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Short Communication

Report on the microbiota of *Melipona quadrifasciata* affected by a recurrent disease

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ABSTRACT

Melipona quadrifasciata is an eusocial stingless bee traditionally used for honey production in Brazil. In the last decades, the species disappeared from the wild in Southern Brazil, being kept exclusively in managed colonies for commercial and recreational purposes. Stingless beekeepers from this region report annual losses of their colonies due to a syndrome of yet unknown causes. We investigate whether it is associated to pathogenic microorganisms already known to cause disease in bees. These results provide a starting point for future studies aimed at clarifying the relationship between the microbial community of stingless bees and their colony collapses.

1. Introduction

Stingless bees (Apidae, Meliponini) are the only highly social bees other than the true honeybees. In Brazil and other parts of South America stingless bees are by far the most abundant bee species, suggesting that they have a prominent role as pollinators in this region (Giannini et al., 2015; Heard, 1999). Before the introduction of honey bees in the 19th century, colonies of stingless bees and wasps were the only sources of honey used in Brazil (Nogueira-Neto, 1997), and stingless bee culture represents an old aboriginal tradition that helps to increase agricultural production by maintaining ecological interactions (Garibaldi et al., 2016). *Melipona quadrifasciata* is one of the most popular stingless bees cultivated in Brazil (Jaffé et al., 2015), where it is called "man daçaia", which in the indigenous language means "beautiful vigilant", referring to the guard that permanently protects the nest entrance. Two *M. quadrifasciata* subspecies that show significant genetic divergence are recognized by their different abdomen coloration patterns, i.e., *M. q. anthidioides*, found in the southeast and northern Brazil, and *M. q. quadrifasciata*, which occurs in the south (Batalha-Filho et al., 2010; Tavares et al., 2013).

In the southernmost sate of Brazil, Rio Grande do Sul, which corresponds to the southern limit of *M. quadrifasciata*'s geographic distribution, wild populations disappeared since more than 50 years, and the species is now regarded as endangered (Blochtein and Marques, 2003; Fundação Zoobotânica, 2014). Furthermore, numerous beekeepers from Rio Grande do Sul have been reporting annual losses of their *M. quadrifasciata* colonies. At the end of summertime, between February and April, workers become unable to fly and crawl with their proboscis everted, leading to massive deaths that ultimately end with the colony collapse. Although such collapses happen synchronously in many different localities, they can't be connected to a common environmental factor, such as a potentially toxic flower or pesticides. Deaths occur in colonies from very dissimilar habitats.

Symbiotic bacteria are known to play an important role in bee health (Hamdi et al., 2011; Vásquez et al., 2012). Pathogens, viruses or other factors may interfere with the normal composition of bacteria associated to the bee gut epithelium (Cariveau et al., 2014;

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Cox-Foster et al., 2007) and in turn the bacterial colonization interferes with subsequent susceptibility to infections (Koch and Schmid-Hempel, 2012; Schwarz et al., 2016). The aim of the present study is to investigate whether the syndrome that annually leads to *M. quadrifasciata* colony collapses in Southern Brazil is correlated to infection with pathogenic bacteria. We describe, for the first time, the bacterial symbiont communities of this stingless bee species based on high throughput sequencing of 16S rDNA.

2. Materials and methods

2.1. Sampling and DNA extraction

M. quadrifasciata adult individuals manifesting symptoms of disease, i.e., disorientation, flight incapacity or proboscis eversion (unhealthy; n = 52) as well as without any detectable symptom (healthy; n = 24) were removed from their colonies for DNA extraction using a clean forceps. Stingless bee colonies were sampled in two summers (February/March) of 2014 and 2015 in two localities of Rio Grande do Sul, i.e., Boqueirão do Leão (30°3′9.7″S; 51°11′6.03″W) and Porto Alegre (30°4′30,3″S; 51°8′4.5″W). DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) from individual abdomens, which were separated from bee bodies in aseptic conditions using sterile scalpels.

2.2. Metabarcording of bacteria, and their possible link to disease

A segment of the bacterial 16S rDNA gene corresponding to the V1-V3 variable region was amplified from the DNA samples with modified barcoded versions of primers 27F (GAGTTTGATCNTGGCTCAG) (Lane, 1991) and 519R (GTNTTACNGCGGCKGCTG) (Turner et al., 1999) and sequenced using Illumina MiSeq technology. Reads were processed with Mothur v. 1.36.1 (Schloss et al., 2009). After filtering out low quality sequences, chimeras were removed with UCHIME (Edgar et al., 2011), as well non-bacterial sequences, based on a preliminary classification using the SILVA v123 nr database (Quast et al., 2013). Only samples containing at least 500× coverage were retained in subsequent analyses (n = 33; see Table S1). Sequences showing ⩾95% identity were clustered in Operational Taxonomic Units (OTUs). A non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities among bacterial communities was performed in PAST version 3.13 (Hammer et al., 2001). To investigate the dynamics of stingless bee microbiota we also performed a Permutational Multivariate Analysis of Variance (PERMANOVA) of Bray-Curtis pairwise distances, in which the factors "sampling year", "colony" and "health status", as well as their interactions, were tested as sources of variation in bacterial community composition. The phylogenetic affinities among principal bacterial OTUs was inferred by maximum likelihood using a dataset of known bee symbiont sequences obtained from GenBank as reference. Alignment was made with MAFTT v. 7.187 (Katoh and Standley, 2013), and phylogenetic analysis was performed with PhyML (Guindon and Gascuel, 2003) using the GTR + G + Inv model ($\alpha = 0.5$), which showed the best AIC score in ModelTest (Darriba et al., 2012). Local support values were estimated by nonparametric bootstrap based on 500 resamplings.

3. Results and discussion

The 16S rRNA amplicons from 33 *M. quadrifasciata* individuals belonging to 11 colonies (Table S1) yielded a total of 52,545 sequences (mean ± SD = 1592 ± 770 per sample) that were binned into 276 OTUs (mean ± SD = 29 ± 12 per sample). Rarefaction curves reach OTU saturation, indicating a good sampling (Fig. S1). The 32 OTUs with ⩾100 sequences (GenBank accesion numbers KX021311-KX021342) that represent 93.8% of the dataset were used for further analyses. The *M. quadrifasciata* bacterial symbiont OTUs were classified in 11 clades (Fig. 1), seven of them belonging to Firmicutes, which correspond to 73.4% of the total sampling.

Overall, the seven clades of Firmicutes and 3 OTUs of Proteobacteria belonging to the family Acetobacteriaceae are the most representative bacteria; Firmicutes Group U, Firmicutes Group Z and Acetobacteraceae correspond respectively to 23%, 23% and 16% of the total sampling. Though OTU frequencies show extensive variation across *M. quadrifasciata* individuals (Fig. 2), the factor that best explains variations in microbiota composition is the colony ($F = 2.752$; $p = 0.0006$); however no significant associations are found between year of sampling ($F = 1.112$; $p = 0.3405$) or stingless bee health status ($F = 0.854$; $p = 0.4915$) and microbiota composition. Interestingly, although main effects of sampling year and health status are not statistically significant, their interaction effect is ($F = 1.146$; $p = 0.0218$), suggesting that the effect of bee health status on microbiota composition is not the same in both years. Overall, there is a crossover effect of sampling year and health status on microbiota composition, which is possibly caused by the higher abundance of Firmicutes group U and Z bacteria in unhealthy bees on 2014, but in healthy bees of 2015 (see Fig. 2). This conclusion is also supported by NMDS, where unhealthy bees of different sampling years fall on opposite sides of coordinate 1 (Fig. S2). Therefore, we tentatively suggest that the syndrome manifested by *M. quadrifasciata* colonies may have a link to its microbiota composition, but we haven't been able to detect it with this preliminary study. Nevertheless, no pathogenic bacteria known for the honeybee, such as *Spiroplasma*, *Melissococcus* and *Paenibacillus* that cause foulbrood disease, and mostly affect honeybee larvae (Bailey and Ball, 1991), were found in our samples.

The main bacterial OTUs identified in our study are phylogenetically related to other known bee symbionts (Fig. 1). Firmicutes group U is related to *Lactobacillus kunkeii*, and belongs to a clade of common symbionts of stingless bee species such as *Tetragonula carbonaria* and *Austroplebeia australis* (Fig. S3; Leonhardt and Kaltenpoth, 2014). Firmicutes group Z clusters with the so-called Firm-5 clade of lactic acid bacteria (Martinson et al., 2011). Different species from this clade were found in *Melipona panamica* (Koch et al., 2013), *Bombus* sp. (Praet et al., 2015) and *Apis mellifera* (Olofsson et al., 2014). The Lactobacillales and the Acetobacteraceae are mostly found in the honeybee stomach and rectum as well as in its hive products (Moran, 2015). Proteobacteria that are dominant in the honeybee ileum, such as *Gilliamella* and *Frischella* (Gammaproteobacteria) or *Snodgrassella* (Betaproteobacteria) are absent in our samples, concordant with previous studies of Meliponini microbiota (Koch et al., 2013). However, a clade of bacteria belonging to the Enterobacteriaceae (Gammaproteobacteria), which are common symbionts of the honeybee ileum, appears in low frequency in our dataset (2% of total sampling), but surprisingly in only four unhealthy individuals (Fig. 2; Table S1). The 16S sequence of this bacterium clusters with another found in the gut of *Eulaema* sp. (Euglossini) from Panama (Fig. 1), and is close to *Yokenella regensburgei* (Koch et al., 2013). Firmicutes group W, which represents 15% of our sampling, is phylogenetically related to *Streptococcus*, a bacterial genus known for causing opportunistic infections in larvae affected by foulbrood disease (Bailey et al., 1973). However, its previously reported presence in *M. panamica* (Koch et al., 2013), as well as the occurrence in healthy and unhealthy *M. quadrifasciata* individuals of the present study suggests a non-pathogenic interaction of *Streptococcus* and *Melipona*. Likewise, we haven't been able to detect other well-known eukaryotic bee pathogens (*Nosema* or *Crithidia*) by PCR (data not shown).

The lack of any direct evidence leaves the question of what is killing *M. quadrifasciata* colonies in Southern Brazil still open. Bee-

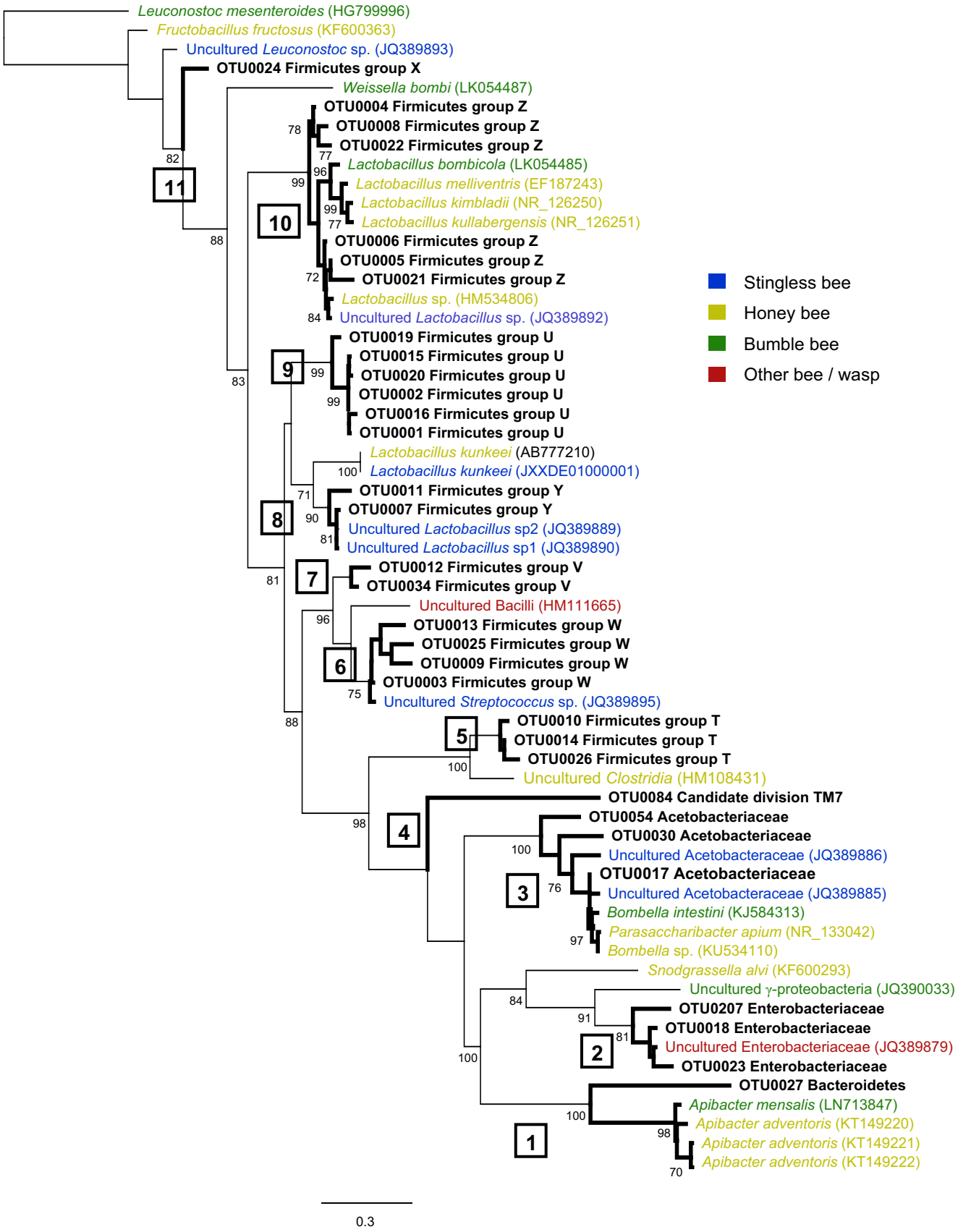

Fig. 1. Phylogenetic reconstruction based on bacterial 16S rRNA sequences. Thirty-two *Melipona quadrifasciata* symbiont OTUs belonging to 11 major clades (shown in bold) are compared to sequences from bacteria previously characterized for other bees, identified by their respective accession numbers. Bootstrap support values larger than 70% are shown at the respective branch nodes.

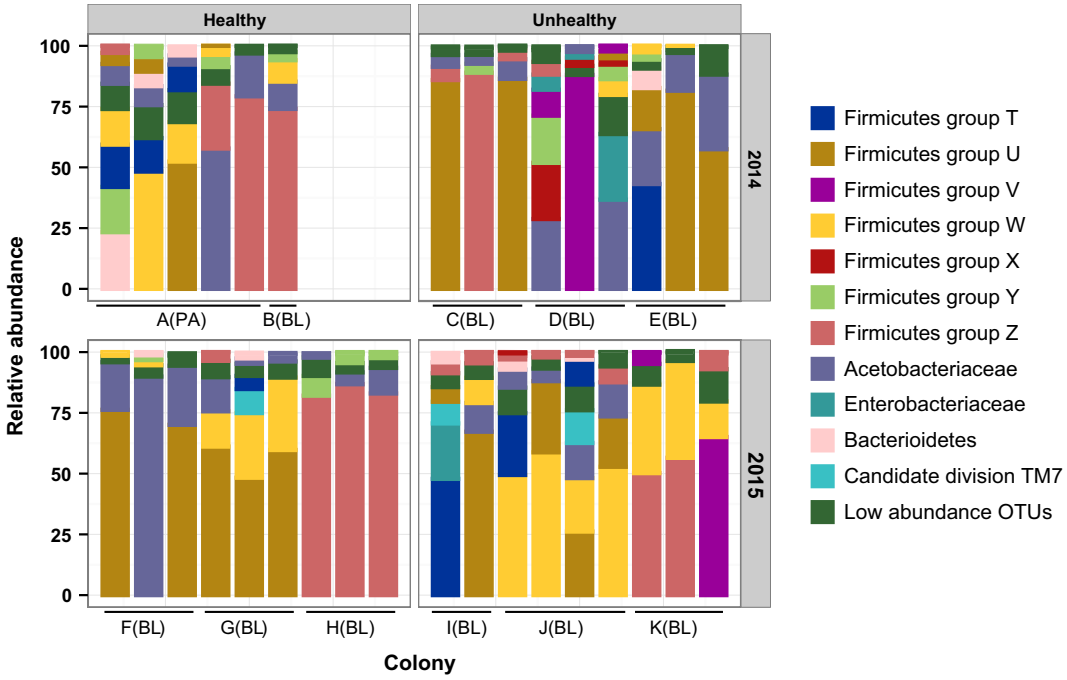

Fig. 2. Relative abundance of the 11 most representative bacterial taxa (clades) of healthy and unhealthy *Melipona quadrifasciata* sampled in two consecutive years. Letters indicate individuals from the same colony; PA and BL refer to the sampling localities, Porto Alegre and Boqueirão do Leão, respectively.

keepers learned that by preventing bees from leaving the colony, or by moving the colony to another locality, it is often possible to avoid the collapse, which makes them suspect that a toxic plant is poisoning their bees, but we observed that the pollen types in the crop of both healthy and unhealthy adults are indistinguishable (data not shown). *M. quadrifasciata* adults seem to forage mostly on *Eucalyptus* spp. – an observation corroborated by other studies on *Melipona* feeding habits in Southern Brazil (Hilgert-Moreira et al., 2014) – and on native Asteraceae, such as *Vernonanthura tweediana*, that bloom in the region in this time of the year. We think that, as already suggested for colony collapses of honeybees (Goulson et al., 2015; Nazzi et al., 2012; VanEngelsdorp et al., 2010), a synergistic effect of multiple factors, such as environmental stresses caused by climatic change, intensive management and the use of pesticides, as well as biological factors that haven't been assessed in the present study, such as viruses or other pathogens, may be at the heart of the problem.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2016.11.012.

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