# Microbiome of the sexual scent organ of Leptonycteris yerbabuenae

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Microorganisms are tightly bounded to the animals on Earth. Bacteria, among other types of microbes, interact with their hosts in several ways regarding metabolic pathways, development, complex behavioral processes such as mate recognition, among others. The adult males of *Leptonycteris yerbabuenae,* a nectarivorous bat, develop an interscapular odoriferous patch during the mating season. Here we present a description of the microbiota associated to this sebaceous patch 11 adult males, and studied it in terms of their taxonomical information. The variability between samples was not relevant to this study, and the most abundant phyla were Firmicutes and Proteobacteria, with dominanting classes including Gammaproteobacteria, Clostridia and Bacilli. The two most abundant species were *Aggregatibacter pneumotropica* and *Actinomyces europaeus* and other *Streptococcus minor*, *Pseudomonas stutzeri*, *P. viridiflava* and *Staphylococcus epidermis,* which are relevant in both normal and wounded human skin. Furthermore, the species present in this mating organ are involved in metabolic pathways related to fatty acid transformation to volatile molecules, which could be playing a key role in mate recognition.

1        Microbiome of the sexual scent organ of *Leptonycteris yerbabuenae*

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15 **Abstract**

16 Microorganisms are tightly bounded to the animals on Earth. Bacteria, among other types of

17 microbes, interact with their hosts in several ways regarding metabolic pathways, development,

18 complex behavioral processes such as mate recognition, among others. The adult males of

19 *Leptonycteris yerbabuenae,* a nectarivorous bat, develop an interscapular odoriferous patch

20 during the mating season. Here we present a description of the microbiota associated to this

21 sebaceous patch 11 adult males, and studied it in terms of their taxonomical information. The

22 variability between samples was not relevant to this study, and the most abundant phyla were

23 Firmicutes and Proteobacteria, with dominanting classes including Gammaproteobacteria,

24 Clostridia and Bacilli. The two most abundant species were *Aggregatibacter pneumotropica* and

25 *Actinomyces europaeus* and other *Streptococcus minor*, *Pseudomonas stutzeri*, *P. viridiflava* and

26 *Staphylococcus epidermis,* which are relevant in both normal and wounded human skin.

27 Furthermore, the species present in this 'mating organ' are involved in metabolic pathways

28 related to fatty acid transformation to volatile molecules, which could be playing a key role in

29 mate recognition.

**Introduction**

30

31 Coevolution between hosts and their microbiota is considered a process of mutual adaptations

32 that leads to specialization, playing a key-role in biological diversity (Moeller et al. 2016;

33 Brockhurst and Koskella 2013). The importance of gut and skin microbiota in the evolution of

34 mate preferences has already been proved (Markov et al. 2009; Sharon et al. 2010; Grice and

35 Segre 2011; Ley et al. 2008; Lee and Mazmanian 2010). Gut and skin microbiota alter the scent

36 of mammals (Archie and Theis 2011; Grice and Segre 2011) and could thus play a significant

37 role in kin recognition (Lizé et al. 2013). Recognition of other individuals is a crucial component

38 of social interactions, which are most often mediated via visual, olfactory or accoustical cues

39 (Bee 2006). Species recognition, selective mating and mate choice are important for maintaining

40 the diversity and stability of populations (Markov et al. 2009). Mate choice includes the ability to

41 find intraspecific sexual partners with certain genetic qualities. This process is intricate and it

42 involves both partners (Andersson 1994; Blows 2007; Kozaketal 2008). Sexual selection

43 determines differences in reproduction that are given by variation among individuals in traits that

44 affect success in completion over mates and fertilization (Andersson 1994). Honest signals and

45 sensory exploitation are included in the models of sexual selection (Muñoz-Romo et al. 2011).

46 The revealing signal theory suggests that only parasite-resistant males would be able to display

47 strong characters to attract females, including the presence of sexual dimorphic ornaments,

48 which are a reflection of health status (Muñoz-Romo et al. 2011). The odors in mammals

49 represent adaptations that allow individuals to choose an appropriate mate (Caspers et al. 2009).

50 Several mammal species including canids, felids, genets, civets and viverrids have oderous

51 secretions rich in volatile fatty acids (Ware and Gosden 1980).

52      Chiroptera are an extremely diverse order of flying mammals. Ecological pressure in a

53      group with different taxa leads to the developement of alternative mechanisms to gather and

54      process information about their environment (Bouchar 2001). Bats communicate using a

55      combination of auditory, visual, and (or) olfactory cues (Voigt et al. 2008). Bats do not usually

56      show conspicuous dimorphism in size, so they develop skin glands as differentiation

57      mechanisms (Fernandez-Vargas et al. 2008). Olfactory systems of bats are based on chemical

58      signals (glands, other odor-producing structures, urine and feces) and are used to locate and

59      distinguish food resources. Olfactory sensory systems are needed as key in the search for food,

60      shelter and parental recognition, communication and mate selection to mantain the ecological

61      balance of the species (Muñoz-Romo et al. 2012; Caspers et al. 2009, 2011; Nassar et al. 2008;

62      Bloss et al. 1999). Males and females of *Mopscondylurus* bats and the male of *Chaerephon*

63      *pumilus* have the ability to distinguish between sexes based on odours collected from the

64      interaural and muzzle glandular areas and also distinguish foreign females. *Pipistrellus*

65      *pipistrellus* is capable to discriminate between odours of a limited number of conspecifics from

66      both their own colony and from a different one (De Fanisand Jones 1995). Chemical signals are

67      an important feature for bats, specially in their dark and crouded roosting conditions where

68      thousands of individuals may live together (Safi and Dechmann 2005). Fish eating bats, *Noctilio*

69      *leporinus* (Noctilonidae) have a typical strong odor related to bacteria such as *Staphylococcus*

70      *aureus*. The males of sac-winged bats, *Saccopeteryx bilineata* (Embalonuridae) have a pouch

71      scent that contains an odoriferous liquid used to courtship (Voigt et al. 2008, Voigt et al. 2005).

72      The big brown bat *Eptesicus fuscus* (Vespertilionidae) share a more common odor signature with

73      roost-mates than with non-roost-mate conspecifics (Bloss et al. 2002).

74     *Leptonycteris yerbabuenae* is a nectarivorous bat, belonging to the Phyllostomidae

75     family, Glossophaginae subfamily. The members of genus *Leptonycteris* are pollinators of

76     *Agave* plants. Population ranges of *L. yerbabuenae* coincide with that of certain species of

77     *Agave*, including *Agave tequilana, A. angustifolia* and *A. salmiana*, from which tequila and

78     mezcal are produced, respectively (Arita 1991; Humphrey 1988). *L. yerbabuenae* latitudinal

79     migrations occur in North America from approximately 30ºN to 21º S, where a resident

80     population is established. Migrations correlate with availability of floral resources at

81     geographical and local scales (Rojas et al. 1999). Mexico harbors two reproductive populations

82     of *L. yerbabuenae* females; a spring-birth population and a winter-birth population. Most roosts

83     of *L. yerbabuenae* vary in sexual composition (Stoner 2003; Ceballos et al. 1997; Cockrum

84     1991). Pregnant females converge in maternity colonies to give birth, breast feed and care for

85     their young (Ceballos et al. 1997); females usually return to the same roost through out the years

86     in different stages of pregnancy and young-care (Hayward and Cockrum 1971). Adult males and

87     non-reproductive females often segregate into groups called "Bachelor colonies". Before

88     foraging at night, both sexes will rest in temporary night roosts. Mating and maternity roosts are

89     separated geographically (Sánchez and Medellín 2007; Stoner 2003; Fleming and Nassar 2002;

90     Cockrum 1971).

91     The adult males of *L. yerbabuenae* develop an interscapular odoriferous patch during the

92     mating season related to the size of testicles (Rincon-Vargas et al. 2013; Muñoz-Romo et al.

93     2012). The sebaceous patch is exclusive of adult males of *L. yerbabuenae*. Individuals show a

94     wound in the interescapular region that is covered with fatty and odoriferous substances (Fig. 1).

95     This sebaceous patch is cyclical and coincides with mating season. Epidermis thickness increases

96     and dermis and hypodermis decrease during sebaceous patch formation during mating period.

97   This is followed by an increase of sebaceous glands in the interescapular zone of individuals that

98   is composed of fatty acids, cholestanes and cholesterol secretions (Nassar 2008). The objective

99   of this study was to explore and describe the microbiome composition of the sebaceous patch, a

100  sexual scent organ in *L. yerbabuenae* males, from a bachelor cave in Mexico.

101

102

103  **Methodology**

104  *Study Site*

105  Bats were sampled in the cave of San Juan Noxchitlan, Oaxaca (18° 03' 00.0" W 97° 40' 00.0"

106  N and). at an altitude of ~1978 masl (Morales et al. 2007; Rojas-Martínez et al. 1999, Rzedowski

107  1978). The resident colony is comprised of ~100,000 bats. All samples were collected in June,

108  2015. This bachelor cave is located in the Tehuacan Valley characterized by an isolated arid-

109  semiarid region (~10,000 Km$^2$), which is considered the most tropical arid zone of North

110  America (Rzedowski 1978). Rainfall average is 495 mm per year, and annual mean temperature

111  of 21 ºC, with very rare frosts (García 1978). The vegetation is arid tropical scrub, with 2750

112  described plant species of which 30% are endemic (Davila et al. 1993). Forest cacti in the area

113  has high densities with ~1800 individual/ha (Valiente-Banuet et al. 1997). (Fig. 2)

114

115  *Bat Sampling*

116  This study was conducted during the mating season in June, 2015. Bats were captured (Kunz et

117  al. 2009), using 12 m, mist nets (Avinet, Dryden, New York, USA) between 18:30 hrs and 07:00

118  hrs in the time of a single night. Standard measurements were taken as follows:  length of

119  forearm was measured using a manual caliper with accuracy at 0.01 mm; body mass was

120   measured with a 100 g manual balance; age of individuals was estimated based on the

121   ossification of wing bones (metacarpals and phalanges) dividing them in the following

122   categories: young, sub-adults, and adults (Anthony 1998; Jones et al. 1996). Condition of

123   testicles (scrotal or inguinal) was recorded.

124   Interescapular patch samples (N=11) were obtained following the guidelines of the American

125   Society of Mammalogists for capture, handling and care mammals (Ganon et al. 2007; Gardner

126   1979).  Each captured bat was placed in a clean sterile plastic bag to obtain interescapular patch.

127   In order to avoid contaminations, we used different sterile plastic bags for each individual.

128   Samples of the interescapular patch were taken using gloves and sterile surgery calipers and

129   scissors, and then placed into eppendorf tubes (5.0 ml). The eleven samples were frozen until

130   processed.

131

132   *DNA extraction*

133   Metagenomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA)

134   according to manufacturer's instructions. Sebaceous patch samples were diluted with 180 μl of

135   extraction buffer ATL, and incubated with lisozyme A (30mg/ml), and proteinase K (10 mg/ml).

136   After enzymatic digestion, the manufacturer´s protocol was followed accordingly. DNA was

137   precipitated with 1 volume of chilled absolute ethanol and 0.1 volume of 3 M sodium acetate

138   then washed with 70% ethanol. Finally, DNA was eluted in 30 μl of molecular gradewater and

139   stored at -20°C until PCR amplification.

140

141   *16S rRNA gene amplification and sequencing*

142    DNA samples were PCR amplified with universal bacteria/archaeal primers 515F/806R

143    (hypervariable region V4) following the procedures reported by Caporaso et al. (2012). PCR

144    reactions (25 µl) contained 2-6 ng of total DNA, 2.5 µl Takara ExTaq PCR buffer 10X, 2 µl

145    Takara dNTP mix (2.5 mM), 0.7 µl bovine serum albumin (BSA, 20 mg ml$^{-1}$), 1 µl primers (10

146    µM), 0.125 µl Takara Ex Taq DNA Polymerase (5 U µl$^{-1}$; TaKaRa, Shiga, Japan) and nuclease

147    free-water. Samples were amplified by triplicate using a PCR protocol including an initial

148    denaturalization step at 95ºC (3 min), followed by 35 cycles of 95ºC (30 s), 52ºC (40 s) and 72ºC

149    (90 s), followed by a final extension (72ºC, 12 min). Triplicates were then pooled and purified

150    using the SPRI magnetic bead, AgencourtAMPure XP PCR purification system (Beckman

151    Coulter, Brea, CA, USA). The purified 16S rRNA fragments (~20 ng per sample) were

152    sequenced on an Illumina MiSeq platform (Yale Center for Genome Analysis, CT, USA),

153    generating ~250 bp paired end reads. The sequence data are available from the NCBI SRA

154    #XXXX.

155    *Sequence analysis*

156    Paired-end sequences were overlapped and merged with FLASH (Magoč & Salzberg 2011).

157    Nucleotide sequences were processed as previously suggested by Caporaso et al. (2010, 2012) in

158    the QIIME pipeline. Quality filtering and demultiplexing were done as suggested by Caporaso et

159    al. (2010) and Bokulich et al. (2013) (Q=19,p=0.75,r=3,n=0). Sequences were then clustered into

160    Operational Taxonomic Units (OTUs) to a 97% sequence identity with UCLUST (Edgar

161    2004,2013). Chimeras were removed using ChimeraSlayer (Haas et al. 2011) and OTUs

162    taxonomically assigned with UCLUST, using the theGreengenes database (release 13_5_8)

163 (McDonald et al. 2012). The taxonomic abundance and statistic analysis were ploted in R with

164 phyloseq (McMurdie and Holmes 2013), ggplot2 (Wickham 2009) and corrplot packages.

165 ***Results and Discusion***

166 *Sebaceous patch microbial composition*

167 Innovations in sequencing technology make possible the exploration of the complexity of the

168 vast human and animal-associated microbiota. Microbial communities are associated with

169 several processes of their host including diseases, metabolic complementation, sexual selection,

170 and many other functions that we are yet to discover. Microbial populations in the skin are

171 related with chemical signals (glands, other odor producing structure, urine and feces) and are

172 imperative for flying mammals in the individual recognition, communication and mate

173 selectiton. It has been shown that the skin microbiota plays an important role in all these social

174 matters (Balter 2012). In this case, the sebaceous patch has the function of mate selection, kin

175 recognition and communication. Moreover, as it is a wound which is mixed with feces, saliva

176 and urine in male bats (Muñoz-Romo et al. 2012), it should have different features than a normal

177 skin microbiota.

178 We calculated estimators for α and β diversities with Shannon entropy indexand Jaccard distance

179 dependent on the abundance, respectively. Shannon indexes were calculated over rarified

180 samples due to the heterogeneity of the sample size, and are represented in Figure 3. As can be

181 seen, all considered samples reached an asymptote. The Jaccard distance was then calculated

182 between each pair of samples in order to obtain a distance matrix, which is represented in Figure

183 3. Both indexes seem to agree in terms of community structure homogeneity, allowing us to join

184 all samples in one single dataset with the phyloseq library. We then identified the OTUs that

185    reached species level classification, resulting in 102 species. The 20 most abundant species were

186    plotted in Figure 4.

187    The sebaceous patch of male *L. yerbabuenae* was composed at the phylum level of Firmicutes

188    (47.9%), Proteobacteria (35.9%), Actinobacteria (3.6%), Fusobacteria (2.8%), Cyanobacteria

189    (2.4%), Bacteroidetes (0.4%), Tenericutes (0.5%) and Verrumicrobia (0.03%). (Fig. 5)

190            These results are consistent with studies in human (and other mammals) skin microbiota

191    where the most representative phyla corresponded to Actinobacteria, Firmicutes, Proteobacteria

192    and Bacteroidetes (Grice and Segre 2011). In the female´s pouch of Tasmanian devil the most

193    abundant phylum are Firmicutes (36.2%) and Proteobacteria (34.4%), Fusobacteria (9.8%),

194    Bacteroidetes (7%) and Actinobacteria (3.3%) (Cheng et al. 2015). The human sebaceous back is

195    predominantly populated by *Propionibacterium* spp. with some representation of Proteobacteria

196    and Bacteroidetes phyla, and *Staphylococcus* spp. (Grice and Segre 2011). Odorous secretions in

197    canids and felids are rich in volatile fatty acids and *Staphylococcus* spp. can also we found in the

198    fish-eating bats (*Noctilioleporinus*). *Staphylococcus* spp. creates the typical strong odor on bats

199    (Voigt et al. 2005; Ware and Gosden1980).

200            When looking at class level, Gammapreotobacterias (40.44), Clostridia (33.70), Bacilli

201    (13.72), Choroplast (2.64), Actinobacteria (0.06), Fusobacteria (2.06), Alphaproteobacteria

202    (1.84), Betaproteobacteria (0.76) and Mollicutes (0.49) were the most abundant. These classes

203    were found in the gut microbiome of Phyllostomid bats (Carrillo et al., 2015). ( Fig. 6).

204            A total of 102 microbial species were identified in the sebaceous patch of *L. yerbabuenae*

205    reproductive males. The two most abundant species in the sebaceous patch of *L. yerbabuenae*

206    were *Aggregatibacter pneumotropica* and *Actinomyces europaeus* (Fig. 6).  *A. pneumotropica* is

207    part of the human microbiota and are occasionally recovered from other body sites, including

208 blood and brain, as causes of endocartitis and abcesses (Norskov-Lauritsen and Kilian 2006). *A.*

209 *europaeus* was detected in patients with urinary infections, and was found in the 33% of the

210 patients with infections related to the skin (Sabbe et al. 1999).

211      The formation of propionic and acetic acid contributes to odor in human axillary by

212 evaporation or promoting bacterial growth. The main species that contribute to this processs are

213 part of the *Staphylococcus* genus (Fredrich et al. 2013). These bacteria are present in different

214 types of odoriferous structures on bats like fish-eating bat and sac-winged bat. In our study we

215 found five species of Staphylococcaceae: *Staphylococcus succinus*, *S. sciurus*, *S. aureus*, *S.*

216 *agalactiae* and *S. epidermis*, a commensal bacterium that modulates the host innate immune

217 response. Phenol-soluble modulins produced by *S. epidermidis* can selectively inhibit skin

218 pathogens, such as *S. aureus* and *Streptococcus anginosus*, *S. minor*, *S. agalactiae*. In this study

219 we only found one specie of the Corynebacteriaceae genus, *Corynebacterium variable*, which is

220 the twentieth most abundant species of bacteria in our results.

221      The sebaceous patch could be considered as a wound. During the mating period, male

222 scratch themselves until bleeding. *Streptococcus minor*, *Pseudomonas stutzeri*, *P. viridiflava* and

223 *Staphylococcus epidermis, S. sciurus* and *S. aureus* are all found in more than 70% of human

224 wounds where they form biofilms to help cicatrization (Schierle et al. 2009). Further, the

225 commensal-induced TLR signalling may be necessary for cell survival and repair during

226 infection. *S. aureus* and *S. epidermis* comprise two of the most common organisms found in both

227 normal cutaneous microbiota and chronic wounds (Schierle et al. 2009; Kloos 1997). *S. sciurus*

228 is considered an animal-related bacterial species and is present on mucosal and skin surfaces of

229 wild, farm animals and pets (Kloos et al. 1997). *Morganella morgani* is found in the intestinal

230 tracts of mammals (humans included) and reptiles as part of the normal microbiota and in some

231 clinical infections which involve the urinary tract or the skin soft tissue (Lin et al. 2014).

232 *Clostridium perfringens*is a frequent postmortem invader from the gut, from tissues of dead

233 animals. Furthermore, it also causes wound infections, and is involved in some necrotizing

234 processes activated by proteolytic enzymes (Niilo 1980). *Clostridium* spp. was found in feces of

235 bears in the wild and captivity, as in Phyllostomids bats (Schwab et al. 2011; Carrillo et al.

236 2015). *Serratia marcescens* is a nosocomial pathogen associated with wounds, urinary tract

237 infections, and bacterimia, and can cause diseases in some patient population like chronica

238 granulomatous disease (Oh et al. 2013).

239

240       The bacterial species found in this study have been reported in healthy and wounded

241 microbiotas. Microbiome plays a crucial role as a resilient factor, healing wounds and sending

242 signals on mating selection and individual recognition. The regulatory mechanism in which these

243 102 bacteria may act is not easy to elucidate based only in their presence or absence. However,

244 the most abundant species that are present in this sebaceous patch are related to the normal skin,

245 wounds and human axila microbiota. These bacteria are usually related to metabolic pathways

246 that transform fatty acid into volatile molecules, which could be the key players in the social

247 processes described above. The results here reported are consistent with other studies regarding

248 bats and other mammals, which have similar secondary organs as *L. yerbabuenae*'s sebaceous

249 patch. The especies that exist in the sebaceous patch of *L. yerbabuenae* represent 12% of the

250 diversity registred in *Saccopeteryx billineata* pouch organ, a secundary structure similar to the

251 sebaceus patch. The bacteria species that are similar between both hosts are: *Bacillus cereus*,

252 *Escherichia coli*, *Rothia dentocariosa*, *Serratia marscescens*, *Staphylococcus aureus* and *S.*

253 *sciuri*. Further, the alpha diversity is similar between this study and the reported by (REF), as

254 well as in the Jaccard distance matrix.

255      Functions conferred by multiple bacteria can be shared across related and unrelated

256 bacterial species (*sensu estricto* Moya and Ferrer 2016). In the gut ecosystem, there is functional

257 redundancy (Moya and Ferrer 2016); here propose the same for sebaceous patches of diferent

258 hosts.

259

260 **Conclusion**

261 The microbiome of the sebaceous patch of *Leptonycteris yerbabuenae* is composed by two

262 principal bacterial phyla: Firmicutes and Proteobacteria. The principal bacterial classes

263 corresponded to Gammapreotobacteria, Clostridia and Bacilli. The most abundant species are

264 *Aggregatibacter pneumotropica* and *Actinomyces europaeus*. Our results are consistent with

265 other studies on chemical signaling on bats. It is the first time that NGS technology has been

266 used for the study of the microbiota in the sexual scent organ. At this moment it is not easy to

267 clarify all the chemical mecanisms involved in the sebaceous patch, but the community

268 composition allows us to follow the trail of the possible metabolic functions involved in the

269 chemical communication between subjects. it is study highlights that it is imperative to consider

270 the functional similarity between bacteria (Moya and Ferrer, 2016), independently of their

271 phylogenetic relatedness.

272

273

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