteris curasoae* and *Leptonycteris yerbabuenae* develop an oderiferous "dorsal patch", a temporary structure in the interscapular region that contains fatty acids and signals mating readiness [22,26,27].

Male *L. yerbabuenae* develop the odoriferous dorsal patch for a short time during the breeding season, using their forelimbs to spread fluids from the anus, penis, and mouth onto their backs in a behavior known as "smearing" [22,28] (Fig 1). The fact that all males with dorsal patches have enlarged testes, and that males with small testes do not develop this trait, suggests

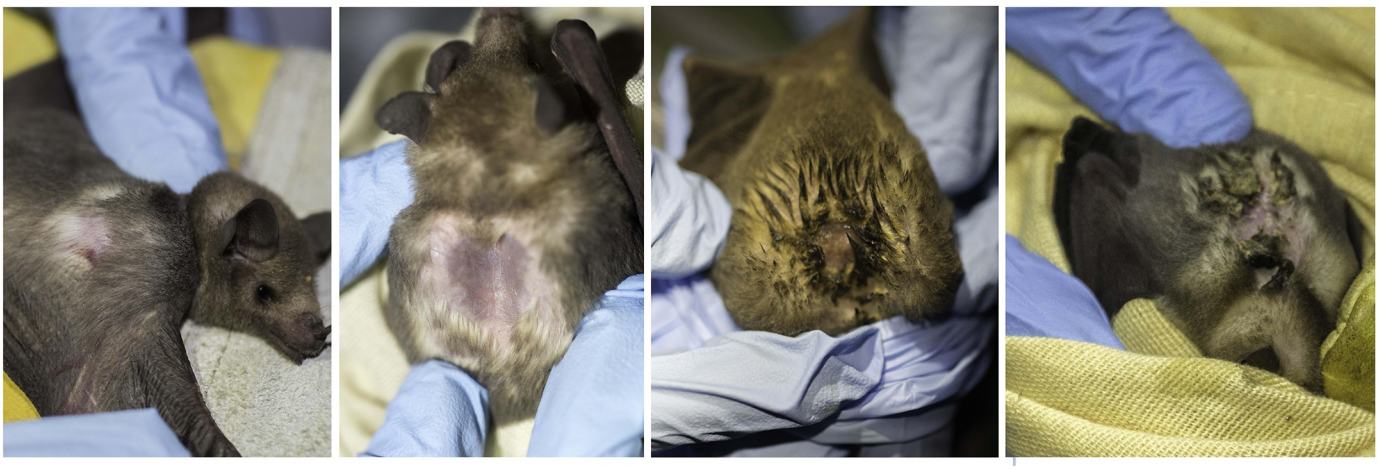

Fig 1. Development of dorsal patch in *L. yerbabuenae*. The dorsal areas of different male bats captured during the same sampling period show patches at different stages of development from a less developed patch (left) to a mature, well-developed patch (right).

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a strong association between dorsal patch development and sexual maturity and possibly readiness for reproduction [22–24,27]. This dorsal patch has a strong odor and a complex chemical profile [22,27], made of compounds that have been reported to be important during the reproductive season of *L.yerbabuenae* and *Leptonycteris curasoae* in female attraction [28,29]. Thus, overall, the available evidence suggests that the dorsal patch is involved in female mate choice mediated through odor [22,24,26,27]. Males with dorsal patches had fewer ectoparasites than those without them, suggesting that this structure might function as a dual signal (odoriferous and visual) of mating readiness and health status [22,26–28]. It has been hypothesized that the odor of the dorsal patch is an honest signal of health status [26].

In this study, we aimed to evaluate the microbial diversity associated with the dorsal patch of reproductive *L. yerbabuenae* males using a high throughput 16S rRNA amplicon sequencing approach.

Materials and methods

Study site

Bats were sampled in the San Juan Noxchitlan cave, Oaxaca (18˚ 03' 00.0" W and 97˚ 40' 00.0" N), at an altitude of 1978 m.a.s.l. [30–32] (Fig 2). The resident colony is comprised of approximately 100,000 bats [31]. This bachelor cave is located in the Tehuacan Valley, characterized as

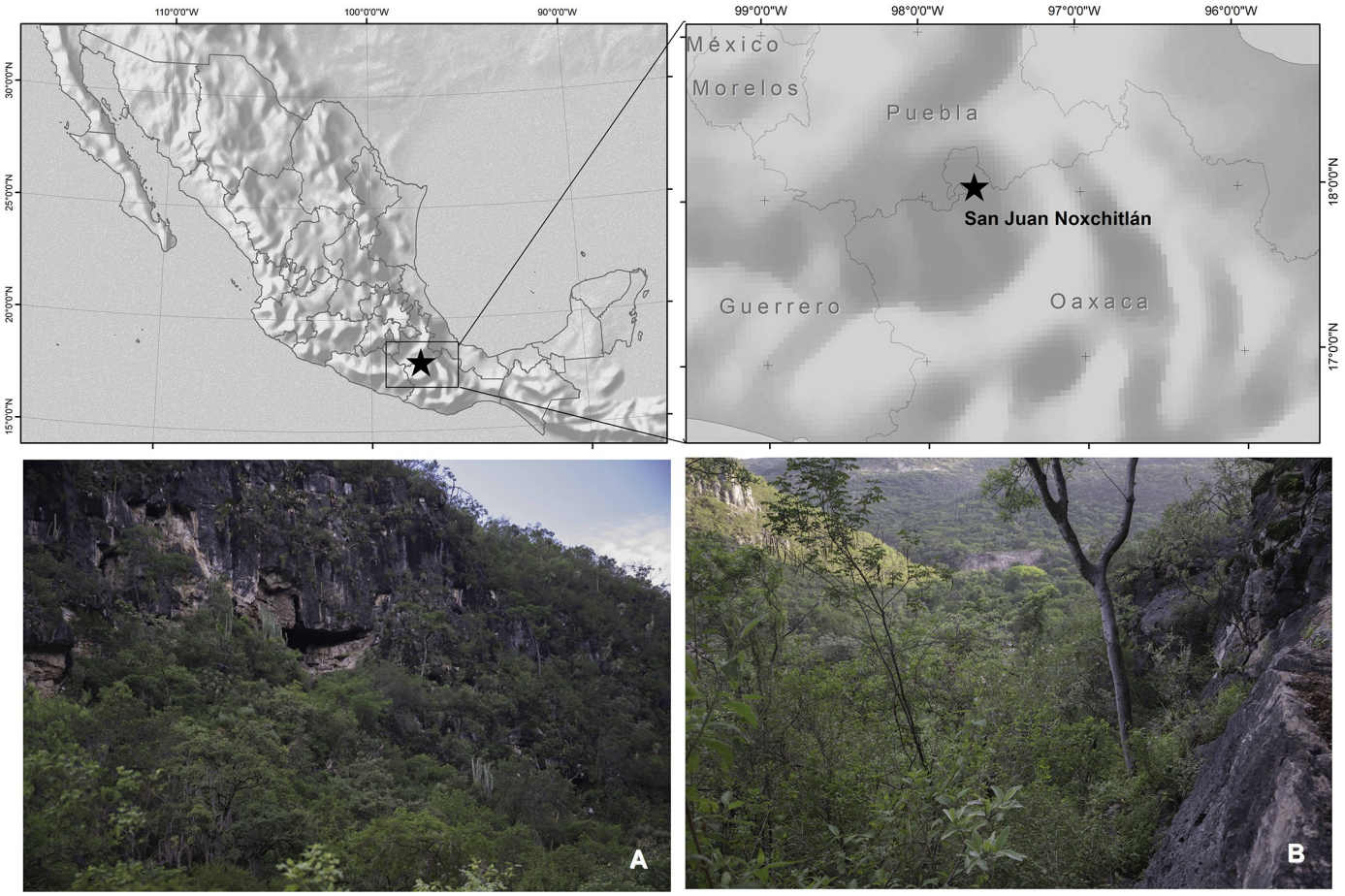

Fig 2. Location of San Juan Noxchitlan cave, Oaxaca, Mexico (18˚ 03' 00.0" W and 97˚ 40' 00.0" N), at an approximate altitude of 1978 m.a.s.l.

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an isolated, arid-semiarid region (10,000 km^2). Average annual rainfall is 495 mm, and annual mean temperature is 21 ˚C, with very rare frosts [33]. The vegetation is mainly tropical deciduous forest [34].

Bat sampling

This study was conducted during the mating season in June 2015. Bats were captured using the methods described by Kunz et al. [35], using 12 m mist nets (Avinet, Dryden, New York, USA) placed at the entrance of the cave from 18:00h in the evening to 06:00h the next day, a sampling effort of 12 hours.

Interscapular dorsal patch samples (n = 11, named with the prefix Oax) were obtained following the guidelines of the American Society of Mammalogists for capture, handling and care of mammals [36,37]. To minimize animal suffering and distress, each animal was processed by experts only, ensuring effective and harmless handling. No anesthetics were administered for chemical immobilization, as they were deemed unnecessary and would only increase the mortality risk. Samples of the crust and fur of the interscapular patch were taken using gloves and sterile surgical calipers and scissors. A 0.25 cm^2 area of the formed crust and hair was cut without touching the skin, and placed into sterile Eppendorf tubes (1.5 ml). Samples were frozen until processing using a field liquid nitrogen dewar.

Ethics statement

Samples were taken from wild bats that were released in the same area as capture, causing no apparent harm to individuals. *Leptonycteris yerbabuenae* is not under federal protection by Mexican law (NOM-059-SEMARNAT-2010). Scientific collection activities were carried out under a scientific collection permit number granted by the Mexican Subsecretary of the Environment and Natural Resources (SEMARNAT), number FAUT-0231, SGPA/DGVS/05780/15. SEMARNAT specifically approved and authorized the tissue sampling methods under this collection permit. Laboratory activities were carried out in the Ecology Institute of the Universidad Nacional Autonoma de Mexico (UNAM), under the authorization of the Biosafety Commission of the Ecology Institute, UNAM; no specific permit was needed because only tissue and skin samples were used (no *in vivo* studies were included). All biosafety standard requirements from the Ecology Institute were satisfied.

DNA extraction

DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) with some modifications. Dorsal patch samples were diluted with 180 μl of Animal Tissue Lysis (ATL) extraction buffer and incubated with lysozyme A (30 mg/ml) and proteinase K (10 mg/ml). After enzymatic digestion, the manufacturer's protocol was followed. DNA was precipitated with 1 volume of chilled absolute ethanol and 0.1 volume of 3 M sodium acetate, then washed with 70% ethanol. Finally, DNA was eluted in 30 μl of molecular grade water and stored at -20˚C prior to PCR amplification.

16S rRNA gene amplification and sequencing

DNA samples were PCR amplified with universal bacteria/archaeal primers 515F/806R (hypervariable region V4) following the procedures reported by Caporaso et al. [38]. PCR reactions (25 μl) contained 2–6 ng of total DNA, 2.5 μl Takara ExTaq PCR buffer 10X, 2 μl Takara dNTP mix (2.5 mM), 0.7 μl bovine serum albumin (BSA, 20 mg ml^{-1}), 1 μl primers (10 μM), 0.125 μl Takara Ex Taq DNA Polymerase (5 U μl^{-1}; TaKaRa, Shiga, Japan) and nuclease-free water.

Samples were amplified in triplicate using a PCR protocol that included an initial denaturation step at 95˚C (3 min), followed by 35 cycles of 95˚C (30 s), 52˚C (40 s) and 72˚C (90 s), followed by a final extension 72˚C (12 min). Triplicates were then pooled and purified using the SPRI magnetic bead, AgencourtAMPure XP PCR purification system (Beckman Coulter, Brea, CA, USA). The purified 16S rRNA fragments (~20 ng per sample) were sequenced on an Illumina MiSeq platform (Yale Center for Genome Analysis, CT, USA), generating ~250 bp paired end reads. The sequence data are available from the NCBI Bioproject PRJNA496019; SUB4625080.

Sequence analysis

The 2x300 MiSeq Illumina paired-end reads were overlapped and merged using FLASH [38,39]. Nucleotide sequences were processed in the QIIME pipeline [40]. Quality filtering and demultiplexing were done as suggested by Caporaso et al. [40] and Bokulich et al. [41] (Q = 19, p = 0.75, r = 3, n = 0). Sequences were then clustered into Operational Taxonomic Units (OTUs) at 97% sequence identity in the open reference mode with USEARCH 6.1[42]. Chimeras were removed using UCHIME2 [43] and OTUs were taxonomically assigned with UCLUST, using the Greengenes database (release 13_5_8)[44]. Sequences were rarefied to 19 000. The taxonomic abundance and statistical analyses were plotted in R with the phyloseq [45] and ggplot2 [46] packages.

OTUs that were shared among samples were searched for their closest OTU using a BLAST search against the RefSeq-NCBI database. The three best hits for each OTU were aligned with MUSCLE [47], and a phylogenetic tree was built with PHYML software [48], using the GTR substitution model and 1000 bootstraps.

Results

A total of 17 male *L. yerbabuenae* individuals were captured, but only 11 samples met the minimal requirements for high-quality DNA extraction. Patches were found on mature males only and covered between 2 and 4 cm^2 of the interscapular area.

Microbiota diversity and composition

In total for the eleven sampled dorsal patches, we found 2,847 phylotypes. The number of observed phylotypes and diversity indices (Shannon and Chao 1) for each individual are provided in Table 1, and similarity among samples based on weighted unifrac distances are given

Table 1. Alpha diversity of phylotypes in the microbiota from each of the 11 dorsal patch samples.

SAMPLE	SHANNON INDEX	Observed OTUs	CHAO1
OAX9	4.55	552	932.68
OAX23	5.00	639	1201.76
OAX26	4.23	523	1245.50
OAX24	4.39	516	857.00
OAX12	4.51	521	1113.88
OAX7	2.02	415	566.08
OAX15	5.39	641	1287.01
OAX11	4.55	464	699.01
OAX16	4.48	529	1017.95
OAX13	4.56	437	909.35
OAX27	7.67	715	946.03

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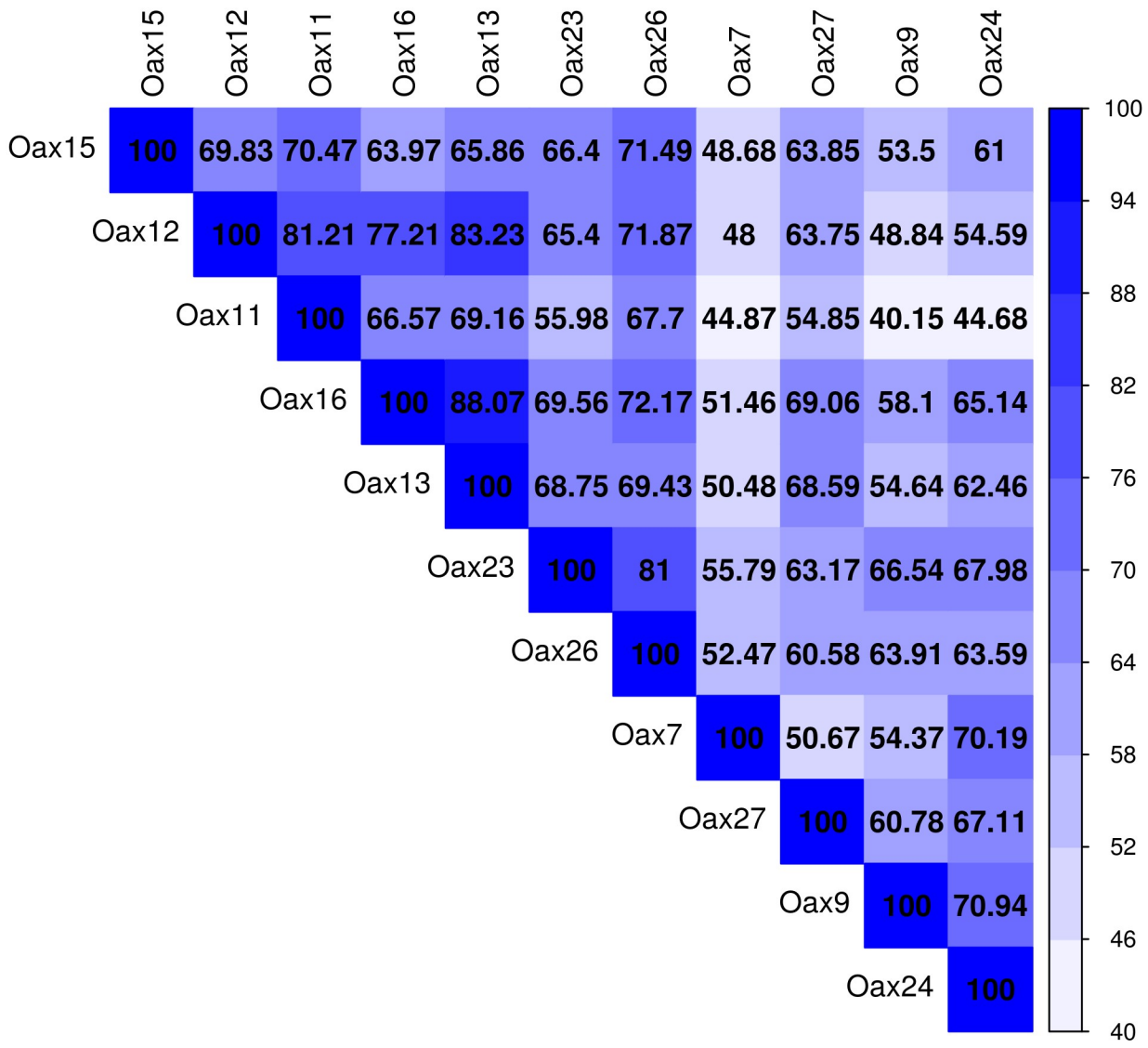

Fig 3. Graphical representation of similarity among the samples from each of the 11 individuals base on weighted unifrac distance.

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in Fig 3. While there were no substantial differences in bacterial groups, the abundance and composition of bacteria varied among individuals (Figs 4–6).

The dorsal patch microbiota of *L. yerbabuenae* was mainly composed of Firmicutes (48%) and Proteobacteria (36%), with smaller contributions from Actinobacteria (3.6%), Fusobacteria (2.8%), Cyanobacteria (2.4%), Tenericutes (0.5%), Bacteroidetes (0.4%), Verrumicrobia (0.03%), and 6.2% corresponding to unassigned bacteria (Fig 3). Class composition was highly skewed towards Gammaproteobacteria (40.44%), Clostridia (33.70%) and Bacilli (13.72) with minor contributions from Fusobacteria (2.06%), Alphaproteobacteria (1.84%), Betaproteobacteria (0.76%), Mollicutes (0.49%) and Actinobacteria (0.06%) (Fig 5).

The OTU level classification of the dorsal patch in *L. yerbabuenae* resulted in 102 recognized OTUs, but over 80% of OTUs were unassigned. The 20 most abundant identified species were: *Acinetobacter johnsonii*, *Actinomyces europaeus*, *Aggregatibacter segnis*, *Brevinema andersonii*, *Bulleidia p-1630-c5*, *Candidatus Nitrososphaera*, *Clostridium perfringens*,

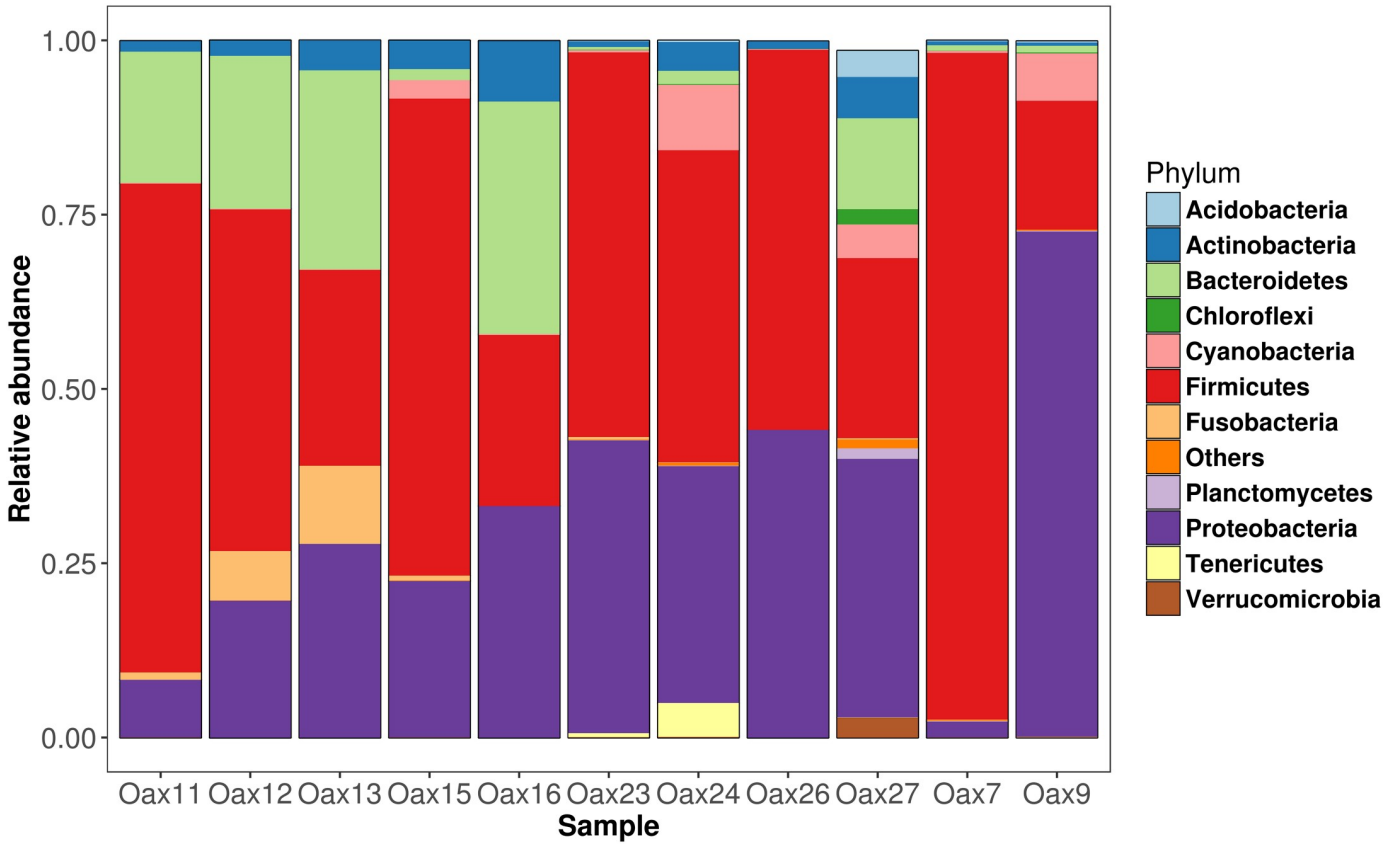

Fig 4. Composition of the dorsal patch at the phylum level.

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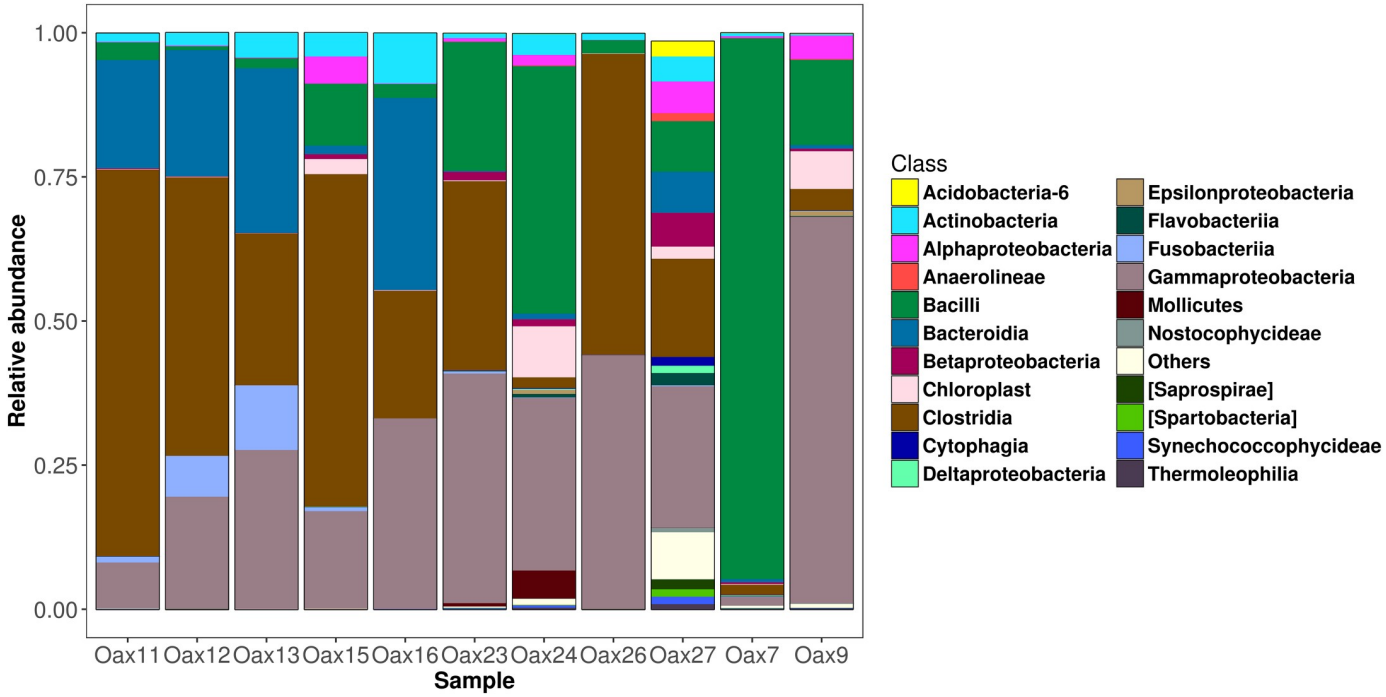

Fig 5. Composition of the dorsal patch at the class level.

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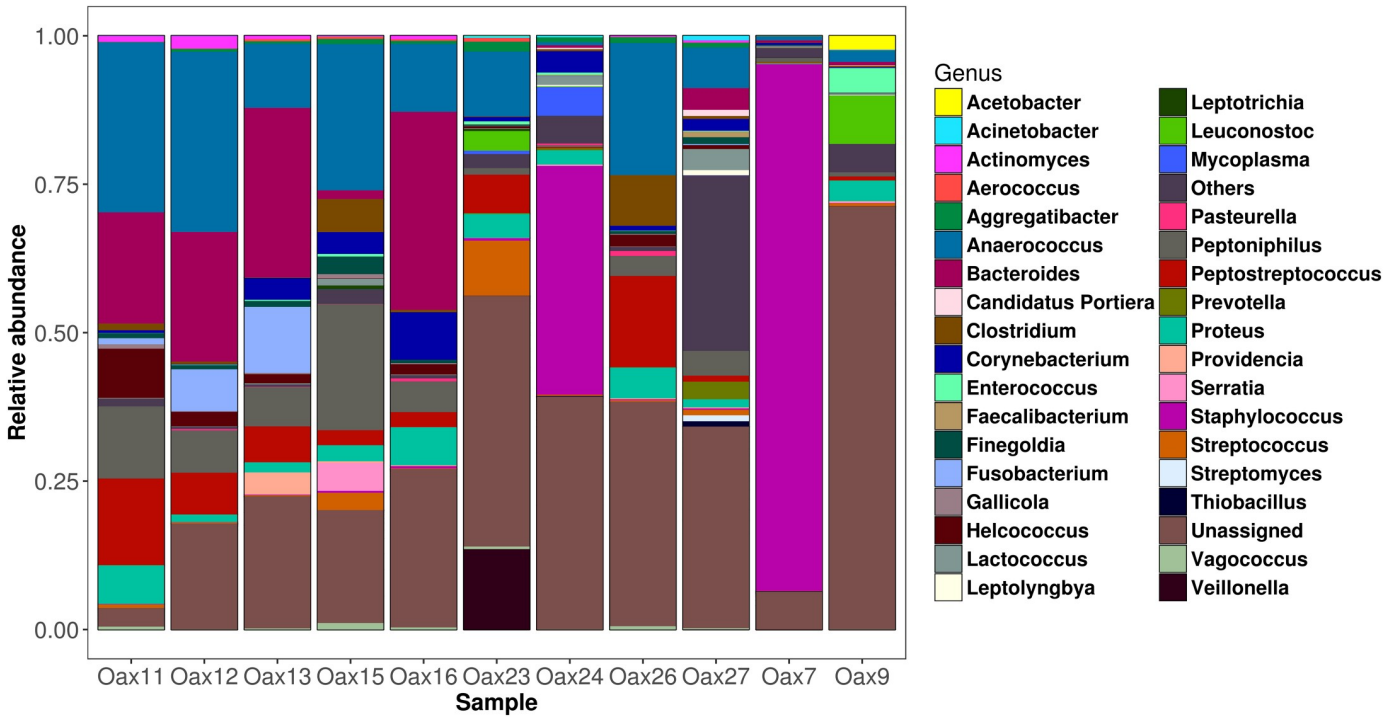

Fig 6. Composition of the dorsal patch at the genus level.

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Corynebacterium variabile, *Faecalibacterium prausnitzii*, *Haemophilus parainfluenzae*, *Lactococcus garvieae*, *Leptolyngbya frigida*, *Methylobacterium adhaesivum*, *Morganella morganii*, *Pasteurella multocida*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Staphyloccocus sciuri*, and *Veillonella parvula* (Fig 6).

A subset of 26 OTUs was shared among all samples, and they collectively accounted for 30–75% of the microbial abundance of the dorsal patch per individual (Figs 7 and 8). Since these were all unassigned OTUs, we generated a phylogenetic tree was generated to associate these 26 shared bacteria to their closest relatives (RefSeq-NCBI Database) (Fig 9). These bacteria are distributed among 13 families; the four with the highest abundance were Tissierellaceae, Enterobacteriaceae, Pasteurellaceae and Streptococcaceae. Three Enterobacteriaceae genera were unassigned, along with one genus each from Pasteurellaceae, Nisseriaceae, Gemellaceae (see supporting information), and Planococcaceae. There was also one unassigned family.

The distribution pattern of 20 of these 26 bacteria was generally similar among samples. Only six showed differential abundance patterns: Pasteurellales OTUs 4466150 and 4353757, Clostridiales OTUs 97301, 768514, 3804335, 4349519 and Bacillales 4454737, 630141 and 4446058. OTUs for which we did not find a species-level relative were: *Clostridum* (97301), *Peptononiphilus* (494906; 4429335; 654307), *Anaerococcus* (4349519; 30062), *Gallicola* (768514), *Peptostreptococcus* (3804335), *Finegoldia* (1096610), *Helcococcus* (New.ReferenceOTU1), *Lactococcus* (4468805), Planococcaceae (630141), *Staphylococcus* (4446058), *Enterococcus* (4453060), Enterobacteriaceae (4425571, 4452613, 4477719, 4385479), Pasteurellaceae (4466150, 4363066, 4353757), Streptococcaceae (4298224), Neisseriaceae (1117566), Gemellaceae (4453535), unassigned (4454737), and Corynebateriaceae (4364814) (Figs 7 and 8).

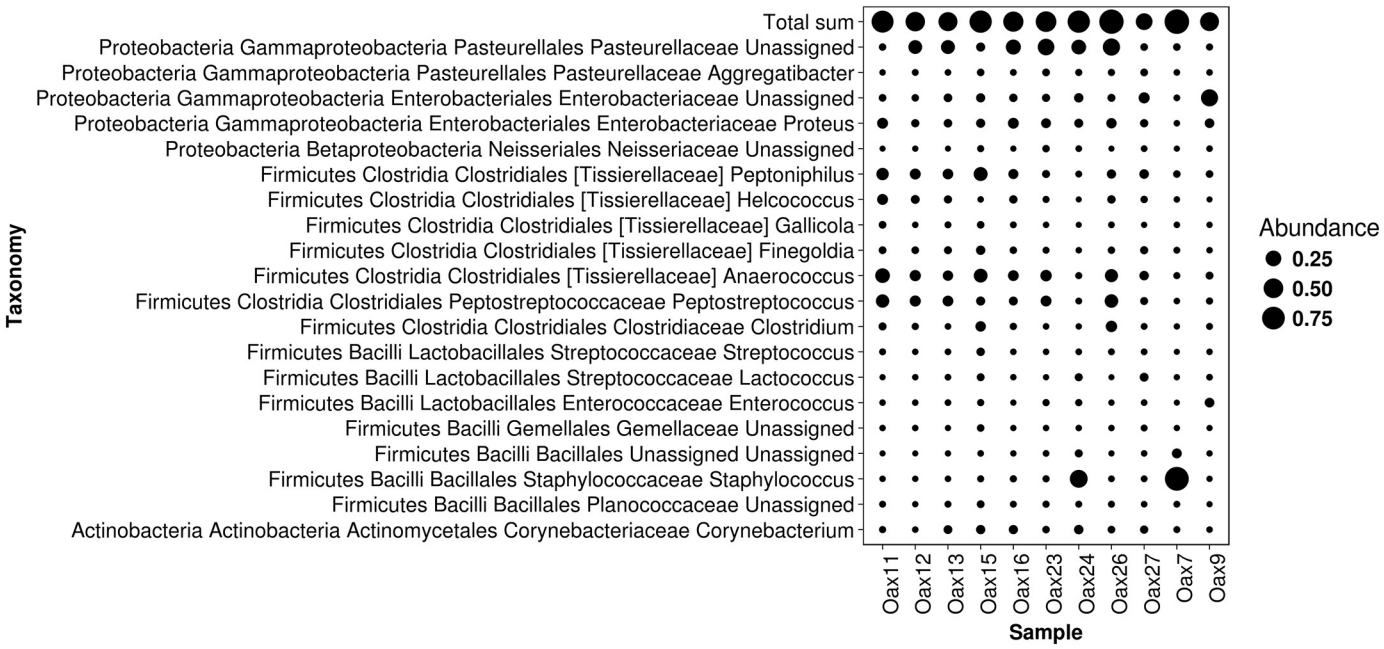

Fig 7. OTUs that were shared among all the samples. Dot sizes represent their relative abundance within the samples. These 26 OTUs combined contribute between 30 and 75% of the total abundance.

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OTU ID	TAXONOMY	GENUS	FAMILY	ORDER	CLASS	PHYLLUM
768514		Gallicola	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
4349519		Anaerococcus	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
30062		Anaerococcus	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
654307		Peptoniphilus	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
4429335		Peptoniphilus	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
494906		Peptoniphilus	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
New.ReferenceOTU1		Helcococcus	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
4364814		Finegoldia	Tissierellaceae	Clostridiales	Clostridia	Firmicutes
4385479		Proteus	Enterobacteriaceae	Enterobacteriales	Gammaproteobacteria	Proteobacteria
4477719		Unassigned	Enterobacteriaceae	Enterobacteriales	Gammaproteobacteria	Proteobacteria
4452613		Unassigned	Enterobacteriaceae	Enterobacteriales	Gammaproteobacteria	Proteobacteria
4425571		Unassigned	Enterobacteriaceae	Enterobacteriales	Gammaproteobacteria	Proteobacteria
4353757		Unassigned	Pasteurellaceae	Pasteurellales	Gammaproteobacteria	Proteobacteria
4363066		Aggregatibacter	Pasteurellaceae	Pasteurellales	Gammaproteobacteria	Proteobacteria
4466150		Aggregatibacter	Pasteurellaceae	Pasteurellales	Gammaproteobacteria	Proteobacteria
4468805		Lactococcus	Streptococcaceae	Lactobacillales	Bacilli	Firmicutes
4298224		Streptococcus	Streptococcaceae	Lactobacillales	Bacilli	Firmicutes
1117566		Unassigned	Neisseriaceae	Neisseriales	Betaproteobacteria	Proteobacteria
4453060		Enterococcus	Enterococcaceae	Lactobacillales	Bacilli	Firmicutes
4453535		Unassigned	Gemellaceae	Gemellales	Bacilli	Firmicutes
4454737		Unassigned	Unassigned	Bacillales	Bacilli	Firmicutes
630141		Unassigned	Planococcaceae	Bacillales	Bacilli	Firmicutes
4446058		Staphylococcus	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
97301		Clostridium	Clostridiaceae	Clostridiales	Clostridia	Firmicutes
3804335		Peptostreptococcus	Peptostreptococcaceae	Clostridiales	Clostridia	Firmicutes
4364814		Corynebacterium	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria

Fig 8. OTU ID taxonomy. Eight (31%) of the genera and one family were unassigned out of the 26 shared OTUs found in the dorsal patch of *L. yerbabuenae*.

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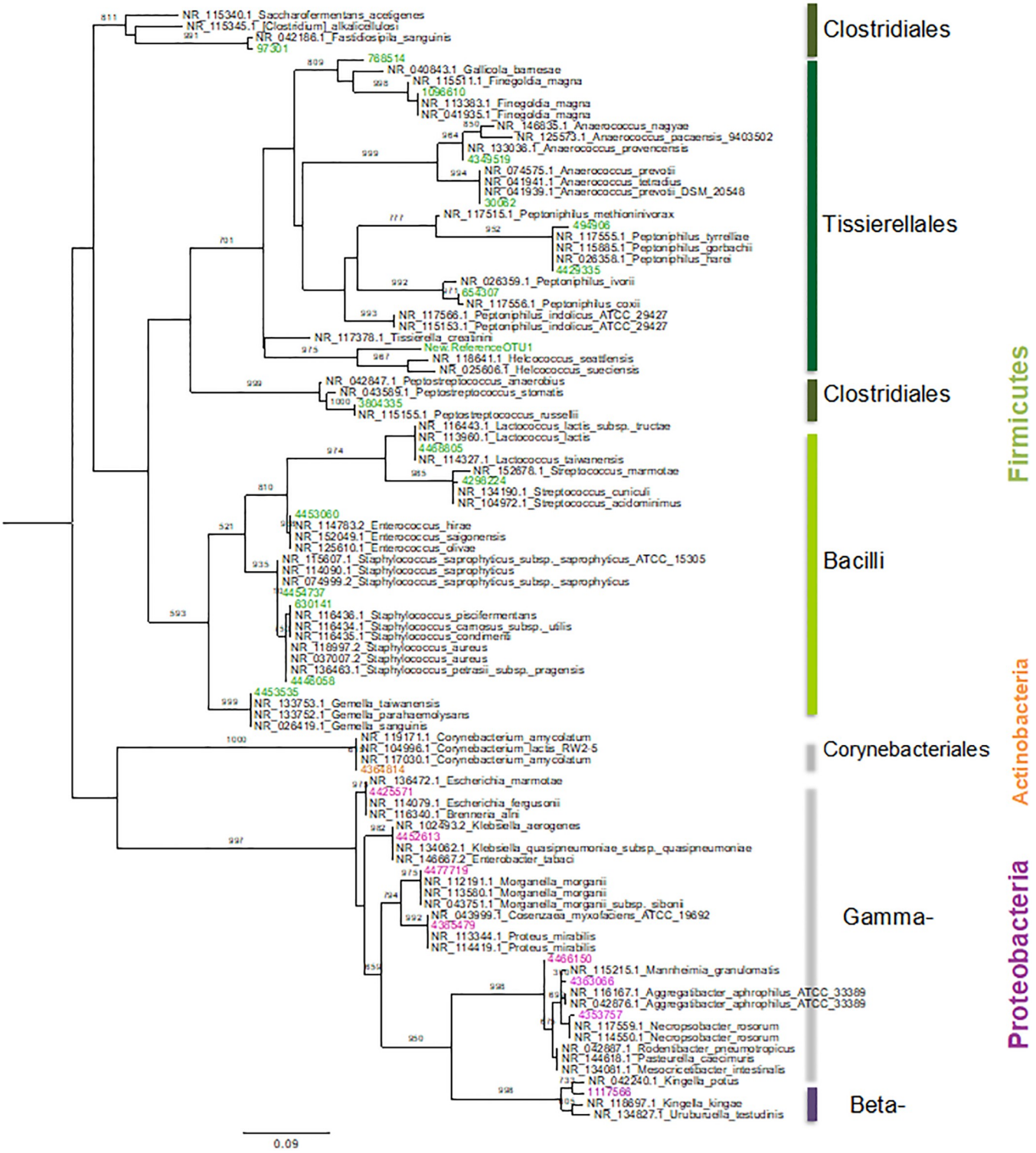

Fig 9. Taxonomic affiliation of each OTU to its three best references (RefSeq-NCBI Database). The code names of related species sequence, shows "NCBI accession id"_"species name".

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Discussion

Dorsal patch core diversity

Although the dorsal patch of *L. yerbabuenae* males harbors a large microbial diversity, A subset of 26 OTUs was shared among all samples, and they collectively accounted for 30–75% of the microbial abundance of the dorsal patch per individual. Within this shared microbiome, 16 phylotypes are fermentative bacteria. These anaerobic bacteria are reported to be relevant in the production of short-chain fatty acids (SCFAs), which in several mammals have been shown to contribute to scent production that influences behavior, for example in bats (*Saccopteryx bilineata* [20] and *Leptonycteris yerbabuenae* [28]), hyenas (*Crocuta crocuta* and *Hyaena hyaena* [49]), and lions (*Panthera leo* [50]). Most of the shared bacteria from the dorsal patch belong to genera and families found and described in humans as part of the skin microbiome (*Finegoldia*, Pasteurellaceae), associated with wounds or infections (*Helcococcus*, *Enterococcus*) [49], or with the production of fermented products or volatile fatty acids (*Peptostreptococcus*, *Anaerococcus*, *Gallicola*, *Peptostreptococcus*, *Lactococcus*, Planococcaceae).

The shared OTUs are shown in the phylogenetic reconstruction, positioned nearest to their closest known relative (Fig 9). Two of the bacteria from the genus *Lactococcus* and *Streptococcus* are associated with wounds [49]. OTUs from the genera *Helcococcus*, *Aggregatibacter*, *Enterococcus*, and the family Corynebacteriaceae and one unassigned OTU include bacteria with pathogenic potential [50]. OTUs from the genus *Finegoldia* and one unassigned OTU were classified as double-function bacteria with fermentative functions [51]. One OTU was unassigned with unknown functions, and OTUs belonging to the genera *Gallicola*, *Anaerococcus*, *Peptoniphilus*, *Proteus*, unassigned *Staphylococcus*, *Clostridium*, *Peptostreptococcus* specialize in fatty acid production through fermentative processes [52]. More specific information on known functions of these groups is summarized in the supporting information (S1 Table).

Diversity and potential implications of common bacteria in the dorsal patch of L. yerbabuenae

The difference observed in the Alpha and Beta diversity analyses of the bacteria (Table 1 and Fig 3) suggest individual-level variation in the assembly of the microbiota in the dorsal patch of each individual. Sixty-one percent of the shared OTUs in the dorsal patch have a fermentative function (Figs 7 and 8). These fermentative bacteria could metabolize short-chain fatty acids (SCFAs), likely contributing to scent production and possibly to mate attraction [17,53–55].

These results are consistent with the first premise of the fermentative hypothesis that volatile odorants are produced by bacteria that colonize mammal scent structures. This premise has also been supported by several other studies [17,20,54]. The second premise, that "individual "odor prints" result from the differences among individuals in the composition of the bacterial communities of these structures" was not tested in this study [20]. To do that, experimental studies are needed to detect the presence of SCFAs and confirm their role in individual recognition.

The recognition of skin microbiota as a main component of odor production is key to further understanding animal behavior. The olfactory receptors, the main olfactory epithelium, and the vomeronasal organ receive the odor signals produced by bacteria, which are then sent to the brain, a chemical signal pathway known as the microbiome-skin-brain axis [55]. The presence of fermentative bacteria in the dorsal patch of *L.yerbabuenae* could be important in generating odor, a potential reproductive signal.

Conclusion

This study contributes to the establishment of baseline knowledge of the bacteria associated with the dorsal patch of *L. yerbabuenae* using molecular methods (16S rRNA sequencing). High-throughput sequencing techniques, along with bioinformatic analysis, allowed us to describe the biodiversity of the bacterial consortium of the dorsal patch of *L. yerbabuenae*. Current microbiome analyses are based on genetic diversity and composition estimations, coupled with information found in the literature. However, a major remaining challenge is that the large majority of the sequences found in this study were unassigned OTUs, making it necessary to rely on assertive association techniques using phylogenetic trees and other informatic comparisons to understand the system. This information could be substantially deepened by using a metagenomic approach to infer functional and taxonomic information as well as metabolic properties present in the bacterial communities associated with this and other systems, as well as their repercussions in the host and its life cycle. There is a long journey ahead to understand how microbiomes intervene in diverse biological systems, and how they contribute to regulating host behavior and reproduction through chemical signaling.

Supporting information

S1 Table. Functions associated with the 26 shared OTUs found in L. yerbabuenae dorsal patch.
(ZIP)

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Capítulo 3. ¿Cómo afecta la separación geográfica la estructura de las comunidades microbianas en dos poblaciones discretas de *Leptonycteris ye*rbabuenae?

How geographical separation in two discrete populations of the bat *Leptonycteris yerbabuenae* affects the structure of its microbial communities

Running title: Microbial communities of two *Leptonycteris yerbabuenae* populations

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ABSTRACT

In this paper, we explore how two discrete and geographically separated populations of the Lesser long-nosed bat (*Leptonycteris yerbabuenae*)—one in central Mexico and the other in the Pacific region of Mexico—differ in their fecal microbiome composition. The V4 region of the 16s rRNA gene from 68 individuals was analyzed using alpha and beta diversity metrics. We obtained a total of 11 566 Amplicon Sequence Variants (ASVs). The bacterial communities in the central and Pacific populations had a diversity of 6939 and 4088 ASVs respectively, sharing a core microbiota of 539 ASVs. Lactating and pregnant females in the two populations had significant differences in Beta diversity patterns compared to other reproductive stages, but the most abundant microbiota is stable and conserved in both populations. This could be a consequence of dietary diversification by pregnant and lactating females to cope with the increased energy requirements of these stages, despite the variation due to geographic separation. Our study assesses the changes in the microbiota during a pre-speciation stage, which has not been explored before in a geographic separation model in bats. The results provide a baseline for future studies of the microbiome in these two wild populations of the lesser long-nosed bat, a main pollinator of the Agaves from which the beverages tequila and mezcal are made.

Key words: reproductive stages, populations, geographic separation, holobiont.

Introduction

The microbial communities that inhabit the guts of mammals are complex, dynamic and critical to the health of the host (Ochman et al, 2010). Microbiota composition and abundance is further determined by several factors such as diet, geography, physiology and health status (Ochman et al., 2010; Ley et al., 2008; Muegge et al., 2011), and even phylogeny. It has been demonstrated, for example, that phylogeny in hominids influences the microbiota at an evolutionary level, suggesting that the microbiota is more than just what the host eats (Ochman et al., 2010).

Microbial symbionts have a variety of roles in the nutrition, immunity, development, reproduction and speciation of their eukaryote hosts, making this symbiosis a major component of eukaryotic fitness and evolution (Brucker & Bordenstein, 2012). Interactions between host and microbiome have coevolved in mutual adaptations into a superorganism association (MacColl, 2011) and are key to biological adaptations (Brockhurst and Koskella, 2013).

The hologenome concept of evolution states that in addition to the host genome, a significant proportion of the microbiome is also transmitted from one host generation to the next and can thus propagate unique properties of the holobiont (Rosenberg & Zilber-Rosenberg, 2019). Furthermore, genetic variation can occur by changes in the host and/or microbiome genomes; the microbiome genome is even expected to be able to adjust to environmental dynamics faster and through more processes that the host, and thus may be playing fundamental roles in the adaptation and evolution of holobionts (Rosenberg & Zilber-Rosenberg, 2019).

Genetic variation in holobionts can occur by mutation and DNA rearrangement, amplification or reduction of specific microbes, acquisition of novel microbes from the environment, and horizontal gene transfer from microbe to microbe or from microbe to host

(Zilber-Rosenberg & Rosenberg, 2008). The multilayered structure of the holobiont consists of a core of host-adapted microbiota assembled from diverse environments and determined by genetic factors, as well as a flexible pool of microbes that depend on environmental diversity and external conditions (Shapira, 2016).

Vertical transmission of the microbiota from parent to progeny allows its maintenance between generations and is likely favored by selection when those microbes are beneficial to the host (Shapira, 2016). The functional profile of the microbiome is probably more constrained by evolution/vertical inheritance than individual bacterial taxa (Martiny et al., 2006; Phillips et al., 2017; Shapira, 2016). Thus, vertical transmission could drive coevolution, resulting in phylogenetic congruence (Shapira, 2016).

The mutualistic and adaptive potential of microbiota offer flexibility and the ability to adapt to a range of ecological niches (Alberdi et al., 2016). Subsequently, mutualist-facilitated adaptations can lead to population fragmentation, isolation and speciation (Shapira, 2016). Speciation—the splitting of a population into two reproductively incompatible populations, each taking a distinct evolutionary path—is a defining process in evolution. Mutualist microbes can drive all modes of isolation, including ecological, behavioural, and developmental asynchronization and genetic divergence, but, as facilitators of niche adaptation, they are frequently associated with ecological isolation (Brucker & Bordenstein, 2012; Shapira, 2016).

In the order Chiroptera, microbiome composition is influenced by the host phylogeny and life history (Phillips et al., 2012). Due to their species-specific feeding strategy specialization, Phyllostomid bats are a model clade to study the relationship of microbiome- host composition, phylogeny and co-evolution (Carrillo-Araujo et al., 2015). Phyllostomid bats are found from the southern USA and northern Mexico to Argentina and show a great evolutionary

diversification of species, in which patterns are dependent on geographic and ecological interactions, resulting in a great diversity of dietary strategies and the most ecologically diverse family within the order Chiroptera (Carrillo-Araujo et al., 2015). They show a remarkable degree of evolutionary diversification of dietary strategies, from insectivory (the ancestral trait) (Monteiro & Nogueira, 2011) to feeding on blood, small vertebrates, nectar, fruit, and complex omnivorous diets (Gardner, 1979).

Leptonycteris yerbabuenae is a migratory phyllostomid bat. It is usually a strict and specialized nectar feeder, though it can occasionally consume fruit, so its diet is generally poor in proteins and minerals (Fleming & Nassar, 2002). *L. yerbabuenae* feeds mainly on nectar from agaves, columnar cacti like *Carnigea gigantea*, different species of Stenocereus and Bombacaceae (*Psedobombax elipticum*), Convolvulaceae and other legumes (Arita, 1991; Arita & Humphrey, 1988; Cole & Wilson, 2006; Valiente-Banuet, Arizmendi, Rojas-Martínez, & Domínguez-Canseco, 1996).

Mexico has two differentiated populations of *L. yerbabuenae*: one along the Pacific coast including Baja California, Sonora and Jalisco states, and the other in the south-central region, including Oaxaca, Morelos, and Guerrero states (Morales-Garza et al., 2007). The two populations are separated geographically, with negligible gene flow, as demonstrated by Morales-Garza et al 2007 using random amplified polymorphic DNA (RAPD) analysis (Morales-Garza et al., 2007).

The population of *L. yerbabuenae* that resides between the latitudes of 30º N and 21º S in North America carry out latitudinal migrations, while populations that reside south of 21º S latitude are year-round residents. Migrations are known to be correlated with the availability of floral resources (Rojas-Martínez, et al., 1999), of which the most predictable are cacti, agave and C3 plant nectar (Burke et al 2019). The central population has more genetic variability

than the Pacific population, likely due to the environmental stability of central Mexico (Morales-Garza et al., 2007; Valiente-Banuet et al., 1996).

In both populations, roosts are differentiated by reproductive stage (Ceballos, Fleming, Chavez, & Nassar, 2006; Cockrum, 1991; Stoner, O.-Salazar, R.-Fernández, & Quesada, 2003), with mating and maternity roosts separated geographically from bachelor and non-reproductive caves (Fleming & Nassar, 2002; Hayward & Cockrum, 1971; Sánchez & Medellín, 2007; Stoner et al., 2003). Pregnant females congregate in maternity colonies to give birth, lactate, and care for their offspring (Ceballos et al., 2006), and they usually return to the same roost year after year in different stages of pregnancy and offspring rearing throughout their lifetimes (Hayward & Cockrum, 1971). Adult males and non-reproductive females often segregate into groups called "bachelor colonies" (Ceballos et al., 2006). Before foraging at night, both sexes rest in temporary night roosts (Ceballos et al., 2006; Cole & Wilson, 2006). A study of the fecal microbiota of different reproductive stages within the central population of *L.yerbabuenae* showed that microbiota diversity is related to reproductive stage rather than geographical distribution within this population (Gaona, Gómez-Acata, Cerqueda- García, Neri-Barrios, & Falcón, 2019). Microbiota composition is consistent in juveniles and non-reproductive females and males, regardless of the roost. Pregnant and lactating females' microbiotas were similar and more diverse than juveniles and non-reproductive adults. One explanation for this is that microbiota evolved with its host to be flexible enough to shift from a specialized diet to a more generalist diet to cope with the increased energy requirements during pregnancy and lactation (Gaona et al., 2019).

The aim of this research is to evaluate the composition of fecal microbiota in two discrete, geographically separated and genetically differentiated lesser long-nosed bat (*L. yerbabuenae)* populations in Mexico (Morales-Garza et al., 2007). Our hypothesis is that the geographical

separation and reproductive isolation of the two *L. yerbabuenae* populations will lead to significant differences in their fecal microbial composition, making it more similar within populations than between them. We also evaluate whether the heterogeneity within the Pacific population is due to differences between the reproductive stages, as occurs in the central population (Gaona et al., 2019).

Methods

Study Site

Bat fecal microbiome samples from the lesser long-nosed bat, *L. yerbabuenae*, were collected at five bat roosts. Two were from the Pacific population—a roost of pregnant and lactating females in Pinacate, Sonora (32º 0' 0"N, 113º 55' 0"W), and a roost of reproductive males and juvenile males and females from Panchito cave, Jalisco (98º, 55'N, 19º 32' W) (Figure 1). The remaining three roosts were from the central population: reproductive and juvenile males were sampled in San Juan Noxchitlan, Oaxaca (97° 40' N, 18° 03' W), a colony of 100,000 bats (Valiente-Banuet et al., 1996). Pregnant and lactating females were sampled in Juxtlahuaca Cave, Guerrero (17° 23" 3' N, 99° 16" 1'W), and juvenile males and females and adult males were sampled in Salitre Cave, Morelos (18° 45" 0.05'N, 99° 11" 23.17'W) (Fig. 1). All fecal samples were collected between January and November, 2015 following the different *L. yerbabuenae* reproductive stages (Table 1) in the two populations.

Bat fecal microbiome sampling

Bats were captured using 12-m mist nets (Avinet, Dryden, New York, USA) at the entrance of the caves using Kunz's technique (Kunz, Betke, Hristov, & Vonhof, 2009) between 18:30 and 7:00 h. Standard measurements were taken in order to confirm identification and

assess age and reproductive stage of individuals. (Anthony, 1988; Kunz, Wemmer, & Hayssen, 1996).. Samples were frozen until processing using a field liquid nitrogen dewar.

Ethics Statement

All fecal samples were obtained following the guidelines of the American Society of Mammalogists for capture, handling and care of mammals (Gardner, 1979; Sikes & Gannon, 2011). To minimize animal suffering and distress, each animal was processed by experts only, ensuring safe and effective handling. Samples were taken from wild bats that were released in the same area of capture, causing no apparent harm to individuals. *Leptonycteris yerbabuenae* is not subject to federal protection under Mexican law (NOM-059-SEMARNAT-2010). All activities were approved and authorized under a scientific collection permit granted by the Mexican Subsecretary of the Environment and Natural Resources (SEMARNAT), number FAUT-0231, SGPA/DGVS/05780/15 SEMARNAT. Laboratory sample processing activities fulfilled all biosafety standard requirements of the Ecology Institute of the Universidad Nacional Autónoma de México (UNAM), under authorization from the Biosafety Commision of the Ecology Institute, UNAM.

Analysis of the sequence data

The paired-end 2x250 reads were processed in QIIME2 (Bolyen et al., 2019). The reads were denoised with the DADA2 (Callahan et al., 2016) plugin to resolve the amplicon sequence variants (ASVs). Both forward- and reverse-reads were truncated at 200 bp, and chimeric sequences were removed using the "consensus" method. Representative ASV sequences were taxonomically assigned using the "classify-consensus-vsearch plugin" with default arguments, using the SILVA 132 database as a reference (Quast et al., 2013). An alignment was performed

with the MAFFT algorithm (Katoh, 2002). After masking positional conservations and gap filtering, a phylogeny was built with the FastTree2 algorithm (Price, Dehal, & Arkin, 2010). The abundance table and phylogeny were exported to the R environment to perform the statistical analysis with the phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen, 2015) and ggplot2 (Wilkinson, 2011) packages. Plastidic ASVs were filtered out of the samples (for subsequent separate analysis, see below), then the samples were rarefied to a minimum sequencing effort of 10 000. A PCoA ordination was performed with the weighted-unifrac distance (Fig. 2). The Shannon alpha diversity index was calculated (Fig. 4). A PERMANOVA with a weighted unifrac distance was performed to assess whether there were significant differences among groups of samples (by region or by stages) with 1000 permutations (Table 2).

To explore the differential abundance of taxa, a LEfSe (linear discriminant analysis of effect size) analysis (Segata et al., 2011) was performed at the family level, first with all samples using the populations as categories, and then within each population, using the reproductive stages as categories, using an LDA cutoff > 2 and p-value < 0.05.

Counts of plastidic ASVs (separated from prokaryotic ASVs before rarefaction) were normalized with the cumulative sum scaling (CSS) method with the metagenomeSeq package (Paulson, Stine, Bravo, & Pop, 2013), a DPCoA (Double Principle Coordinate Analysis) and the Shannon index were calculated to assess the likely variation in the diet of the bats in the different stages within the regions. A PERMANOVA was carried out with the DPCoA distance matrix to assess the Beta diversity between populations. The data are deposited in the NCBI platform, in the bioprojects PRJNA508738 and PRJNA543523. A Pearson correlation analysis was performed with the observed ASVs of plastid and prokaryotic 16S to assess the relationship between microbiota diversity (prokaryotic 16S ASVs) and diet diversity (plastidic ASVs).

Results

Microbiome composition in the two populations of *Leptonycteris yerbabuenae*

Of the total samples collected, 68 were positively PCR amplified: 30 for the Pacific population (12 juvenile, 7 adult, 6 pregnant, and 5 lactating), and 38 for the central population (6 juvenile, 20 adult, 6 pregnant, and 6 lactating). We obtained a total of 11 566 ASVs after rarefying to a sampling depth of 10 000 reads (see the accumulation curve in Fig. S1 of suppl. Material).

The Principal Coordinates Analysis (PCoA) showed beta diversity difference between the central (Guerrero, Morelos, Oaxaca) and Pacific (Sonora and Jalisco) *L. yerbabuenae* populations (Fig. 2, bottom). Within each population, pregnant and lactating females formed one group, and juveniles and non-reproductive adults another (Fig. 2. Top left and right).

Figure 3 shows the 40 most abundant bacterial classes, showing the differences between the composition within each population. There was a trend toward higher diversity among pregnant and lactating females in the central population, but higher diversity among juveniles in the Pacific population (Figures 3 and 4.). The Shannon index shows that the Alpha diversity was more homogeneous within the Pacific population (Fig. 4) than in the central population (Gaona et al., 2019).

Comparison using a PERMANOVA test showed differences among reproductive stages; there were significant differences in pairwise comparisons of lactating and pregnant females versus juveniles and adults, and a significant effect of reproductive stage nested within

population (Table 2), showing a clear difference both between the two populations and among stages within each population.

The LeFSe analysis shows differences among reproductive stages within each population at the family level in bacterial communities (Fig. 5 A) as well as between the two populations (Fig. 5 B). The Venn diagram (Fig. 5 B) shows 539 ASVs that are shared between the two populations. The central population had 6939 ASVs and the Pacific 4088 ASVs, a difference of 2,851 ASVs (Fig. 5 B). The two populations also differed in family-level abundance. The LefSe analysis identified six differentiated families for each population, in the central population these were Chtoniobactereaceae, Leptolyngbyaceae, Phormidiaceae, Microscillaceae, and WD2101 soil group; and in the Pacific population, these were Dietziaceae, Porphyromonadaceae, Brevibacteriaceae, Mycobacteriaceae, Nocardiaceae, Khizobiaceae (Fig 5. A).

Bat diet variability between the two regions

The amplification of plastid 16S genes is incidental in fecal microbiome studies. Since chloroplasts are plant organelles acquired by endosymbiosis, their amplification is due to contamination when the host feeds on plant material. Since different plant species' chloroplasts differ genetically, plastidic 16S gene diversity may provide a relative index of plant diversity in the diet (Knight, 2018). The number of plastidic reads in the 68 samples ranged from 17 to 76,979, with a mean of 5,589 (see Suppl. Material). We used the entire plastid dataset with the rationale that although some samples had very low plastid reads, the total reads per sample including bacterial 16S had more than 10 000 reads. After normalization by the CCS method to avoid the effect of differences in the libraries' sample sizes, we calculated the Shannon index and performed a DPCoA and a PERMANOVA (Fig. 6 and Table 2).

Similar to the microbiota of the two populations, the Beta diversity of chloroplast ASVs was higher in the central population (variance within population), but alpha diversity was similar (Fig. 6). Chloroplast diversity also differed among reproductive stages, with pregnant and lactating females having the highest beta chloroplast diversity (Fig. 6).

Discussion

Changes in microbiome composition could direct speciation, given that the functions of the microbiome have evolutionary consequences in the host (Suzuki, 2017). A central issue in the hologenome concept is whether the hologenome can be considered a unit of selection, in other words, whether the three components of natural selection—variation, differential success, and inheritance—apply to the holobiont (Cerqueda-García & Falcón, 2016; Moran & Sloan, 2015; Suzuki, 2017). The differences we found between the two populations of *L. yerbabuenae* in Mexico suggest that in this case there is variation in the holobiont. There is variation between the two populations, shown in the PCoA plot; the diversity within bacterial communities in the central population is larger than in the Pacific population (6,939 ASV, compared to 4,088 ASV), and the beta diversity among groups within the central population is more heterogeneous. In addition, Morales-Garza (Morales-Garza et al., 2007), found higher genetic variability in the central population, associated with year-round availability of pollen and nectar (Fleming & Nassar, 2002; Rojas-Martínez et al., 1999; Villaseñor, Dávila, & Chiang, 2017). It remains to be tested whether these inter-population microbiome differences could indicate differential success if the changes in microbial composition are related to losses or gains of metabolic capabilities that contribute to host fitness (Hooper, Littman, & Macpherson, 2012)

We found that the two populations share a core microbiota of only 539 ASV, and that these are the most abundant bacterial groups for both populations, accounting for about 75 %

of the relative abundance (Fig. 7). This suggests stability in the microbiome within the populations over time, and consistent with findings in hominids, the microbiome is more related to the species than to diet (Ochman et al., 2010). Each population has six families with differential abundance detected by the LeFSe analysis (Fig. 5 A), and a difference of 2,851 ASVs. In addition, ontological characteristics of bats, such as the rearrangement and growth of organs, including the stomach and the enlargement of the intestine, during pregnancy (Speakman, 2018), constrain differences and similarities in the microbiome (Gaona et al., 2019). Despite the changes in Beta diversity due to geographical separation, the reproductive stages are more similar between the populations (Fig. 3). Flexibility in the microbiome has been confirmed in experiments in which individuals of different species are given a similar diet, their microbiota tend to homogenize (Xiao, Xiao, Liu, Zhao, Sun, Tan, Sun, Liu & Feng, 2019). Our results partially support this result, since the populations we have studied are more similar within populations . However, we also found a core microbiome that also supports microbiota stability (Coyte, Schluter, & Foster, 2015) and ontogenetic differences in both populations (Gaona et al. 2019). The correlation between microbiota ASVs and plastid ASVs was positive and significant in the adult and juvenile stages (Fig. S3, Suppl. Material), suggesting a more direct diet-microbiota relationship, and thus a lamarckian acquisition of microbiota. However, this positive correlation is relaxed or broken in the pregnant and lactating stages in both populations. This suggests that in the demanding stages of pregnancy and lactation, the alpha diversity of microbiota increases more steeply than the plastids (diet), suggesting that the physiological state is driving the changes more than the diet. Thus the core microbiota is present throughout the life cycle, but in pregnancy and lactation the microbiota is more divergent (Fig 7). The PERMANOVA analysis of only pregnant and lactating females between the two populations showed that these stages have a higher coefficient of variance (R^2 ~0.13) than the

adult and juvenile stages ($R^2 \sim 0.10$)(Fig S4 and S5, Suppl. Material).

The second and third requirements— differential success and evidence of inheritance of the microbiome—remain to be evaluated. One of the main predictions of microbiome heritability is that the offspring's microbiome will resemble the parental microbiome. Thus, a progenitor holobiont (species A with its microbiome) will resemble its holobiont offspring (species B with its microbiome). We are unable to directly evaluate that statement here, since we do not have samples of a parental-progeny holobiont and this study solely focused in one bat species with geographic separation.

The term phylosymbiosis is used to describe the congruence between the differences in bacterial communities and the phylogenetic divergences among species (Brooks et al., 2016; Kohl et al., 2018; Mazel et al., 2018), which in a broad sense corresponds to the inheritance of the microbiome. While the two populations we sampled in this study are the same species, our data show a relationship between differences in host bacterial communities and genetic differences among populations (Morales-Garza et al., 2007), which could suggest an early process of phylosymbiosis, or a pre-speciation stage which has not been explored before in a geographical separation model. As described above, the differences between the two populations of *L. yerbabuenae* show higher variation (Beta diversity) in the microbiota the central population (Fig. 2, and 4), which also has a higher genetic diversity and is a more stable population (Morales-Garza et al., 2007).

Macroecological theory indicates that the highest genetic diversity will be found in areas with better access to resources and the best ecological conditions for the species. For this species, the available resources have determined two main divergence routes and very likely the segregation of the species, as confirmed by stable isotope analysis of mitochondrial DNA

(Burke, Frey, Ganguli, & Stoner, 2019), and these changes correspond with orography (Burke et al, 2019). *L. yerbabuenae* are reliant on the availability of flowers, and the central region has year-long availability (Fleming & Nassar, 2002; Rojas-Martínez et al., 1999; Villaseñor et al., 2017) in contrast to the seasonal availability of the Pacific region (Petit, Excoffier, & Mayer, 1999). Thus, the reproductive peaks of bats and the population composition is different between the populations, apparently supported by the Beta diversity of the plastid ASVs, and the variance between the two populations is high ($R^2 \sim 0.2$ from the PERMANOVA, Table 2).

Our results show a differentiation of the microbiota composition between populations and among reproductive stages. One explanation for these trends is the differentiation of the diet, which has been demonstrated to be a main factor shaping the functionality and diversity of the gut microbiome, resulting in convergence between microbial communities and their hosts' foraging habits (Muegge et al., 2011). There is evidence that the diet shapes the functionality, diversity, and relative abundance of dominant phyla, as well as the populations of specific bacterial groups; this is influenced in part by the composition of macronutrients consumed, and in part by the introduction of new microbes from food itself (Muegge et al., 2011; Voreades, Kozil, & Weir, 2014).

In the two populations of *L. yerbabuenae,* our results show significant differences in the Beta diversity of lactating and pregnant females, perhaps associated with the high energy, protein and calcium requirements during these life stages (Gaona et al., 2019; Speakman, 2008). Previous work has shown that the diversity of plants consumed by *L. yerbabueanae* is 2.4 times higher among pregnant and lactating females than among non-reproductive females and males (Riechers, Martínez-Coronel, & Vidal, 2003). This increased foraging diversity is also reflected in our results, with a higher diversity of gut microbes in pregnant and lactating females in the

central population, likely due to the diversified diet due to the physiological conditions of pregnancy and lactation and the high plant diversity in the central region (Fig. 6).

The analysis of chloroplasts showed a difference between the two populations; while there was some overlap between the central population and Pacific population adults, the two populations tended to separate, with lower diversity in the Pacific (Fig. 6).

Conclusion

The differential fecal microbiota composition of the two *L. yerbabuenae* populations, central and Pacific, which inhabit geographically disjunct ranges that differ in their availability of nectar and pollen, opens the door to exploring microbiome-bat relationships that may be influenced by natural selection. Both variation and and stability are present, suggesting that it is not just the host, but the host-microbiome unit, the "holobiont," which could be subject to natural selection and evolutionary processes (Cerqueda-García & Falcón, 2016; Suzuki, 2017). Also, this study suggests an early process of pre-speciation phylosymbiosis, since diversity was highest in the central population, where there is also higher genetic diversity, perhaps due to the unrestricted availability of foraging resources. This is in contrast to the Pacific population, which had lower microbiome and genetic variability, probably because resources are only available seasonally. These resource availability interactions influence reproduction and population size, with increased genetic diversity, both in the host and in the microbiome.

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Competing interests

The authors declare no competing interests.

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Data Accessibility Statement

The raw reads are available in the NCBI bioprojects PRJNA508738 and PRJNA543523.

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Contributed new reagents or analytical tools: L. Falcón

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Writing – Original Draft: O. Gaona, X. Neri-Barrios, D. Cerqueda-García

Writing – Review & Editing: O. Gaona, D. Cerqueda-García, A. Moya

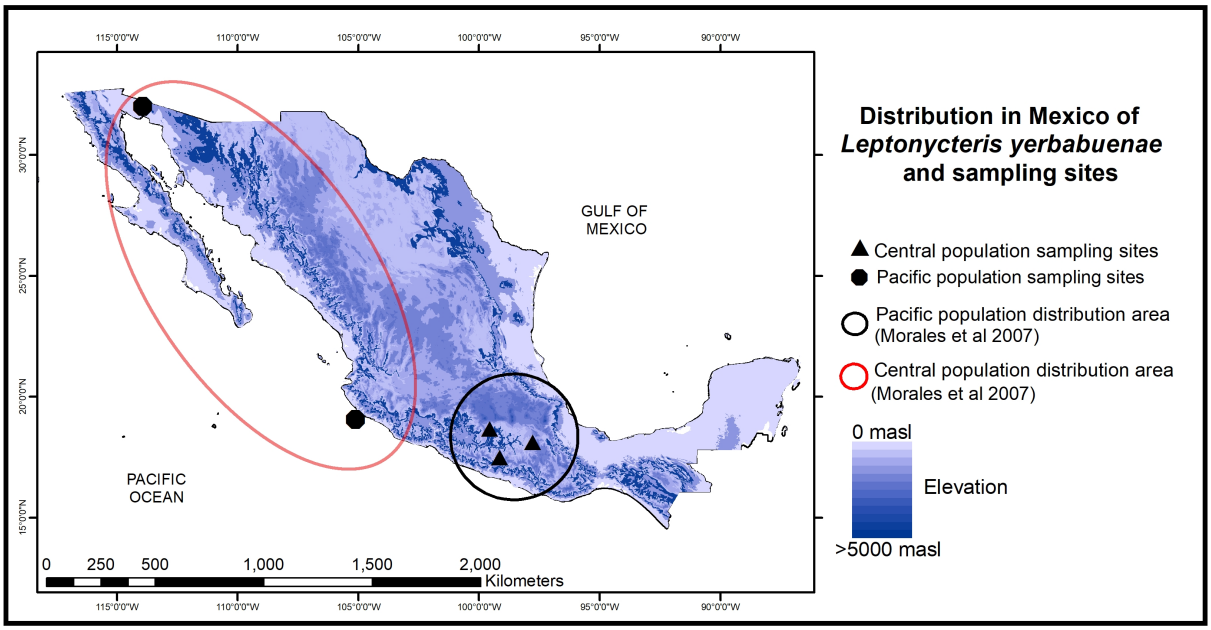

Figure 1. Distribution of *L. yerbabuenae* in Mexico. The central population is found in Morelos, Guerrero and Oaxaca, and the Pacific population in Baja California, Sonora and Jalisco.

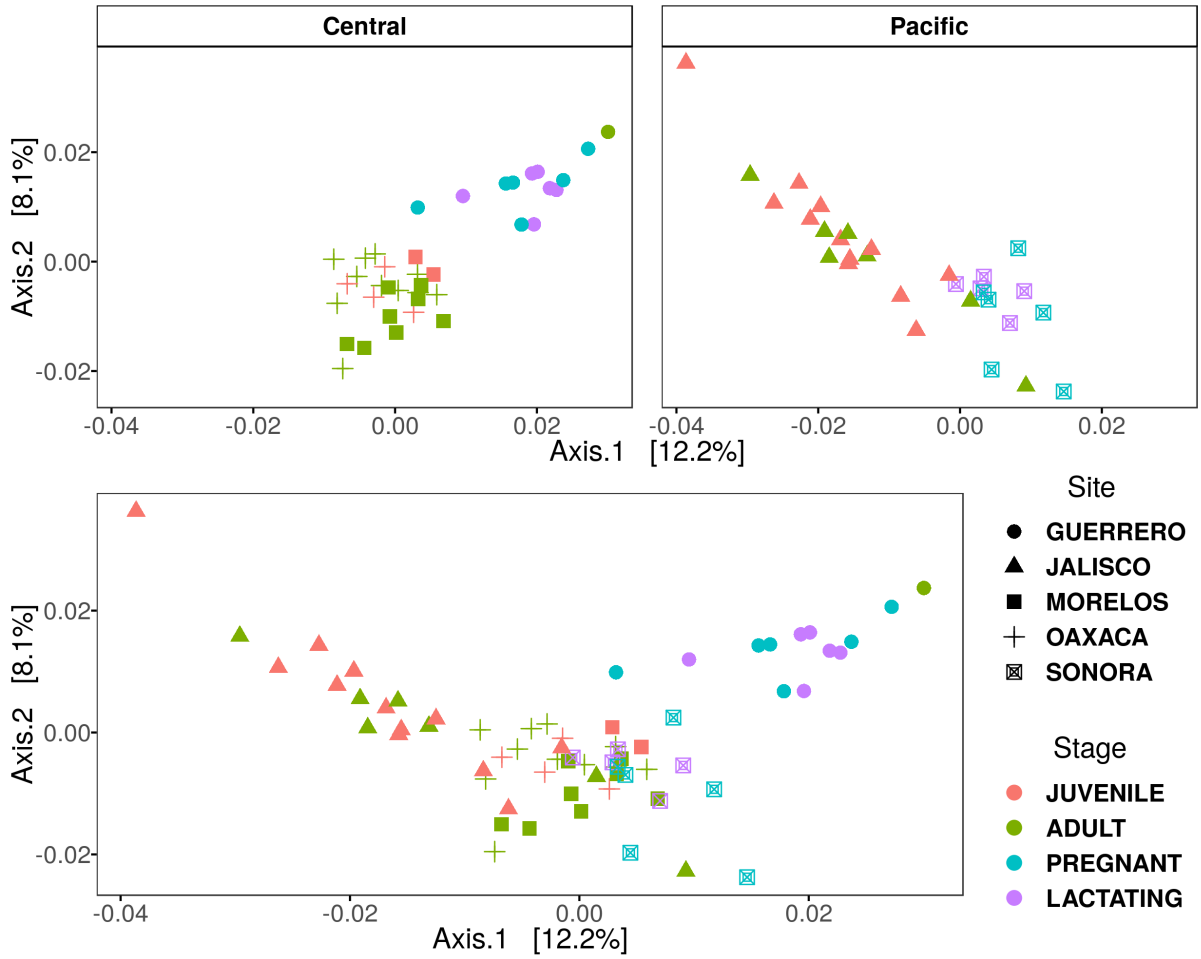

Figure 2. PCoA of weighted unifrac distance. In the upper panels, each population is shown separately; in the bottom panel the two populations are shown together.

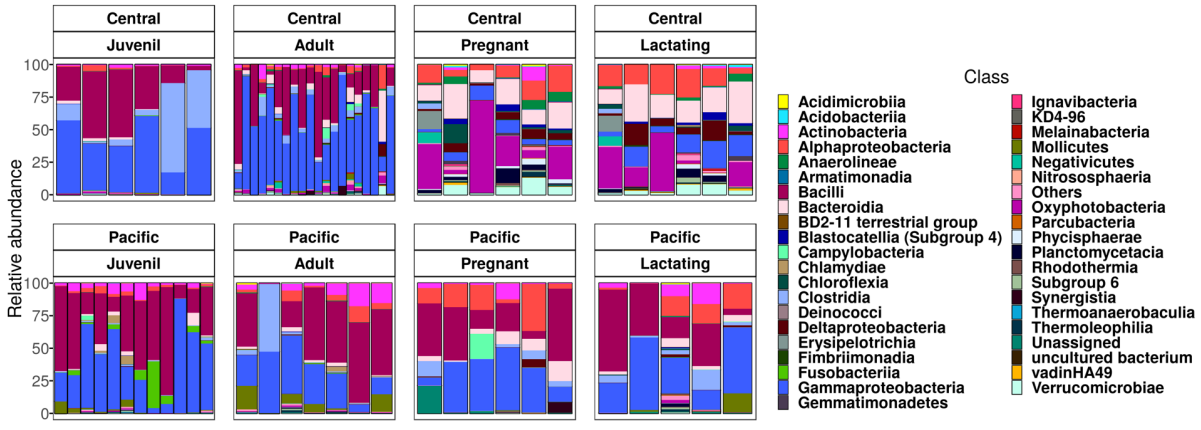

Figure 3. Abundances of the 40 most abundant classes; the remaining classes were grouped in the “others” category.

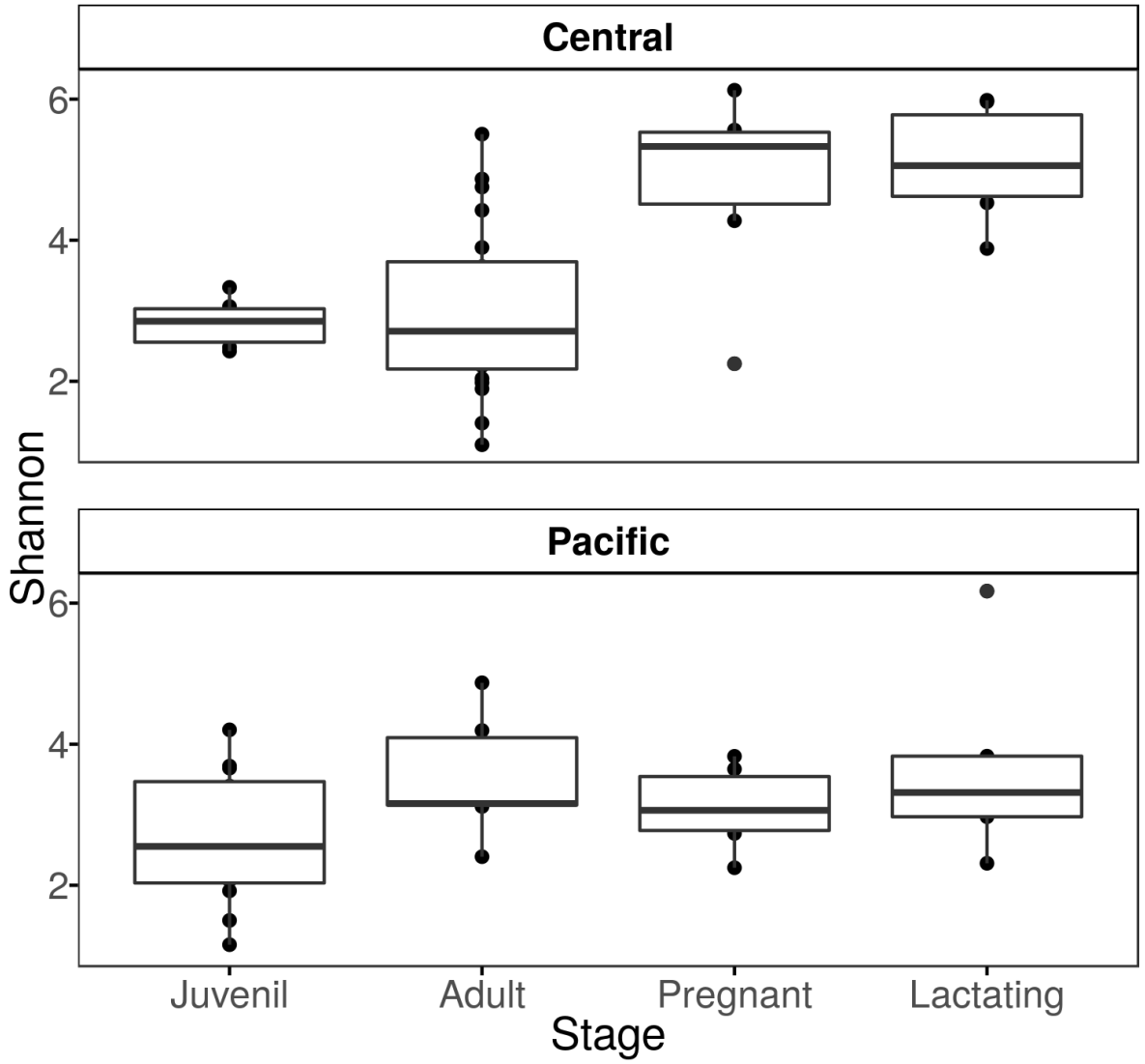

Figure 4. Shannon index of samples grouped by reproductive stage and population. In the central population there were significant differences among stages (Kruskal test *P* value < 0.01).

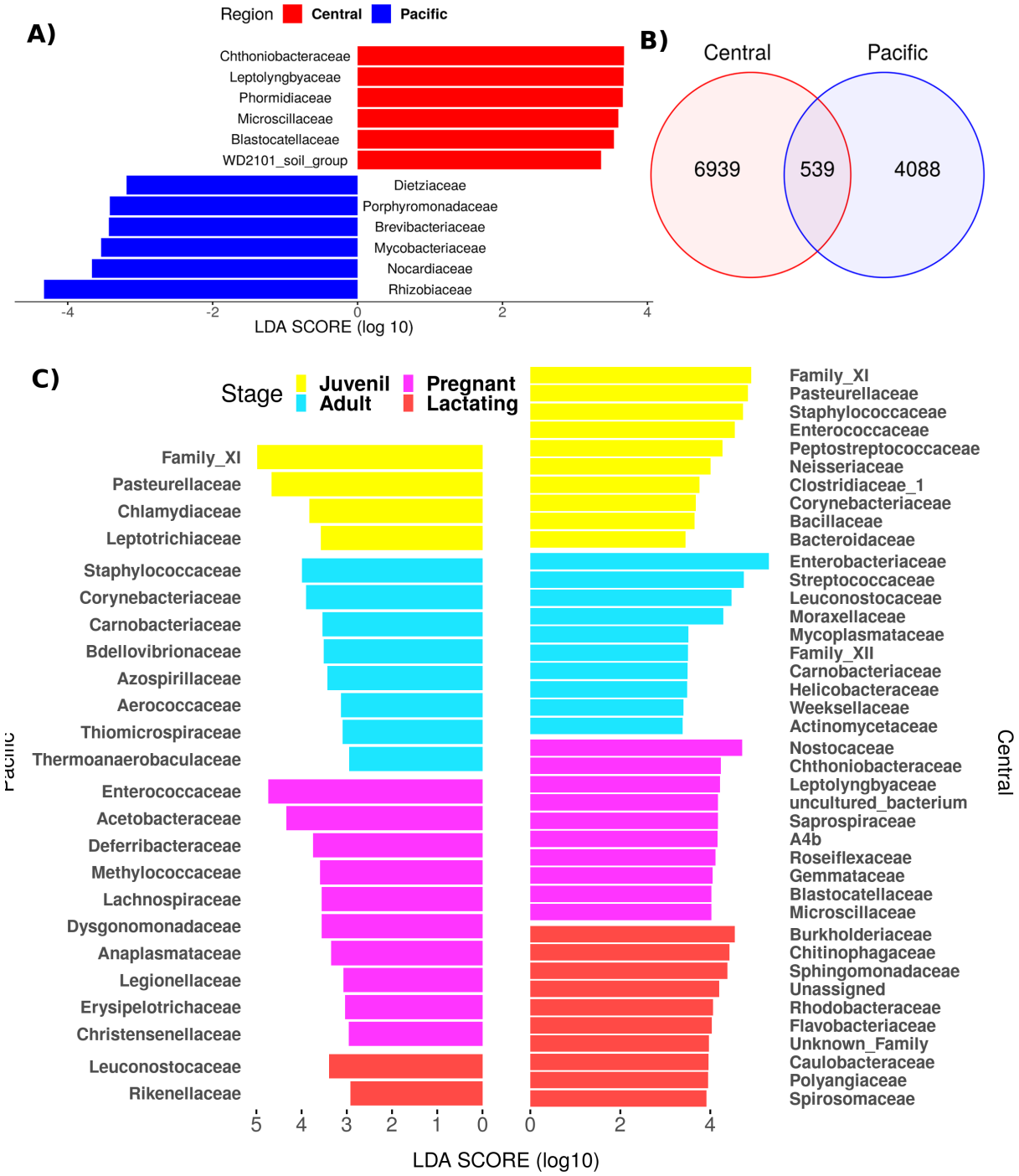

Figure 5. LeFSe analysis of samples with an LDA cutoff > 2 at the family level. A) Families with differential abundance between populations; B) Venn diagram showing the ASV shared between populations; C) Families with differential abundance detected by the LeFSe analysis between reproductive stages within each population—for the central population, just the 10 families with the highest LDA score are shown for each stage (full list provided in the suppl. material).

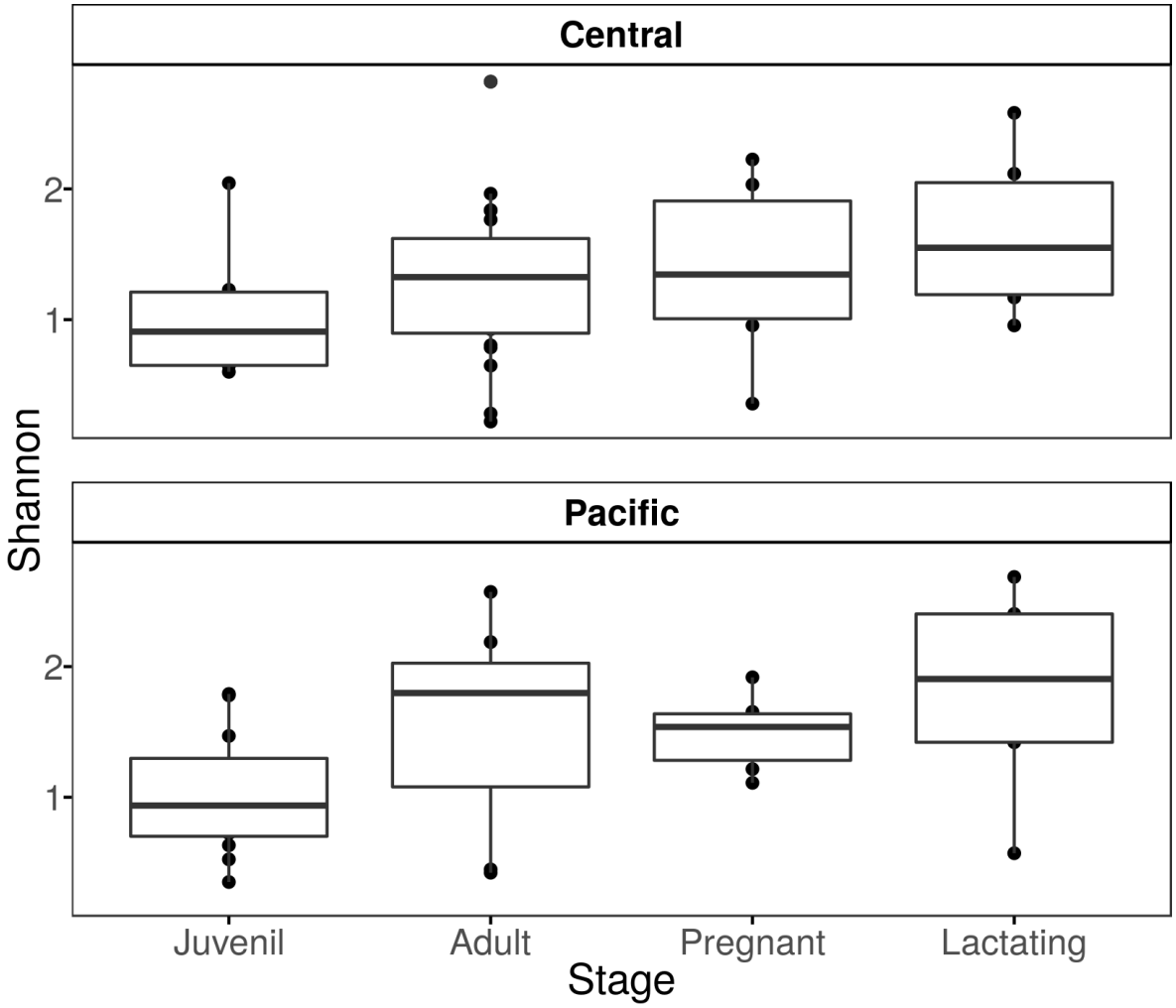

Figure 6. Diversity of plastidic ASVs in each stage within each population. A) DPCoA ordination showing the distance among the samples; B) Boxplots of Shannon index among reproductive stages within each population.

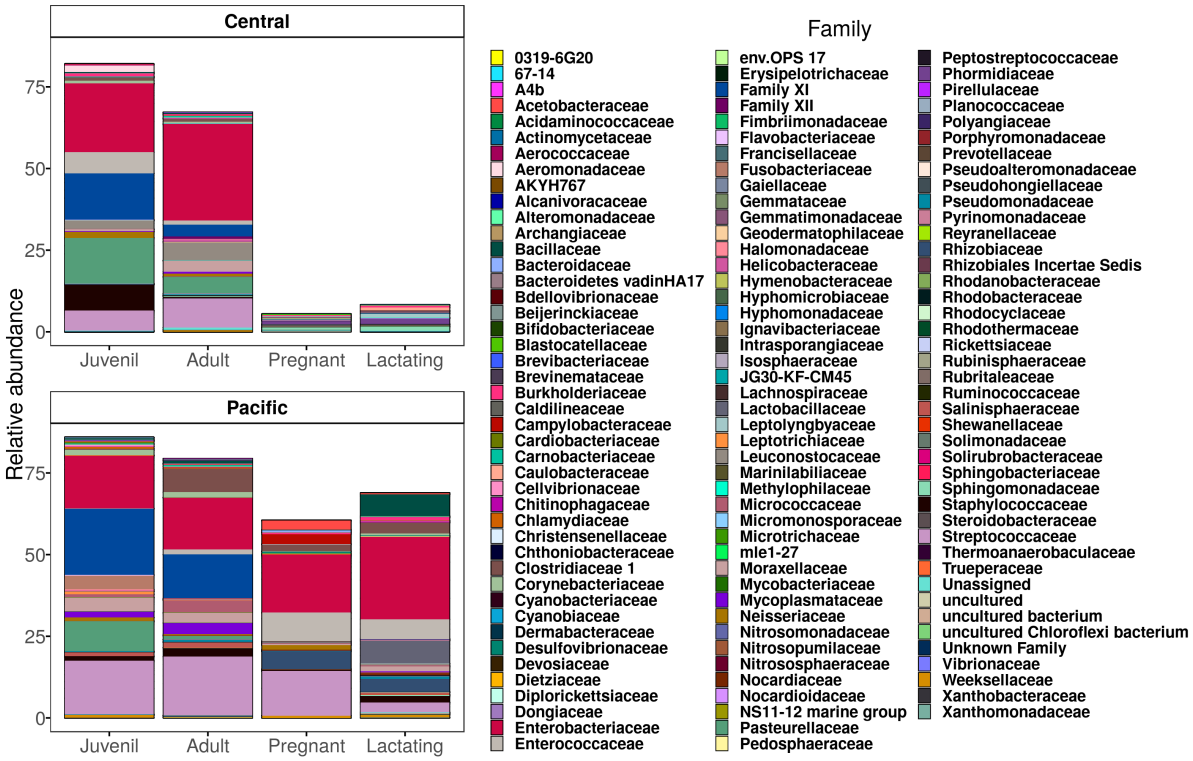

Figure 7. Family classification of the 539 shared ASVs among the two populations. The families with abundance below 1% were combined in the "others" category. The full list of families per sample are shown in the suppl. material (Fig. S1).

Table 1. Fecal samples collected from the Pacific population (n= 31) and central population (n= 51).

Sample	Counts	Region	Stage
C11	1386	Central	Adult
C13	331	Central	Adult
C15	551	Central	Adult
C17	1421	Central	Adult
C19	13395	Central	Adult
C21	3747	Central	Adult
C23	12756	Central	Adult
C26	2881	Central	Juvenil
C27	10388	Central	Juvenil
C28	5026	Central	Juvenil
C29	913	Central	Juvenil
C3	732	Central	Adult
C30	59	Central	Juvenil

C32	57	Central	Juvenil
C33	24607	Central	Adult
C34	15030	Central	Adult
C35	6045	Central	Adult
C36	5791	Central	Adult
C41	3075	Central	Adult
C42	7323	Central	Adult
C43	16467	Central	Adult
C44	14362	Central	Adult
C5	997	Central	Adult
C57	184	Central	Lactating
C62	96	Central	Pregnant
C65	211	Central	Pregnant
C67	17	Central	Pregnant
C69	258	Central	Lactating
C7	603	Central	Adult
C71	780	Central	Pregnant
C73	126	Central	Lactating
C74	109	Central	Lactating
C76	30	Central	Adult
C77	322	Central	Lactating
C79	76979	Central	Pregnant
C81	511	Central	Pregnant
C83	178	Central	Lactating
C9	1412	Central	Adult
P1	1204	Pacific	Lactating
P10	603	Pacific	Pregnant
P11	512	Pacific	Pregnant
P2	282	Pacific	Lactating
P20	146	Pacific	Juvenil
P21	1366	Pacific	Juvenil
P22	1425	Pacific	Adult
P23	40	Pacific	Adult
P24	27831	Pacific	Juvenil
P25	47656	Pacific	Adult
P27	4636	Pacific	Adult
P28	1564	Pacific	Adult
P29	913	Pacific	Adult
P3	5953	Pacific	Lactating
P30	7114	Pacific	Juvenil
P31	39840	Pacific	Juvenil
P32	420	Pacific	Juvenil
P33	179	Pacific	Juvenil
P34	736	Pacific	Adult
P35	181	Pacific	Juvenil
P36	90	Pacific	Juvenil
P37	334	Pacific	Juvenil
P38	299	Pacific	Juvenil

P39	1999	Pacific	Juvenil
P4	290	Pacific	Pregnant
P5	2093	Pacific	Lactating
P6	1907	Pacific	Pregnant
P7	362	Pacific	Pregnant
P8	432	Pacific	Pregnant
P9	978	Pacific	Lactating

Supplementary methods

Extraction of DNA from feces

Metagenomic fecal DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, feces collected into 1.5 ml sterile tubes were diluted with 180 µl of ATL extraction buffer with 20µl proteinase K (10 mg ml^{-1}). Tubes were mixed thoroughly by vortexing and were incubated at 56°C at 1500 rpm for 50 min. 200µl of AL Buffer with 200 µl ethanol (96-100%) were added and mixed thoroughly by vortexing. The mixture was transferred into the DNeasy Mini spin column, washed with Buffer AW1 and then with AW2. The DNA was eluted with 200 µl of AE Buffer and precipitated with absolute ethanol, 0.1 volume 3 M sodium acetate and 2 µl glycoblue. DNA was resuspended in 30 µl of molecular grade water and stored at -20°C until PCR amplification.

16S rRNA gene amplification and sequencing

DNA samples were PCR-amplified using the hypervariable V4 region of the 16S rRNA gene with universal bacterial/archaeal primers 515F/806R following the procedures reported by Caporaso et al. (Caporaso et al., 2012) and Carrillo et al. (Carrillo-Araujo et al., 2015). PCR reactions (25 µl) contained 2-6 ng of total DNA, 2.5 µl Takara ExTaq PCR buffer 10X, 2 µl Takara dNTP mix (2.5 mM), 0.7 µl bovine serum albumin (BSA, 20 mg ml^{-1}), 1 µl primers (10 µM), 0.125 µl Takara Ex Taq DNA Polymerase (5 U $µl^{-1}$) (TaKaRa, Shiga, Japan) and nuclease-

free water. Samples were amplified in triplicate using a PCR protocol consisting of an initial denaturation step at 95ºC (3 min), followed by 35 cycles of 95ºC (30 s), 52ºC (40 s) and 72ºC (90 s), followed by a final extension (72ºC, 12 min). Triplicates were then pooled and purified using the SPRI magnetic bead, AgencourtAMPure XP PCR purification system (Beckman Coulter, Brea, CA, USA). The purified 16S rRNA fragments (~20 ng per sample) were sequenced on an IlluminaMiSeq platform (Yale Center for Genome Analysis, CT, USA), generating ~250 bp paired-end reads.

Discusión

De acuerdo a su función, la microbiota es considerada un órgano esencial, ubicuo y simbiótico (Moya y Ferrer, 2016). En esta tesis se propone que los estadíos reproductivos en *L. yerbabuenae* modelan la microbiota en las hembras preñadas y lactantes, cambiando de una dieta generalista a especialista, cambio de dieta que se debe a las necesidades fisiológicas del hospedero. Existen rearreglos de los órganos internos del hospedero. Algunos de esos cambios se refieren al crecimiento del intestino, fenómeno observado en mamíferos pequeños (Koren et al., 2012), dando una mayor superficie a la colonización de nuevas bacterias que facilitan la asimilación de energía de las moléculas obtenidas del cambio de dieta (Nicholson et al., 2005). La relación hospedero-microbiota es más evidente en las hembras preñadas y lactando que en los otros estadíos reproductivos, independientemente de la geografía (Gaona et al., 2019). Las hembras preñadas y lactantes son más generalistas durante el período de maternidad y lactancia para compensar la demanda energética buscando néctar en plantas diferentes de las que usualmente se alimentan (Riechers-Pérez, 2003; Gaona et al., 2019). Esta modificación en la dieta sugeriría que los individuos no reproductivos mantienen su estrategia especialista y podrían ser polinizadores más eficientes, sugiriendo que los machos son mejores polinizadores. Por otro lado, la respuesta de la microbiota intestinal, que es inmediata para poder cambiar de una dieta especialista a generalista, pudo haber evolucionado para garantizar el éxito reproductivo anual en las hembras (Gaona et al., 2019).

Los cambios en las abundancias y diversidad en la microbiota no se limitan a las hembras, hemos observado que suceden en la microbiota en los machos a nivel cutáneo. Cuando los machos de *L. yerbabuenae* están aptos para reproducirse, desarrollan un parche interescapular en la zona dorsal; se rascan con las patas, el pelo se cae completamente, y llenan esta zona con líquidos urogenitales y saliva, conducta denominada "*smearing*" (Muñoz et al., 2009). Aunque los murciélagos de otras especies tienen estructuras similares en otras partes del cuerpo, hemos observado una diferencia en los análisis de diversidad que sugiere un ensamblaje de microbiota distinto para cada individuo en el parche dorsal. De las OTU (Unidades Taxonómicas Operacionales) compartidas en el parche dorsal, el 61% de ellas tienen alguna función fermentativa, que probablemente podría metabolizar los ácidos grasos de cadena corta (AGCC), y quizá contribuir a la producción de olores. Estos

resultados son compatibles con la primera premisa de la hipótesis fermentativa que indica que “los metabolitos volátiles son producidos por bacterias que interactúan con las estructuras olfativas de los mamíferos” (Albone et al., 1974; Theis et al., 2012; Leclaire et al., 2014).

De igual forma, durante este trabajo se analizó el cambio en el microbioma de las poblaciones del norte y centro de *L. yerbabuenae*. Pudimos observar que se comparte sólo un 4.88% de la composición del microbioma entre ellas, en donde los grupos bacterianos que tienen en común son: Family_XI, Pasteurellaceae, Leptotrichiaceae, Staphylococcaceae, Corynebacteriaceae, Carnobacteriaceae, Bdellovibrionacae, Azospirillaceae, Aerococcaceae, Thiomicrospiraceae, Thermoanaerobaculaceae, Enterococcaceae, Acetobacteraceae, Deferribactereaceae, Methylococcaceae, Lachnospiraceae, Disgonomonadaceae, Anaplasmataceae, Legionellaceae, Erysipelotrichaceae, Christensenellaceae, Leuconotocaceae, Rikenellaceae. Las familias únicas para la población del centro son: Chtoniobactereaceae, Leptolyngbyaceae, Phormidiaceae, Microscillaceae, y WD2101; las de la población del Pacifico corresponden a Dietziaceae, Porphyromonadaceae, Brevibacteriaceae, Mycobacteriaceae, Nocardiaceae, Khizobiaceae. Es interesante observar que las poblaciones tienen microbiomas diferentes, lo cual se puede asociar con el cambio de dieta dependiente del alimento al que tienen acceso. Los resultados obtenidos de la composición diferencial de microbiota fecal de las dos poblaciones de *L. yerbabuenae*, central y del Pacífico, que habitan en zonas independientes y tienen diferencias en la disponibilidad de néctar y polen, abre la puerta para explorar las relaciones microbioma-murciélago que pueden verse influenciadas por la selección natural. En nuestro estudio se cumplen dos de los tres requisitos para la selección natural: variación y éxito diferencial, lo que sugiere que no solo el huésped, sino la unidad de microbioma del huésped, el "holobionte", podría estar sujeto a selección y a procesos evolutivos (Suzuki, 2017). El tercer requisito, la herencia, es difícil de evaluar.

Se ha discutido sobre la multifuncionalidad de las bacterias, ya que dependiendo de sus abundancias podrían llevar a la disbiosis del hospedero en cuestión (Moya, 2016), o ser parte del microbioma del hospedero sano. Faltan estudios más profundos para tratar de dilucidar cómo las bacterias cambian sus abundancias, bajo qué señales químicas, funcionales y ambientales los grupos bacterianos hacen esa sucesión bacteriana en las

diferentes superficies del cuerpo. Hay hipótesis que sugieren que existe herencia vertical y del microbioma. La transmisión vertical de simbiontes del progenitor a la progenie permite el mantenimiento de la microbiota entre generaciones (Shapira, 2016). El perfil funcional del microbioma probablemente esté más limitado por la evolución y la herencia vertical que los taxones bacterianos individuales (Martiny et al., 2006; Phillips et al., 2017; Shapira, 2016), ya que la transmisión vertical obliga a la coevolución, se hereda y prevalence en la descendencia por generaciones (Moeller et al., 2016), lo que resulta en congruencia filogenética (Shapira, 2016).

La aproximación de los análisis que realizamos para el caso particular de *L. yerbabuenae*, sugiere que la microbiota es un reflejo tanto de los estadíos reproductivos del individuo no sólo en el intestino para el caso de hembras, sino en la piel para los machos, lo cual repercute en la conducta y atracción sexual, y se observan familias de bacterias únicas para cada población. Aún quedan muchas preguntas por responder, ante lo cual nuestro estudio representa una línea base en el estudio de dos poblaciones en vida silvestre de *L. yerbabuenae*.

Los vertebrados más estudiados sobre microbioma han sido los mamíferos y la mayoría de los artículos científicos se enfocan en el intestino, principalmente de animales en cautiverio. Los estudios en animales en vida silvestre son escasos así como aquellos sobre asociaciones microbióticas que podrían tener gran relevancia; tal es el caso de los estudios en mucosas, piel, estadios reproductivos, ontogenia, etc. Las bacterias más representativas en microbioma conocidas en la actualidad, se refieren a Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria y Tenericutes (Sullam et al., 2015).

Los estudios en ecología de vertebrados pueden enriquecerse al incorporar el conocimiento del microbioma, y la investigación microbiológica puede cambiar fundamentalmente la forma en que se abordan las preguntas en biología evolutiva. Existe un vínculo indirecto entre la composición microbiana y las respuestas metabólicas, influenciado fuertemente por la plasticidad metabólica y la redundancia funcional de las bacterias (Moya y Serrato, 2016). El microbioma es un ejemplo de sucesión ecológica y dinámica poblacional. Los engranes de estas interacciones ecológicas aún no son claros, sin embargo, la abundancia de las poblaciones de bacterias parece determinar cambios que

pueden repercutir en la salud o estabilidad de los individuos y en procesos importantes como la especiación.

Es necesario invertir más esfuerzos en el estudio del microbioma en vertebrados en vida silvestre, no únicamente en el intestino, sino en diferentes órganos como piel, mucosas y otras partes del organismo para realizar comparaciones con lo que sucede en cautiverio. La rama de microbioma en vida silvestre puede dar luz sobre las interacciones y simbiosis que están relacionadas con la evolución de la vida misma. Por otro lado también puede ser útil en la resolución de problemas que atañen a la sociedad sobre contaminación, cambios de uso de suelo, especies invasoras, reintroducción de especies, falta de alimento, estudios de remediación o restauración ecológica, entre otros. Poder entender y discernir el mecanismo de las asociaciones simbióticas que hacen posible el microbioma en una combinación exuberante de organismos diferentes como arqueas, bacterias y virus hará posible la construcción de sistemas alternativos para sanar y mejorar varios ambientes ecológicos y la salud humana.

Para dar continuidad a los estudios de vida silvestre deben también mejorarse los métodos de colecta y plantearse objetivos precisos sobre las respuestas que se buscan, ya que al parecer la alimentación es un factor determinante en el microbioma intestinal.

Conclusiones

Este estudio aporta información valiosa para el conocimiento básico de las bacterias asociadas al microbioma fecal y cutáneo de dos poblaciones de *L. yerbabuenae.* Hemos tratado de responder preguntas de historia natural de esta especie, al evaluar su microbiota intestinal y la asociada al parche dorsal utilizando métodos moleculares (secuenciación de 16S rDNA). Las técnicas de secuenciación de alto rendimiento, junto con el análisis bioinformático, nos permitieron describir la biodiversidad del microbioma en los diferentes estadíos reproductivos de *L. yerbabuenae*, el parche dorsal de machos, así como en las dos poblaciones de esta especie conocidas para México. Los análisis actuales de microbioma se basan en estimaciones de diversidad genética y composición, junto con información encontrada en la literatura. Sin embargo, un gran desafío es que la mayoría de las secuencias encontradas en estos estudios no están asignadas, por lo que es necesario entender en qué se basan las técnicas de asociación que usan árboles filogenéticos y otras

comparaciones informáticas. Es fundamental profundizar esta información utilizando un enfoque metagenómico para inferir información funcional y taxonómica, así como las propiedades metabólicas presentes en las comunidades bacterianas asociadas con este y otros sistemas, así como sus repercusiones en el huésped y su ciclo de vida. Hay un largo viaje por delante para comprender cómo interviene el microbioma en diversos sistemas biológicos y cómo contribuirían a regular el comportamiento del huésped y el éxito reproductivo a través de la señalización química.

Por otro lado, el mayor número de estudios en microbioma se centra en humanos y con un enfoque particular en salud. Se ha demostrado que la alimentación es uno de los factores determinantes en éstos cambios de abundancia y biodiversidad en el microbioma, aunque deben considerarse los factores exógenos y endógenos de estas comunidades. Este nicho complejo está regulado por condiciones locales y ambientales.

La aproximación de los análisis que realizamos para el caso particular de *L. yerbabuenae* sugiere que el microbioma puede ser considerado de manera funcional como un órgano altamente flexible, y que la microbiota en éste caso particular es un reflejo tanto de los estadíos reproductivos como de sus poblaciones, y que tiene efecto sobre el parche sebáceo de machos, repercutiendo en la conducta y atracción sexual. Aún quedan muchas preguntas por responder, pero este estudio representa una línea base en el estudio de dos poblaciones en vida silvestre de *Leptonycteris yerbabuenae*.

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