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SOCIAL WASPS AS A MODEL TO STUDY HOST-MICROBE INTERACTIONS

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SUMMARY

Social insects represent the most widespread group of organisms on the Earth. They proved to be adapted to a wide number of different ecological niches, playing a central role in the ecosystem balance. The understanding of the ecology of social insects, such as social wasps must not disregard from the study of their microbial communities. The effects of this interaction influence the ecology of both sides, social insect and microorganisms. In particular, the insect intestinal tract has evolved structures and physiological conditions that have allowed the development of microbial symbioses.

In this thesis, I have treated the microbial symbioses developed in social wasps under different points of view and in different conditions.

Concerning the first task, I investigated how the gut microbial communities (bacteria and fungi) switch during the hornet ontogenetic development (from juvanile to adult life stages), and this phenomenon was studied in the invasive alien species (IAS), *Vespa velutina nigrithorax,* currently present in Italy. *V. velutina* is an Asian hornet, accidentally introduced in Europe becoming a threat for the ecosystems due to the strong predation on pollinators, and competition with native hornet species, thus determining high social-economic consequences. I demonstrated that the gut microbial communities of *V. velutina* were signficantly affected by the phenotypic variability and ontogenetic dynamics. Furthermore, the hornet nest microbial composition does not influence the microbiota and mycobiota compositions of the hornets. Thus, the stage-related gut physiological changes during *V. velutina* development according to the stage-related diet could play a combined role in the evolution of the gut microbial communities.

Regarding the second task, I performed the comparison of the gut microbial communities composition between the worker stages of the alien species *V. velutina* mentioned above, the native hornet *Vespa crabro* and their main prey *Apis mellifera*. The structure of the microbiota was significant associated with the insect species (*V. crabro*, *V. velutina* and *A. mellifera*), highlighting the presence of enriched Lactic Acid Bacteria (LAB) -related filotypes, differentially associated to the hornets and honey bees.

Instead, the organization of the mycobiota appeared significantly related to the insect genus (*Apis* or *Vespa*) but not with the insect species. Thus, suggesting that the gut fungal communities of the hornets could be more affected by the environmental stimuli. Furthermore, unlike the honey bees, *Saccharomycetales* appeared as the comprehensive order of the entire mycobiota composition in both hornet species.

Concerning the third task, I evaluated the effect of different environmental stimuli on the gut microbial composition of the native hornet species *V. crabro*, comparing to honey bee *A. mellifera*. Three different environmental stimuli were evaluated for each insect species, maintaining a similar environmental patterns for both species. Doing that, I tested the effect of hornets foraging behavior on ripe grape berries in the vineyard at the end of Summer, in the period close to the grape harvest. The hornets who spent their time foraging on the ripe grape in the vineyard were significantly enriched in *Saccharomycetes* when compared to hornets

sampled from olive tree bark hollow 50 days far from the harvest and compared to the hornets that spent their time predating the honey bees in apiary in the vineyard environmental context.
The fourth task concerned the understanding of the effect of two particular strains of *Saccharomyces cerevisiae*, proved to induce trained immunity in mammal and human monocytes, on the immune system and intestinal microbial composition of social wasp *Polistes dominula*. I tested these effects on foundress and worker stages, more easily manipulated compared to the hornets, and knowon for their interaction with *S. cerevisiae* as demonstrated by Stefanini et al. in 2012 and 2016. I have shown that, following the oral administration, these two peculiar strains of *S. cerevisiae* enhance the immune system in foundresses but not in workers, helping the wasps in the elimination of *Escherichia coli* introduced by hemolymph infection. Furthermore, both yeast strains induced in the most immune-responsive group of foundresses, a shaping of the gut microbiota. Thus, this study demonstrated the presence of a castal –dependent interaction between *S. cerevisae* and social wasps *P. dominula*. This important relationship established between yeast and social wasps rovides significant insights in understanding the ecological role of these two very important organisms.

Gli insetti sociali rappresentano il gruppo più diffuso di organismi sulla terra. Si sono adattati a nicchie ecologiche molto diverse, svolgendo un ruolo centrale nell'equilibrio degli ecosistemi. La comprensione dell'ecologia degli insetti sociali, come le vespe sociali, non deve prescindere dallo studio delle loro comunità microbiche. Gli effetti di questa interazione influenzano l'ecologia di entrambe le parti. In particolare, il tratto intestinale dell'insetto ha evoluto strutture e condizioni fisiologiche che hanno permesso lo sviluppo di simbiosi microbiche.
In questa tesi, ho trattato le simbiosi microbiche sviluppate nelle vespe sociali sotto diversi aspetti e in diverse condizioni.
Per quanto riguarda il primo punto, ho studiato come le comunità microbiche intestinali (batteri e funghi) si sono sviluppate nelle fasi di vita dei calabroni, quindi ho studiato questo fenomeno nella specie esotica invasiva (IAS) Vespa velutina nigrithorax, attualmente presenti in Italia. V. velutina è un calabrone asiatico, introdotto casualmente in Europa ed è diventato una minaccia per gli ecosistemi a causa della forte predazione sugli impollinatori, della concorrenza con le specie di calabrone autoctone, quindi determinando forti conseguenze socio-economiche. Ho dimostrato che le comunità microbiche intestinali di V. velutina sono state significativamente influenzate dalla variabilità fenotipica e dalla dinamica ontogenetica. Inoltre, la composizione microbica del nido non influenza i filotipi trovati nelle fasi di vita degli insetti. Pertanto, i cambiamenti fisiologici intestinali legati allo stadio durante lo sviluppo di V. velutina in accordo con la dieta legata allo stadio potrebbero svolgere un ruolo combinato nell'evoluzione delle comunità microbiche intestinali.
Per quanto riguarda il secondo punto, ho eseguito il confronto della composizione delle comunità microbiche intestinali tra le caste operaie della specie aliena V. velutina mensionata sopra, il calabrone nativo Vespa crabro e la loro preda principale Apis mellifera. La struttura

del microbiota era significativamente associata alle specie di insetti (V. crabro, V. velutina e A. mellifera), evidenziando la presenza di filotipi arricchiti correlati ai batteri lattici (LAB), differenzialmente associati ai calabroni e alle api da miele.
Invece, l'organizzazione del micobiota appariva significativamente correlata al genere degli insetti (Apis o Vespa) ma non alla specie di insetto. Pertanto, suggerendo che le comunità fungine intestinali dei calabroni potrebbero essere più colpite dagli stimoli ambientali. Inoltre, a differenza delle api mellifere, Saccharomycetales appariva come l'ordine globale dell'intera composizione del micobiota in entrambe le specie di calabroni.
Per quanto riguarda il terzo punto, ho quindi valutato l'effetto di diversi stimoli ambientali sulla composizione microbica intestinale della specie di calabrone nativa V. crabro, confrontandola con l'ape mellifera. Sono stati valutati tre diversi stimoli ambientali per ciascuna specie di insetto, mantenendo modelli ambientali simili per entrambe le specie. In questo modo, ho testato l'effetto del foraggiamento dei calabroni sugli acini maturi in vigna alla fine dell'estate, nel periodo vicino alla vendemmia. I calabroni che hanno speso più tempo foraggiando sull' uva matura in vigna erano notevolmente arricchiti di Saccharomycetes rispetto ai calabroni campionati dall' incavo della corteccia di olivo a 50 giorni dalla raccolta e rispetto a i calabroni che trascorrevano il loro tempo a predare le api da miele nell'apiario nel contesto ambientale della vigna.
Il quarto punto riguardava la comprensione dell'effetto di due particolari ceppi di Saccharomyces cerevisiae, dimostrati in grado di indurre trained immunity nei mammiferi e nei monociti umani, sul sistema immunitario e sulla composizione microbica intestinale della vespa sociale Polistes dominula. Ho testato questi effetti sulle caste fondatrice e operaia, più facilmente manipolabile rispetto ai calabroni e note per la loro interazione con S. cerevisiae, come dimostrato da Stefanini et al. nel 2012 e nel 2016. Ho dimostrato che, in seguito alla somministrazione orale, questi due ceppi speciali di S. cerevisiae migliorano nelle fondatrici, ma non nelle operaie il sistema immunitario, aiutando le vespe nell'eliminazione di Escherichia coli a seguito dell' infezione nell' emolinfa. Inoltre, entrambi i ceppi di lievito hanno indotto nel gruppo più immuno-rispondente di fondatrici, un rimodellamento del microbiota intestinale. Pertanto, questo studio ha dimostrato la presenza di un'interazione dipendente dalla casta tra S. cerevisae e le vespe sociali P. dominula. Questa importante relazione stabilita tra lievito e vespe sociali fornisce spunti significativi nella comprensione del ruolo ecologico di questi due organismi molto importanti.

CHAPTER I

GENERAL INTRODUCTION

Social insects and microbiota

Insects are an extremely diverse and widely represented group of organisms on Earth, composed of over a million of described species able to colonize almost every global ecosystems (Misof et al., 2014; Larsen et al., 2017). Similarly to plant, mammals and humans, insects host a variety of symbiotic microorganisms, usually referred as microbiota, that contribute to many insects physiological processes and overall to their ecology, playing a central role in their survival and evolution. The success of an organism within the ecological dynamics, such as survival, competition for resources, reproduction and invasive displacement, is often determined by symbiotic microbial communities that together with the host constitute a whole organism so called "holobiont" (Zilber-Rosenberg and Rosenberg, 2008). Thus, over time insects have developed symbiosis with many different types of microorganisms and these interactions have allowed insects to benefit from them at different levels. Symbioses can occur in many different insect tissues but the intestinal tract represents the anatomical part that constitutes the microenvironment where a large number of symbioses have evolved (Engel and Moran, 2013). Moreover, the insect intestine constitutes an environment involved in the microbial conservation, proliferation and finally dispersion and this is even more evident for the hymenoptera that act as natural vectors (Roxo et al., 2010; Sühs et al., 2009).

A striking example of the success of symbiosis is *Buchnera aphidicola,* which has lost fundamental genes for pathogenicity, thus developing a close mutualistic relationship with aphids (Douglas et al., 1998). The functional contributions of the symbiotic microbial communities in insects are actually widely described; for example, the food processing by making nutrients accessible (Klepzig et al., 2009) as described in honey bees during pollen digestion (Roulston and Cane, 2000), polysaccharides degradation, such as cellulose and xylans (Hosokawa et al., 2007; Adams et al., 2011), detoxification from toxic compounds from plants (Hansen and Moran, 2014) and protection against infection and parasites (Koch and Schmid-Hempel, 2011). Therefore, gut microbial symbionts can affect many insects' functional and crucial processes such as the immune system maintenance (Schneider and Chambers 2008), manipulate insects' behavioral patterns (Lewis and Lizé, 2015), they can also induce resistance against insecticides (Xia et al. 2018), increase the nutrient uptake, such as vitamin B (Nikoh et al., 2014), and at last can determine a drastical reduction of the levels

of Zika virus (infecting agents that cause Dengue fever in humans) in *Wolbachia*-harbored mosquitoes (Dutra et al., 2016).

The ecological relevance for the ecosystem services combined with the possible agrotechnical implications have led to increased knowledge about the hymenoptera gut microbiota, in particular about two of the most studied hymenoptera, such as honey bee and bumble bee (Martinson et al., 2011; Engel et al., 2012; Koch et al., 2012). Hence, the study of the microbiota has extended to many other social and not-social insects.

Over time, the study of the insect-microbial symbiotic interaction has become one of the great challenge in the 'omic' era. The development of sequencing techniques allowed us to widely describe the microbial profiles, bringing out crucial mutualistic host-microbes interactions. The identification of new species of microorganisms, often together with their isolation, has improved the understanding of the insect-microbial symbiosis phenomenon. Thus, the study of the insect microbiota and mycobiota represent a foundamental process in the understanding of insect ecology with a wide applicative implications. Sometimes, many of the insects that develop symbiosis with microorganisms are studied because of their possible harmful effect towards human, pet and plants (Webber and Gibbs, 1989; Hosokawa et al., 2007; Van Den Abbeele et al., 2010; Eigenbrode et al., 2018), but these symbiosis can also represent an important source of positive applications, indeed, enzymes produced from commensal biomass-degrading microorganisms could be employed in the industrial polysaccharide degradation (Liang et al., 2018), microbial symbiomes could be used as a strategy for pest management (Arora and Douglas, 2017; Itoh et al., 2018), and often they can provide molecules with antimicrobial activity, thus helping in the development of antibiotics (Matsui et al., 2012) or antifungals (Haeder et al., 2009; Van Arnam et al., 2016, Florez et al., 2018), and even up to the production of molecules with antitumoral activity (Piel, 2002).

Despite the wide number of studies about insect-microbes relationship, this area still needs to be developed. Hence, the importance of the study and comparison of both bacterial and fungal communities, extending the interest also to the insect species that have not been yet fully described, among them the social wasps.

The insect-fungi interaction

The study of symbiotic microorganisms in insects has mainly focused on bacterial communities, however, fungi and insects coevolved in the same ecological contexts for millennia (Vega and Blackwell, 2005) and this interaction has given rise to crucial symbiotic relationships. Indeed, some insect surprisingly represented a wide source of new yeasts species (Sung-Oui et al., 2005).

The role of fungi, and among these the true yeasts (*Ascomycota: Saccharomycotina*) in host-interaction appears crucial for the understanding of evolution and ecology of social insects. Fungi often play a bivalent role, they can engage in mutualistic relationships giving benefits to the insect ecology but can also act as insect pathogens, as reported for *Beauveria, Acremonium, Metarhizium, Clonostachys ,Cladosporium and Paecilomyces* genera (Vega et al., 2005). Beyond the negative interactions, the largest part of yeast evolve in mutualistic interactions becoming crucial for the insect life. Many studies have focused on the interaction between yeasts and hymenoptera, a very important insect's order, deeply connected to the pollination and overall to the ecosystem services. These yeast can help the hymenoptera providing or helping in many insect functional processes such as the food digestion and nutrient assimilation. Leaf-cutter ants grow basidiomyceteus fungus *Leucoagaricus gongylophorus* responsible for the biomass degradation (Aylward et al., 2013) and another midgut-associated fungus in *Macrotermes natalensis* appears to be related to the production of the enzymes involved in nutrients acquisition (Martin et al., 1978). This interaction has induced sometimes the development of specialized anatomical traits in some insects such as ambrosia beetle that have developed aspecific structure called mycangia able to collect and transport yeast spores (Stone et al., 2005).

Some symbioses have been previously described also in wasps, as the wood wasp *Sirex noctilio* that use the fungus *Amylostereum areolatum* in the colonization of healthy trees through the fungus cellulose degrading enzymes (Nielsen et al., 2009). It is plausible that, as describedin the insects mentioned above, these symbioses can also occur in other social insects such as the social wasps (social wasps and hornets).

In that, the social wasps have always been studied in order to understand more about their behavior and for the related pest management, but currently we need to understand more about their microbial symbiotic interactions.

Role of *Saccharomyces cerevisiae* in the ecology of social wasps

As already mentioned, yeasts have established a close interaction with a large number of insects (Vega and Dowd, 2005). Unlike many other microorganisms, yeasts strictly dependent on the presence of vectors for their dispersion in the environment (Goddard et al., 2010; Francesca et al., 2012) and this represents a fundamental condition for the yeast ecological success. Among the yeasts found in association with the Hymenoptera, *Sacchromycetes*, especially the species *S. cerevisiae*, has been already studied and described (Stefanini et al., 2012, Stefanini et al., 2016). The wasp's intestine represent an important source of fungal biodiversity providing a large number of *S. cerevisiae* strains with variable phenotype traits (Dapporto et al., 2016). The hymenopteras intestinal tract seems to be a suited place for the yeast reproduction; spore of *S. cerevisiae* can survive during the passage through the gut of *D. melanogaster* increasing the outbreed probability (Coluccio et al., 2008), likely increasing the yeast biodiversity.

In the last years, studies demonstrated that the social wasp *P. dominula* host *S. cerevisiae* in their gut during the winter hibernation (Stefanini et al. 2012) and the queen wasp can transfer this yeast vertically within the colony to the workers and larvae, guaranteeing the yeast propagation and thus the dispersion (Stefanini et al., 2012). As already mentioned, social wasps also act as a natural reservoir favoring the yeast interspecific hybridization, indeed, *S. paradoxus* does not survive as a pure strain in the wasp gut but only as a *S. cerevisiae* hybrid (Stefanini et al., 2016). These conditions allowed us to understand the central role of *S. cerevisiae* in the ecology of this hymenoptera and speculate about the ecological consequences of this relationship.

These studies provide a broad description of the ecological advantage for the yeast *S. cerevisiae* within this symbiosis, but it is not currently known what is the advantage for the wasp in hosting this yeast. The recent discovery about the immunomodulatory effect of *S. cerevisiae* in inducing trained immunity in mammals through innate immune system activation have led to study the yeast-related immunomodulatory capacities in other organisms. The innate immune component of the immune system would seem to have evolved mechanisms shared among vertebrates and invertebrates, involved in the recognition and defence against microbial pathogens (Lehrer et al., 1999). However, the insects immune system and its interaction with yeasts still needs to be better explored.

Invasive species and their microbiota

Invasive alien species (IAS) represent a current problem affecting the balance of ecosystems determining the loss of biodiversity (Clavero and García-Berthou, 2005) in both animal and plant kingdoms. This dramatic effect is also due to the increase of invasive species, causing damage to public health, threats against local species, loss of resources from the primary productions and overall to the global economy (Chapman and Bourke, 2001; Pejchar and Mooney, 2009; Butchart et al., 2010; Vilà et al., 2011). The impact of the IAS is expressed in the loss of biodiversity and alteration of ecosystems, in that, the introduction of IAS in the ecosystems could fuel the dispersion of parasites; and increase the predation thus the pressure towards native species (Mooney and Cleland, 2001; Tompkins et al., 2003; Clavero and García-Berthou, 2005; Salo et al., 2007). The invasion strategies can sometimes be intensified through the introduction of pathogenic microorganisms, and it is also shown that the introduction of alien microorganisms in an ecosystem can produce dramatic effects (Litchman, 2010) and the adaptation to a new niche and overall the interaction with a new environment often performed by hymenoptera (including invasive hymenoptera species), can also pass through the symbiotic microorganisms interactions (Zientz et al., 2005; Hughes et al., 2008).
Thus, the study of microbial communities associated with invasive species can therefore be crucial in the understanding of invasive species success and their impact on the environment.
Thus, in my thesis project I also studied the gut microbial composition of a very important European IAS, *Vespa velutina nigrithorax*, performing the analysis of the microbial and fungal communities considering the intraspecific phenotypic variability and ontogenetic dynamics. *V. velutina* is an invasive hornet species native of South East Asia accidentally introduced in France and in neighboring countries in Europe (Villemant et al., 2011; Monceau et al., 2014, Monceau and Thiéry, 2017; Robinet et al., 2019). Recently, the expansion of the Asian hornet *V. velutina* within the European countries has also involved the northern Italy (Bartolotti and Cervo, 2016; Granato et al., 2019) becoming a threat for the ecosystems due to the strong impact on pollinating insects and other artropods involved in the ecosystem services. Furthemore, *V. velutina* forages in the same ecological niche of the native hornet *Vespa crabro* competing for the resources. Currently, the largest part of the knowledge about *V. velutina* has been developed on its behavior, ecological and social impact but actually we do not know enough about its microbiota, particularly, we do not know anything about V. velutina gut fungal composition. The only study of *V. velutina* microbiota was performed only on

microbial communities in a *V. velutina* population in Korea (Kim et al., 2018). Thus, I investigated the microbiota and mycobiota of the Asian hornet during its ontogenetic development, trying to identify the patterns of microbial communities within the different life stages, providing a first comprehensive description of the gut microbial communities in this important invasive species. This knowledge could be fundamental for the understanding of *V. velutina* ecology and likely for possible future pest management applications.

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Aim of the thesis

(i) Perform the analysis of the gut microbial and fungal communities associated with the Asian hornet *Vespa velutina nigrithorax* in the related life stages, such as reproductive phenotypes (workers and future queens), life stages (larvae, newly emerged individuals and adults) and non-living samples (nest paper and larval faeces) providing a comprehensive description of the gut microbiota and mycobota compositions.

(ii) Compare the gut microobiota and mycobiota structures of three different but ecologically related hymenoptera, the worker life stages of *Vespa velutina*, *Vespa crabro* and *Apis mellifera*. Thus, understanding how different the hornet alien and native species are from each other and from their usual prey *Apis mellifera*.

(iii) Evaluate the effect of different environmental stimuli on gut microbiota and mycobiota organization of *Vespa crabro* and *Apis mellifera*. Among these, understand the effect of ripe berries foraging behavior on the hornet microbial communities composition, in compariosn with the hornets collected and influenced by other environmental matrices. the same experimental approach was also carried out for *Apis mellifera*, understanding how the effects of the different environmental patterns are transversal within insects from different genera.

(iv) Evaluate the role of two *Saccharomyces cerevisiae* strains, proved to induce trained-immunity in mammals and human monocytes, on two life stages (workers and future foundresses) of social wasps *Polistes dominula*. Determine the immunomodulatory effect and the gut microbial shaping performed by *S. cerevisiae* strains.

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CHAPTER II

CASTE AND DEVELOPMENT AFFECT GUT MICROBIAL COMPOSITION OF THE INVASIVE HORNET VESPA VELUTINA NIGRITHORAX

Abstract

Social insects represent a dominant component of biodiversity in most terrestrial habitats and their impact on ecosystem functioning is often mediated by symbiosis with microorganisms. Here, we provided the first comprehensive characterization of the gut microbial communities of the invasive yellow-legged hornet *Vespa velutina nigrithorax*. The species recently colonized Europe, becoming a high ecological and economic concern, as it threatens pollinator survival and competes with native hornet species. We used meta-genomics to describe the yeasts and bacteria gut communities of individuals of different reproductive phenotypes (workers and future foundresses), life stages (larvae, newly emerged individuals and adults) and non-living samples (nest paper and larval faeces). We showed that the microbiological signature changes in accordance with the hornet developmental stages but nevertheless maintains a clear division from the non-living samples. *Bacilli*, *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria* were the most abundant classes of bacteria, and *Saccharomycetes*, *Dothideomycetes*, *Tremellomycetes* and *Eurotiomycetes* were the most represented yeast classes. We found that while overall OTU richness in yeast and bacteria communities does not significantly differ across phenotypes, composition strongly does, with yeast and bacterial communities switching in frequency and abundance during ontogeny (from larvae to adults) and according to reproductive phenotype (future foundresses *vs* workers). Moreover, the gut microbial communities poorly mirrors the environmental (e.g nest) yeast and bacterial communities, suggesting that hornets possess a specific microbial signature which dynamically changes during life and according to individual phenotype. Our data provide the springboard for future assessment of the functional role of gut microbial organism in hornets and pave the way to link gut microbiota to the role played by hornets in the ecosystems.

Introduction

A major feature of the current 'Anthropocene' epoch is the fast and increasing loss of biodiversity, which affects the functioning of natural ecosystems and threatens human well-being. Insects are among the animal groups that are suffering the most marked decrease in their abundance and diversity: a recent study estimated a loss of 75% of the biomass of flying insect in protected areas in Germany over the last 30 years (Leather, 2017). Such a huge loss is especially worrying when concerning groups of insects which provides crucial ecosystem services, as the case of social insects. Bees, wasps, ants and termites are indeed important ecosystem services providers, being involved in pollination, predation, seed dispersal and nutrient recycling to such an extent that social insects are considered among "the little things that run the world" (Wilson 1987, Del Toro et al., 2012). In addition to providing essential ecosystem services with consequential positive impacts on human beings, some social insect species also cause severe negative impacts on human economy, such as in the case of accidental species translocation from native to new habitats (i.e. biological invasions) (Chapman and Bourke, 2001). The changes in social insect diversity, abundance and communities composition is likely mirrored by changes in ecosystem functioning, potentially leading to what could be seen as an "Ecological Armageddon" (Leather 2017).

The interaction between social insects and their environment, and consequently their impact on it, is largely mediated by symbiosis with microorganisms (Zientz et al., 2005; Hughes et al., 2008). Therefore, gut microbial communities strongly influence social insect survival and fitness, by mediating crucial life history traits such as nutritional ecology, immunity and reproduction (Koch and Schmid-Hempel, 2011; Mutinelli, 2011; Rosengaus et al., 2011; Engel and Moran, 2013). Moreover, symbiosis with microorganisms underlie some of the most astonishing and important social insects provides. For example, cellulose digestion from wood fibers in termites is mediated by gut-dwelling microbes, as well as nitrogen fixation in herbivorous ants is mediated by gut-specific microbial communities (Radek, 1999; Pinto-Tomás et al., 2009; Suen et al., 2010; Brune and Dietrich, 2015). Moreover, social insects may also positively affect reproduction, survival and dispersal of gut microorganisms, as it has been suggested for the economically significant *Saccharomyces* yeast which inhabit social wasps guts (Stefanini et al., 2012; Dapporto et al., 2016).

Understanding the diversity and composition of gut microbial communities, as well as how they change and interact with their social insect host features is thus of overwhelming

importance in order to understand the interaction between social insects and their environment. There is also potential to use this knowledge to manage the positive and negative impacts of social insects on ecosystems and human economy. For example, the understanding of honey bee gut microbiota is a promising tool to improve its health, thus securing and increasing its pollination services (Anderson and Ricigliano, 2017). On the other hand, the manipulation of the microbiota in pest and invasive species may result in significant practical applications for the development of management strategies (Microbial Resource Management) (Douglas, 2007; Crotti et al., 2012).

Despite the growing body of knowledge on social insect gut microbiota that has been gathered during the last decades, the variability and functions of social insects' microbial communities still suffers from some crucial limitations. First, the taxonomic coverage is still limited, with most knowledge focused on model organisms or few particularly economically relevant species (taxonomic limitation). For example, while more than 15000 species of social insects have been described, detailed studies on the gut microbiota have been carried out only for a handful of species (mainly the honey bee *Apis mellifera*, and few species of ants and termites) (Engel and Moran, 2013). Moreover, there is also a poor phenotypic coverage (phenotypic limitation), i.e. only few studies examined in detail the intraspecific variation in microbial communities by sampling many different phenotypes (such as different morphs, or different castes) and/or investigating the dynamic changes through individual life (Berlanga et al., 2011; Diouf et al., 2015; Kapheim et al., 2015; Otani et al., 2019)

Here we provide a comprehensive characterization of the gut microbial communities (fungi and bacteria) of a social insects of recent economic and ecological interest, the yellow legged hornet *Vespa velutina nigrithorax,* with a detailed sampling which considered intraspecific phenotypic variability and ontogenetic dynamics. *Vespa velutina* (hereafter Vv) is an invasive hornet species native of South East Asia (Monceau et al., 2014). Its presence was first recorded in South of France, in 2004 since then the species rapidly spread across most Europe (Villemant et al., 2011; Robinet et al., 2019). Vv has significant ecological, economic and sanitary impacts, such that it has been listed among the invasive alien species of most concern for Europe (Haxaire and Villemant, 2010; Requier et al., 2019) (COMMISSION IMPLEMENTING REGULATION (EU) 2016/1141- The species significant ecological impact is due to its predation on a vast array of insect species (Spradbery, 1973; Matsuura and Yamane, 1990), some of which (honey bees included, but also many diptera and other

hymenoptera) provide valuable ecosystem services, such as pollination and predation on pest species (e.g. other wasps). Moreover, due to competition for a similar ecological niche, Vv may also be a threat for the native hornet species, the European hornet, *V. crabro* (hereafter Vc), which by the way is protected in some Eu countries (e.g. Germany) (Cini et al., 2018). Economically, the main threat posed by Vv is on beekeeping activities, as the yellow-legged hornet is a specialized predator of honey bees (Monceau et al., 2014) and can have high impact on honey bee colonies survival and a dramatic increase of hive managing time and effort. Finally, Vv might also have a potential impact on human health since envenomation of Vv can induce severe allergic or toxic reactions, resulting in organ failure and death (Chugo et al., 2015; Liu et al., 2015) and Vv colonies are usually large (hosting up to thousands of workers), and built close to urban environments (Liu et al., 2015; Monceau and Thiéry, 2017). The great concern due to Vv arrival, diffusion and impact in Europe and other invaded countries (Korea, Japan) boosted research. However, most of the gathered knowledge is related to life history trait, ecology and behaviour of V*espa velutina*. Currently, there is almost no informations about gut hornet's microbial composition in general, and about Vv in particular. The only study so far performed on *Vv* investigated gut bacterial communities, but not yeast, and was performed in Korea, another invaded region (Kim et al., 2018). Regarding the native hornets, only yeasts have been investigated (Stefanini et al., 2012; Dapporto et al., 2016).

By using metatassonomic approach, we aim at addressing three main research questions, which concern the gut microbial composition (yeast and bacteria) of *V. velutina*, its variation across life stages and castes, and its resemblance/dependence with nest-related microorganisms.

First, we aim at providing a comprehensive characterization of bacterial and yeast gut communities of this species in the new introduced range (Europe), evaluating the richness, diversity and composition. This information is crucial to understand the potential role of the different microorganisms, and to enable future comparison with native or non-invasive hornet species. This will pave the pathway to understand the microbial feature that might influence species success and impact on ecosystems. A second question is to what extent the gut microbiota is conserved or not across life stages and across different phenotypes. Understanding the extent and the nature of these changes will highlight which are the sets of microorganisms which are stage and/or phenotype-specific, and which allow them to tackle

the challenges faced by the specific lifestage/phenotype. Finally, we are interested in understanding how much the species core microbiota are shared with the nest, which acts as an indicator of environmental microbial communities. In addition to providing shelter and protection for the brood, the nest also represents the locus of *Vespidae* social life (Starr, 1991). At the same time, the nest is a product of wasp activity (the paper of the nest is made by vegetal fibers chewed by wasps) and is filled with hornet products, the main of which is the meconium, i.e. the residues of larval faeces. It is clear thus that the environment in which hornets live can be both a potential source of microbes and reflect the microorganisms harboured by hornets. We compared the gut microbial communities of living hornets with the microbial communities of non-living samples, i.e. nest paper and meconia.

These three research questions are instrumental in order to understand the variation in gut microbiota in Vv and thus potentially identifying putative candidate microbial communities/elements specifically linked and beneficial for specific life stages. In addition to the information of generic importance (i.e. the dynamics of insect gut microbiota across life stages and phenotypes and its resemblance with the environment), our results provide the first characterization of the gut microbiota of this invasive pest species during their developmental stages. This has the potential to open future avenues of research for applied perspectives on this species. Overall, this study provides the springboard for future assessment of the functional role of gut microbial organism in hornets, thus enabling to link gut microbiota to the role played by hornets in the ecosystems, as well as enabling the first steps toward possible management strategies of this invasive pest.

Matherials and Methods

The model species Vespa velutina: life-history and colonial cycle

Vespa velutina is a social species with an annual colony cycle (Spradbery, 1973; Matsuura and Yamane, 1990; Monceau et al., 2014). In the European invasive population, new colonies are founded in spring by single mated queens that survived the wintering diapause (Monceau et al., 2014; Cini et al., 2018). By early summer workers start to emerge and take over the duties of rearing the immature brood and providing for the growing colony. Workers unceasingly forage in search of nest-building material and prey to feed the larvae, which, unable to leave their cells, entirely depend on workers for their development. Adult workers catch and manipulate prey given to developing larvae, which, in exchange, regurgitate drops of liquid, rich in semi-digested nutrients (aminoacids), for the colony adults (throphallaxis). Before reaching the pupal stage, larvae expel the entire content of their digestive system which remains at the bottom of the nest cell (meconium). At the end of summer, the colony reaches its peak in terms of number of individuals and the new generations of sexuals (males and reproductive gynes) are produced. After emergence reproductive individuals stay in the nest, where throphallaxis exchange frequently occur also among adults; mating takes place in autumn, after that, workers and males die before the winter while mated future queens enter hibernation. As other social wasps species, Vv build their nest combs with paper material made by collecting plant fibres in the surrounding environment and then by mixing this plant material with saliva (Matsuura and Yamane, 1990; Monceau et al., 2014).

Insects and nest sampling

Vv combs with sealed brood were collected during the months of 2016 in the surroundings of Ventimiglia (Imperia, Liguria, Italy), from 3 nests that were gathered by local beekeepers. We collected from each nest the following samples: paper nest (a piece of approximately 1 cm side, from the wall of a cell randomly chosen); meconium, i.e. the waste products of larvae, randomly chosen by a cell; newly emerged females: these adult females were sampled while they were emerging, to be sure they did not have the chance to exchage liquid with other individual or get “contaminated” in other way, e.g by licking the nest surface. We uncapped pupal cocoons and sampled those individual which were ready to emerge (easily recognized by their exoschelteon cosnistence and colour, and by the fact that they were moving and

chewing the pupal cap to emerge); adult gynes: these were sampled directly from the nest comb, their reproductive phenotype (i.e. that they were gynes) was assessed during dissection by looking at the abundance and colour of fat bodies: as gynes has to overwinter while workers don't, fat storage allow to distinguish between workers (no or almost absent fat storage) and gynes (abundnant fat storage) (Cini et al., 2018). Workers were sampled at apiaries, in order to be sure about their phenotype (i.e. while they were foraging to collect bees). Paper nest (approximately 1 cm2), meconium (the mecomium found in a single cell) and gut from Larvae, Gynes, Workers and Newly emerged females were collected and stored in RNAlater solution until DNA extraction.

Totally, we gathered 32 samples covering 20 insect guts, distributed as it follows: 8 from workers, 5 from adult foundresses, 3 from newly emerged females, 4 from larvae, and 12 non-insect samples, including 9 meconium samples and 3 paper nest samples.

DNA extraction and rRNA genes sequencing

The insects surface was sterilized with 70% ethanol. All insect samples were dissected and the guts were collected in storage and stabilization solution RNAlater (Invitrogen – Thermo Fisher Scientific) in sterile microcentrifuges tubes and stored at -20°C until DNA extraction. Meconium and paper nest samples were collected and stored using the same protocol. Microbial DNA extraction from all samples were performed using DNeasy PowerLyzer PowerSoil Kit (QIAGEN) following the manufacturer's protocol. Total genomic DNA were then quantified by Tecan Quantification Device (Life Sciences). DNA sequencing were carried out by MiSeq - Illumina platform at BMR Genomics (BMR Genomics sequencing service of University of Padova, Italy, https://www.bmr-genomics.it/). After sequencing the fastq sequences files were demultiplexed. DNA sequencing were carried out on V3-V4 region of the 16S rRNA genes using the primers Pro341F: 5′-CCTACGGGNBGCASCAG-3′ and Pro805R: 5′-GACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014). The same guideline were used for ITS2 rRNA genes sequencing performed using the primers ITS3: 5'-GCATCGATGAAGAACGCAGC-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3' (White et al., 1990).

Sequences clustering

Overall quality of samples was evaluated with FastQC program (Andrews, 2010) and low-quality end of forward and reverse reads were trimmed using Sickle (Joshi and Fass, 2011; quality cut-off of 20 and a length threshold after trimming of 200). MICCA pipeline (ver. 1.7.2, Albanese et al., 2015) was used for OTUs/SVs picking as follows: forward and reverse reads were joined with "micamergepairs" command, reads with N bases were discarded with command micca filter. OTUs/SVs picking and chimera checking was performed with "miccaotu" command and the UNOISE3 protocol as picking algorithm. Taxonomy was assigned to the representative sequences of the identified OTUs/SVs classified using the RDP classifier (ver 2.11, Wang et al., 2007).

Statistical analysis

All statistical analyses were performed into R environment (version 3.4.4 ref), Ordinational analyses were computed using Principal Coordinate Anlysis based on Bray-Curtis distance with "ordinate" function (phyloseq package version 1.23). Ordinations were analysed using Permutational Multivariate Analysis of Variance (PEMANOVA) using distance matrices as implemented in the "adonis2" function (vegan package version 2.5). beta diversity between developmental stages was computed using the Sorensen index as $S = (B + C)/(2A + B + C)$, with A = shared species among groups, B = species from group B and C = species from groups C ("betadiver" function of vegan package 2.5). Beta diversity indices were calculated using the veg dist as reported in Bacci et al., 2018. The influence of castal stages on alpha diversity was inspected thorough one-way analysis ov variance (ANOVA) as implemented in the "aov" function of stats native R package. Differentially abundance OTUs detected assessed using DESeq2 (Love et al., 2014). The influence of sample type was assessed using likelihood ratio test (LRT, "deseq" function).

Results

Microbial communities composition

The bacterial taxa obtained from 16S rRNAs detected in all samples corresponded to the following main phylotypes belonging to the phylum by the order of rank abundace, *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. At the class-level the main taxa are mainly represented by *Bacilli*, *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria* (Figure 2f). The yeast taxa obtained from ITS rRNAs detected in all samples corresponded to the following main phylotypes belonging to the phylum, *Ascomycota*, *Basidiomycota and Zygomycota.* The main related class are *Saccharomycetes*, *Dothideomycetes*, *Tremellomycetes* and *Eurotiomycetes* (Figure 2f).

Gut microbiota shapes across life stages and phenotypes

'Sample type' (life stage samples, as well as non-insect samples) significantly affected the microbial distribution for bacteria and yeast. PERMANOVA using distance matrices is statistically significant for the *insect type* ($p < 0.001$ and R^2 statistic indices 0.45 and 0.4 respectively for bacteria and fungi; Table 1). Thus, the analysis showed that the *insect type* influenced the microbial composition and this occur for each developmental stage and for the not-insect samples (meconium and paper nest).

Table 1: PERMANOVA performed on the *insect type* variable for both bacterial (16S) and fungal (ITS) communities .

	Term	Df	SumOfSqs	R^2	F	Pr(>F)
	insect type	5	5.240	0.459	4.417	<0.001
16S	Residual	26	6.169	0.541	/	/
	Total	31	11.409	1	/	/
	insect type	5	4.632	0.401	3.486	<0.001
ITS	Residual	26	6.910	0.599	/	/
	Total	31	11.542	1	/	/

Principal Coordinate Analysis (PCoA; Bray-Curtis distance) showed a sharp separation for each developmental stage as showed in Figure 2a,b,c and Figure 3a,b,c. However, a more evident division was reported for the fungal communities that showed a strong association to each developmental stage (Figure 3a,b,c). Even if this effect was evident for all sample types, the non-insect samples (meconium and paper nest) reported a more clear separation from all insect samples (Figure 2b,c and Figure 3b,c,).Results from PCoA were confirmed using beta diversity analysis performed using Sorensen similarity index which showed pattern of clustering for both bacteria and yeast (Figure 2e and Figure 3e) displaying a subset of matches based on similarity that shift according to the PCoA analysis. The paper nest displayed a high level of dissimilarity compared to all other samples, therefore foundresses paired with newly emerged and the same condition was visible for larvae and meconium. Foundresses and newly emerged showed more shared phylotypes (Sorensen similarity index, Figure 2 and 3) compared to the other samples and the same condition was reported for larvae and meconium (Sorensen similarity index, Figure 2 and 3). 'Sample-type' significantly affected alpha-diversity indices though the effect was more relevant regarding the mycobiota (Table 2). Then, the microbial diversity indices linked to the *insect type* variable is much more influenced by yeasts than bacteria, indicating that yeasts actually constitute a better signature of gut microbiota comparing to the bacteria. Hierarchical clustering analysis using UPGMA based on Bray-Curtis distance showed a similarity pattern according with the Sorensen's index analysis. The workers and foundresses stages resulted to be dominated by *Saccharomycetes* (>99%) that hierarchically decrease in newly emerged, larvae, meconium and finally appear almost absent in paper nest. The main bacterial class across all samples results to be the following class, *Bacilli* and *Gammaproteobacteria* mainly represented in workers, foundresses and newly emerged.

Table 2: Alpha diversity indices table reporting Evenness, Inverse Simpson (InvSimpson), Richness (Number of OTUs) and Shannon diversity for *insect type* and *environmental site* variables. All diversity indices were reported for both bacterial (16S) and fungal (ITS) communities.

	Diversity index	Term	Df	sumsq	meansq	statistic	p.value	adj.p
16S	Evenness	*insect type*	5	0,11	0,02	0,77	0,58	0,58
	Evenness	*environmental site*	5	0,21	0,04	1,43	0,26	0,51
	InvSimpson	*insect type*	5	4900,5	980,1	4,2	0,01	0,02
	InvSimpson	*environmental site*	5	1140,1	228,01	0,98	0,46	0,46
	Number of OTUs	*insect type*	5	428145	85629	5,71	0	0
	Number of OTUs	*environmental site*	5	61736	12347,19	0,82	0,55	0,55
	Shannon diversity	*insect type*	5	4,41	0,88	0,84	0,53	0,53
	Shannon diversity	*environmental site*	5	6,15	1,23	1,18	0,35	0,53
ITS	Evenness	*insect type*	5	0,9	0,18	4,57	0,01	0,01
	Evenness	*environmental site*	5	0,35	0,07	1,77	0,16	0,16
	InvSimpson	*insect type*	5	3429,7	685,93	11,21	2,33E-05	4,65E-05
	InvSimpson	*environmental site*	5	270,51	54,1	0,88	0,51	0,51
	Number of OTUs	*insect type*	5	180574	36114,87	37,79	8,31E-10	1,66E-09
	Number of OTUs	*environmental site*	5	9512,4	1902,48	1,99	0,12	0,12
	Shannon diversity	*insect type*	5	36,27	7,25	12,2	1,25E-05	2,50E-05
	Shannon diversity	*environmental site*	5	5,37	1,07	1,81	0,16	0,16

The workers showed a significant high richness rank compared to the meconium, newly emerged and nest samples but it does not involve in a higher level of diversity compared to the other sample types (Figure 2g). The paper nest bacterial communities appeared statistically significant compared to all other sample type as displayed by Inverse Simpson index (Figure 2g).

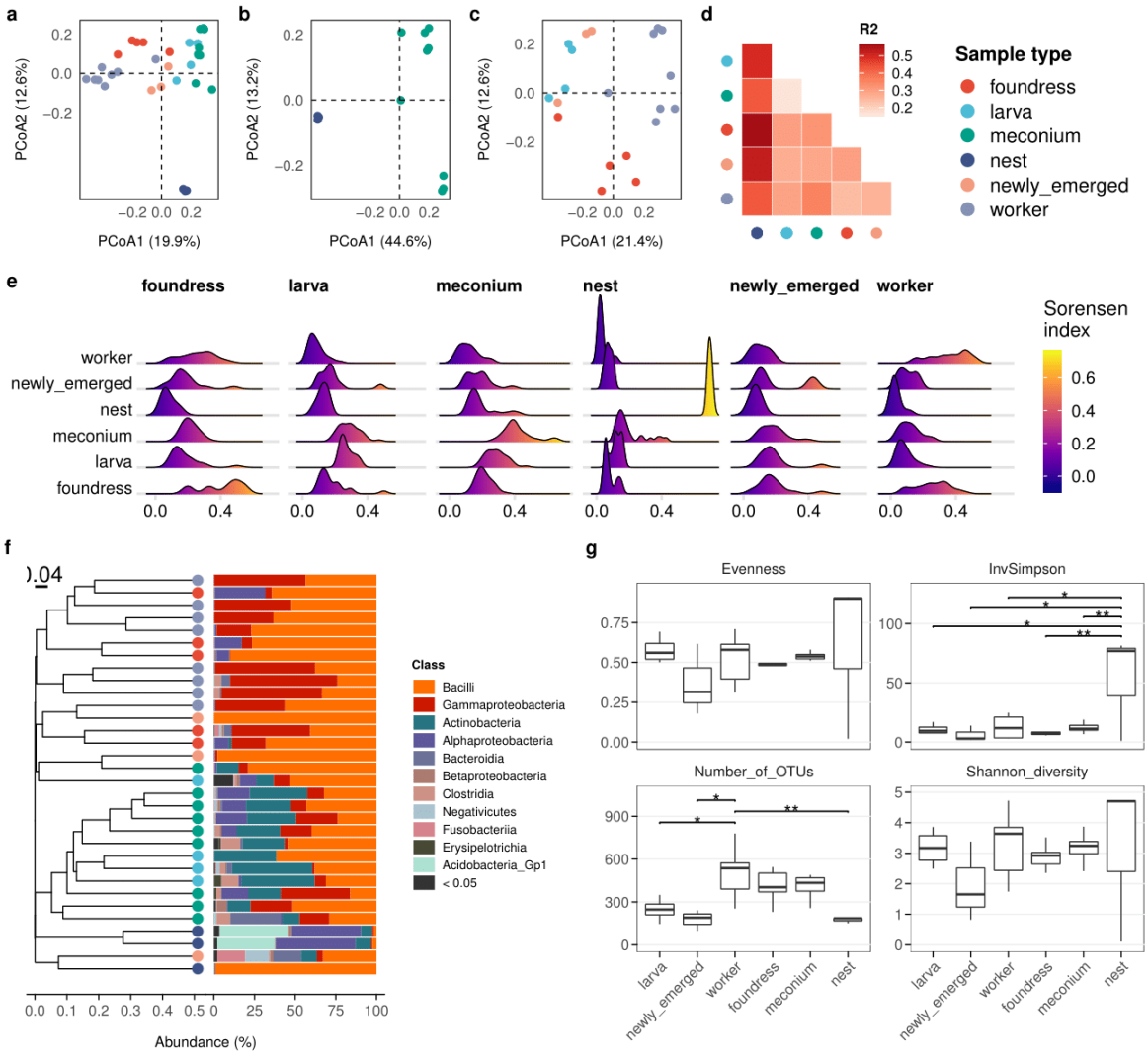

Figure 2: Bacterial communities diversity and composition. Principal coordinate analysis (PCoA) based on Bray-Curtis distance reported for the whole sample types (a), only meconium and paper nest samples (b) and only foundress, worker, newly emerged and larvae samples (c). R^2 values from pairwise adonis permutational analysis of variance test on communities composition was displayed as a 'red' gradient cells (d). Pattern of similarity between all different sample types performed using Sorensen index was displayed as area plot and the relative index rank reported as color gradient from 'blue' to 'yellow' (e). Relative abundances (%) of bacterial communities were displayed in the class-level bar plots, the related hierarchical clustering analysis (Bray-Curtis distance) was also reported (f) (∗ $p < 0.05$, ∗∗ $p < 0.01$).

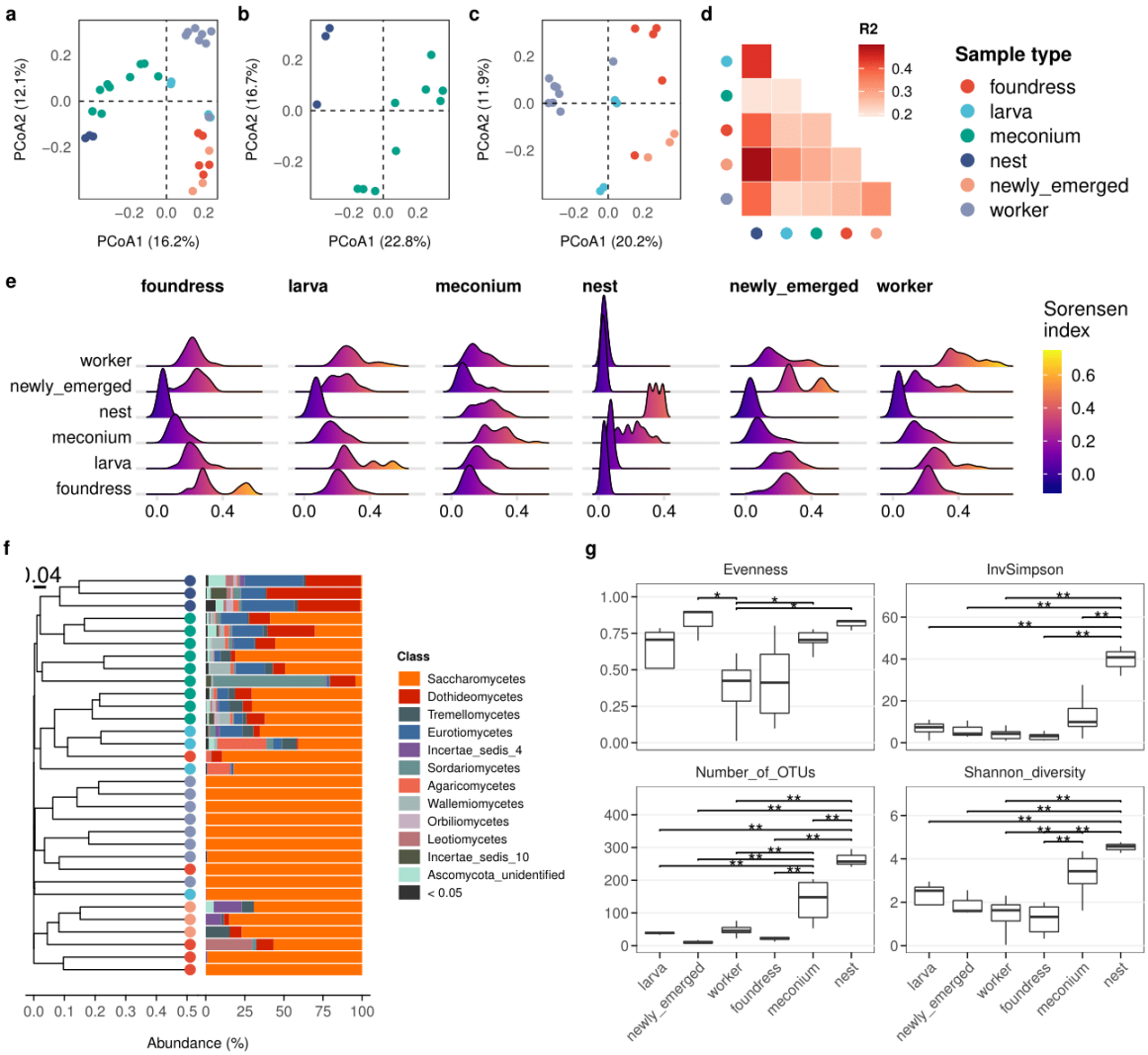

Figure 3: Fungal communities diversity and composition. Principal coordinate analysis (PCoA) based on Bray-Curtis distance reported for the whole sample types (a), only meconium and paper nest samples (b) and only foundress, worker, newly emerged and larvae samples (c). R^2 values from pairwise adonis permutational analysis of variance test on communities composition was displayed as a 'red' gradient cells (d). Pattern of similarity between all different sample types performed using Sorensen index was displayed as area plot and the relative index rank reported as color gradient from 'blue' to 'yellow' (e). Relative abundances (%) of fungal communities were displayed in the class-level bar plots, the related hierarchical clustering analysis (Bray-Curtis distance) was also reported (f) (* $p < 0.05$, ** $p < 0.01$).

Relative abundance and taxonomic distribution

We detected average differential relative abundant OTUs among all sample types using DESeq2 analysis (alpha = 0.05) approach. Foundresses, larvae and meconium showed as the most representative differentially abundant genus *Lactobacillus* and *Bifidobacterium* (Figure 4 F,L,M). *Lactococcus* is one of the leading abundant genera with *Lactobacillus*, and *Bifidobacterium.* Meconium result featured by *Actinobacteria* genus such as *Bifidobacterium*, *Alphaproteobacteria*, as well as genera inside bacilli phylum (*Lactobacillus, Fructobacillus, Leuconostoc, Enterococcus, Staphylococcus, Streptococcus and Bacillus*) with the exception of *Lactococcus*. Futhermore, meconium showed a relative high abundant level of *Betaproteobacteria, Clostridia*, and other genera from the *Gammaproteobacteria* phylum such as *Frischella*, *Gilliamella* and *Diplorickettsia,* thus contributing to represent and describe the microbial communitites displayed among all sample types (Figure 4).

Larvae and meconium showed the most close pattern of abundance, even if the larvae samples result less represented in *Alphaproteobacteria*, *Betaproteobacteria*, *Gilliamella* and *Diplorickettsia* (Figure 4 L,M). Newly emerged and paper nest showed the most poor relative abundance distribution of the entire dataset (Figure 4 N,NE). Foundresses and workes castal stage mainly harbored as a main phyla *Bacilli* and *Gammaproteobacteria*, however, both showed some quite distinct imbalance in characteristic differentially abundant taxonomic groups displayed, in workers, by a significant decrease in *Lactobacillus.*, *Alpharoteobacteria* and *Actinobacteria* with a raise up in the genera from phylum *Gammaproteobacteria*, such as *Buttiauxella* (Figure 4 F,W). Looking inside *Gammaproteobacteria*, overall mainly represented by workers, a discrete pattern of differentially enriched genera were displayed, which are *Klebsiella*, *Enterobacter* and *Escherichia/Shigella*, most represented in foundresses with the exception for Buttiauxella average more abundant in the worker castal stage (Figure 4 F,W).

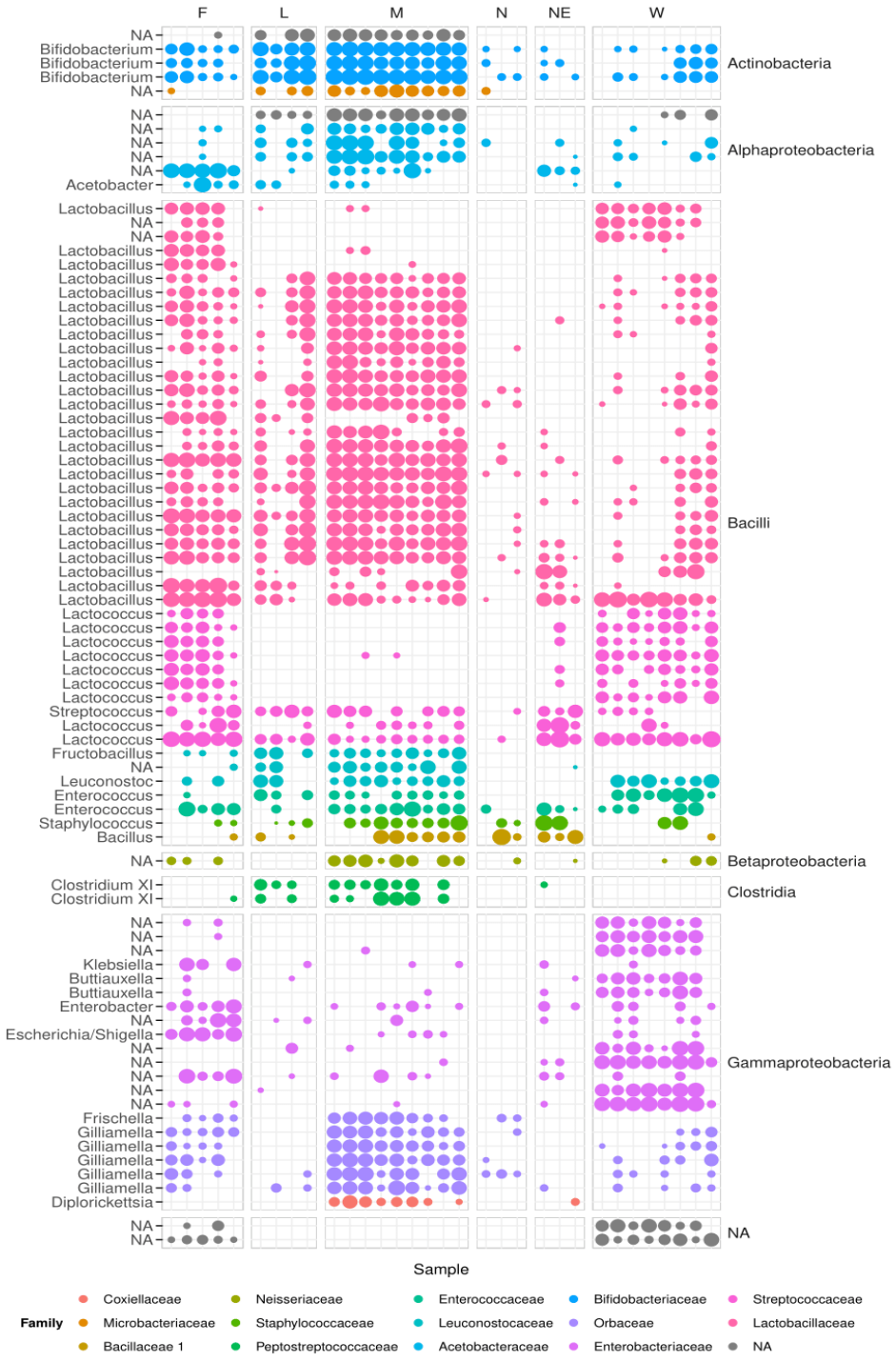

Figure 4: Phylotypes from DESeq2 analysis displayed as a bubble chart shows enrichment of differentially abundant bacterial taxonomy groups, reported for family, class and genus, (NA: not assigned) for all sample types. F: foundress, L: larvae, M: meconia, N: nest, NE: Newly emerged, W: worker. The color of bubble represents the bacterial genus and the size the amount of differentially enriched phylotypes in the bacterial dataset.

The DEseq2 analysis on fungal communitites reported *Saccharomycetes* as a main representative class of the entire ITS dataset. *Metschnikowia* And *Hanseniaspora thailandica* prove to be widespread across all sample groups, even if they result to be less differentially abundant in the nest paper samples (Figure 5 N,E). The analyses also showed differentially average abundant species within sample types, *Metschnikowia cibodasensis* absent in foundresses result mostly related to meconium and workers, then decreasing in the other sample types. *Pichia fermentans* highly differentially abundant in the workers, decreased in meconium and foundresses, resulting then absent in newly emerged and paper nest samples (Figure 5 M,F,NE). *Debaryomyces prosopidis* results to be strongly represented in larvae, meconium and worker samples, as well as *Saccharomycetales* order mirroring the same pattern of relative abundance with the exeption for foundresses where this phylotipe was deeply represented. *Tremellomycetes* is the only Basidiomycetes dispalyed in the entire ITS dataset showing a high ranking abundance in meconium, larvae, nest and only one foundress sample. As it happened for the microbiota also in the mycobiota, the meconium still the most representative sample, in term of diversity, of the entire stages dataset.

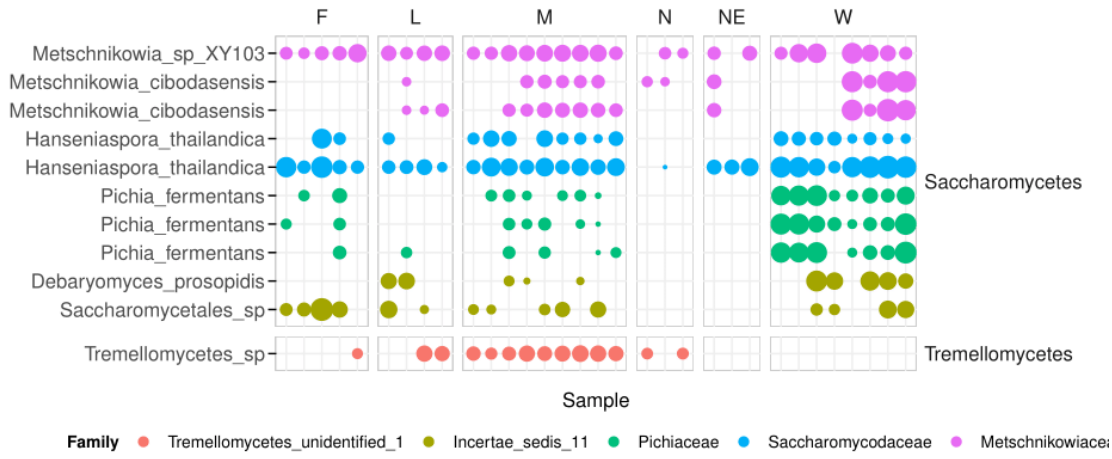

Figure 5: Phylotypes from DESeq2 analysis displayed as a bubble chart shows enrichment of differentially abundant fungal taxonomy groups, reported for family, class and genus/species, (NA: not assigned) for all sample types. F: foundress, L: larvae, M: meconia, N: nest, NE: Newly emerged, W: worker. The color of bubble represents the fungal genus and the size the amount of differentially enriched phylotypes in the fungal dataset.

Discussion

Here, we provided the first comprehensive characterization of the bacterial and fungal communities of an introduced European population of the Asian hornet *V. velutina nigritorax.* We carried out the microbiota and mycobiota analyses on different hornet life stages (Larvae, newly emerged and adult females), on two different reproductive phenotypes (workers and foundresses) and comparing these with two type of not-living colonial samples, namely the meconial and the nest material (referred as paper nest).

Overall, we identified *Bacilli*, *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria* as the most abundant classes of bacteria, and *Saccharomycetes*, *Dothideomycetes*, *Tremellomycetes* and *Eurotiomycetes* as the most abundant yeast classes. Alpha diversity indices showed that the worker stage present a significant high number of OTUs compared to all other sample types, however there is no differences displayed by Shannon and Evenness indices between all sample types, except for Inverse Simpson index that suggest a significant higher diversity composition of the nest compared to the other sample types. Nevertheless, the alpha diversity indices as a whole suggest that the number and distribution of bacterial species

among all sample types are rather uniform. Similarly, the same indication is displayed in alpha diversity indices analysis of sample's yeast communities composition that follow the trend described above but including meconium as well as nest sample.

A striking result of this study is that microbial communities show significant sample-specific signature for both bacteria and yeasts, showing differences in composition across life stages and among reproductive phenotypes. Indeed, beta diversity analyses based on relative abundance and presence/absence of OTUs, showed a pattern of sample-relted clustering composition among all sample types in agreement with the ecology of each developmental stage, indicating the presence of sample-type related microbiota and mycobiota. Moreover, all sample's communities composition grouped according to the sample type developmental stage. The switching of the microbial communities is therefore consistent with the hornet's phenotypic change, showing a displacement of the communities from the larval stage with the related meconium up to the both adult stages (wrokers and foundresses) passing through the newly emerged. The ontogenetic development of the hornets from the larva to the adult stages leads to a change in the intestinal microbial communities. Differently from *Apis mellifera*, in the hornet adult stages we can see an enrichment in Lactococci and the polarization of Lactobacilli and Bifidobacteria, significantly rapresented in the larvae and in the foundresses but almost absent in the newly-emerged.

The main bacterial classes found in insect samples are *Bacilli* and *Gammaproteobacteria*. *Actinobacteria* mostly present in the meconium decreased in larvae then declined in the adult stages that clearly appear colonized by *Bacilli* and *Gammaproteobacteria*. The presence of *Actinobacteria* in juvenile stages may play a role in the developmental process or nutrients digestion and defence against parasites. Cases of *Actinobacteria* symbionts have been found in pyrrhocorid bugs demonstrating their role in the sterilization of the egg surface (Salem et al., 2013). Different phylotipes of *Actinobacteria* have been found to defend ants, beetles and wasps against detrimental microorganisms by producing antibiotics (Kaltenpoth, 2009; Seipke et al., 2012; Visser et al., 2012) and they might be also involved in the nutrient processing and acquisition (Salem et al., 2014; Hanshew et al., 2014). *Orbacea*e, *Lactobacillus* and *Acetobacteraceae* were reported as the main enriched families in adult stages (foundresses and workers) as previously found in *Drosophila melanogaster*, explained ad bacterial communitites affected by fruit-sugar rich diet and involved in the maintenance of the immune system and mating preferences (Broderick and Lemaitre, 2012). These communities associated

with the fruit-based diet are in agreement with the hornets lifestyle, then certainly the nutrition plays a fundamental role in the microbial communities selection. The diet would also explain the high presence of *Saccharomycetes* in adult stages, whereas they feed on sugary sources such as fruits and reinforcing the importance of the Hymenoptera in the dispersion and overall in the ecology of these sugar-related yeast (Stefanini, 2018).

Bifidobacterium and *Lactobacillus*, already extensively represented in *Apis mellifera* gut microbiota are the main bacterial communities responsible for a subset of functions, essential for the insect development and overall for the maintenance of the health of the entire colony (Vásquez et al 2012). Here, *Bifidobacterium* and *Lactobacillus* were transversally distributed through the insect samples but mostly enriched in the adult stages, cosnolidate the role of these very important microorganisms as essential bacterial communities in the ecology of social hymenoptera. However, the same result was not described in the gut microbiota of *V. velutina* sampled in Korea as reported in Kim et al. in 2018. The Asian hornets sampled in Korea did not showed the communities described above, instead they were mainly characterized by *Flavobacteriales* (Kim et al. 2018).

The *V. velutina* mycobiota results mainly characterized by *Saccharomycetes* strongly represented in adult stages and less represented in larvae and meconium where we found as main communities, *Dothidomycetes*, *Tremellomycetes* and *Eurotiomycetes* described as a leading classes, which strongly participate in the nest mycobiota composition with the almost absence of *Saccharomycetes*. The nest showed a completely different communities composition compared to the other sample types as displayed by diversity and composition analyses. We can again assert that the hornet gut mycobiota as well as the microbiota was not affected by the nest-associated microbial communities. This aspect is extremely interesting cosidering that the nest represent the environment where the larvae grow, develop in adult stages and take part in the expansion, nursing and maintenance of the colony. This shade light on the evolution concept of the microbiota in a species-specific sense and even more in a caste-specific sense suggesting an even closer link of the intestinal microbial communities and their host regardless the environment.

The main relatively abundant taxa representative of the ITS dataset are *Metschnikowia cibodasensis*, *Hanseniaspora thailandica*, *Pichia fermentans*, *Debaryomyces prosopidis*, *Metschnikowia*, *Saccharomycetales* and *Tremellomycetes.*

Pichia fermentans is an Ascomycete knowon for its fermentative traits and the role in the fermentation of many fermentative substrates but recenty was also found in association with the insects, such as *D. melanogaster* and social wasps (Fogleman et al., 2014; Stefanini et al., 2018). This makes *P. fermentans* a yeast mutually able of living and adapting to the gut of different insects genera.

Tremellomycetes found in paper nest are known aa plant –associated class (Zhang et al., 2015). The presence of *Tremellomycetes* in the nest paper is in accordance with the nest building matrix usually made by environmental wood debris and this offers interesting insights to consider the analysis of the nest microbial composition as a biomarker assay in the study of plant pathogens.

The evolution of microbial communities during ontogenetic development were previously described in other social insects, such as *Apis mellifera* (Martinson et al., 2012). The physiochemical conditions of the gut in invertebrates is able to drive the development of the microbial communities (Lemke et al., 2003). Thus, the stage-related physiological changes during insect development combined to the stage-related diet could play a combined role in the selection of intestinal microbial communities. This effect could be the basis of the diversification that we highlighted between immature and adult stages.

Our results provide the "first microbial composition resource" of the microbiome of *V. velutina* in Europe, and also suggests the importance of considering life stages and reproductive phenotypes as well as nest influence in order to obtain comprehensive picture of insect gut microbial communities. This will provide the springboard for future assessment of the functional role of gut microbial organism in hornets, thus enabling to link gut microbiota to the role played by hornets in the ecosystems, as well as also enabling the first steps toward possible management strategies of this invasive pest.

Author Contributions

This chapter version represents a first draft of the work not actually published, the authors contributed as follows: *Niccolò Meriggi*[1], *Alessandro Cini*[2], *Federico Cappa*[1], *Francesco Vitali*[3], *Rita Cervo*[1] and *Duccio Cavalieri*[1]: experimental design; *Alessandro Cini*[2] and *Federico Cappa*[1]: sampling; *Francesco Vitali*[3] and *Giovanni Bacci*[1]: Data analyses; *Niccolò Meriggi*[1] and *Alessandro Cini*[2]: Manuscript drafting.

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CHAPTER III

COMPARATIVE ANALYSIS OF INTESTINAL BACTERIAL AND FUNGAL COMMUNITIES OF VESPA CRABRO, VESPA VELUTINA AND APIS MELLIFERA

Abstract

The gut microbiota is a fundamental component in the life of the hymenoptera, contributing to process food, to their immune competence and behavior. Monitoring the presence of alien species in an environmental niche means not only knowing their impact on the ecosystem but also the symbiontic microorganisms they carry in. Here, we analyzed the gut bacterial and fungal communities of a native and an invasive species of hornets, *Vespa crabro* and *Vespa velutina,* respectively, during the predation of *Apis mellifera*. Comparison of gut microbiota of these two hornet species with that of the prey honey bee showed different microbial profiles affected by food sources and by environment. Hornets host a more diversified gut mycobiota composed by a greater number of fungal OTUs compared to bacterial communities, while *Apis mellifera* presents a polarized gut microbiota composed by a few but more abundant taxa. We also identified a key role of Lactic acid Bacteria (LAB) in the intestinal core microbiota either in honey bees or in hornets. Interestingly, despite the gut bacterial communities significantly differed in each insect species (*V. crabro*, *V. velutina* and *A. mellifera*), the fungal communities composition appeared significantly related to the insect genus (*Apis* or *Vespa*), showing no significant differences between the two hornet species. Thus, the gut fungal communities, so far unexplored in insects, resulted an interesting microbial marker for hymenoptera, probably strongly affected by the environmental stimuli.

Introduction

The symbiontic relationships between insects and microrganisms influence nutrient acquisition (Douglas et al., 2009), behaviour (Lewis and Lize, 2015; Weiss and Aksoy, 2011), mating preferences (Sharon et al., 2012), sexual selection (Otti, 2015), immune system modulation and the overall insect health (Weiss et al., 2011; Engel et al., 2016; Douglas et al., 2009).

To better understand the success of an organism within the ecological dynamics, such as survival, competition for resources, reproduction and invasive displacement, we cannot ignore the concept of *holobiont* that includes the host and its symbiontic microbial communities as a whole organism (Zilber-rosenberg and Rosenberg, 2008; Bosch and McFall-Ngai, 2011).

Symbiotic microorganisms and their genome, known as microbiome, are widly described in hymenoptera, such as bumble bees (Koch and Schmid-hempel, 2011; Schmid-hempel, 2016) and honey bees (Weiss et al., 2011; Engel et al., 2016; Kwong et al.; 2017). The honey bee, *Apis mellifera* is one of the most studied social insect and its gut microbiota has been well described by Engel and Moran (Engel et al., 2014; Kwong and Moran, 2016; Engel et al., 2016, Martinson et al., 2011). *Apoidea,* such as honey bees and bumble bees, showed a distinctive microbiota with few enriched phylotypes found worldwide. However, the same condition has not been observed in solitary bees (Martinson et al., 2011, Mohr and Tebbe, 2006; Koch and Schmid-Hempel, 2011). Moreover, social insects seem to have acquired gut microbial communities specialized for their social behaviour. The whole microbiome composition seems to be little affected by environmental microorganisms (McFrederick et al., 2013). However, the relationships between social Hymenoptera and their microbiome, are not yet fully known in others families, such as *Vespidae*. Social hornets belonging to the *Vespa* genus (Hymenoptera, Vespidae) display different social habits comparing to social bees, including diverse behavioural strategies related to colony foundation and development, offspring sustenance and feeding. *Vespa* species in both temperate and tropical regions display annual nesting cycles (Matsuura, 1991). In temperate regions, the new colony is founded between Spring and early Summer by a single queen that mated during the preceding autumn and has survived hibernation. The colony develops rapidly after the first workers emerge. The number of workers reaches its maximum between late summer and early autumn, when also reproductives (i.e. males and future queens) are produced (Greene, 1984). Soon after the emergence of sexuals, the founding queen dies, workers' emergence ceases, and the colony

starts to decline rapidly (Matsura 1991). All hornets are predators and can be stratified as generalists, semi-specialists, or specialists based on their preferences for different prey and hunting strategies (Matsuura 1984; Matsuura 1991). Adult hornets, similarly to honey bees, are essentially glucivores. While bees prefer floral nectar, hornets are usually more generalist and the main carbohydrate sources are tree sap, honeydew, flower nectar, ripe fruit, mushrooms and other sugary secretions (Matsura, 1991). Protein sources are instead obtained from different substrates; honey bees eat pollen, whereas hornets hunt various arthropods (including honey bees, which often represents the favourite prey). However, adult hornets do not directly consume their prey, but they instead deliver the protein food to larvae, that give back nitrogen to adults, in the form of aminoacids, through trophallassis exchanges (Takashi et al., 1991).

Invasive alien species (IAS) often represent intense stressing agents for the native ecosystems. Competition and predation on native species drastically disrupts the ecological equilibrium of the ecosystem (Mooney and Cleland, 2001; Tompkins et al., 2003; Clavero and García-Berthou, 2005; Salo et al., 2007). Furthermore, IAS are responsible for loss of animals and plants biodiversity and various ecological negative consequences, as well as economic damage and threat for the human health (Pejchar and Mooney, 2009; Lockwood et al., 2005). *V. velutina nigrithorax* is an IAS which is rapidly spreading in Europe after its accidental introduction in 2004 (Monceau et al., 2004; Keeling et al., 2017; Granato et al., 2019; Rodriguez-Flores et al., 2019). This species represents an important threat against pollinators and other native species (Mooney and Cleland, 2001; Salo et al., 2007) and it could also represent a serious issue in terms of economic impact and public safety (Liu et al., 2015). Invasive *V. velutina nigrithorax* is likely to compete for resources with the native hornet species *Vespa crabro.*

Alien and native species may carry out gut microbial signature able to affect the ecosystem that they inhabit. The behaviour of an invasive species unavoidably may lead to a reassortment in the hosted-microbial communities (Lester et al., 2017). Moreover, the knowledges about the host-associated microorgnisms of invasive species has allowed the control of spreading of pathogens and their impact on the ecosystem (Crotti et al., 2012).

The knowledge concerning the behavioral traits of *V. velutina* has widly increased in the last years but, at present, we do not know enough about its gut microbiota composition. Since honey bees (*Apis mellifera*) represent a preferential prey for the two hornet species (Ono et al.,

1987; Matsuura, 1991; Shah and Shah, 1991; Ono et al., 1995; Couto et al., 2014; Cini et al., 2018), we wondered if consumption of this specific prey could affect the gut microbial communities.

Understanding how the gut microbiota of invasive and native species differ from each other, and how it changes in comparison to other hymenoptera, such as *A. mellifera*, connected by a prey-predator relationship, can provide crucial informations about the invasive success and overall on ecology of these insects. Here, we showed that the microbiota structure of these three insects was significantly related to the insect species variable, thus the microbial communities composition statisticantly differ between *V. crabro, V. velutina* and *A. mellifera*. In contrast, the fungal communities change in accordance with the insect genus (*Apis* and *Vespa*), showing a sharp separation between the two genera *Apis* and *Vespa*, indeed, no statistical significant differences in the fungal communities composition were displayed between the two hornet species. Interestingly, unlike honey bees, all taxonomic biomarkers identified in the hornets were associated with the *Saccharomycetales* order. This happened in accordance with the current knowledge concerning the ecology of social wasps and yeasts (Stefanini et al., 2012; Stefanini et al., 2018), contributing to further define the relationship between this very important yeasts and their natural carriers.

Materials and Methods

Sampling

Workers of the two hornet species, *Vespa velutina nigrithorax* and *Vespa crabro*, were collected while foraging for honey bees from three different apiaries in the surroundings of Ventimiglia (Imperia, Liguria- Italy). Honey bee foragers returning from their foraging flights were collected from the same apiaries. Honey bee foragers as well as bee-hawking hornets, flying in front of the hive entrances, were captured with an insect net, separated based on species into plastic boxes (dim fauna-box) and transferred to the laboratory.

Genomic DNA extraction

Insects abdomen were sterilized (washed in 70% etanhol soluzion) dissected and the gut tissues were collected and stored in RNAlater (Invitrogen – Thermo Fisher Scientific) at -20°C until DNA extraction. Microbial DNA extraction was by using DNeasy PowerLyzer PowerSoil Kit (QIAGEN) according to the manufacturer's protocol.

Bacterial and fungal communities sequencing

DNA sequencing were performed on MiSeq platform (Illumina) at BMR Genomics sequencing service (University of Padova, Italy, https://www.bmr-genomics.it) by following the Illumina's protocols for bacterial and fungal communities sequencing (www.illumina.org). For microbiome 16S rRNA gene (V3-V4 regions) was sequenced by using primers Pro341F: 5′-CCTACGGGNBGCASCAG-3′ and Pro805R: 5′-GACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014); for mycobiome ITS2 region was sequenced by using primers ITS3: 5'- GCATCGATGAAGAACGCAGC-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3' (White et al., 1990).

Clustering an statistical analysis

Demultiplexed sample libraries were visually inspected with the FastQC program (Andrews, 2010); low-quality ends of forward and reverse reads were trimmed using Sickle (Joshi and Fass, 2011) with a quality cutoff of 20 and a length threshold after trimming of 200. MICCA pipeline v. 1.7.2 (Albanese et al., 2015) was used for operational taxonomic unit

(OTU)/sequence variant (SV) picking, as previously reported in Meriggi et al. 2019 (Meriggi et al., 2019).

Alpha and beta diversity analyses were performed in R (v.3.42; R Core Team, 2018), employing package *phyloseq* v.1.22.3 (McMurdie and Holmes, 2013) and *microbiome* package (Lahti et al., 2017), as previously indicated in Meriggi et al. (Meriggi et al., 2019). Overall differences were tested with ANOVA, while species accumulation curves were calculated with the *ranacapa* package (Kandlikar et al., 2018). Prior to any analysis, count data were scaled (Paulson et al., 2013). Heatmaps reporting OTU distribution in different samples were created with the pheatmap package (Kolde, 2012). For heatmap construction, rows (i.e., OTUs/SVs) were ordered based on the results of the *plot_heatmap* command in the phyloseq package, using PCoA as the ordination method and Bray–Curtis as the distance measure, while columns (i.e., samples) were ordered based on Euclidean distance (as implemented in the pheatmap package). Permutational multivariate analysis of variance (PERMANOVA) were calculated using 999 permutation with *adonis* function in vegan package (Oksanen et al., 2018). Core microbiome was calculated with microbiome package as the OTUs present in all but one samples of a category (i.e. in the case of VV worker ijn 7 out of 8 samples). Venny (Oliveros, 2007-2015) was used to construct venn diagram showing intersections between core components. Correlation analysis were performed wit *corr.test* function in the psych package (Revelle, 2018) using Holm correction. Correlations were calculated on the trans-kingdom community data, obtained by joining OTU tables of Bacteria and Fungi communities. This analysis was performed using both the Pearson and the Spearman correlation coefficient, ultimately selecting only significant correlations (after Holm correction). Results were then imported in cytoscape to build two distinct correlation networks, one from Pearson and one with Spearman correlations. In both of them, nodes represents OTUs, connected with edges if a significant correlation was found. Finally, we used the function merge networks in Cytoscape (Shannon et al., 2003) to calculate the union between the above reported networks. To investigate modules in the correlation network, we performed cluster analysis using ClusterMaker2 (Morris et al., 2011) plugin in cytoscape, with Glay community clustering algorithm.

Microbial biomarker discovery and related statistical significance were assessed by using the linear discriminant analysis (LDA) effect size (LEfSe) method, based on the bacterial and fungal OTUs relative abundances. An alpha significance level of 0.05, either for the factorial

Kruskal-Wallis test among classes or for the pairwise Wilcoxon test between subclasses, was used. A size-effect threshold of 2.0 on the logarithmic LDA score was applied for discriminative microbial biomarkers.

LAB (Lactic Acid Bacteria) identification was performed using Basic Local Alignment Search Tool nucleotide (BLASTn) software in the National Center for Biotechnology Information (NCBI) database, basing on the highest percentage of identity (Query cover 100% and Identity 100%). Statistical analysis was performed using *ggpubr* package (Kassambara, 2019).

Results

Gut microbiota and mycobiota structure in hornets and honey bees

In order to characterize the gut microbiota and mycobiota of an invasive hornet species in comparison with the native one, and their main prey the honey bee *A. mellifera*, we collected a total of 8 *V. velutina var. nigrithorax* (Vv) and 8 *V. crabro* (Vc) during a foraging process on a total of 6 *A. mellifera* (Am) samples that were preyed in front of their beehive in Liguria region (Italy).

Alpha diversity analysis was carried out in order to verify differences in richness and diversity of the gut fungal and bacterial communitites between the two predator hornet species (native vs alien invasive), in comparison with the honey bee prey. For mycobiota, we did not observe significative differences among the three sample groups by using 4 different estimation indeces, such as OTUs number (richness), Chao 1, Sahnnon H diversity and Pielou's J Evenness (Figure 2A-D). Principal Coordinates Analysis (PCoA) performed on Bray-Curtis distances showed a clear separation between mycobiome of the honey bee group and the two hornets' groups (Figure 2E; PERMANOVA R^2= 0.26, p-value < 0.001). Pairwise PERMANOVA comparisons showed no differences between fungal community in the both hornet groups (PERMANOVA VV vs VC: R^2=0.10, p-value = 0.257), while significant differences between hornets and honey bees were reported (PERMANOVA VV vs AM: R^2=0.22, p-value < 0.001; PERMANOVA VC vs AM: R^2=0.30, p-value < 0.001). Hierachical Clustering based on Bray-Curtis distances (Figure 2F) confirmed the observed separation according to the two different Hymenoptera genera. Samples of Am group clustered in a separate branch from both native and alien hornet sample groups.

When gut bacterial communities were compared among groups, alpha diversity analysis showed clear differences in terms of richness and diversity with respect to the fungal ones. Richness showed a significant higher number of OTUs in both hornets' microbiomes than honey bees' one (*Vv* vs *Am*: p=0.01; *Vc* vs *Am*: p<0.01). Chao 1 index showed significant differences between both hornets and honey bees (*Vv* vs *Am*: p<0.01; *Vc* vs *A m*: p<0.01), as well as Shannon H diversity indices between *Vc* and *Am* (p=0.02) (Figure 1A-D). No significant differences were reported for Pielou's J Evenness index for all group comparisons. As observed by mycobiome composition, PCoA ordination (PERMANOVA R^2= 0.36, p-value < 0.001) and Hierarchical Clustering, performed on bacterial communities, confirmed the distinct pattern of separation among both hornets and honey bee groups (Figure 1E-G). Unlike the mycbiota, pairwise PERMANOVA performed on gut microbiota also showed a significant difference between the two hornets species (PERMANOVA VV vs VC: R^2=0.15, p-value = 0.014; PERMANOVA VV vs AM: R^2=0.31, p-value = 0.001; PERMANOVA VC vs AM: R^2=0.43, p-value = 0.001).

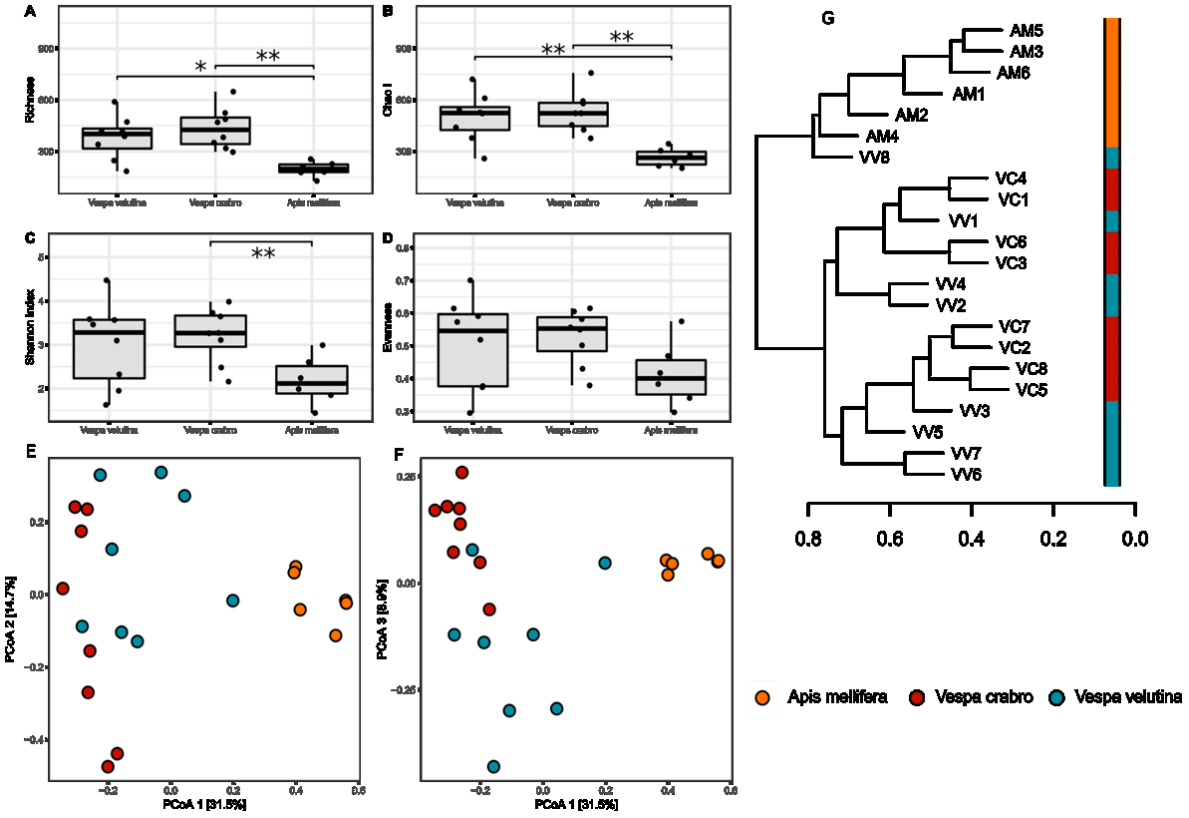


Figure 1. Gut bacterial communities exploration in two hornet species (*V. crabro* and *V. velutina*) and *A. mellifera*. Microbiota characterization dispalyed by (A-D) alpha diversity analysis by using four different indices, richness (A), Chao-1 (B), Shannon diversity index (C) and Evenness (D) (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ns = not statistically significant). PCoA analysis (Bray-Curtis distances) (E-F) and Hierarchical Clustering analysis (Bray-curtis distances) (G).

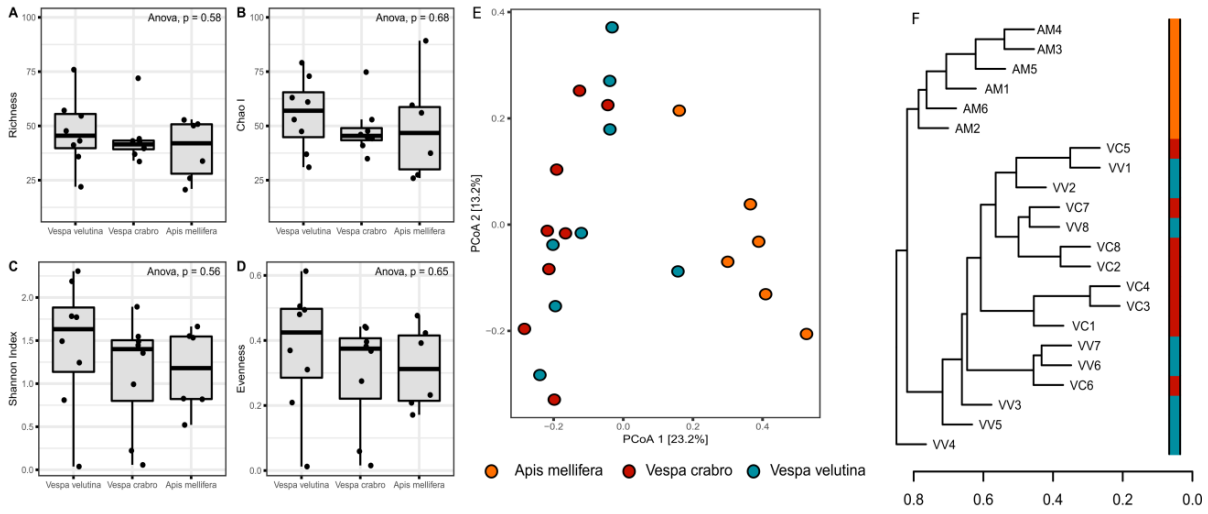


Figure 2. Gut fungal communities exploration in two hornet species (*V. crabro* and *V. velutina*) and *A. mellifera*. Microbiota characterization dispalyed by (A-D) alpha diversity analysis by using four different indices, richness (A), Chao-1 (B), Shannon diversity index (C) and Evenness (D) (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ns = not statistically significant). PCoA analysis (Bray-Curtis distances) (E) and Hierarchical Clustering analysis (Bray-curtis distances) (F).

In order to describe the microbial composition profile and structure of the three insect species, we evaluated either rank abundance curves of the OTUs and the distribution of taxa relative abundances among sample groups by means of the heatmap (Figure 3A-B). The rank abundance curves performed on bacterial communities showed that the species rank in *A. mellifera* group ended under 600, while in hornets, a less abundant number of species continued up to 1000-1200. Considering the fungal counterpart, in all insect groups, a few of rank species, but more abundant than bactaria, ended to 100-140.

By heatmap, OTUs from 16S dataset appear to be similarly distributed among hornet samples compared to the honey bees. *A. mellifera* group showed a lower number of OTUs (generally distributed at the extremes of the heatmap), but with higher relative abundances compared to the OTUs of hornet groups (Figure 3B). A similar trend of OTUs distribution and abundance among gropus was also reported in the heatmap of the fungal dataset (Figure 3B).

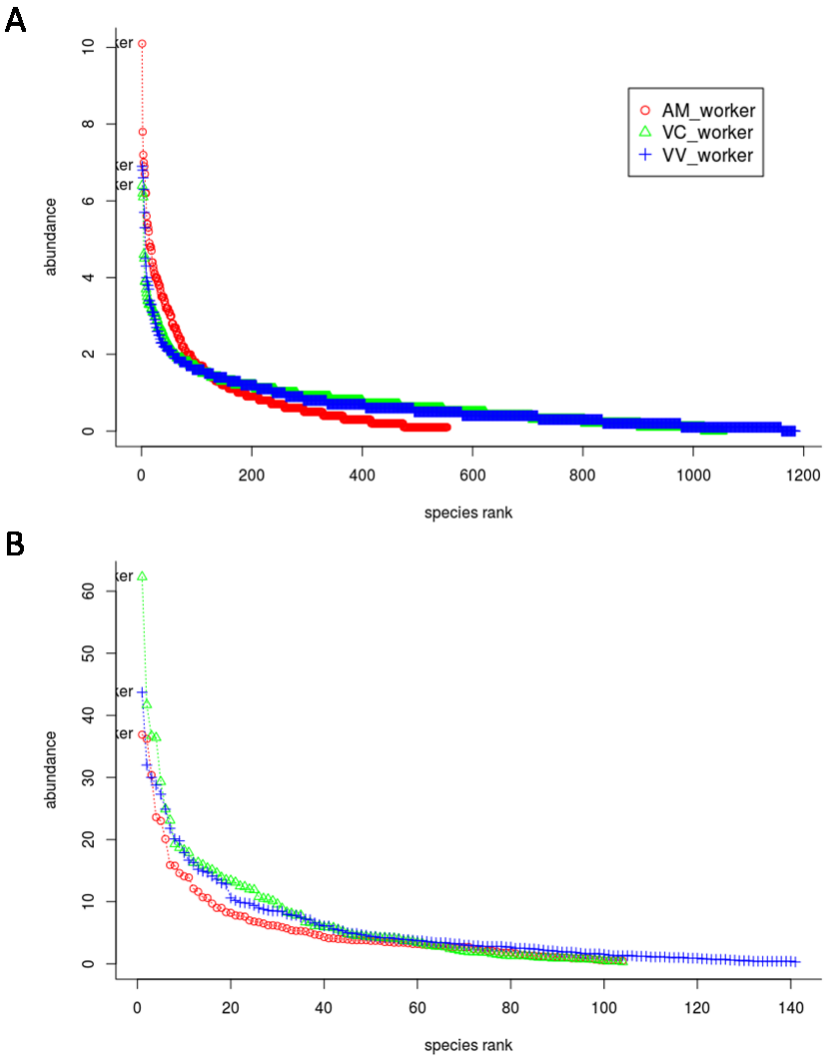


Figure 3: Rank abundance curves of (A) bacterial and (B) fungal communities. In Y- and X-axis species abundances and species rank were reported, respectively. Rank abundance curves were performed for each samples among groups (Red: *A. mellifera*; green: *V. crabro*; blue: V. velutina).

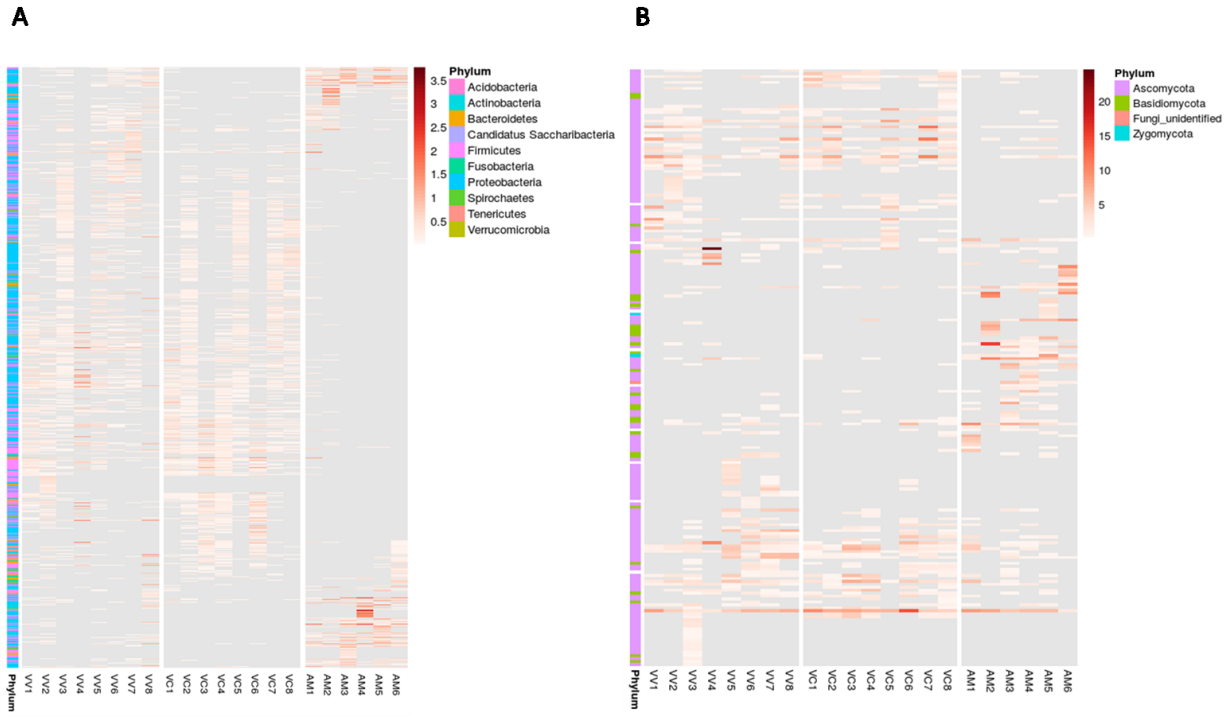


Figure 4. Heatmap of taxonomic composition profiles based on OTUs relative abunce. Heatmaps reported the OTUs relative abundance and distribution of (A) bacterial and (B) fungal communities among the groups. Relative abundance of each OTUs among groups was displayed using color gradient from white to red, while grey color represents 0.

Core gut microbiota and mycobiota of hornets and honey bees

To explore the symbiotic relationships between the host and its gut bacterial and fungal communities, we performed the analysis of the core gut microbiota and mycobiota of the three insect species. We considered the core OTUs as those present in all but one samples of a category (i.e. in the case of VV worker ijn 7 out of 8 samples) .

Regarding core bacterial community, we identified 30 core OTUs in Am, 44 in Vc and 9 in Vv (Figure 5A-B). Interestingly, no common core OTUs were reported among hornets and honey bees, while 7 OTUs (1 from *Lactobacillus* and 3 *Lactococcus* genera and 3 *Enterobacteriaceae* family) were shared between Vc and Vv, and only 2 (from *Enterobacteriaceae* family) were found as exclusively associated to *V. velutina*-core (Figure 5A-B). Overall, we observed that the hornets- or honey bee- associated core bacterial OTUs were assigned to no more than 7 genera (Figure 5 A,C), such as *Bifidobacterium* (Actinobacteria), *Lactobacillus* (Firmicutes), *Streptomyces* (Actinobacteria), *Lactococcus* (Firmicutes), *Gilliamella* (Proteobacteria), and *Enterococcus* (Firmicutes).

Based on these results, we could hypothesize that the core gut microbiota of hornets and honey bees is populated from a substantial Lactic Acid bacteria (LAB) communities and the related phylotipes seem to be exclusively associated with the insect species. The BLAST alignment of the first most abundant OTUs assigned to the LAB genera, showed a clear different composition pattern between hornets and honey bees as reported in Figure 6. The aligned OTUs sequences (referred as DENOVO) were associated with 100% of both Identity and Query Cover (see Materials and Methods section) to these three following species: *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactococcus lactis*. After Kruskal-Wallis test (Denovo 1_*Lactobacillus plantarum*, p=0.083; Denovo 2_*Lactocuccus lactis*, p=0.0085; Denovo 7_*Lactobacillus brevis*, p=0.006) the comparisons of means among groups by Wilkoxon test were performed (Table 1). *Lactobacillus plantarum* was significantly more abundant in *V. crabro* comparing with Am (Wikoxon, p=0.029), the same trend was also found between Vv and Vc, despite this comparison was not significant (Wilkoxon, p=0.063). *Lactobacillus brevis* was significantly more abundant in Vc than Vv (Wilkoxon, p=0.013) and *A. mellifera* (Wikoxon, p=0.01). *Lactococcus lactis* was significantly enriched in both hornets when compared to Am (Wilkoxon, Vc*Am: p=0.005; Vv*Am: p=0.029).

Similary, we explored the gut mycobiota core. In the fungal community of the three groups of insects, only a small number of core OTUs were identified (5 in Am, 9 in Vc and 6 in Vv, respectively; Figure 5). Interestingly, only one core OTU (belonging to *Hanseniaspora thailandica*|SH237946.06FU) was shared among the two hornets and honey bees, and 3 OTUs core (assigned to *Pichia fermentans* species) were shared between Vc and Vv.

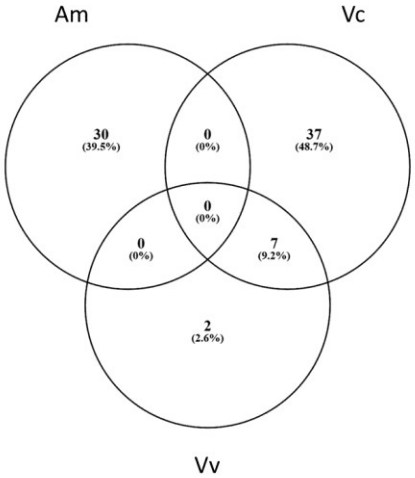
A

AM		VC		VV	
OTU	Taxonomy	OTU	Taxonomy	OTU	Taxonomy
DENOVO3	Actinobacteria_Bifidobacteriaceae_Bifidobacterium	DENOVO17	Firmicutes_Enterococcaceae_Enterococcus	DENOVO1	Firmicutes_Lactobacillaceae_Lactobacillus
DENOVO14	Actinobacteria_Bifidobacteriaceae_Bifidobacterium	DENOVO1	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO2	Firmicutes_Streptococcaceae_Lactococcus
DENOVO21	Actinobacteria_Bifidobacteriaceae_Bifidobacterium	DENOVO7	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO1082	Firmicutes_Streptococcaceae_Lactococcus
DENOVO2785	Actinobacteria_Bifidobacteriaceae_NA	DENOVO44	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO1905	Firmicutes_Streptococcaceae_Lactococcus
DENOVO255	Actinobacteria_Streptomycetaceae_Streptomyces	DENOVO46	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO5	Proteobacteria_Enterobacteriaceae_NA
DENOVO2736	Actinobacteria_Streptomycetaceae_Streptomyces	DENOVO271	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO8	Proteobacteria_Enterobacteriaceae_NA
DENOVO336	Bacteroidetes_Flavobacteriaceae_NA	DENOVO327	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO43	Proteobacteria_Enterobacteriaceae_NA
DENOVO25	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO496	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO51	Proteobacteria_Enterobacteriaceae_NA
DENOVO26	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO523	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO2422	Proteobacteria_Enterobacteriaceae_NA
DENOVO37	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO779	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO53	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO799	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO55	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO1130	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO58	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO1171	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO65	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO1316	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO83	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO1390	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO106	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO1971	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO159	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO2015	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO196	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO2362	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO1337	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO2433	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO1676	Firmicutes_Lactobacillaceae_Lactobacillus	DENOVO2802	Firmicutes_Lactobacillaceae_Lactobacillus		
DENOVO4	Proteobacteria_Enterobacteriaceae_NA	DENOVO641	Firmicutes_Lactobacillaceae_NA		
DENOVO656	Proteobacteria_Enterobacteriaceae_NA	DENOVO846	Firmicutes_Lactobacillaceae_NA		
DENOVO941	Proteobacteria_Enterobacteriaceae_NA	DENOVO1000	Firmicutes_Lactobacillaceae_NA		
DENOVO1566	Proteobacteria_Enterobacteriaceae_NA	DENOVO2	Firmicutes_Streptococcaceae_Lactococcus		
DENOVO1613	Proteobacteria_Enterobacteriaceae_NA	DENOVO890	Firmicutes_Streptococcaceae_Lactococcus		
DENOVO1961	Proteobacteria_Enterobacteriaceae_NA	DENOVO901	Firmicutes_Streptococcaceae_Lactococcus		
DENOVO138	Proteobacteria_Neisseriaceae_NA	DENOVO976	Firmicutes_Streptococcaceae_Lactococcus		
DENOVO77	Proteobacteria_Orbaceae_Gilliamella	DENOVO1082	Firmicutes_Streptococcaceae_Lactococcus		
DENOVO95	Proteobacteria_Orbaceae_Gilliamella	DENOVO1111	Firmicutes_Streptococcaceae_Lactococcus		
DENOVO139	Proteobacteria_Orbaceae_Gilliamella	DENOVO1468	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO1718	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO1820	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO1905	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO2040	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO2074	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO2151	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO2262	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO2343	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO2373	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO2425	Firmicutes_Streptococcaceae_Lactococcus		
		DENOVO73	Proteobacteria_Enterobacteriaceae_Buttiauxella		
		DENOVO5	Proteobacteria_Enterobacteriaceae_NA		
		DENOVO8	Proteobacteria_Enterobacteriaceae_NA		
		DENOVO43	Proteobacteria_Enterobacteriaceae_NA		

B

AM		VC		VV	
OTU	Taxonomy	OTU	Taxonomy	OTU	Taxonomy
DENOVO1	**Ascomycota_Hanseniaspora_thailandica|SH237946.06FU**	**DENOVO1**	**Ascomycota_Hanseniaspora_thailandica|SH237946.06FU**	**DENOVO1**	**Ascomycota_Hanseniaspora_thailandica|SH237946.06FU**
DENOVO6	Ascomycota_Kluyveromyces_marxianus|SH232098.06FU	DENOVO2	Ascomycota_Pichia_fermentans|SH223345.06FU	DENOVO2	Ascomycota_Pichia_fermentans|SH223345.06FU
DENOVO8	Ascomycota_Davidiella_tassiana|SH196750.06FU	DENOVO3	Ascomycota_Metschnikowia_sp_XY103|SH197258.06FU	DENOVO5	Ascomycota_Pichia_fermentans|SH223345.06FU
DENOVO11	Zygomycota_Mucor_brunneogriseus|SH200020.06FU	DENOVO4	Ascomycota_Pichia_fermentans|SH223345.06FU	DENOVO17	Ascomycota_NA
DENOVO12	Ascomycota_Saccharomycetales_sp|SH223340.06FU	DENOVO5	Ascomycota_Pichia_fermentans|SH223345.06FU	DENOVO43	Ascomycota_Hanseniaspora_thailandica|SH237946.06FU
		DENOVO17	Ascomycota_NA	DENOVO64	Ascomycota_Lachancea_thermotolerans|SH205946.06FU
		DENOVO28	Ascomycota_NA		
		DENOVO33	Ascomycota_Saccharomyces_cerevisiae|SH241331.06FU		
		DENOVO36	Ascomycota_Dipodascaceae_sp|SH197414.06FU		

C



D

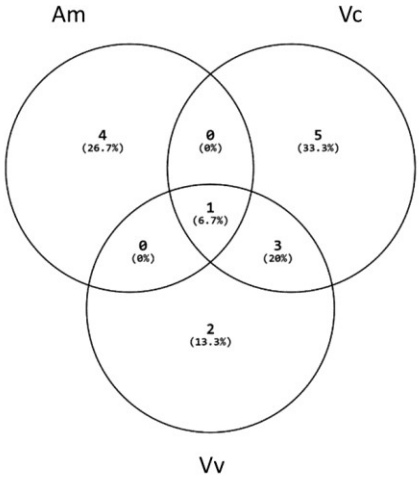


Figure 5. Core gut microbiota and mycobiota perfomed on the both hornets (Vv and Vc) and honey bee (Am). Table A reported each OTUs (referred as DENOVO) composing the core microbiota of the three insect groups (AM, VC, VV). Table B reported each OTUs (referred as DENOVO) composing the core mycobiota of the three insect groups (AM, VC, VV). Red labelled DENOVO correspond to the shared OTUs between at last two groups. Venn diagram performed on bacterial communities (C) shows the number of OTUs associated with the percentage value, exclusive and shared for each insect group. Venn diagram performed on fungal communities (D) shows the number of OTUs associated with the percentage value, exclusive and shared for each insect group.

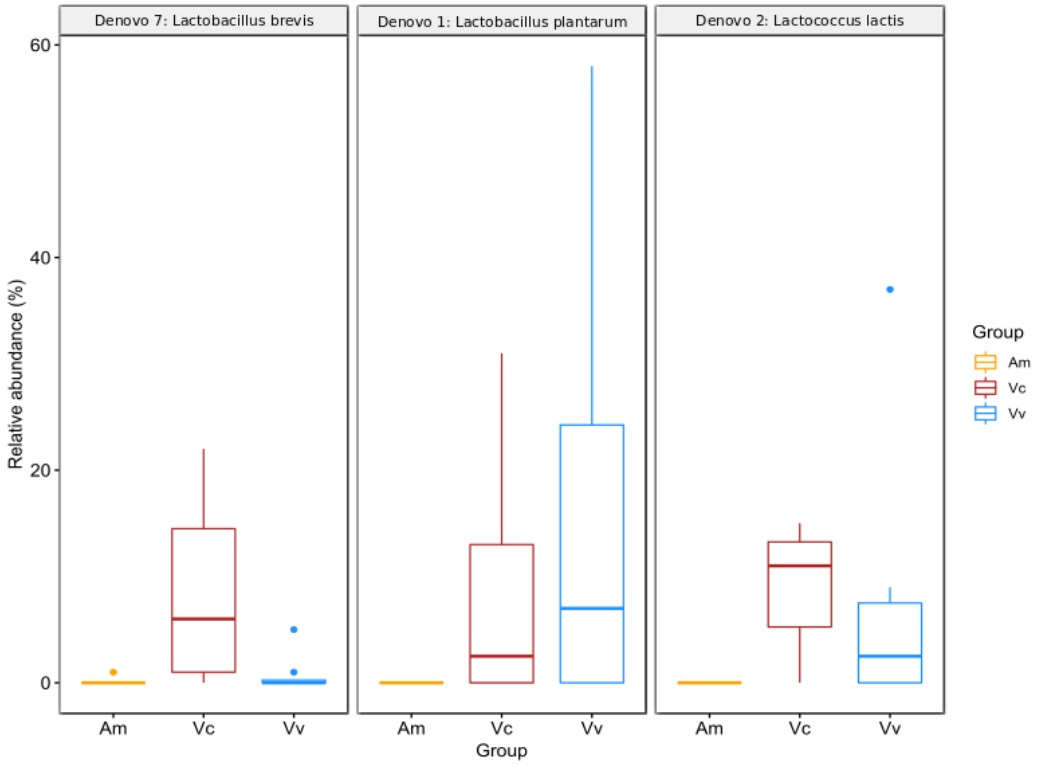


Figure 6. Comparisons among the most abundant LAB-related OTUs from BLAST alignment. The Box plots showed the relative abundance (%) per group (Am: *Apis mellifera*, Vc: *Vespa crabro*, Vv: *Vespa velutina*) for the three main abundant LAB-related OTUs referred as Denovo 7 (*Lactobacillus brevis*), Denovo 1 (*Lactobacillus plantarum*) and Denovo 2 (*Lactobacillus lactis*).

Table 1. Multiple comparisons using Wikoxon test among the three insect groups. DENOVO relative abundance (%) was compared among groups (Am: *Apis mellifera*, Vc: *Vespa crabro*, Vv: *Vespa velutina*). Denovo alignment: taxonomic species obtained from BLAST analysis on DENOVO (OTU); Sample: sample groups in different comparison combinations; P: p-value; p.sign: significance of each p-value (∗ $p < 0.05$, ∗∗ $p < 0.01$, and ns = not statistically significant).

Denovo alignment	Sample	p	p.sign
Lactobacillus plantarum	Am*Vc	0.029	*
Lactobacillus plantarum	Am*Vv	0.063	ns
Lactobacillus plantarum	Vc*Vv	0.869	ns
Lactobacillus brevis	Am*Vc	0.01	*
Lactobacillus brevis	Am*Vv	0.719	ns
Lactobacillus brevis	Vc*Vv	0.013	*
Lactococcus lactis	Am*Vc	0.005	**
Lactococcus lactis	Am*Vv	0.029	*
Lactococcus lactis	Vc*Vv	0.169	ns

Different bacterial and fungal profiles characterize hornet and honey bee guts

To determine significant different microbial profiles related to the hornet and honey bee guts, we performed LEfSe analysis in order to discover microbial biomarkers among the three groups.

LEfSe analysis showed that *Streptomyces*, *Bifidobacterium*, *Sporosarcina*, *Faecalibacterium*, *Saccharibacter*, *Pantoea*, *Frischella* and *Gilliamella* were the bacterial genera signifcantly associated to Am. *Dysgonomonas*, *Vagococcus*, *Leuconostoc, Asaia, Lonsdalea*, Obesumbacterium, *Raoultella* and *Serratia* genera were significantly associated to Vv and *Rothia*, *Porphyromonas*, *Alloprevotella*, *Lactococcus*, *Acetobacter*, *Buttiauxella*, *Morganella* and *Providencia* genera were found in Vc (Figure 7A).

Unlike the microbiota, LEfSe analysis performed on mycobiota shows a sharp division in accordance to the insect genus (*Apis* and *Vespa*), most of the biomarkers displayed in Vc were also detected in Vv and are all related to *Saccharomycetales* order. Particularly, *Lachancea thermotolerans* was found as a microbial marker in Vv, while *Candida bracarensis*, *Pichia fermentans* and *Hanseniaspora guillermondii* species were detected as a markers in Vc.

The genus *Wallemia* and *Davidiella tassiana, Penicillium chrysogenum, Penicillium polonicum, Cordyceps bassiana, Cryptococcus victoria, Mucor brunneogriseus* species appear

significantly associated to Am., therefore, the only *Saccharomycetales* detected as a microbial marker in Am was *Kluyveromyces marxianus* (Figure 7B).

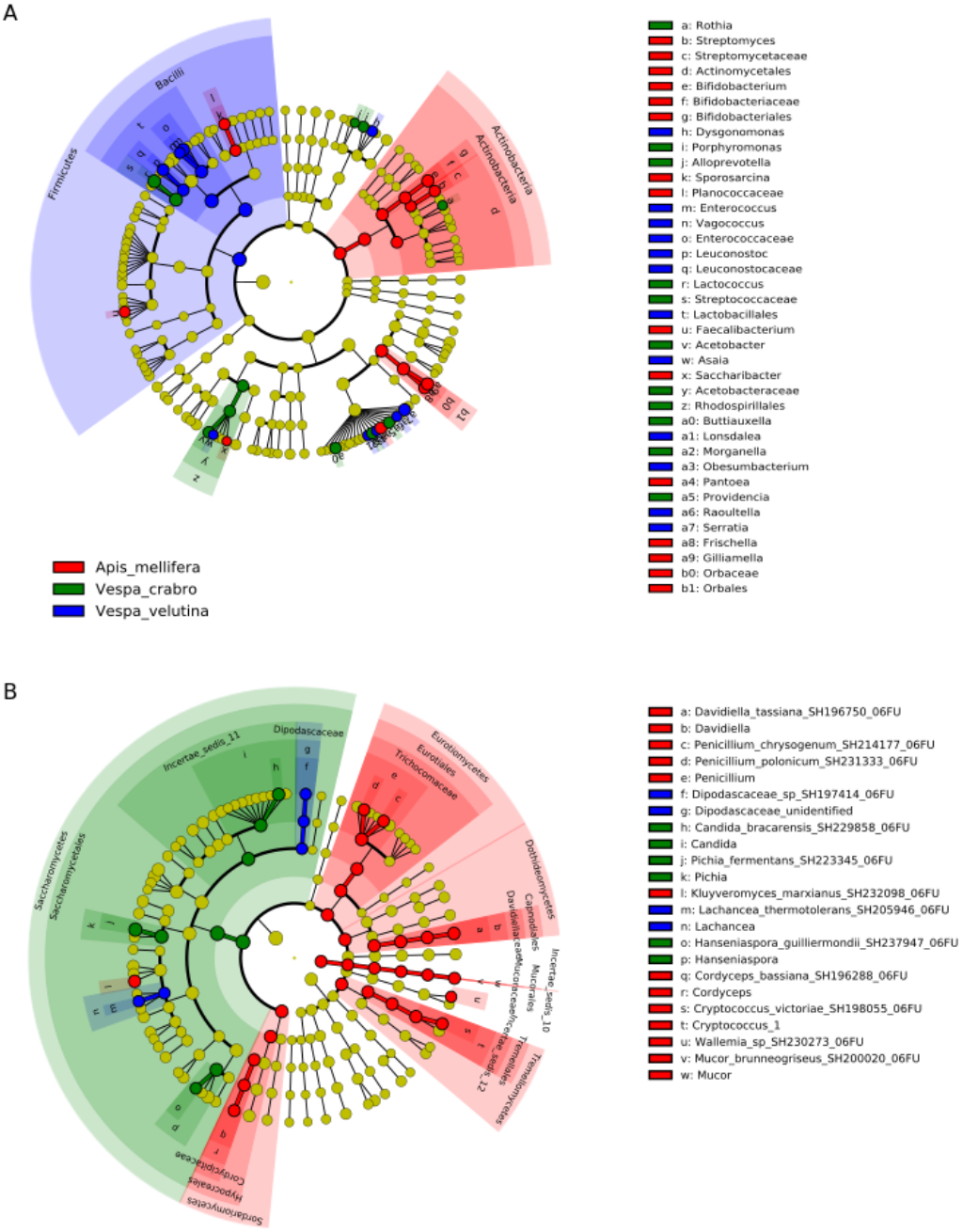


Figure 7. Bacterial and fungal biomarker discovery by LEfSe analysis. (A) bacterial and (B) fungal taxa associated to hornets (*V. crabro* in blue, and *V. velutina* in green) and honey bees (red). Results indicated the statistically significant taxa enrichment among groups (Alpha value = 0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

In addition to searching for significant different microbial profiles, we also explored cross-kingdom interaction in the gut microbial community of hymenoptera. To do so, we firstly joined data of bacterial and fungal communities (i.e. the OTUs tables from the two kingdoms) and performed multiple correlation analysis between all OTUs. The analysis were performed using both Pearson and Spearman correlation coefficient, selecting only significant correlations (after Holm correction) with R value greater than 0.8. Thus, we built a network based on both Pearson and Spearman correlation, and finally reported the merged (UNION) network of the two correlation coefficients. The results of this analysis were imported to Cytoscape to construct two separated correlation networks and merged these to build Figure 8A, in which bacterial and fungal OTUs represented the nodes (rectangles indicate fungal OTUs while ellipses indicate bacterial OTUs; node size is proportional to node degree) connected by an edge if a significant Pearson or Spearman correlation was found. Relative abundances have not been mapped in the network since they deeply change between the three insect groups, thus, the addition of the average abundance mapped on the node size, would not been so informative. The median (non zero) abundance of the OTUs were displayed in the barplot representing the cluster compositions (Figure 8).

Inspection of network structure, suggested certain modularity and the presence of distinct sub-cluster correlations, despite the presence of a unique connected component. To further investigate such modularity, we performed cluster analysis using ClusterMaker2 (Matherials and Methods) plugin in Cytoscape, with Glay community clustering algorithm. Clustering analysis divided the network in 8 clusters, which are reported in panel B to G of Figure 8 (light-blue rectangles indicate fungal OTUs while orange ellipses indicate bacterial OTUs; size is proportional to node degree), and indicated as node color in Figure 8A. Barplots in panel B to G of Figure 8, display the non-zero median CSS-scaled abundance of the OTUs composing a network cluster in the three hymenoptera.

Overall, almost all correlations were positive, indicating co-occurrence between OTUs and suggesting a diffused commensalist or mutualistic relationship between different bacterial OTUs as well as between bacterial and fungal OTUs. Also, cross-Kingdom commensalist or mutualistic relationship seems to be diffused as all clusters were composed of both bacteria and fungi. Nevertheless, cluster 7 (panel G) is and exception to this observation being composed of only one fungal OTU and 21 bacterial OTUs. Notably, we consider this cluster associated to Vc, as OTUs median abundance is almost 0 in all other sample groups. The two largest clusters, clusters 2 and 3, are composed of OTUs that were found at median abundance different to 0 in all the insect samples. Nevertheless, we observe that cluster 3 is the only one with OTUs enriched in Am respect to Vc and Vv and, accordingly, panel A showed that this cluster tend to be splitted from the rest of the network. The other main cluster, cluster 2, contains OTUs widly represented in Am and Vv, while the other clusters are all more represented in hornets, either Vv (cluster 5 and 6) or Vc (cluster 1 and 7).

Concerning the network phylotypes composition, the network-based analysis on microbiota and mycobiota of the three insect groups showed, as mentioned above, an heterogeneous composition, with the presence of 2 clusters associated with the insect genus (*Vespa* or *Apis*) (Figure 8B,F), one associated only with Vv and the other comprensive of all groups. In that, cluster 'C' was related only with Vv microbial communities composed by *Carnobacterium, Kregervanrija fluxuum* and *Zymobacter* (Figure 8C).

Candida bracarensis, Hanseniaspora guillermondii, *Kluyveromyces dobzhanskii* and *Rothia* grouped into a cluster (Figure 8B) related to both hornets, furthermore, Dysgonomonas appear related just to hornets samples as well (Figure 8F). These last two clusters take part in the network that contains microorganisms that are probably central in the hornets ecology but not in honey bees ecology. Cluster 'D' and 'E' were displayed as the two largest (connected) clusters composing the network structure and appeared equally composed by both bacteria and fungi.

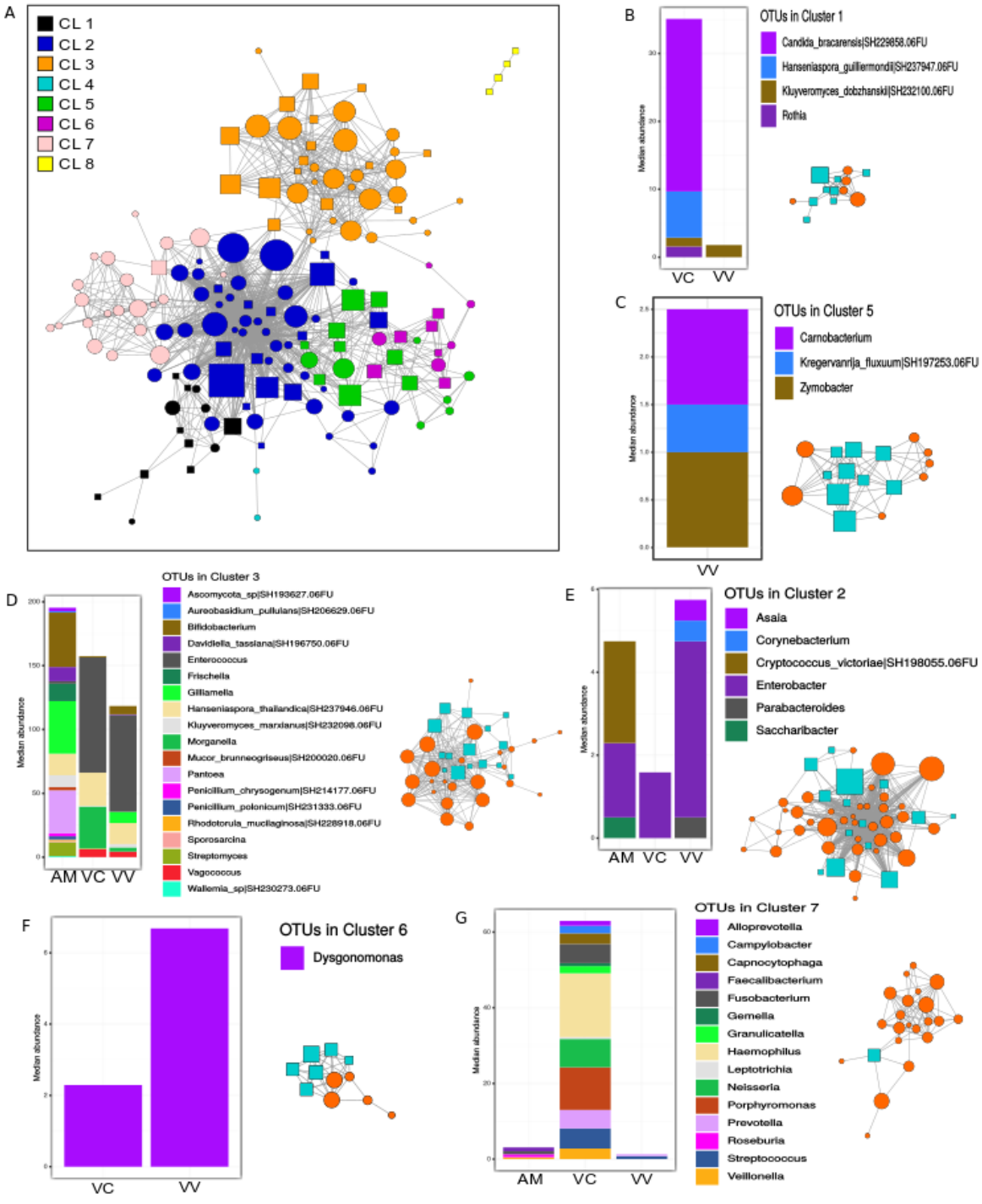

A
CL 1
CL 2
CL 3
CL 4
CL 5
CL 6
CL 7
CL 8
B
OTUs in Cluster 1
Candida_bracarensis|SH229858.06FU
Hanseniaspora_guilliermondii|SH237947.06FU
Kluyveromyces_dobzhanskii|SH232100.06FU
Rothia
Median abundance
VC VV
C
OTUs in Cluster 5
Carnobacterium
Kregervanrija_fluxuum|SH197253.06FU
Zymobacter
Median abundance
VV
D
OTUs in Cluster 3
Ascomycota_sp|SH193627.06FU
Aureobasidium_pullulans|SH206629.06FU
Bifidobacterium
Davidiella_tassiana|SH196750.06FU
Enterococcus
Frischella
Gilliamella
Hanseniaspora_thailandica|SH237946.06FU
Kluyveromyces_marxianus|SH232098.06FU
Morganella
Mucor_brunneogriseus|SH200020.06FU
Pantoea
Penicillium_chrysogenum|SH214177.06FU
Penicillium_polonicum|SH231333.06FU
Rhodotorula_mucilaginosa|SH228918.06FU
Sporosarcina
Streptomyces
Vagococcus
Wallemia_sp|SH230273.06FU
Median abundance
AM VC VV
E
OTUs in Cluster 2
Asaia
Corynebacterium
Cryptococcus_victoriae|SH198055.06FU
Enterobacter
Parabacteroides
Saccharibacter
Median abundance
AM VC VV
F
OTUs in Cluster 6
Dysgonomonas
Median abundance
VC VV
G
OTUs in Cluster 7
Alloprevotella
Campylobacter
Capnocytophaga
Faecalibacterium
Fusobacterium
Gemella
Granulicatella
Haemophilus
Leptotrichia
Neisseria
Porphyromonas
Prevotella
Roseburia
Streptococcus
Veillonella
Median abundance
AM VC VV

Figure 8. Network representation of microbiota and mycobiota correlations using Pearson and Spearman coefficient. Nodes represente bacterial (circles) and fungal (squares) OTUs, with size representing node degree. Edges connect the OTUs showing a significant correlation. Network clusters were represented using different colors (A), and reported separately using colors to highlight bacteria (orange) and yeast (light-blue) in each network. A barplot representing the median (non zero) abundance of OTUs composing the cluster in the three hymenoptera groups, is reported for every cluster (except cluster 8 and 4 which were to small to be considered).

Discussion

Evidences showed that the gut microbiota of the hymenoptera, especially that of honey bee, characterized by a low diversity, would seem to be very stable worldwide (Corby-Harris et al., 2014; Kwong et al., 2017), and strongly influenced by sociality (Kwong and Moran, 2016; Kwong et al., 2017. The honey bee microbiota has been widely explored but it is not known actually enough about the hornet microbiota. Thus, we carried out the analysis of the gut microbial communities of the two hornet species currently present in Europe, including Italy, Vv and Vv.

Vc and Vv showed significant differences in some behavioral traits, such as foraging activity colony size. Therefore, alien and native species may carry out with them a microbial signature able to affect the ecosystem that they inhabit. The migratory behaviour of an invasive species unavoidably may lead to a reassortment in the hosted-microbial communities and at the same time, the competition and predation of invasive alien species towards native ones may drastically disrupt the ecological equilibrium of the ecosystems. Thus, understand the impact that alien species may have on the ecosystem appear crucial and the study of the composition of their symbiontic microorganisms can explain better their ecology and success.

The main differences displayed by alpha diversity analyses were related to the bacterial communitities and showed a significant differences between both hornets and honey bee, despite no significant differences were reported for Shannon diversity index between Vv and Am. No differences were reported among the two hornets. Moreover, no significant differences were displayed in alpha diversity analyses performed for fungal communities.

PERMANOVA analysis showed the presence of a different community structure between both hornets and honey bees. Interesting, when PERMANOVA was performed between each individual hornet against Am, the comparison appear statistically significant for bacterial but not for fungal communitities. This data, was supported by beta diversity analyses, suggests that the microbial communities are widly diversified following the insect species. Therefore, the presence of a bacterial signature appear evident and it differs between hornets and bees but also between the two different hornets species. The same condition does not appear evident for the fungal communities that seem to follow a composition based on the insect genus rather than on the insect species, in fact, the two hornets do not appear to have a significant different mycobiota composition when compared between them.

Analyses of the gut microbial population structures of the two hornets and honey bee, showed, as expected, that Am displayed a polarized gut microbiota and mycobiota, consisting of a few taxa, much more enriched than the hornets. This could be the expression of the high level of specialization of the microbial communities with important functional potential in the ecology and social behaviour of the honey bee colony, acquired in centuries of evolution and domestication.

Unlike honey bees, hornets do not have clear age-dependent division of labor, and sterile individuals can perform various activities both inside and outside of their nest. The microbial composition and structures of the two *Vespa* species seem to be characterized by a high number of microbial species (not so abundant), probably widly acquired by the environment, giving not very specilized functional patterns to their ecology as happen for honey bees.

Thus, the honey bee microbiota could be described as a rigid system, polarized, composed by a few specialized phylotypes. On the other side, the hornets displayed a more flexible microbiota composed by very different but less enriched communities which we hypothesize may be the expression of the typical social wasps nomadic behaviour, building primary nests followed by the secondary ones and carrying out an intense predatory activity towards different arthropods. Hence, the microbial biodiversity found between the two different insect genera could be attributable to their interaction with the environment and overall to their social behavior.

By comparison of core microbiome of hornets and honey bee, we observed a simplified core community (no more than 7 genera) exclusively associated to the different insect species. No common core of bacterial OTUs were found among hornets and honey bees, while 7 OTUs

(including *Lactobacillus, Lactococcus* genera and *Enterobacteriaceae* family) were shared between Vc and Vv. Regarding core mycobiota of the three insect groups, only one core OTU (belonging to *Hanseniaspora thailandica* species) was shared among the two hornets and honey bees, and 3 OTUs (assigned to *Pichia fermentans* species) were shared between Vc and Vv.

Our results showed that *Lactobacillus*, *Bifidobacterium*, *Gilliamella* and *Pantoea* were bacterial genera enriched in Am. Unlike honey bee, the hornets showed a drastic reduction of *Bifidobacterium* and increased abndance of *Lactococcus* genus.

The presence of *Bifidobacterium* in both hornets appeared almost absent, as also occurred in two other hornets species from Asia, *V. mandarinia* and *V. simillima* (Suenami et al., 2019). Thus, seems that bifidobacteria do not play a central role in the ecology of the hornet as happen for the honey bee, however, *Lactobacillus* genus was confirmed as a central taxa in the microbial composition of gut of hymenoptera. It is possible that, like in honey bee, it provides functional traits to the immune system and in general to the health of the hornets. Overall, we observed that the core gut microbiota of hornets and honey bee seems to be populated from a substantial Lactic Acid bacteria (LAB) community. Despite *Lactobacillus* appears as a widely shared genus between honey bees and hornets, these seem to be selectively associated with the two insect genera, displayed a distinctive abundance pattern when OUT-level analysis was performed.

Lactobacillus genus amerged as an insect species-specific phylotipe, indeed, OTUs assigned to *Lactobacillus plantarum* and *Lactococcus lactis* were associated with both hornet species but were absent in honey bee. OTU of *Lactobacillus brevis* appears significantly enriched only in Vc compared to Am and Vv. Therefore, the LAB were confirmed as central microorganisms in the insect ecology (Vásquez et al., 2012). Indeed, symbiotic LAB communities such as *Lactobacillus* genus, were frequently found in the honey bee gastroenteric tract (e.g crop), as proved for the honey bee *A. mellifera* (Olofsson and Vásquez, 2008; Vásquez et al., 2012). LAB communities play a key role in the production of honey and beebread (Olofsson and Vásquez, 2008) and were proved to inhibit one important honey bee pathogen, *Paenibacillus larvae* (Forsgren et al., 2010). LAB possess antimicrobial properties against environmental microorganisms present in nectars and on pollen in order to defend their niche (the honey crop) and prevent spoilage of honey and bee bread during their production.

Evidences suggest that LAB antimicrobial mechanisms have evolved in synergy with bees to defend themselves and their hosts from environmental threats, and possibly for defence against specific honey bee pathogens. These results suggest that the symbiotic relationship between LAB and hymenoptera could be extended to other insects such as hornets.

Therefore, the presence of LAB in the gut microbiota of hornets, even if assigned to different OTUs with respect to that of honey bees, suggests that these important microbial communities could easily adapt themselves to the intestine of different hymenoptera, possibly providing central benefits for the insect health.

Regarding the gut fungal communites, these results represent the first comprehensive characterization of gut mycobiota in hornets. The mycobiota appears to be correlated to the insect genus rather than the insect species, in fact, the biomarkers of the hornets appear in complete overlap under the order of the *Saccharomycetales*, showing a sharp division respect to the mycobiota of honey bee. *Pichia fermentans* (Stefanini et al., 2012; Meriggi et al., 2019), *Lachancea* (Stefanini et al., 2012) and *Hanseniaspora* (Jimenez et al., 2017; Stefanini et al., 2012; Meriggi et al., 2019), enriched in hornets, were previously found as social wasps associated communities. Moreover, *Hanseniaspora* was identified in social wasps by Jimenez et al in Canada, thus suggests that this association may also occur outside of Europe (Jimenez et al., 2017) and this could represent a permanent and non-transitory relationship. Am shows a different microbial-associated pattern, represented by genera *Penicillium* (*P. chrysogenum* and *P. polonicum*), *Davidiella* (*D. tassiana*), *Crytococcus* (*C. victoria*), *Mucor* (*M. brunneogriseus*), *Kluyveromyces* (*K. marxianus*) and *Cordyceps* (*C. bassiana*) which emerge as honey bee markers. *Hanseniaspora thailandica* appears as a core among all three species of *Hymenoptera*, suggesting that this phylotpe could be equally adapted to the enteric environment of hornets and honey bee. Interestingly, *C. bassiana* was found among the fungal biomarkers in honey bee and we think this may be an effect of the extensive usage of this entomopathogenic microorganism in the cultivated fields for the pest control in the agroecosystems services (Gul et al., 2014). We hypothesize that the presence of *C. bassiana* spores in sublethal doses may be due to the strong presence of this fungus in our honey bee sampling set hence the significance in the association with the honey bee gut fungal composition. The honey bee –associated genus *Cryptococcus* was already found in termites (König, 2006) and was caught to Lepidoptera in association with *Metschnikowia related strains* (Brysch-Herzberg, 2004).

Cryptococcus was previously detected in floral nectar visited by bumble bees, suggesting that could be transported by pollinating hymenopterans and that may be well adapted to their intestinal environment. Even, bacterial markers appear to follow a similar association pattern. *Saccharibacter* reported as honey bee markers was already isolated from pollen (Jojima et al., 2004), other honey bee bacterial markers such as *Pantoea* isolated from floral nectar and *Frischella* and *Gilliamella* already described as part of honey bee microbiota (Fridman et al., 2012; Loncaric et al., 2009; Engel et al., 2016; Ludvigsen et al., 2018) confirm the stability of the Am microbiota and its correlation with flower nectars and floral / plant framework.

In this, the social behavior and particularly the diet can play a central role in the development of fungi-insect associations.

Indeed, *Hanseniaspora guillermondi* and *Pichia fermentans*, an hornet biomarkers, were previously isolated from fruits and fruit trees and not from blossoms (Vadkertiová et al., 2012). *Saccharomyces cerevisiae* and *Metschnikowia* displayed in Vc core mycobiota appeared associated with fruits, though *S. cerevisiae* were isolated from both blossoms and fruits (Vadkertiová et al., 2012).

Moreover, Vv bacterial marker *Serratia* was described as an oak trees–associated genus, suggesting that the oak trees may represent a suitable environment for the hornets' nest. This further supports how the hornets microbiota in combination with other factors is strongly influenced, more than in honey bee, by the environment where they feed and develop the nest (Poza-Carrión et al., 2008).

The network analysis confirm the central role of *Saccharomycetales* in the structuring of the hornets' gut microbial communities. The two main clusters that make up the network and that involve all three insect species were equally composed of bacteria and yeasts and these may represent the filotypes that better explain the gut microbial composition of these hymenopterans.

Our results showed that the gut microbiota composition is specific for each insect species, instead the mycobiota result associated to the insect genus highlighting a clear division between honey bee and hornet. We believe that this can be strongly influenced by the intestinal physiochemical characteristics and by the different diet exerted by honey bees and hornets (nectariferous sources vs fruits). Bacteria, on the contrary, seem to be associate to each of the three insect species as a "microbial signature", however, which factors were related to this condition need to better deepen.

Author Contributions

This chapter version represents a first draft of the work not actually published, the authors contributed as follows: *Niccolò Meriggi*[1], *Alessandro Cini*[2], *Federico Cappa*[1], *Francesco Vitali*[3], *Rita Cervo*[1] and *Duccio Cavalieri*[1]: experimental design; *Alessandro Cini*[2] and *Federico Cappa*[1]: sampling; *Francesco Vitali*[3], *Giovanni Bacci*[1]and *Niccolò Meriggi*[1]: Data analyses; *Niccolò Meriggi*[1] and *Monica Di Paola*[1]: Manuscript drafting.

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CHAPTER IV

EFFECT OF DIFFERENT ENVIRONMENTAL STIMULI ON THE GUT MICROBIAL COMPOSITION OF VESPA CRABRO AND APIS MELLIFERA

Abstract

Insects are the most widespread animals on the planet, occupying many of the environmental niches usually considered inhospitable. Their ability in the environment colonization allows them to exert a continuous interaction with a wide number of microorganisms. Mutualistic interactions with symbiotic microorganisms allow insects to acquire nutrients from environmental substrates, resist against pathogens and overall guarantee their state of health. however, how the environment affects these mutualistic relationships, therefore, what changes it produce on the insect gut microbial composition?.

We tested the effect of different environmental stimuli on the gut microbial composition of two insect species, *Apis mellifera* and *Vespa crabro*.

We observed that foraging activity in the vineyard during grape ripening has a different effect on the gutmicrobial communities of these two social insects. Indeed, hornets isolated during foraging in the vineyard in the ripe berries period were showed to be significantly enriched in *Saccharomycetes*. Analougsly, honey bees collected from olive tree bark hollow appeared statistically enriched in *Saccharomycetes* and *Tremellomycetes*, a fungus widly present in the olive trees. The presence of *Saccharomycetes* in the group of hornets that fed on ripe grapes confirms the role of social wasps in the ecology of *Saccharomycetes* yeasts, laying the groundwork for further considerations regarding their interaction with the vineyard environment.

Introduction

Insects are a large group of organisms widely distributed on earth, able to colonize a wide variety of different environments (Misof et al., 2014; Larsen et al., 2017). The great dispersion of insects worldwide and their interaction with the environment makes them in direct contact with environmental microbial communities. This level of interaction could be the cause of the wide number of host-microbial symbiosis developed by insects in the course of evolution. It is actually known that the environment exert a strong impact on the development of vertebrate gut microbiota (Spor et al., 2011). Frequently, the symbiotic interactions arising from microbial-host mutualism exerted a central role in the protection against pathogens (Koch and Schmid-Hempel 2011), nutrient acquisition (Klepzig et al., 2009, Douglas et al., 2009) and modulate the insect immune system (Schneider and Chambers 2008).

The environmental microorganisms can be integrated in the insects' life cycle or can inhabit the gut environment and overall in their ecology, giving benefits for a health status. For example, stinkbug *Riptortus clavatus* host specific bacteria from the genus *Burkholderia*, essential for the host insect fitness, and these microorganisms were not vertically transmitted but acquired from the environment (Kikuchi et al., 2007). The insects gut appear a niche in which a high number of symbiosis occurred (Hooper and Gordon, 2001). We actually know that the environment can affect the structure of the insect gut microbiota (Yun et al., 2014). However, the gut microbiota can be also influenced by dietary habits of the host (Colman et al., 2012). Indeed, it has been described how changes in the host's diet lead to a structural change in the intestinal microbial composition and the related metabolic traits associated to the hosted microorganisms (Kane & Breznak, 1991; Santo Domingo et al., 1998; Broderick et al., 2004). Environment and diet seem not to exert a steady action on all the insect species but act in the insect-depend manner. However, there is not actually enough knowledges about the correlation of these two important variables on fungal communities. For this reason, we provided a comprehensive analysis of the effect of different environments on both gut bacterial and fungal communities in two insect species, *Apis mellifera* and *Vespa crabro*. The choice of the hornets and the vineyard environment in this study is not by chance.

The hypothesis that social wasps could carry the yeasts on grapes originated from the studies (Mortimer and Polsinelli, 1999) of Prof. M. Polsinelli (Dept of Biology, University of Florence- Italy) and Prof. B. Mortimer (Harvard University, USA) in Chianti vineyard. Polsinelli and Mortimer observed that before ripening, *S. cerevisiae* cells are almost absent in

the grape berries (~0,05%), while a significantly higher percentage of *S. cerevisiae* (~25%) was observed in the damaged ripe berries . In recent years, it has been discovered that hymenoptera, among these wasps (*Polistes sp*.) and hornets (*Vespa crabro*) are closely related to the ecology of *Saccharomycetes*, especially *S. cerevisiae* (Stefanini et al., 2012), the most frequently used yeast in the wine production. The life cycle of wasps and hornets is perfectly overlapping with the maturation cycle of the grapes and, interestingly, *V. crabro* is proven to have a suitable buccal apparatus to break the skin of grape berries (Stefanini et al., 2012). Therefore, the gut of *Vispidae* also act as a niche where the yeast of *Saccharomyces* genus mate between themselves with an increased rate of interspecific hybrids, thus increasing the yeast biodiversity (Stefanini et al., 2016). These findings supported the hypothesis that wasps, *Saccharomycetes* and vineyard could live in a synergic relationship that is not yet fully understood. We think that hymenoptera, in particular hornets (*V. crabro*), play a crucial role as vectors of *Saccharomyces* strains, spreading them from rural environments to the vineyard. Thus, wasps guarantee a *continuum* between the wild environment and vineyard, and consequently shaping the microbial terroir.

Therefore, considering the knowledge previously mentioned, in this study, we would like to test the following hypotheses: (i) could the vineyard represent an environment that differently influence the gut microbiota composition of the two insect species due to the diversified nutritional behavior of *Apoidea* and *Vespidae.* (ii) Could the environment exert different effects on microbiota and mycobiota composition of both the two insect species. (iii) Could help to better understand the origin of the yeasts present in the vineyard involved in the fermentation process and their natural carriers. (iv) could provide further informations regarding the development of wine terroir.

We performed the characterization of gut microbiota and mycobiota of *V. crabro* and *A. mellifera*, captured in different environmental niches around the vineyard in different seasons. We tested the effect of different environmental stimuli, demonstrating that the hornets and honey bees gut microbial communities were differently affected from the different environmental conditions. Indeed, The hornets catched during their foraging activities on ripe grape berries were significantly enriched in *Saccharomycetes*, demonstrating that hornets and vineyard interact each other influencing their microbial compositions. We suggest that the hornets that act as a natural vectors transporting yeasts on ripe grape berries might be also

affected by the vineyard and this can occur possibly *via* sugar intake from ripe berries or *via* microbial exchange with the natural terroir of the vineyard.

Matherials and methods

Insect sampling

In order to analyze the environmental effect in a natural and non-experimental context the insects sampling were performed from nests founded in different environmental. Insect collection was performed in two different locations belonging to the Ca' Marcanda winery and the sampling areas were located in Castagneto Carducci (Tuscany - Italy) and Bibbona (Tuscany - Italy), far each other around 7 km as the crow flies.

The hornets *V. crabro* were collected considering three different environmental patterns: (i) a group of 8 hornets catched during their foraging activity on ripe grape berries at the end of Summer in vineyard area in Bibbona countryside (Tuscany- Italy), (ii) a group of 7 hornets collected from their nest built inside an olive tree bark hollow, close to the vineyard area in Bibbona. In order to exclude any occasional effect of nutrition, considering the hornets flight range, we performed the sampling at the end of Autumn (around 50 days after the grape harvest), excluding the presence of ripe berries from the panel of food sources present in the environment. (iii) a group of 8 hornets collected during their foraging activity on honey bees in front of apiary at the end of Summer in a winery area in Castagneto Carducci, around 7 km as the crow flies from Bibbona countryside. Similarly, we performed the gut microbial communities analysis of *A. mellifera*, a social insect that has different diet and social behavior. It is known that the honey bees present a stable microbiota with a few phylotypes present in other honey bees worldwide (Weiss et al., 2011; Engel et al., 2016; Kwong et al.; 2016). Therefore, we carried out the sampling considering three different environmental patterns for hoenybee: (i) a group of 8 honey bees collected from the apiary moving outside of the hive for their foraging flight and predated by the hornet groups mentioned above in (iii), at the end of Summer in winery area in Castagneto Carducci winery. (ii) a group of 6 honey bees collected from colony swarmed in olive trees bark hollow at the end of Summer in Castagneto Carducci. (iii) a group of 7 honey bees collected from colony swarmed in the cavity of winery cellar wall at the end of Summer in Castagneto Carducci .

DNA extraction and sequencing

For gut microbiota DNA extraction, insect surface was sterilized by washing in 70% etanhol solution. Insects were then dissected and the gut collected and stored in RNAlater buffer (Invitrogen – Thermo Fisher Scientific) at -20°C until DNA extraction. Microbial DNA extraction was performed using DNeasy PowerLyzer PowerSoil Kit (QIAGEN) according to the manufacturer's protocol. Next generation sequencing was carried out on MiSeq platform (Illumina) at BMR Genomics sequencing service (University of Padova, Italy, https://www.bmr-genomics.it/) by following the Illumina's protocols for bacterial and fungal communities sequencing (www.illumina.org). The sequencing of rRNA genes for both bacterial and fungal communities was performed using the following primers, Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014) for 16S rRNA genes (V3-V4 regions) and ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al., 1990) for ITS2 region.

Amplicon sequencing analysis

In this chapter we used a different approach called DADA2 which detects single-nucleotide differences in biological sequences not observed in standard OTUs clustering methods (e.g. *micca* performed in chapters II, III and V). DADA2 outputs counts for biological sequences detected called amplicon sequence variants (ASV). Hence, ASVs are high resolution variants of OTUs making marker-gene sequencing analysis more precise and reusable as mentioned in Callahan et al. 2017. Both fungal and bacterial amplicon sequences were clustered into Amplicon Sequence Variants (ASVs) using the DADA2 package (version 1.6.0) (Callahan et al., 2016) in R version 3.4.3 (http://www.R-project.org). Primers used for PCR amplification were removed using cutadapt version 1.15 (Martin, 2011) in paired-end mode. If a primer was not found, the sequence was discarded together with its mate to reduce possible contamination. The big-data approach reported at https://benjjneb.github.io/dada2/bigdata.html was followed to reconstruct ASVs. Forward and reverse reads were trimmed at 280bp and 190 bp, respectively. Reads (and their respective forward or reverse read) with more than two expected errors were filtered out. Denoised reads were merged while removing all reads with at least one mismatche or an overlap length shorter than 20bp. Chimeric sequences were identified and removed using the consensus method

"removeBimeraDenovo" function. All sequence variants were taxonomically annotated using "DECIPHER" package (version 2.6.0) (Wright, 2016). Bacterial sequences were classified using the Silva SSU (Quast et al., 2012) reference database version 132 whereas fungal sequences were classified using the Warcup ITS training set version 2 (Deshpande et al., 2016). Sequences aligning to chloroplasts, mitochondria, Archaea and Eukaryotes were removed.

Microbial communities analysis

Microbial diversity was assessed using the "vegan" package (version 2.5) (Oksanen et al., 2018). To detect microbial pattern along environmental gradient, non-metric multidimensional scaling was used (Bray-Curtis distance) (Bray and Curtis, 1957) together with permutational multivariate analysis of variance (PERMANOVA) based on the same distance index. Microbial communities were considered as a single entity composed of bacteria and fungi but they were also analyzed separately to inspect peculiar pattern related to single taxonomic groups. To detect ASVs significantly affected by the environment the "DESeq2" package (Love et al., 2014) was used with the Likelihood Ratio Test (LRT). Variants significantly affected by the environment were grouped using a "dynamic branch cutting" methods implemented in the "dynamicTreeCut" package (version 1.63) (Langfeld et al., 2007). The presence of peculiar taxonomic groups was inspected using hypergeometric test (Lundberg et al., 2012) on the groups defined above.

Results

In order to explore differences in the microbial structures of gut communities of insects living in different environmental niches, ordination analysis by using non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances were performed on both fungal and bacterial communities. As expected, the insect genus (*Apis* vs *Vespa*) appears as the main variable that causes a sharp separation in both bacterial and fungal datasets. Therefore, in order to observe the effect of each environment pattern on gut microbial composition, we performed the analysis of each case study separately (Figure 1A). Ordination analysis performed on honey bees showed a clear division for fungal dataset, showing a pattern of clusters that switch in accordance with the *environment* variable. Similarly, even the hornets showed a more clear

cluster following the *environment* variable when the analysis was performed only on fungal communities (Figure 1A). When the two insect species where analyzed separately, PERMANOVA analysis showed significant differences according the *environment* variable in both honey bees and hornets for both fungal and bacterial datasets as reported in Table 1. As indicated by the R^2, the environment variable appears to be more related to the fungal communities, which are largely influenced by the different environmental patterns comparing to the bacteria. Thus, the environmental substrate able to affect the microbial composition in both insect species appear to be more relevant in the development of mycobiota composition. Indeed, when PERMANOVA where performed on the two single variables (*insect species* and *environment*), without considering honey bee and hornet separately, the *insect species* and *environment* variables were proved to influenced the gut bacterial and fungal compositions (Table 2). The effect of the environment was greater when the two datasets, bacteria and fungi, where analyzed separately (Table 2).

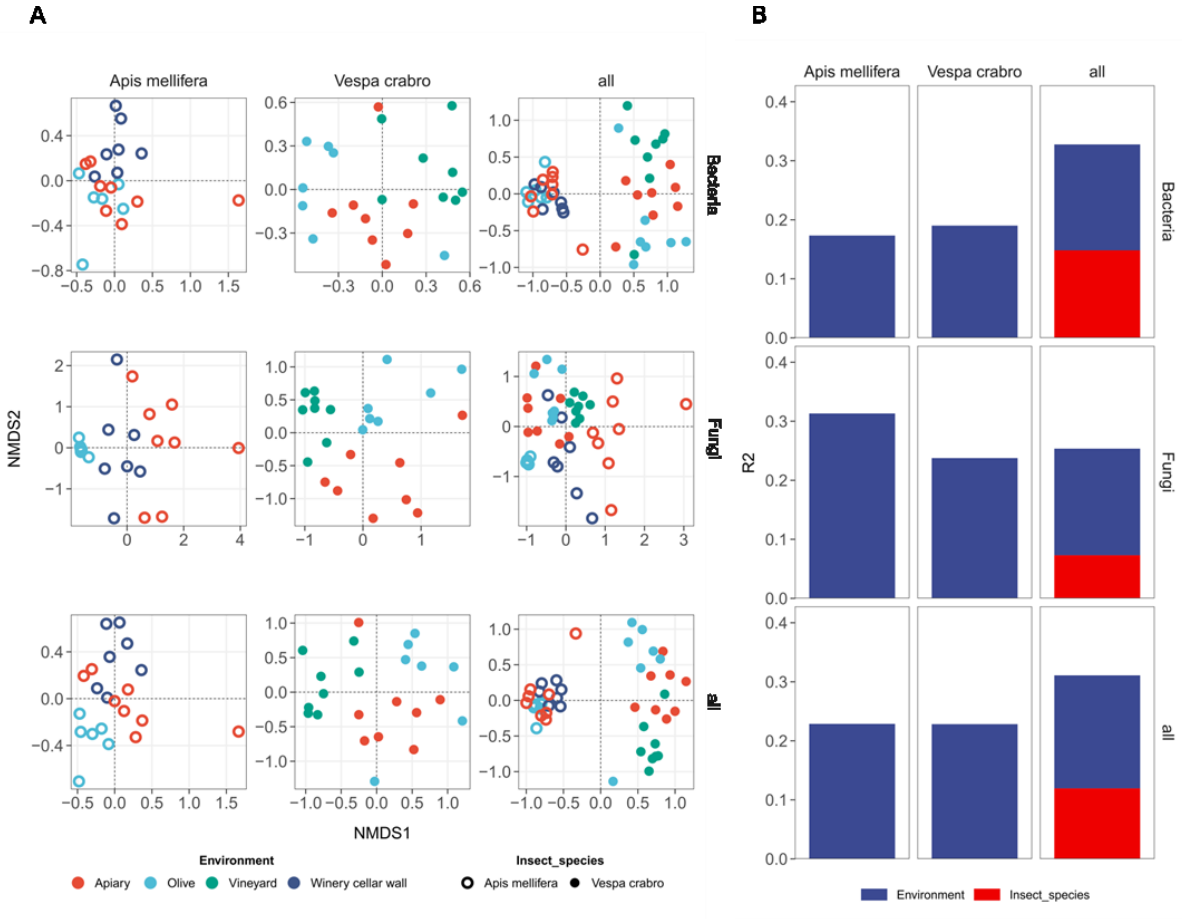

Figure 1. Ordination analysis using non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances performed on *Apis mellifera* and *Vespa crabro* for bacterial (Bacteria), fungal (Fungi) datasets and both datasets joined together (all) (A). Permutational multivariate analysis of variance (PERMANOVA) score is reported on the right part of the panel (B), the score is displayed as barplot with y-axis = R^2 and x-axis = tested variable and splitted for bacteria, fungi and both analyzed together (all).

Table 1. PERMANOVA performed considering the *environment* variable for *Apis mellifera* and *Vespa crabro*, for bacterial (16S), fungal (ITS) datasets, separately, and both joined together (all).

	Term	Df	SumOfSqs	R^2	F	Pr(>F)
			Apis mellifera			
	Environment	2	0.918	0.173	1.886	0.001
	Residual	18	4.382	0.827	/	/
16S	Total	20	5.300	1.000	/	/
			Vespa crabro			
	Environment	2	1.650	0.190	2.347	0.001
	Residual	20	7.029	0.810	/	/
	Total	22	8.678	1.000	/	/
			Apis mellifera			
	Environment	2	2.774	0.313	4.105	0.001
	Residual	18	6.081	0.687	/	/
	Total	20	8.855	1.000	/	/
ITS			***Vespa crabro***			
	Environment	2	2.091	0.238	3.118	0.001
	Residual	20	6.706	0.762	/	/
	Total	22	8.797	1.000	/	/
			Apis mellifera			
	Environment	2	1.465	0.229	2.668	0.001
	Residual	18	4.942	0.771	/	/
	Total	20	6.408	1.000	/	/
all			***Vespa crabro***			
	Environment	2	1.990	0.228	2.957	0.001
	Residual	20	6.731	0.772	/	/
	Total	22	8.721	1.000	/	/

Table 2. PERMANOVA performed considering the *environment* and *insect species* variables for bacterial (16S), fungal (ITS) datasets, separately, and both joined together (all).

Term	Df	SumOfSqs	R^2	F	Pr(>F)
		16S			
Environment	3	3.203	0.179	3.468	0.001
Insect species	1	2.647	0.148	8.598	0.001
Residual	39	12.004	0.672		
Total	43	17.853	1.000		
		ITS			
Environment	3	3.437	0.181	3.144	0.001
Insect species	1	1.391	0.073	3.818	0.001
Residual	39	14.210	0.746		
Total	43	19.039	1.000		
		all			
Environment	3	3.482	0.191	3.612	0.001
Insect species	1	2.173	0.119	6.763	0.001
Residual	39	12.532	0.689		
Total	43	18.188	1.000		

Furthermore, DESeq analysis (Likelihood Ratio Test) performed only on ASVs that were significantly influenced by the environment, showed that the greatest percentage of sequence variants was obtained from the two insect species when analyzed separately. The percentage drastically decreased when the two insects were analyzed joined together (Figure 2, Barplot). Overall, the analysis showed that the highest percentage of ASVs significantly influenced by the environment were related to fungal taxa. Therefore, the number of ASVs displayed in Venn diagram showed that the number of variants significantly influenced by the environment are strongly affected by the insect species. Indeed, the number of significantly associated ASVs shared between the two insect species appear unfair (Figure 2. Venn diagram).

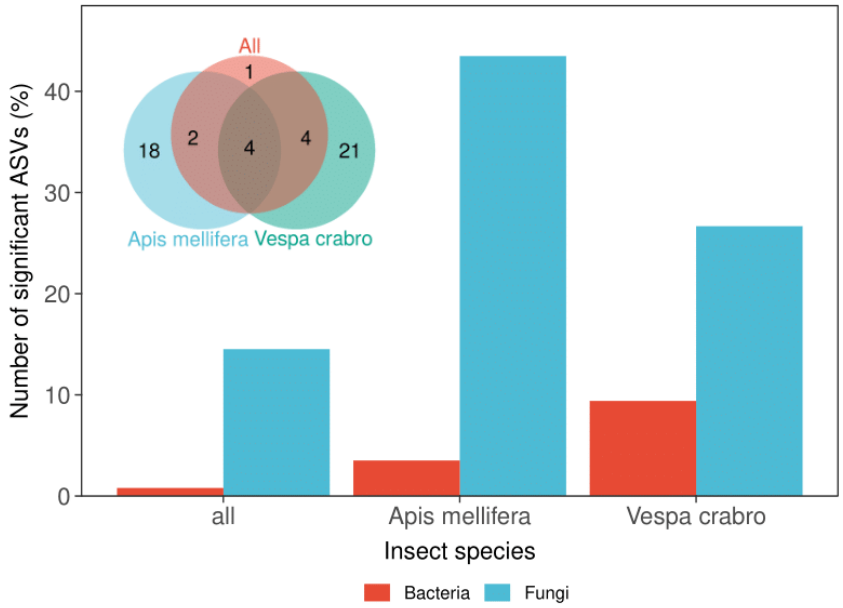

Figure 2. DESeq analysis of the ASVs that were significantly affected by the environment. Barplot reported on y-axis the percentage of significant ASVs and on x-axis the insect groups, *Apis mellifera*, *Vespa crabro* and both insect species joined (all). Barplot were colored in relation to bacterial (red) and fungal (blue) communities. Venn diagram shows the number of significant ASVs shared among all groups.

ASVs from DESeq analysis, mentioned above, were tested using dynamic branch cutting methods (see Matherial and Methods) to obtain a clustering of the significant ASVs for each environmental pattern, in both honey bees and hornets (Figure 3 and 4). ASVs relative abundances were displayed by variance stabilizing transformation (VST). All significant ASVs were clustered in 3 groups for *A. mellifera* and 4 groups for *V. crabro*. ASVs appeared related to specific environment, with the exception of group 4 in *V. crabro,* which showed a wide number of ASVs slightly detected in all environments (Figure 4). Therefore, group 1 in honey bees and group 4 in hornets appear significantly enriched only in fungi (Figure 3 and 4). To understand what was the level of enrichment of the fungal phylotypes in groups 3 (honey bee) and 4 (hornet) and if this represented a random event, we performed the hypergeometric test (Table 5).

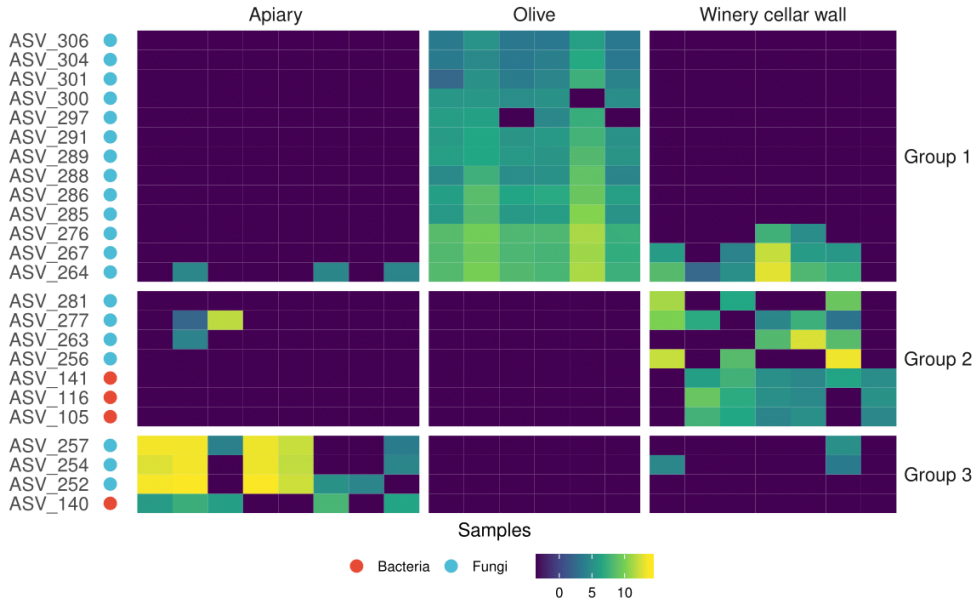

Figure 3. Heatmap from DinamiCutTree analysis in *A. mellifera*. The heatmap showed the ASVs significant affected from the environment variable and the related clusters. The ASVs relative abundances were displayed in the color scale, from blue to yellow, based on variance stabilizing transformation (VST).

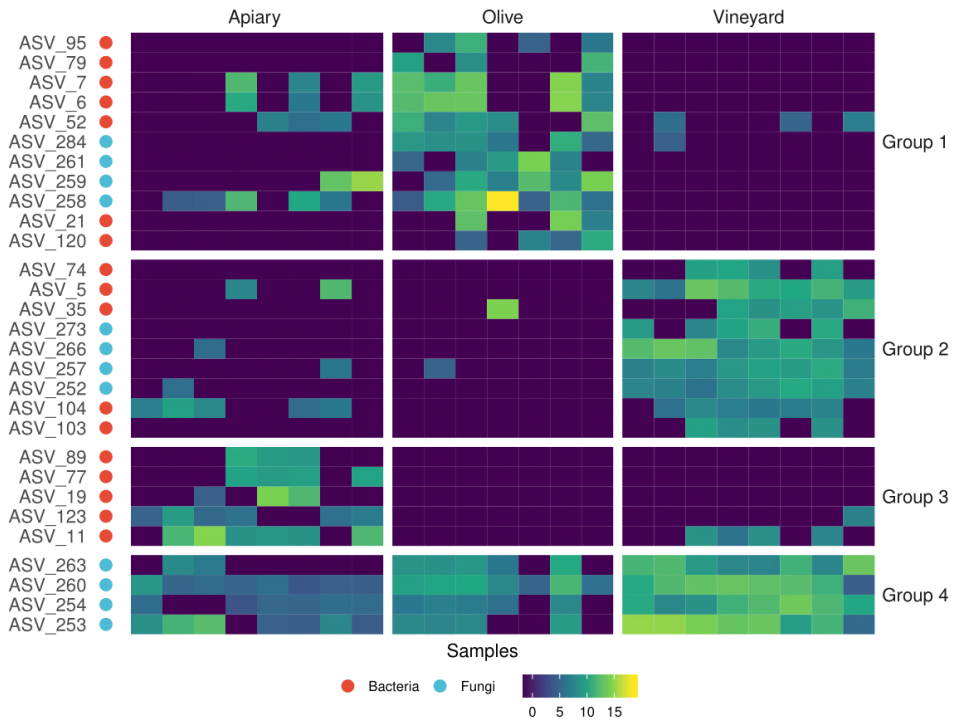

Figure 4. Heatmap from DinamiCutTree analysis in *V. crabro*. The heatmap showed the ASVs significant affected from the environment variable and the related clusters. The ASVs relative abundances were displayed in the color scale, from blue to yellow, based on variance stabilizing transformation (VST).

Table 3: Teable with the taxonomic assignment of each ASV included in the DinamiCutTree analysis in *A. mellifera* reported in the heatmap in Figure 3.

ASV	taxonomic assignment	Group cluster	Insect species
ASV_306	*Debaryomyces carsonii*	Group 1	*A. mellifera*
ASV_304	*Hanseniaspora osmophila*		
ASV_301	*Tremellomycetes*		
ASV_300	*Metschnikowia reukaufii*		
ASV_297	*Metschnikowia*		
ASV_291	*Saccharomycetales*		
ASV_289	*Saccharomycetales*		
ASV_288	*Saccharomyces*		
ASV_286	*Metschnikowia*		
ASV_285	*Cryptococcus flavescens*		
ASV_276	*Metschnikowia*		
ASV_267	*Metschnikowia*		
ASV_264	*Metschnikowia*		
ASV_281	*Debaryomyces*	Group 2	
ASV_277	*Alternaria*		
ASV_263	*Hanseniaspora*		
ASV_256	*Lachancea thermotolerans*		
ASV_141	*Enterobacteriaceae*		
ASV_116	*Enterobacteriaceae*		
ASV_105	*Enterobacteriaceae*		
ASV_257	*Pichia*	Group 3	
ASV_254	*Pichia*		
ASV_252	*Pichia*		
ASV_140	*Gilliamella*		

Table 4: Teable with the taxonomic assignment of each ASV included in the DinamiCutTree analysis in *V. crabro* reported in the heatmap in Figure 4.

ASV	taxonomic assignment	Group cluster	Insect species
ASV_95	*Enterococcus*		
ASV_79	*Enterococcus*		
ASV_7	*Enterobacteriaceae*		
ASV_6	*Hafnia-Obesumbacterium*		
ASV_52	*Enterococcus*		
ASV_284	*Rhodotorula mucilaginosa*	Group 1	
ASV_261	*Mucor*		
ASV_259	*Kluyveromyces dobzhanskii*		
ASV_258	*Candida*		
ASV_21	*Enterobacteriaceae*		
ASV_120	*Brenneria*		
ASV_74	*Lactobacillus*		
ASV_5	*Lactobacillus*		
ASV_35	*Pediococcus*		
ASV_273	*Pichia*		*V. crabro*
ASV_266	*Hanseniaspora guilliermondii*	Group 2	
ASV_257	*Pichia*		
ASV_252	*Pichia*		
ASV_104	*Lonsdalea*		
ASV_103	*Lactobacillaceae*		
ASV_89	*Carnobacterium*		
ASV_77	*Lactococcus*		
ASV_19	*Enterobacteriaceae*	Group 3	
ASV_123	*Brenneria*		
ASV_11	*Lactococcus*		
ASV_263	*Hanseniaspora*		
ASV_260	*Pichia*	Group 4	
ASV_254	*Pichia*		
ASV_253	*Hanseniaspora*		

After the determining of significant ASVs, we tried to establish a functional relationship between taxa and the role of the environment. We used the Hypergeometric test to identify potential interesting taxa sets among significant ASVs. This analysis allows us to understand which sub-set of microbial phylotypes within the significant ASVs are over- or under-represented. Hypergeometric test showed a statistical significance in *Saccharomycetes* and *Tremellomycetes* in group 1 (honey bee) and likewise only in *Saccharomycetes* in group 4

(hornet). The fungal population fraction observed for both categories was significantly much higher than the expected (Table 5). This analysis highlight the massive presence of members of this important fungal class in honey bees from olive environment (Group 1 of *A. mellifera*) and in hornets from vineyard-ripe berries environment (Group 4 of *V. crabro*).

Table 5. Hypergeometric test table. Fraction.pop: calculated fraction inside the population, fraction.obs: fraction observed within the population, exp.q: number of expected ASVs belonging to the selected categories, p-value and adjusted p-value (Benjamini–Hochberg correction). Insect species, group (grp) and categories were reported on left side of the table.

Insect species	grp	categories	fraction.pop	fraction.obs	exp.q	p.value	adj.p.value
Apis mellifera	1	*Saccharomycetes*	0.212	0.846	2.762	0.000	0.000
Apis mellifera	1	*Tremellomycetes*	0.012	0.154	0.162	0.006	0.006
Vespa crabro	4	*Saccharomycetes*	0.164	1.000	0.655	0.001	0.001

The enrichment in the observed *Saccharomycetes* was confirmed by ternary plot based on their geometric mean in the three analyzed environments. In the ternary plot analysis, we assumed the vineyards and the winery cellar wall as a winery-like environment (V|W in Figure 5). The ASVs distribution reflected the analyses described above, showing the statistically significant association between *Saccharomycetes* and *Tremellomycetes* in group 1 of the honey bees, and only *Saccharomycetes* in group 4 of the hornets.

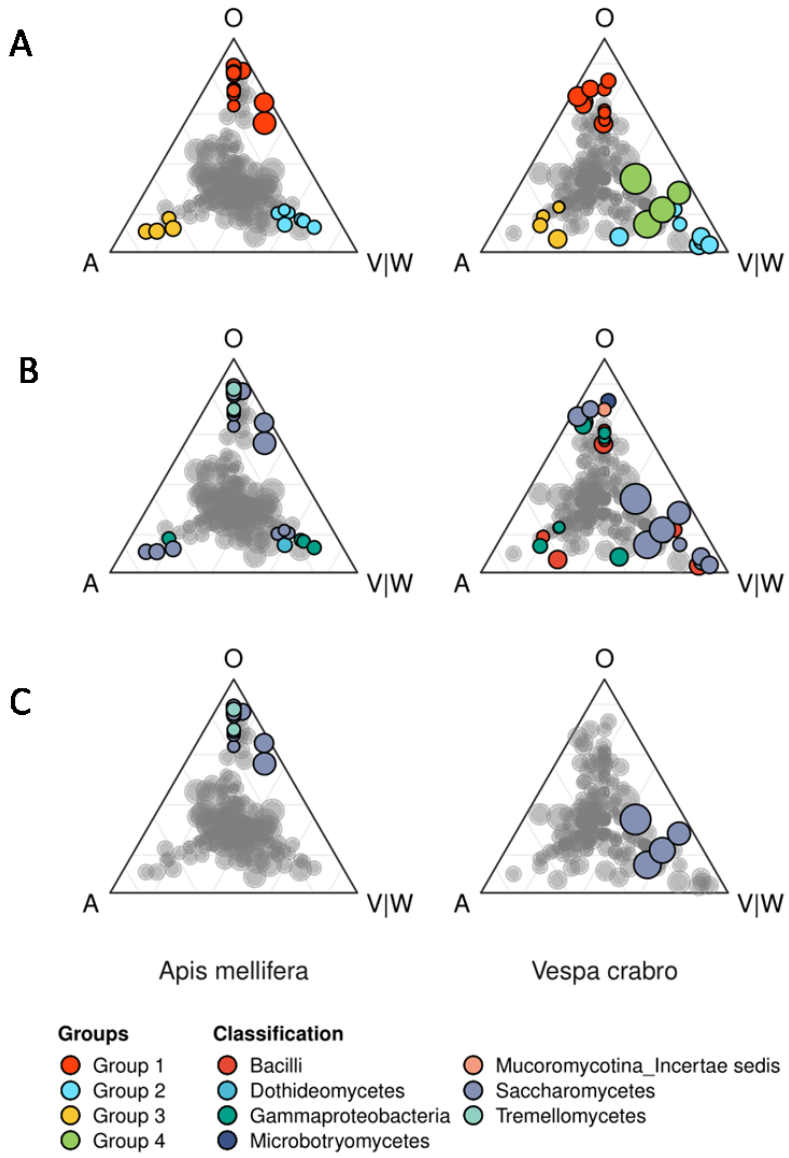

Figure 5. Ternary plot reported the taxa distribution of the 6 groups of insects (*A. mellifera* and *V. crabro*) in the three environmental patterns (corners, A: Apiary, O: Olive tree, V|W: vineyard and Winery cellar wall). (A) the plenary distribution of the four groups obtained from dynamic branch cutting analysis. (B) taxonomic composition of the four groups of insects displayed above. (C) statistically significant enriched phylotypes related to the groups mentioned above. Groups and Classification were reported in the legend below the ternary plot.

Discussion

In the last years the role of social wasps has been explored and their ecology was deeply connected to the ecology of *Saccharomycetes*, such as *S. cerevisiae*, describing their ability to host yeasts during the winter, transmit them vertically within the colony, carry the yeast cells in the environment and contribute in the increasing os *Saccharomyces* yeast biodiversity (Stefanini et al., 2012; Stefanini et al., 2016). This makes social wasps, such as *V. crabro*, an ideal carrier closely connected with the yeast ecology, able to guarantee the propagation and dispersion of yeast cells within the environment. Stefanini et al. suggested that the hornets, due to their buccal apparatus, might be able to break the grape berries, then inoculate the microorganisms inside berries and ensuring them the access to the sugary sources. This supported the evidence described by Polsinelli et al who showed that *S. cerevisiae* was not easily found on undamaged berries but on broken ones. Here, we hypothesized that this close connection between wasps, yeasts and environment such as vineyard could determine an important effect on the gut microbial communities. Thus, we have shown that the insect gut microbiota and mycobiota can be differentially affected from different environmental stimuli and this happen differently in the two insect species *V. crabro* and *A. mellifera*.

Insect species and *environment* variables significantly influence the diversity of both micorbiota and the mycobiota. Despite, the fungal composition was more influenced than the bacterial composition in both insect species. Furthermore, ASVs significantly affected by the *environment* variable associated with honey bees were substantially different from those associated with hornets, this showed that the insect microbial communities might respond differently to the different environmental stimuli.

Through the dynamic branch cutting method it was possible to group the ASVs significantly influenced by the environment into a discrete clusters (referred to as ‘group’). Among these, two groups emerge as significantly associated with *A. mellifera* (Tremellomycetes and Saccharomycetes) and one with *V. crabro* (Saccharomycetes) and following the Hypergeometric test these taxa associated to group 1 in honey bess and the group 4 in hornets appeared as signifcantly over-represented compared to the expected rate. The ASVs from the group described above were releted to *Saccharomycetes* in hornets collected during their foraging flight on ripe grape berries in the late Summer (Vineyard pattern), while, *Saccharmycetes* and *Tremellomycetes* were significantly enriched in swarmed honey bees collected from olive tree hollow in the late Summer (Olive tree pattern). The presence of

Tremellomycetes significantly associated with honey bees that founded their nest in the olive tree hollow is easily explained by the results of Varanda et al. which showed that the *Tremellomycetes* were one of the most represented fungal filotypes in olive trees (Varanda et al., 2019). Therefore, we believe that this effect can be explained by the ability of the *Tremellomycetes* to colonize the gastrointestinal tract of swarming bees that founded their colony inside olive trees.

Furthemore, we think that the presence of *Saccharomycetes* significantly enriched in hornets was an effect of the feeding on with ripe grape berries. The level of *Saccharomycetes* decreased in the group of hornets that had no access to ripe grapes after about 50 days (hornets from olive trees). Similarly, the hornets that forage in the apiary spend most of their time predating honey bees which has been shown to be a preferential prey compared to other generic protein sources (Cini et al., 2108). Moreover, in the same study Cini et al. showed that the time spent by the hornets manipulating sugar sources such as honey and grapes was significantly higher than candy (Cini et al., 2018).

Thus, *V. crabro* and vineyard appear connected by a mutualistic relationship that allow the hornets to act as vectors of *Saccharomycetes* transporting the yeasts in the vineyard, meanwhile, increasing the *Saccharomycetes* intestinal abundance likely by increasing food sugars intake by the ripe grape berries feeding.

The hypothesized mechanisms that determine the enrichment of *Saccharomycetes* in the intestinal tract of the hornets associated with vineyard environment might be a result of a selection of the microorganisms who benefit from a sugar-rich diet. Therefore, the yeasts present on the ripe broken berries (likely broken by the hornets) could trigger fermentative processes and produce volatile aromas attracting the hornets on vineyard (Tabata and Kitamoto, 2018), thus determining a feedback effect that fuels the mutualistic interaction between yeasts and social wasps. Hence, the *Saccharomycetes* enriched by the diet can in turn be inoculated on the rape grape barries finding a sugary substrate advantageous for their growth.

We know that the microbiota and mycobiota of social insects is represented by indispensable communities responsable for the insects' health, however many of these could prove to be "passengers" easily influenced by different environmental stimuli.

Finally, the ecological role of the hornet-associated gut microrganisms and the way in which they communicate to the environment or be affected by the environment appears to be much

more complex than expected, thus, the importance to study these interactions to better define the symbiosis between *Saccharomycetes* and social insects.

Author Contributions

This chapter version represents a first draft of the work not actually published, the authors contributed as follows: *Niccolò Meriggi*[1], *Monica Di Paola*[1] and *Duccio Cavalieri*[1]: experimental design; *Niccolò Meriggi*[1]: sampling; *Giovanni Bacci*[1]: Data analyses; *Niccolò Meriggi*[1]: Manuscript drafting.

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CHAPTER V

SACCHAROMYCES CEREVISIAE INDUCES IMMUNE ENHANCING AND SHAPES GUT MICROBIOTA IN SOCIAL WASPS

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Abstract

Trained immunity is the enhanced response of the innate immune system to a secondary infection after an initial encounter with a microorganism. This non-specific response to reinfection is a primitive form of adaptation that has been shown to be conserved from plants to mammals. Insects lack an acquired immune component and rely solely on an innate one, and they have expanded it upon traits of plasticity and adaptation against pathogens in the form of immune priming. The recent discoveries of the role of *Saccharomyces cerevisiae* in the insect's ecology and the ability of this yeast to induce trained immunity in different organisms suggest that insects could have developed mechanisms of adaptation and immune enhancing. Here, we report that two yeast strains of *S. cerevisiae*, previously shown to induce trained immunity in mammals, enhance resistance to Escherichia coli infection in the paper wasp Polistes dominula. The reduction of injected E. coli load by *S. cerevisiae* strains was statistically significant in future foundresses but not in workers, and this occurs before and after hibernation. We thus investigated if the effect on E. coli was mirrored by variation in the gut microbiota composition. Foundresses, showing immune enhancing, had statistically significant changes in composition and diversity of gut bacterial communities but not in the fungal communities. Our results demonstrate that *S. cerevisiae* can prime insect responses against bacterial infections, providing an advantage to future foundress wasps to carry these microorganisms. Understanding the mechanisms involved in the generation of specific and long-lasting immune response against pathogenic infections in insects and the influence of the induction of trained immunity on the commensal gut microbiota could have a major impact on modern immunology.

Introduction

Immunological memory is an important evolutionary trait that improves host survival upon secondary infection (Netea et al., 2011, 2019). Trained immunity is a newly discovered mechanism of innate immune memory, causing metabolic and epigenetic reprograming of innate immune cells [monocytes/macrophages, natural killer (NK) cells] (Netea et al., 2011, 2019). This non-specific response describes an inflammatory protection against a microorganism upon a second encounter, independently from adaptive immunity, and it has been shown to be present not only in mammals but also in plants (Durrant and Dong, 2004) and insects (Pham et al., 2007; Rodrigues et al., 2010; Norouzitallab et al., 2016). This discovery represents a paradigm change in the biology of immunity with respect to the rigorous division between innate and adaptive immune responses.

Viruses, parasites, and many bacterial or fungal cells or components of their cell wall [lipopolysaccharide (LPS), β-glucan, and chitin] represent strong stimuli of innate immune memory (Mulder et al., 2019). In mice models deficient for functional T and B lymphocytes, β-glucans from fungal cell walls induce trained immunity against non-pathogenic Candida albicans via functional reprograming of monocytes, through histone methylation, leading to enhanced cytokine production in vivo and in vitro (Quintin et al., 2012).

Recent experiments from our group showed that human monocytes stimulated by chitin from *Saccharomyces cerevisiae* lead to enhanced ability to eliminate a wide range of microorganisms, such as *C. albicans* (an opportunistic fungus), *Staphylococcus aureus* (Gram-positive bacterium), or *Escherichia coli* (Gram-negative bacterium) (Rizzetto et al., 2016).

The study of the occurrence of trained immunity in different organisms can be a leading approach to unravel the robustness and plasticity of this phenomenon. An insect model of immune memory is important for a deeper understanding of host defense and thus identifying the most effective approaches to modulate it. Insects evolved a complex innate immune system allowing the rapid elimination of unwanted microorganisms (Rosengaus et al., 1999). Defensive mechanisms against pathogens are mainly related to the activity of phagocytic cells present in the hemolymph and to the production of antimicrobial peptides (Gillespie et al., 1997; Lehrer and Ganz, 1999). Indeed, the activation of innate immunity in vertebrates and invertebrates showed shared mechanisms in the defense against microbial pathogens (Hoffmann et al., 1999), such as the observed activation of nuclear factor (NF)-κB family in Toll-like receptor (TLR) signaling pathways in *Drosophila melanogaster* after fungal

interaction (Silverman and Maniatis, 2001). Although lacking elaborate immune-specific reactions based on the "memory" of previously encountered pathogens, as in vertebrates, insects can show an increased resistance upon a secondary exposure to a microorganism (Little and Kraaijeveld, 2004; Sadd and Schmid-Hempel, 2006).

To date, the plasticity of the immune response in invertebrates has been mainly associated with a species-specific response against the pathogen responsible for the primary infection (immune priming) (Milutinović and Kurtz, 2016; Cooper and Eleftherianos, 2017). This immunization can last for days or weeks after the primary infection (Sadd and Schmid-Hempel, 2006) and can be vertically transferred across generation (Sadd et al., 2005; Cooper and Eleftherianos, 2017; Bordoni et al., 2018). Recently, it has been shown that immune priming in *D. melanogaster* is related to the activation of the phagocytes acting as effectors of the response against E. coli (Elrod-erickson et al., 2000). This finding suggests that immune priming determines an increase of immune activity with a reduced probability of reinfection through an improved clearance of pathogens. However, many aspects related to trained immunity in invertebrates remain unclear.

Recent reports have shown that the yeast *S. cerevisiae* exploits social wasps as vectors and natural reservoir for winter survival and summer dispersal (Stefanini et al., 2012). Polistes dominula paper wasps, in particular, preserve yeasts in their guts and presumably spread them in the environment but also favor hybridization in their intestinal environment, allowing the formation of genetically recombined strains of *S. cerevisiae*, and other yeasts (Stefanini et al., 2016). It is known that gut microbial communities locally influence immune response (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Arnolds and Lozupone, 2016). Less clear is if the yeasts determine physiological responses in the insects, also through modulation of gut microbiota.

According to present knowledge (Stefanini et al., 2012), S. cerevisiae is a natural component of paper wasps' gut microbiota, and it plays a crucial role in the insects' ecology. The occurrence of a non-pathogenic microorganism in the gut could have the potential to alter the immunocompetence and gut microbial balance of its host. Here, we investigated if two strains of S. cerevisiae, previously demonstrated able to induce trained immunity in mammals (Rizzetto et al., 2016), elicit an immunization in *P. dominula* against bacterial infections, and promote gut microbiota modulation. These findings allow us to elucidate the complex

relationships between host immunocompetence and its microbiota in the context of wasp natural history.

Materials and Methods

Insect Sampling and Housing Maintenance

Wasps of the species *P. dominula* were sampled from the "insect open lab," an experimental wasp orchard grass located in a field at the university campus in Florence (Sesto Fiorentino, Central Italy, 43°50′7″N, 11°11′46″E). Two different wasp castes, workers (collected before sexual emergence and at the beginning of summer) and future foundresses (female wasps that had not yet found a nest), were considered for immunological trials. The foundresses were tested before and after the winter diapause, a life condition in which different hormonal balance and fluctuation of insect immune system occur (Kubrak et al., 2014).

After sampling, the wasps were pooled and maintained in cohousing under controlled conditions (according to environmental photoperiod and room temperature) in sterile plastic cages and fed with autoclaved water and 40% D-glucose solution, referred to as sugar solution (SS) ad libitum for 7 days. After this period, each wasp was randomly assigned to yeast treatment (SS plus yeast) or to a control group (only SS), split, and kept separately in sterile 35-mm Petri dishes for the entire trial period, in order to avoid trophallaxis or any wasp–wasp interactive behavior.

S. cerevisiae and E. coli Strain Cultivation

Two *Saccharomyces cerevisiae* strains (YP4 and YH1) belonging to a clinical yeast collection of isolates from human fecal samples (Rizzetto et al., 2016; Ramazzotti et al., 2019) were tested as enhancers of resistance to bacterial infection in paper wasp P. dominula.

The two strains were previously proven for their ability to induce training immunity in human cells and mice (Rizzetto et al., 2016) and for different T-polarizing cytokine production in humans (Ramazzotti et al., 2019). These strains also differ in genetic background, as observed by previous analysis of *S. cerevisiae* population structure (Ramazzotti et al., 2019). YP4 strain belongs to the "Human Gut 2" cluster (referred to as HG2), which includes strains isolated from wasps and human intestine, with a common ancestral lineage, whereas YH1 belongs to the HG3 cluster, which includes wasps and human gut strains and other strains isolated from

bakery, wine, and other fermentations, descending from a common ancestor, previously identified as West Africa (Liti et al., 2009).

The *Saccharomyces cerevisiae* strains were grown on yeast peptone dextrose (YPD) broth medium (2% yeast extract, 1% peptone, and 2% D-glucose) at 30°C. Yeast cells were counted with a Bürker chamber to determine the title and washed using sterile phosphate-buffered saline (PBS) solution and then were resuspended in SS and orally administered with sterile tips at a concentration of 10^8 cells per wasp (Stefanini et al., 2016).

For bacterial infection, non-pathogenic *E. coli*, an immune elicitor commonly used to test immune competence in insects (Cappa et al., 2015; Cini et al., 2018), was used. *E. coli XL-1 Blue*, tetracycline resistant {chromosomal genotype: mutant alleles: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]}, was grown on Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl), added with tetracycline 10 μg/ml in order to exclude any other microbial contaminants. This microorganism was selected because it is not naturally found in *P. dominula*; thus, we can exclude its presence in our wasps prior to infection.

Table 1. Summary of genetic, phenotypic, and immunomodulatory traits of the two tested *Saccharomyces cerevisiae* strains.

	***S. cerevisiae* strains**		**References**
	YP4	**YH1**	
Source	Human feces	Human feces	Ramazzotti et al., 2019
Genetic background (microsatellite loci and whole-genome sequencing)	Human and insect gut (HG2) cluster; HG2 ancestor, and pure lineage	Human and insect gut (HG3) cluster; West Africa ancestor lineage	Liti et al., 2009; Ramazzotti et al., 2019
Phenotypic traits	Low sporulation (<25%)	High sporulation (>25%)	Ramazzotti et al., 2019
	Low invasiveness	Invasive	Ramazzotti et al., 2019
	No pseudohyphal formation	With pseudohyphal formation	Ramazzotti et al., 2019
	Cell wall composition: chitin, 15.84 ± 3.77; glucan, 56.04 ± 5.17; mannan, 28.11 ± 0.43	Cell wall composition: chitin, 12.31 ± 1.68; glucan, 51.06 ± 1.79; mannan, 36.62 ± 8.0	Rizzetto et al., 2016; Ramazzotti et al., 2019
Trained immunity			
Human monocytes [test of training with *S. cerevisiae* strain toward lipopolysaccharide (LPS), Pam3Cys4, or *Candida albicans* stimulation; cytokine profiles]	Infection agent: LPS → high level of interleukin (IL)-6 and production tumor necrosis factor (TNF)-α P3C → high level of TNF-α and IL-6 *C. albicans* → high level of IL-6	Infection agent: LPS → IL-6 and TNF-α production P3C → high level of IL-6 and TNF-α production *C. albicans* → not performed	Rizzetto et al., 2016
C57BL/6 mouse model	Chitin training before fungal infection mediates resistance against *C. albicans* systemic infection. Treatment with chitin markedly enhanced survival of infected mice	Not performed	Rizzetto et al., 2016
Immunophenotyping [T-polarizing cytokine levels upon human peripheral blood mononuclear cell (PBMC) challenge]	high IL-17, low interferon (IFN)-g	low IL-17, high IFN-g	Ramazzotti et al., 2019

Insect Model P. dominula: Housing, Feeding, and Infection

In order to observe the effect of administration of *S. cerevisiae* strain on bacterial clearance in an insect model, the first group of autumn foundresses (N = 40 pre-hibernation foundresses) was preliminary tested. The wasps were divided into two subgroups: 20 foundresses fed with SS to which yeast strain YP4 was added, while the remaining 20 wasps were fed with SS without any yeast added, as a control group (Supplementary Figure S1: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material).

A second experiment (Figure 1) was conducted with wasps of different castes and in different seasons. A group of autumn foundresses (pre-hibernation foundresses) was divided into three subgroups (N = 62): 19 foundresses were fed with SS added with YP4 strain, 20 with SS and YH1 strain, and the remaining 23 with SS without any yeast added (control group). A group of spring foundresses (N = 84) was collected after the winter diapause and similarly divided into three subgroups: 31 were fed with SS added with YP4 strain, 33 with SS and YH1 strain, and 20 with SS only. Workers (n = 53) were collected in summer from 10 colonies (at least five workers per colony) and treated as follows: 18 workers were fed with SS and YP4 strain, 19 workers with SS and YH1 strain, and 16 workers with SS without yeast (control group).

After feeding, foundresses were maintained at 8°C in the dark and fed ad libitum with sterile SS for 10 days (Stefanini et al., 2016). Workers were maintained in similar conditions to foundresses except for temperature, which was kept around 20°C, according to the seasonal temperature in which the workers emerge in the colony. After 11 days of feeding, bacterial infection with *E. coli* was performed. Furthermore, in order to exclude the presence of naturally occurring tetracycline-resistant microorganisms, five wasps were randomly taken from the same collection groups. Their whole body, gut included, was dissected and plated on LB agar, added with tetracycline 10 μg/ml, and incubated for 72 h at 37°C.

Escherichia coli cells were grown aerobically in LB medium plus tetracycline 10 μg/ml at 37°C. Cells were counted on a Bürker chamber, washed using PBS solution, and resuspended in PBS. Then, E. coli (10^5 cells) was injected in each wasp with a HamiltonTM micro syringe through the intersegmental membrane between the second and third abdominal tergites (Cini et al., 2018).

After injection, wasps were maintained in the dark at room temperature (20°C) for 24 h. The dead wasps were removed. Each wasp was then dissected under sterile conditions and homogenized in 1 ml of PBS solution using a sterile pestle. Before homogenization, the gut

was collected for subsequent metagenomic analysis while the sting and the venom sac were removed in order to avoid possible reduction of the bacterial count due to the presence of antimicrobial peptides in the wasp venom. The homogenate was serially diluted and plated on LB solid medium plus tetracycline 10 µg/ml and then incubated at 37°C overnight.

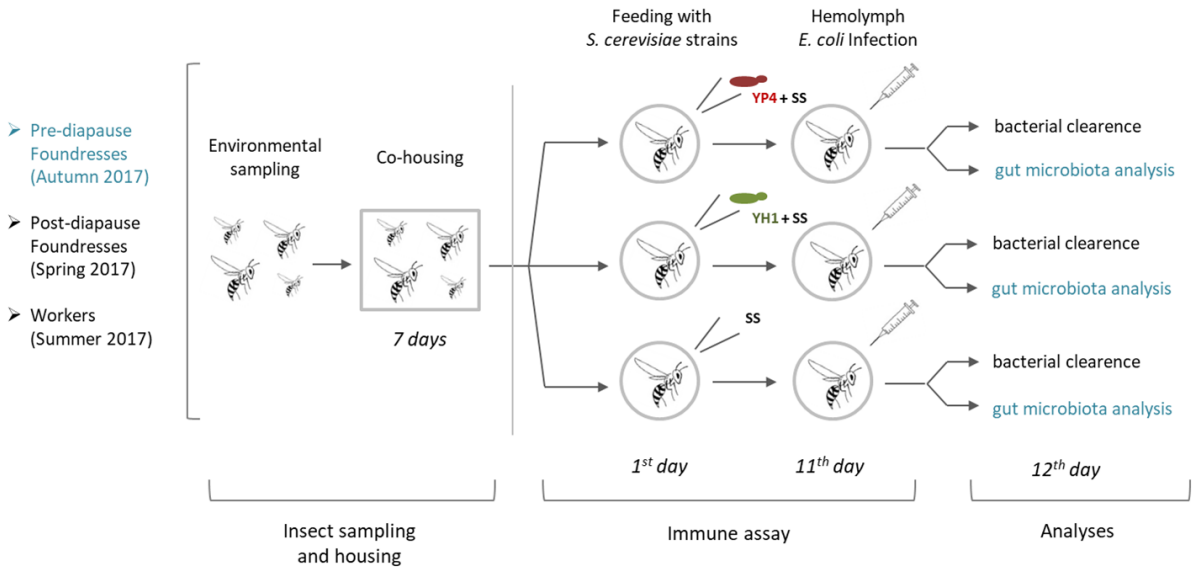

Figure 1. Schematic representation of experimental workflow. After environmental sampling and cohousing for 7 days, Saccharomyces cerevisiae strains (YP4 and YH1) were administered in foundresses of *Polistes dominula* wasps collected in different seasons (autumn and spring) and life stages (pre- and post-diapause) and workers collected in summer. At the 11th day, bacterial clearance was compared among treated and control groups. In the pre-diapause foundresses collected in autumn (in blue), gut microbiota analysis was also performed. SS, sterile sugar solution (40% D-glucose). Details were reported in the section "Materials and Methods."

Bacterial Clearance Evaluation

The day after infection (12th day), bacterial clearance was evaluated by counting bacterial colony-forming units (CFUs) per milliliter per wasp. We performed the bacterial clearance test as a good proxy of insect immunity, since injection of live bacteria provides an integrative view of the activation of the organismal immune system (Charles and Killian, 2015). Bacterial

clearance was compared among wasps belonging to different castes (foundresses and workers), treatments (control and YP4 and YH1 strains), and seasons for foundresses (autumn and spring), by using generalized linear mixed models (GLMMs) where the experiments (three for foundresses and one for workers) were included as a random effect. The effect of fixed factors and their interaction have been tested, obtaining a type III analysis of variance table, by using the Anova function of the "car" R package (Fox and Weisberg, 2019). Moreover, the effect of individual yeast strains compared to the control has been also assessed by using the summary function of the "stat" R package. The generalized linear models (GLMs) have been carried out, grouped and separated for the two experiments as well as for the two castes (foundresses and workers).

Genomic DNA Extraction From Gut Bacterial and Fungal Communities and Sequencing

The wasp guts were collected and stored in RNAlater Stabilization Solution (Invitrogen, Thermo Fisher Scientific) in sterile microcentrifuge tubes at −20°C until DNA extraction. DNA extraction from gut samples was performed using DNeasy PowerLyzer PowerSoil Kit (QIAGEN) following the manufacturer's protocol. The total DNA was quantified by a Tecan quantification device (Life Sciences). The sequencing was carried out by an Illumina MiSeq platform (BMR Genomics sequencing service of the University of Padova, Italy). DNA sequencing on bacterial communities was performed on the V3–V4 region of the 16S ribosomal RNA (rRNA) genes by using the primers Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014). The same guidelines for fungal communities were carried out on internal transcribed spacer (ITS) 2 rRNA genes by using the primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Illumina sequencing reads are available at the European Nucleotide Archive2 under accession study PRJEB32390.

Gut Microbiota Analysis

Demultiplexed sample libraries were visually inspected with the FastQC program (Andrews, 2010); low-quality ends of forward and reverse reads were trimmed using Sickle (Joshi and Fass, 2011) with a quality cutoff of 20 and a length threshold after trimming of 200. MICCA pipeline v. 1.7.2 (Albanese et al., 2015) was used for operational taxonomic unit

(OTU)/sequence variant (SV) picking as follows: forward and reverse reads were joined with micca “mergepairs” command, and reads with N bases were discarded with command “micca filter”. OTU/SV picking and chimera checking were performed with the “miccaotu” command and the UNOISE3 protocol as a picking algorithm. Taxonomy was assigned to the representative sequences of the identified OTUs/SVs classified using the RDP classifier v. 2.11 (Wang et al., 2007).

Subsequent analyses were performed in R (v.3.42; R Core Team, 2018), employing package “phyloseq v.1.22.3” (McMurdie and Holmes, 2013) to import data [as biological observation matrix (BIOM) files], to perform PCoA ordination analysis (using the Bray–Curtis distance measure), and to plot microbiota composition as a bar plot. Alpha diversity indices were calculated with the microbiome package (Lahti et al., 2017), and overall differences were tested with ANOVA, while species accumulation curves were calculated with the “ranacapa” package (Kandlikar et al., 2018). Prior to any analysis, count data were scaled with CSS transform as implemented in the “metagenomeSeq” (Paulson et al., 2013). Heatmaps reporting OTU distribution in different samples were created with the “pheatmap” package (Kolde, 2012). For heatmap construction, rows (i.e., OTUs/SVs) were ordered based on the results of the “plot_heatmap” command in the “phyloseq” package, using PCoA as the ordination method and Bray–Curtis as the distance measure, while columns (i.e., samples) were ordered based on Euclidean distance (as implemented in the pheatmap package). Color maps of OTU/SV abundance were scaled on the rows. Association between gut microbial community diversity and bacterial CFUs found in the hemocele post infection (a proxy for immune response priming and activation) were evaluated with distance-based permutational multivariate analysis of variance (PERMANOVA; Bray–Curtis distance; 9,999 permutations) using the adonis function in the “vegan v.2.5-2” (Oksanen et al., 2018) package, as well as employing multivariate-structure-based GLMs (negative binomial), as implemented by the “manyglm” command in the package “mvabund” (Wang et al., 2012). STAMP (Parks et al., 2014) software was used to compare relative abundance of OTUs at different taxonomic levels and to create plots. We used Welch’s t test (with no correction for multiple comparison) and displayed only OTUs with a $p < 0.05$.

Results

S. cerevisiae Strains, Able to Induce Trained Immunity in Mammals, Enhance Bacterial Clearance in Foundresses of Social Wasp *P. dominula.*

In order to test the ability of *S. cerevisiae*, a generally recognized as safe (GRAS) yeast, to enhance resistance to bacterial infection, we selected two *S. cerevisiae* strains based on their previously observed ability to induce training immunity in human cells and mice (Rizzetto et al., 2016).

In a first experiment, bacterial clearance was preliminarily tested on one group of autumn foundresses after administration of the YP4 yeast strain (Supplementary Figure S1: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material).

Subsequently, an immune trial was performed on two different wasp castes (future foundresses and workers) in three different seasons (summer workers and autumn and winter foundresses, see section "Materials and Methods"). The experimental design is depicted in Figure 1. By immune trials performed on foundress wasps, we observed that the bacterial clearance was lower in the control group than in the treated groups (Figure 2, Supplementary Datasheet S2, and Supplementary Figure S4: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material).

Firstly, data of the bacterial clearance from the two different experiments (including the preliminary test with the sole YP4 strain administration, as reported in Supplementary Figure S1: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material) were analyzed separately, showing a statistically significant effect of yeast treatment on the reduction of *E. coli* load (Table 2). Then, we have extended the analysis to all groups by integrating all variables (treatments, caste state, and season) into a GLMM (Table 3; see section "Materials and Methods"). The GLMM showed that the treatment with *S. cerevisiae* decreased the infection burden (measured as *E. coli* CFUs per milliliter as reported in Table 3 and shown in Figure 2). The treatment effect was significant in the reduction of *E. coli* load regardless the two different trials, also when the foundresses from the preliminary experiment were added in an overall analysis (Table 3).

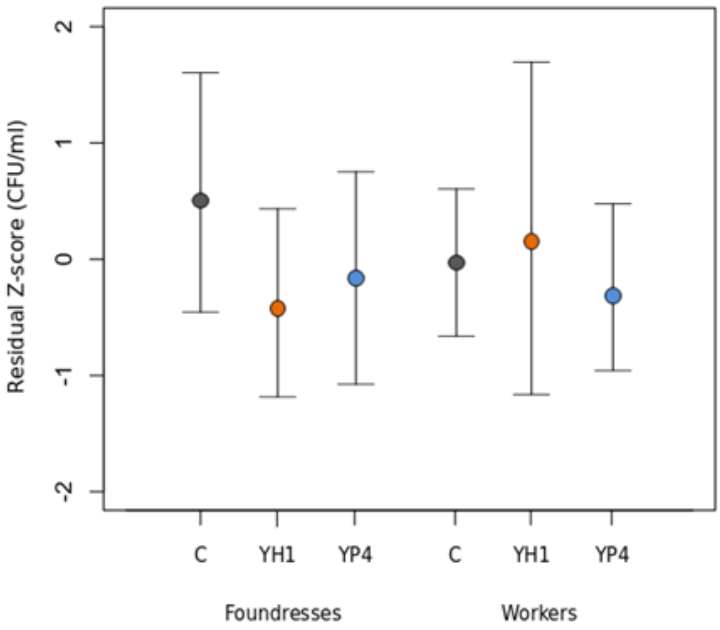

Figure 2. Bacterial clearance in *P. dominula* in foundresses and workers after yeast administration. Mean and standard deviation of residual load of *Escherichia coli* colony-forming units (CFUs) per milliliter in control (C) and treated groups (YH1 strain and YP4 strain administration) in foundresses and workers, at 24 h after bacterial infection. Data are representative of the bacterial clearance measure, reported as z-score transformation (residual z-score of CFUs per milliliter).

Table 2: ANOVA of deviance for 217 total observations (173 foundresses and 44 workers)

	Variables	df	χ^2	*P*
Preliminary immune trial in foundresses (YP4 strain)	Treatment	1	5,336	0.027
Complete immune trial in foundresses and workers (YP4 vs. YH1 strains)	Treatment	2	10,096	0.006
	Caste	1	2,585	0.108
	Treatment * Caste	2	5,547	0.060
Both preliminary and complete immune trials in foundresses and workers (YP4 vs. YH1 strains)	Treatment	2	16,485	<0.001
	Caste	1	2,572	0.109
	Treatment * Caste	2	5,563	0.062
Foundresses	Treatment	2	14,233	<0.001
	Season	1	228,375	<0.001
	Treatment * Season	2	2,714	0.257
Workers	Treatment	2	1,099	0.577

Data are separated for preliminary test (only autumn foundresses and YP4 yeast strain), the complete test (treatment and caste after YP4 and YH1 yeast strain administration), and both preliminary and complete immune trials. Separated analyses among foundresses and workers are also reported. df, degree freedom; P, p-value.

The experiments and analyses have been repeated for foundresses and workers separately (Table 3), revealing a caste-dependent effect, statistically significant in foundresses but not in workers (Figure 2). Furthermore, we showed a significant interaction effect for the sole strain YH1 ($p = 0.019$, Table 3) that increased bacterial clearance in foundresses but decreased resistance to the infection in workers (higher CFU-per-milliliter counts; Figure 2).

The experiments on the foundresses were performed in autumn (entry into diapause) and spring (exit to the diapause). We found that the levels of CFUs per milliliter were significantly higher in the spring than in the autumn ($p < 0.001$; Table 3). However, the treatment affects the CFU-per-milliliter levels regardless of the season, as shown by the lack of significance of the interaction effect in Table 3 ($p = 0.785$ for YH1 $*$ season and $p = 0.251$ for YP4 $*$ season).

Bacterial Clearance Induced by S. cerevisiae Treatment Is Associated to Shaping of Gut Microbiota Composition

In order to evaluate the effect of *S. cerevisiae* administration on the gut microbial communities, either *via* direct interaction or as a consequence of the immune training, we performed characterization of gut microbiota on the autumn foundress group, in which the effect of yeast administration on bacterial clearance was more evident. Autumn was also preferred because it is the season with a higher isolation rate of *S. cerevisiae* strains from wasp guts (Stefanini et al., 2012).

We selected nine gut samples of foundresses per group (treatments and control), pooling them together in a total of three gut samples per group, as representative gut microbiota collection of all tested samples of the respective groups (Supplementary Figure S2: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material). The criteria for sample selection were in accordance with the coefficient-of-variation (CV) analysis calculated by comparison of intragroup and intergroup (intragroup CV%: control group = 15.4; YP4-treated group = 38.2; YH1-treated group = 36.2; intergroup CV%: control vs. YP4 = 46.9; control vs. YH1 = 48; as reported in Supplementary Table S1: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material).

By 16S rRNA V3–V4 region sequencing, a total of 214,089 sequence reads were obtained after quality filtering, clustering in a total of 495 OTUs. The number of 16S rRNA sequences per sample ranged from 1,607 to 51,340. Although the control group showed a low amount of sequence reads, no statistical differences in read number were observed among groups (Supplementary Figure S5: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material).

Table 3: Generalized Linear Mixed Model (GLMM) analysis.

	Variables	Estimate	Standard error	*Z* Values	*P*
Complete immune trial in foundresses and workers (YP4 vs. YH1 strains)	YH1-C	−0.511	0.179	−2.86	0.004
	YP4-C	−0.374	0.183	−2.04	0.041
	Caste	−2,894	1.800	−1.61	0.108
	YH1 * Caste	0.815	0.345	2.34	0.019
	YP4 * Caste	0.079	0.350	0.23	0.820
Foundresses	YH1-C	−0.425	0.213	−2.00	0.046
	YP4-C	−0.490	0.161	−3.04	0.002
	Season	3,424	0.227	15.11	< 0.001
	YH1 * Season	−0.083	0.303	−0.27	0.785
	YP4 * Season	0.312	0.271	1.15	0.251
Workers	YH1-C	0.304	0.404	0.75	0.45
	YP4-C	−0.295	0.404	−0.73	0.46

Comparison of single variables (combination of treatment, caste, and season) by using GLMM, on bacterial clearance observed in all tested foundresses and workers.

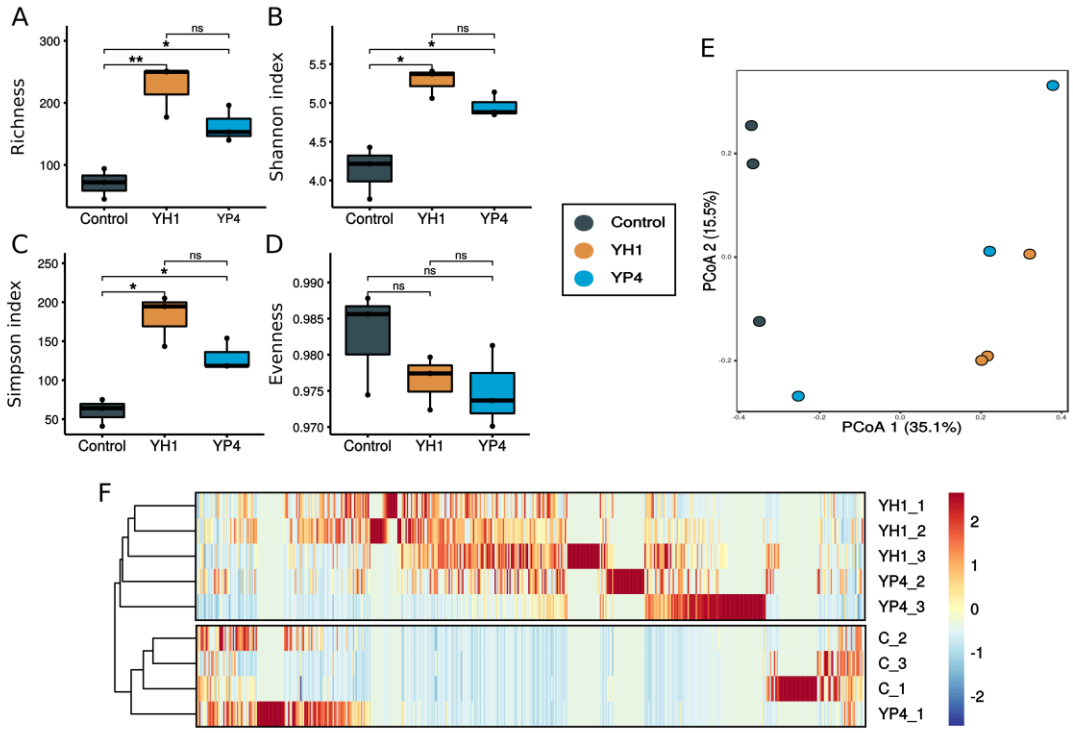

Figure 3. Alpha and beta diversity of gut microbial communities. (A–D) Alpha diversity measures: (A) richness, (B) Shannon diversity index, (C) Simpson index, and (D) evenness of control and treated (YH1 and YP4) groups. Statistically significant comparisons by t test (∗p < 0.05, ∗∗p < 0.01, and ns = not statistically significant). (E) Principal coordinate analysis (PCoA) ordination based on the Bray–Curtis distance. (F) Heatmap reported the frequency of operational taxonomic unit (OTU) abundance within treatment and control groups.

By comparison of the alpha diversity indices among the three groups (control and YH1- and YP4-treated groups), we observed a reduction in richness (Figure 3A) and diversity (i.e.,

Shannon and inverse Simpson indices; Figures 3B,C) of gut bacterial communities in the control group, with respect to the two treated groups (Figure 3; ANOVA test; richness: $F = 16.91$, $p = 0.0034$; Shannon's index: $F = 17.6$, $p = 0.0031$; inverse Simpson index: $F = 18.32$, $p = 0.0027$; a pairwise t test is reported for every comparison in Figure 3). Moreover, as indicated by a higher evenness index, the microbial community of the control group had more homogeneous abundance distribution than the treated groups (Figure 3D).

PCoA ordination analysis based on Bray–Curtis distances showed a clear separation of gut samples, along the first ordination axis (explaining 35.1% of the variability), according to the type of treatment (Figure 3E). In particular, samples from the YH1-treated group showed a clear separation and a more defined grouping than did the control and YP4 groups (Figure 3E). PERMANOVA was used to test the different distributions of samples, as observed in ordination analysis (Supplementary Table S2: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material).

Statistical analysis showed that *S. cerevisiae* treatment has an effect, as a whole, on microbiota composition, when compared to the control group (control vs. treatment: $R2 = 0.3$, $p = 0.01$), and the treatments with the two *S. cerevisiae* strains differentially affect the microbiota composition (control vs. YH1 treatment vs. YP4 treatment: $R2 = 0.4$, $p = 0.03$). In Figure 3F, the heatmap displays the OTU frequencies and abundance distribution among control and treated samples. We observed different trends of OTU pattern distribution between control and treated groups (separated branches in hierarchical clustering as reported in Figure 3F), as well as between the two different yeast-treated groups.

Generalized linear models analysis (mvabund R package) was performed in order to assess the multivariate association between residual CFUs per milliliter (following the bacterial clearance) and gut bacterial community composition, showing significant association (likelihood ratio statistic: 857.4, $p = 0.002$). We found 75 OTUs (Supplementary Datasheet S1: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material) with a significant univariate association to (log)CFUs per milliliter of bacterial infectious agent, regardless of treatments or control. When correction for multiple comparison was applied, *Campylobacter* was still significantly associated to the count of infectious agent (adjusted $p = 0.013$; Supplementary Datasheet S1: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material).

To evaluate the overall effect of *S. cerevisiae* administration and the subsequent resistance to *E. coli* infection on the wasp gut microbiota composition, we compared the microbial community's relative abundance (at phylum and genus levels) among treated and control groups, as shown in Figure 4 and Supplementary Figure S3 (https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material). At the phylum level, an increase of *Bacteroidetes* and *Fusobacteria* was observed in treated groups (Figure 4A). At the genus level, 17 genera showed significant different abundances between control and treatment groups (Figure 4B; Welch's t test). Among them, *Staphylococcus* (*Firmicutes* phylum) and *Morganella* (*Proteobacteria* phylum) were significantly enriched in the control group. Interestingly, *Escherichia/Shigella* genus was significantly enriched in the gut community of the controls compared to the treated groups (Figure 4C; ANOVA p = 0.025).

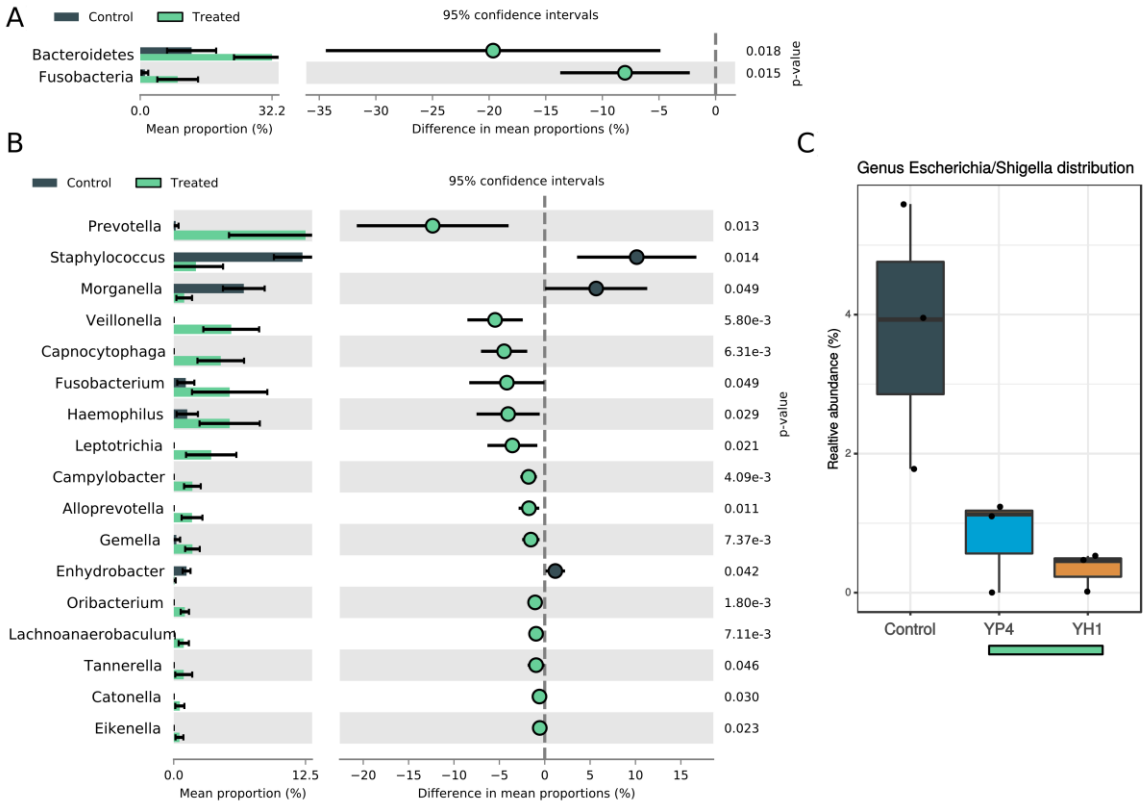

Figure 4. Comparison of statistically significant taxa in treated and control groups. Mean proportion and confidence intervals (%) in control vs. treated (YH1 and YP4) groups explored

at phylum (A) and genus (B) levels (p-values by Welch's t test). (C) Relative abundance (%) of *Escherichia/Shigella* genus distribution between control and treated groups (ANOVA p = 0.025).

In treated groups, *Prevotella (Bacteroidetes), Veillonella (Firmicutes), Capnocytophaga (Bacteroidetes), Fusobacterium (Fusobacteria), Haemophilus (Proteobacteria),* and *Leptotrichia (Fusobacteria)* were found to be significantly abundant (Figure 4B; Welch's t test).

The two yeast strains highly differed in their ability to change the microbial profiles (Figure 5). While administration of both *S. cerevisiae* strains reduced the levels of *Staphylococcus* and *Morganella*, when compared to the control group (Figures 5A,B), the single comparison between strain administration and control (YH1 vs. control in Figure 5A and YP4 vs. control in Figure 5B) showed a strain-dependent effect on gut microbiota composition. To note, YH1 treatment (Figure 5A) was associated to an enrichment of a higher number of bacterial genera, such as *Fusobacterium, Veillonella, Alloprevotella, Capnocytophaga, Porphyromonas, and Campylobacter*, compared to YP4 treatment (Figure 5B; Welch's t test).

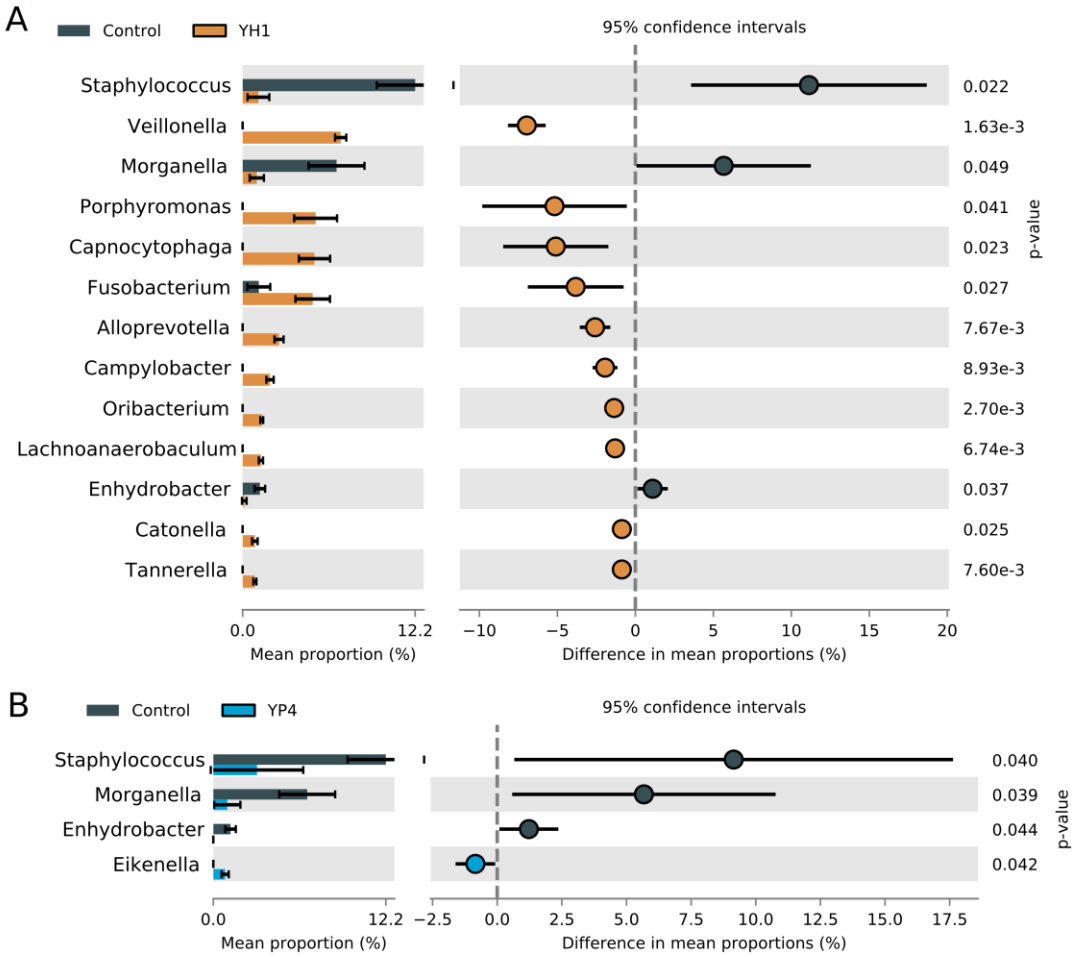

Figure 5. Comparison of statistically significant taxa in YH1- and YP4-treated and control groups. Mean proportion and confidence intervals (%) in (A) control vs. treated YH1 and (B) control vs. YP4, explored at genus level (p-values by Welch's t test).

S. cerevisiae Treatment Does Not Affect the Gut Fungal Communities

For the fungal ITS2 region sequencing, we obtained a total of 14,646 sequences after quality filtering, clustering in a total of 63 OTUs. The number of sequence reads per sample ranged from 202 to 3,024. Contrary to the gut bacterial community, the mycobiota composition did not show significant differences in richness and biodiversity between control and treated groups, as observed by alpha diversity and PCoA ordination analysis based on the Bray–Curtis distance (Figure 6). Accordingly, PERMANOVA analysis used to test the differences in fungal community composition among groups showed no effect of treatment on gut mycobiota (Supplementary Table S3: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material). In

Figure 7A, we reported an overview of relative abundances of fungal taxa (>1%) annotated at the species level in the three groups. No significant association between fungal community composition and bacterial infection load (CFUs per milliliter of *E. coli*) have been observed. The fungal community abundances (Figure 7A) appeared unaffected by the *S. cerevisiae* administration and by bacterial infection in hemocele. Only Metschnikowia sp. was found significantly enriched in the control group compared to both treated groups (Figures 7B,C; ANOVA $p < 0.001$).

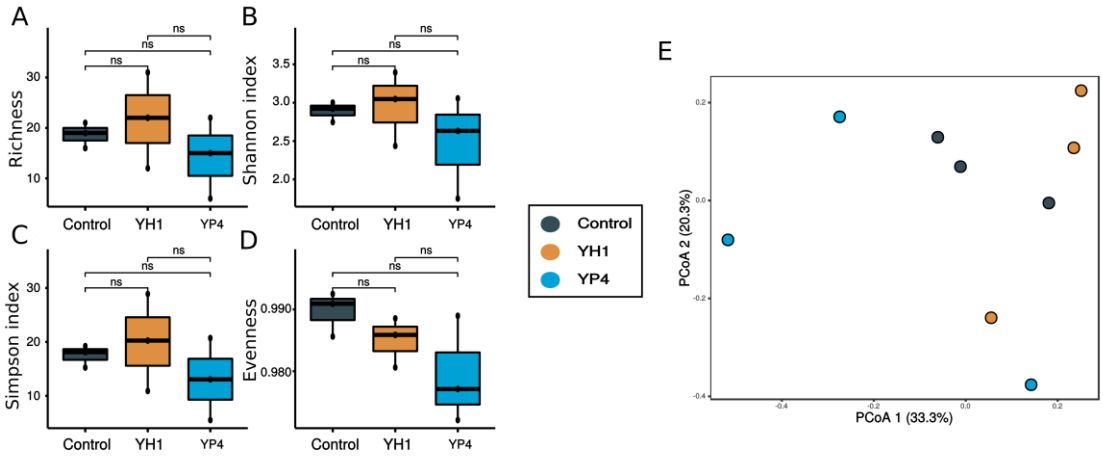

Figure 6. Alpha and beta diversity of gut mycobiota. (A–D) Comparison of alpha diversity measures: (A) richness, (B) Shannon diversity index, (C) Simpson index, and (D) evenness among treated (YH1 and YP4) and control groups. Statistically significant comparisons by t test (∗$p < 0.05$, ∗∗$p < 0.01$, and ns = not statistically significant). (E) Principal coordinate analysis (PCoA) ordination based on the Bray–Curtis distance among fungal communities between control and treated groups.

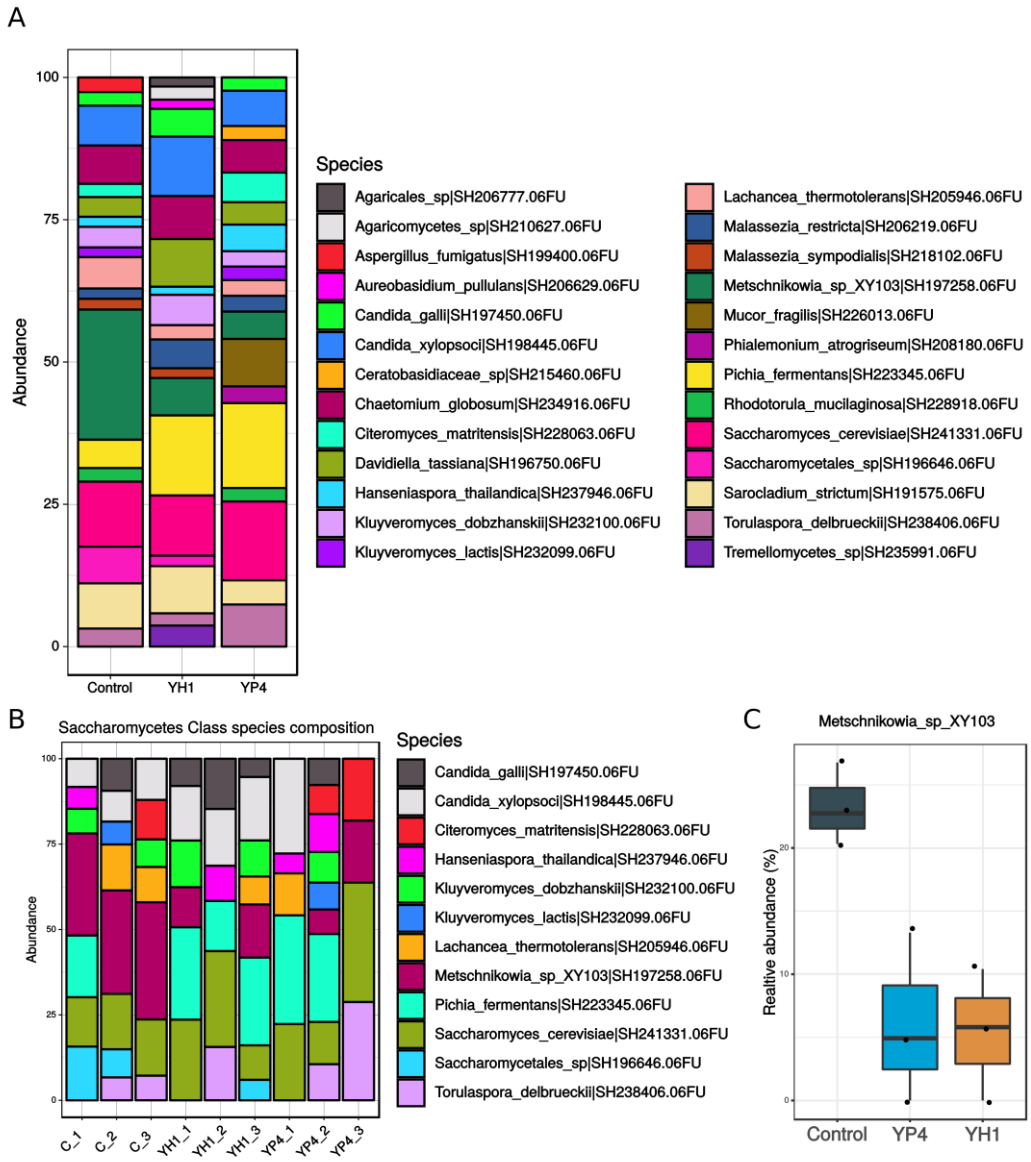

Figure 7. Gut mycobiota community abundance. (A) Comparison of gut mycobiota composition in treated and control groups. Bar plot showed the taxa with >1% of relative abundance annotated at species level. (B) Focus on Saccharomycetes fungal class composition reporting species taxa with >1% of relative abundance. (C) Relative abundance (%) of *Metschnikowia sp.* distribution between control and treated groups.

Discussion

In recent years, the physiological and ecological relationships between hymenopterans, in particular social wasps, and the yeast *S. cerevisiae* have become evident (Stefanini, 2018). The wasps likely act as natural vectors, hosting and transporting *S. cerevisiae* between different environmental niches and favoring mating among strains (Stefanini et al., 2012, 2016; Ramazzotti et al., 2019). While the advantage provided to the yeast is well understood, the benefit for the wasp to carry *S. cerevisiae* remains unclear. In this study, we tested the hypothesis that *S. cerevisiae* could help the insect to resist infections during hibernation. This hypothesis is supported by previous studies (Quintin et al., 2012; Rizzetto et al., 2016; Rusek et al., 2018; Mulder et al., 2019) showing that fungal cells or components of cell wall (i.e., chitin) are potent stimuli of innate immune memory in mammals. To this aim, we investigated whether two well-characterized *S. cerevisiae* strains, by genetic, phenotypic, and immunophenotypic assays (Ramazzotti et al., 2019), previously proved to induce trained immunity in mammals Rizzetto et al. (2016), are also able to enhance resistance of the invertebrate immune system against bacterial infection and shape the gut microbial communities. A few studies have focused on the nature of the insect immune system, revealing that it can be efficiently primed upon exposure to microorganisms, acquiring a protective effect upon subsequent challenge with the same, and/or different microbes (Cooper and Eleftherianos, 2017). We used the social wasp *P. dominula* as an animal model for innate immune trials. They evolved mechanisms to tolerate and benefit from the presence of this GRAS microorganism, providing protection against pathogens and maintaining health status, through the development and boosting of the immune system and providing protection against pathogens. Our results showed that *S. cerevisiae* induces in foundresses, but not in workers, a higher response against bacterial infection compared to the controls, possibly via the activation of strain-dependent innate immunization. In fact, following YH1 administration when compared to YP4 strain, we observed significant differences in immune enhancing, as well as in the changes of the gut microbiota. The data presented here, alongside the immune training induced by the same yeast strains in mouse models and human monocytes (Rizzetto et al., 2016), show that the yeast strains can provide protective mechanisms shared between different subphyla, potentially through analog immune training mechanisms occurring in mammals, and insects. The yeast-mediated protection against pathogens could be important to protect hibernating foundresses during overwintering, simultaneously providing yeasts with

the environmental niche where they mate and survive, thus suggesting the existence of a complex symbiotic interaction. Complex communication systems have been evolved between the host immune system and the intestinal microbiota (Belkaid and Hand, 2014). Evidence is mounting in support of a dominant and decisive role of gut in shaping and modulating immune responses in the prevention of disease (Hand, 2016). However, the effect of trained immunity on commensal microbial communities, that is, the microbiota, has not been shown yet. The gut microbiota plays a fundamental role in the education and functional tuning of the host immune system, thereby acting as adjuvant to the host immune system and continually driving the nature of immune responses, providing protection against pathogens. In turn, the host immune system has evolved multiple means by which to maintain its symbiotic relationship with the microbiota (Dethlefsen et al., 2007; Cho and Blaser, 2012; Lynch and Pedersen, 2016; Gupta et al., 2018). Hymenoptera's gut contains a relatively simplified microbiota, with lesser microbial species as compared to that of mammals, yet social insects harbor microbial communities with highly specialized and beneficial functions in nutrition, protection from parasites and pathogens, and modulation of immune responses (Engel and Moran, 2013).

In both yeast-fed groups, we also observed reduction in potential pathogenic bacterial genera, such as *Staphylococcus, Morganella, and Escherichia/Shigella,* compared to the control group. The observed reduction of *Staphylococcus* load, following yeast feeding, may be also associated with the ability of *S. cerevisiae* to counteract and inhibit *Staphylococcus* biofilm making, as previously observed (Walencka et al., 2007).

The two yeast strains showed different abilities to change the bacterial microbiota composition. The YH1 strain displays the strongest caste-dependent immunomodulatory effect, to the detriment of the resistance to *E. coli* infection in the workers, and shows increased ability in changing the gut microbiota, suggesting that the changes in the bacterial communities are related to strain-specific immunomodulation. While this effect is evident for bacterial communities, *S. cerevisiae* administration does not alter the diversity of the fungal communities. In the latter, only the Metschnikowia genus was reduced. Yeast–yeast competition for nutrients are well-known, especially regarding *S. cerevisiae* and *Metschnikowia* growing in fermentative processes (Alexandre et al., 2004; Fleet, 2008; Albergaria and Arneborg, 2016; Ciani et al., 2016; Sadoudi et al., 2017). Our results suggest that the competition between these two yeasts is not limited to grape must but may occur also in the insect gut.

It is unlikely that the observed effects are influenced by a role of *S. cerevisiae* as nutrient supplementation: (i) it is known that in *P. dominula*, the green fluorescent protein (GFP)-labeled *S. cerevisiae*, ingested by wasps, populate the gut for at least 4 months (Stefanini et al., 2012, 2016) (thus, the microorganism is still alive after treatment and survives after passage through the wasps' digestive tract); (ii) immune training occurred only in foundresses and not in workers, showing a caste-dependent effect in insects that received the same amount of *S. cerevisiae* cells (if the effect would have been related to nutrition, one would expect this to be equal regardless of the caste); (iii) the two strains showed significant differences in the ability to improve the bacterial clearance (a strain-dependent immune-mediated event excludes a general nutritional effect of the yeast); and (iv) the differences in immune enhancing are mirrored by differences in the changes of the microbiota composition (this finding corroborates our conclusion that the effect is mediated by immune responses and not just by feeding).

Overall, this study provides a preliminary indication of the complex interactions existing between insects' immune responses and their symbiotic gut communities. Future studies using insects as models promise stimulating information that will potentially uncover the relationship between immune priming and physiological functions, as well as their effect on gut microbiota. Our results suggest that yeasts and social insects possess key synergistic elements and that a deeper understanding of these interactions, including the molecular determinants of the priming and trained immunity, may provide significant insights on the host immune system–microbe evolution.

Data Availability Statement

Data are available as electronic Supplementary Material. Illumina sequencing data are available at the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB32390) under accession study PRJEB32390.

Author Contributions

DC, ST, and NM conceived the study. NM, DR, FC, FT, and AG carried out the experiment. LD and NM performed the statistical analyses. FV performed the metagenomic and data analysis. NM, MP, LB, and DC drafted the manuscript. All authors critically revised the

manuscript, approved the final version of the manuscript, and agreed to be held accountable for the content therein.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material

Footnotes

^ https://www.bmr-genomics.it/

^ http://www.ebi.ac.uk/ena/data/view/PRJEB32390

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CHAPTER VI

GENERAL CONCLUSIONS

General conclusions

The data obtained from the first task showed that the microbial communities development in the invasive hornet *V. velutina* follow a sample-specific signature, showing differences in composition across life stages and among reproductive phenotypes. The hornets' ontogenetic development from the larva to the adult stages plays acrucial role in the gut microbial communities composition. The microbiota switching across life stages related to the ontogenetic development were previously described also in other social insects, such as *Apis mellifera* (Martinson et al., 2012). I think that the variations of the intestinal physiological and chemical conditions during the insect development can drive the gut microbiota organization as already demonstrated by Lemke et al. in the insect *Pachnoda ephippiata*. Therefore, the stage-related physiological condition combined to the stage-related diet could determine the selection of intestinal microbial communities. These results also suggest the importance to consider the insect life stages and reproductive phenotypes to obtain a wide overview of the evolution of social insect gut microbial communities. The future prospects are, extend the analysis to native communities of the same insect species by sampling them in Asia and generally improve the sampling spectrum in Italy and Europe.

From the comparison performed in the second chapter, I showed that the two hornets *V. velutina* and *V. crabro* and the honey bee *A. mellifera*, displayed a gut microbial population structures. *A. mellifera* showed a very polarized gut microbial structure, consisting of a few taxa, much more enriched comparing to the hornets phylotipes distributions. Instead, the hornets showed a more widespread microbiota and mycobiota with less enriched but more diversified taxonomic profile. Interesting, the gut microbiota composition was affected by insect species determining a different bacterial composition among the three analyzed insects. Moreover the mycabiota composition was affected by the insect genus (*Apis* or *Vespa*) and the hornet-associated phylotypes were all belonging to the order *Saccharomycetales*. In my opinion, this occurs due to the effect of different environmental stimuli and ecological traits, such as the different diet and the intestinal physiochemical characteristics associated to the hornets and honey bees. These conditions could also be responsible for the different levels of lactic acid bacteria (LAB) displayed between hornets and honey bees. In the future, it would be interesting to assess whether the differences between the two hornet species are stable or transient, for this reason it may be necessary to extend the sampling, thus include populations of hornets from other sites in Italy.

In my third task, I showed how an environmental stimulus differentially influences the gut microbial composition of the hornet *V. crabro* and honey bee *A. mellifera*. We know that the diet can influence the intestinal microbial composition, in particular the omnivorous diet in insects was associated with an higher richness and Shannon index comparing to the stenophagous diet (Yun et al., 2014). I evaluated the effect of specific environmental stimuli on two insects with different diets and behavoir, particularly I showed the effect fo the foraging activity on the gut microbial communities during the seasonal period of maximum grape ripeness on the hornets and honey bees, comparing it with other environmental stimuli.

The different environmental patterns corresponded to significant variations in the micorbiota and the mycobiota organization. However, the most evident effect was exerted by the ripe grapes feeding as showed by the differences of mycobiota composition of the hornets that foraged in the vineyard during the period of maximum ripening. Honey bees, isolated from olive tree bark hollow showed significant variations in the mycobiota through the enrichment of *Saccharomycetes* and *Tremellomycetes*. In particular, the presence of *Tremellomycetes* seems to be easily correlated with the frequent isolation of these fungal taxa in the olive trees (Varanda et al., 2019). I suggest that this is a result of a mutualistic relationship that allow the hornets to act as vectors of *Saccharomycetes*, transporting the yeasts in the vineyard, meanwhile, enriching the *Saccharomycetes* intestinal abundance likely by increasing food sugars feeding on ripe grapes is the component of a feedback effect that sees the hornets enrich their gut with *Saccharomycetes* through grape sugar intake and yeasts ensure access to sugary sources by means of hornets transport. In the future, it would be interesting isolate the cultivable *Saccharomycetes* from hornets and grape berries and evaluate the genetic similarity using microsatellite or whole genome analysis.

Recently the ecological relationships between hymenopterans, such as social wasps, and fungi have become evident (Stefanini et al., 2018). We know that the social wasps act as natural vectors, hosting and carring *Saccharomycetes* such as *S. cerevisiae* between different environmental niches and favoring the production of interspecific hybrids increasing the yeast biodiversity (Stefanini et al., 2012, Stefanini et al., 2016; Ramazzotti et al., 2019). In my fourth task, i demostrated that *S. cerevisiae* strains, proved to induce trained immunitity in mammals (Rizzetto et al., 2016) help the social wasp *Polistes dominula* resisting against *E. coli* infection and shapes its gut microbial comosition and this occured only in foundresses and not in workers. This has shown that yeasts able to stimulate mammals immune system can also enhance the invertebrates immune system. Therefore, this suggests that the immune mechanisms involved in the recognition fo the microorganisms are in a certain part shared between Vertebrates and Invertebrates. The yeast-mediated protection against pathogens could be essential in the surviving of foundresses during wasp's winter hibernation, and provide an environmental niche where the yeasts can mate, survive, and be passed within the colony thus defining the existence of a complex symbiotic interaction. In the future, it would be important to test other strains and evaluate the functional contribution of the yeast *S. cerevisiae* to the wasp's immune system likely by whole genome analysis and transcriptomic assays on wasp immune system gene markers.

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APPENDIX

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Saccharomyces cerevisiae Induces Immune Enhancing and Shapes Gut Microbiota in Social Wasps

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Trained immunity is the enhanced response of the innate immune system to a secondary infection after an initial encounter with a microorganism. This non-specific response to reinfection is a primitive form of adaptation that has been shown to be conserved from plants to mammals. Insects lack an acquired immune component and rely solely on an innate one, and they have expanded it upon traits of plasticity and adaptation against pathogens in the form of immune priming. The recent discoveries of the role of *Saccharomyces cerevisiae* in the insect's ecology and the ability of this yeast to induce trained immunity in different organisms suggest that insects could have developed mechanisms of adaptation and immune enhancing. Here, we report that two yeast strains of *S. cerevisiae*, previously shown to induce trained immunity in mammals, enhance resistance to *Escherichia coli* infection in the paper wasp *Polistes dominula*. The reduction of injected *E. coli* load by *S. cerevisiae* strains was statistically significant in future foundresses but not in workers, and this occurs before and after hibernation. We thus investigated if the effect on *E. coli* was mirrored by variation in the gut microbiota composition. Foundresses, showing immune enhancing, had statistically significant changes in composition and diversity of gut bacterial communities but not in the fungal communities. Our results demonstrate that *S. cerevisiae* can prime insect responses against bacterial infections, providing an advantage to future foundress wasps to carry these microorganisms. Understanding the mechanisms involved in the generation of specific and long-lasting immune response against pathogenic infections in insects and the influence of the induction of trained immunity on the commensal gut microbiota could have a major impact on modern immunology.

Keywords: innate immunity, immune training, *Saccharomyces cerevisiae*, gut microbiota, *Polistes dominula*

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INTRODUCTION

Immunological memory is an important evolutionary trait that improves host survival upon secondary infection (Netea et al., 2011, 2019). Trained immunity is a newly discovered mechanism of innate immune memory, causing metabolic and epigenetic reprograming of innate immune cells [monocytes/macrophages, natural killer (NK) cells] (Netea et al., 2011, 2019). This non-specific

response describes an inflammatory protection against a microorganism upon a second encounter, independently from adaptive immunity, and it has been shown to be present not only in mammals but also in plants (Durrant and Dong, 2004) and insects (Pham et al., 2007; Rodrigues et al., 2010; Norouzitallab et al., 2016). This discovery represents a paradigm change in the biology of immunity with respect to the rigorous division between innate and adaptive immune responses.

Viruses, parasites, and many bacterial or fungal cells or components of their cell wall [lipopolysaccharide (LPS), β-glucan, and chitin] represent strong stimuli of innate immune memory (Mulder et al., 2019). In mice models deficient for functional T and B lymphocytes, β-glucans from fungal cell walls induce trained immunity against non-pathogenic *Candida albicans via* functional reprograming of monocytes, through histone methylation, leading to enhanced cytokine production *in vivo* and *in vitro* (Quintin et al., 2012).

Recent experiments from our group showed that human monocytes stimulated by chitin from *Saccharomyces cerevisiae* lead to enhanced ability to eliminate a wide range of microorganisms, such as *C. albicans* (an opportunistic fungus), *Staphylococcus aureus* (Gram-positive bacterium), or *Escherichia coli* (Gram-negative bacterium) (Rizzetto et al., 2016).

The study of the occurrence of trained immunity in different organisms can be a leading approach to unravel the robustness and plasticity of this phenomenon. An insect model of immune memory is important for a deeper understanding of host defense and thus identifying the most effective approaches to modulate it. Insects evolved a complex innate immune system allowing the rapid elimination of unwanted microorganisms (Rosengaus et al., 1999). Defensive mechanisms against pathogens are mainly related to the activity of phagocytic cells present in the hemolymph and to the production of antimicrobial peptides (Gillespie et al., 1997; Lehrer and Ganz, 1999). Indeed, the activation of innate immunity in vertebrates and invertebrates showed shared mechanisms in the defense against microbial pathogens (Hoffmann et al., 1999), such as the observed activation of nuclear factor (NF)-κB family in Toll-like receptor (TLR) signaling pathways in *Drosophila melanogaster* after fungal interaction (Silverman and Maniatis, 2001). Although lacking elaborate immune-specific reactions based on the "memory" of previously encountered pathogens, as in vertebrates, insects can show an increased resistance upon a secondary exposure to a microorganism (Little and Kraaijeveld, 2004; Sadd and Schmid-Hempel, 2006).

To date, the plasticity of the immune response in invertebrates has been mainly associated with a species-specific response against the pathogen responsible for the primary infection (immune priming) (Milutinović and Kurtz, 2016; Cooper and Eleftherianos, 2017). This immunization can last for days or weeks after the primary infection (Sadd and Schmid-Hempel, 2006) and can be vertically transferred across generation (Sadd et al., 2005; Cooper and Eleftherianos, 2017; Bordoni et al., 2018). Recently, it has been shown that immune priming in *D. melanogaster* is related to the activation of the phagocytes acting as effectors of the response against *E. coli* (Elrod-erickson et al., 2000). This finding suggests that immune priming determines an increase of immune activity with a reduced probability of reinfection through an improved clearance of pathogens. However, many aspects related to trained immunity in invertebrates remain unclear.

Recent reports have shown that the yeast *S. cerevisiae* exploits social wasps as vectors and natural reservoir for winter survival and summer dispersal (Stefanini et al., 2012). *Polistes dominula* paper wasps, in particular, preserve yeasts in their guts and presumably spread them in the environment but also favor hybridization in their intestinal environment, allowing the formation of genetically recombined strains of *S. cerevisiae*, and other yeasts (Stefanini et al., 2016). It is known that gut microbial communities locally influence immune response (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Arnolds and Lozupone, 2016). Less clear is if the yeasts determine physiological responses in the insects, also through modulation of gut microbiota.

According to present knowledge (Stefanini et al., 2012), *S. cerevisiae* is a natural component of paper wasps' gut microbiota, and it plays a crucial role in the insects' ecology. The occurrence of a non-pathogenic microorganism in the gut could have the potential to alter the immunocompetence and gut microbial balance of its host. Here, we investigated if two strains of *S. cerevisiae*, previously demonstrated able to induce trained immunity in mammals (Rizzetto et al., 2016), elicit an immunization in *P. dominula* against bacterial infections, and promote gut microbiota modulation. These findings allow us to elucidate the complex relationships between host immunocompetence and its microbiota in the context of wasp natural history.

MATERIALS AND METHODS

Insect Sampling and Housing Maintenance

Wasps of the species *P. dominula* were sampled from the "insect open lab," an experimental wasp orchard grass located in a field at the university campus in Florence (Sesto Fiorentino, Central Italy, 43°50′7″N, 11°11′46″E). Two different wasp castes, workers (collected before sexual emergence and at the beginning of summer) and future foundresses (female wasps that had not yet found a nest), were considered for immunological trials. The foundresses were tested before and after the winter diapause, a life condition in which different hormonal balance and fluctuation of insect immune system occur (Kubrak et al., 2014).

After sampling, the wasps were pooled and maintained in cohousing under controlled conditions (according to environmental photoperiod and room temperature) in sterile plastic cages and fed with autoclaved water and 40% D-glucose solution, referred to as sugar solution (SS) *ad libitum* for 7 days. After this period, each wasp was randomly assigned to yeast treatment (SS plus yeast) or to a control group (only SS), split, and kept separately in sterile 35-mm Petri dishes for the entire trial period, in order to avoid trophallaxis or any wasp–wasp interactive behavior.

S. cerevisiae and *E. coli* Strain Cultivation

Two *Saccharomyces cerevisiae* strains (YP4 and YH1) belonging to a clinical yeast collection of isolates from human fecal samples (Rizzetto et al., 2016; Ramazzotti et al., 2019) were tested as enhancers of resistance to bacterial infection in paper wasp *P. dominula*.

The two strains were previously proven for their ability to induce training immunity in human cells and mice (Rizzetto et al., 2016) and for different T-polarizing cytokine production in humans (Ramazzotti et al., 2019). These strains also differ in genetic background, as observed by previous analysis of *S. cerevisiae* population structure (Ramazzotti et al., 2019). YP4 strain belongs to the "Human Gut 2" cluster (referred to as HG2), which includes strains isolated from wasps and human intestine, with a common ancestral lineage, whereas YH1 belongs to the HG3 cluster, which includes wasps and human gut strains and other strains isolated from bakery, wine, and other fermentations, descending from a common ancestor, previously identified as West Africa (Liti et al., 2009).

Table 1 summarizes the different genetic, phenotypic, and immunophenotypic characteristics of the two tested strains, as reported by our previous studies (Rizzetto et al., 2016; Ramazzotti et al., 2019).

The *Saccharomyces cerevisiae* strains were grown on yeast peptone dextrose (YPD) broth medium (2% yeast extract, 1% peptone, and 2% D-glucose) at 30°C. Yeast cells were counted with a Bürker chamber to determine the title and washed using sterile phosphate-buffered saline (PBS) solution and then were resuspended in SS and orally administered with sterile tips at a concentration of 10^8 cells per wasp (Stefanini et al., 2016).

For bacterial infection, non-pathogenic *E. coli*, an immune elicitor commonly used to test immune competence in insects (Cappa et al., 2015; Cini et al., 2018), was used. *E. coli* XL-1 Blue, tetracycline resistant {chromosomal genotype: mutant alleles: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]}, was grown on Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl), added with tetracycline 10 μg/ml in order to exclude any other microbial contaminants. This microorganism was selected because it is not naturally found in *P. dominula*; thus, we can exclude its presence in our wasps prior to infection.

Insect Model *P. dominula*: Housing, Feeding, and Infection

In order to observe the effect of administration of *S. cerevisiae* strain on bacterial clearance in an insect model, the first group of autumn foundresses ($N = 40$ pre-hibernation foundresses) was preliminary tested. The wasps were divided into two subgroups: 20 foundresses fed with SS to which yeast strain YP4 was added, while the remaining 20 wasps were fed with SS without any yeast added, as a control group (**Supplementary Figure S1**).

A second experiment (**Figure 1**) was conducted with wasps of different castes and in different seasons. A group of autumn foundresses (pre-hibernation foundresses) was divided into three subgroups ($N = 62$): 19 foundresses were fed with SS added with YP4 strain, 20 with SS and YH1 strain, and the remaining 23 with SS without any yeast added (control group). A group of spring foundresses ($N = 84$) was collected after the winter diapause and similarly divided into three subgroups: 31 were fed with SS added with YP4 strain, 33 with SS and YH1 strain, and 20 with SS only.

Workers ($n = 53$) were collected in summer from 10 colonies (at least five workers *per* colony) and treated as follows: 18 workers were fed with SS and YP4 strain, 19 workers with SS and YH1 strain, and 16 workers with SS without yeast (control group).

After feeding, foundresses were maintained at 8°C in the dark and fed *ad libitum* with sterile SS for 10 days (Stefanini et al., 2016). Workers were maintained in similar conditions to foundresses except for temperature, which was kept around 20°C, according to the seasonal temperature in which the workers emerge in the colony. After 11 days of feeding, bacterial infection with *E. coli* was performed. Furthermore, in order to exclude the presence of naturally occurring tetracycline-resistant microorganisms, five wasps were randomly taken from the same collection groups. Their whole body, gut included, was dissected and plated on LB agar, added with tetracycline 10 μg/ml, and incubated for 72 h at 37°C.

Escherichia coli cells were grown aerobically in LB medium plus tetracycline 10 μg/ml at 37°C. Cells were counted on a Bürker chamber, washed using PBS solution, and resuspended in PBS. Then, *E. coli* (10^5 cells) was injected in each wasp with a Hamilton™ micro syringe through the intersegmental membrane between the second and third abdominal tergites (Cini et al., 2018).

After injection, wasps were maintained in the dark at room temperature (20°C) for 24 h. The dead wasps were removed. Each wasp was then dissected under sterile conditions and homogenized in 1 ml of PBS solution using a sterile pestle. Before homogenization, the gut was collected for subsequent metagenomic analysis while the sting and the venom sac were removed in order to avoid possible reduction of the bacterial count due to the presence of antimicrobial peptides in the wasp venom. The homogenate was serially diluted and plated on LB solid medium plus tetracycline 10 μg/ml and then incubated at 37°C overnight.

Bacterial Clearance Evaluation

The day after infection (12th day), bacterial clearance was evaluated by counting bacterial colony-forming units (CFUs) per milliliter per wasp. We performed the bacterial clearance test as a good proxy of insect immunity, since injection of live bacteria provides an integrative view of the activation of the organismal immune system (Charles and Killian, 2015). Bacterial clearance was compared among wasps belonging to different castes (foundresses and workers), treatments (control and YP4 and YH1 strains), and seasons for foundresses (autumn and spring), by using generalized linear mixed models (GLMMs) where the experiments (three for foundresses and one for workers) were included as a random effect. The effect of fixed factors and their interaction have been tested, obtaining a type III analysis of variance table, by using the Anova function of the *car* R package (Fox and Weisberg, 2019). Moreover, the effect of individual yeast strains compared to the control has been also

TABLE 1 | Summary of genetic, phenotypic, and immunomodulatory traits of the two tested *Saccharomyces cerevisiae* strains.

	***S. cerevisiae* strains**		**References**
	YP4	**YH1**	
Source	Human feces	Human feces	Ramazzotti et al., 2019
Genetic background (microsatellite loci and whole-genome sequencing)	Human and insect gut (HG2) cluster; HG2 ancestor, and pure lineage	Human and insect gut (HG3) cluster; West Africa ancestor lineage	Liti et al., 2009; Ramazzotti et al., 2019
Phenotypic traits	Low sporulation (<25%)	High sporulation (>25%)	Ramazzotti et al., 2019
	Low invasiveness	Invasive	Ramazzotti et al., 2019
	No pseudohyphal formation	With pseudohyphal formation	Ramazzotti et al., 2019
	Cell wall composition: chitin, 15.84 ± 3.77; glucan, 56.04 ± 5.17; mannan, 28.11 ± 0.43	Cell wall composition: chitin, 12.31 ± 1.68; glucan, 51.06 ± 1.79; mannan, 36.62 ± 8.0	Rizzetto et al., 2016; Ramazzotti et al., 2019
Trained immunity			
Human monocytes [test of training with *S. cerevisiae* strain toward lipopolysaccharide (LPS), Pam3Cys4, or *Candida albicans* stimulation; cytokine profiles]	Infection agent: LPS → high level of interleukin (IL)-6 and production tumor necrosis factor (TNF)-α P3C → high level of TNF-α and IL-6 *C. albicans* → high level of IL-6	Infection agent: LPS → IL-6 and TNF-α production P3C → high level of IL-6 and TNF-α production *C. albicans* → not performed	Rizzetto et al., 2016
C57BL/6 mouse model	Chitin training before fungal infection mediates resistance against *C. albicans* systemic infection. Treatment with chitin markedly enhanced survival of infected mice	Not performed	Rizzetto et al., 2016
Immunophenotyping [T-polarizing cytokine levels upon human peripheral blood mononuclear cell (PBMC) challenge]	high IL-17, low interferon (IFN)-g	low IL-17, high IFN-g	Ramazzotti et al., 2019

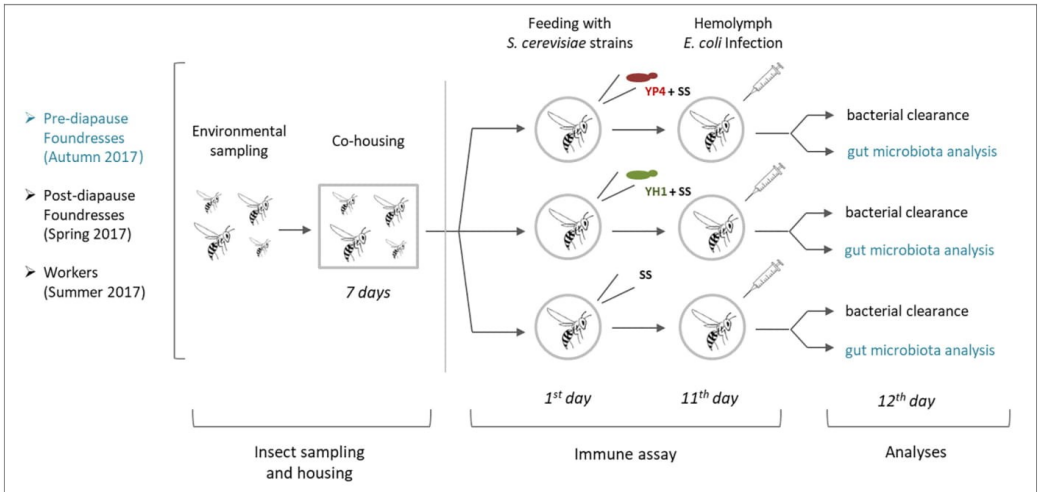

FIGURE 1 | Schematic representation of experimental workflow. After environmental sampling and cohousing for 7 days, *Saccharomyces cerevisiae* strains (YP4 and YH1) were administered in foundresses of *Polistes dominula* wasps collected in different seasons (autumn and spring) and life stages (pre- and post-diapause) and workers collected in summer. At the 11th day, bacterial clearance was compared among treated and control groups. In the pre-diapause foundresses collected in autumn (in blue), gut microbiota analysis was also performed. SS, sterile sugar solution (40% D-glucose). Details were reported in the section "Materials and Methods."

assessed by using the summary function of the *stat* R package. The generalized linear models (GLMs) have been carried out, grouped and separated for the two experiments as well as for the two castes (foundresses and workers).

Genomic DNA Extraction From Gut Bacterial and Fungal Communities and Sequencing

The wasp guts were collected and stored in RNAlater Stabilization Solution (Invitrogen, Thermo Fisher Scientific) in sterile microcentrifuge tubes at −20°C until DNA extraction. DNA extraction from gut samples was performed using DNeasy PowerLyzer PowerSoil Kit (QIAGEN) following the manufacturer's protocol. The total DNA was quantified by a Tecan quantification device (Life Sciences). The sequencing was carried out by an Illumina MiSeq platform (BMR Genomics sequencing service of the University of Padova, Italy[1]). DNA sequencing on bacterial communities was performed on the V3–V4 region of the 16S ribosomal RNA (rRNA) genes by using the primers Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014). The same guidelines for fungal communities were carried out on internal transcribed spacer (ITS) 2 rRNA genes by using the primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Illumina sequencing reads are available at the European Nucleotide Archive[2] under accession study PRJEB32390.

Gut Microbiota Analysis

Demultiplexed sample libraries were visually inspected with the FastQC program (Andrews, 2010); low-quality ends of forward and reverse reads were trimmed using Sickle (Joshi and Fass, 2011) with a quality cutoff of 20 and a length threshold after trimming of 200. MICCA pipeline v. 1.7.2 (Albanese et al., 2015) was used for operational taxonomic unit (OTU)/sequence variant (SV) picking as follows: forward and reverse reads were joined with *micca mergepairs* command, and reads with N bases were discarded with command *micca filter*. OTU/SV picking and chimera checking were performed with the *miccaotu* command and the UNOISE3 protocol as a picking algorithm. Taxonomy was assigned to the representative sequences of the identified OTUs/SVs classified using the RDP classifier v. 2.11 (Wang et al., 2007).

Subsequent analyses were performed in R (v.3.42; R Core Team, 2018), employing package *phyloseq* v.1.22.3 (McMurdie and Holmes, 2013) to import data [as biological observation matrix (BIOM) files], to perform PCoA ordination analysis (using the Bray–Curtis distance measure), and to plot microbiota composition as a bar plot. Alpha diversity indices were calculated with the *microbiome* package (Lahti et al., 2017), and overall differences were tested with ANOVA, while species accumulation curves were calculated with the *ranacapa* package (Kandlikar et al., 2018). Prior to any analysis, count data were scaled with CSS transform as implemented in the *metagenomeSeq* (Paulson et al., 2013). Heatmaps reporting OTU distribution in different samples were created with the *pheatmap* package (Kolde, 2012). For heatmap construction, rows (i.e., OTUs/SVs) were ordered based on the results of the *plot_heatmap* command in the *phyloseq* package, using PCoA as the ordination method and Bray–Curtis as the distance measure, while columns (i.e., samples) were ordered based on Euclidean distance (as implemented in the *pheatmap* package). Color maps of OTU/SV abundance were scaled on the rows. Association between gut microbial community diversity and bacterial CFUs found in the hemocele post infection (a proxy for immune response priming and activation) were evaluated with distance-based permutational multivariate analysis of variance (PERMANOVA; Bray–Curtis distance; 9,999 permutations) using the *adonis* function in the *vegan* v.2.5-2 (Oksanen et al., 2018) package, as well as employing multivariate-structure-based GLMs (negative binomial), as implemented by the *manyglm* command in the package *mvabund* (Wang et al., 2012). STAMP (Parks et al., 2014) software was used to compare relative abundance of OTUs at different taxonomic levels and to create plots. We used Welch's *t* test (with no correction for multiple comparison) and displayed only OTUs with a $p < 0.05$.

[1]https://www.bmr-genomics.it/

[2]http://www.ebi.ac.uk/ena/data/view/PRJEB32390

RESULTS

S. cerevisiae Strains, Able to Induce Trained Immunity in Mammals, Enhance Bacterial Clearance in Foundresses of Social Wasp *P. dominula*

In order to test the ability of *S. cerevisiae*, a generally recognized as safe (GRAS) yeast, to enhance resistance to bacterial infection, we selected two *S. cerevisiae* strains based on their previously observed ability to induce training immunity in human cells and mice (Rizzetto et al., 2016).

In a first experiment, bacterial clearance was preliminarily tested on one group of autumn foundresses after administration of the YP4 yeast strain (**Supplementary Figure S1**). Subsequently, an immune trial was performed on two different wasp castes (future foundresses and workers) in three different seasons (summer workers and autumn and winter foundresses, see section "Materials and Methods"). The experimental design is depicted in **Figure 1**. By immune trials performed on foundress wasps, we observed that the bacterial clearance was lower in the control group than in the treated groups (**Figure 2**, **Supplementary Datasheet S2**, and **Supplementary Figure S4**). Firstly, data of the bacterial clearance from the two different experiments (including the preliminary test with the sole YP4 strain administration, as reported in **Supplementary Figure S1**) were analyzed separately, showing a statistically significant effect of yeast treatment on the reduction of *E. coli* load (**Table 2**). Then, we have extended the analysis to all groups by integrating all variables (treatments, caste state, and season) into a GLMM (**Table 3**; see section "Materials and Methods"). The GLMM showed that the treatment with *S. cerevisiae* decreased the

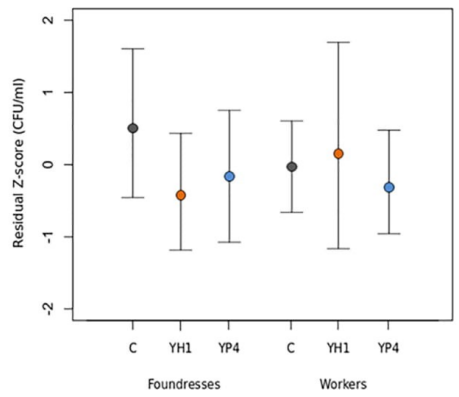

FIGURE 2 | Bacterial clearance in *P. dominula* in foundresses and workers after yeast administration. Mean and standard deviation of residual load of *Escherichia coli* colony-forming units (CFUs) per milliliter in control (C) and treated groups (YH1 strain and YP4 strain administration) in foundresses and workers, at 24 h after bacterial infection. Data are representative of the bacterial clearance measure, reported as *z*-score transformation (residual *z*-score of CFUs per milliliter).

TABLE 2 | ANOVA of deviance for 217 total observations (173 foundresses and 44 workers).

	Variables	df	χ^2	*P*
Preliminary immune trial in foundresses (YP4 strain)	Treatment	1	5,336	0.027
Complete immune trial in foundresses and workers (YP4 vs. YH1 strains)	Treatment	2	10,096	0.006
	Caste	1	2,585	0.108
	Treatment * Caste	2	5,547	0.060
Both preliminary and complete immune trials in foundresses and workers (YP4 vs. YH1 strains)	Treatment	2	16,485	<0.001
	Caste	1	2,572	0.109
	Treatment * Caste	2	5,563	0.062
Foundresses	Treatment	2	14,233	<0.001
	Season	1	228,375	<0.001
	Treatment * Season	2	2,714	0.257
Workers	Treatment	2	1,099	0.577

Data are separated for preliminary test (only autumn foundresses and YP4 yeast strain), the complete test (treatment and caste after YP4 and YH1 yeast strain administration), and both preliminary and complete immune trials. Separated analyses among foundresses and workers are also reported. df, degree freedom; P, p-value.

infection burden (measured as *E. coli* CFUs per milliliter as reported in **Table 3** and shown in **Figure 2**). The treatment effect was significant in the reduction of *E. coli* load regardless the two different trials, also when the foundresses from the preliminary experiment were added in an overall analysis (**Table 3**).

The experiments and analyses have been repeated for foundresses and workers separately (**Table 3**), revealing a caste-dependent effect, statistically significant in foundresses but not in workers (**Figure 2**). Furthermore, we showed a significant interaction effect for the sole strain YH1 ($p = 0.019$, **Table 3**) that increased bacterial clearance in foundresses but decreased resistance to the infection in workers (higher CFU-per-milliliter counts; **Figure 2**).

The experiments on the foundresses were performed in autumn (entry into diapause) and spring (exit to the diapause). We found that the levels of CFUs per milliliter were significantly higher in the spring than in the autumn ($p < 0.001$; **Table 3**). However, the treatment affects the CFU-per-milliliter levels regardless of the season, as shown by the lack of significance of the interaction effect in **Table 3** ($p = 0.785$ for YH1 * season and $p = 0.251$ for YP4 * season).

Bacterial Clearance Induced by *S. cerevisiae* Treatment Is Associated to Shaping of Gut Microbiota Composition

In order to evaluate the effect of *S. cerevisiae* administration on the gut microbial communities, either via direct interaction or as a consequence of the immune training, we performed characterization of gut microbiota on the autumn foundress group, in which the effect of yeast administration on bacterial clearance was more evident. Autumn was also preferred because it is the season with a higher isolation rate of *S. cerevisiae* strains from wasp guts (Stefanini et al., 2012).

We selected nine gut samples of foundresses per group (treatments and control), pooling them together in a total of three gut samples per group, as representative gut microbiota collection of all tested samples of the respective groups (**Supplementary Figure S2**). The criteria for sample selection were in accordance with the coefficient-of-variation (CV) analysis calculated by comparison of intragroup and intergroup (intragroup CV%: control group = 15.4; YP4-treated group = 38.2; YH1-treated group = 36.2; intergroup CV%: control vs. YP4 = 46.9; control vs. YH1 = 48; as reported in **Supplementary Table S1**).

By 16S rRNA V3–V4 region sequencing, a total of 214,089 sequence reads were obtained after quality filtering, clustering in a total of 495 OTUs. The number of 16S rRNA sequences per sample ranged from 1,607 to 51,340. Although the control group showed a low amount of sequence reads, no statistical differences in read number were observed among groups (**Supplementary Figure S5**).

By comparison of the alpha diversity indices among the three groups (control and YH1- and YP4-treated groups), we observed a reduction in richness (**Figure 3A**) and diversity (i.e., Shannon and inverse Simpson indices; **Figures 3B,C**) of gut bacterial communities in the control group, with respect to the two treated groups (**Figure 3**; ANOVA test; richness: $F = 16.91$, $p = 0.0034$; Shannon's index: $F = 17.6$, $p = 0.0031$; inverse Simpson index: $F = 18.32$, $p = 0.0027$; a pairwise t test is reported for every comparison in **Figure 3**). Moreover, as indicated by a higher

TABLE 3 | Generalized linear mixed model (GLMM) analysis.

	Variables	Estimate	Standard error	*Z* Values	*P*
Complete immune trial in foundresses and workers (YP4 vs. YH1 strains)	YH1-C	−0.511	0.179	−2.86	0.004
	YP4-C	−0.374	0.183	−2.04	0.041
	Caste	−2,894	1.800	−1.61	0.108
	YH1 * Caste	0.815	0.345	2.34	0.019
	YP4 * Caste	0.079	0.350	0.23	0.820
Foundresses	YH1-C	−0.425	0.213	−2.00	0.046
	YP4-C	−0.490	0.161	−3.04	0.002
	Season	3,424	0.227	15.11	< 0.001
	YH1 * Season	−0.083	0.303	−0.27	0.785
	YP4 * Season	0.312	0.271	1.15	0.251
Workers	YH1-C	0.304	0.404	0.75	0.45
	YP4-C	−0.295	0.404	−0.73	0.46

Comparison of single variables (combination of treatment, caste, and season) by using GLMM, on bacterial clearance observed in all tested foundresses and workers.

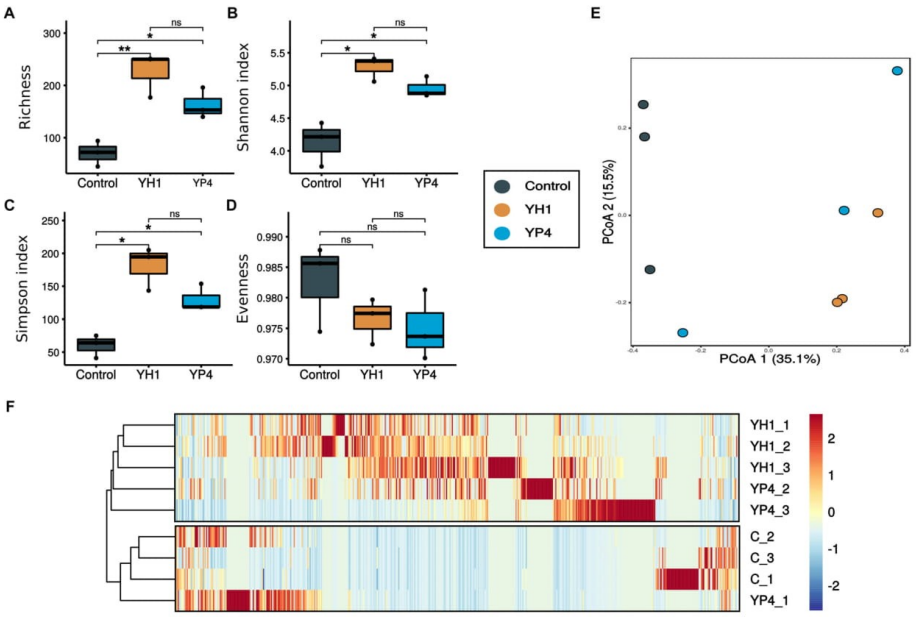

FIGURE 3 | Alpha and beta diversity of gut microbial communities. **(A–D)** Alpha diversity measures: **(A)** richness, **(B)** Shannon diversity index, **(C)** Simpson index, and **(D)** evenness of control and treated (YH1 and YP4) groups. Statistically significant comparisons by *t* test (*$p < 0.05$, **$p < 0.01$, and ns = not statistically significant). **(E)** Principal coordinate analysis (PCoA) ordination based on the Bray–Curtis distance. **(F)** Heatmap reported the frequency of operational taxonomic unit (OTU) abundance within treatment and control groups.

evenness index, the microbial community of the control group had more homogeneous abundance distribution than the treated groups (**Figure 3D**).

PCoA ordination analysis based on Bray–Curtis distances showed a clear separation of gut samples, along the first ordination axis (explaining 35.1% of the variability), according to the type of treatment (**Figure 3E**). In particular, samples from the YH1-treated group showed a clear separation and a more defined grouping than did the control and YP4 groups (**Figure 3E**). PERMANOVA was used to test the different distributions of samples, as observed in ordination analysis (**Supplementary Table S2**). Statistical analysis showed that *S. cerevisiae* treatment

has an effect, as a whole, on microbiota composition, when compared to the control group (control vs. treatment: $R^2 = 0.3$, $p = 0.01$), and the treatments with the two *S. cerevisiae* strains differentially affect the microbiota composition (control vs. YH1 treatment vs. YP4 treatment: $R^2 = 0.4$, $p = 0.03$). In **Figure 3F**, the heatmap displays the OTU frequencies and abundance distribution among control and treated samples. We observed different trends of OTU pattern distribution between control and treated groups (separated branches in hierarchical clustering as reported in **Figure 3F**), as well as between the two different yeast-treated groups.

Generalized linear models analysis (*mvabund* R package) was performed in order to assess the multivariate association between residual CFUs per milliliter (following the bacterial clearance) and gut bacterial community composition, showing significant association (likelihood ratio statistic: 857.4, $p = 0.002$). We found 75 OTUs (**Supplementary Datasheet S1**) with a significant univariate association to (log)CFUs per milliliter of bacterial infectious agent, regardless of treatments or control. When correction for multiple comparison was applied, *Campylobacter* was still significantly associated to the count of infectious agent (adjusted $p = 0.013$; **Supplementary Datasheet S1**).

To evaluate the overall effect of *S. cerevisiae* administration and the subsequent resistance to *E. coli* infection on the wasp gut microbiota composition, we compared the microbial community's relative abundance (at phylum and genus levels) among treated and control groups, as shown in **Figure 4** and **Supplementary Figure S3**. At the phylum level, an increase of Bacteroidetes and Fusobacteria was observed in treated groups (**Figure 4A**). At the genus level, 17 genera showed significant different abundances between control and treatment groups (**Figure 4B**; Welch's *t* test). Among them, *Staphylococcus* (Firmicutes phylum) and *Morganella* (Proteobacteria phylum) were significantly enriched in the control group. Interestingly, *Escherichia/Shigella* genus was significantly enriched in the gut community of the controls compared to the treated groups (**Figure 4C**; ANOVA $p = 0.025$).

In treated groups, *Prevotella* (Bacteroidetes), *Veillonella* (Firmicutes), *Capnocytophaga* (Bacteroidetes), *Fusobacterium* (Fusobacteria), *Haemophilus* (Proteobacteria), and *Leptotrichia* (Fusobacteria) were found to be significantly abundant (**Figure 4B**; Welch's *t* test).

The two yeast strains highly differed in their ability to change the microbial profiles (**Figure 5**). While administration of both

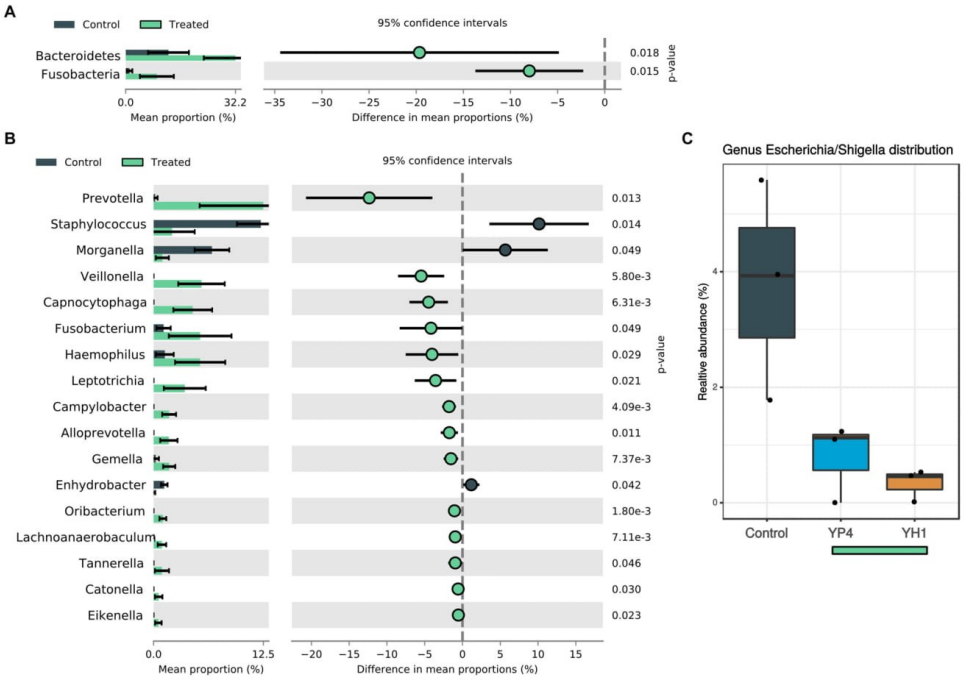

FIGURE 4 | Comparison of statistically significant taxa in treated and control groups. Mean proportion and confidence intervals (%) in control vs. treated (YH1 and YP4) groups explored at phylum **(A)** and genus **(B)** levels (*p*-values by Welch's *t* test). **(C)** Relative abundance (%) of *Escherichia/Shigella* genus distribution between control and treated groups (ANOVA $p = 0.025$).

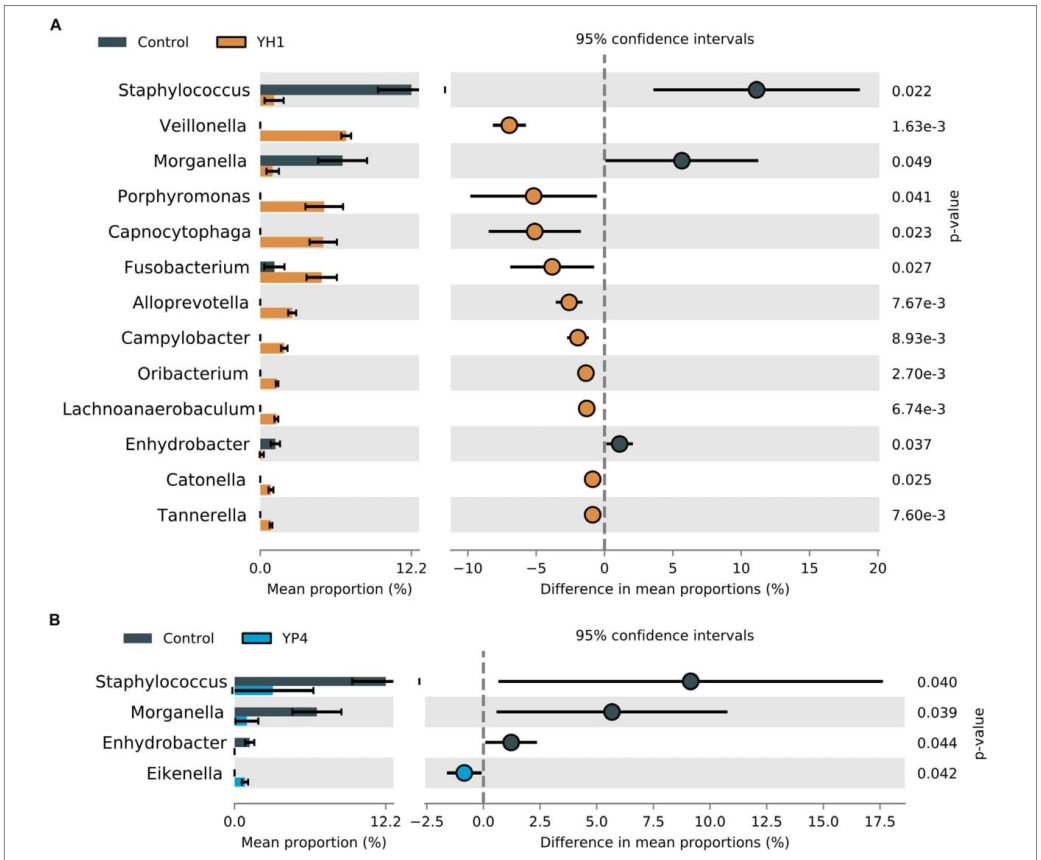

FIGURE 5 | Comparison of statistically significant taxa in YH1- and YP4-treated and control groups. Mean proportion and confidence intervals (%) in **(A)** control vs. treated YH1 and **(B)** control vs. YP4, explored at genus level (*p*-values by Welch's *t* test).

S. cerevisiae strains reduced the levels of *Staphylococcus* and *Morganella*, when compared to the control group (**Figures 5A,B**), the single comparison between strain administration and control (YH1 vs. control in **Figure 5A** and YP4 vs. control in **Figure 5B**) showed a strain-dependent effect on gut microbiota composition. To note, YH1 treatment (**Figure 5A**) was associated to an enrichment of a higher number of bacterial genera, such as *Fusobacterium*, *Veillonella*, *Alloprevotella*, *Capnocytophaga*, *Porphyromonas*, and *Campylobacter*, compared to YP4 treatment (**Figure 5B**; Welch's *t* test).

S. cerevisiae Treatment Does Not Affect the Gut Fungal Communities

For the fungal ITS2 region sequencing, we obtained a total of 14,646 sequences after quality filtering, clustering in a total of 63 OTUs. The number of sequence reads per sample ranged from 202 to 3,024. Contrary to the gut bacterial community, the mycobiota composition did not show significant differences in richness and biodiversity between control and treated groups, as observed by alpha diversity and PCoA ordination analysis based on the Bray–Curtis distance (**Figure 6**). Accordingly, PERMANOVA analysis used to test the differences in fungal community composition among groups showed no effect of treatment on gut mycobiota (**Supplementary Table S3**). In **Figure 7A**, we reported an overview of relative abundances of fungal taxa (>1%) annotated at the species level in the three groups. No significant association between fungal community composition and bacterial infection load (CFUs per milliliter of *E. coli*) have been observed. The fungal community abundances (**Figure 7A**) appeared unaffected by the *S. cerevisiae* administration and by bacterial infection in hemocele. Only

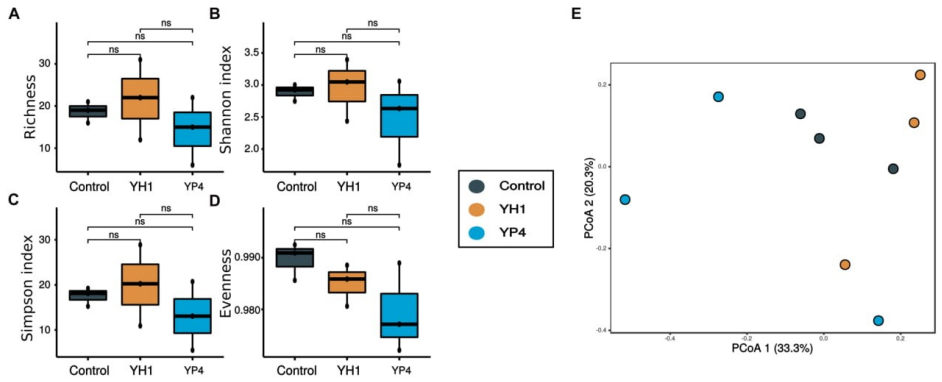

FIGURE 6 | Alpha and beta diversity of gut mycobiota. **(A–D)** Comparison of alpha diversity measures: **(A)** richness, **(B)** Shannon diversity index, **(C)** Simpson index, and **(D)** evenness among treated (YH1 and YP4) and control groups. Statistically significant comparisons by *t* test (*$p < 0.05$, **$p < 0.01$, and ns = not statistically significant). **(E)** Principal coordinate analysis (PCoA) ordination based on the Bray–Curtis distance among fungal communities between control and treated groups.

Metschnikowia sp. was found significantly enriched in the control group compared to both treated groups (**Figures 7B,C**; ANOVA $p < 0.001$).

DISCUSSION

In recent years, the physiological and ecological relationships between hymenopterans, in particular social wasps, and the yeast *S. cerevisiae* have become evident (Stefanini, 2018). The wasps likely act as natural vectors, hosting and transporting *S. cerevisiae* between different environmental niches and favoring mating among strains (Stefanini et al., 2012, 2016; Ramazzotti et al., 2019). While the advantage provided to the yeast is well understood, the benefit for the wasp to carry *S. cerevisiae* remains unclear. In this study, we tested the hypothesis that *S. cerevisiae* could help the insect to resist infections during hibernation. This hypothesis is supported by previous studies (Quintin et al., 2012; Rizzetto et al., 2016; Rusek et al., 2018; Mulder et al., 2019) showing that fungal cells or components of cell wall (i.e., chitin) are potent stimuli of innate immune memory in mammals. To this aim, we investigated whether two well-characterized *S. cerevisiae* strains, by genetic, phenotypic, and immunophenotypic assays (Ramazzotti et al., 2019), previously proved to induce trained immunity in mammals Rizzetto et al. (2016), are also able to enhance resistance of the invertebrate immune system against bacterial infection and shape the gut microbial communities. A few studies have focused on the nature of the insect immune system, revealing that it can be efficiently primed upon exposure to microorganisms, acquiring a protective effect upon subsequent challenge with the same, and/or different microbes (Cooper and Eleftherianos, 2017).

We used the social wasp *P. dominula* as an animal model for innate immune trials. They evolved mechanisms to tolerate and benefit from the presence of this GRAS microorganism, providing protection against pathogens and maintaining health status, through the development and boosting of the immune system and providing protection against pathogens. Our results showed that *S. cerevisiae* induces in foundresses, but not in workers, a higher response against bacterial infection compared to the controls, possibly *via* the activation of strain-dependent innate immunization. In fact, following YH1 administration when compared to YP4 strain, we observed significant differences in immune enhancing, as well as in the changes of the gut microbiota.

The data presented here, alongside the immune training induced by the same yeast strains in mouse models and human monocytes (Rizzetto et al., 2016), show that the yeast strains can provide protective mechanisms shared between different subphyla, potentially through analog immune training mechanisms occurring in mammals, and insects. The yeast-mediated protection against pathogens could be important to protect hibernating foundresses during overwintering, simultaneously providing yeasts with the environmental niche where they mate and survive, thus suggesting the existence of a complex symbiotic interaction. Complex communication systems have been evolved between the host immune system and the intestinal microbiota (Belkaid and Hand, 2014). Evidence is mounting in support of a dominant and decisive role of gut in shaping and modulating immune responses in the prevention of disease (Hand, 2016). However, the effect of trained immunity on commensal microbial communities, that is, the microbiota, has not been shown yet. The gut microbiota plays a fundamental role in the education and functional tuning of the host immune system, thereby acting as adjuvant to the host immune system and continually driving the nature of immune responses, providing protection against pathogens. In turn, the host immune system has evolved multiple means by which to maintain its symbiotic relationship with the microbiota (Dethlefsen et al., 2007; Cho and Blaser, 2012; Lynch and Pedersen, 2016; Gupta et al., 2018).

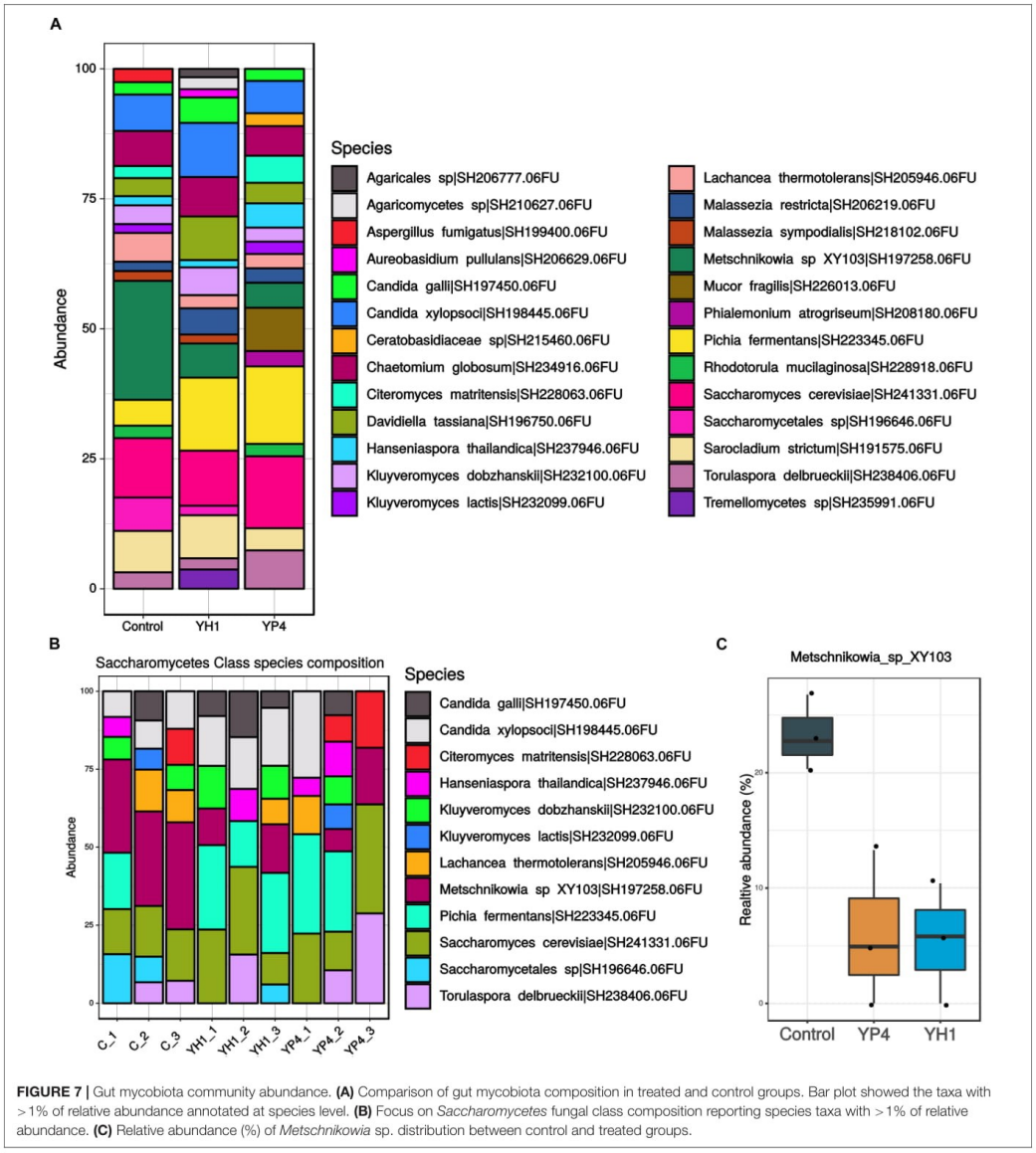

FIGURE 7 | Gut mycobiota community abundance. **(A)** Comparison of gut mycobiota composition in treated and control groups. Bar plot showed the taxa with >1% of relative abundance annotated at species level. **(B)** Focus on *Saccharomycetes* fungal class composition reporting species taxa with >1% of relative abundance. **(C)** Relative abundance (%) of *Metschnikowia* sp. distribution between control and treated groups.

Hymenoptera's gut contains a relatively simplified microbiota, with lesser microbial species as compared to that of mammals, yet social insects harbor microbial communities with highly specialized and beneficial functions in nutrition, protection from parasites and pathogens, and modulation of immune responses (Engel and Moran, 2013).

In both yeast-fed groups, we also observed reduction in potential pathogenic bacterial genera, such as *Staphylococcus*, *Morganella*, and *Escherichia/Shigella*, compared to the control group. The observed reduction of *Staphylococcus* load, following yeast feeding, may be also associated with the ability of *S. cerevisiae* to counteract and inhibit

Staphylococcus biofilm making, as previously observed (Walencka et al., 2007).

The two yeast strains showed different abilities to change the bacterial microbiota composition. The YH1 strain displays the strongest caste-dependent immunomodulatory effect, to the detriment of the resistance to *E. coli* infection in the workers, and shows increased ability in changing the gut microbiota, suggesting that the changes in the bacterial communities are related to strain-specific immunomodulation. While this effect is evident for bacterial communities, *S. cerevisiae* administration does not alter the diversity of the fungal communities. In the latter, only the *Metschnikowia* genus was reduced. Yeast–yeast competition for nutrients are well-known, especially regarding *S. cerevisiae* and *Metschnikowia* growing in fermentative processes (Alexandre et al., 2004; Fleet, 2008; Albergaria and Arneborg, 2016; Ciani et al., 2016; Sadoudi et al., 2017). Our results suggest that the competition between these two yeasts is not limited to grape must but may occur also in the insect gut.

It is unlikely that the observed effects are influenced by a role of *S. cerevisiae* as nutrient supplementation: (i) it is known that in *P. dominula*, the green fluorescent protein (GFP)-labeled *S. cerevisiae*, ingested by wasps, populate the gut for at least 4 months (Stefanini et al., 2012, 2016) (thus, the microorganism is still alive after treatment and survives after passage through the wasps' digestive tract); (ii) immune training occurred only in foundresses and not in workers, showing a caste-dependent effect in insects that received the same amount of *S. cerevisiae* cells (if the effect would have been related to nutrition, one would expect this to be equal regardless of the caste); (iii) the two strains showed significant differences in the ability to improve the bacterial clearance (a strain-dependent immune-mediated event excludes a general nutritional effect of the yeast); and (iv) the differences in immune enhancing are mirrored by differences in the changes of the microbiota composition (this finding corroborates our conclusion that the effect is mediated by immune responses and not just by feeding).

Overall, this study provides a preliminary indication of the complex interactions existing between insects' immune responses and their symbiotic gut communities. Future studies using insects as models promise stimulating information that will potentially uncover the relationship between immune priming and physiological functions, as well as their effect on gut microbiota. Our results suggest that yeasts and social insects possess key synergistic elements and that a deeper understanding of these interactions, including the molecular determinants of the priming and trained immunity, may provide significant insights on the host immune system–microbe evolution.

DATA AVAILABILITY STATEMENT

Data are available as electronic **Supplementary Material**. Illumina sequencing data are available at the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB32390) under accession study PRJEB32390.

AUTHOR CONTRIBUTIONS

DC, ST, and NM conceived the study. NM, DR, FC, FT, and AG carried out the experiment. LD and NM performed the statistical analyses. FV performed the metagenomic and data analysis. NM, MP, LB, and DC drafted the manuscript. All authors critically revised the manuscript, approved the final version of the manuscript, and agreed to be held accountable for the content therein.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02320/full#supplementary-material

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