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INVERTEBRATE MICROBIOLOGY

Impact of Nutritional Stress on Honeybee Gut Microbiota, Immunity, and *Nosema ceranae* Infection

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Abstract

Honeybees are important pollinators, having an essential role in the ecology of natural and agricultural environments. Honeybee colony losses episodes reported worldwide and have been associated with different pests and pathogens, pesticide exposure, and nutritional stress. This nutritional stress is related to the increase in monoculture areas which leads to a reduction of pollen availability and diversity. In this study, we examined whether nutritional stress affects honeybee gut microbiota, bee immunity, and infection by *Nosema ceranae*, under laboratory conditions. Consumption of *Eucalyptus grandis* pollen was used as a nutritionally poor-quality diet to study nutritional stress, in contraposition to the consumption of polyfloral pollen. Honeybees feed with *Eucalyptus grandis* pollen showed a lower abundance of *Lactobacillus mellifer* and *Lactobacillus apis* (Firm-4 and Firm-5, respectively) and *Bifidobacterium* spp. and a higher abundance of *Bartonella apis*, than honeybees fed with polyfloral pollen. Besides the impact of nutritional stress on honeybee microbiota, it also decreased the expression levels of vitellogenin and genes associated to immunity (glucose oxidase, hymenoptaecin and lysozyme). Finally, *Eucalyptus grandis* pollen favored the multiplication of *Nosema ceranae*. These results show that nutritional stress impacts the honeybee gut microbiota, having consequences on honeybee immunity and pathogen development. Those results may be useful to understand the influence of modern agriculture on honeybee health.

Keywords *Apis mellifera* · Colony losses · Pollen · Nutritional stress · Pathogens · Microbiota

Introduction

Western honeybees *Apis mellifera* are important managed pollinators worldwide, having an essential role in the ecology of natural environments and agricultural production [1, 2]. In recent years, large-scale colony losses have been reported in different countries [3–6]. These losses have been associated with the infection by multiple pests and pathogens (such as the mite *Varroa destructor*, the microsporidia *Nosema ceranae*, and different RNA viruses), intoxication *with* pesticides, and nutritional stress [7–9]. Nutritional stress is linked to the increase of monoculture areas which leads to a reduction of pollen availability and diversity for honeybees [7, 9].

Adult honeybee nutrition relies on the intake of honey and pollen. Pollen provides proteins, lipids, and vitamins necessary for the healthy growth and development [reviewed at 10, 11]. Its composition, quality, or quantity influences honeybee physiology (protein concentrations, macromolecule

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metabolism, hypopharyngeal glands size, tissue growth and development, glutathione S-transferase activity, immunocompetence) [12–15], longevity [16], and resistance to pathogens including *Nosema apis* and *Nosema ceranae* [17–19].

Previous studies have shown that honeybee colonies subjected to nutritional stress, located in a *Eucalyptus grandis* plantation during the flowering period in autumn, became rapidly infected by *N. ceranae*, decreasing brood and adult population [20, 21] and undergoing high colony losses [21, 22]. These losses may be related with the poor-nutritional quality of *Eucalyptus* spp. pollen, since protein percentage decreases during the flowering period to values under 20% [20, 21], they have low content of lipids [20, 23] and essential fatty acid omega 3 [24], and they have deficiency in the essential amino acid isoleucine [25, 26].

Besides that, it has been proposed that environmental landscape affects the honeybee gut microbiota, influencing the relative abundance of some of its members [27]. This microbiota is dominated by eight core bacterial clusters, which comprise 95–99% of bacteria in the gut [28–31], including *Gilliamella apicola* [32], *Snodgrasssella alvi* [32], *Lactobacillus* Firm-4 [33], Lactobacillus Firm-5 [33], Bifidobacterium spp. [33], *Frischella perrara* [34], *Bartonella apis* [35], and *Parasaccharibacter apium* [36]. It has been involved in the defense against pathogens, metabolism, growth, development, and immunity [30]. Disruption of the microbiota, named dysbiosis, may have consequences on the bee development and immunity, affecting the ability of bees to respond to environmental stressors [30].

The honeybee immune system is composed of a complex network of mechanisms, including cellular and humoral immune defenses reviewed in [37]. In particular, humoral defenses rely on the production of antimicrobial peptides (apidaecins, abaecin, hymenoptaecin, and defensins), which act generating leaks in prokaryotic membranes; and inhibiting bacterial protein translation or folding [38]. Besides that, honeybees have evolved a social immunity, a collective defense that arises from the behavioral cooperation among individuals, such as grooming, hygienic behavior or food sterilization [37, 39]. Doublet et al. [40], identified a common set of genes that respond to the infection by different pathogens (*V. destructor*, *Nosema apis*, *N. ceranae*, and RNA viruses), including hymenoptaecin, defensin, and abaecin, among others.

We hypothesize that nutritional stress shapes the composition of the honeybee gut microbiota having consequences on honeybee immunity and favoring the infection by pathogens. Therefore, we evaluated the effect of two different pollen diets (*E. grandis* or polyfloral pollen) in the composition of the gut microbiota, on the expression of different genes involved in the humoral and cellular immune response, and in the infection by *N. ceranae*.

Methods

Pollen Samples

E. grandis monofloral stored pollen samples (bee bread) were manually collected from frames of colonies located in *E. grandis* plantations during its flowering period (Rivera, Uruguay), in April and May (autumn) 2014, for trial 1 or 2, respectively. Polyfloral stored pollen (bee bread) was manually collected from colonies located in the experimental apiary at the Instituto Nacional de Investigación Agropecuaria (INIA "La Estanzuela", Colonia, Uruguay) in April (autumn) and October (spring), 2014 for trials 1 and 2, respectively. In all cases, one frame per colony, from five colonies, were collected and mixed. Pollen samples were homogenized making a paste, and no water or syrup was added. Samples were kept at −20 °C until used or analyzed. Palynological analyses were performed to validate their botanical composition [41]. At least 1200 pollen grains per sample were identified [42]. Although there is a low risk of pesticide pollen contamination, we cannot completely rule out this possibility.

Two independent trials were performed in 2014 (trials 1 and 2), and different pollen samples were used in each trial (Fig. 1A suppl. data).

Trial 1

In spring, three healthy *A. mellifera* colonies, local hybrids between *A. m. scutellata*, *A. m. ligustica*, and *A. m. mellifera,* were randomly selected from the experimental apiary at INIA "La Estanzuela", Colonia, Uruguay. Colonies had been treated against the mite *V. destructor* in the previous autumn (with Amitraz in stripes) and did not show clinical symptoms of diseases.

One frame of brood containing last-stage pupae was removed from each hive, taken to the laboratory, and incubated at 34 ± 1 °C, 60% relative humidity in the darkness until honeybee emergence, as recommended by Williams et al. [43]. Immediately after emergence, bees from different frames were collected. Only bees emerged within the first 24 h were used, in order to standardize the age. After that time, bees from different colonies were mixed, placed in cages, and incubated at 30 ± 1 °C, 60% relative humidity in the darkness. Six cages of bees (n = 40 per cage) were fed with *E. grandis* stored pollen and other six with polyfloral stored pollen. About 3 g of pollen was served per cage in a sterile plastic recipient, and it was replaced every 48 h. A sucrose solution was also provided to bees in Pasteur pipettes (ad libitum, 50% w/w in water). Every day, bees were monitored, dead individuals were counted and removed, and sucrose solution was replaced (Fig. 1B suppl. data).

Trial 2

An independent second trial was performed using three healthy colonies from the experimental apiary of the Universidad Nacional de Mar del Plata (Mar del Plata, Argentina), local hybrids between *A. m. ligustica* and *A. m. mellifera*. As in trial 1, recommendations by Williams et al. [43] were followed. Six cages of bees ($n = 80$ per cage) were fed with *E. grandis* stored pollen while other six were fed with polyfloral pollen, *ad libitum*. About 3 g of pollen was served per cage in a sterile plastic recipient, and it was replaced every 48 h. Three days post-emergence, honeybees from three cages per nutritional regimen were individually infected with 100,000 spores of *N. ceranae* in 10 μl of sucrose solution [44] and returned to the same cage with the same diet. Honeybees from the other cages were individually fed with the same volume of sugar syrup, as controls. Every day, bees were monitored, dead individuals were removed, pollen consumption was estimated (by weight), and sucrose solution was replaced (Fig. 1C suppl. data).

Nosema ceranae spores used for infection were obtained from foraging honeybees from a naturally infected colony and purified by the triangulation method [45]. The spore suspension was quantified using a hemocytometer [46] and immediately used for experimental infection (Fig. 1C. suppl. data). The species identification was carried out by multiplex PCR according to Martin-Hernández et al. [47] (Table 1 suppl. data).

Microbiota Analyses

DNA Extraction from Pollen

Three replicates of *E. grandis* and polyfloral pollen samples (0.2 g/sample) used in trials 1 and 2 were processed according to Anderson et al. [48]. DNA was quantified using a NanoDrop1000 spectrophotometer (Thermo Scientific™) and concentrations were normalized to 10 ng/μl.

DNA Extraction from Honeybees

Ten days after emergence, 20 honeybees/cage from 3 cages/treatment from trials 1 and 2 were collected. Honeybees were externally sterilized using a chlorine solution 1% [48]. Guts were extracted, pooled ($n = 20$/cage), and homogenized using a glass rod and a plastic tube. DNA was extracted using the SDS-CTAB method [49], quantified using a NanoDrop1000 spectrophotometer (Thermo Scientific™), and concentrations were normalized to 20 ng/μl.

PCR for Detection of Pathogens

DNAs obtained from stored pollen and honeybees were subjected to PCR for the detection of *N. ceranae*, *N. apis*, and *Lotmaria passim*, as previously described [47, 50] (Table 1 suppl. data). PCR reactions were carried out using a MultiGeneOptiMax Thermal Cycler (Labnet International, USA). Positive controls (using DNA from *N. ceranae*, *N. apis*, or *L. passim*) and negative controls were included. Amplified products were analyzed by electrophoresis in a 2% agarose gel in Tris/borate/EDTA (TBE) at 120 V for 30 min, stained with GelRed (Biotum, USA), and visualized by UV light.

Quantitative PCR

The quantity of 16S rRNA genes (bacterial load) in honeybees was determined through ratio calculations using universal 16S rRNA gene [51] and a honeybee gene (RPS5 gene [52], Table 1 suppl. data). To quantify the total bacterial number in pollen, a standard curve from DNA obtained from an *Escherichia coli* XL1 Blue culture was used. Cycle threshold (Ct) values of each sample were then compared to the standard curve to approximate the number of bacteria per sample, according to Ott et al. [53]. In both cases, PCR were carried out in a final volume of 20 μl, comprising 1× SYBR (Power SYBR ® Green PCR Master Mix, Applied Biosystems), 0.3 μM of each primer, 40 ng of DNA and RNAse-free water, and PCR cycling described before. All qPCR reactions were performed in triplicate, in a BIO-RAD CFX96™ Real-Time system, and two negative controls were included in each run.

16S rRNA Amplicon Sequencing

DNA obtained from stored pollen and honeybees was analyzed by sequencing of V4 region of 16S rRNA gene using an Illumina MiSeq platform and 250 paired-end (PE) cycles (University of Texas at Austin, USA).

Immune Gene Expression Analyses

RNA Extraction and cDNA Synthesis

Ten days after emergence, ten honeybees per nutritional regimen from trial 1 were collected and stored at −80 °C. Individual honeybees were homogenized in RLT buffer (Qiagen) and subjected to RNA extraction using the RNAeasy Plus minikit (Qiagen), according to the manufacturer's instructions. One microliter of total RNA was treated with DNAse and used to generate first-strand cDNA using the Quantitec Reverse Transcription kit, also according to the manufacturer's instructions (Qiagen).

Quantitative PCR

Relative expression of abaecin, defensin, lysozyme, glucose dehydrogenase, glucose oxidase, hymenoptaecin, and vitellogenin genes were assessed using previously reported primers. Ribosomal protein S5 (RPS5) and ß-actin were used as housekeeping genes to normalize the variation of cDNA levels ([52, 54, 55]; Table 1 suppl. data). The reaction mix consisted of 1X QuantiTect SYBR Green PCR MasterMix (Qiagen), 0.5 μM of each primer, RNAse-free water and 5 μl of 1:10 diluted cDNA in a final volume of 25 μl. PCR reactions were carried out using a BIO-RAD CFX96™ Real-Time system and the cycling program consisted of an initial 95 °C for 15 min, and 40 cycles of three-step PCR at 94 °C for 15 s, 52 °C for 30 s and 72 °C for 30 s. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product (from 65 to 95 °C, with increments of 0.5 °C every 0.05 s.).

Fluorescence was measured in the elongation step and negative controls (without DNA) were included in each reaction run.

Quantification of *N. ceranae* Spores

At 4, 7, and 12 days post infection, five honeybees per cage from trial 2 were sampled. Midguts were removed, and spore number/honeybees (intensity of parasite infection) were quantified by using a hemocytometer (Neubauer improved) [46].

Data Analysis

Honeybee Survival

The impact of nutrition and infection by *N. ceranae* on honeybee survival was analyzed using the Kaplan-Meier method, and statistical differences were compared using the Log-rank test.

16S rRNA Amplicon Sequencing

For analysis of bacterial community data, the protocol suggested by Engel et al. [56] was followed. Paired-end reads were joined using the fastq-join method and demultiplexed with QIIME software package (Qiime.org) [57]. Sequences were analyzed using the QIIME software tool with default parameters for each step. Reads were screened for chimeras using the software program USEARCH 6.1. De novo operational taxonomic unit (OTU) picking was performed with the uclust option in QIIME [58]. Assignment of taxonomy to representative OTUs was carried out with the Greengenes database classifier [59] at the default 97% sequence identity. Sequences matching plant chloroplast or mitochondrial 16S rRNA were filtered from the dataset. The optimal sampling depth was determined through examination of exploratory rarefaction curves of observed OTUs plotted against sampling depth, and the dataset was rarefied.

To evaluate the impact of nutrition/*Nosema* spp. infection on the composition of the gut microbiota, dissimilarity matrices Unifrac weighted (by OTUs abundance), Unifrac unweighted (presence/absence of OTUs), and Bray-Curtis were built. Matrices were used to produce classical multidimensional scaling (principal coordinates analysis). Statistical differences between groups were tested using PERMANOVA (using 1000 permutations). The Shannon diversity index was also calculated, and differences between groups were assessed using the Student *t* test or Mann-Whitney Test.

Besides that, differences between abundance of the different OTUs were examined using the DESeq2 software [60], as described by Jones et al. [27]. Statistical analyses were performed using R software [61].

Gene Expression Analyses

The geometric mean of the Ct (threshold cycle number) of reference genes (RPS5 and β-actin) was calculated and used for normalization. The expression ratio was analyzed as described by Pfaffl [62]. The data corresponding to each gene were analyzed to determine if they fitted a normal distribution (Kolmogorov-Smirnov test) and whether there was a homogeneous variance (Levene test).

Gene transcript levels, bacterial abundance, and *N. ceranae* infection between different groups were evaluated by Student *t* test in those variables which fit parametric assumptions, and by the Mann-Whitney test when the variables did not. *P* values below 0.05 were considered significant. Statistical analyses were performed using Past 3x version 2.17c [63, 64].

Results

Pollen Diets

In order to evaluate the impact of nutritional stress on honeybees, newly emerged individuals were fed with two different diets: *E. grandis* monofloral stored pollen and polyfloral stored pollen (bee bread) (Fig. 1 suppl. data). Two independent trials were performed; *Eucalyptus grandis* stored pollen samples used in both trials had a purity of 99%, while polyfloral stored pollen samples were composed by 18 or 23 different species (trial 1 and 2, respectively; Table 2 suppl. Data).

Both pollen types showed similar bacterial loads and composition, although subtle differences were found (Table 3 and Figs. 2 and 3, suppl. data). *E. grandis* pollen showed a lower OTU diversity than polyfloral pollen, according to rarefaction curves (Fig. 4 suppl. data) and Shannon diversity index (*t*-test,

$t = -15.65$; $t = -8.28$, for trials 1 and 2, respectively, $p < 0.01$ in both cases).

According to DESeq2, abundance of *Lactobacillus* Firm-4 and Firm-5 was similar in both pollen samples in trial 1 and trial 2, and *Bifidobacterium* spp. and *Bartonella apis* were not detected.

Besides that, *N. apis*, *N. ceranae*, and *L. passim* were not detected in any pollen sample (data not shown).

Those results indicate that both groups of honeybees received pollen with different botanical species compositions and subtle differences on associated microbiota and absence (or undetectable level) of the studied pathogens were observed.

Impact of Nutritional Stress on Honeybee Survival and Pollen Consumption

Honeybees fed on *E. grandis* and polyfloral pollen survived almost 60 days under laboratory conditions, and survival curves were similar in both groups. No difference was observed after 30 (Log-rank test; Statistic = 0.02; $p = 0.88$, Fig. 5 suppl. data) or 60 days (Log-rank test; Statistic = 5.56; $p = 0.35$).

Impact of Nutritional Stress on the Gut Microbiota

The impact of different diets on the honeybee gut microbiota was assessed through the analysis of the bacterial community size and community composition, by 16S rRNA gene amplicon sequencing. No significant changes in the bacterial community size were found in the gut microbiota honeybees fed with *E. grandis* monofloral stored pollen and polyfloral stored pollen (Table 4 suppl. data). Regarding OTUs diversity, in trial 1, polyfloral pollen intake increased the diversity according to rarefaction curves and Shannon diversity index, meanwhile in trial 2, no significant difference was observed (MW test, $U = 0.00$ $p = 0.05$; $U = 0.00$ $p = 0.14$ for trials 1 and 2 (control bees), respectively (Fig. 1; Fig. 6 suppl. data).

The gut microbiota was composed by *Lactobacillus* Firm-4 and Firm-5, *Bifidobacterium* spp., *Snodgrassella alvi*, *Pasteurellales/Orbales*, *Bartonella apis*, and *Parasaccaribacter apium*.

The diet did not alter the bacterial general composition of the gut microbiota (trial 1, Unifrac weighted analysis PERMANOVA test statistic = 5.22, $p = 0.09$; Unifrac unweighted analysis PERMANOVA test statistic = 2.21, $p = 0.10$; Bray-Curtis PERMANOVA analysis test statistic = 4.57, $p = 0.11$; non-infected honeybees (control) from trial 2, Unifrac weighted analysis PERMANOVA test statistic = 3.44, $p = 0.1$; Unifrac unweighted analysis PERMANOVA test statistic = 2.08, $p = 0.1$; Bray-Curtis PERMANOVA analysis test statistic = 2.24, $p = 0.1$; Fig. 2).

However, significant differences at OTU level were observed according to DESeq2 results. Consumption of *E. grandis* pollen (model of nutritional stress) decreased the abundance of *Lactobacillus mellifer* and *L. apis* (*Lactobacillus* Firm-4 and Firm-5) compared with polyfloral pollen in trial 1 (Fig. 3). *E. grandis* pollen also decreased the abundance of *Bifidobacterium asteroids and Bifidobacterium corineforme* compared to polyfloral pollen, in trial 2 (non-infected honeybees). On the other hand, *E. grandis* pollen increased the abundance of *Bartonella apis* in both trials.

Impact of Nutritional Stress on Immune Gene Expression

Diets altered the expression of genes involved in honeybee physiology, according to quantitative PCR results (Fig. 4). *E. grandis* pollen consumption decreased the expression level of vitellogenin, glucose oxidase, hymenoptaecin, and lysozyme (MW test, $U = 13$ $p = 0.03$; $U = 6$ $p = 0.05$, $U = 2$ $p = 0.02$, and $U = 11$ $p = 0.01$; respectively), in comparison to polyfloral pollen. On the other hand, expression of abaecin, defensin, and glucose dehydrogenase genes was not affected ($p \geq 0.05$ in all cases).

Impact of Nutritional Stress on *N. ceranae* and the Gut Microbiota

To evaluate the impact of nutritional stress on pathogen infections, honeybees fed on different diets were infected with *N. ceranae* spores. A higher level of *N. ceranae* spores was observed in honeybees fed with *E. grandis* pollen compared to bees fed on polyfloral (4 days post-infection $5.9 \times 10^4 \pm 1.2 \times 10^4$ and $2.4 \times 10^4 \pm 8.6 \times 10^3$ spores/bee, *t*-test $t = -4.01$ $p = 0.02$; 7 days post-infection $2.5 \times 10^6 \pm 9.8 \times 10^5$ and $6.2 \times 10^5 \pm 4.95 \times 10^5$ spores/bee, *t*-test $t = -3.02$ $p = 0.04$, Fig. 5). Twelve days after infection, the number of spores was similar in both groups, reaching $2.6 \times 10^7 \pm 2.9 \times 10^6$ and $2.3 \times 10^7 \pm 2.2 \times 10^6$ spores/bee in honeybees fed with *E. grandis* or polyfloral pollen, respectively. No *Nosema* spp. spores were detected in the honeybees that belonged to the control groups (non-infected honeybees).

Nosema ceranae infection together with diet generated significant alterations on the composition of the gut microbiota (Figs. 1 and 2, Unifrac weighted analysis PERMANOVA test statistic = 4.99, $p = 0.001$; Unifrac unweighted analysis PERMANOVA test statistic = 2.56, $p = 0.004$; Bray-Curtis PERMANOVA analysis test statistic = 5.50, $p = 0.001$).

Infection increased the abundance of two OTUs of *Acetobacteriaceae* in honeybees fed on polyfloral pollen (Fig. 6). However, in the case of honeybees fed on *E. grandis* pollen, the impact was higher since the abundance of five different OTUs increased (*Acetobacteraceae*,

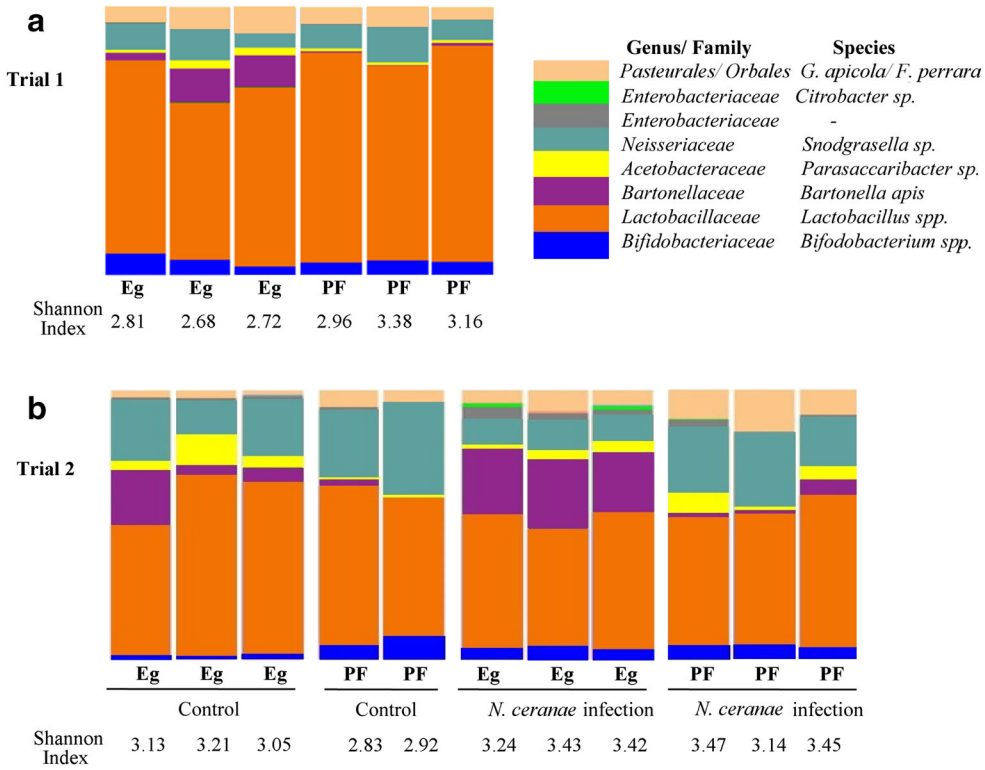

Fig. 1 Relative abundance of bacterial genera and Shannon diversity index of gut bacterial community of honeybees analyzed through 16S rRNA amplicon sequencing (Illumina MySeq). **a** Trial 1, honeybees were fed with *E. grandis* (Eg) or polyfloral (PF) stored pollen. A single MiSeq PE run of 250 cycles resulted in 232,345 reads, of which 221,576 (95%) passed stringent quality thresholds. The data set was rarefied to 29,372 sequences, and after alignment and clustering, we identified a total of 221,560 unique sequences and 159 OTUs (at 97% identity) across the entire data set. **b** Trial 2, honeybees were fed with *E. grandis* or polyfloral stored pollen and infected with *Nosema* spp. spores. In total, 712,706 reads were obtained of which 712,600 passed the quality filters. Subsequently, the data set was normalized to 23,399 sequences per sample, with remaining 112 OTUs

Enterobacteraceae, *Bifidobacterium* spp., *Bartonella apis*, and *G. apicola*).

Interestingly, *N. ceranae*-infected honeybees fed with *E. grandis* pollen showed a lower abundance of *Lactobacillus helsingborgensis* (*Lactobacillus* Firm-5) and a higher abundance of *Bartonella apis* than infected honeybees fed on polyfloral pollen (Fig. 3).

Discussion

Nutritional stress has been proposed as an important driver of honeybee colony losses [7, 9]. In a previous study, we demonstrated that under field conditions, nutritional stress (feeding of colonies mainly on *E. grandis* pollen) promoted the reproduction of *Nosema* spp. and weakened the colonies, decreasing the adult honeybee and brood populations [20]. However, the mechanisms underlying this interaction remain elusive.

In this study, we show that nutritional stress alters the composition of the honeybee gut microbiota and immunity, favoring the infection by *N. ceranae*.

Eucalyptus grandis pollen was used as a model to study nutritional stress, since it has a low lipid content, the protein percentage decreases during the flowering period to values under 20% and it is deficient in isoleucine [20, 21].

Two independent trials using newly emerged honeybees under controlled laboratory conditions were performed. Although the natural establishment of the microbiota can take about 4 days after emergence [63, 64], those bees were able to develop a typical microbiota [28–31]. Bacteria could be acquired by direct contact with the frames after emergence or with pollen ingestion [63, 64].

Nutritional stress (consumption of *E. grandis* pollen) induced the decrease of the abundance of *Lactobacillus mellifer*, *Lactobacillus apis*, or *Bifidobacterium* spp. and increased the abundance of *Bartonella apis*, in healthy bees. Those results were consistent when the microbiota of *N. ceranae*-infected honeybees was assessed.

Pollen bacteria did not explain the differential abundance of these bacterial species in the honeybee gut, since species of *Lactobacillus* Firm-4 and Firm-5 abundances were similar in both pollens from each trial, and *Bifidobacterium* spp. and *Bartonella apis* were not detected.

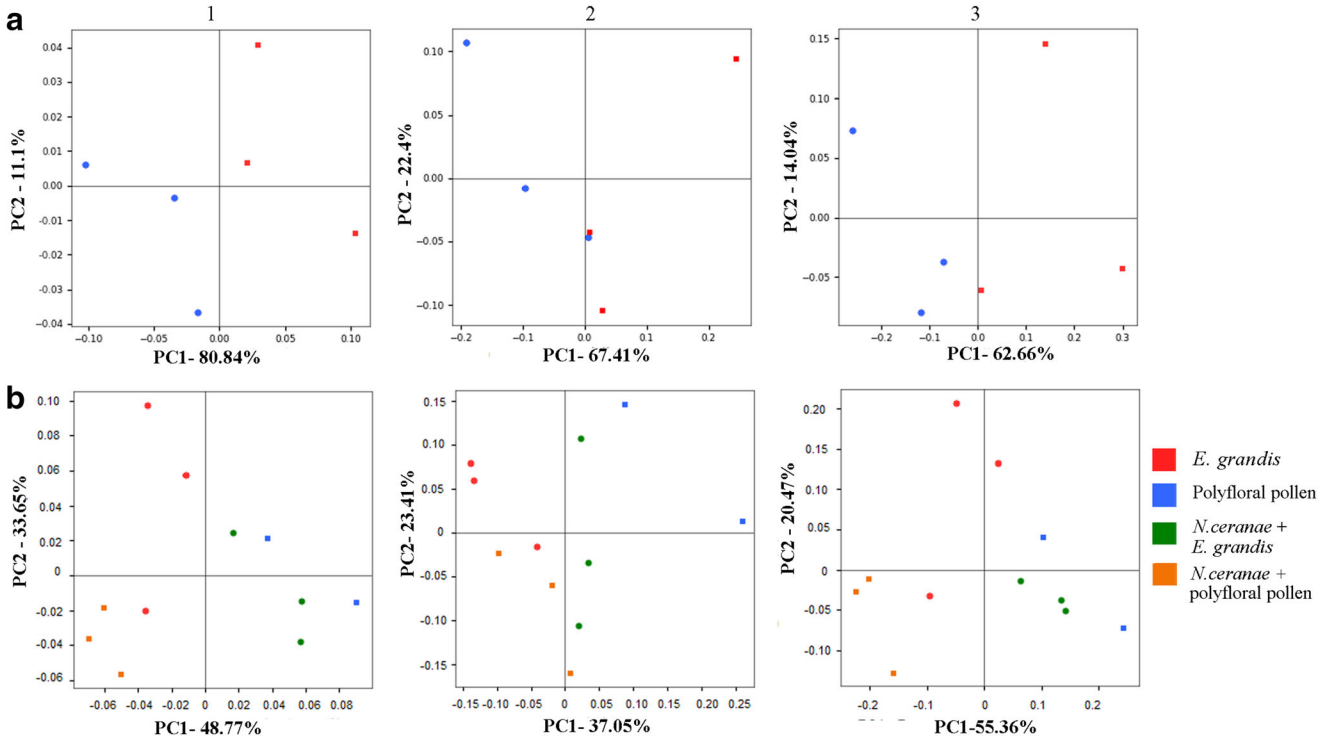

Fig. 2 Principal coordinate analysis (PCoA) of Unifrac weighted (1), Unifrac unweighted (2), and Bray Curtis (3) analyses of bacterial communities of honeybees fed with *E. grandis* (red) and polyfloral (blue) stored pollen from trial 1 (**a**) and infected with *Nosema* spp. from trial 2 (**b**)

Analysis of complete genomes of *Lactobacillus* spp. revealed that these bacteria possess numerous phosphotransferase systems involved in the uptake of sugars, while *Bifidobacterium* spp. has abundant genes for carbohydrate utilization [33, 65, 66]. Both genera are all able to utilize glucose and fructose, the most abundant sugars in the honeybee diet. These bacteria have large putative surface proteins which may be related to adhesion or degradation of plant compounds, and gene clusters for biosynthesis and utilization of trehalose, a disaccharide used for energy storage in insects [33, 65, 66]. Besides that, it has been proposed that *Lactobacillus* spp. and *Bifidobacterium* spp. have in vitro inhibitory effect against *P. larvae* and *Melissococcus plutonius*, causative agents of American and European foulbrood, respectively [67–69].

Bartonellaceae is a bacterial family that includes facultative intracellular pathogens present in a variety of mammals, usually acquired by vector transmission or by animal bites or scratches [70, 71]. Hubert et al. [72] showed that *V. destructor* could act as a reservoir of these bacteria, but their significance in the bee microbiota is still unknown.

Those changes in the honeybee gut microbiota in bees subjected to nutritional stress may represent an important disadvantage regarding the bee physiology and defense against pathogens.

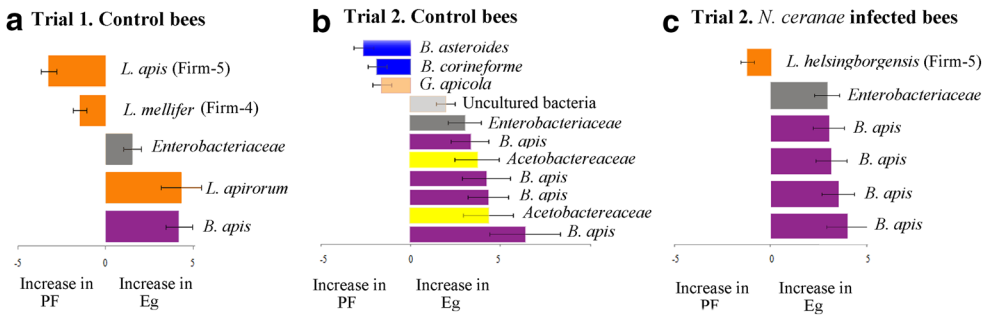

Fig. 3 Comparison of abundance of different OTUs between honeybees fed with *E. grandis* and polyfloral stored pollen, by using DESeq2 analysis. A log2-fold change of >0 indicates that abundance was higher in honeybees fed on *E. grandis* pollen. **a** Samples from trial 1; **b** samples from non-infected honeybees (control) from trial 2; **c** samples from *N. ceranae*-infected honeybees from trial 2. OTUs with less than 60 reads in total were not considered

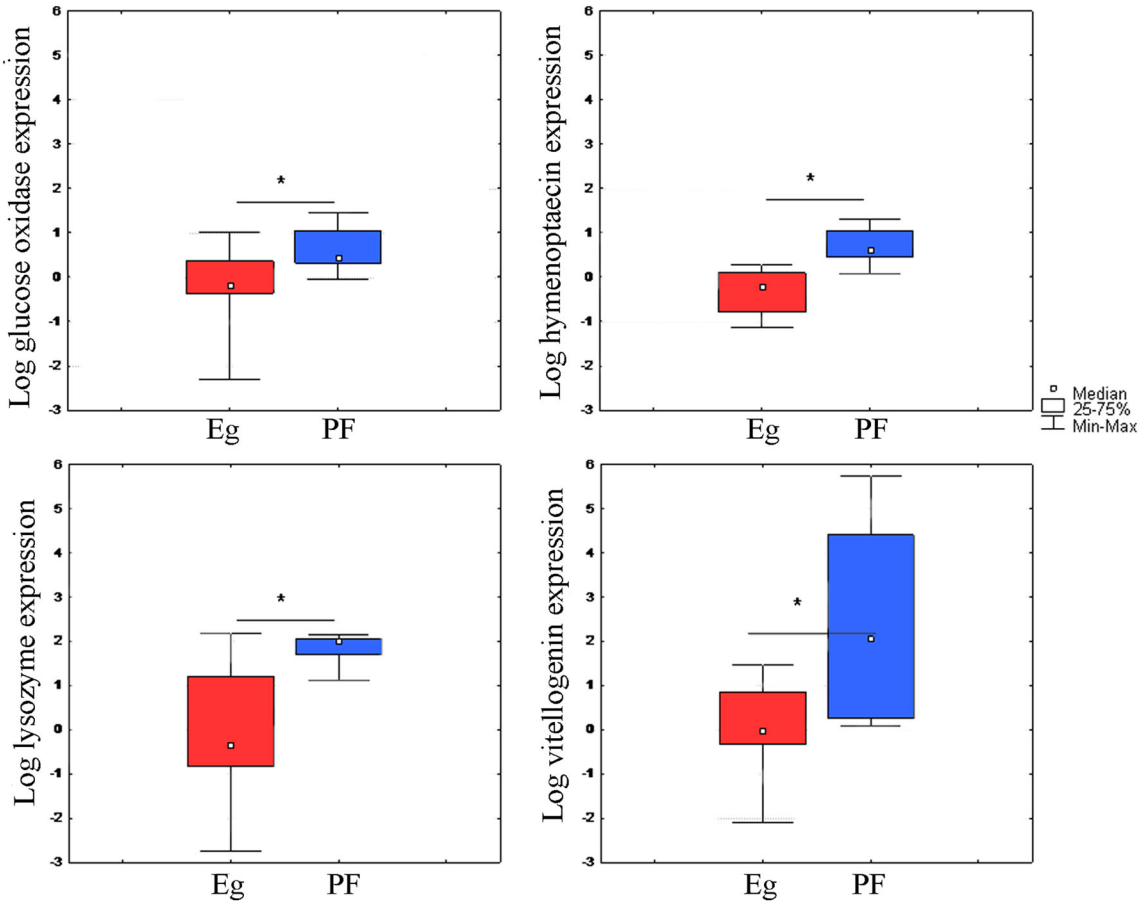

Fig. 4 Relative expression of glucose oxidase, hymenoptaecin, lysozyme, and vitellogenin genes in honeybees fed with *E. grandis* (Eg) or polyfloral (PF) stored pollen. Quantification of gene expression was carried by qPCR. Ten individual honeybees per treatment were used. Asterisks indicate significant differences (p values under 0.05)

Although under laboratory conditions consumption of *E. grandis* pollen did not affect bee lifespan, it decreased the expression level of vitellogenin, compared to polyfloral pollen. Vitellogenin has an important role in the division of labor, foraging specialization, queen longevity, and resistance to oxidative stress [55, 73–75]. A low vitellogenin level is associated with precocious foraging, low protection against oxidative stress and a shorter lifespan [74]. Previous studies have also evidenced that vitellogenin expression changes in response to diet, suggesting it could be an interesting marker of the honeybee nutritional status on healthy honeybees [13, 76–78]. The low vitellogenin expression level found on honeybees fed on *E. grandis* pollen confirmed that honeybees are under nutritional stress.

Eucalytpus grandis pollen consumption also generated a decrease in the glucose oxidase expression, in accordance with Alaux et al. [15]. This enzyme catalyzes the oxidation of β-d-glucose to gluconic acid and hydrogen peroxide, which has antiseptic properties. Those products reach the larval food and honey, contributing to food sterilization and prevention of

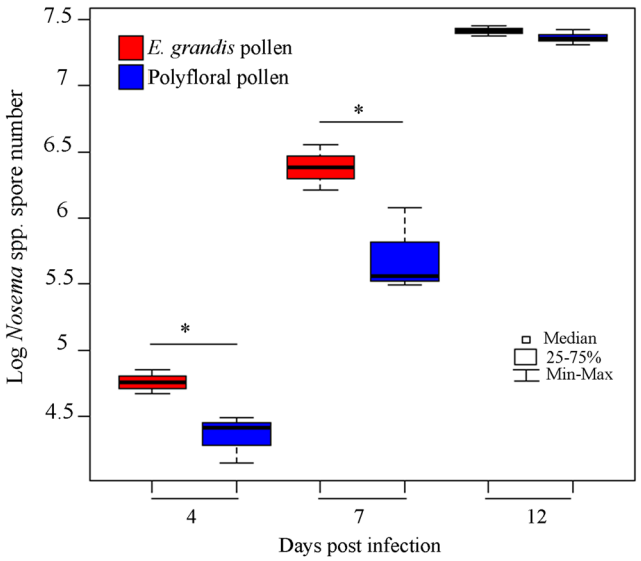

Fig. 5 *Nosema* spp. infection level per honeybee, in honeybees fed with *E. grandis* (Eg) or polyfloral (PF) stored pollen. Asterisks indicate significant differences (p values under 0.05)

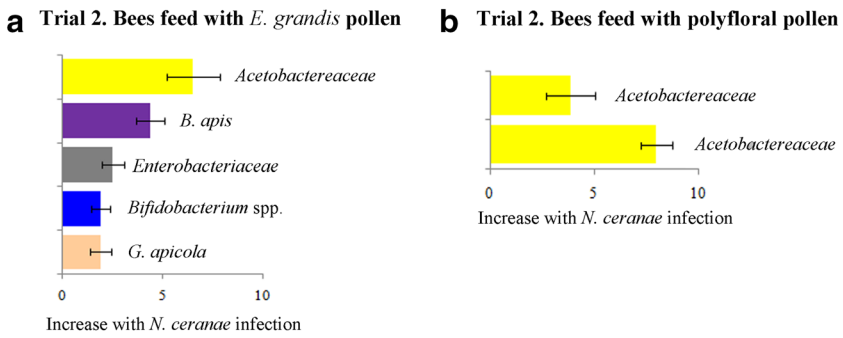

Fig. 6 Comparison of abundance of different OTUs between honeybees fed with *E. grandis* (a) or polyfloral stored pollen (b) and infected by *N. ceranae*, by using DESeq2 analysis. A log2-fold change of >0 indicates that abundance was higher in infected honeybees. OTUs with less than 60 reads in total were not considered

contamination with pathogenic microorganisms [39]. For this reason, glucose oxidase activity is considered as a marker of social immunity [15].

Furthermore, in this study, we confirmed that nutritional stress also decreased the expression of genes involved in individual immunity (hymenoptaecin and lysozyme). Hymenoptaecin is an antimicrobial peptide [79] and lysozyme is a non-specific immunity factor that hydrolyzes (1, 4)-glycosidic bonds in the peptidoglycan layer of the bacterial cell wall [80]. Lysozyme also promotes the expression of antimicrobial peptides, optimizing the immune response [81]. The decrease in the expression of those peptides in honeybees under nutritional stress may be associated with a diminished defensive ability against pathogens.

Finally, nutritional stress promoted the multiplication of *N. ceranae*, during the first stages of infection. Although previous studies had proposed that rich pollen diets stimulated the development of *Nosema* spp. [18, 19, 82], comparisons were performed between honeybees feed with sugar syrup and honeybees fed with different amounts of pollen, but not using honeybees fed with pollen with different botanical composition. In this case, nutritional stress may accelerate *N. ceranae* reproduction, although at the end of the experiment all bees reached were infected by 2×10^7 spores/bee.

The increase of *N. ceranae* spores in honeybees under nutritional stress may be associated with an alteration of the honeybee gut microbiota and a depression of the immune system, which might accelerate the microsporidium multiplication.

In this regard, previous studies have reported that dietary supplementation with *Lactobacillus* spp. or *Bifidobacterium* spp. (or their metabolites) can reduce the infection level of this microsporidium [82–84].

Infection by *N. ceranae* seems to generate a stronger impact on the gut microbiota of nutritional stressed honeybees, compared to honeybees fed on polyfloral pollen. In particular, *N. ceranae* infection increased the abundance of five different OTUs, including *G. apicola*. The association between *G. apicola* and *N. ceranae* has previously been reported by Rubanov et al. [85], and further studies should be carried out to explain this interaction.

Results obtained in the present study contribute to the understanding of the influence of agriculture intensification on honeybee colony health. Vast monoculture areas might cause honeybee's nutritional stress, decreasing the abundance of potentially beneficial microorganisms of the honeybee gut microbiota, decreasing the expression of honeybee immune-related genes, and favoring the multiplication of pathogens like *N. ceranae*. Besides that, *N. ceranae* infection can also depress the honeybee immune system [86], subtly alter the honeybee gut microbiota [85], cause energetic stress [87] and digestive problems affecting the nutritional status of honeybees [47].

Those results evidence the existence of a complex network between nutrition, gut microbiota, immunity, and pathogen infection. These links, which have been profusely described in other animal species and humans, encourage the design of strategies for the improvement of honeybee health through nutritional approaches or modulation of the gut microbiota using beneficial microbes.

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