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Abstract

Infestations of the ectoparasitic mite *Varroa destructor* cause significant honeybee colony weakening, due to both host-feeding and virus transmission effects. In addition to the natural (i.e., behavioral) and innate (i.e., cellular and humoral response) mechanisms of defense against pathogens and parasites, a contribution to maintain a good colony health may derive from the resident microbial community.

The present study investigated the variations in the immune-related gene expression levels and in the relative abundance of representative bacterial taxa of the core honeybee microbiota in colonies infested by *Varroa* mites in comparison with honeybees from non-infested colonies. Significant changes in the selected bacterial taxa were detected in immune-suppressed individuals from *Varroa*-infested colonies. While a high variability characterized different insect stages randomly sampled from infested and non-infested hives, emerging adults bearing sucking *Varroa* mites on their body showed a considerably higher DWV titer and a more important immunosuppression compared with adults without mites. On the other hand, the abundance of the overall bacterial community and of specific bacterial taxa increased significantly in parasitized adults. Target microbes included lactobacilli, bifidobacteria, *Brevibacillus laterosporus*, Gammaproteobacteria and Betabroteobacteria species. A possible compensation mechanism involving the core bacterial community in *Varroa*-infested hives is discussed.

Key words: Mite; DVW; AMP; Immunosuppression; Lactobacillus; Bifidobacterium; Snodgrassella; Social immunity.

Introduction

Health preservation of the western honey bee *Apis mellifera* is a global issue to ensure efficient crop pollination services and to support a range of bee-associated products like honey, royal jelly, bee bread, propolis, venom, and wax. Several efforts have been devoted to understanding the mechanisms contributing to the regulation of unusually high numbers of colony mortalities, often associated with diverse pathogens, parasites and, more in general, stress factors (VanEngelsdorp et al., 2009; Hedtke, Jensen, Jensen, & Genersch, 2011; Dainat, Evans, Chen, Gauthier, & Neumann, 2012; Nazzi et al., 2012). Among them, infestations of the ectoparasitic mite *Varroa destructor* are cause of significant colony weakening, due to both host-feeding and virus transmission effects (Le Conte, Ellis, & Ritter, 2010; Nazzi & Le Conte, 2017). Although a variety of viruses can be transmitted by *Varroa* to *A. mellifera*, the deformed wing virus (DWV) is one of the most widespread species whose prevalence and persistence in symptomatic or asymptomatic colonies is strictly dependent on the presence of the mite (Martin, Ball, & Carreck, 2010). *Varroa* life cycle includes a phoretic phase in which mites spread out riding on adult workers or drones, and a reproductive phase that starts when mites invade uncapped brood cells to feed on honeybee immature stages and to reproduce, after the cell has been sealed (Rosenkranz, Aumeier, & Ziegelmann, 2010). The natural mechanisms of defense against the mite include the ‘‘grooming behavior” and the ‘‘removal of parasitized brood cells” (Guzman-Novoa, Emsen, Unger, Espinosa-Montaño, & Petukhova, 2012). An additional hygienic behavior of the honey bee is the collection of resin that is incorporated into the nest as propolis, exhibiting broad-spectrum antimicrobial properties (Simone-Finstrom & Spivak, 2010; Pusceddu et al., 2017). Innate mechanisms of defense, normally acting at individual level, are also involved in the colony response to high *Varroa* infestation (varroosis). These include the production of antimicrobial peptides, melanization, phagocytosis and the enzymatic degradation of pathogens, in response to the combined action of the mite and DWV, which normally causes immunosuppressive effects on different insect stages (Di Prisco et al., 2016). An additional contribution to insect innate mechanisms of defense might be

given by the beneficial microbial community inhabiting their body (Engel & Moran, 2013). The core bacterial community of honeybees resides in the intestine and is represented by 8-10 species with more than 97% 16S ribosomal DNA sequence identity (Moran, 2015). They include the two Gammaproteobacterial species *Gilliamella apicola* and *Frischella perrara* (Orbaceae), the betaproteobacterial species *Snodgrassella alvi* (Neisseriaceae) in the midgut, and the Gram-positive bacteria *Lactobacillus spp.* and *Bifidobacterium spp.* in the rectum, as the most abundant (Kwong & Moran, 2016). While the specific function of these species and their actual symbiotic relationship with the host are still unclear, in analogy with the vertebrate gut community, they are supposed to contribute to honeybee health, buffering the adverse action of environmental stress factors, including pathogens and parasites (Engel et al., 2016; Alberoni, Gaggìa, Baffoni, & Di Gioia, 2016). Recent metagenomic studies are providing evidence of the contribution of gut bacteria to food detoxification and to the immune system improvement (Kwong, Engel, Koch, & Moran, 2014; Kwong, Mancenido, & Moran, 2017).

In addition to this core bacterial community, other bacterial residents of the bee body may become significantly important to counteract against certain pathogens. This is the case of the endospore former *Brevibacillus laterosporus* (Ruiu, 2013; Marche, Mura, & Ruiu, 2016), whose antimicrobial properties and the inhibitory effects on the honeybee pathogen *Paenibacillus larvae* have been documented (Alippi & Reynaldi, 2006; Hamdi & Daffonchio, 2011).

Whilst the beneficial role of a good microbial balance for honeybee colony health is well recognized (Budge et al., 2016), available information on the dynamics of core bacterial species in honeybees parasitized by DWV-carrying *Varroa* is still poorly understood. Significant changes in the honeybee microbiome were observed in honeybees parasitized by *Varroa* (Sandionigi et al., 2015), and some bacterial species were reported to be shared by both the parasite and its host (Hubert et al., 2016). Moreover, microbial infections were found to be associated with the wounds caused by mite punctures (Kanbar & Engels, 2013).

On the other hand, most data on honeybee response to DWV-*Varroa* combination derive from laboratory studies (Di Prisco et al., 2016), therefore, investigations on the actual scenario at the colony level are needed.

The present study describes the variations in the immune-related gene expression levels and in the relative abundance of representative bacterial taxa of the core honeybee microbiota in colonies infested by *Varroa* mites in comparison with honeybees from non-infested colonies. The aim of the present investigation was to test the hypothesis that significant changes in the core microbial community occur in immune-suppressed individuals from *Varroa*-infested colonies.

Materials and methods

Sample collection and processing

Honey bee samples were collected in 2014 from an experimental apiary consisting of 10 beehives located in Nuoro (Central Sardinia, Italy), an area characterized by common *Varroa* infestations. During the experimental period, a group of five beehives (non-infested) underwent routine antivarroa treatments based on applications of amitraz (Apivar®) and thymol (Apiguard®) in summer. A second group of five beehives (infested) did not receive any acaricidal treatment in order to facilitate a natural mite population development. *Varroa* management in the first group was suspended a month before honeybee sampling activities began (October). At this date, *Varroa* infestation level in each beehive was evaluated, determining the percentage of infested workers (Pappas & Thrasyvoulou, 1988). On the same date, colony strength was determined through an estimation of the sealed brood extent and of the amount of adult bees. For this purpose, one-sixth of a Dadant-Blatt frame (188 cm^2) was used as a unit of measure, converted in the table of the results section in number of sealed cells and adult bees obtained by multiplying the number of sixth of each matrix for 780 and 254, respectively, according to the method described by Marchetti (1985).

At the same time, three replicated pools (1 pool= 10 specimens) of the following insect samples were collected from each infested or non-infested hive: 1) honeybee workers, 2) emerging adults, 3)

pupae, and 4) larvae (5-6 day old). None of these samples bore *Varroa* mites on their body. Additional samples of emerging adults were collected from infested hives distinguishing between insects bearing (*Varroa* attached) or not (without *Varroa*) a sucking mite on their body. After collection, each individual was immediately frozen, before being surface-sterilized with sodium hypochlorite (0.2 %) (Genersch et al., 2013), rinsed in sterile and cold phosphate-buffered saline (PBS), pooled (n = 10) and homogenized in PBS using sterile plastic pestles. The homogenate was filtered through sterile gauze to remove any debris, and the remaining suspension was centrifuged at 15,000 x g for 15 min at 4 °C. After discarding the supernatant, aliquots of the remaining pellet were used for either DNA or RNA extraction.

RNA extraction, retro-transcription and relative quantification of immune-related genes

Total RNA extraction from homogenized pools was routinely conducted with TRIzol® Reagent (Life Technologies) according to manufacturer's instructions (Chomczynski & Sacchi, 1987). All RNA samples, after being quantified and purity checked with NanoDrop ND-1000 Spectrophotemeter (Thermo Scientific), were treated with RQ1 RNase-Free DNase (Promega). An aliquot (2 µg) of each sample was used for first-strand cDNA synthesis using SuperScript® II Reverse Transcriptase (Life Technologies), oligo dT (Promega), and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Life Technologies) according to the manufacturers' protocol. *Power* SYBR® Green PCR Master Mix (Life Technologies) was used for quantitative PCR experiments on an Applied Biosystems 7900HT Fast Real-Time PCR System, with the following cycle conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, annealing for 1 min at a variable temperature depending on the primer pair, and extension at 60 °C for 1 min. A list of genes and primer pairs used in qPCR reactions with 300 ng cDNA template in a total volume of 20 µl, is provided in Table 1. Forward and reverse primers were selected from previous reports or newly designed on gene sequences deposited in GenBank (National Center for Biotechnology Information, NCBI) using Primer3web (version 4.0.0) (Untergasser et al., 2012).

After being validated, each primer set was tested for PCR efficiency by standard curve and dissociation curve analyses (Pfaffl, 2001).

Additional quantitative reverse transcription PCR (RT-qPCR) analyses were conducted for a relative quantification of the deformed wing virus (DWV).

Each analysis was performed in three technical replicates and included at least three biological replicates (each biological replicate included 10 insects).

DNA extraction and relative quantification of the core bacterial community

Homogenized honeybee pools were resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 8), 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100. Then, total DNA extraction was routinely conducted as described in Evans et al. (2013). After DNA quantification with NanoDrop ND-1000 Spectrophotemeter (Thermo Scientific), samples were normalized for qPCR analyses.

For a relative quantification of the core bacterial community in different honeybee stages either from infested or non-infested hives, normalized DNA samples (20 ng), were used as templates in Quantitative Real-Time PCR (qPCR) reactions employing *Power* SYBR® Green PCR Master Mix (Life Technologies) and primer pairs targeting specific regions of 16S rRNA gene of the following representative taxa, including honeybee core bacterial species: Gammaproteobacteria, Betaproteobacteria, *Lactobacillus* spp., *Bifidobacterium* spp. Universal bacterial primers were also used to assess total bacterial numbers (Table 1, Supplementary file S1). For relative quantification of *B. laterosporus* in honeybee, species-specific primer pair targeting *cpbA* gene were used (Marche et al., 2017).

Relative q-PCR reactions were conducted in a total volume of 20 µl in an Applied Biosystems 7900HT Fast Real-Time PCR System with the following cycle conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, annealing for 1 min at a variable temperature depending on the primer pair, and extension at 60 °C for 1 min.

Primers efficiency was preliminarily tested by standard curve and dissociation curve analyses (Pfaffl, 2009). Each sample was run in technical triplicates and at least three biological replicates were involved in analyses.

Additional PCR analyses were conducted on all samples to verify the possible presence of the honeybee pathogens *Paenibacillus larvae* and *Nosema ceranae*, employing primer pairs shown in Table 1.

Statistical analysis

Statistical analyses were performed with SAS software (version 9.1) with significance level set at $\alpha = 0.05$ (SAS, 2004).

Data on *Varroa* infestation level and colony strength were compared between hive groups using Wilcoxon rank-sum test.

The relative abundance of the target genes was analyzed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) for gene expression normalization to the endogenous reference genes *5S rRNA* and *β-actin* that were previously validated as suitable honeybee internal control gene for PCR normalization (Ryabov et al., 2014). A one-way ANOVA was used to test for significant differences across the treatments according to Bonferroni's test. T-test was used for comparison between two groups. Data are presented as the fold change relative to the control.

The CANCORR procedure of SAS, including canonical correlation (CCA) and redundancy analysis (RDA), was performed for analyzing the relationship between environmental variables (infestation level, family strength, stage of the bee, DWV load) and the relative abundance of immune-related gene transcripts and of selected bacterial taxa.

Linear regression analyses were used for analyzing the relationship between the DWV load and the relative expression of immune related genes or the relative abundance of bacterial taxa.

Linear regression and RDA analyses were completed using Vegan package (Oksanen et al., 2013) of R (version 3.4.0; R Core Team, 2013). Data matrix was square root transformed before running these analyses.

RESULTS

Varroa infestation and colony strength

Colony strength level, determined using one-sixth of a Dadant-Blatt frame (188 cm^2) as a unit of measure (Marchetti, 1985), and the percentage of *Varroa* infestation in the infested and non-infested hive groups of the experimental apiary, are shown in Table 2. Comparing the two groups, no significant differences emerged for colony strength, expressed in terms of number of sealed cells ($W = 20$, $P = 0.1508$) and of adult bees ($W = 21$, $P = 0.0952$), while a significantly higher (more than a hundred-fold) *Varroa* infestation level ($W = 0$, $P = 0.0079$) was detected in the infested compared with the non-infested group.

Relative abundance of the Deformed Wing Virus (DWV) and of the overall bacterial community

The relative abundance of the Deformed Wing Virus in different honeybee stages from infested compared with non-infested hives, including emerging adults bearing mites on their body, is shown in Fig. 1. DWV was detected in all samples from infested and non-infested hives with a generally increased virus load in samples from infested hives ($F_{4,40} = 14.56$, $P < 0.0001$). The virus load was significantly increased in larvae ($F_{1,16} = 17.44$, $P = 0.0007$), pupae ($F_{1,16} = 5.21$, $P = 0.0456$), emerging adults ($F_{1,16} = 9.32$, $P = 0.0076$), and workers ($F_{1,16} = 25.11$, $P = 0.0002$). A significantly higher load was found in *Varroa*-bearing emerging adults ($F_{1,16} = 14.60$, $P = 0.0015$).
Although DWV was detected in both *Varroa*-infested and non-infested hives, no signs or symptoms associated with the disease it causes were observed.

The variations in the relative abundance of the overall bacterial number in honeybee samples from infested in respect to non-infested hives are shown in Fig. 2. While differences were not detected for the diverse insect stages analyzed (larvae: $F_{1,16} = 1.03$, $P = 0.3245$; pupae: $F_{1,16} = 0.37$, $P = 0.5523$; emerging adults: $F_{1,16} = 1.33$, $P = 0.2654$; workers: $F_{1,16} = 2.66$, $P = 0.1222$), a significant increase was found in emerging adults bearing sucking *Varroa* mites $F_{1,16} = 6.74$, $P = 0.0195$.
No symptoms related to the two common honeybee pathogens *Paenibacillus larvae* and *Nosema ceranae* were observed in the colony, and these species were never molecularly detected in samples used in this study.

Relative expression of immune-related genes in honeybees from infested and non-infested colonies

Canonical correlation using the four environmental variables (infestation level, family strength, stage of the bee, and DWV load) as predictors of the relative expression of immune-related genes disclosed a statistically significant full model (Wilks' Lambda = 0.08849820, F (20, 209.90) = 11.31, $p < 0 .0001$), revealing four canonical functions with squared canonical correlations of 0.81, 0.35, 0.22, 0.05, respectively. Function 1 was considered the most important in the model, contributing more than 80 % of the variance. The first canonical variable of this correlation function is a weighed sum of all environmental variables (standardized canonical coefficients), with a heavier contribution of DWV load (0.72), a moderate influence of the honeybee stage (0.34) and an insignificant weight of family strength (0.1355) and hive infestation level (-0.1448). On the other side, the standardized canonical coefficients for the immune-gene variables show that PGRP-S1, Nimrod-C2, and Defensin-2 gave a greater contribution to the second canonical variable (0.68, 0.41, and 0.39, respectively). Figure 3 displays the correlations between all variables involved in the model.

Relative abundance of core bacterial community in honeybees from infested and non-infested colonies

Canonical correlation using infestation level, family strength, stage of the bee, and DWV load as environmental variables revealed significant effects on the relative abundance of selected bacterial taxa (Wilks' Lambda = 0.06334807, F (20, 209.90) = 13.62, p < 0 .0001), disclosing four canonical functions with squared canonical correlations of 0.89, 0.39, 0.05, 0.01, respectively.

The first function of the correlation model contributed more than 90 % of the variance. Based on the standardized canonical coefficients, the first canonical variable received a different contribution by environmental variables, including DWV load (0.82), honeybee stage (0.23), family strength (-0.05) and hive infestation level (-0.21). On the other side, the standardized canonical coefficients for the bacterial taxa variables show that Betaproteobacteria, *B. laterosporus*, and *Lactobacillus* spp. gave a greater contribution to the second canonical variable (0.94, 0.26, and 0.22, respectively). Figure 4 displays the correlations between all variables involved in the model.

Relative expression of immune-related genes in emerging adults bearing sucking mites.

Relative expression of immune-related genes in emerging adults with *Varroa* mites attached in respect to those without *Varroa* from the same infested hive are shown in Fig. 5.

In general, honeybees with *Varroa* mites attached to their body showed a significant decrease in the relative expression level of these target genes. More in detail, a significant decrease in the transcript level of *Hymenoptaecin* (t = 10.92, P < 0.0001), *Defensin-2* (t = 8.77, P < 0.0001), *Apidaecin* (t = 8.80, P < 0.0001), *PGRP-S1* (t = 5.02, P = 0.0005), and *Nimrod-C2* (t = 4.59, P = 0.0009), was observed.

Canonical correlation using the presence/absence of *Varroa* on bee body and DWV load as environmental variables revealed a significant effect of these variables on the expression level of immune-related genes (Wilks' Lambda = 0.06662225, F (10, 22) = 6.32, p = 0 .0002), with a

canonical correlation function explaining more than 99 % of the variance. The two environmental variables presence/absence of *Varroa* and DWV load significantly contributed to the first canonical variable, as represented by their standardized canonical coefficients (-0.8980 and -0.1035, respectively), and appeared obviously correlated with each other.

The effects of DWV load on the expression of each selected gene were further examined using linear regression analysis, which highlighted a significant negative correlation for *Hymenoptaecin* (R^2 = 0.78, F = 57.93, P < 0.0001), *Defensin-2* (R^2 = 0.83, F = 79.11, P < 0.0001), *Apidaecin* (R^2 = 0.77, F = 52.70, P < 0.0001), *PGRP-S1* (R^2 = 0.63, F = 26.76, P < 0.0001), *Nimrod-C2* (R^2 = 0.59, F = 23.23, P < 0.001) (Fig. 7A).

Relative abundance of core bacterial community in emerging adults bearing sucking mites

Relative abundance of selected bacterial taxa in emerging adults from infested hives comparing honeybees with and without *Varroa* attached are shown in Fig. 6. A significantly increased relative abundance of Gammaproteobacteria (t = -7.14, P < 0.0001), Betaproteobacteria (t = -5.13, P = 0.0004), *Lactobacillus spp*. (t = -8.81, P < 0.0001), *Bifidobacterium spp*. (t = -6.19, P = 0.0001), and *B. laterosporus* (t = -4.35, P = 0.0012) was detected in emerging adults with attached mites compared with adults without mites.

Canonical correlation showed that the presence/absence of *Varroa* on bee body and DWV load (environmental variables) significantly influenced the relative abundance of selected bacterial taxa (Wilks' Lambda = 0.03471774, F (10, 22) = 9.61, p < 0 .0001), and the model explained more than 80 % of variance. Based on the values of standardized canonical coefficients, the DWV load (1.8178) appeared to contribute more significantly to the first canonical variable in respect to the presence/absence of *Varroa* (-0.8431).

As a result of linear regression analysis the DWV load was shown to be positively correlated with the relative bacterial abundance for all selected taxa, including Gammaproteobacteria (R^2 = 0.71, F = 39.72, P < 0.0001), Betaproteobacteria (R^2 = 0.67, F = 33.00, P < 0.0001), *Lactobacillus* spp. (R^2

= 0.81, F = 66.25, P < 0.0001), *Bifidobacterium* spp. (R^2 = 0.71, F = 38.82, P < 0.0001), *B. laterosporus* (R^2 = 0.65, F = 29.72, P < 0.0001) (Fig. 7B).

Figure 8 shows a RDA ordination plot where the direction of the arrows for individual factors describe how the factors are correlated with each other, and the magnitude of the arrows determines the relative importance of that factor or interaction. The RDA ordination explained 84.3 % of the variance. The distribution of honeybee sample pools was mostly explained by the presence/absence of *Varroa* mites on their body, which led to a significant separation of these samples on the diagram. Pools of emerging adults bearing mites appeared associated with higher DWV load and higher abundance of selected bacterial taxa, thus confirming the pattern described by canonical correlation. Instead, a greater expression of immune related genes appeared associated with samples without *Varroa*.

Discussion

The impact of *V. destructor* on the honeybee is not limited to the direct consequences of its sucking activities, but a significant contribution to colony weakening phenomena derives from the pathogenic action of mite-carried viruses, among which DWV plays a major role (Wilfert et al., 2016). As a result of the intimate relationship between this virus and *Varroa*, it is difficult to differentiate between specific pathogenic and parasitic effects on individual bees (Nazzi et al., 2012). Furthermore, a higher complexity characterizes the hive ecosystem (Nazzi & Pennachio, 2014). It is generally accepted that the combined *Varroa*-virus action causes a general honeybee immunosuppression (Di Prisco et al., 2016) and these effects are significantly correlated with the virus titer in the host (Kuster, Boncristiani, & Rueppell, 2014).

Accordingly, we observed a general downregulation of immune-related genes in most honeybee samples from *Varroa*-infested compared with non-infested hives. However, a high variability among samples lacking a *Varroa*-mite attached to their body was observed, and such variations were mostly explained by the honey bee stage and the DWV load. A clear immune-impairing effect

was instead associated with bees bearing mites on their body. As expected, a higher DWV load was detected in samples from infested compared with non infested hives, and a significantly higher virus titer was found in *Varroa*-bearing emerging adults, which confirms that *Varroa* and virus actions are tightly correlated (Di Prisco et al., 2016).

The quantitative variation in the overall bacterial community in different honeybee stages from infested in respect to non-infested hives, was not significant, while a significant increase in its abundance was found in emerging adults bearing *Varroa* mites. As expected, variations in the abundance of specific bacterial taxa were significantly related to the honey bee stage. In addition differences were correlated to the DWV load, which aligns with the assumption that the bacterial balance in the honeybee body is an indicator of the colony health conditions (Kwong & Moran, 2016). When no significant changes were detected for the total bacterial number, we could speculate that the reduction in specific bacterial groups were balanced by the increase in other species groups. However, such changes were highly variable among different stages of honeybees and no clear interpretation could be made, suggesting that they might just be related to an altered colony health status (Budge et al., 2016). A clear increase of the relative abundance of selected bacterial taxa was instead associated with emerging adults bearing mites on their body, and this trend was significantly correlated with the combined DWV-*Varroa* action.

The inconsistency of fold changes observed comparing different honeybee stages is likely to be directly related to the bacterial community diversity characterizing each development stage (Martinson et al., 2012). Accordingly, pupae are expected to lose most of the gut bacterial community acquired during the larval stage. However, our study did not keep into account a direct comparison between insect stages, but the relative changes in *Varroa*-infested in respect to non-infested colonies.

Other differences may derive from the behavior of *Varroa* mites that feed on the host only during a specific period of its developmental life cycle (Rosenkranz et al., 2010). On the other hand, *Varroa* microbiome may directly influence the resident microbial community composition in a parasitized

honeybee (Sandionigi et al., 2015), including a possible bacterial transfer via mites (Hubert et al., 2016). The increase in core bacterial species we detected in emerging honeybees bearing mites on their body, is in line with the relative increase in several taxa including *S. alvi* and some *Lactobacillus* species that was observed in colonies with high *Varroa* infestation levels (Hubert et al., 2017).

The interactions of honeybees between each other and with the hive or the outside environment are other factors affecting the individual and colony microbiota (Powell, Martinson, Urban-Mead, & Moran, 2014). Everything considered, it is difficult to give a conclusive interpretation of the causes of microbiome changes in colonies infested by *Varroa*. In the attempt to overcome such complexity, we conducted a direct comparison between emerging adults bearing or lacking *Varroa* mites on their body, collected from the same infested hives, thus reducing the number of variables affecting the microbiome composition. Adults emerging with a *Varroa* mite attached to their body were characterized by a considerably higher DWV titer and a more important immunosuppression compared with adults without mites from the same hive. In parasitized adults, the abundance of the overall bacterial community and of each taxa targeted in this study, increased significantly. Gammaproteobacteria include the two Gram-negative species *G. apicola* and *F. perrara*. The first has been characterized as a fermentative bacterium involved in the carbohydrate metabolism (Kwong and Moran, 2013) including the ability of breaking down potentially toxic carbohydrates, and the second is believed to be directly involved in melanization immune response through scab formation in the gut of its host (Engel, Bartlett, & Moran, 2015). Betaproteobacteria mainly refer to Neisseriaceae, that includes *S. alvi*, a species involved in the formation of biofilm-like layers on the ileum epithelium (Engel, Martinson, & Moran , 2012). This betaproteobacterium, like the previously mentioned species, is a dominant member of the honey bee gut microbiota and appears to be involved in both nutrition and stress response (Kwong et al., 2014; Zheng et al., 2016). Lactobacilli and bifidobacteria include lactic acid bacterial (LAB) symbionts of *A. mellifera*, that play an important role in honeybee nutrition and antimicrobial response against pathogens (Vasquez

et al., 2012; Corby-Harris, Maes, & Anderson, 2014; Killer, Dubná, Sedláček, & Švec 2014), in analogy with their ability to modulate the host immune response in humans and animals (Servin, 2004; Audisio, 2017). The relative increase in the abundance of *B. laterosporus* in emerging adults parasitized by *Varroa* supports its possible involvement in the response to an altered health status (Hamdi & Daffonchio, 2011).

A first and intuitive explanation for the increase in our target bacterial taxa, that represent the core bacterial community of the honeybee, may relate to the fact that individuals with a lowered immune capacity would reduce their barriers against a variety of bacterial species whose proliferation is consequently favored. However, everything might be under the control of a more sophisticated mechanism developed through evolution. It has been reported that the composition and proportion of resident bacterial species living in the honeybee gut are the result of a co-evolution process (Kwong & Moran, 2015) and that the susceptibility to parasites is affected by microbiota composition (Schwarz, Moran, & Evans, 2016). This implies that symbiotic bacteria and their host have established an equilibrium under the control of the insect immune system (Anderson, Sheehan, Eckholm, Mott, & DeGrandi-Hoffman, 2011), while other bacterial species have instead developed strategies to overcome insect immune defenses, thus evolving toward a pathogenic interaction (Hornef, Wick, Rhen, & Normark, 2002). It can be inferred that when individual honeybees are immune-impaired, the beneficial inhabitants of their gut go through a natural increase. Such dynamics, observed as a result of the combined parasitic and pathogenic action of DWV and *Varroa*, may constitute a natural mechanism of compensation. Accordingly, it was reported that *Varroa* infestation affects the carbohydrates and protein metabolism in parasitized bees, which would be compensated by the increase in the proportion of core gut bacteria involved in the regulation of these metabolic functions (Hubert et al., 2017). Moreover, some of the honeybee bacterial symbionts can directly stimulate the immune-impaired immune system of their host inducing the production of AMPs (Kwong et al., 2017). Such mechanisms are the result of an evolutionary selection process that led to establish a native honeybee bacterial community where

the prevailing species are few and beneficial (Kwong & Moran, 2016). Everything considered, the quantitative variations in representative taxa of the honeybee core bacterial community support their direct or indirect contribution to the honeybees health in colonies infested by *Varroa* mites. Given the functional diversity within the honeybee microbiome (Engel et al., 2016), further studies are needed to clarify the actual role of each bacterial species and to identify those that may represent the best candidates in programs aiming at maintaining and improving bee health.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgements

This study was financially supported by the Italian Ministry of Education, University and Research (MIUR) (PRIN Project 2012 “Honeybee social immunity: behavioral, chemical and microbiological aspects”, 2012RCEZWH); and by Autonomous Region of Sardinia LR 7/07 TENDER 2013 (Project: Studio, definizione e applicazione di mezzi e strategie per il controllo biologico delle batteriosi delle api in un ambiente mediterraneo).

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Table 1 - Oligonucleotide sequences used for analyses.

Species	Gene[a]	Primer sequence[b]		Annealing temperature (°C)	Reference[c]
		Sense 5'- 3'	Antisense 5'- 3'		
Apis mellifera	*Hymenoptaecin*	5' CTCTTCTGTGCCGTTGCATA3'	5'GCGTCTCCTGTCATTCCATT3'	60	Evans et al., 2006
Apis mellifera	*Defensin-2*	5'GGGTAACGTGCGACGTTTTA3'	5'GCAACTACCGCCTTTACGTC3'	60	NM_001011638.1
Apis mellifera	*Apidaecin*	5'TTTTGCCTTAGCAATTCTTGTTG5'	5'GGATGAGGTGGTCTTGGTT3'	63	Simone et al., 2009
Apis mellifera	*PGRP-S1*	5'GAGGCTGGTACGACATTGGT3'	5'TTATAACCAGGTGCGTGTGC3'	60	XM_001121036.4
Apis mellifera	*Nimrod C2*	5'GCGTGGAGGACGGGAAACCG3'	5'ACATCGATGGCAGAGCGGCG3'	60	Nazzi et al., 2012
Apis mellifera	*5S rRNA*	5' TTAGGAGACAGGCGGTGGA3'	5' TAGACGAACCTTTGGCAGCA3'	60	XM_006570236
Apis mellifera	*B-actin*	5'AGGAATGGAAGCTTGCGGTA3'	5'AATTTTCATGGTGGATGGTGC3'	60	Ryabov et al., 2014
Universal bacteria	16S rRNA	5'AGAGTTTGATCCTGGCTCAG3'	5'ACGGCTACCTTGTTACGACTT3'	60	Weisburg et al., 1991
Gammaproteobacteria	16S rRNA	5'TTGTTGCCAGCGATTAGG3'	5'ATTCTGATTCACGATTACTAGC3'	60	Li et al., 2012
Betaproteobacteria	16S rRNA	5'AAGCGGTGGATGATGTGG3'	5'TGATGGCAACTAATGACAAGG3'	61	Li et al., 2012
Lactobacillus spp.	16S rRNA	5'TAACGCATTAAGCACTCC3'	5'GCTGGCAACTAATAATAAGG3'	63	Li et al., 2012
Bifidobacterium spp.	16S rRNA	5'CAAGCGAGAGTGAGTGTACC3'	5'GCCGATCCACCGTTAAGC3'	57	Li et al., 2012
B. laterosporus	*cpbA*	5'GCTTCACACGATCAGCAACC3'	5'TGTAGGCGGGCAGCTAAAAA3'	60	Marche at al., 2017
Nosema ceranae	*large subunit rRNA*	5'CGGATAAAAGAGTCCGTTACC3'	5'TGAGCAGGGTTCTAGGGAT3'	57	Nazzi et al., 2012
Deformed Wing Virus	*Polyprotein helicase domain*	5'GCGCTTAGTGGAGGAAATGAA3'	5'GCACCTACGCGATGTAAATCTG3'	60	Nazzi et al., 2012
Paenibacillus larvae	*gyrA*	5'ATGCGGTCATCCCTATTGAG3'	5'GGTCATCTTCCCGCAAATTA3'	60	De Graaf et al., 2013

[a]*PGRP-S1* = Peptidoglycan recognition protein S1

[b]For validation, primer pairs were preliminarily checked in end-point PCRs followed by sequencing.

[c]In the case of newly designed primers, GenBank accession numbers are shown.

Table 2 – Mean (± S.E.) of colony strength and *Varroa destructor* mite infestation percentage in hive groups from the experimental apiary at the sampling time.

Hive group	Colony strength[b]		*Varroa* infestation %
	No. Adults	No. Sealed cells	
Infested	3794.76 ± 901.7a[c]	3728.40 ± 663.0a	9.53 ± 1.45a
Non-infested[a]	6705.60 ± 1135.3a	7363.20 ± 1567.8a	0.06 ± 0.05b

[a] Subjected to antivarroa treatments (suspended 1 month before sampling)

[b] Determined employing one-sixth of a Dadant-Blatt frame (188 cm^2) as a unit of measure (Marchetti, 1985).

[c] Different letters within the same column indicate significantly different means ($p < 0.05$)

Fig. 1 - Relative abundance (mean ± S.D.**) of the Deformed Wing Virus (DWV) in different honey bees stages sampled from hives infested by *Varroa destructor*.** Fold changes were calculated relative to the abundance in honey bees from non infested hives**. For each mean,** asterisk (*) indicates a significant difference with the non-infested group, while different letters indicate significant differences between stages (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Em. Adults: Emerging Adults; Em. Adults+V: Emerging adults with attached *Varroa* mites.

Fig. 2 - Relative abundance (mean ± S.D.) **of the overall bacterial community in different honey bees stages sampled from hives infested by *Varroa destructor*.** Fold changes were calculated relative to the abundance in honey bees from non-infested hives**. For each mean,** asterisk (*) indicates a significant difference with the non-infested group, while different letters indicate significant differences between stages (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Em. Adults: Emerging Adults; Em. Adults+V: Emerging adults with attached *Varroa* mites.

Fig. 3 - Scatter plot matrix summarizing the correlation between all environmental variables (infestation level, family strength, stage of the bee, and DWV load) and immune-related gene expression levels .

Fig. 4 - Scatter plot matrix summarizing the correlation between all environmental variables (infestation level, family strength, stage of the bee, and DWV load) and the abundance of selected bacterial taxa.

Fig. 5 - Relative expression fold change (mean ± S.D.) **for different immune-related genes in honey bee emerging adults from the *Varroa*-infested hive group bearing *Varroa* mites on their**

body. Fold changes were calculated relative to the expression level in **emerging adults from the same hives, but lacking *Varroa* mites.** Asterisks (*) indicate significant differences between samples with and without attached mites (t-test; * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$).

Fig. 6 - Relative abundance (mean ± S.D.) **of selected bacterial taxa representing the core bacterial community of honey bee emerging adults from the *Varroa*-infested hive group, bearing *Varroa* mites on their body.** Fold changes were calculated relative to the abundance in **emerging adults** from the same hives**, but lacking *Varroa* mites.** Asterisks (*) indicate significant differences between samples with and without attached mites (t-test; * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$).

Fig-7 - Linear regression plots with 95% confidence intervals (shaded areas) showing the predicted relationship between the DWV load and the relative abundance of immune-related gene transcripts (A) or bacterial taxa (B).

Fig. 8 - Redundancy analysis (RDA) ordination diagram of the emerging adult data for the effect of presence/absence of *Varroa* mites and DWV load on the expression of immune related genes and on the relative abundance of selected bacterial taxa. V1-9= sample pools with *Varroa;* C1-9= sample pools without *Varroa*.

Fig. 1

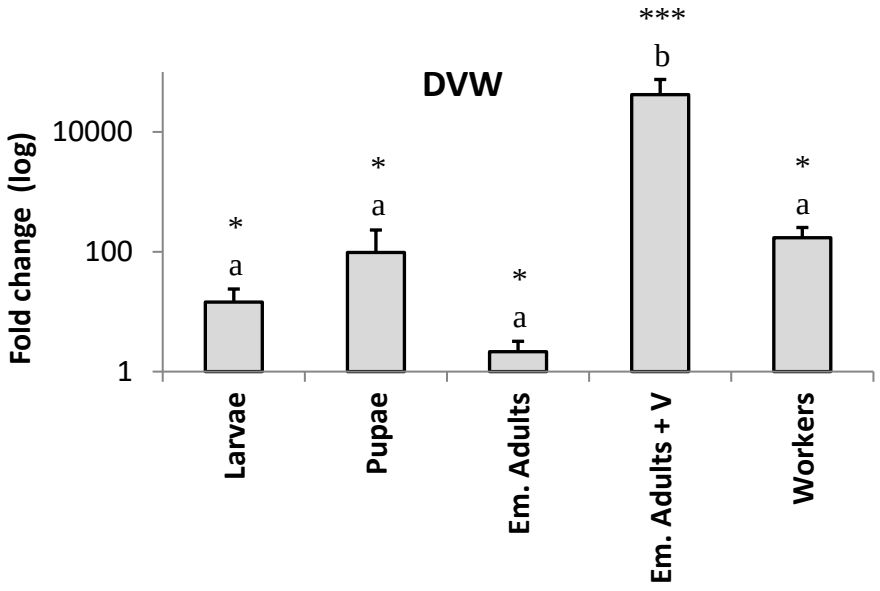

DVW
Fold change (log)
10000
100
1
*
a
*
a
*
a
***
b
*
a
Larvae
Pupae
Em. Adults
Em. Adults + V
Workers

Fig. 2

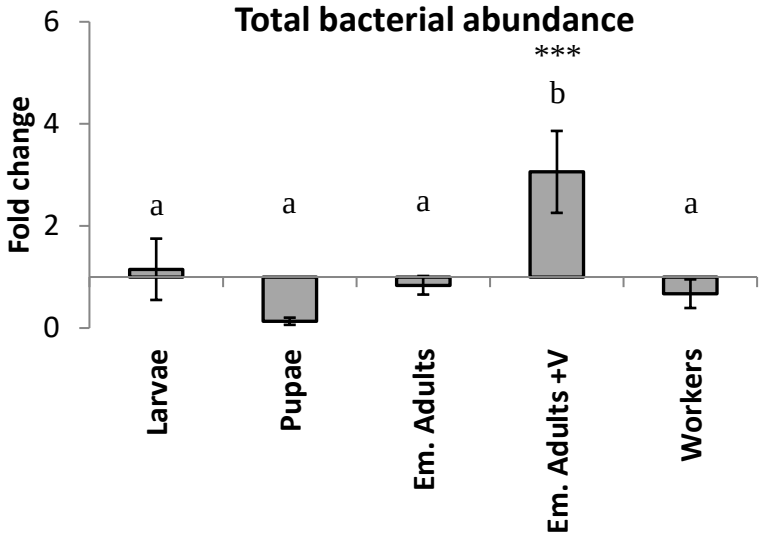
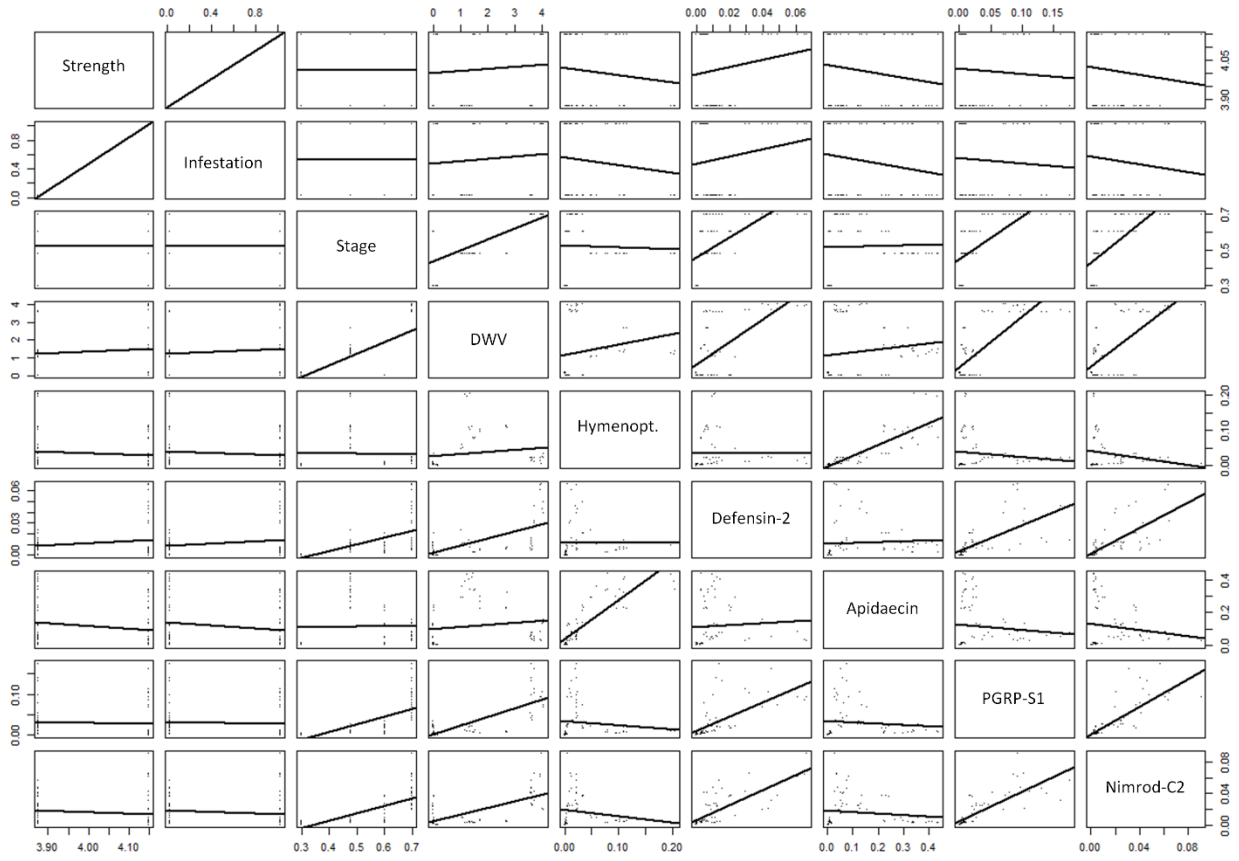

Total bacterial abundance
Fold change
6
4
2
0
***
b
a
a
a
a
Larvae
Pupae
Em. Adults
Em. Adults +V
Workers

Fig. 3


Strength
Infestation
Stage
DWV
Hymenopt.
Defensin-2
Apidaecin
PGRP-S1
Nimrod-C2

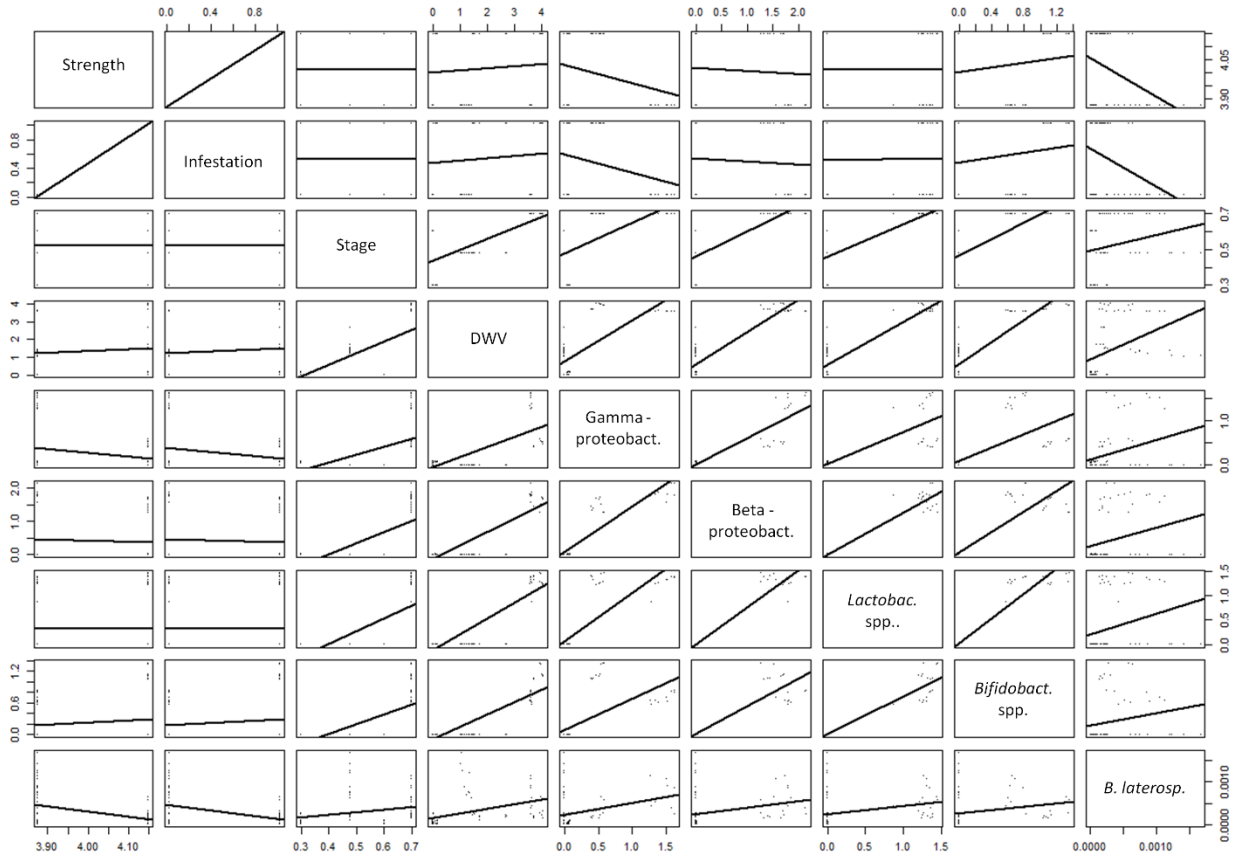

Strength
Infestation
Stage
DWV
Gamma - proteobact.
Beta - proteobact.
Lactobac. spp..
Bifidobact. spp.
B. laterosp.

Fig. 5

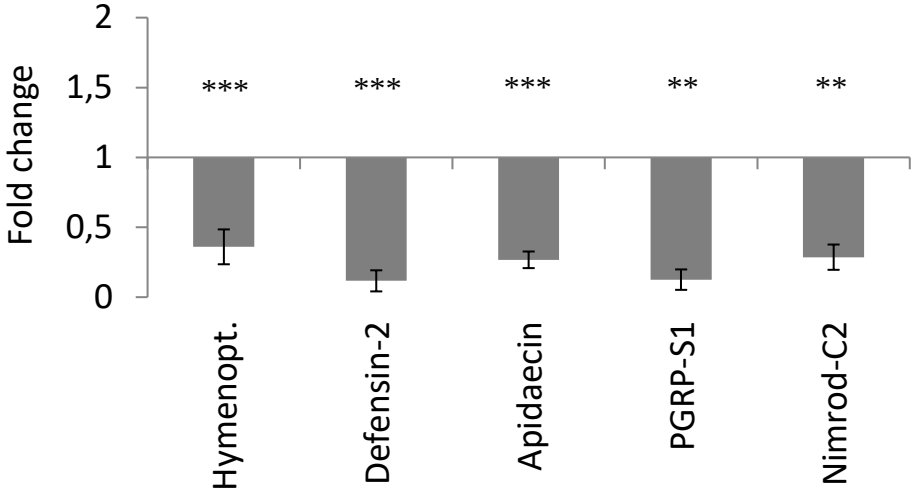

Fold change
2
1,5
1
0,5
0
***
***
***
**
**
Hymenopt.
Defensin-2
Apidaecin
PGRP-S1
Nimrod-C2

Fig. 6

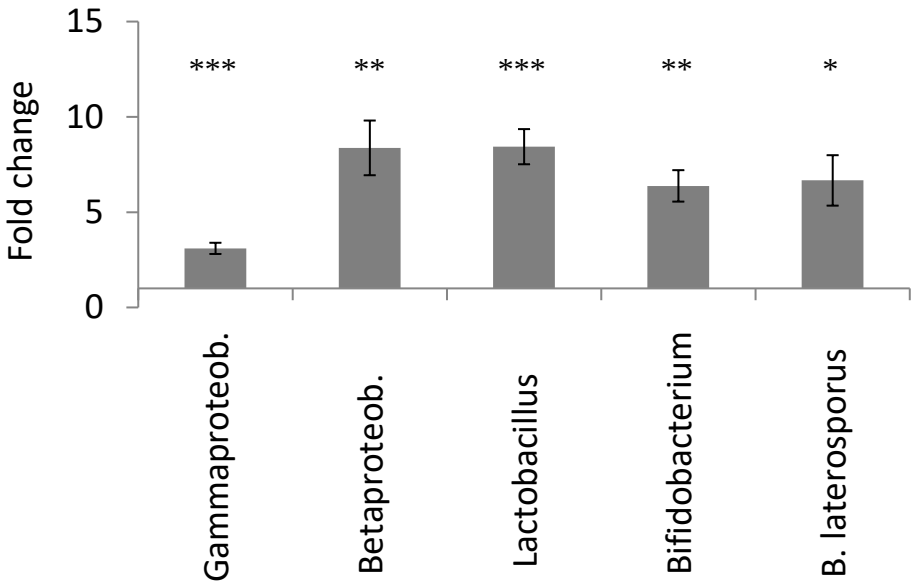

15
10
5
0
Fold change
***
**
***
**
*
Gammaproteob.
Betaproteob.
Lactobacillus
Bifidobacterium
B. laterosporus

Fig. 7

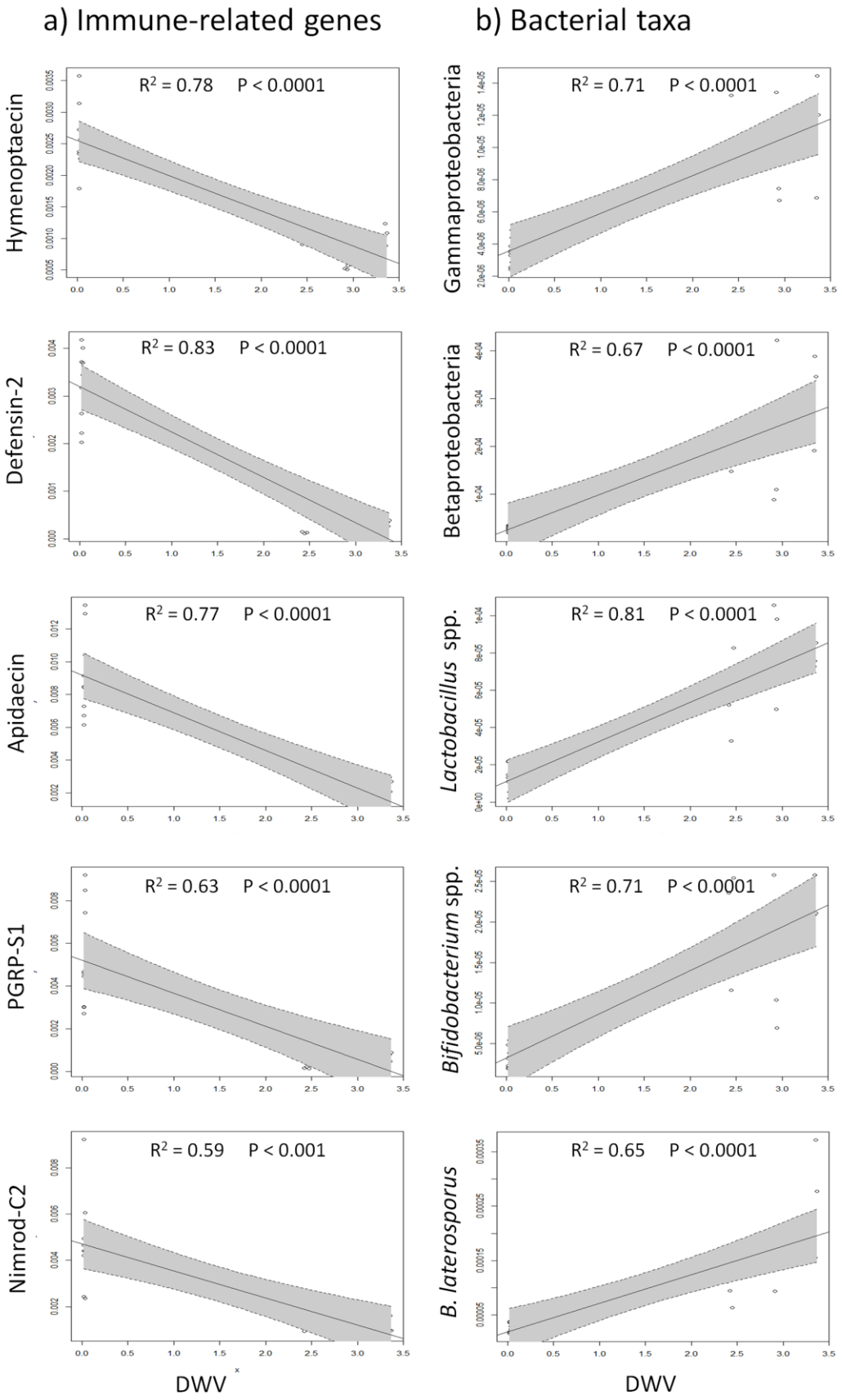
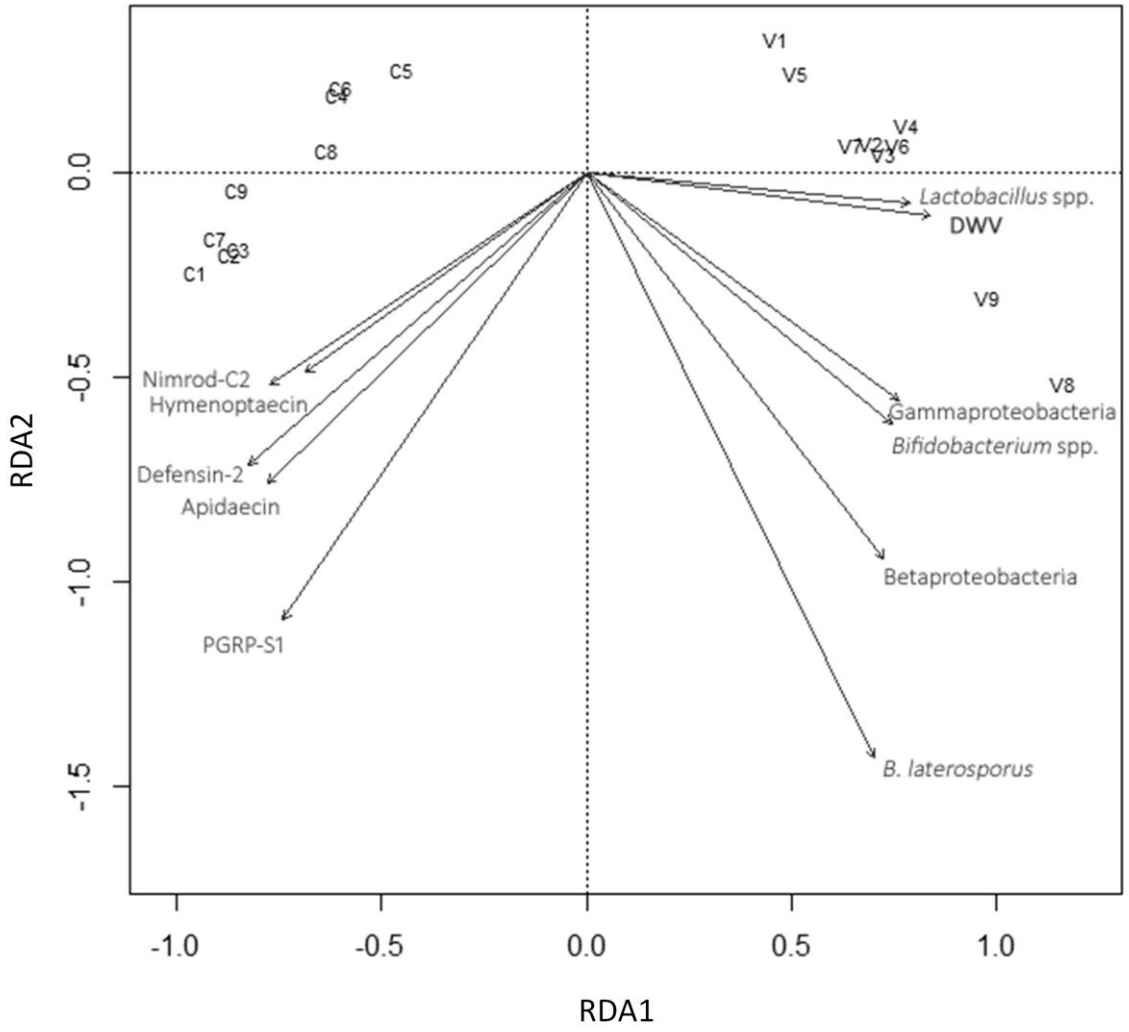

a) Immune-related genes
b) Bacterial taxa
Hymenoptaecin
R² = 0.78 P < 0.0001
Gammaproteobacteria
R² = 0.71 P < 0.0001
Defensin-2
R² = 0.83 P < 0.0001
Betaproteobacteria
R² = 0.67 P < 0.0001
Apidaecin
R² = 0.77 P < 0.0001
Lactobacillus spp.
R² = 0.81 P < 0.0001
PGRP-S1
R² = 0.63 P < 0.0001
Bifidobacterium spp.
R² = 0.71 P < 0.0001
Nimrod-C2
R² = 0.59 P < 0.001
B. laterosporus
R² = 0.65 P < 0.0001
DWV
DWV

Fig. 8


V1
C5
V5
C6
C4
V4
V7
V2
V6
V3
C8
0.0
C9
Lactobacillus spp.
DWV
C7
C3
C2
C1
V9
-0.5
Nimrod-C2
Hymenoptaecin
V8
Gammaproteobacteria
Bifidobacterium spp.
Defensin-2
Apidaecin
Betaproteobacteria
-1.0
PGRP-S1
B. laterosporus
-1.5
RDA2
-1.0
-0.5
0.0
0.5
1.0
RDA1