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RELAÇÃO DA NUTRIÇÃO APÍCOLA COM A MICROBIOTA DO PÓLEN E DO SISTEMA DIGESTÓRIO DE ABELHAS MELÍFERAS VERIFICADA POR SEQUENCIAMENTO DE NOVA GERAÇÃO

São Gabriel, RS, Brasil
2015

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Dissertação apresentada ao Programa de Pós-graduação *Stricto Sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Dr. Luiz Fernando Wurdig Roesch

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RESUMO

A microbiota e os genes funcionais ativamente envolvidos no processo de decomposição e utilização de grãos de pólen em pão de mel e no trato digestório de abelha ainda não são completamente compreendidos. O objetivo deste trabalho foi avaliar a estrutura e diversidade da comunidade de bactérias e Archaeas em amostrasde pão de mel e sistema digestório de abelhas africanizadas, bem como para prever os genes envolvido na bioprocessamento microbiano do pólen, usando a tecnologia de seqüenciamento de nova geração. Um total de 11 filos bacterianos foram encontrados dentro do sistema de digestório de abelhas e 10 filos bacterianos foram encontrado dentro pão de mel. Embora a comparação a nível de filo mostre mais filos em comum, a análise filogenética mais profunda mostrou maior variação de composição taxonômica. A família Enterobacteriaceae, Ricketsiaceae, Spiroplasmataceae e Bacillaceae, foram os principais grupos responsáveis por a especificidade do intestino de abelhas, enquanto as principais famílias responsáveis pela especificidade do pão de mel foram Neisseriaceae, Flavobacteriaceae, Acetobacteraceae e Lactobacillaceae. Em termos da estrutura da comunidade microbiana, a análise mostrou que as comunidades dos dois ambientes foram bastante diferentes umas das outras, com apenas 7% dos táxons a nível de espécies compartilhados entre o sitema digestório de abelhas e o pão de mel. Os resultados indicaram a presença de um elevado nível de especialização e uma microbiota intestinal bem adaptada dentro de cada abelha e do pão de mel.A comunidade associada ao pão de mel, apresentou maior abundância relativa de genes relacionados com a degradação de aminoácidos, carboidratos, e o metabolismo lipídico, sugerindo que biodegradação do pólen ocorre predominantemente pela microbiota associada ao pão de mel. Estes resultados sugerem uma complexa e importante relação entre nutrição de abelhas e suas comunidades microbianas.

Palavras-chave: Post-Light™ Ion *Sequenciador por Semiconductor*; Nutrição apícola; Xenobióticos; Predição Funcional

ABSTRACT

The microbiota and the functional genes actively involved in the process of breakdown and utilization of pollen grains in beebread and beeguts are not yet understood. The aim of this work was to assess the diversity and community structure of bacteria and archaea in Africanized honeybee guts and beebread as well as to predict the genes involved in the microbial bioprocessing of pollen using state of the art 'post-light' based sequencing technology. A total of 11 bacterial phyla were found within bee guts and 10 bacterial phyla were found within beebread. Although the phylum level comparison shows most phyla in common, a deeper phylogenetic analysis showed greater variation of taxonomic composition. The families Enterobacteriaceae, Ricketsiaceae, Spiroplasmataceae and Bacillaceae, were the main groups responsible for the specificity of the bee gut while the main families responsible for the specificity of the beebread were Neisseriaceae, Flavobacteriaceae, Acetobacteraceae and Lactobacillaceae. In terms of microbial community structure, the analysis showed that the communities from the two environments were quite different from each other with only 7 % of species-level taxa shared between beegut and beebread. The results indicated the presence of a highly specialized and well-adapted microbiota within each bee gut and beebread. The beebread community included a greater relative abundance of genes related to amino acid, carbohydrate, and lipid metabolism, suggesting that pollen biodegradation predominantly occurs in the beebread. These results suggests a complex and important relationship between honeybee nutrition and their microbial communities.

Keywords: Post-Light™ Ion Semiconductor Sequencing; Honeybee Nutrition; Xenobiotics; Functional Prediction

SUMÁRIO

INTRODUÇÃO

As abelhas (*Apis mellifera*) desenvolvem um papel significativo para a lucratividade e produtividade agrícola através da polinização de diversos cultivares que tem a sua produção elevada, visto que a polinização aumenta as chances de ocorrer a formação de frutos. Estima-se que 70% dos cultivares agrícolas dependem destes insetos para completar o seu desenvolvimento e metade desta produção desapareceria sem a polinização realizada pelas abelhas (KLEIN *et al.*, 2006). Isto afetaria a economia, com conseqüente escassez de alguns alimentos, aumento dos custos para a população e a modificação da dieta humana como se conhece hoje (McGREGOR 1976, O' TOOLE C 1993).

As abelhas presentes no Brasil e analisadas neste estudo são uma variedade africanizada do gênero *Apis* que foi trazida para o Brasil na década de 1950 e cruzou com outras subespécies de abelhas europeias também introduzidas no Brasil no século XIX. Tais cruzamentos originaram abelhas hibridas, com características predominantes africanizadas, como a capacidade de enxamear rapidamente, adaptação ao clima local e expansão do enxame em curto período de tempo (OLIVEIRA *et al.*, 2005). De acordo com as características destes insetos, sua capacidade rápida de enxamear exige um grande aporte energético. Tal aporte é obtido pelo consumo de mel e de pólen extraído de plantas silvestres e muitas vezes de cultivares agrícolas.

Os grãos de pólen são fonte de carboidratos, proteínas e aminoácidos utilizados como fonte de nutrição principal para estes insetos. Os grãos de pólen coletados durante os meses de floração são armazenados dentro de pequenas cavidades, construídas com cera pelas abelhas nos favos, denominados alvéolos. Os alvéolos não são apenas um local de armazenagem dos grãos, mas também um ambiente que apresenta condições favoráveis a sua biotransformação em pão de mel, que ocorre devido a ação de enzimas presentes na saliva das abelhas que são depositadas sobre os alvéolos, selando sua cavidade e alterando o pH do meio (GILLIAM, 1979). As enzimas presentes na saliva das abelhas, como invertases, diastases e glicose oxidase são componentes essenciais para a quebra da parede celular de cada grão de pólen (BRODSCHNEIDER & CRAILSHEIM, 2010).

Após este período de armazenagem em que ocorre a quebra das proteínas, o pólen torna-se pão de mel que apresenta quantidades de aminoácidos ideais para a nutrição das abelhas BRODSCHNEIDER & CRAILSHEIM, 2010). Durante o tempo de armazenagem esta mistura

de grãos de pólen e saliva fica exposta ao contato dos insetos dentro da colmeia (BRODSCHNEIDER & CRAILSHEIM, 2010). Devido ao hábito forrageiro dos mesmos e a intensa atividade de trabalho dentro da colmeia, muitos micro-organismos são levados para o interior da colônia, seja através dos produtos coletados ou agregados a superfície corporal do inseto. Estes micro-organismos também utilizam os produtos estocados pelas abelhas como fonte energética (GILLIAM *et al.,* 1989). A atividade de degradação torna o pólen armazenado um composto mais fácil de ser assimilado. Sua composição química com carboidratos menores é mais simples podendo ser imediatamente consumido, tanto pelas abelhas quanto pela comunidade microbiana que se estabelece junto ao pão de mel, utilizando-o como alimento e auxiliando a quebra (GILLIAM, 1997).

O estudo de comunidades microbianas associadas a animais relatou diversas interações mutualísticas estabelecidas entre o hospedeiro e o simbionte, sendo a principal representada pela degradação e assimilação de nutrientes para a alimentação (MARTINSON *et al.,* 2011, OLOFSSON & VASQUEZ, 2008, VASQUEZ *et al.,* 2009). A presença e estrutura das comunidades microbianas observadas neste estudo seria responsável pelo bom funcionamento entre a dieta a base de pão de mel (pólen processado) e a formação de uma comunidade microbiana que habita o interior do trato digestório destes insetos, contribuindo para um melhor estado de saúde (OLOFSSON & VASQUEZ, 2008).

Devido a ampla função que cada microrganismo pode desenvolver, uma comunidade microbiana formada por diversas espécies é importante para manter o inseto saudável (BRODSCHNEIDER & CRAILSHEIM, 2010). A presença de uma comunidade microbiana com ampla diversidade de espécies torna o trato digestório do inseto mais capacitado para realizar a degradação dos mais diversos compostos oriundos de sua dieta. Por outro lado, um dos maiores desafios para a microbiologia tem sido estimar a diversidade microbiana de forma adequada (BUNGE *et al.*, 2014).

Devido a características morfológicas e fisiológicas dos micro-organismos, há necessidade da utilização de análises indiretas, envolvendo o cultivo *in vitro* ou o sequenciamento de genes marcadores, para a condução de inventários microbianos nos ecossistemas. Atualmente existe um consenso entre ecologistas microbianos de que a chave para predizer a diversidade microbiana é evitar o isolamento, focalizando os estudos no DNA

microbiano (CURTIS & SLOAN, 2005). Tal consenso emerge do conceito de que sequências de DNA podem ser usadas na identificação de micro-organismos.

Neste trabalho, a hipótese norteadora foi a existência de uma microbiota exclusiva, encontrada no pólen armazenado pelas abelhas, responsável pela biodegradação deste até pão de mel. Esta comunidade microbiana seria diferente daquela encontrada no sistema digestório das abelhas o que reforça a necessidade do armazenamento do pólen e a incapacidade das abelhas em digerir pólen não processado usando recursos próprios ou através da microbiota do sistema digestório.

1.2 OBJETIVO

O objetivo deste estudo foi estimar a diversidade e a abundância das comunidades microbianas presentes nas amostras de pólen e sistema digestório de abelhas melíferas através da amplificação do gene 16S rRNA, as informações obtidas com o processamento dos dados do sequenciamento foram também utilizados para construir um perfil funcional para estas comunidades, buscando identificar suas funções nos ambientes.

1.3 APRESENTAÇÃO DO MANUSCRITO

O presente manuscrito está apresentado na forma de artigo científico, disponível online na revista Antonie van Leeuwenhoek (ISSN 1572-9699). O trabalho foi submetido a revisão no dia 30 de outubro de 2014 e foi aceito para publicação no dia 12 de janeiro de 2015 (DOI 10.1007/s10482-015-0384-8).

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ORIGINAL PAPER

Relationship between honeybee nutrition and their microbial communities

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Abstract The microbiota and the functional genes actively involved in the process of breakdown and utilization of pollen grains in beebread and bee guts are not yet understood. The aim of this work was to assess the diversity and community structure of bacteria and archaea in Africanized honeybee guts and beebread as well as to predict the genes involved in the microbial bioprocessing of pollen using state of the art 'post-light' based sequencing technology. A total of 11 bacterial phyla were found within bee guts and 10 bacterial phyla were found within beebread. Although the phylum level comparison shows most phyla in common, a deeper phylogenetic analysis showed greater variation of taxonomic composition. The families Enterobacteriaceae, Ricketsiaceae, Spiroplasmataceae and Bacillaceae, were the main groups responsible for the specificity of the bee gut while the main families responsible for the specificity of the beebread were Neisseriaceae, Flavobacteriaceae, Acetobacteraceae and Lactobacillaceae. In terms of microbial community structure, the analysis showed that the communities from the two environments were quite different from each other with only 7 % of species-level taxa shared between bee gut and beebread. The results indicated the presence of a highly specialized and well-adapted microbiota within each bee gut and beebread. The beebread community included a greater relative abundance of genes related to amino acid, carbohydrate, and lipid metabolism, suggesting that pollen biodegradation predominantly occurs in the beebread. These results suggests a complex and important relationship between honeybee nutrition and their microbial communities.

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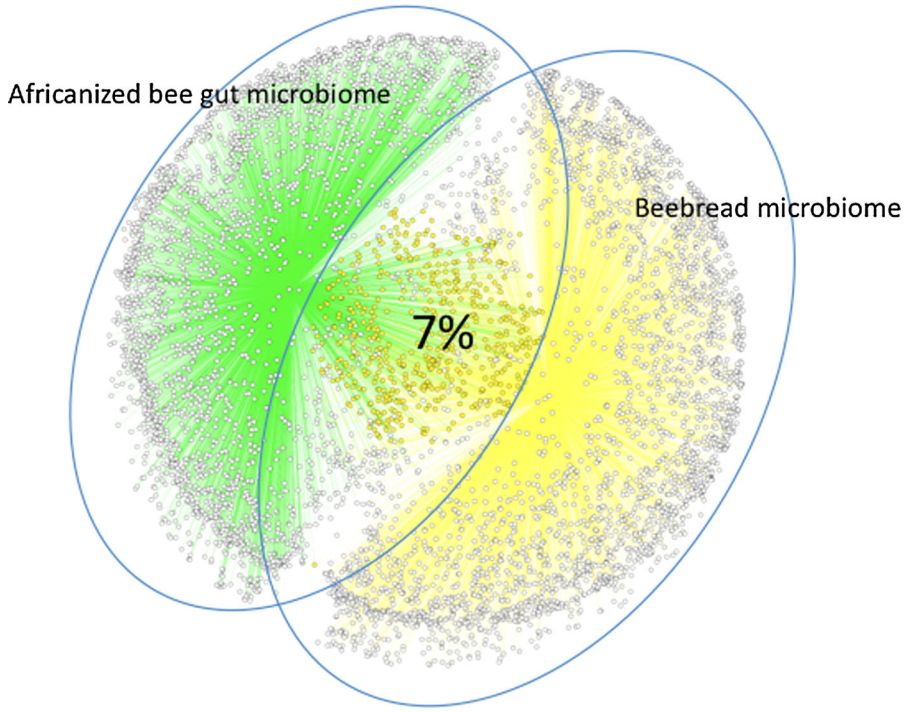
Graphical abstract



Keywords Post-Light^{TM} ion semiconductor sequencing · Microbial functional prediction · Microbial phylogeny · Africanized honeybee · *Apis mellifera*, 16S rRNA

Introduction

Honeybees are considered the most important group of plant pollinators in many ecosystems (Bawa et al. 1985; Bawa 1990; Shipp et al. 1994; Heard 1999). They contribute significantly to agricultural productivity and profitability.

The microbial gut community of *Apis mellifera* might be involved in nutritional processes, such as breakdown and utilization of pollen grains as well as degradation of toxic compounds found in the environment (Engel et al. 2012). These gut bacteria may also provide defense responses against pathogens by producing inhibitory compounds or by monopolizing nutrients within the gut (Forsgren et al. 2010; Koch and Schmid-Hempel 2011; Engel et al. 2012). Although many functions of the bee microbiota have been discovered, adult honeybees and bumblebees harbor a specialized but surprisingly species-poor community of bacteria in their guts (Koch and Schmid-Hempel 2011; Martinson et al. 2011).

The best food sources for bee colonies are nectar and pollen, and various insects and vertebrates are able to use pollen as food source (Roulston and Cane 2000). However, pollen is difficult to digest and honeybees are unable to consume fresh unprocessed pollen. The potential microbial involvement in pollen breakdown is especially important for supplemental feeding of honeybee colonies during non-foraging periods. Many artificial high-protein diets containing no pollen but rather protein from soybean are currently commercialized. Although the soybean contains all essential amino acids required by the bee's diet, the lack of a microbial pre-fermentation might limit the total use of this protein source. This observation might also explain why fermented diets are consumed more readily than unfermented ones (Ellis et al. 2006). Although pollen is nutrient-rich, the carbohydrate exine, which is refractory to most digestive systems, protects pollen from digestion (Roulston and Cane 2000). Pollen stored in the honeycomb receives the

addition of digestive enzymes through the workers' saliva (Gilliam 1979). These enzymes' actions are capable of changing the pH in the honeycomb, making the environment selective to some microbial taxa. While changes in the nutritional composition of pollen appear to be guided by microbial activity, the microbiota and the functional genes actively involved in this process in beebread and bee guts are not yet understood. Pioneer studies characterized the microbiota of pollen and beebread by culture-dependent approaches (Gilliam 1979). Those studies were very important to access the cultivable microorganisms; however, many microorganisms defy standard cultivation approaches. As a result, molecular methods to sequence 16S rRNA genes by high-throughput sequencing and the phylogenetic investigation of communities by reconstruction of unobserved states should enhance our understanding of the bee microbiome.

Most of the studies involving associations with bees and microorganisms have been conducted with European bees or bumblebees. However, studies of Africanized bees, which are more resistant to diseases and more productive than European bees, are scarce. The intent of this work was to enhance our knowledge about microbial communities inhabiting both bee guts and beebread by assessing their similarities and/or differences as well as predict the genes involved in the microbial bioprocess of pollen using state of the art 'post-light'-based sequencing technology.

Materials and methods

Ethics Statement

All colonies were sampled in an apiary of the Cooperativa Apícola do Pampa Gaúcho located in São Gabriel, Rio Grande do Sul State, Brazil. Our field collections did not involve endangered or protected species and no specific permissions were required since the responsible beekeeper granted access to the apiary. The authors declare no conflict of interest.

Experiment design and DNA extraction

An apiary containing 10 honeybee colonies (located 5 m away from each other) was established during springtime (September–December, 2012) from queens that had been inseminated naturally. According to Whitfield et al. (2006), honeybee colonies in Southern Brazil are product of a genetic mixture of 25 % European and 75 % African populations as a result of the introduction of *A. mellifera scutelatta* in 1956. The apiary was located at the boundary between a pristine forest segment and a 7-year old *Eucalyptus* sp. forest. A general scheme of the experimental design and analysis is shown on Fig. 1. Bee gut samples were obtained from 10 worker nurse bees, removed from each one of the colonies ($n = 100$) after a 30-day period after the establishment of the colony. Worker bees were placed in a 15 mL tube (1 tube per colony), kept on ice, brought to the lab, and immediately dissected. Gut organs (crop, hindgut, midgut, ileum, and rectum) were humidified with sterile saline solution (0.9 % w/v NaCl) and removed using sterile tweezers and scissors. After dissection, gut organs were stored at −20 °C for a maximum of 24 h. For beebread samples, a frame containing combs filled with beebread was removed from each colony, also after a 30-day period from colonies' establishment. These combs were placed inside a sterile plastic bag, kept on ice, and brought to the lab. Three samples of beebread were taken from the combs using sterile tweezers and needles and kept at −20 °C until further use. Worker bees (=bee gut) and beebread were sampled in the same day. Microbial DNA extraction from gut and beebread samples was carried out using the QIAamp DNA Stool kit (QIAGEN, USA), according to the manufacturer's instructions. For each sample type per colony, individual DNA extractions were pooled. The nucleic acid concentration and purity of each sample was evaluated by the NanoVue spectrophotometer (GE Life Sciences, USA). Extracted DNA samples were kept at −20 °C until further use.

For the overall analysis of the microbial diversity and structure comparisons from each bee gut or beebread, the 16S rRNA gene from the V3 region was amplified using the primer fusion technique and sequenced with the Ion Torrent Personal Genome Machine (Life Technologies, USA). Finally, to obtain high sequence coverage and to detect the rare microbes inhabiting the samples, a second sequencing run was performed using one single sample randomly chosen from each bee gut and beebread. The 16S rRNA gene from the V6 region was amplified using the adaptor ligation technique and sequenced with the Ion Torrent Personal Genome Machine (Life Technologies, USA).

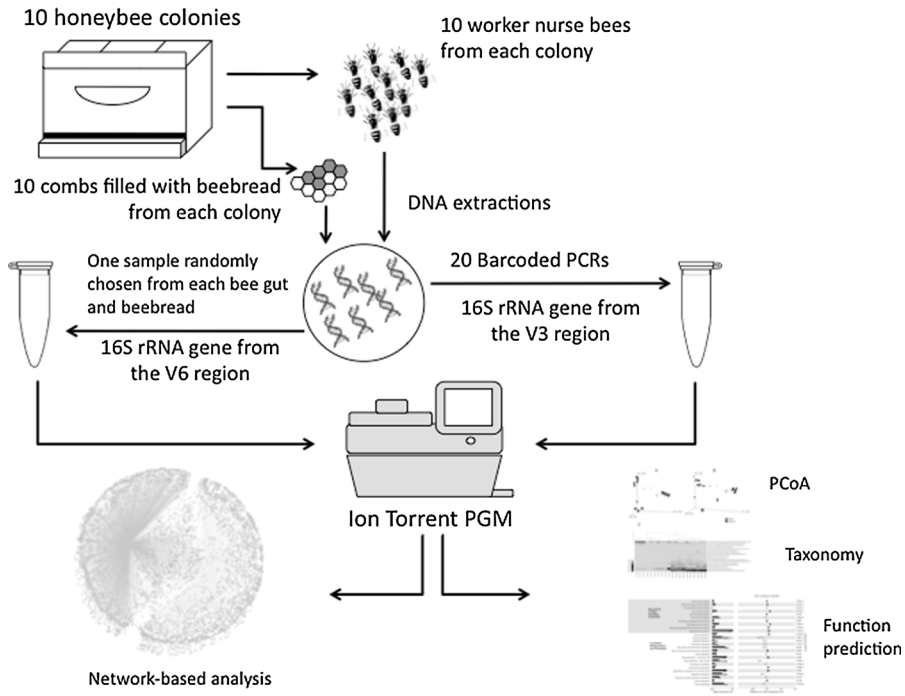


Fig. 1 General scheme of the experimental design and workflow conducted for the analysis of the microbial communities in the bee guts and beebread

Amplicon libraries preparation using primer fusion

Three independent PCR reactions were performed for each of the 20 samples with the 515F and 806R primers (Caporaso et al. 2012). The forward primer was modified adding the P1 adaptor sequence (5′-CCTCTCTATGGGCAGTCGGTGAT-3′) at the 5′-end of the primer. To reduce any effect the composite primer might have on PCR efficiency, a two-base linker (GT) was inserted between the primer and the P1 adaptor. Barcoded primers were used to multiplex the amplicon pools so they could be sequenced together and computationally separated afterwards. To do this, 12-base barcodes were added at the 5′-end of the reverse primers using the self-correcting barcode method of Hamady et al. (2008). A two-base linker (CC) was inserted between the primer and the barcode. The A1 adapter (5′-CCATCTCATCCCTGCGTGTCTCCGA CTCAG-3′) was then added to the 5′-end of the barcoded primer.

PCR was performed with the High Fidelity PCR Enzyme Mix (Thermo Scientific, USA). The mixtures contained 5 µl of 10X high fidelity PCR buffer with 15 mM $MgCl_2$, 0.2 mM of each dNTP, 100 mM of each primer, 2.5 U of high fidelity PCR enzyme mix, and approximately 100 ng of DNA template in a final volume of 50 µl. The PCR conditions were 94 °C for 2 min, 25 cycles of 94 °C for 45 s; 55 °C for 30 s; and 72 °C for 30 s extension, followed by 72 °C for 4 min. The PCR products for each of the 20 samples were purified and combined in equimolar ratios using the quantitative DNA binding method (SequalPrep Kit, Invitrogen, Carlsbad, CA, USA) to create a DNA pool for template amplification (EmPCR) and sequencing.

Short amplicon library preparation using adaptor ligation

Three independent PCR reactions were performed for each sample with the primers 917F (Keijser et al. 2008) and 1046R (Sogin et al. 2006) primers, for the amplification of approximately 130 base pairs of the V6 region of the 16S rRNA gene. PCR was performed with the High Fidelity PCR Enzyme Mix (Thermo Scientific, USA). The mixtures contained 5 µl of 10× high fidelity PCR buffer with 15 mM $MgCl_2$, 0.2 mM of each dNTPs, 100 mM of each primer, 2.5 U of high fidelity PCR enzyme mix, and approximately 100 ng

of DNA template in a final volume of 50 µl. The PCR conditions were 94 °C for 2 min, 25 cycles of 94 °C for 45 s; 56 °C for 30 s; and 72 °C for 30 s extension, followed by 72 °C for 4 min.

Prior to Ion Torrent PGM sequencing, the short amplicon libraries were processed in order to add the barcoded adaptors, A and P1, necessary for sequencing. The adaptors were added to the amplicons using the Ion Plus Fragment Library Kit and the Ion Xpress™ Barcode Adapters (Life Technologies, USA). The reactions were performed based on the user bulletin MAN0006846 revision 3.0 available at http://ioncommunity.lifetechnologies.com, with minor modifications during the amplicon purification step as follows: the bead suspension with the DNA was incubated with the Agencourt® AMPure® XP Reagent (Beckman Coulter, USA) (2× sample volume) at room temperature for 10 min and all washing steps were performed with 500 µl of freshly prepared 80 % ethanol during 30 s. All other steps for preparing short amplicon libraries (end-repair, barcoded adaptors ligation and nick-repair) were performed according to the user bulletin mentioned above. The following barcodes TTGGAGTGTC and TCTAGAGGTC were added to the short amplicons from bee gut and beebread samples, respectively.

All barcoded amplicons were quantified by quantitative real-time PCR using Ion Library Quantitation Kit (following the user guide Ion Library Quantitation Kit TaqMan® assay quantitation of Ion Torrent libraries Publication Part Number 4468986 Rev. A) and the Applied Biosystems® 7500 Fast Real-Time PCR System according to the manufacturer's instructions. The samples were adjusted to 15×10^6 molecules per microliter and mixed in equal amounts to obtain an equimolar pool of amplicons that was used for template amplification onto Ion Sphere™ Particles (ISPs).

Template amplification and sequencing

The template-positive ISPs containing clonally amplified 16S rRNA genes (either fusion primer technique or adaptors ligation technique) was prepared with the Ion OneTouch™ System using the Ion OneTouch™ 200 Template Kit v2 following the user guide Publication Number 4478372 Revision B (available at http://ioncommunity.lifetechnologies.com). The resulting ISPs were sequenced on Ion 316™ microchip using the Ion Torrent Personal Genome Machine (Life Technologies, USA) and the Ion PGM™ 200 Sequencing Kit following the workflow suggested in the user guide Part Number 4474246. After sequencing, the sequence reads were filtered within the PGM software that removed low quality and polyclonal sequences. All PGM filtered data were exported as Fastq file that was used for the subsequent bioinformatics analysis.

16S rRNA reads processing for downstream analyses

A fundamental problem with the use of next-generation sequencing for single read analysis is the number of artifacts that might exacerbate biases via the presence of chimeric sequences and sequence errors (Schloss et al. 2011). In attempt to reduce the sequencing error, a stringent quality-filtering method was applied to improve our downstream analyses. The Fastq file exported from the Ion PGM™ System was processed using mothur v.1.30.2 (Schloss et al. 2009). The multiplexed reads were first filtered for quality and assigned to the starting samples. The filtering criteria removed any sequence that: (1) contained a homopolymer greater than 8 bases, (2) contained any ambiguous base call, (3) had more than one mismatch to the barcode sequence, and (4) was shorter than 100 bases in length. Also, the sequences were quality screened using a moving window that was 50 bases long. Within that window, any read was removed with an average quality score (inferred as Phred score) below 25. Finally the chimeric sequences were checked using the chimera.slayer command and any sequences that were of mitochondrial or chloroplast origin were removed from the dataset.

Library comparisons

For the overall comparison of significant differences among bacterial communities from bee guts and beebread, Principal Coordinates Analysis (PCoA) was performed with the amplicon libraries. PCoA is an ordination method based on multivariate statistical analysis that maps the samples in different dimensions and reflects the similarity of the biological communities. The calculations were performed within the QIIME pipeline (Caporaso et al. 2010). A matrix using the UniFrac metric (weighted and unweighted) for

each pair of environments was calculated. The distances were converted into points in space with the number of dimensions one less than the number of samples. The first three principal dimensions were used to plot a three-dimensional graph that was visualized using KING (Chen et al. 2009). To test whether the results were robust, a sequence-jackknifing technique was used in which the PCoA clusters were regenerated using a subset corresponding to about 70 % of the total number of sequences obtained in the sample with the smaller number of sequences randomly selected from each sample for 100 replicate trials. In addition, to see which taxa were more prevalent in different areas of the PCoA plot, the ten most abundant taxa were added to the PCoA plots. Ellipses were drawn around the mean values to represent the interquartile ranges (IQRs), which measures the statistical dispersion between the upper and lower quartiles. If the ellipses are small, the same result would likely to be achieved with a different set of sequences from the same environment. Alternatively, if the ellipses are large, a different result might be expected. In addition, to determine which taxa were more prevalent in different areas of the PCoA plot, the nine most abundant family-level taxa were combined with the jackknifed principal coordinates.

To identify the organisms whose abundances differ between the bee gut and beebread samples, each sequence was assigned to an OTU at 95 % similarity by using uclust (Edgar 2010). The number of sequences in each OTU found in each sample was used to construct a table with OTUs (lines) and samples (columns). This table was used to generate a color-coded Clustered Image Map (heat map) with CIMminer (Weinstein et al. 1997). Finally, representative sequences of each OTU were subjected to the RDP naïve Bayesian rRNA Classifier (Wang et al. 2007), which attaches complete taxonomic information from domain to species for each sequence in the database with 80 % taxonomy confidence and an *e*-value of 0.001. That means some OTUs might only be classified at certain coarse taxonomic level because a deeper level (e.g. genus) would not present the required degree of confidence.

To examine the co-occurrence of OTUs between the samples a network-based analysis was applied. The network was performed using the dataset generated with the short amplicon libraries because these libraries had greater sequencing coverage (Lemos et al. 2011). The network allows for the visualization of the OTUs that are either unique or shared by specific groups of bee gut and beebread samples. Connections were drawn between samples and OTUs, with edge weights defined as the number of sequences from each OTU that occurred in each sample. To cluster the OTUs from the bee gut or beebread samples in the network, the spring embedded algorithm layout was used. In this algorithm, the nodes act like steel rings that exert mechanical forces creating an attractive force between nodes that are far apart, and a repulsive force between nodes that are close together (Shannon et al. 2003). In the resultant graph, samples that share more OTUs cluster closer together. The network diagram was visualized with Cytoscape (Shannon et al. 2003) with two kinds of nodes; OTU-nodes, grouped using 97 % similarity cutoff, and bee gut and beebread sample nodes.

Metagenome prediction

The 16S rRNA database was used to predict the microbial functions within the bee gut and the beebread using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). The software uses an extended ancestral-state reconstruction algorithm to predict which gene families are present and then combines genes families to estimate the composite metagenome (Langille et al. 2013). Briefly, the demultiplexed reads were subjected to a closed-reference OTU picking protocol (Caporaso et al. 2010) where the sequences were searched against a greengenes reference database at 97 % identity. Any read that did not hit the reference collection was discarded. The outputted OTU table was normalized according to the 16S rRNA gene copy number per genome and a final metagenome functional prediction was created.

To determine statistical differences between the bee gut and the beebread metagenomes, the statistical analysis of metagenomic profiles (STAMP) software package was used (Parks and Beiko 2010). The statistical hypothesis tests were performed using the Welch's *t* test (Bluman 2007) while confidence intervals were calculated using the Welch's inverted method, and correction was made using Bonferroni multiple test correction (Adbi 2007).

Data accessibility

Raw sequences were submitted to the NCBI Sequence Read Archive under the study number SRP043294, experiment number SRX603452.

Results

Using the library prepared with the primer fusion technique as described in the "Amplicon libraries preparation using primer fusion" section, a total of 505,606 reads were obtained. After filtering the reads by base quality and removing reads shorter than 100 bases, a total of 207,628 high-quality 16S rRNA gene sequences were obtained in this study (76,628 and 131,000 sequences for bee gut and beebread samples, respectively). After taxonomic assignment and chimera removal, a total of 11 phyla (Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria, TM7, Tenericutes and Verrucomicrobia) were found within bee guts and 10 phyla (Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Gemmatimonadetes, OD1, Planctomycetes, Proteobacteria, Tenericutes and Verrucomicrobia) were found within beebread. Firmicutes and Proteobacteria were the most abundant phyla within both beebread (29 % of Firmicutes and 62 % of Proteobacteria) and bee guts (16 % of Firmicutes and 19 % of Proteobacteria). No archaeal sequences were found in the beebread samples and only one sequence of the phylum Crenarchaeota and five sequences of the phylum Euryarchaeot*a* were found in the bee gut samples.

In terms of microbial community structure, the analysis showed that the communities from the two environments were quite different from each other (Fig. 2a, b). The first three axes of the Jackknifed unweighted PCoA explained 42 % of the total variation found within microbial communities while the first three axes of the weighted Jackknifed PCoA accounted for 89.3 % of the variation. The results indicated that the overall differences between the clusters were related to both presence/absence and the abundance of specific OTUs. The group made up by the microbial communities from the beebread is illustrated as a dense cluster indicating that the microbial communities from this environment were closely related in terms of membership and structure. On the other hand, the group represented by the microbial communities from the bee guts is shown as a dispersed cluster indicating a greater variation of microbial membership and structure when compared with the beebread. The families Enterobacteriaceae, Ricketsiaceae, Spiroplasmataceae and Bacillaceae, were the main groups responsible for the specificity of the bee gut with the first two families being the most abundant (Fig. 2c). The main families responsible for the specificity of the beebread were Neisseriaceae, Flavobacteriaceae, Acetobacteraceae and Lactobacillaceae.

To examine the co-occurrence of OTUs between the samples, a network-based analysis was applied using the dataset generated with the short amplicon libraries as described in the "Short amplicon library preparation using adaptor ligation" section. From this library, a total of 448,117 and 360,797 high-quality sequences from the V6 region were obtained from the bee guts and beebread, respectively. These data provided coverage greater than 99 % as calculated by the Good's coverage (Good 1953). The network consisted of 6,208 OTUs (called nodes), in which 2,646 OTUs were found exclusively in the bee guts and 3,032 were present only in the beebread (Fig. 3). The network analysis revealed that only 7 % (530 OTUs) were shared between bee gut and beebread, confirming the very distinctive microbiota of bee guts and beebread.

On the basis of the PICRUSt analysis, 6,909 enzymes involved in metabolism processes were predicted (please refer to "Discussion" section for more information about the limitations and interpretation of these results) within the bee guts and beebread. These enzymes were grouped in 114 functional profiles related to 12 main metabolic functions (Figs. 4, 5). Those metabolic functions were found in both bee gut and beebread. However, it was possible to detect statistically significantly higher abundance of sequences matching the following functional profiles/pathways in the beebread: (a) amino acid metabolism—11 pathways; (b) carbohydrate metabolism—10 pathways; (c) glycan biosynthesis and metabolism—7 pathways; (d) lipid metabolism—11 pathways; (e) metabolism of cofactors and vitamins—7 pathways; (f) metabolism of other amino acids—7 pathways; (g) nucleotide metabolism—2 pathways; (h) xenobiotics biodegradation and metabolism—10 pathways. In contrast, the following functional profiles/pathways were predicted to

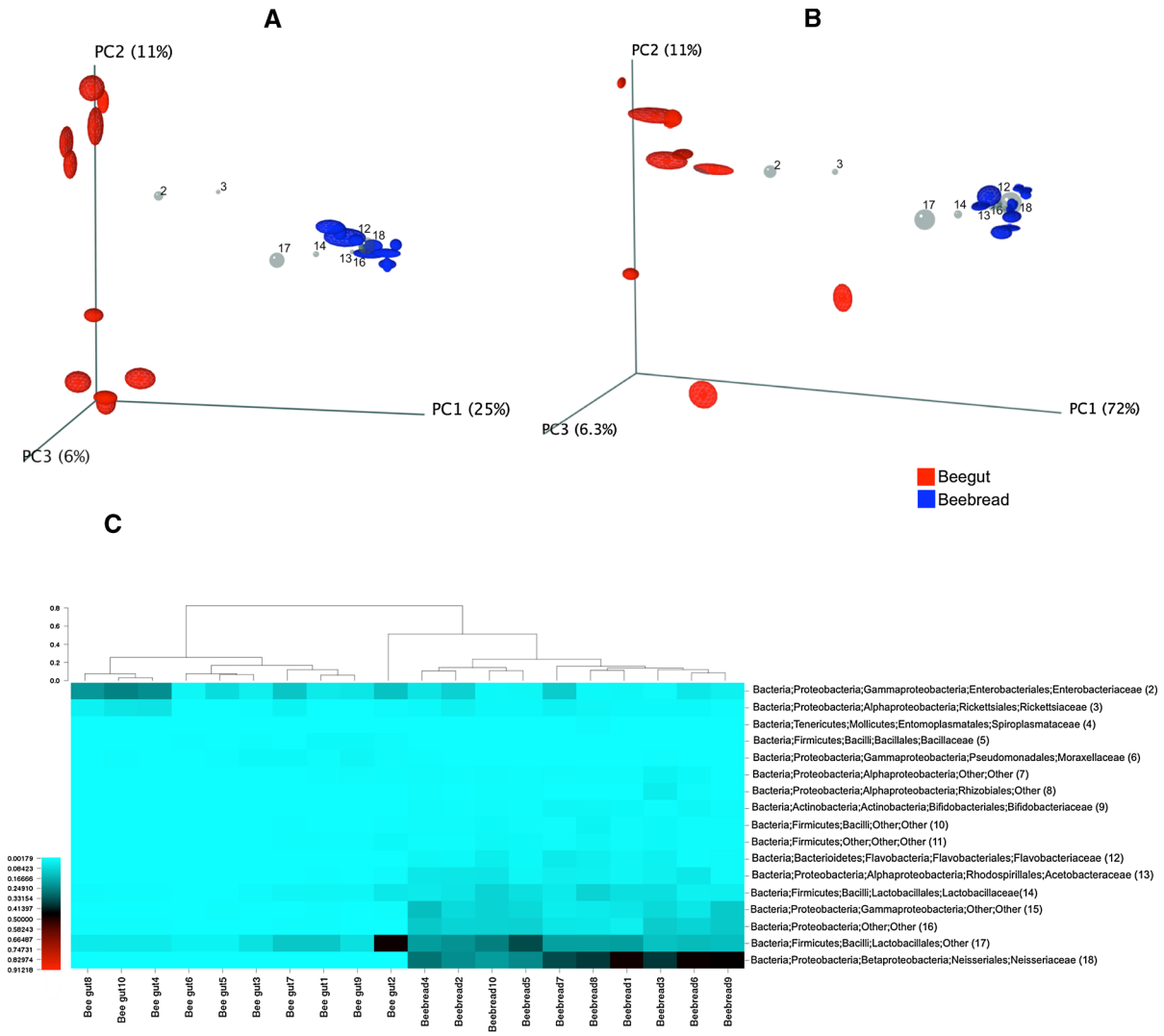


Fig. 2 Overall comparisons of microbial communities based on their composition. **a**, **b** 3D Jackknifed Principal Coordinates (PCoA) biplots depicting the clusters of bacterial communities within 20 samples each from the bee guts (*red*) and beebread (*blue*). **a** Unweighted UniFrac distance metrics. **b** Weighted UniFrac distance metrics. The positions of the points are the average for the jackknife replicates and *ellipses around points* represent the interquartile range (IQR) for the 1,000 jackknife replicates. *Each point* represents a microbial community. *Points* closer to each other represent similar microbial communities while *points* far from each other represent dissimilar microbial communities. The *gray circles* (sized according to the bacterial relative abundance) represent the bacterial taxa, plotted in the same PCoA space, contributing to the PCoA clusters. The numbers next the *gray circles* correspond to bacterial families as depicted in the heatmap. **c** Heat map generated from 16S rRNA data reflecting the major differences in taxa abundance across bee gut and beebread samples. Note that only the most representative taxa are shown in the heat map. A complete heat map with all taxa grouped by family can be found in the Fig. S1

be statistically significantly more abundant in the bee guts: (a) biosynthesis of other secondary metabolites—8 pathways (mainly related to resistance and biosynthesis of antibiotics); (b) energy metabolism—5 pathways; (c) enzyme families—2 pathways (peptidases and protein kinases).

Discussion

In this study, we focused on the analysis and comparison of *Apis mellifera* gut and beebread microbiome by using state of the art 'post-light' based sequencing technology (Rothberg et al. 2011). We

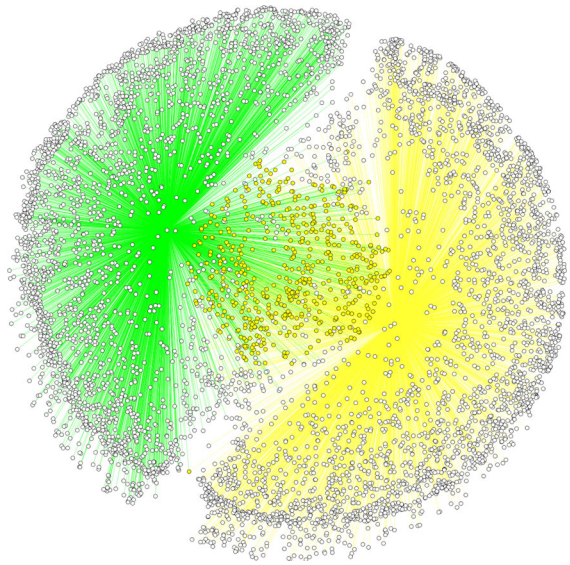


Fig. 3 Network based analysis of microbial communities. *White circles* are representative sequences from each OTU clustered based on the relatedness of the sequences (97 % similarity). *Yellow circles* are representative sequences shared between bee gut and beebread. Each bee gut or beebread samples are connected with the OTUs through edges color-coded according to the origin of the sequence. *Green* bee gut and *yellow* beebread

investigated the presence/absence of microbial taxa, the microbial community structure and its possible involvement with nutritional processes such as the breakdown and utilization of pollen grains.

The relative abundance of archaea in these samples was very low within the guts of *Apis mellifera.* Only 6 of 207,628 high-quality 16S rRNA gene sequences were classified as Archaea. In fact, Archaea do not appear to be important members of these communities. However, it should be mentioned that biases at the steps of DNA extraction, PCR amplification, primer choice and sequencing, might present some degree of interference in the results obtained. Nevertheless, as Probe Match (http://rdp.cme.msu.edu/probematch/search.jsp) predicted that the primers used in this study would amplify 16S rRNA from Archaea, these biases were unlikely to have missed many archaeal taxa.

Large variability of the bee microbiome within different colonies was observed through the PCoA analysis (Fig. 2). Several exogenous as well as endogenous factors could affect the intestinal microbiota in these bees. The environment of the sampled insects was the same, thus minimizing its contributions to microbiome variability. Another important factor in determining the microbial diversity and community structure inside the bee guts is the age of the insects and their respective function in the colony. Younger workers may have maternally inherited microbiomes while older workers, which leave the hive to forage, might present microbiomes acquired from the environment or via flowers (Martinson et al. 2011; Mcfrederick et al. 2012). Finally, the microbiome acquisition might be determined by the bees' health status (Cox-Foster et al. 2007).

The microbial communities of the bee guts and the beebread were easily distinguished (Figs. 2, 3). Such large differences were expected since both guts and beebread are from environments that differ in temperature, pH and osmotic potential. Thus, only seven percent of the total OTUs were found in common between guts and beebread. Both environments presented high diversity of taxa and those taxa present different functions involving nutrient processing. The exclusive conditions of each environment supported a set of distinct microbial functions.

The Lactobacillaceae dominated beebread samples (Fig. 2c). The presence of representatives of Lactobacillaceae has already been reported in honeybee workers (Vojvodic et al. 2013). In fact, Lactobacillales were also found in association with the bee guts (see Fig. 2c). Recent evidence point to the presence of a microbial core community within the European bee guts and the bacteria belonging to the Lactobacillales order appear to be present in this core community (Moran et al. 2012). Sampling two localities in USA and five bees from four colonies, Moran et al. (2012), found *Gilliamella* (Gamma 1) *Snodgrassella* (Beta), Firm4 and Firm5 present in all European bees and Bifido, Gamma2 and Alpha2 present in most European bees (for detailed phylogenetic identification of those groups see Martinson et al. 2011). In our work, Africanized bees were collected from only one locality in Brazil but from ten colonies. At the phylum level, the same representatives of the microbial core were found in Africanized bees, indicating that this bacterial core might also be present in Africanized populations. Within the beebread, Lactobacillaceae might be involved in fermentative processes or in the production of secondary metabolites responsible for changes in the pH of the beebread. Studying the origin of *Lactobacillus* in the *Apis* genus, McFrederick et al.

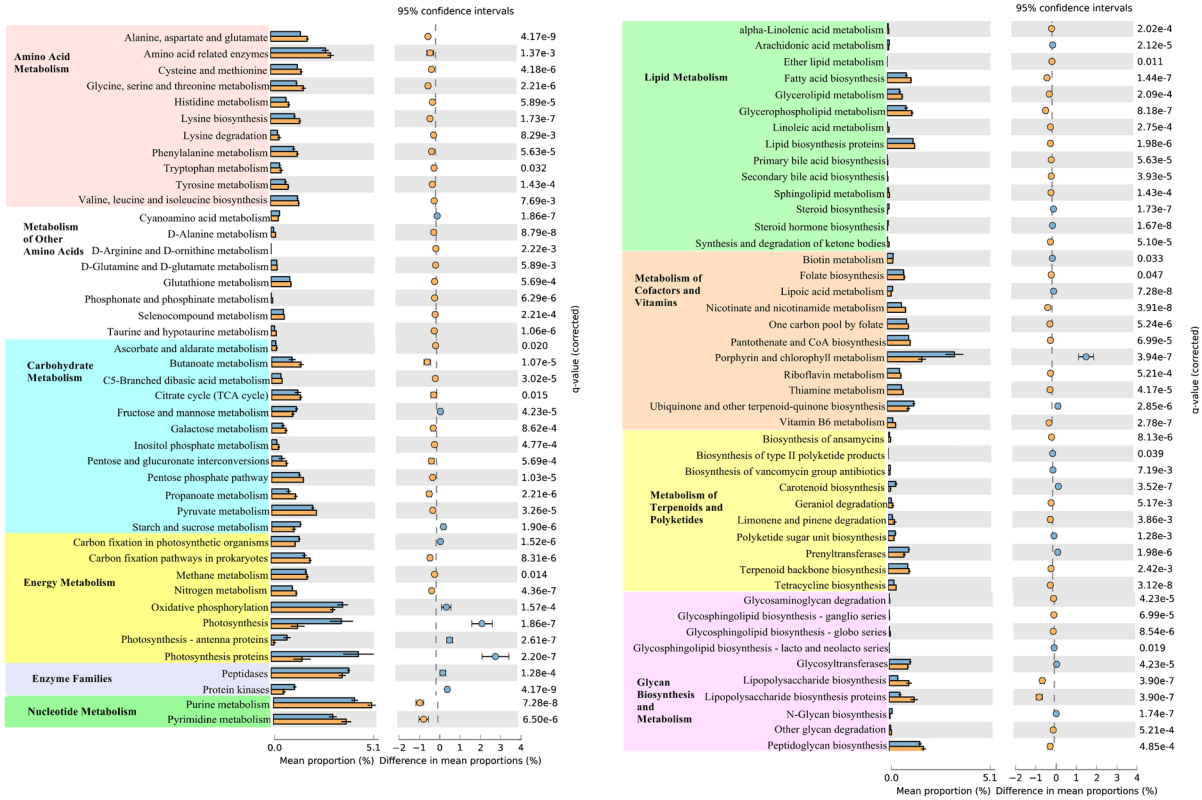


Fig. 4 Relative abundance of microbial metabolic functions from 10 composite bee guts (*blue*) and 10 composite beebread (*orange*) samples. The *error bars* show calculated standard variation. The *colored circles* represent the 95 % confidence intervals calculated using the Welch's inverted method. Corrected q-values were calculated using Bonferroni Multiple test correction (q-value < 0.05)

(2012) reported the presence of close relatives of several bacteria that have been isolated from flowers. In addition to Lactobacillaceae, Enterobacteriaceae were abundant within bee guts, while Neisseriaceae were common within beebread. Working with European bees captured in two different seasons (spring and fall) in an apiary at Arizona, USA, Corby-Harris et al. (2014) also found *Lactobacillus* and sporadically abundant Enterobacteriaceae in crops of foragers' bees. Such observations are in line with the hypothesis that at least Lactobacillaceae is frequently found in bee guts irrespective of the geographic localization, environmental conditions (Moran et al. 2012) or bee ganetics (European bees from USA or Africanized bees form Brazil as found in our study). Anderson et al. (2013) sampled beebread and bee gut of honey bees from apiaries located at the USDA Carl Hayden Bee Research Center in Tucson AZ, USA. Using cultivation techniques and rRNA and rDNA sequencing, the researchers compared bacterial diversity from floral nectar, different segments of the honey bee alimentary tract, and beebread. Similar to our findings, within the beebread they found Firmicutes (*Lactobacillus*), Actinobacteria, Proteobacteria (Acetobacteriaceae) and Bacteroidetes.

An important challenge faced by microbial ecologists is to discover the metabolic differences that are affected by the differences in microbial taxa. This can be assessed with metagenomic or metatranscriptomic approaches. However, in this work we took advantage of a bioinformatics tool developed by Langille et al. (2013), capable of predicting the metagenome based on phylogenetic information. According to Langille et al. (2013), with only 105 assigned 16S rRNA gene reads, PICRUSt can provide the same accuracy of 15,000 annotated metagenomic sequences. PICRUSt

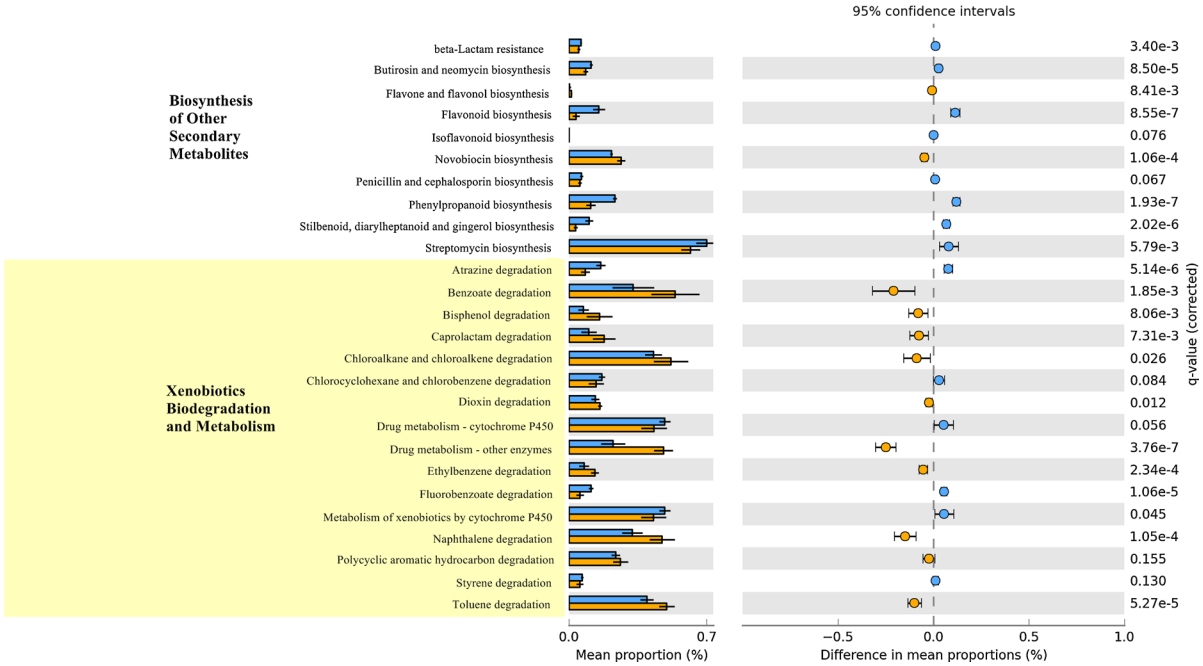


Fig. 5 Relative abundance of microbial genes related to biosynthesis of secondary metabolites and xenobiotics biodegradation and metabolism from 10 composite bee guts (*blue*) and 10 composite beebread (*orange*) samples. The *error bars* show calculated standard variation. The *colored circles* represent the 95 % confidence intervals calculated using the Welch's inverted method. Corrected q-values were calculated using Bonferroni Multiple test correction (q-value < 0.05)

does not provide direct evidence of a community's functional capabilities but might provide useful insights about the functions the microbial community might be able to perform (Langille et al. 2013).

Genes involved in the biosynthesis of streptomycin were found in greater proportion than the genes related to the biosynthesis of other antibiotics. Streptomycin is an antibiotic that belongs to the aminoglycoside category and acts by inhibiting the protein synthesis commonly against gram-negative bacteria (Singh and Mitchison 1954). The presence of such genes raises the hypothesis about the possible role of commensal or normal microbiota in protecting *Apis mellifera* against pathogenic bacteria and in maintaining the healthy status of the hive.

In addition to the possibility of protection against pathogens, the microbiota from the beebread possessed many genes related to xenobiotics biodegradation, including phase I detoxification systems such as cytochrome P450 (CYP). The superfamily of CYP proteins is responsible for the catabolism of 90 % of all drugs (Liu et al. 2013). Thus, these genes may provide important defense mechanisms against exogenous chemicals (Guengerich 2007). The presence of xenobiotic substances in a given organism is usually associated with environmental exposures to a range of compounds including drugs, natural compounds, as well as synthetic substances such as pesticides. Pollinators, such as honeybees, are directly exposed to chemical agents used in agriculture. Our metagenome prediction suggested the presence of many genes that can degrade the most commonly used pesticides in agriculture such as atrazine and fluorobenzoate. Such compounds are routinely used for weed control.

This work represents the evidence for the presence of microbial genes related to the pollen degradation in the beebread highlighting the importance of the microbiota associated with the beebread and its potential role in pollen breakdown. In addition, the results highlight the possible involvement of the microbiota associated with the bee guts in building up a defense system based on the biosynthesis of antibiotics and xenobiotics biodegradation. Xenobiotic degradation may be important in the

degradation of herbicides or insecticides carried with pollen and deposited in the combs.

In summary, the microbial communities of the bee guts and the beebread were very distinctive in terms of community structure and composition indicating the presence of a highly specialized and well-adapted microbiota within each bee gut and beebread. In fact, only a small proportion of the microbial taxa were found in common between bee guts and beebread. These differences might be related with the role of these communities within each environment. For instance, the microbial community from the beebread works as a bioreactor for pollen digestion, and contributes to the detoxification processes that provide bees with an increased ability to eliminate the toxic compounds carried with pollen.

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3. CONSIDERAÇÕES FINAIS

Este estudo analisou as comunidades microbianas associadas ao pão de mel, coletado e estocado pelas abelhas para sua alimentação, bem como a comunidade microbiana presente no sistema digestório destes insetos. As amostras coletadas no Pampa Gaúcho indicaram que as comunidades microbianas destes dois ambientes com características diversas, como temperatura, umidade e pH abrigam comunidades microbianas distintas que desenvolvem papeis diferentes nos dois ambientes estudados.

Os resultados encontrados estão de acordo com a hipótese que foi testada neste projeto, comprovando que a microbiota associada ao pólen estocado participa da sua biotransformação em pão de mel. Outro achado importante está relacionado com a capacidade que a microbiota, associada ao sistema digestório das abelhas possui para degradar os agrodefensivos.

O estudo da microbiota associada as abelhas melíferas é um campo muito vasto, muitas pesquisas ainda precisam ser realizadas, visando complementar o conhecimento nesta área e com isto auxiliar na manutenção de enxames saudáveis e produtivos.

Para dar continuidade a esta pesquisa, as próximas questões que precisam ser elucidadas tratam sobre o impacto que a modificação da estrutura da comunidade microbiana, causaria no processo de biotransformação dos grãos de pólen em pão de mel e testar se a microbiota associada ao trato digestório das abelhas melíferas, desempenharia o mesmo papel na degradação de agrotóxicos, se esta fosse constituída por outras espécies.

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