Evolutionary biology

Honeybee microbiome is stabilized in the presence of propolis

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Honeybees have developed many unique mechanisms to help ensure the proper maintenance of homeostasis within the hive. One method includes the collection of chemically complex plant resins combined with wax to form propolis, which is deposited throughout the hive. Propolis is believed to play a significant role in reducing disease load in the colony due to its antimicrobial and antiseptic properties. However, little is known about how propolis may interact with bee-associated microbial symbionts, and if propolis alters microbial community structure. In this study, we found that propolis appears to maintain a stable microbial community composition and reduce the overall taxonomic diversity of the honeybee microbiome. Several key members of the gut microbiota were significantly altered in the absence of propolis, suggesting that it may play an important role in maintaining favourable abundance and composition of gut symbionts. Overall, these findings suggest that propolis may help to maintain honeybee colony microbial health by limiting changes to the overall microbial community.

1. Introduction

Honeybees (*Apis mellifera*) as eusocial, cavity-nesting organisms have evolved many mechanisms to maintain a homeostatic nest environment. For proper development of larvae and pupae, temperature and humidity need to remain relatively constant. In addition, the nest architecture must support comb attachment, be waterproof and restrain detritus. Finally, it is critical that microbial growth be controlled in such an environment. Honeybees collect and deposit plant resins on the hive walls to help maintain optimal nest conditions and use these resins to restrict nest entrances to reduce predation and parasitism [1]. As resin is brought into the hive, it is mixed with varying amounts of bee-produced wax and is then termed 'propolis' [1,2].

Propolis not only has an architectural purpose but it also functions as a type of social immune defence—a colony-level defense mechanism against pathogens and parasites that arises due to the collective behavior of individuals [3–6]. Resins are collected as a type of social medication with foragers increasing resin collection when the colony is pathogen challenged [7–9]. In addition, the presence of a propolis envelope in pathogen-challenged colonies appears to increase the antimicrobial activity of the glandular secretions that workers feed to developing larvae [10]. Furthermore, bees from healthy colonies with more propolis in the nest interior have decreased investment in their immune response, which may lead to increased lifespan [11]. As reviewed in Simone-Finstrom *et al.* [5], propolis has both direct effects against pathogens and more subtle effects on individual bees that may translate to reduced disease at the colony and individual levels. However, the specific mechanisms explaining these effects are yet to be elucidated.

Plant exudates incorporated into propolis are rich in secondary metabolites that are not involved in primary plant biochemical processes such as growth

and development, but are important for mediating interactions with other organisms (e.g. insects, microbes) [12]. The most well-characterized plant secondary metabolites are essential oils (EOs), which are complex mixtures of volatile aromatic compounds. Natural EO mixtures have a broad range of anti-septic and antimicrobial activities due to the fact that the differing components often exhibit multiple modes of action [12]. EOs, thus, exert pressure on animal microbiota to tolerate, use, or detoxify secondary plant compounds that are encountered in the environment [13,14]. EOs have been exper-imentally shown to increase host weight gain and improve resistance to infection in some animals via microbiota alterations that impact community structure and function [15].

The honeybee gut and hive environments are colonized by distinct microbial communities that impact individual and colony-level health. Therefore, the collective honeybee gut micro-biota and hive microbiota can be considered an extension of the colony phenotype [16–18]. The gut harbours a core community that includes ubiquitous animal gut bacteria (i.e. *Lactobacillus* and *Bifidobacterium*) as well as specialized clades that are shared with other corbiculate bees (i.e. *Snodgrasella* and *Gillia-mella*) [17–19]. Gut bacteria are present at low levels in hive materials such as food stores and comb, but core hive bacteria (i.e. *Bombella apis* (formerly *Parasacharribacter apium* [20]) and *L. kunkeii*) can survive the extreme conditions of the hive and there-fore are present at significantly higher concentrations [21]. Both gut and hive bacteria are adapted to survive bee social immune defenses such as glucose oxidase production and innate immune functions such as antimicrobial peptides [17,22]. Even though honeybees do not consume propolis, we hypothesized that the honeybee microbiota has co-evolved to thrive in the pres-ence of propolis. In this way, we postulate that the role of propolis as a social immune defense may extend to influencing microbial homeostasis in the hive and collective gut of the colony.

Since it has previously been documented that a propolis-enriched environment influences total bacterial loads of honeybee colonies [6], the goal of this study was to explore how propolis may specifically influence microbial community structure in honeybees. As our knowledge of the importance of the honeybee microbiota to bee health has increased rapidly over the last several years, investigations regarding how the hive environment influences honeybee microbiota have increased significance.

2. Methods

(a) Environmental parameters and study design

Experimental design was described previously by Borba *et al.* [23]. Briefly, 12 colonies were provided with commercially available propolis traps (Mann Lake Ltd, MN, USA) stapled to the four inner walls of each bee box to encourage the bees to construct a propolis envelope within the nest. This treatment resulted in pro-polis-rich colonies. Twelve additional colonies served as controls; no propolis trap was provided and the bees deposited propolis in the cracks and crevices within the box and were left with smooth interior walls, and therefore were propolis-poor. Exper-imental measures quantifying population and brood size, *Varroa* infestation, *Nosema* spp., bacterial load and viral titre can be found in Borba *et al.* 2015 from the 2012 September cohort [23]. There were no differences in colony size, parasite or pathogen load, or colony bacterial load (as assessed by 16S gene expression) in the colonies at the time of sampling for the current study. How-ever, immune-gene expression was different between the two colony treatments [23], consistent with previous work [6].

Newly emerged bees (noted by their location near eclosing adults from pupal cells, and by their fuzzy appearance [24]) were painted using enamel paint markers and collected after six days [25]. The marked 7-day-old bees were stored at −80°C until analysis. Seven-day-old bees were chosen for analysis as differ-ences in total bacterial loads in bees collected from propolis-rich or propolis-poor colonies were previously observed at this time point [6]. This is also after the age at which the characteristic gut bacterial communities are established [26].

(b) Sample processing and DNA extraction

An average of five to six whole bees from six propolis-rich and six propolis-poor colonies (total $N = 62$ samples) were processed indi-vidually. DNA extraction was carried out following established methods [27,28]. Briefly, whole bees were flash-frozen in liquid nitrogen and ground using sterilized pestle into a fine powder. Following grinding, Tris–EDTA and lysozyme (at 20 mg ml^{-1}) were added and the sample was incubated at 37°C for 30 min. After incubation, proteinase K (at 50 mg ml^{-1}) was added and the sample incubated at 50°C overnight. Phenol–chloroform extrac-tion was performed twice before ethanol precipitation and DNAs were re-suspended in Tris–EDTA and cleaned using a column-based genomic clean-up kit (Zymo) according to manufacture instructions. DNAs were quantified using a NanoDrop2000 instru-ment (Thermo Scientific Inc., Grand Island, NY, USA). PCR was performed using barcoded Illumina primers following the Earth Microbiome protocols [22,23], with HF Phusion polymerase mix (New England BioLabs, Ipswich, MA, USA) and 3% dimethylsulf-oxide (DMSO). Amplifications were performed in triplicate and pooled before normalization based on PicoGreen quantification.

(c) 16S rRNA gene amplicons community analysis and data filtering

PCR and sequencing were performed using a modified version of the protocol presented in Caporaso *et al.*, adapted for the Illumina MiSeq 300 bp paired-end sequencing. The V4 region of the 16S rRNA gene was amplified with region-specific primers that included the Illumina flowcell adapter sequences [27]. Samples were multiplexed and then sequenced on a single flowcell. The software Quantitative Insights Into Microbial Ecology (QIIME 2), an open-source microbiome data science platform, was used for data preprocessing and sequence analysis [29]. Raw sequence data files were de-multiplexed and low-quality reads were removed using the default parameters for QIIME2 for paired-end data. Chimeric sequences were corrected using the DADA2 plugin [30]. Alpha and beta diversity analyses were done using the q2-diversity plugin and to generate the principal coordinates analysis with a minimum sampling depth of 15 000 reads and con-cordance of features shared by at least two samples. Taxonomic analysis was done with the q2-feature-classifier plugin to map sequence data to taxonomic features. Classifiers were trained directly from the sample data with a 99% similarity to the Silvia 16S sequence database [31]. Representative features were then sub-ject to BLASTn query using the NCBI database to assign specific isolates. QIIME2's diversity plugin was used to perform permuta-tional multivariate ANOVA (PERMANOVA) and a test for homogeneity of multivariate dispersions (PERMDISP) to deter-mine statistical differences in group clustering and dispersion. Identification of differentially abundant features across samples was done using the statistical framework Analysis of Composition of Microbes (ANCOM) and Gneiss [32,33].

3. Results

(a) Bacterial sequences and classification

We obtained 3 103 363 reads sequenced from the 16S rRNA V4 region from 62 samples. Quality filtering reduced the total
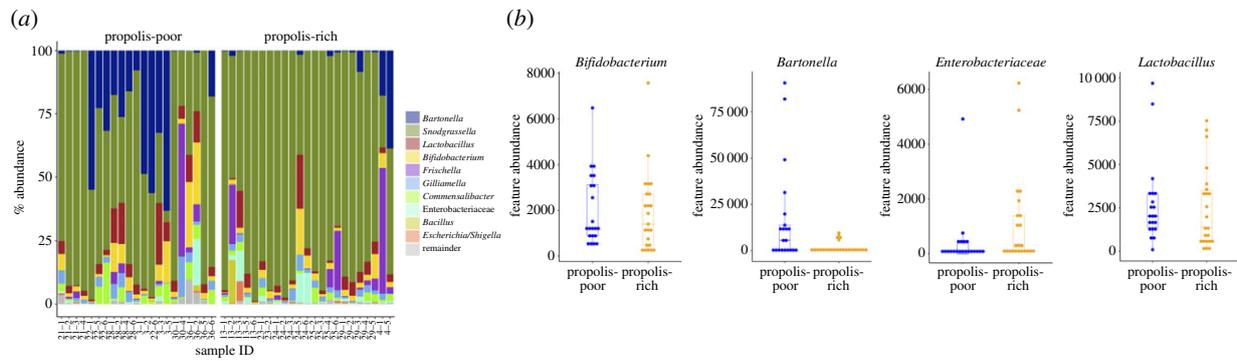
Figure 1. (*a*) Taxonomic composition of the microbial communities of bees from propolis-rich and propolis-poor colonies; (*b*) comparison of the number of times each taxa was observed by a given read (feature abundance) of four significantly differentiated taxa by ANCOM analysis.
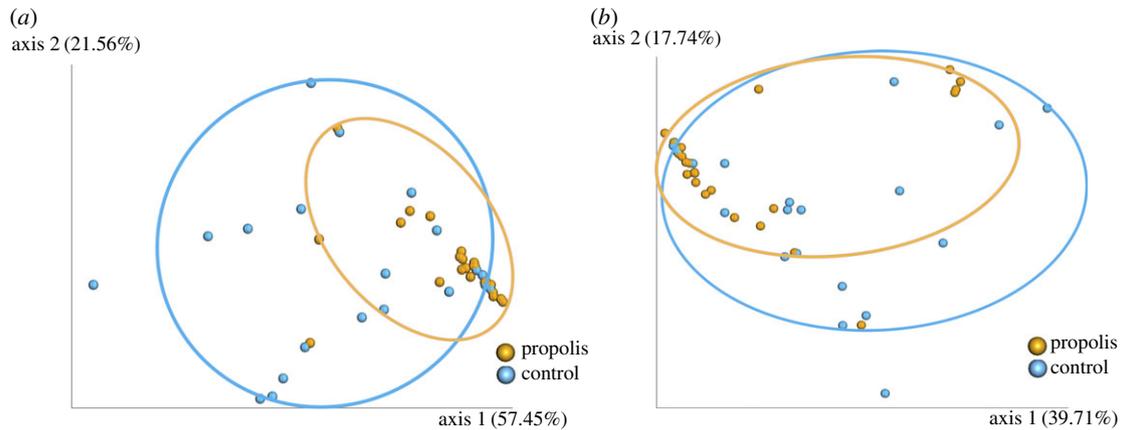

Figure 2. (*a*) Principal coordinates analysis (PCoA) using Bray–Curtis and (*b*) weighted UniFrac measures of dissimilarity showing greater levels of similarity (clustering) among propolis-rich samples as compared to samples from propolis-poor colonies along with the 95% confidence interval indicated by the respective circles. Significance determined through PERMANOVA: Bray–Curtis: $p = 0.027$, weighted UniFrac: $p = 0.002$.

number of samples to 44 (23 propolis-rich, 21 propolis-poor), with a sequences per sample range from 15 208 to 165 115 (median = 39 448). Sequences clustered in a total of 415 unique taxonomic features. Sampling depth was sufficient to capture similar levels of feature diversity for both treatment groups (electronic supplementary material, figure S1).

(b) Taxa identification

Sequenced reads were resolved down to the family or genus level to maintain confidence in taxonomic designation. The main taxa present across both groups are presented in figure 1*a* and include taxa such as *Snodgrassella*, *Bartonella*, *Lactobacillius*, *Bifidobacterium*, *Frischella* and *Gilliamella* (figure 1*a*). These taxa are considered part of the dominant bacteria of the honeybee gut [26,28,34–36].

(c) Microbial community differences

PERMANOVA and PERMDISP identified significant differences in microbial communities between bees from propolis-rich and propolis-poor colonies using two different dissimilarity measures (PERMANOVA: Bray–Curtis: $p = 0.027$, $r^2 = 0.06$, weighted UniFrac: $p = 0.002$, $r^2 = 0.15$; PERMDISP: Bray–Curtis: $p = 0.045$, weighted UniFrac: $p = 0.004$). All alpha diversity and unweighted UniFrac distances between the two sample groups were not significant. Principal coordinate analysis (PCoA) plots for Bray–Curtis and weighted UniFrac shows microbial community diversity with significant clustering of the propolis-rich group compared to propolis-poor (figure 2*a,b*).

(d) Taxa differences

Identification of taxa that were differentiated between bees reared in the propolis-rich versus propolis-poor environments was done using the ANCOM and Gneiss methods. Two taxa (*Bartonella* and *Lactobacillus*) were identified as significantly differentiated by both methods. *Bartonella* and *Lactobacillus* were both more abundant in the propolis-poor group as compared to the propolis-rich group. ANCOM analysis found a total of eight taxa that were significantly differentiated between the two treatments (*Bartonella*, *Lactobacillus*, *Bifidobacterium*, Enterobacteriaceae, *Bombella*, Corynebacteriales, *Methylobacterium* and *Dietzia*) (figure 1*b*). However *Bombella*, Corynebacteriales, *Dietzia* and *Methylobacterium* had extremely low abundance and differences could be artefacts of sampling (electronic supplementary material, figure S2). *Bartonella* and *Lactobacillus* were more abundant in bees from propolis-poor colonies, while *Bifidobacterium* and Enterobacteriaceae were more abundant in bees from propolis-rich colonies (figure 1*b*). The Gneiss method identified three significantly differentiated taxa (*Bartonella*, *Lactobacillus*, *Snodgrassella* (electronic supplementary material, figure S3)). *Bartonella* and *Lactobacillus* were more abundant in the propolis-poor treatment (as also indicated by ANCOM analysis), while *Snodgrassella* was enriched in the propolis-rich treatment.

4. Discussion

We examined the microbial community composition of honeybees in propolis-rich and propolis-poor environments. As bees

collected from propolis enriched environments have previously been shown to harbour fewer bacteria (based on 16S rRNA abundance [6] but see also [23]), our aim here was to determine the effect of propolis on specific taxa of the honeybee microbiota. The honeybee microbiota from propolis-rich colonies were more similar to each other in taxonomic composition, compared to propolis-poor colonies. We found that the honeybee microbiota was more consistent between bees collected from propolis-rich colonies, while those from propolis-poor colonies exhibited greater taxonomic diversity. Several bacterial groups were also found to have different relative abundances with respect to the amount of propolis in the colonies. The results presented herein suggest that propolis may support regulation of colony microbiota by maintaining a stable or homeostatic microbial community.

Bray–Crutis and weighted UniFrac measures of dissimilarity found significant differences in the overall taxonomic microbial diversity between bees from propolis-rich and propolis-poor colonies. Significance in the PERMANOVA tests demonstrates that there were overall localized differences between the two groups across the two diversity measures. PERMDISP provides additional evidence that the two groups were also significantly different in terms of the variance distribution that exists within propolis-rich and propolis-poor colonies. The latter of the two tests provides subsequent evidence that the microbial population variation is limited in the propolis-rich colonies and thus more stable across colonies as compared to the propolis-poor colonies. Given that honeybee-associated microbes have likely co-evolved with their hosts in the presence of propolis, as propolis use is ubiquitous in feral colonies nesting in tree cavities [1], some microbial symbionts may be more or less sensitive to its chemical properties. While this has yet to be explored, these results raise the question of whether specific ratios of particular taxa are particularly important for bee health or if the stability of representation of specific taxa is key to preventing dysbiosis [19]. While it is likely a combination of the two, variation in community composition and diversity can dramatically affect the overall health of the host [37]. Four of the eight significantly differentiated taxa we identified in this study are considered to be core members of the honeybee gut microbiome (*Lactobacillus*, *Bifidobacterium*, *Bartonella* and *Snodgrassella*) [34]. *Lactobacillus* and *Bifidobacteriaceae* both belong to taxonomic groups that are implicated in carbohydrate transport and polysaccharide processing [38]. *Lactobacillus* were primarily comprised of the 'Firm-5' phylotype, and understanding how its differential abundance with respect to propolis or other environmental conditions could impact colony health is important for future work. Bifidobacteriaceae were more abundant in the propolis group and have been previously shown to be important to maintaining colony health [17,26,35,37,39]. *Bartonella* is a gut symbiont [40] that has been shown to differ seasonally [41] and in honey bees exposed to various types of landscapes [34], raising the question of its functional roles and how propolis may interact with it.

Other significant taxa identified in the study, such as Enterobacteriaceae, are facultative anaerobes that are suggested to be involved in metabolic processes such as including sugar and nitrogen processing [41]. Therefore, alterations in the abundances of this family of microbes may have an impact on host metabolism. *Snodgrassella* was the most abundant taxa identified in our study. *Snodgrassella* is a common honeybee symbiont which modulates the gut environment by consuming O_2 to maintain anaerobic conditions in the gut lumen [19,42]. The significant abundance of this bacterium in the bee gut suggests that it may play a significant role in maintaining the overall homeostasis of the gut microflora. Although we were unable to detect strain-level differences within *Snodgrassella*, it may be interesting to explore how the specific strains regulate gut community dynamics and host physiology. Further study is also necessary to better characterize the specific effects of microbiota shifts and how they are related to functional changes in host physiology and health (see electronic supplemental material, PICRUSt analysis), particularly in this context of strain diversity.

5. Conclusion

In total, our results demonstrate that honeybees in a propolis-rich environment differ in their relative abundances of core microbial community members. These findings provide interesting and novel insight into how a feature of the nest environment can influence the community structure of co-evolved bee-associated microbes, and suggest an additional mechanism by which propolis may contribute to overall colony health. Future work should address if disruption of gut microbiome homeostasis influences the establishment of pathogens or growth of opportunistic species and if this is mediated by differential responses to the antimicrobial activity of propolis or indirectly through the bees' physiological responses to propolis in the hive environment. Additionally, we have identified candidate taxa for future functional investigations that may help to further understand complex microbial dynamics involved with regulating the health of honeybee colonies.

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