STRESS, IMMUNITY AND HONEYBEE HEALTH: NEW STRATEGIES TO LIMIT COLONY LOSSES

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Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza»
Dante Alighieri
(Divina Commedia - Inferno, c. XXVI, vv. 118-120)

A chi è stato sempre al mio fianco.
A chi se né andato.
A chi ha creduto in me, forse più di me stessa.
A tutti coloro che mi hanno permesso di realizzare questo lavoro.
A me, che alla fine ce l'ho fatta.

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RIASSUNTO

Le api mellifere sono di fondamentale importanza per il mantenimento della biodiversità e dell'economia umana in tutto il mondo, non solo per il loro ruolo centrale nel settore dell'apicoltura, ma, soprattutto, per il servizio ecologico che forniscono come impollinatori dei prodotti agricoli e della maggior parte delle piante selvatiche.
Nell'ultimo decennio, si è registrato un aumento significativo delle perdite di colonie in tutto il mondo, in particolare nell'emisfero settentrionale. La drammatica entità di questo fenomeno ha attirato l'attenzione dell'opinione pubblica e il crescente interesse della comunità scientifica per le conseguenze dannose sul settore apistico, sulla produzione alimentare e sulla stabilità degli ecosistemi.
L'intensa attività di ricerca condotta non ha identificato uno specifico agente causale per le diffuse perdite di colonie, piuttosto è stata proposta un'origine multifattoriale per questa sindrome. Il coinvolgimento di molti fattori, biotici (patogeni e parassiti) e abiotici (insetticidi, pratiche di apicoltura, cambiamenti climatici, diminuzione dell'abbondanza e della diversità delle risorse floreali), come possibili cause della perdita delle colonie di api, rende complicata la diagnosi e il controllo di questo fenomeno. Numerosi studi di monitoraggio hanno messo in evidenza un elemento comune alle perdite delle colonie, ovvero l'elevata carica di parassiti e patogeni. In particolare, la perdita delle colonie è stata associata alla compresenza del *virus delle ali deformi* (*Deformed Wing Virus*, DVW) e del suo vettore, l'acaro ectoparassita *Varroa destructor*. Il DWV è un virus appartenente alla famiglia degli *Iflaviridae* la cui epidemiologia e patogenicità sono cambiate drasticamente con la diffusione globale di *V. destructor.* Questo acaro, infatti, favorisce non solo la diffusione del virus, ma anche la sua replicazione incontrollata e il passaggio dalle comuni infezioni asintomatiche a devastanti infezioni conclamate. Recenti studi hanno descritto come la simbiosi stabilita tra l'acaro e il virus abbia un impatto negativo sull'immunità e sulla salute delle api. La capacità delle api di difendersi da parassiti e agenti patogeni si basa su adattamenti comportamentali, noti come immunità sociale, e su barriere di difesa sia umorali che cellulari. A livello cellulare, la via Toll contribuisce alla difesa antivirale contro l'infezione da DWV, mantenendo sotto controllo la replicazione virale.
Tuttavia, qualsiasi fattore di stress che compete per le risorse metaboliche o che indebolisce le barriere antivirali agendo negativamente sul fattore di trascrizione immuno-correlato NF-kB, favorisce la replicazione virale incontrollata. Inoltre, lo stesso DWV

riduce l'immunocompetenza dell'ospite, prendendo di mira la segnalazione di NF-kB attraverso un meccanismo ancora sconosciuto, che ha conseguenze devastanti sulla salute delle api. Infatti, elevati livelli virali sono associati a una ridotta espressione del gene *dorsal-1A*, che codifica il fattore di trascrizione NF-κB coinvolto nella risposta a diversi fattori di stress, inclusi i patogeni. Il gene *dorsal-1A* fa parte della via di segnalazione Toll e regola la secrezione di peptidi antimicrobici, come la defensina e l'apidaecina.
L'immunosoppressione indotta dal DWV abbassa le difese immunitarie contro gli agenti patogeni, ma può anche avere un impatto sulla capacità delle api di regolare la comunità batterica intestinale, che partecipa all'omeostasi fisiologica dell'ospite. Infatti, le poche specie che compongono la comunità intestinale delle api mellifere sono controllate dall'immunità dell'ospite e possono, a loro volta, migliorare la risposta immunitaria, esercitando un ruolo protettivo nei confronti degli agenti patogeni attraverso meccanismi non ancora completamente chiari. La disbiosi intestinale, che può esacerbare lo stato immunocompromesso, è stata osservata nelle api in seguito all'esposizione a xenobiotici e agenti patogeni intestinali, come il microsporidio *Nosema apis*, ma è stata scarsamente caratterizzata in risposta alle infezioni virali. Una comprensione più approfondita dei meccanismi molecolari alla base dell'infezione da DWV, come l'interazione proteina-proteina tra ospite-patogeno, oppure di come l'infezione da DWV altera la composizione della comunità intestinale può fornire informazioni sui processi biologici presi di mira dal virus e può offrire l'opportunità di sviluppare nuove strategie di gestione e approcci terapeutici volti a ricostituire la competenza immunitaria delle api e limitare la proliferazione virale.
Il presente lavoro di tesi si è concentrato su due obbiettivi principali atti a sviluppare strategie finalizzate a tenere sotto controllo l'effetto negativo del virus: il primo è stato la caratterizzazione dell' effetto dell'infezione da DWV sulla comunità microbica intestinale delle api, il secondo si è concentrato sull'individuazione di interattori molecolari virus-ospite coinvolti nei processi biologici di patogenesi virale.

Le attività di ricerca relative al primo obiettivo, descritte nel Capitolo 2, hanno fornito nuove informazioni riguardo l'impatto dell'infezione da DWV sul microbiota intestinale delle api. Per questo studio sono stati combinati esperimenti di campionamento in condizioni naturali con esperimenti di infezioni controllate in laboratorio. Nel primo esperimento sono state raccolte api in due diverse fasi di sviluppo: larve e nutrici. I campionamenti sono stati eseguiti durante la stagione

estiva in due periodi differenti, giugno e luglio, da due diversi alveari degli apiari sperimentali di Portici. Ciascuna ape è stata dissezionata separando l'intestino, per analizzare il microbiota mediante sequenziamento del gene dell'RNA ribosomiale 16S (rRNA), dal resto del corpo dell'ape, da cui è stato isolato l'RNA per quantificare il titolo virale tramite qRT-PCR. Il raggruppamento dei campioni sulla base della composizione del microbiota batterico ha permesso di identificare gruppi associati con elevati titoli di DWV, caratterizzati da deplezione di Bacilli e aumento di taxa opportunisti come *Bartonella* e *Tyzzerella*, rispettivamente in api adulte e larve. Per valutare se l'alterazione del microbiota osservata in condizioni naturali fosse dipendente dall'infezione da DWV, è stato allestito un esperimento in ambiente controllato per stabilire una relazione causale tra l'infezione da DWV e le alterazioni del microbiota. A questo scopo è stata eseguita un'infezione artificiale su individui appena emersi dalle celle di covata (2-18 ore) mediante l'iniezione di un lisato virale ottenuto da api infette (sintomatiche). Dopo l'iniezione, è stato somministrato a tutte le api una dose di estratto intestinale, per inocularle con il microbiota di api sane. Le api sperimentali sono state raccolte a cinque giorni dall'emergenza e processate come negli esperimenti di campo. Anche in questo caso si è osservato un impatto negativo dell'infezione da DWV sulla comunità intestinale dei *Bacilli* (*Fructobacillus, Lactobacillus*), mentre aumentava l'abbondanza relativa del genere *Bartonella*. Inoltre, in entrambi gli esperimenti, sia di api raccolte sul campo, in cui il carico DWV è stato trattato come una variabile continua, sia di api infette artificialmente, in cui la carica virale è stata analizzata in termini categorici di "basso" o "alto", è stata evidenziata la correlazione negativa tra il carico di DWV e l'espressione di *dorsal-1A*, fattore di trascrizione coinvolto nella risposta immunitaria. La ridotta espressione di *dorsal-1A* in risposta all'infezione da DWV suggerisce un potenziale meccanismo attraverso il quale il DWV compromette la risposta immunitaria dell'ospite, rendendo le api più suscettibili sia all'infezione da DWV stessa che alla disbiosi microbica.

Sulla base dei risultati ottenuti, è stato eseguito un biosaggio per testare la capacità di ceppi di *Lactobacillus*, uno dei generi ridotti dall'infezione di DWV, di ripristinare le difese antivirali. In questo esperimento api adulte provenienti da una colonia altamente infestata dall'acaro *V. destructor* (tasso di infezione > 20%) e, pertanto, naturalmente infette dal DWV, sono state alimentate con una soluzione di saccarosio al 50% contenente un mix di due ceppi *Lactobacillus*, L. *fermentum* (AA) e *L. casei* (EV). La valutazione del

titolo virale delle api sperimentali ha mostrato una ridotta infezione virale in api alimentate per 14 giorni con i probiotici, suggerendo un possibile recupero della competenza immunitaria mediante il ripristino del profilo batterico intestinale. I risultati ottenuti in questo studio potranno essere di supporto per lo sviluppo di nuovi probiotici e diete in grado di rinforzare le difese contro i patogeni e di migliorare la salute delle api.

Il secondo tema di ricerca, trattato nel Capitolo 3 di questo lavoro di tesi, ha fornito nuove informazioni circa i meccanismi molecolari alla base della patogenesi del DWV. Per identificare gli interattori che mediano il processo infettivo del DWV nell'ospite *A. mellifera*, è stato adottato un approccio proteomico. Estratti proteici ottenuti sia da api con ali deformi sia da api asintomatiche sono stati utilizzati per eseguire esperimenti di immunoprecipitazione accoppiati ad analisi di spettrometria di massa. Il suddetto approccio ha identificato 142 proteine delle api che interagiscono con DWV e coinvolte in fondamentali processi biologici, come la traduzione, il trasporto e il catabolismo delle proteine.
Tra le proteine identificate è stata selezionata l'arginina chinasi come candidato promettente per ulteriori saggi funzionali volti a caratterizzare il suo ruolo nell'infezione dell'ospite da parte di DWV. È stato osservato che, sia in condizioni di infezione naturale sia in seguito a infezione artificiale in laboratorio, il livello di trascrizione del gene che codifica per l'Arginina Chinasi (*ArgK*) e la carica virale sono positivamente correlati. Allo scopo di studiare l'impatto del silenziamento genico sulla carica virale, è stato allestito un esperimento di silenziamento in vitro su cultura di cellule primarie ottenute da pupe di ape. L'esperimento ha mostrato una significativa riduzione del titolo virale in seguito al knockdown di *ArgK* in cellule infettate artificialmente con il lisato virale. I risultati ottenuti suggeriscono che *ArgK* è un promettente candidato per lo sviluppo di nuove strategie terapeutiche basate sul knockdown genico volte a ridurre la replicazione del DWV. Nel complesso, questi risultati rappresentano un nuovo contributo all'area di ricerca in rapida crescita dei probiotici e delle terapie volte a preservare la salute degli impollinatori contro i patogeni.

SUMMARY

Honey bee health decline poses a significant global issue due to the profound impact of these pollinators on the environment and the human economy. Reduced bee survival is the ultimate outcome of a multifactorial syndrome triggered by various stressors that interact synergistically. A common factor among all collapsing colonies is the high load of parasites and associated pathogens, such as *Deformed Wing Virus* (DWV). DWV is an endemic immunosuppressive virus that causes asymptomatic covert infections, usually kept under control by the bees' immune system unless exposed to stressors that weaken antiviral defenses. DWV-induced immunosuppression not only affects individual defense against pathogens but may also disrupt the regulation of the gut bacterial community, which plays a crucial role in gut physiology and immunity. The limited understanding of the molecular mechanisms underlying DWV infection hampers the development of new management strategies and therapeutic approaches aimed at mitigating the negative impact of pathogens on honey bees.

In the first chapter of the present thesis, we explored the effects of DWV infection on the modulation of bee gut microbiota. We analyzed bacterial gut community via 16S rRNA amplicon sequencing under natural and artificial infection conditions. Both approaches indicated the occurrence of gut dysbiosis in infected bees, characterized by a depletion of Bacilli and an increase of *Alphaproteobacteria* and *Clostridia.* A preliminary experiment demonstrated that DWV infection can be limited by supplementing honey bees with *Lactobacillus* species, a taxon which is negatively impacted by DWV infection.

The second chapter focuses on the identification of protein interactors between the virus and host involved in DWV pathogenesis. Our proteomic approach, consisting in co-immunoprecipitation and mass spectrometry analysis of bee protein extracts, identified 142 interactor proteins. Among these, arginine kinase, was selected for functional studies, to assess its role in DWV infection. Arginine kinase expression correlated positively with DWV load and its knockdown in honey bee-infected cells reduced DWV load after artificial infection. These results make it a potential candidate for developing new therapeutic strategies based on gene knockdown aimed at reducing DWV replication. Collectively, these results represent a novel contribution to the fast-growing research area of probiotics and therapeutics aimed at preserving pollinators' health against pathogens.

CHAPTER 1

GENERAL INTODUCTION

1.1 Ecological and economic importance of honey bee

The Western honey bee (*Apis mellifera*) is a globally recognized pollinator species that plays a crucial role in maintaining biodiversity and supporting the global economy *(Allen-Wardell et al., 1998; Klein et al., 2007)*.

The honey bees are a source of profit in beekeeping industry, contributing significantly to the global economy. The production of hive products (honey, propolis, wax, venom, royal jelly) generates income for beekeepers worldwide, supporting livelihoods and contributing to the agricultural industry *(Potts et al., 2016)*. In Italy, the economic impact of beekeeping, constituting one million hives, was estimated at approximately 1.5 billion euros annually by the Italian Beekeepers Federation (FAI). The United States, with the highest total number of colonies, around 2.6 million, was estimated at a value greater than 14 billion dollars (*Ellis et al., 2015)*.

Managed honey bee colonies provide economic benefits to farmers by pollinating dependent crops, including fruits, vegetables, and nuts *(vanEngelsdorp & Meixner, 2010).* Honey bees are a common and effective visitor to crops worldwide contributing about 13% of floral visits to 5% of plant species in all plant networks *(Hung et al., 2018)*.

Approximately 80 crop species cultivated for food global rely heavily on insect pollination, with bees being primary contributors *(EFSA, 2017)*. The calculated total economic value of global agricultural production dependent on pollinating insects is 9.5%, or USD 200 billion globally and over EUR 20 billion for Europe. valued at €153 billion annually to agricultural crop output worldwide, and more than €20 billion to that sector for Europe *(Gallai et al., 2009; Hristov et al., 2020; Klein et al., 2007)*. Moreover, it has been estimated that crop

productivity can increase by over 90% if pollinated by insects; this makes the western honey bee by far the most important commercial pollinator *(Klein et al., 2007)*.
The ecological service of pollination extends beyond crop production, impacting entire ecosystems and the diversity of wild plant populations. Honey bees, along with other pollinating insects, contribute to the reproductive success of numerous plant species, playing a vital role in maintaining the balance of ecosystems *(Potts et al., 2010)*. Diversification of pollinator species is crucial for ensuring the resilience of ecosystems in the face of environmental challenges *(Klein et al., 2007).* The ecosystem service of bees contributes so significantly to crop pollination that it is also utilized as a management tool. Honeybees, bumblebees, and other bee species are bought or hired by farmers in many countries to supplement the local pollinator fauna *(Gallai et al., 2009)*. While their role in apiculture is significant, the ecological service as pollinators is their most important contribution because it favours increased agricultural production, quality, shelf life, and economic value *(Klatt et al., 2014)*. This results in increased revenue for farmers and sustained financial growth in the beekeeping sector (*Geslin et al., 2017)*.
In recent years, urban beekeeping has garnered increasing interest as a rewarding pastime, fostering biodiversity, local food production, and a reconnection with nature *(Moore & Kosut, 2013)*.
Additionally, Apis mellifera serves as an important bioindicator of environmental pollution *(Celli & Maccagnani, 2003)* and is utilized as a model organism for studying various biological aspects, including behavior, immunity, toxicity, microbiota, and genetics *(Eban-Rothschild & Bloch, 2012; Kohno & Kubo, 2019; Łoś et al., 2019; Zheng et al., 2018)*.

1.2 Biology of honey bee

Apis mellifera (*Hymenoptera*: *Apidae*) is a eusocial insect, forming matriarchal, monogenic, and multiannual societies comprising 50,000 to 80,000 individuals. Each colony is led by a single queen (fertile female), along with thousands of adult workers (non-reproductive females), worker brood (eggs, larvae, and pupae), and a few hundred drones (reproductive males) *(Bailey & Ball, 1991)*. Sex determination in honey bees is based on the haplodiploid system, which is common

among insects of the order Hymenoptera, where males are haploid and females are diploid *(Heimpel & de Boer, 2008)*.
Honey bee colonies reside in nests (in their natural habitat) or hives (in managed conditions), consisting of vertically and parallelly constructed wax combs. Each comb is composed of a double series of hexagonal cells, with approximately 750 cells per square decimetre, modelled from wax produced by worker bees themselves. These cells vary in size, with smaller cells used for brood rearing and food storage (pollen or honey), larger cells reserved for rearing drones and queen cells, which are acorn-shaped and built along the lower edge of the combs *(Contessi, 2016)*.
Within each colony, three polymorphic castes can be distinguished (see Figure 1), all of which are winged and possess specific roles within the hive.

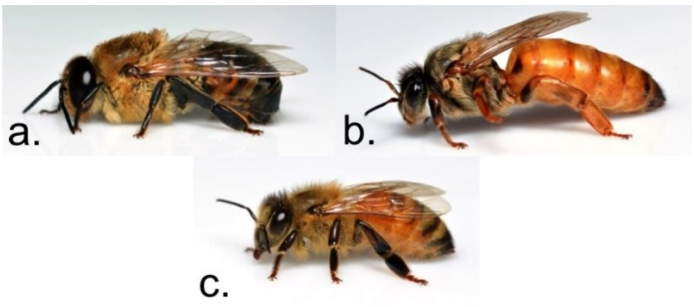


Figure 1 - Honey bee castes: a) drone (male), b) queen (reproductive female), and c) worker (non-reproductive female). Credit: Mike Bentley, UF/IFAS

Drones are larger than workers but smaller than queens, and their sole responsibility is to pass the colony's genes on to the next generation by mating with queens from other colonies during the nuptial flight. They do not participate in the collection of nectar and pollen and are expelled from the hive by the workers at the end of the season. The queen is the largest bee in the colony and her primary role is egg laying *(Contessi, 2016)*. She may leave the colony to mate during swarming (colony division) or when a colony relocates to a new nest site. After emerging as a new queen, she must mate with multiple drones within a few days to store sufficient spermatozoa in her spermatheca for her entire lifespan, typically 3-4 years. The queen can control egg fertilization during oviposition, thereby determining the sex of her offspring. The queens may measure the cell size with their front legs to decide whether to fertilize an egg or not. A fertilized egg can develop into either a fertile queen or a sterile worker, two castes genetically identical but differing only in the type and amount of food

received during the larval stage, while unfertilized eggs develop in drones *(Contessi, 2016)*.
Workers are the smallest of the three castes and perform various tasks within the hive *(Contessi, 2016)*. They do not specialize in a single task but undergo age-correlated behavioral division of labor, known as temporal (or age) polyethism *(Johnson, 2010)*.
During the summer, drones and workers typically live no longer than 4-5 weeks, while workers during hibernation can survive for up to 8 months. All three castes undergo four stages of development: egg, larva, pupa, and adult. With the exception of the adult stage, which occurs outside the cell, all stages take place within hexagonal wax cells built inside the nest *(Contessi, 2016)*.
The development of a worker bee takes approximately 21 days (15 days for queens, 24 for drones). The cycle begins when a fertile queen lays an egg in a cell prepared by the bees (Figure 2). After about three days, the larva hatches and is fed royal jelly secreted by the pharyngeal glands of nurse bees. The larvae grow rapidly over the next 6 days before being capped with wax by nurse bees. The larva then spins a cocoon around itself and develops into a pupa, a stage that lasts about 12 days. Upon emerging from the cell, the young adult immediately begins cleaning the cell for reuse *(Kilani, 1999)*. The first tasks of young workers are related to the brood nest, such as cleaning cells. Around four days after hatching, they become nurses and begin feeding older larvae. As their hypopharyngeal, wax, and venom glands develop, they transition to feeding younger larvae, processing food, building the nest, and protecting the colony. At around 20 days of age, they become foragers, leaving the hive to collect nectar, pollen, propolis, and water to feed the entire colony. This polyethism schedule is flexible, allowing bees to engage in other duties as needed according to the colony's requirements *(Johnson, 2010)*.

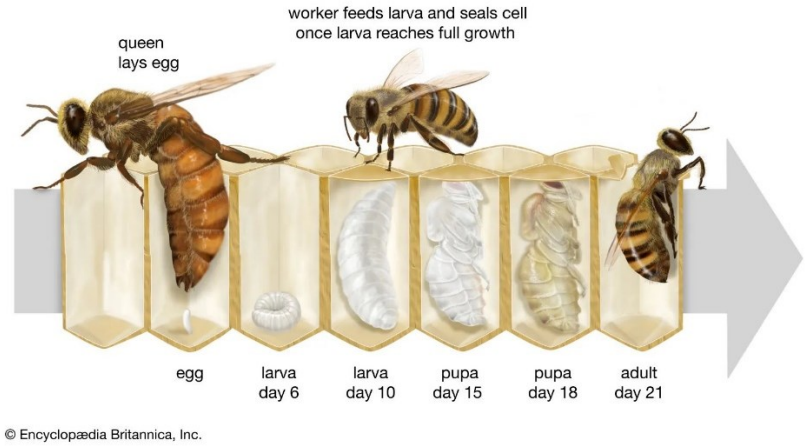


Figure 2 - Life cycle of the honeybee. Encyclopædia Britannica, Inc.

1.3 Honey bee colony losses

In the last decade, a significant increase in colony losses has been registered worldwide, in particular in the Northern Hemisphere *(K. V. Lee et al., 2015; Neumann & Carreck, 2010; Van Der Zee et al., 2012).* In the United States, an annual colony loss of 45% was recorded between 2012 and 2013 *(Steinhauer et al., 2014)* and 34% the following year *(K. V. Lee et al., 2015)*, while in some European countries, the winter loss of colonies reached 30% *(Zee et al., 2014)*. Periodic and extensive losses of this type are not entirely unusual phenomena; numerous testimonies have been brought over centuries and in many places in beekeeping *(Aizen & Harder, 2009; Ellis et al., 2010; vanEngelsdorp et al., 2008; vanEngelsdorp & Meixner, 2010).* However, the gravity of recent phenomena has attracted the attention of public opinion and the increasing interest of the scientific community for the harmful impact on the beekeeping sector, on food production and on ecosystem stability *(Stokstad, 2007; Underwood & vanEngelsdorp, 2007)*. The intense monitoring programmes *(Van Der Zee et al., 2012, 2015)* did not identify a specific causal agent for the widespread colony losses, but rather a multifactorial origin was proposed for this syndrome *(Ratnieks & Carreck, 2010).* Various factors, such as pathogens, parasites, insecticides, beekeeping practices, climate change, abundance, and diversity of floral resources *(Baker & Schroeder, 2008; Y. Chen et al., 2004; Spleen et al., 2013; Steinhauer et al., 2014)*, represent possible causes of honey bee colony losses, making the diagnosis and control of this phenomenon complicated, especially because bees are exposed

simultaneously or successively to several stress factors *(vanEngelsdorp et al., 2009)*. Although each of these stressors has a poorly understood relative importance, a common element in all collapsing colonies are the high loads of parasites and pathogens *(Kielmanowicz et al., 2015; Neumann & Carreck, 2010; Ratnieks & Carreck, 2010)*. In particular, the mutualistic symbiosis between the *Deformed Wing Virus* (DVW) and its vector, the ectoparasitic mite *Varroa destructor*, has been associated with a negative impact on honey bee immunity and health *(Di Prisco, Desiderato, et al., 2016; Kielmanowicz et al., 2015; Nazzi et al., 2012)*.

V. destructor is an obligate ectoparasite of *A. mellifera* feeding on the haemolymph of adult bees, larvae and pupae *(Aronstein et al., 2012; Bailey & Ball, 1991; Francis et al., 2013)*. By feeding on bees' liquid tissues, this mite has a strong negative impact on honey bee physiology, both for the direct effects due to uptake of nutritional resources *(Di Prisco et al., 2016; Genersch, 2010; Wilfert et al., 2016)* and, indirectly, for the role of vector and/or activator of viral pathogens, which aggravates the immune stress induced by mite infestation *(Nazzi & Le Conte, 2016)*. This mite, in fact, favours not only the spread of the virus but also its uncontrolled replication and the transition from common asymptomatic infections to devastating overt infections *(de Miranda & Genersch, 2010; Martin et al., 2012; Wilfert et al., 2016)*.
In order to have a better understanding of the mechanisms underlying this dramatic effect on honeybee colonies, in the next sections, the details of the *Deformed Wing Virus* (Section 1.4) and the immunity of honeybees (Section 1.5) will be discussed.

1.4 Deformed Wing Virus (DWV) and immunesuppresion syndrome

Deformed Wing Virus (DWV) is an RNA virus member of the order *Picornavirales*, family *Iflaviridae*.
DWVs are small, non-enveloped viruses with an isometric particle that encloses a single-stranded, positive-sense RNA (ssRNA [+]) genome. The viral capsid has an icosahedral structure with a diameter of approximately 30 nm and is made up of three structural proteins, VP1, VP2, and VP3. The organisation of the genome is typical of the genus Iflavirus and is constituted by a large open reading frame (ORF),

which encodes both structural and non-structural proteins. The ORF is flanked by a long 5' untranslated region (5' UTR) and a short and highly conserved 3' untranslated region (3' UTR), which ends with a tail formed by numerous adenine bases (tail poly-A+) *(Van Oers, 2010; de Miranda & Genersch, 2010).*
DWV was firstly isolated in Japan from adult Varroa-infested bees (A. mellifera) that showed a serological relationship the *Egyptian bee virus (EBV)* *(Bailey & Ball, 1991)* . Subsequently, it was differentiated and named Deformed Wing Virus due to typical symptom, as vestigial and crumpled wings (Figure 3), which were observed in bees from which it was isolated. Other common symptoms associated with its infection include the presence of bloated abdomens, paralysis, and a severely shortened adult lifespan for emerging worker and drone bees. *(Benaets et al., 2017; de Miranda & Genersch, 2010)*. DWV is widespread not only in *A. mellifera* but also in *A. cerana* and *A. florea*, and it has been identified in a range of invertebrates, including bumble bees, wasps, beetles, and wax moths *(Martin & Brettell, 2019).*

Figure 3 - Adult bee with deformed wings and one of the mites which parasitized the pupa is still clinging to the bee (de Miranda & Genersch, 2010).

The DWV was localised in the abdomen, thorax, and brain of adult bees, in particular at the level of the optic neuropils, antennal lobes, and fungiform bodies *(Chen et al., 2006b)*. It was also detected in the seminal vesicles of drones and in the ovaries of queens *(Fievet et al., 2006)*, indicating transmission during natural mating in honey bees *(Chen et al., 2006b).*
It can be transmitted between honeybees through various routes, both horizontally between adult bees via the faecal-oral pathway and vertically between generations via egg or sperm *(Chen et al., 2006a)*. Under field conditions, the parasitic mite Varroa destructor is the main vector of DWV between bees, with adult female mites obtained from

infected honeybee pupae containing high virus titers (Wilfert et al., 2016).
Although the virus can be transmitted in different routes, its virulence is low *(,De Miranda & Fries, 2008; Yue et al., 2007),* allowing it to remain within the colony in a latent state, as happens for other insect viruses *(Bonsall et al., 2005; Burden et al., 2003; de Miranda & Genersch, 2010; Dimmock et al., 2007)*. It was demonstrated that DWV replication is kept under control by the immune system, resulting in asymptomatic covert infections when bees are not exposed to stress agents that weaken antiviral barriers *(Annoscia et al., 2020; Nazzi et al., 2012; Nazzi & Pennacchio, 2018)*. However, transmissions associated with Varroa were associated with an increase in virulence and evident symptoms *(Mockel et al., 2011)*.
The complex network of relationships that exist between the bee and its pathogens has as centre the bee's immune system and, above all, NF-κB, a transcription factor involved in a number of immune responses in insects *(Nazzi & Pennacchio, 2018; Reichhart et al., 1993; Zambon et al., 2005),* including the secretion of antimicrobial peptides, such as *defensin* and *apidaecin (Di Prisco et al., 2016)*.

Indeed, DWV negatively modulates the transcription of the *dorsal-1A* gene, which encodes a member of the NF-κB family of transcription factors, causing an immunosuppression syndrome *(Nazzi et al., 2012)*.
Like *Varroa*, several other stress factors can interfere with the equilibrium underpinning DWV covert infections, promoting uncontrolled viral replication. In particular, any stress factor competing for metabolic resources or negatively acting on NF-κB signalling can trigger unbound replication *(Di Prisco, 2016; Di Prisco et al., 2013; Nazzi et al., 2012)*. Moreover, the increasing viral infection is a self-boosted process, as the growing DWV titer generates an escalating immunosuppression by targeting NF-κB signalling through an as yet unknown mechanism, which has devastating consequences for bee health.

1.5 Honey bee immune defence

Like other insects, honey bees possess a complex and efficient immune system consisting of both physical barriers and a sophisticated array of cellular and humoral defence mechanisms

(innate immunity) *(Strand, 2008)*, Additionally, social insects, including honey bees, exhibit colony-level responses (social immunity) that involve behavioural adaptations *(Cremer et al., 2007)*. The interplay of these factors determines the honey bees' ability to defend themselves against parasites and pathogens *(Nazzi & Pennacchio, 2014).* A comprehensive understanding of the immune characteristics of bees is essential for elucidating the immunosuppressive effects associated with DWV infection and other stressors. Particularly in conjunction with the virus, these stressors can disrupt immune defences and contribute to colony collapse *(Nazzi et al., 2012)*.

1.5.1 Social immunity

Social immunity in honey bees involves various responses, including behavioural, physiological, and organizational, aimed at reducing the transmission and severity of pests and pathogens within the hive *(Cremer et al., 2007)*.
Grooming and hygiene are two examples of behavioural defence. Grooming is a mechanism used to remove foreign particles *(Boecking & Spivak, 1999)* and parasites from bees' bodies, reducing Varroa infestations *(Evans & Spivak, 2010)*, while hygiene involves the removal of infected larvae from healthy brood cells, limiting disease spread *(Rothenbuhler, 1964)*. "Social fever" is observed in response to fungal infections, where bees collectively increase nest temperature to inhibit pathogen spread. Honey bees also use propolis and substances like honey and royal jelly, which possess antimicrobial properties, to combat pathogens *(Starks et al., 2000*). These social defence mechanisms expose honey bees to fewer pathogens compared to solitary insects, possibly explaining the reduction in gene families associated with individual immune responses (*Evans et al., 2006*).

1.5.2 Individual immunity

The insect immune response has developed multiple strategies to defend against attacks by bacteria, fungi, parasites, and viruses. The first line of defence is provided by the epithelia, which maintain constant contact with a vast array of microorganisms. Structures such as the exoskeleton's cuticle, the peritrophic membrane lining the digestive tract, and the tracheal epithelia serve as both physical barriers and local defence mechanisms by producing antimicrobial

peptides and reactive oxygen species *(W.-J. Lee & Hase, 2014; You et al., 2014)*.

When these morphological defences prove insufficient to halt pathogen attacks, the second line of defence, known as innate immunity, is activated. Innate immunity is characterized by cellular and humoral responses *(Evans & Spivak, 2010; Lemaitre & Hoffmann, 2007).* Haemocytes, circulating cellular components in the haemolymph, play roles in phagocytosis, nodulation, and encapsulation of pathogens. Humoral responses encompass molecules produced and released into the hemolymph to target invading parasites or pathogens. These include reactive oxygen or nitrogen intermediates, proteolytic cascades regulating coagulation and melanization, lysozymes, cytokines, and antimicrobial peptides (AMPs). AMPs, predominantly synthesized by fat body cells and haemocytes, serve as potent molecules against bacterial and fungal pathogens *(Wilson-Rich et al., 2008).*

Immune response is activated through the sensing of molecular patterns typical of pathogens (PAMPs - Pathogen Associated Molecular Patterns), which can be recognized by receptors present on host cells (PRRs - Pathogen Recognition Receptors). The pathogen-host interaction triggers specific pathways in immune cells that regulate cellular behaviour and the immune system *(Lemaitre & Hoffmann, 2007)*. Evans and colleagues *(2006)* identified four immunity pathways implicated in inducible host defence: Toll, IMD, Janus kinase (JAK) STAT, and JNK, based primarily on extensive searches for orthologs to well-studied fruit fly, mosquito, and moth species.

Insect innate immunity primarily tackles viral infections through the RNA interference (RNAi) pathway. RNAi is a highly conserved, sequence-specific post-transcriptional gene and virus silencing mechanism triggered by double-stranded RNA (dsRNA) *(Brutscher et al., 2015)*. It is also a valuable gene silencing tool frequently used to understand gene function in various organisms, including bees at different developmental stages *(Niu et al., 2014)*. The effectiveness of silencing can be gene-, dsRNA-, and tissue-specific *(Brutscher & Flenniken, 2015)*.

Studies in the field and laboratory, particularly in A. mellifera and *A. cerana*, suggest that the RNAi-mediated antiviral response plays a significant role in bee defence *(Niu et al., 2014)*. Furthermore, the application of RNAi technology against specific viruses in honey bees has demonstrated a reduction in viral titers, suggesting that these

insects possess the machinery necessary to mount an antiviral response based on RNAi *(Hunter et al., 2010)*.

1.6 Gut microbiota

The gut microbiota is a microbial community residing in the digestive tract that establishes symbiotic interactions with the host. Similar to other metazoans, the gut microbiota of insects is involved in important physiological aspects and the health status of its host *(Engel et al., 2012)*. The main roles identified include nutrient metabolism, detoxification, development, behavior, and protection from parasites and pathogens, modulating immune response *(Engel & Moran, 2013; W.-J. Lee & Hase, 2014; Siddiqui et al., 2022).*

The microbiota of *A. mellifera* is dominated by 8 to 10 bacterial species or phylotypes, categorized by clustering at 97% sequence identity of the 16S rRNA, belonging to three different phyla: Proteobacteria, Actinobacteria, and Firmicutes *(Moran et al., 2012).* Among them, five taxa compose the core members: *Gilliamella apicola* (*Gammaproteobacteria*), *Snodgrassella alvi* (*Betaproteobacteria*), two species of *Lactobacillus* spp. (*Firm-4* and *Firm-5*), *Bifidobacterium asteroides* (*Actinomycetes*), along with other non-core members with restricted niches in the bee gut *(Bonilla-Rosso & Engel, 2018; Kwong & Moran 2016; Moran, 2015).* These species clusters typically account for 95% of the entire gut community, with the remaining 5% likely consisting of pathogenic or environmental organisms.

The relative simplicity and stability of the composition depend on transmission routes, diet (exclusively composed of nectar and pollen), and host selection through specific immune responses and physiochemical and structural characteristics of the gut *(Bonilla-Rosso & Engel, 2018)*.

The development and maintenance of the specialized microbial community in the honey bee gut are facilitated by their social behaviours, which represent reliable transmission mechanisms *(Kwong et al., 2017a)*. Direct contact with food resources and hive environmental factors favor colonization and transfer of beneficial bacteria *(Engel et al., 2016)*. The abundance and structure of the microbiota change throughout the *A. mellifera* life cycle and are dependent on the gut anatomy *(Martinson et al., 2012)*.. Bacterial

species may be absent or sporadically present in larvae and newly emerged workers. However, through contact with the hive and trophallaxis between nestmates, the microbiota becomes established in the adult worker gut between three and five days after emergence, with characteristic phylotypes maintained throughout the worker's life, spanning diverse tasks and dietary regimens *(Martinson et al., 2012; Powell et al., 2014)*.

The adult gut is divided into four major organs (crop, midgut, ileum, and rectum), each providing different functions in the catabolism and absorption of food, as well as different environments for bacterial symbionts *(Snodgrass RE., 1984)*. The crop, used for the storage and transport of nectar for feeding larvae and the production of honey, and the midgut, responsible for digestion and absorption of food, host few but important bacteria meanwhile, the distal section of the gut is colonized by the majority of bacterial biomass, which is differentially distributed between the ileum and rectum *(Martinson et al., 2011)*. Within the ileum, *Snodgrassella alvi* and *Gilliamella* spp. thrive and form a stable biofilm, while *Lactobacillus* and *Bifidobacterium* species predominate in the rectum *(Motta & Moran, 2024)*.

Factors such as social contact, environmental exposure, diet, and host physiology affect microbial composition and formation of highly specific biochemical interactions occupying distinct niches *(Kwong, Medina, et al., 2017; Martinson et al., 2011; Miller et al., 2019)*.

Numerous metagenomic, metatranscriptomic, and whole-genome sequencing studies have been conducted on the bee gut microbial community to elucidate the potential metabolic roles of the gut microbiota *(Ellegaard et al., 2015; Engel et al., 2012)*.

Due to the highly specific diet of bees, the gut microbial community has adapted much of its genetic heritage to metabolize carbohydrates present in honey and nectar, as well as proteins contained in pollen and bee bread *(Bonilla-Rosso & Engel, 2018; Engel et al., 2012; F. J. Lee et al., 2015)*.

Different strains of dominant bacteria have shown the ability to utilize various food components or neutralize food toxins, exhibiting highly complementary metabolic capabilities, as observed in *G. apicola* and *S. alvi* *(Kwong et al., 2014)*.

Another potential role for the gut community is providing host protection against parasites and pathogens, acting as regulators of stress tolerance and disease resistance *(Ellegaard et al., 2015; Raymann & Moran, 2018; Steele et al., 2021)* The gut microbiota confers colonization resistance against potentially harmful microbes such as the parasite *Lotmaria passim* *(Schwarz et al., 2016)* and the

fungal pathogen *Nosema apis* *(Maes et al., 2016a)*. Research has demonstrated that the gut microbiota of honey bees can stimulate and regulate the expression of antimicrobial peptides (AMPs) in the hemolymph *(Kwong, et al., 2017a)*, highlighting the role of gut symbionts in the stimulation of the insect immune system. Specific strains of bee gut bacteria, such as *Lactobacillus*, have been found to stimulate the host's immune system, providing protection against pathogenic infections *(Lang et al., 2022; Vásquez et al., 2012)*.

The proper functioning of the gut microbiota relies on a stable and specific composition of the bacterial community *(Kwong et al., 2017a)*, Despite some minor differences related to analysis methods, numerous sequencing studies worldwide have shown remarkable consistency in microbial community structure and membership. This consistency is in part due to the maintaining of microbiota homeostasis by the host's immune system, which promotes the colonization of symbionts and the exclusion of non-symbionts through several pathways, including IMD, Toll, and dual oxidase *(Guo et al., 2023; Schmidt & Engel, 2021; You et al., 2014)*.

The disruption of gut homeostasis, characterized by alterations in the microbial community and loss of function (dysbiosis), has important consequences on honey bee health, although its full extent is unknown *(Maes et al., 2016a; Zheng et al., 2017)*.

Honey bee gut dysbiosis can be triggered by several factors, including climate change, poor nutrition, pesticides, antibiotics, parasites, herbicides, and infectious diseases *(Daisley et al., 2020a; Maes et al., 2016a; Motta et al., 2018; Raymann et al., 2017; Raymann & Moran, 2018; Vázquez et al., 2023).* However, the impact of viral pathogens, which in some cases induce host’s immunosuppression (e.g. DWV), on honey bee gut microbiota has been poorly explored.

AIMS OF THE RESEARCH

Multiple stressors can destabilise the immune system and promote the uncontrolled proliferation of DWV. The virus can directly affect the genes responsible for immune defence responses *(Di Prisco et al., 2016)*, promoting its own replication and potentially impacting immune competence *(Nazzi et al., 2014)*. Considering the close relationship between immune competence and the regulation of the intestinal microbiota, it was hypothesised that DWV's immune-suppressive effect could destabilise the intestinal immunity that regulates the homeostasis of the intestinal microbiota. This alteration can lead to dysbiosis and amplify the immunosuppressive phenomenon, thereby reducing the ability to defend against pathogens.
Furthermore, although numerous studies have demonstrated that DWV is capable of interfering with the host's defence mechanisms, little is known about the complex network of interactions established between the virus and the host's cellular components underlying DWV infection.
The aim of the present work was to investigate some aspects of the host-virus interaction whose functional and molecular details could be used to the development of new control strategies of the DWV infection. The work was divided in two main objectives:

1) The study of the potential impact of DWV infection on the gut microbial community and its physiological homeostasis, aimed at evaluating whether and how immunocompetence can be recovered through the restoration of a functional microbiota using tailor-made nutritional or probiotic plans (Chapter 2).

2) The study of the protein-protein interaction at the host-pathogen interface in order to identify potential molecular targets involved in the biological process of viral proliferation, whose manipulation may limit DWV replication (Chapter 3).

CHAPTER 2

IMPACT OF DWV INFECTION ON HONEY BEE GUT MICROBIOTA AND IMMUNITY

2.1 INTRODUCTION

Honey bee (*Apis mellifera*) health decline is a problem of global concern, as these pollinators have a remarkable impact on the environment and human economy. Several monitoring programs reported a high proportion of colony losses at a local scale *(Kulhanek et al., 2017)*, largely varying in different countries and years. Recent monitoring programs reported an annual colony loss proportion of 17% in Italy in 2018-2019 *(Gray et al., 2020)*, and 45% in the USA, in 2020-2021 *(Bruckner et al., 2023)*. The lower survival rate of bee colonies is the result of a multifactorial syndrome triggered by several stress factors that may interact synergistically and cause a reduction of bee immunocompetence *(Goulson et al., 2015; Nazzi & Pennacchio, 2014; Neumann & Carreck, 2010).* Indeed, a common element to all collapsing colonies is the high load of parasites and pathogens, often including the Deformed Wing Virus (DVW) *(Dainat et al., 2012; Genersch et al., 2010; Highfield et al., 2009)*. DWV (family Iflaviridae; genus *Iflavirus*) is an endemic immunosuppressive virus of honey bees which is vectored by the parasitic mite *Varroa destructor (Di Prisco et al., 2016; Wilfert et al., 2016).* When transmitted by the mite or experimentally by injection, the two widespread genotypes of DWV, genotypes A and B, are both highly virulent pathogens of *A. mellifera (McMahon et al., 2016).* DWV replication is kept in check by the immune system, resulting in asymptomatic covert infections, when bees are not exposed to stress agents which weaken antiviral barriers *(Annoscia et al., 2020; Nazzi et al., 2012; Nazzi & Pennacchio, 2018)*. Under stress conditions, DWV undergoes an uncontrolled viral

replication, a self-boosted process, as the growing DWV titer generates an escalating immunosuppression *(Nazzi & Pennacchio, 2014)*. Increasing viral levels are associated with a reduced expression of *dorsal-1A* gene, which encodes a NF-κB transcription factor, activating antiviral and antimicrobial barriers, and, more in general, stress responses *(Barroso-Arévalo et al., 2019; Nazzi et al., 2012; Di Prisco et al., 2016; Lourenço et al., 2018).*

Immunity is tightly connected to a functional gut microbiota. Indeed, the few species which compose honey bee gut community *(Engel et al., 2012; Kwong & Moran, 2016*) are controlled by host immunity *(Guo et al., 2023),* and can, in turn, enhance the immune response *(Kwong et al., 2017a),* exerting a protective role against pathogens *(Bonilla-Rosso & Engel, 2018).* However, the gut microbiota of honey bees is susceptible to several perturbing factors, such as antibiotics *(Raymann et al., 2017)*, agrochemicals *(Motta et al., 2018)* and nutritional imbalances *(Maes et al., 2016)*, suggesting that gut dysbiosis can be an important pacemaker of pollinators' health *(Daisley et al., 2020a)*, which has been largely overlooked so far. Indeed, the effect of gut dysbiosis, defined as an alteration in microbial balance and functionality in the gut, on bee health is not well characterized *(Daisley et al., 2020a)*. However, the effects of honey bee gut dysbiosis can range from reduced fitness of individuals *(Zheng et al., 2018)* to dysfunctional social behaviour *(Liberti et al., 2022; Vernier et al., 2020)*, suggesting its possible contribution to colony health decline and eventual collapse *(Anderson & Ricigliano, 2017).*

The negative impact of DWV infection on honey bee immunity can, in principle, potentially affect the gut microbiota composition *(Bai et al., 2021; You et al., 2014, 2014*), and the resulting dysbiosis could favour the progress of viral infection, as suggested also by a recent study indicating that dysbiotic honey bees are less tolerant to DWV infection *(Dosch et al., 2021)*. Here we address this research issue, assessing if DWV infection, by downregulating immune response, may alter honey bee gut microbiota. To test this hypothesis, we adopted a double and complementary approach based on the characterization of the bacterial gut community of field collected bees and of bees subjected to controlled infection under laboratory conditions. Field collected bees were used to assess if different viral loads were associated with different gut microbiota compositions. This was complemented by analyses of the gut microbiota changes in honey bees which were artificially infected with DWV, by injecting viral lysate in newly emerged adults.

2.2 MATERIALS AND METHODS

2.2.1 Biological material

The honey bees used in this study were collected from *A. mellifera* colonies maintained at experimental apiary of the Department of Agricultural Sciences, University of Naples "Federico II". Colonies were regularly monitored for evaluating overall health and treated against *Varroa* mites. The strength of bee colonies was estimated by checking the presence of abundant population and provisions. All experiments were conducted during the warm season, from April to October.

2.2.2 Honey bee collection for field condition experiment

To investigate the relationships between DWV infection and the gut microbiota in natural condition, two different stages of bees were collected: larvae and nurses. Samples were collected from two hives in similar conditions of strength and provisions, in two sampling dates: 9th June 2020 and 30th July 2020. A total of 60 samples for each stage, except for nurse bees (N = 44), were collected, using different methods hereafter described. A section of a frame containing open brood, measuring approximately 15 x 6 cm, was cut out and transported in a plastic box (dimensions: 22,5 x 19 x 7,7 cm), to prevent dehydration. Fifth instar larvae were removed from the comb with a soft forceps and placed in a plastic Petri dish with wet filter paper and kept in an incubator at 33°C and 80% of Relative Humidity (RH) for 1 hour. Larvae without signs of melanization were transferred to individual microcentrifuge tubes and stored at -20°C.
Nurse bees were identified by observing their activity of nursing immatures on the frame. Asymptomatic bees were collected by using a plastic tube and transported in a thermic bag. In the laboratory, they were immobilised by chilling to be transferred in individual microcentrifuge tubes and stored at -20°C.

2.2.3 Artificial infection experiment

To study the causal effect of DWV infection on honey bee gut community, artificial infections were performed by injecting viral lysate in emerged honey bees from a low mite-infested hive.

To obtain naturally emerged bees, a piece of approximately 15 x 6 cm from a frame of capped brood was cut and placed in aerated plastic box (dimensions: 22,5 x 19 x 7,7 cm) to be kept in a dark incubator at 32°C and 60% of RH. The bees which emerged during the transport were discarded, to use in the experiment only bees of known age (from newly emerged to 16 hours).

2.2.3.1 Virus lysate preparation

The DWV lysate for the following virus infectivity assay was obtained from an adaptation of the method described in de Miranda et al. *(2013)*. Honey bees with deformed wings were collected from a mite-infested colony and stored at -80°C until they were singly ground with a pestle in a 1.5 mL microtube and 500 µL of 1X Phosphate Buffer Saline (PBS) (137 mM NaCl, 2.7 mM Potassium Chloride, 10 mM Phosphate Buffer; pH 7.4). The tubes were spun in a centrifuge, at 4°C, for 5 minutes at 13,000 g to separate the debris from the aqueous part which was transferred to a new tube. To each tube, 100 µL of chloroform were added, then vortexed for 30 seconds and centrifuged at 4°C for 15 minutes at 8,000 g. The supernatant was transferred in a new tube and filtered through a 0.22 µm syringe filter tip (VWR) to remove bacteria contaminants and non-viral pathogens. From each filtered lysate, 100 µL were removed to check the possible presence of common bee viruses by PCR according to *(Chen et al., 2006b),* and to quantify the DWV load by RT-qPCR, as described in section 2.2.5. The filtered lysates were aliquoted into sterile tubes of 50 µL, then glycerol was added to a final concentration of 40% and stored at -80°C.

2.2.3.2 Gut homogenate preparation

Gut homogenate was prepared following an adapted method from Dosh et al. *(2021),* to inoculate the same gut community in all experimental individuals (Powell et al., 2014; Kwong et al., 2017; Dosch et al., 2021). This step is important because bees emerged in the lab lack their gut community (Powell et al., 2014), a condition which expose them to gut colonization by opportunistic microorganisms with a possible negative impact on their health during the bioassay. Moreover, by inoculating the same background microbiota in all bees, we reduce experimental error and are able to observe how DVW infection during gut colonization shapes the microbiota. Briefly, homogenized whole guts, dissected from four

nurse bees, were homogenated with a pestle in 500 µL 50% (w/v) sucrose solution and then diluted 1:1 with 50% (w/v) sucrose solution. The gut homogenate was kept on ice and used to inoculate all the bees of a single experiment. To assess presence/absence of DWV infection in gut homogenate we analyzed RNA isolated from the rest of the body (carcass) after gut dissection by qRT-PCR, as described below.

2.2.3.3 Viral infection by injection

To cold-anaesthetize bees prior to injection, in order to prevent further damage to experimental animals, naturally emerged bees were taken from the incubator in groups of 10 and transferred in a sterile box (12x12x12 cm), which was kept at -20°C for 3 minutes, until the bees were immobile.
Under a stereomicroscope (SteREO Discovery V8, Zeiss, Jena, Germany), each immobilized bee was injected with 2.5 µL of 1X PBS containing 10^4 DWV copies. Viral lysates were injected between 4^{th} and 5^{ft} abdominal segments (**Figure 4**), using a beveled 33G NanoFil needle (NF33BV-2), mounted on a 10 µL NanoFil syringe (World Precision Instruments, Berlin, Germany).

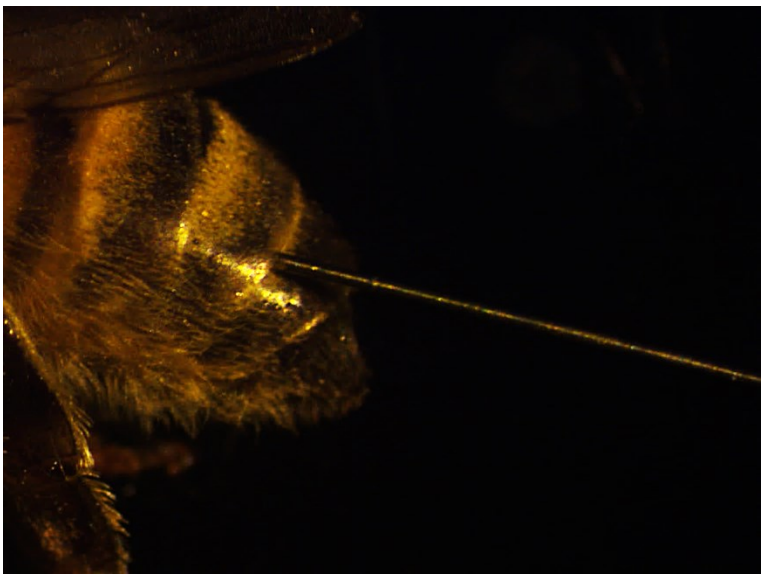


Figure 4 - Injection of an adult bee with the viral lysate. Injections were performed between 4th and 5ft abdominal segments, using a beveled 33G NanoFil needle (NF33BV-2).

Following injection bees were monitored until waking up to check that they had not suffered any injury. All bees that showed evident damage were excluded from the experiment. Non-injected (NI) bees, used as control group, and DWV-injected (DWV) bees were manually fed with 5 µL of honey bee gut homogenate, using a micropipette (**Figure 5**), to establish the gut community, as described elsewhere *(Dosch et al., 2021; Kwong et al., 2017a; Powell et al., 2014)*.
DWV- and NI- control bees were placed in separated plastic cages (28x11x11 cm) with pollen paste (80% w/w fresh corbicular pollen with tap water) and 50% filtered sucrose/water solution (w/v) *ad libitum*.
Sucrose solution (50% w/v) was filtered through a 0.22 µm syringe filter tip (VWR) to remove bacteria contaminants and non-viral pathogens and stored at -20°C until use.
Pollen paste and sucrose solution represent respectively proteins and carbohydrate source commonly used in bee experiments. Pollen paste feeder was made by cutting a section from a 2 mL microcentrifuge plastic tube, while sucrose feeder was obtained by cutting away the plastic holder of a 5 mL syringe.
Plastic cages, with approximately 15 bees per cage, were maintained in a dark incubator at 32°C and 60% RH for the duration of the experiment (Figure 6). These conditions are in agreement with suitable method for honey bee research set out by Williams et al., *(2013)*.

Figure 5 - Newly emerged honey bee (< 6 hours after emergence) carefully removed from brood comb and fed with 5 µL of gut homogenate, delivered with a micropipette.

Figure 6 - Plastic cages with a removable bottom used to contain emerged bees. Each cage has feeding devices and a ventilation hole. Plastic cages are kept in an incubator at constant temperature (32°C ± 1°C) and relative humidity (60% ± 2% RH) .

Bees were monitored each day for the duration of experiment and dead bees were discarded. Every two days, pollen paste and sugar solution were replaced. After 5 days from the injection, 15 experimental bees per treatment were collected and stored at -20°C until the dissection.
The whole experiment was repeated to have a duplicate of each treatment.

2.2.4 Effect of *Lactobacillus* as probiotics on honey bee survival and DWV load

To understand whether administration of *Lactobacillus* strains as probiotics may influence the levels of DWV infection, we conducted a preliminary test on bees naturally infected by DWV. Two strains of *Lactobacillus*, *L. fermentum* (AA) and *L. casei* (EV), were previously isolated from dairy products and donated by Prof. Danilo Ercolini, (Department of Agricultural Sciences, University of Naples Federico II).
AA and EV were cultured at 35 °C using MRS broth (catalogue number 288130; BD Difco) under aerobic conditions. The concentration of bacterial culture was estimated by OD_{600} readings using Varioskan Flash spectrophotometer (Thermo Fisher Scientific). Bacteria were pelleted at 10,000 g for 10 min and resuspended in 50% (w/v) sucrose solution to obtain a final concentration of $1x10^7$ CFU/mL of both strains. Subsequently, the bacterial suspensions" were mixed 1:1 (v/v) to obtain the bacterial mix AAEV, containing $0.5x10^7$ CFU/mL of each strain.
Brood frames from an apiary with high *Varroa* infestation (about 20% infested brood), thus naturally infected with DWV, were collected and kept in an incubator until adults emergence (32 ± 2 °C and 60% RH). Newly emerged honey bees were carefully removed from brood combs from the same colony, manually fed with 5 µL of freshly prepared gut homogenate and maintained as described above. Plastic cages, with approximately 20 bees per cage, were maintained in a dark incubator at 32°C and 60% RH for the duration of the experiment. After 24 hours from emergence, bees were fed with sucrose solution alone (CT) or supplemented with the bacterial mix (AAEV = $1×10^7$ CFU/mL). Both solutions (with and without bacteria) and corbicular pollen were provided *ad libitum*. Sucrose solution and pollen paste were replaced every two days. Honey bee survival was checked daily for 25 days, while eight honey bees per experimental

condition were sampled at day 7 and 14 from the first probiotics administration, to be processed as described below in order to estimate DWV load.

2.2.5 Molecular analysis

To study the impact of DWV infection on honey bee gut microbiota and immunity, the DNA was extracted from each isolated gut to determine the microbiota composition by 16S amplicon sequencing, while the carcass (rest of the body), from which RNA was isolated, was used to quantify DWV infection and gene expression by RT-qPCR.

2.2.5.1 Dissection

Under the stereomicroscope (SteREO Discovery V8, Zeiss, Jena, Germany), each experimental individual was immobilised on a piece of styrophor with entomology needles and the abdomen was ventrally opened using sterilized microforceps and microscissors to expose the internal organs. For each bee, the whole gut, excluding the crop, and the rest of the body were collected and placed in two different 1.5 mL individual tubes chilled on ice and stored at -20 °C until nucleic acids extraction.

2.2.5.2 RNA Extraction

Carcass samples were homogenized in 250 µL of TRIzol Reagent (Thermo Fisher Scientific) with plastic pestles and processed according to the manufacturer's instructions. Following isopropanol precipitation and ethanol (70%) washing, the resulting RNA pellet was resuspended in 400 µL diethyl pyrocarbonate (DEPC) treated water, made by using Milli-Q (Elix® Advantage Systema with vent filter Type 2 (pure) water). The quality and quantity of the isolated total RNA were evaluated by measuring the absorbance with Varioskan Flash spectrophotometer (Thermo Fisher Scientific).
The Turbo DNase-free kit (Invitrogen) was used to remove traces of DNA from the extracted RNA. The treatment was performed on 50 µL volumes (150 ng/µL) in a 0,5 mL tubes by adding 5 µL of 10X Buffer and 1 µL of DNase I (2 U) and incubating at 37°C for 30 minutes. To inactivate DNase, 5 µL of DNase inactivation reagent was added and the incubation was carried out at room temperature for 5 minutes. After a brief centrifugation at 10,000 g, 50 uL were transferred in a

new tube. The RNA samples were stored at -80°C until DWV quantification and gene expression analysis.

2.2.5.3 Screening for virus of the colony by end-point PCR

The RNA extracted from 100 µL of filtered lysate (see subsection 2.2.3.1) was used as template for a retrotranscription reaction using cDNA High capacity RT-Kit (Applied Biosystems™) to test the presence of Deformed Wings Virus (DWV) and the absence of other common bee viruses (**Table 1**) by PCR before the artificial infection, as described elsewhere *(Chen et al., 2006b)*.

Table 1 – PCR primers used for evaluating the presence/absence of common bee virus.

Primer Name	Sequence	Target organism	Amplicon size (bp)	Tm °C	Reference
DWV_F	CTTACTCTGCCGTCGCCCA	Deformed wing virus	194	61.6	(Bradford et al., 2017)
DWV_R	CCGTTAGGAACTCATTATCGCG			60.3	
IAPV_F	GCGGAGAATATAAGGCTCAG	Israeli acute paralysis virus	586	58.5	(vanEngelsdorp et al., 2009)
IAPV_R	CTTGCAAGATAAGAAAGGGGG			54.2	
SBV_F	GCTGAGGTAGGATCTTTGCGT	Sacbrood virus	824	61.2	(Chen et al., 2006b)
SBV_R	GCTGAGGTAGGATCTTTGCGT			55.5	
KBV_F	GATGAACGTCGACCTATTGA	Kashmir bee virus	415	54.3	(Chen et al., 2006b)
KBV_R	TGTGGGTTGGCTATGAGTCA			57.8	
BQCV_F	TGGTCAGCTCCCACTACCTTAAAC	Black queen cell virus	700	63.0	(Chen et al., 2006b)
BQCV_R	GCAACAAGAAGAAACGTAAACCAC			58.9	
CBPV_F	AGTTGTCATGGTTAACAGGATACGAG	Chronic bee paralysis virus	455	60.6	(Chen et al., 2006b)
CBPV_R	TCTAATCTTAGCACGAAAGCCGAG			60.8	
ABPV_F	TTATGTGTCCAGAGACTGTATCCA	Acute Bee Paralysis Virus	900	59.2	(Chen et al., 2006b)
ABPV_R	GCTCCTATTGCTCGGTTTTTCGCT			64.0	

2.2.5.4 Quantification of DWV load and dorsal-1A expression by RT-qPCR

The quantification of DWV genome copies in honey bee carcasses was performed using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems) as described elsewhere *(Annoscia et al., 2020)*. All primers used are shown in **Table 2**. Titers of DWV-A and DWV-B were determined by relating the Ct values of unknown samples to an established standard curve. The standard curve was established by plotting the logarithm of 10-fold dilutions of a starting solution containing 0.01 ng of purified PCR product (PureLink PCR Purification Kit, Thermo Fisher Scientific), against the corresponding Ct value as the average of three repetitions. The PCR efficiency was calculated based on the slope and coefficient of correlation (R^2) of the standard curve, according to the following formula: $E = 10^{(-1/\text{slope})} - 1$ (**Table 3**).

Table 2 - Primers used for quantification of DWV load and gene expression by qPCR

Primer Name	Sequence	Target organism	Target gene	Amplicon size (bp)	Tm (C°)	Reference
qDWV-Af	GCGCTTAGTGGAGGAAATGAA	DWV-A	helicase	69	65.5	(Di Prisco, Annoscia, et al., 2016)
qDWV-Ar	GCACCTACGCGATGTAAATCTG				65.8	
qDWV-Bf	GCAAGTTGGAGATAATTGTA	DWV-B	NTR (IRES region)	116	54.9	(Bradford et al., 2017)
qDWV-Br	CGATACTTACATTCTTCAAGAT				54.8	
qDORf	ACAGGCAGAAGCTGAGAAGC	Apis mellifera	dorsal 1A	143	64.2	(Lourenço et al., 2018)
qDORr	TTGCCATCGGATACAAGGAT				64.5	
qACTf	TTGTATGCCAACACTGTCCTTT	Apis mellifera	β-actin	120	65.2	(Simone et al., 2009)
qACTr	TGGCGCGATGATCTTAATTT				66.8	

Table 3 - Standard curves used for absolute DWV quantification by qPCR

Target	Equation	R^2	Efficiency %
DWV-A	y = -3.168x + 38.625	0.99	106.85
DWV-B	y = -3.3048x + 36.805	0.99	100.72

DWV load was calculated as the sum of DWV-A and DWV-B loads, expressed as viral genome copies per nanogram of total RNA. Annealing temperature were set at 60°C and 55°C for DWV-A and DWV-B, respectively.
Differential relative expression of *dorsal-1A* was measured by one-step qRT-PCR, using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. Each reaction was prepared in 20 µL and contained 10 µL qRT-PCR mix 2X, 100 nM of forward and reverse primers, 0.16 µL of 125X RT enzyme mix, DEPC treated water and 50 ng of total RNA. All samples were analyzed in duplicate on a Step One Real Time PCR System (Applied Biosystems). *β- actin* was used as endogenous control for RNA loading. Relative gene expression data were analyzed using the ΔΔCt method *(Livak & Schmittgen, 2001)*.

2.2.5.5 DNA Extraction

Genomic DNA was isolated from the gut samples as described in Evans et al., 2013. Each sample was homogenized in 250µL of lysis buffer CTAB-based (cationic detergent cetyltrimethylammonium bromide) (0.1 M Tris-HCl, pH 8; 1.4 M NaCl; 0.02 M EDTA, pH 8; 2% CTAB, w/v; 0.25% DTT, v/v; 40 µg proteinase K, 20µg RNase A) with a sterile plastic pestle. Gut homogenate (100 µL), two pollen samples (~ 100 mg), sucrose (200 µL) and one blank control were processed for DNA extraction. Negative controls were used to monitor for reagent and environmental contaminations: plastic pestles were immersed in the CTAB buffer without gut tissues. The resulting pellets were resuspended in 30 µL nuclease-free water, while the pellets from pollen extraction were resuspended in 100 µL nuclease-free water.
The quantity and the quality of all DNA extracted were assessed using Varioskan Flash spectrophotometer (Thermo Fisher Scientific). The concentration was adjusted to a standard value for all samples (20 ng/µL) and was stored at -20 °C until 16S sequencing analysis.

2.2.5.6 16S Amplicon Sequencing and Data Processing

To characterize the microbial communities associated with honey bee gut, the sequencing of bacterial 16S rRNA gene was carried out, using high throughput MiSeq technology (Illumina, San Diego, CA, USA) by Genomix4Life srl (Baronissi, Salerno, Italy).

DNA templates were used for amplification of ~300 bp of the V3-V4 hypervariable region of the bacterial 16S rRNA gene employing fusion primers with partial Illumina adaptors. The universal bacterial 341F forward primer (5′-CCTACGGGNGGCWGCAG) and the 785R reverse primer (5′-GACTACHVGGGTATCTAATCC) were used. PCR reactions, library preparation and sequencing were performed by IGATech (Udine, Italy). Amplicons were paired-end sequenced (2 × 250 bp) on an Illumina NovaSeq 6000 platform using standard protocols.

Raw fastq files were processed using the QIIME2 platform *(Bolyen et al., 2019)*. The field conditions and artificial infection experiments were denoised and taxonomically annotated separately. For denoising and estimation of amplicon sequence variants (ASVs) in each sample, we used DADA2 algorithm *(Callahan et al., 2016)* through QIIME2. Primers were trimmed while forward and reverse reads were not truncated. After training the naïve Bayes classifiers on SILVA reference sequences (SILVA release 138.1) *(Quast et al., 2013)*, trimmed for the amplified region, the fit-classifier-sklearn method *(Bokulich et al., 2018)* was employed for the taxonomic annotation of the ASVs (confidence=0.95). Subsequently, the ASVs assigned to mitochondria and chloroplasts were excluded, as well as the ASVs belonging to the six most abundant genera identified in negative controls (pestle samples), including human/contaminants-associated genera such as uncultured *Caulobacteraceae, Cutibacterium, Nevskia, Variovorax, Ralstonia, Staphylococcus*. Subsequent analyses were performed using R packages ‘phyloseq’ and ‘vegan’. Samples with less than 70,000 reads were excluded from the dataset. To plot relative abundances at class level, only taxa with more than 1% abundance in at least one sample were retained. ASV tables were rarefied to 80,000 and 200,000 reads for field conditions and artificial infection experiments, respectively. Rarefied tables were used as input for alpha and beta diversity analyses. To identify differentially abundant (DA) taxa, we integrated three methods which represent different approaches with diverse false discovery rates and considered only taxa shared between at least two DA methods. ANCOM-BC (H. Lin & Peddada, 2020) and DESeq2 *(Love et al., 2014)* methods were performed in R, while LEfSe *(Segata et al., 2011)* was installed via conda from a Github repository (https://github.com/SegataLab/lefse). To perform ANCOM BC, rarefied table was used as input, while, to reduce dataset complexity for DESeq2 analysis, all ASVs with a variance greater than 10-5 were

removed. As input for LefSE the ASV table resulting from Qiime2 pipeline, cleaned from undesired taxa and samples, was used.
Differentially abundant genera were selected to test their correlation with DWV load, using Spearman's rank correlation. Significant correlations were incorporated in a Dysbiosis Index (DI), to describe the dysbiotic status of each bees with a single value (Vujkovic et al., 2020). DI was calculated as the log ratio of geometric means of taxon abundances that were positively correlated with DWV load (having $P < 0.05$ and $r > 0$) over taxa that were negatively correlated with DWV load (having $P < 0.05$ and $r < 0$).

$$DI = log_{10}\left(\frac{\sqrt[n]{x_1\ x_2\ \dots x_n}}{\sqrt[m]{y_1\ y_2\ \dots y_m}}\right),$$

where each x denotes read counts for taxa positively correlated with DWV load and n is the total number of such taxa, whereas each y denotes read counts for taxa negatively correlated with DWV load and m is the total number of these taxa. Taxon read counts were used with an added pseudocount of 1, in order to accommodate the geometric mean by removing zeroes.

2.2.6 Statistical analysis

To analyze correlation between DWV infection and *dorsal-1A* expression we used Spearman's rank correlation. Wilcoxon-test was used to test differences in α-diversity metric (Shannon index), relative abundances (after centered log-ratio scale transformation) and DWV load of DWV-injected and non-injected bees. Student's t-test was used to analyze the effect of artificial infection on the expression of *dorsal-1A.* The assumption of normal distribution of data was tested and met via Shapiro-Wilk test. Each dataset was checked for homoscedasticity using Levene's test. Community differences were verified by permutational multivariate analysis of variance (PERMANOVA), using the 'adonis2' package. All statistical analyses were performed using R software (version 4.0.3) and Prism v.7 (software GraphPad, San Diego, CA, USA), setting the significance level at 0.05.

2.3 RESULTS

2.3.1 Gut microbiota associated with DWV infection in natural conditions

To study the relationship between DWV and gut microbiota we sampled mature larvae (N=60) and nurse bees (N=44, from two hives (hive1 and hive2) with similar brood and food conditions, at two different dates (June and July). RNA samples, isolated from bee carcasses after gut dissection, were used to quantify DWV load via RT-qPCR.

A preliminary analysis conducted on nurse bees pointed out a co-infection by genotypes A and B. However, in almost all samples DWV-B load was lower than DWV-A load (**Figure 7**A), with a rate of DWV-A/DWV-B ranging from 0.01- to 10^{10}-fold (median: 696; **Figure 7**B). Moreover, we observed a significant positive correlation between the two genotypes (Spearman's rank correlation: R = 0.345, S = 9285.8, *P* = 0.021; **Figure 7**C). Thus, we used total DWV (DWV-A + DWV-B) genome copies, normalized on the nanograms of total RNA isolated, as a measure of DWV load.

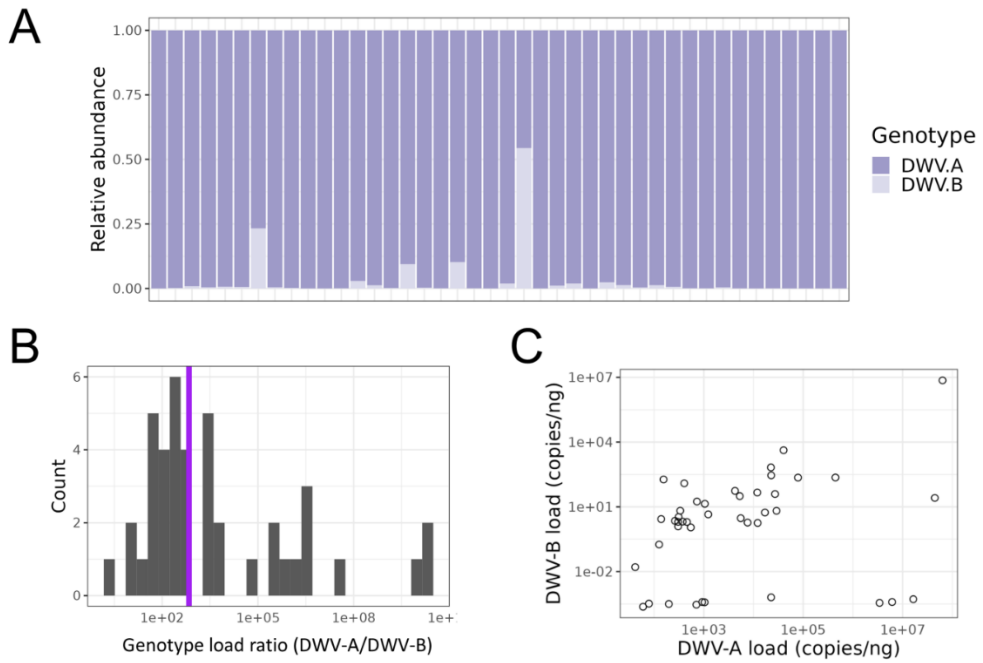


Figure 7 - Quantification of DWV genotype A and B in field-collected bees. A) Relative abundance of DWV genotypes obtained from absolute quantification by RT-qPCR. B) Distribution of genotype load ratios (DWV-A/DWV-B). Purple vertical line indicates median (=696). C) DWV-A and DWV-B loads are positively correlated (Spearman's rank correlation: R = 0.345, S = 9285.8, P = 0.021).

2.3.1.1 Mature larvae

DWV load in field collected mature larvae, measured as total (DWV-A + DWV-B) genome copies/ng of total RNA, ranged from 1.90 x 10^1 to 1.72 x 10^4 in 60 samples collected. After filtering for sequencing depth, the final dataset included 47 samples, which were clustered on the basis of their microbiota composition *(Sirisena et al., 2018)*. Hierarchical clustering (**Figure 8**) revealed two main groups, A and B, and four minor subgroups (A1, B1, B2, B3). Groups A and B were associated with two different sampling dates, July and June, respectively.

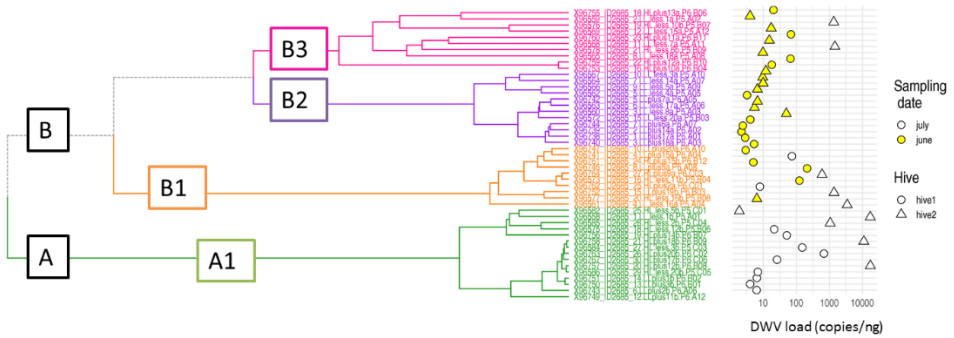


Figure 8 - Hierarchical clustering using Bray-Curtis distance (microbiota composition) and Ward's clustering algorithm. The analysis clustered samples on the basis of their gut bacterial community composition, identifying four subgroups and two main groups (A and B). These two latter, are associated with different sampling dates (June and July), as indicated by Pearson's Chi-squared test (X-squared = 24.09, df = 1, p-value = 9.2x10-7).

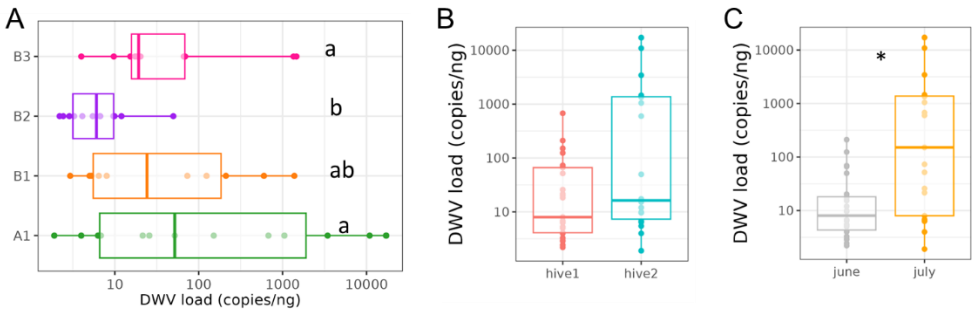


Figure 9 - DWV load in field-collected larvae. The four biogroups identified showed different viral levels (A), with B2 having lower DWV load than A1 and B3, but not B1 (Kruskal-Wallis chi-squared = 9.84, df = 3, p-value = 0.0199; Pairwise comparisons using Wilcoxon rank sum exact test with Benjamini-Hochberg p-value correction). Biogroup B1 had an intermediate DWV load, compared to the other groups. DWV load was not statistically different between sampled hives (B, Wilcoxon Ranks Sum exact test: W = 183, p-value = 0.05045), while it was higher in larvae sampled in July, compared to those sampled in June (C, Wilcoxon Ranks Sum exact test: W = 424, p-value = 0.0009334).

Although DWV load was not different between the two macro-groups A and B (Wilcoxon rank sum exact test: W = 318, p-value = 0.077), the four subgroups identified were associated with different viral loads.

In particular, B2 resulted associated with a lower DWV load compared to A1 and B3, but not B1 (**Figure 9**A). DWV load was not statistically different between the two sampled hives (**Figure 9**B), while it was higher in July, compared to June (**Figure 9**C).
As expected, the analysis of β-diversity showed a clear separation of the four identified clusters (**Figure 10**A). Moreover, sampling date had a significant effect on gut community composition. Shannon index (α-diversity) was higher in the gut of larvae with lower DWV load (**Figure 10**B).

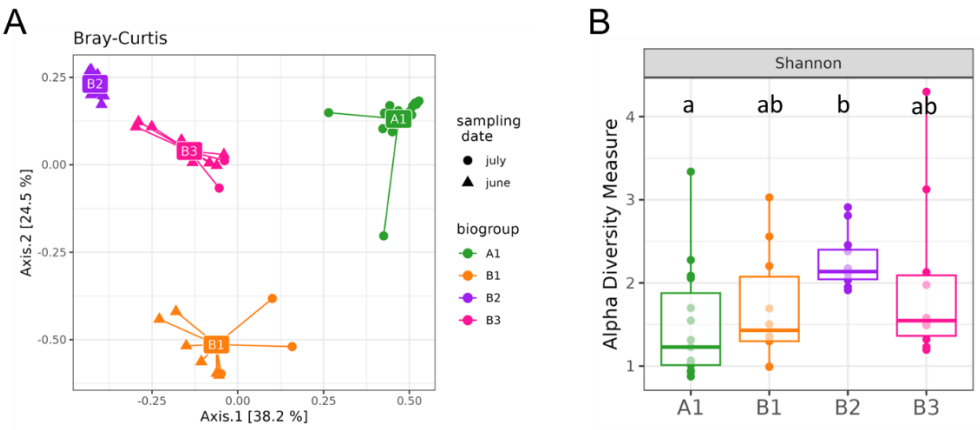


Figure 10 - Alpha and beta-diversity of gut bacterial community in mature larvae. (A) Beta-diversity analysis (Bray –Curtis dissimilarity) revealed a significantly different composition between the four biogroups analyzed (PERMANOVA: df=3, F=29.834, p-value= 0.001), as well as between June and July (PERMANOVA: df=1, F=17.984, p-value= 0.001). No differences in Beta-diversity were observed between hive 1 and hive 2. (B) Alpha-diversity analysis revealed a significant higher Shannon index in B2 group, which was associated with a lower DWV load (Kruskal-Wallis chi-squared = 11.275, df = 3, p-value = 0.0103; Pairwise comparisons using Wilcoxon rank sum exact test with Benjamini-Hochberg p-value correction).

Our analysis reported the core bacterial genera of the honey bee larval microbiota. Barchart of relative abundance at class level (**Figure 11**A) showed that the biogroup with lower DWV loads, B2, is associated with higher abundance of Bacilli (Red). At genus level, B2 Group showed a higher abundance of *Apilactobacillus* (Fig. 12B).

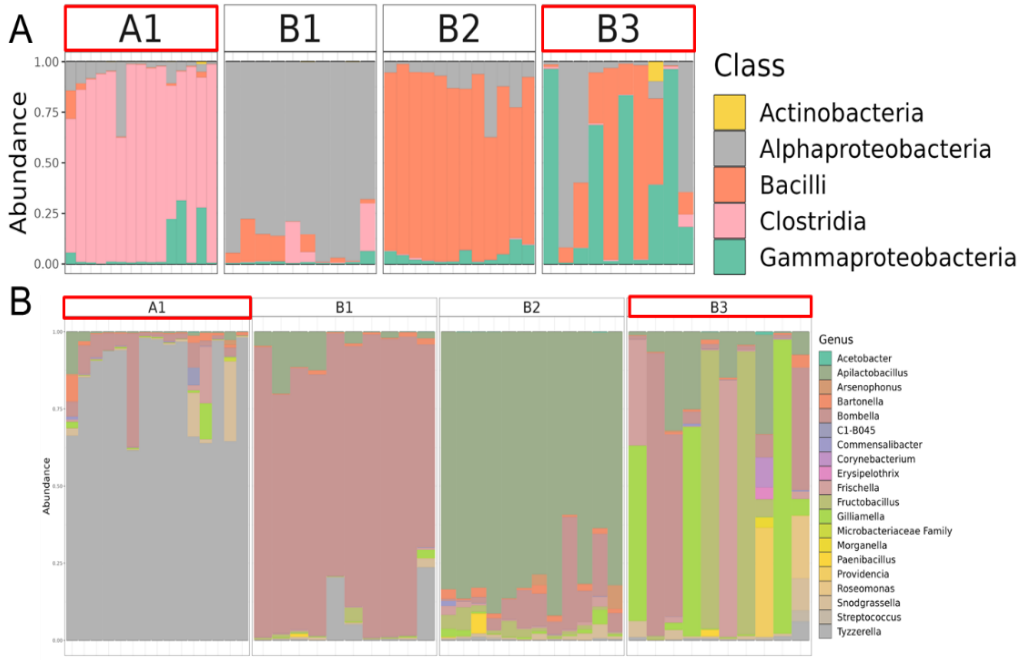


Figure 11 - Barchart of relative abundance at class (A) and genus level (B). Biogroups with higher DWV loads are highlighted in red.

This observation was confirmed by differential abundant analysis (**Figure 12**) which identified *Apilactobacillus* (Bacilli) as significantly more abundant in larvae with lower DWV loads (B2). On the other hand, DA analysis indicated that *Tyzzerella* (Clostridia) was more abundant in one of the groups with higher DWV loads (A1), compared to B2.

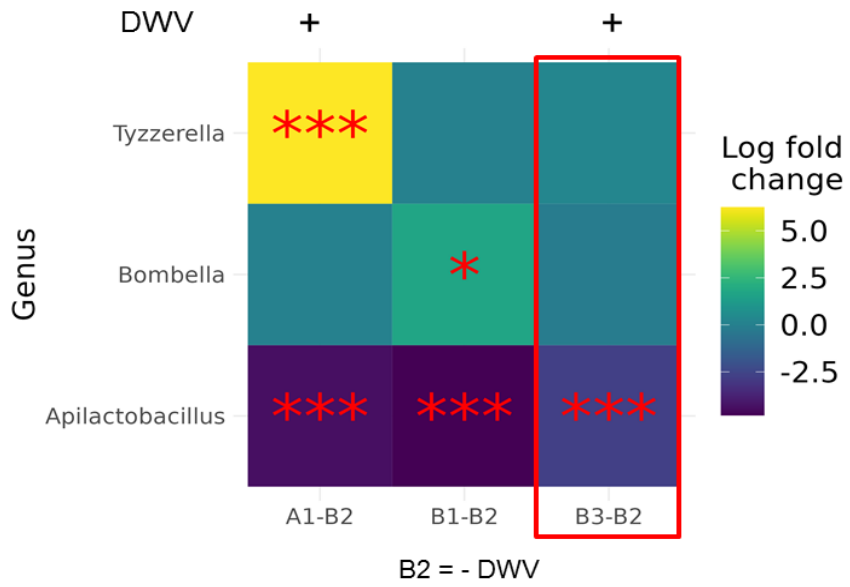


Figure 12 - Heatmaps showing differentially abundant genera identified by ANCOM-BC analysis. Compared to B2, B3 (higher DWV load, in the same macrogroup/period) showed a lower abundance of *Apilactobacillus*. Compared to B2, A1 (higher DWV load) showed higher abundance of *Tyzzerella*.

Indeed, considering the whole set of analyzed larvae, DWV load was positively correlated with *Tyzzerella* abundance (Figure 13A) and negatively correlated with *Apilactobacillus* abundance (Figure 13B). Then, Disbiosys Index (DI) was used to describe the observed gut microbiota changes as a single numerical value, negative DI indicates normobiosis, whereas a positive DI indicates dysbiosis. DI was calculated using the formulaDI = log10 ((*Tyzzerella* abundance)/(*Apilactobacillus* abundance)).
DI resulted positively correlated with DWV load (**Figure 13**C). Moreover, as a proxy for measuring host immunocompetence we used the expression of a gene involved in the activation of the immune response, *dorsal-1A* *(Nazzi et al., 2012)*, which was not correlated with DWV load (**Figure 13**D).

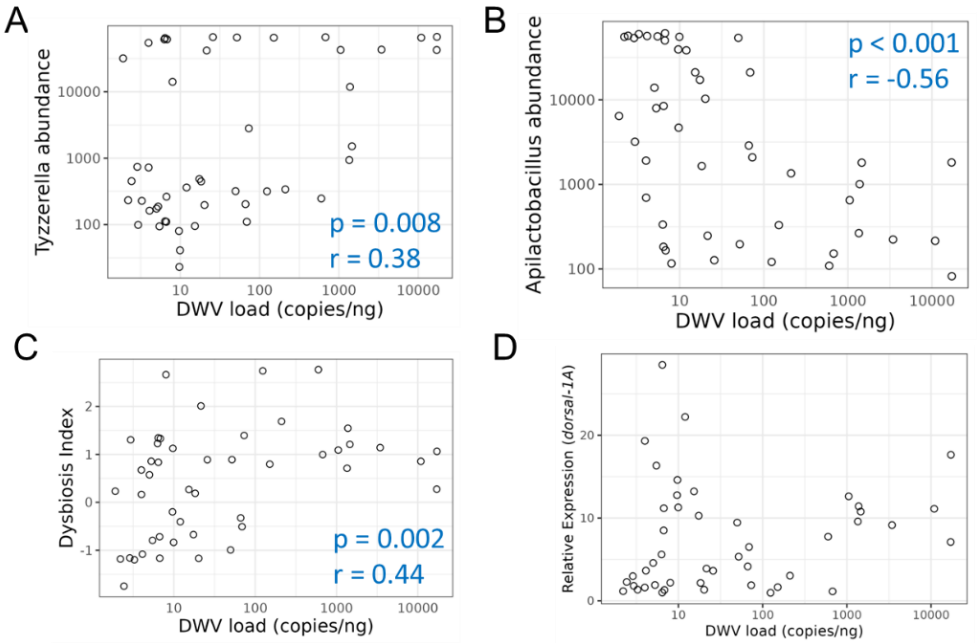


Figure 13 - Spearman correlations between DWV load and microbiota/immunity features. *Tyzzerella* abundance correlates positively with DWV load (A), while *Apilactobacillus* abundance correlates negatively with DWV load (B). *Tyzzerella* and *Apilactobacillus* abundances were incorporated in a dysbiosys index (DI) using the formula: DI = log10 ((*Tyzzerella* abundance)/(*Apilactobacillus* abundance)). DI resulted positively correlated with DWV load (C), which, however, was not correlated with the expression of *dorsal-1A*, a key gene of the immune response (D). In blue are indicated Spearman's rank correlation p-values (p) and rho (r).

2.3.1.2 Nurse bees

DWV load in field-collected nurse bees, measured as total (DWV-A + DWV-B) genome copies/ng of total RNA, ranged from 4.17 x 10^1 to 7.07 x 10^7 in 42 samples collected from two hives. After filtering for sequencing depth, the final dataset included 34 samples.
Hierarchical clustering (**Figure 14**) revealed two main groups, A and B, and five minor subgroups (A1, A2, B1, B2, B3). Groups A and B were associated with two different sampling dates, June and July, respectively. DWV load was not different between A and B macro-groups, but it was higher in subgroups A1 and B2, compared to B3 and A2 (**Figure 15**A). DWV load was not affected by hive (**Figure 15**B) or by sampling date (**Figure 15**C)

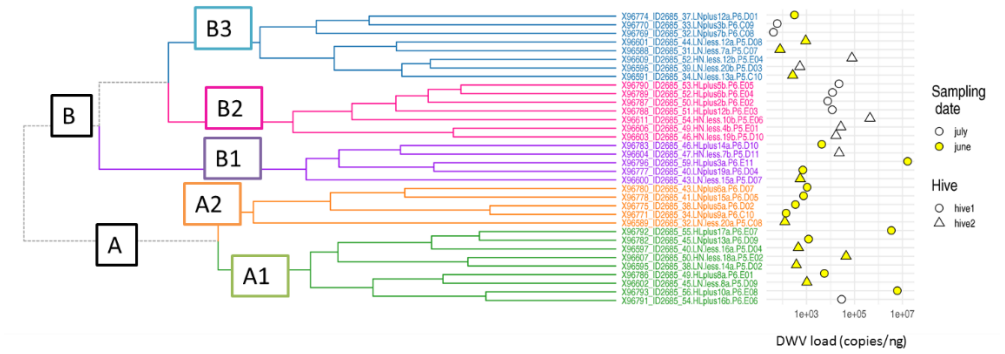


Figure 14 - Hierarchical clustering using Bray-Curtis distance (microbiota composition) and Ward's clustering algorithm. The analysis identified 5 biogroups and two main biogroups (A and B). These two latter, are associated with two different sampling dates (June and July), as indicated by Pearson's Chi-squared test (X-squared = 7.6331, df = 1, p-value = 0.005731).

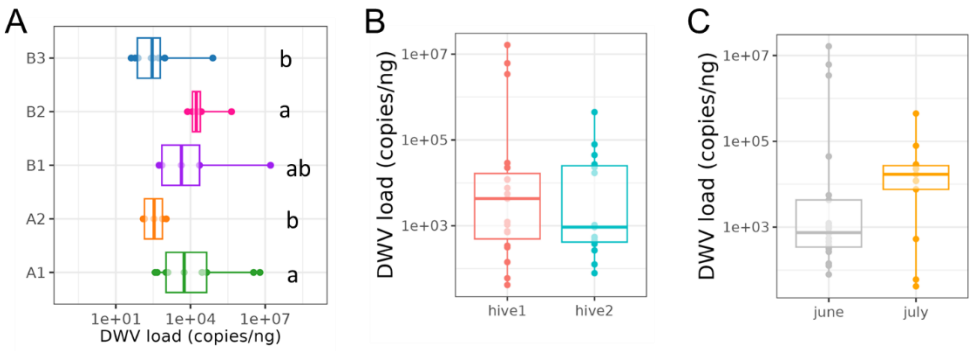


Figure 15 - DWV load in field-collected nurse bees. The 5 biogroups identified showed different viral levels, with A1 and B2 having higher DWV load than A2 and B3 (Kruskal-Wallis chi-squared = 14.271, df = 4, p-value = 0.006479; Pairwise comparisons using Wilcoxon rank sum exact test with Benjamini-Hochberg p-value correction). Biogroup B1 had an intermediate DWV load, compared to the other subgroups. DWV load was not different between A and B groups (Wilcoxon rank sum exact test: W = 318, p-value = 0.077), between hive 1 and hive 2 (Wilcoxon rank sum exact test: W = 152, p-value = 0.758) or between June and July (Wilcoxon rank sum exact test: W = 180, p-value = 0.129).

As expected, the analysis of β-diversity showed a clear separation of the five identified clusters (**Figure 16**A). Moreover, sampling date had a significant effect on gut community composition. α-diversity analysis

(Observed species, Shannon, Simpson) revealed no differences between the 5 biogroups (B) (**Figure 16**B).

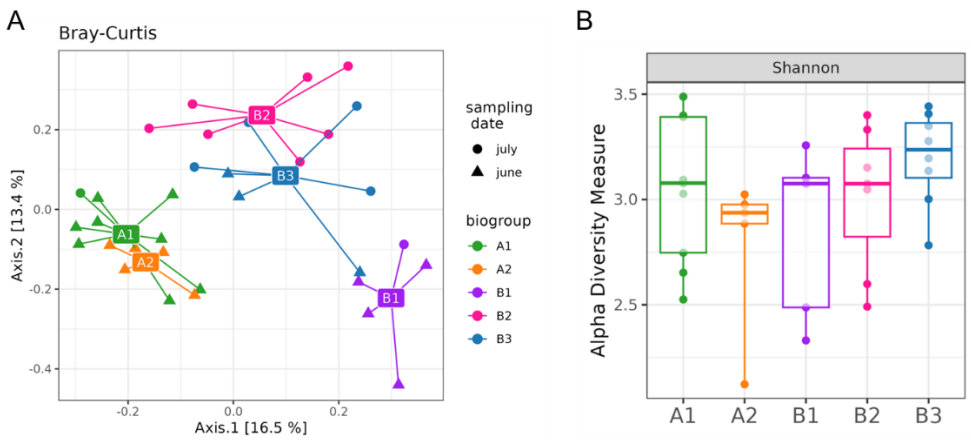


Figure 16 - Beta-diversity analysis (Bray-Curtis dissimilarity) revealed a significantly different composition between the 5 biogroups analyzed (PERMANOVA: df=4, F=4.069, p-value= 0.001), as well as between June and July (PERMANOVA: df=1, F=3.589, p-value= 0.001) (A). No difference on Beta-diversity was observed between the two different hives sampled (hive 1 and 2). α-diversity analysis (Shannon) revealed no differences between the 5 biogroups (B).

Our analysis reported the core bacterial genera of the honey bee adult microbiota. Barchart of relative abundance at class level (**Figure 17**A) showed that the biogroups with higher DWV loads, B2 and A1, are associated with higher abundance of *Alphaproteobacteria* (Grey). At genus level, B2 Group showed a higher abundance of *Bartonella* (**Figure 17**B, red). Indeed, differential abundant analysis indicated that *Bartonella* is significantly more abundant in nurse bees with higher DWV loads, such as A1 (**Figure 18**A) and B2 (**Figure 18**B). On the other hand, *Lactobacillus* (Bacilli) was less abundant in one of the groups with higher DWV loads (B2), compared to B3 (**Figure 18**B). Moreover, compared to A2, A1 (higher DWV load, in the same macrogroup/period) showed a higher abundance of *Snodgrassella* (**Figure 18**A).

Figure 17 - Barchart of relative abundance at class level (A) showed that biogroups with higher DWV loads, A1 and B2, are associated with a higher abundance of *Alphaproteobacteria* (Grey). At genus level, B2 Group showed a higher abundance of *Bartonella* (B). All the core genera of the honey bee gut microbiota are reported. Biogroups with higher DWV loads are highlighted in red.

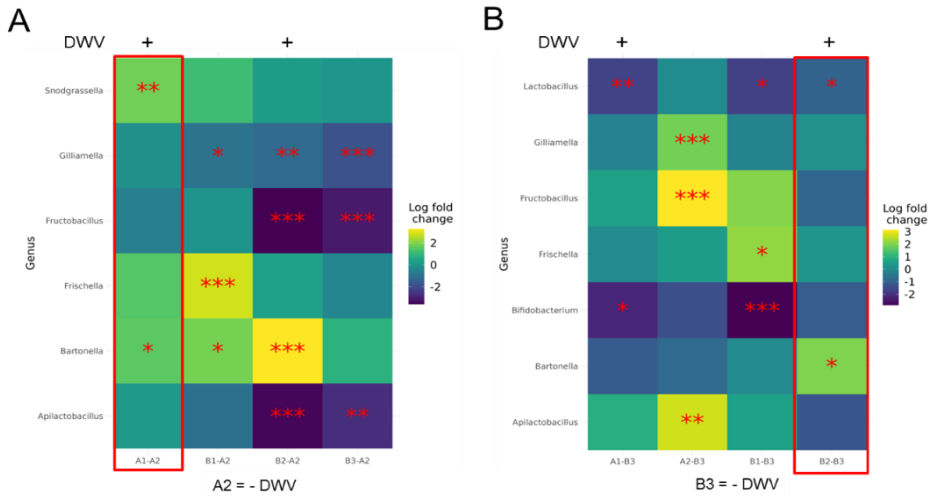


Figure 18 - Heatmaps showing Differentially abundant genera identified by ANCOM-BC analysis. Compared to A2, A1 (higher DWV load, in the same macrogroup/period) showed a higher abundance of *Bartonella* and *Snodgrassella* (A). Compared to B3, B2 (higher DWV load, in the same macrogroup/period) showed higher abundance of Bartonella, and a reduced load of *Lactobacillus* (B).

When testing these genera for correlation across all sampled nurse bees only *Bartonella* (

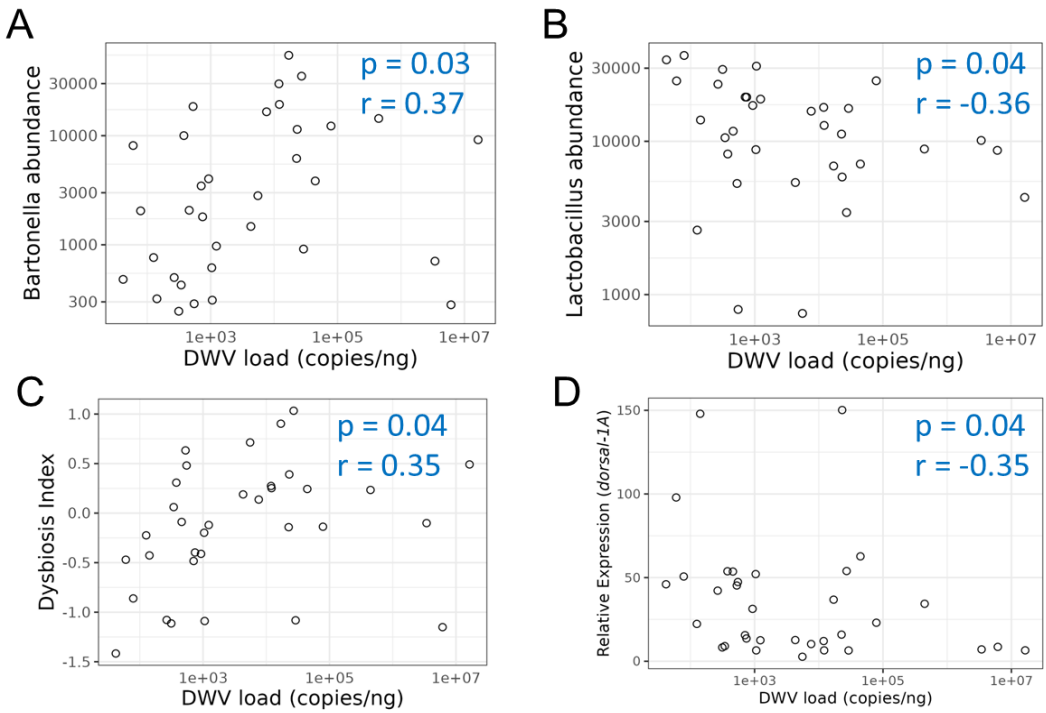
Figure 19A) and *Lactobacillus* (

Figure 19B) abundance resulted positively and negatively correlated with DWV load, respectively. We included both *Bartonella* and *Lactobacillus* abundance in a Disbiosys Index (DI), which resulted positively correlated with DWV load (

Figure 19C). Moreover, as a proxy for measuring host immunocompetence we used the expression of a gene involved in the activation of the immune response, *dorsal-1A* *(Nazzi et al., 2012)*, which was negatively correlated with DWV load (

Figure 19D).

Figure 19 - Spearman correlations between DWV load and microbiota/immunity features. A common feature of A1 and B2 gut communities, associated with higher DWV loads, is the high abundance of *Bartonella* species. Indeed, *Bartonella* abundance correlates positively with DWV load (A). On the other hand, *Lactobacillus* abundance correlates negatively with DWV load (B). The abundances of the other core microbiota components were not significantly correlated with DWV load. Thus, *Bartonella* and *Lactobacillus* abundances were incorporated in a dysbiosys index (DI) using the formula : DI = log10((*Bartonella* abundance)/(*Lactobacillus* abundance)). DI resulted positively correlated with DWV load (C), which correlated negatively with the expression of *dorsal-1A*, a key gene of the immune response (D). In blue are indicated Spearman's rank correlation p-values (p) and rho (r).



2.3.2 Gut microbiota associated with DWV infection under controlled conditions

In a second experiment we performed artificial infection of honey bees to assess the effect of DWV on gut microbiota. To simulate the role of viral infection through transmission by *V. destructor*, we either injected or not newly emerged individuals with a known quantity of DWV particles. In the injected viral lysate DWV-B load was marginal compared to DWV-A load, representing only 0.003% of total DWV genome copies (

Table 4). Indeed, injected bees were prevalently infected by DWV-A genotype, at 5 days from injection (Figure 20).

Table 4 - Quantification of specific DWV genotypes in the injected viral lysate

Genotype	Ct	Viral copies/µL	Viral copies/µL in stock	Injected copies	Relative abundance (%)
DWV-A	11.88	3.61E+08	6.40E+07	1.60E+04	99.997
DWV-B	23.08	8.07E+04	4.54E+04	1.14E+00	0.003

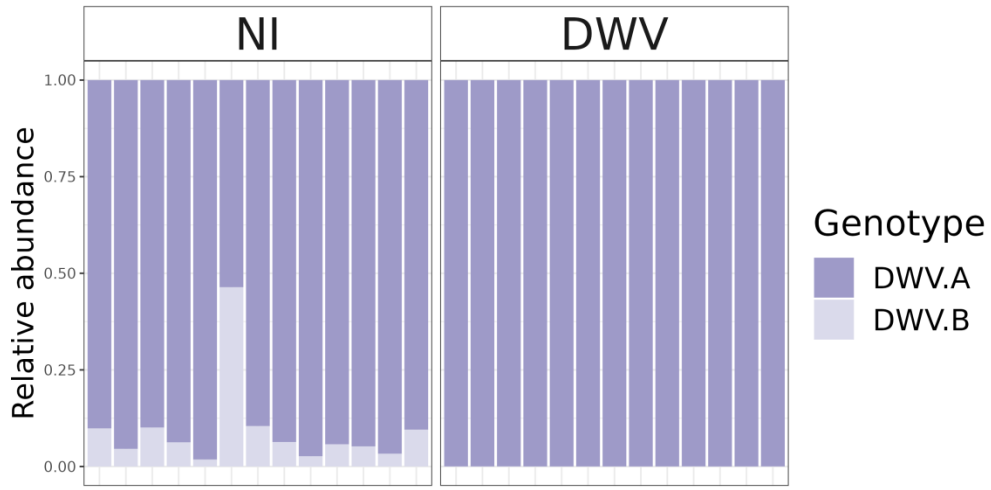


Figure 20 - Relative abundance of DWV genotypes in injected (DWV, n=13) and non-injected bees (NI, n=13). After 5 days from treatment, injected bees showed a prevalence of DWV-A genotype. DWV-B relative abundance is higher in NI bees, although total DWV levels were significantly lower than those of DWV bees (Figure 21A).

The analyzed dataset included non-injected bees (NI, n=13), with total DWV loads ranging from 0.17 to 2.30, and bees injected with 10^4 viral particles (DWV, n=13), with total DWV loads ranging from 8.40 x 10^5 to 2.47 x 10^6 copies per nanogram of total RNA (approximately equal to 10^8-10^9 copies/bee) (Figure 21A). We scored the effect of artificial infection on the expression of *dorsal-1A*, which was significantly reduced in DWV bees Figure 21B).

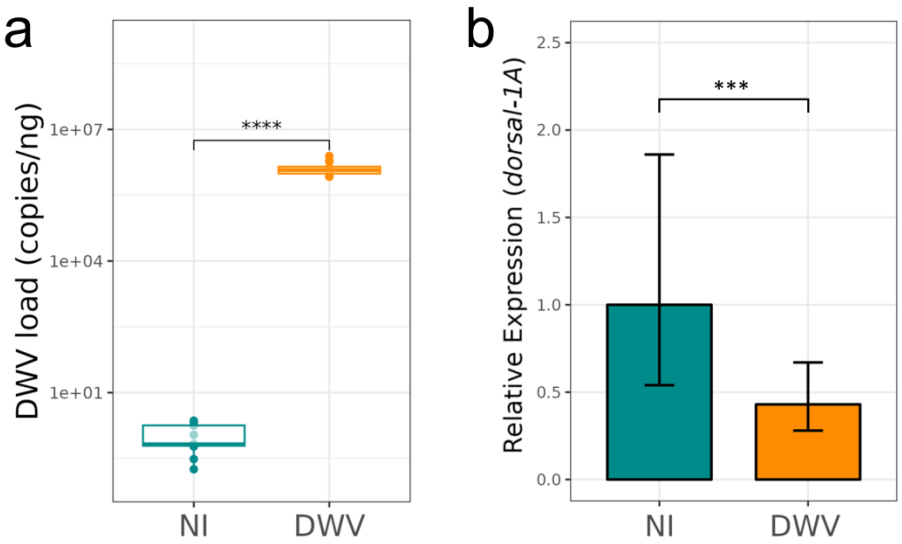


Figure 21 - DWV load and immunocompromission in artificially infected bees. DWV load was significantly higher in DWV-injected (DWV, n=13), compared to non-injected (NI, n=13) bees (Wilcoxon-test: W = 0, P = 3.644e-05) (A). Values on Y-axis are reported in Log10 scale. Dorsal-1A expression was significantly lower in DWV bees (B), compared to NI (Student's t-test: t(23.4) = -4.07, P = 0.0004). ***P < 0.001, ****P < 0.0001.

A total of 26 samples were sequenced, generating an average of 389,906 reads per sample. The final OTU table included 1483 ASVs. At the class level, members of the *Alphaproteobacteria* were more relatively abundant in DWV-injected bees compared with NI (Wilcoxon-test: W = 132, *P* = 0.014) (Figure 22A and Figure 23), whereas Bacilli were more relatively abundant in NI bees (Wilcoxon-test: W = 16, *P* < 0.001) (Figure 22A and Figure 23). These differences were associated with a significant loss of alpha diversity in DWV injected bees (inverse Simpson index, Figure 22B). PERMANOVA revealed that gut community composition was significantly different between NI and DWV (Figure 22C). Our integrated differential abundance analysis identified gut bacterial taxa that differed in abundance between NI and DWV (Figure 22D and Figure 22E). Among bacterial genera associated with NI bees (Figure 22D and Figure 24), *Lactobacillus* and *Fructobacillus* were shared between the three methods used, while no other genus resulted shared. Among genera associated with DWV bees (Figure 22E and Figure 24), *Bartonella* and *Commensalibacter* were identified as differentially abundant by all the methods used, while *Enterococcus* was shared between ANCOM-BC and LEfSe, and *Snodgrassella* was

shared between DESeq2 and LEfSe. Moreover, Wilcoxon-test of relative abundances confirmed that Lactobacillus (W = 15, $P < 0.001$) and *Fructobacillus* (W = 8, $P < 0.001$) were more abundant in NI, while Bartonella (W = 143, $P = 0.002$) and *Commensalibacter* (W = 140, $P = 0.003$) were more abundant in DWV bees (Figure 25).

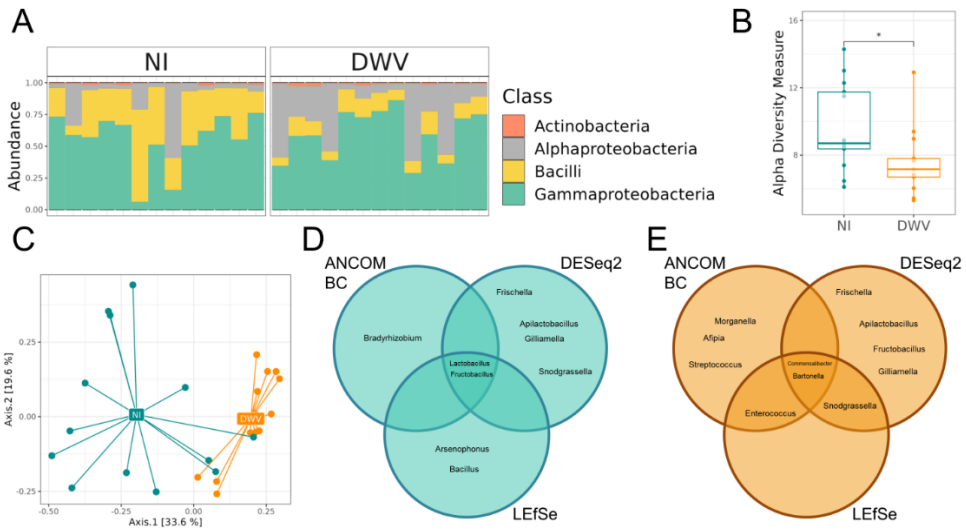


Figure 22 - DWV infection alters gut microbiota composition of honey bees. (A) Relative abundances at Class level. (B) α-diversity (inverse Simpson index) per infection group (Wilcoxon-test: W = 44, $P = 0.039$). Boxes denote inter-quartile range, bar denotes median, and whiskers denote range.(C) Principal coordinate analysis of bacterial compositions per infection group. Centroids are depicted as labels for NI and DWV groups. PERMANOVA indicated a significant difference between groups ($F_{(1, 24)} = 6.6744$, $P = 0.001$). (D) and (E) Differentially abundant genus associated to low and high infection group, respectively. The integrated analysis included significant results using ANCOM-BC ($P < 0.05$), DESeq2 ($P < 0.01$) and LEfSe ($P < 0.05$, LDA score > 2). n.s.: non-significant, *P < 0.05, **P < 0.005, ***P < 0.001.

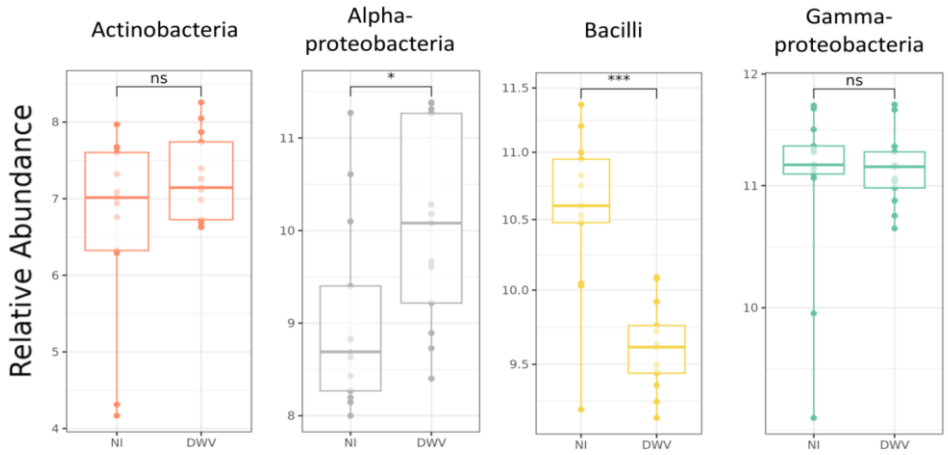


Figure 23 - Relative abundances of most representative bacterial classes in bees injected with DWV lysates (DWV) and non-injected (NI). Alphaproteobacteria were more abundant in DWV-injected bees (Wilcoxon-test: W = 132, P = 0.014) , while Bacilli were more abundant in non-injected bees (Wilcoxon-test: W = 16, P < 0.001). Relative abundance data were transformed to the centered log-ratio scale (clr) before Wilcoxon-test. n.s.: non-significant, *P < 0.05, **P < 0.005, ***P < 0.001.

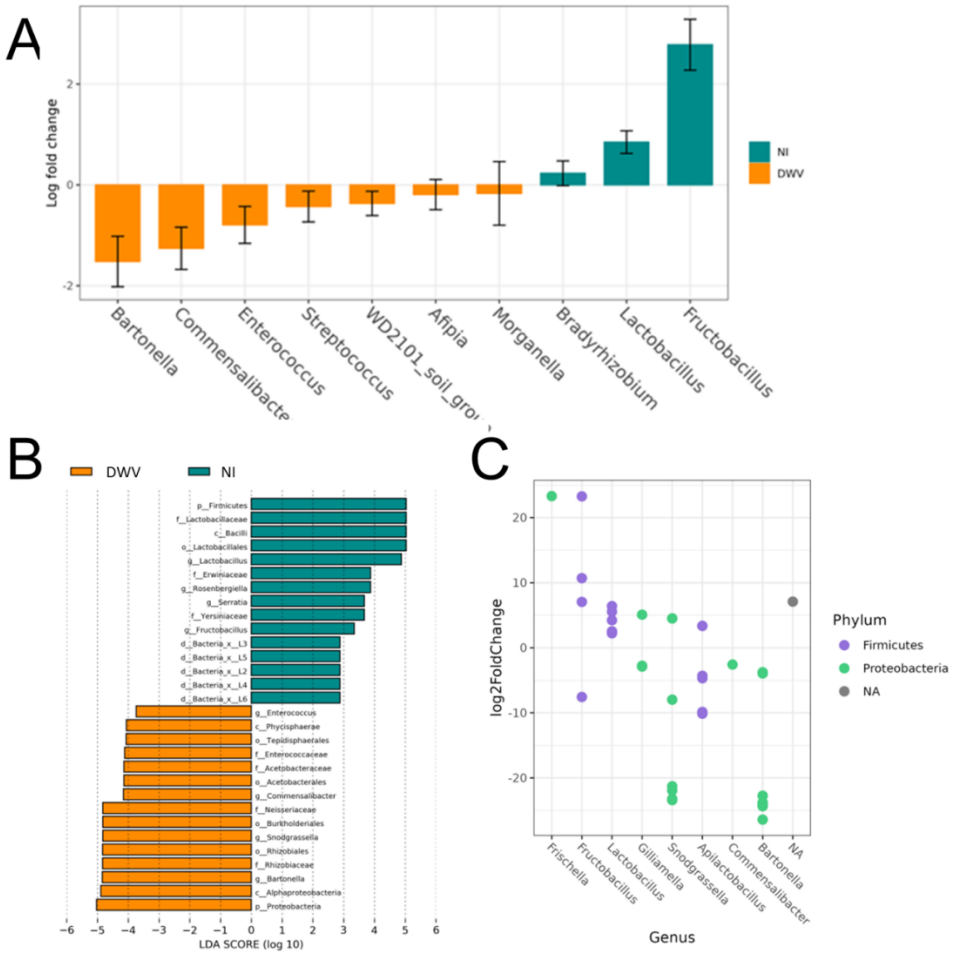


Figure 24 - Differential abundance analyses of gut community between non-injected (NI) and DWV-injected bees (DWV). (A) Significant ANCOM-BC results ($P < 0.05$) included seven and three genera associated to DWV and NI, respectively. (B) At the genus level, LEfSe analysis ($P < 0.05$, LDA score > 2) identified four and four taxa associated to DWV and NI, respectively. (C) Significant DESeq results ($P < 0.01$) of NI vs. DWV bees. Firmicutes were associated with low DWV loads, such as *Lactobacillus* and *Fructobacillus*, while Proteobacteria were associated with high DWV loads, such as *Bartonella* and *Commensalibacter.*

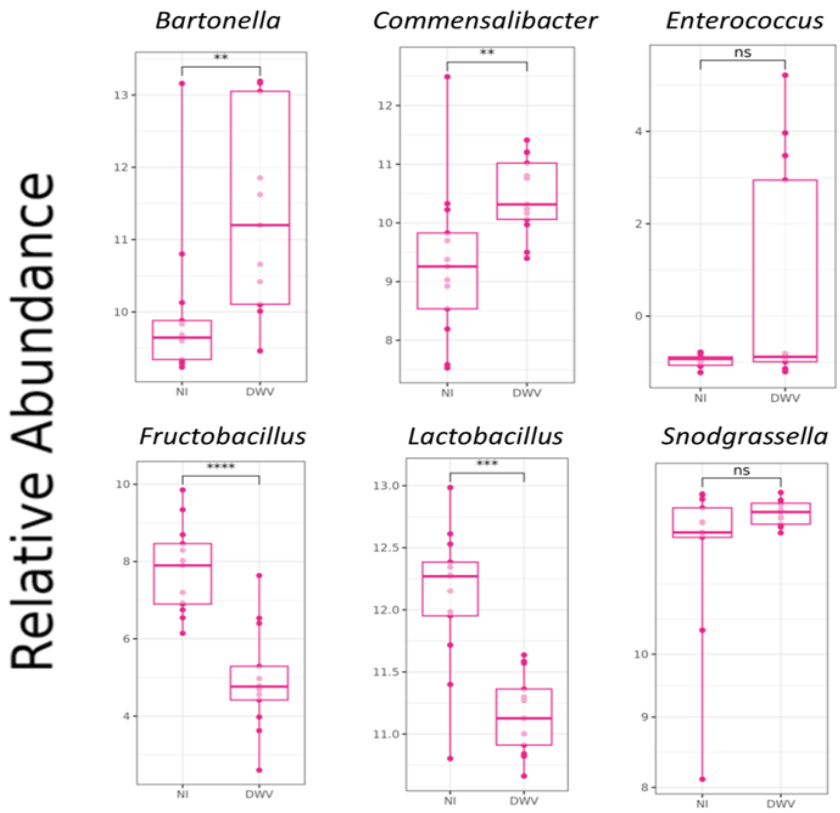


Figure 25 - Relative abundances of differentially abundant bacterial genera in DWV-injected (DWV) and non-injected bees (NI). Wilcoxon-test revealed that Lactobacillus (W = 15, P < 0.001) and *Fructobacillus* (W = 8, P < 0.001) were more abundant in NI, while Bartonella (W = 143, P = 0.002) and *Commensalibacter* (W = 140, P = 0.003) were more abundant in DWV bees. No significant differences were observed for *Enterococcus* and *Snodgrassella*. Relative abundance data were transformed to the centered log-ratio scale (clr) before Wilcoxon-test. n.s.: non-significant, **P* < 0.05, ***P* < 0.005, ****P* < 0.001.

2.3.3 Effect of *Lactobacillus* probiotics on survival and DWV load in naturally infected bees

To provide direct experimental evidence supporting the potential link between the DWV infection and the alteration of the gut microbiota composition, we performed rescue experiments to recover honey bee immunity.

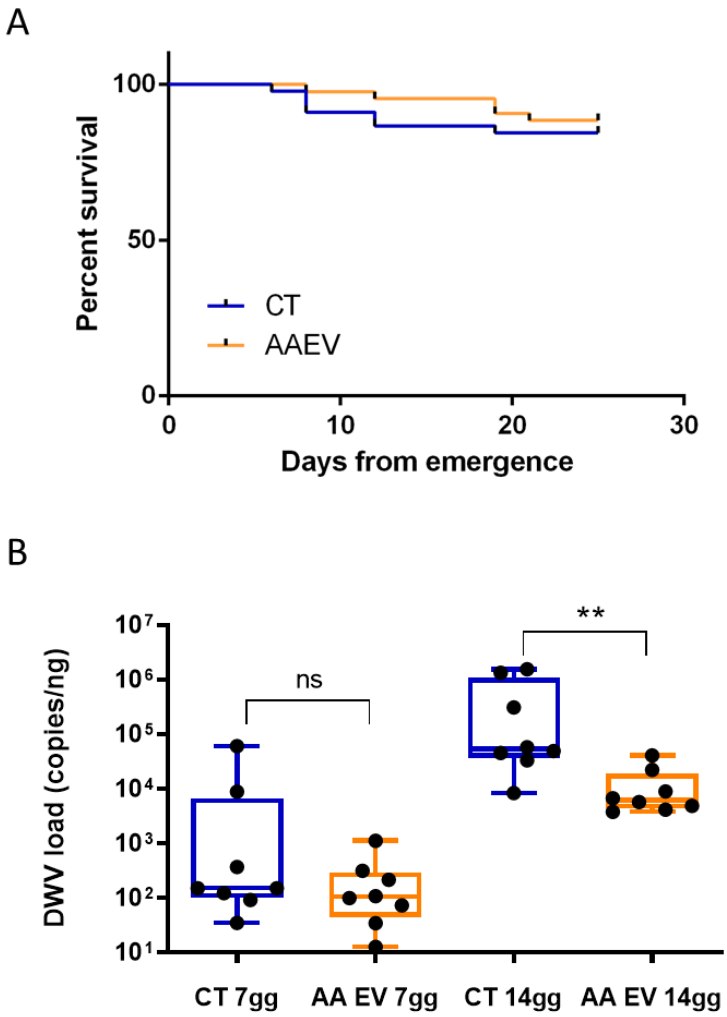


Figure 26 - Effects of *Lactobacillus* probiotics on survival and DWV load in naturally infected bees. (A) Survival rate of adult bees fed with sucrose solution alone (CT) or with a *Lactobacillus* supplement (AA EV) did not show significantly different (log-rank test: $\chi 2(1) = 0.361$, P value = 0.548). (B) Number of DWV copies in experimental adult bees at 7th and 14th day of feeding with sucrose solution alone (CT) and with a *Lactobacillus* supplement (AA EV). After 14 days bees which were fed *Lactobacillus* probiotics (10^7 CFU/mL) daily showed a reduced DWV load (Wilcoxon test W= -34.00, P = 0.0156 or Mann Whitney test: n = 8, P = 0.0019). Values on Y-axis are reported in Log10 scale. Boxes denote inter-quartile range, bar denotes median, and whiskers denote range. Points represent individual bees. n.s.: non-significant, *P < 0.05, **P < 0.005, ***P < 0.001.

We attempted to rescue honey bee health through the administration of different strains of *Lactobacillus*, a genus which is depleted in

DWV-infected bees. The survival rate was not significantly different between group of adult bees fed with a 1:1 mix of *L. fermentum* (AA) and *L. casei* (EV) bees fed without *Lactobacillus* supplement (CT) (Figure 26A). After 14 days of administration of mixture of *Lactobacillus* (AAEV), naturally infected honey bees show a reduced DWV load, compared to control bees fed with sucrose solution alone (Figure 26B). No significant influence of probiotics was observed after 7 days of food supplementation.

2.4 DISCUSSION

In this study, we investigated the impact of DWV infection on the honey bee gut microbiota using a combination of field sampling and controlled experimental infections. In a first experiment, involving nurse bees sampled during the summer, we identified different groups of samples based on their microbiota profile and compared their DWV loads through RT-qPCR. In a complementary experiment involving artificial DWV infection, we validated our findings by analyzing the gut microbiota composition of artificially infected bees, providing a controlled setting to establish a causal relationship between DWV infection and microbiota changes.

Genotype specific quantification of DWV pointed out the co-occurrence of both DWV-A and DWV-B variants, with DWV-A significantly higher than DWV-B load in almost all bee samples collected. This, to our knowledge, represents the first report of DWV-B infection in honey bee hives from Campania (South Italy). Considering that DWV-A and DWV-B loads were positively correlated, as observed in other studies *(Dubois et al., 2020; Ryabov et al., 2017)*, we quantified DWV load as the sum of the two genotype-specific loads.

In both scenarios of field-sampled bees, in which DWV load was treated as a continuous variable, and artificially infected bees, where we analyzed the viral load in categorical terms of “low” or “high”, our study pointed out a negative correlation between DWV load and the expression of dorsal-1A, a member of NF-kB family, playing a central role in the immune response *(Evans et al., 2006)*. This aligns with previous studies that have highlighted the immunosuppressive effects of DWV infection on honey bees *(Barroso-Arévalo et al., 2019; Nazzi et al., 2012).* A recent study suggested that dorsal-1A may participate in the regulation of Dual Oxidase (Duox) for ROS production in the

honey bee gut, which acts as the effector in inhibiting non-native bacterial strains *(Guo et al., 2023)*.

The reduced expression of dorsal-1A in response to DWV infection suggests a potential mechanism through which DWV compromises the host immune response, rendering the bees more susceptible to both DWV infection itself and microbial dysbiosis.

Indeed, our microbial community analysis, based on Bray-Curtis dissimilarity, revealed significant differences in core gut microbiota composition between bees with varying levels of DWV infection, in both field-sampled and artificially infected bees. In artificially infected bees experiment, aimed at simulating DWV transmission through *V. destructor* feeding, PCA indicated a clear separation of gut community composition between infected and non-infected bees, which were all fed with an equal dose of the same gut homogenate, highlighting the potential impact of DWV infection on shaping the gut microbiota landscape. This community shift is likely the result of an active DWV infection during microbiota colonization and establishment in the gut. A common feature of the observed compositional shifts is the increase in *Bartonella* (*Alphaproteobacteria*) and *Tyzzerella* (*Clostridia*) a depletion of *Fructobacillus*, *Lactobacillus* and *Apilactobacillus* (*Firmicutes*, Bacilli), as indicated by our differential abundance analysis. This kind of compositional shift is typical of honey bee gut dysbiosis, characterized by a drastic depletion in certain core taxa (e.g., *Betaproteobacteria* and *Lactobacillus* spp.) coupled with a bloom in other core species (e.g., *Alphaproteobacteria*) *(Daisley et al., 2020a; Raymann et al., 2017)*. *Lactobacillus* depletion is not a novel feature of honey bee dysbioses. Indeed, dysbioses deriving from xenobiotics, such as the herbicide glyphosate, are characterized by a depletion of *Lactobacillus* species (*Lactobacillus Firm-4* and *Firm-5*) at five days from treatment *(Motta et al., 2018)*. *Lactobacillus* depletion occurs also in response to nutritionally poor-quality diet. Indeed, the consumption of *Eucaliptus grandis* pollen was associated with a low abundance of *Lactobacillus mellifer*, *Lactobacillus apis* (*Firm-4* and *Firm-5*, respectively) and *Bifidobacterium* spp., and a high abundance of *Bartonella apis* *(Castelli et al., 2020)*, a compositional shift very similar to what we observed.

Lactobacillus species are known probiotics with a potential use in beekeeping *(Daisley et al., 2020b),* and their depletion in highly infected bees is indicative of their putative role in conferring protection against DWV infection. Indeed, we demonstrated that DWV infection can be limited by supplementing honey bees with *Lactobacillus*, through an experiment under lab conditions. The

treatment with probiotics has no negative effect on survival rate of experimental bees over 25 days. The reduction of DWV observed at 14th day of treatment and not after 7 days may reflect the time it takes for the immune system to be stimulated by the probiotic supplement and act on the viral load.

Our results raise interesting questions about the potential role of *Alphaproteobacteria* in responding to or interacting with DWV infection. In particular, *Bartonella* *(Kešnerová et al., 2016)* was associated with high DWV loads in both experiments. Interestingly, a capture-release study based on 14-day old adult bees from Varroa-resistant and -susceptible colonies in Gotland (Sweden) showed that *Bartonella apis* was consistently associated with colonies susceptible to the parasite *(Thaduri et al., 2021),* which is the most important vector of DWV. Blooming of *Bartonella* in honey bee gut is associated with wintering bees *(Kešnerová et al., 2020)* and seems to be related to the capacity of this species in using more diverse energy substrates, such as metabolic wastes lactate and ethanol *(C. Li et al., 2022)*, suggesting a sort of opportunistic behaviour *(Anderson & Ricigliano, 2017)*. Such behaviour is not surprising if we consider that Bartonella apis, as well as the recently described *Bartonella choladocola* sp. nov. and *Bartonella apihabitans* *(Liu et al., 2022)*, represents the closest relative of pathogenic *Bartonella species* *(Segers et al., 2017).*

In larvae, *Tyzzerella* was associated with high DWV loads. The abundance of this genus increased significantly in larvae exposed to the herbicide Glyphosate as reported by a recent study *(Vázquez et al., 2023)*. Further research is needed to elucidate the functional roles of these taxa in relation to DWV susceptibility and possible contributions to the observed immunosuppression. Considering that our study, limited to 16S rRNA amplicon sequencing analysis, cannot provide reliable information beyond the genus level, where a great functional diversity is expected *(Ellegaard & Engel, 2019)*, future studies would benefit from an approach based on isolation and genomic or shotgun-metagenomic analysis.

The overall impact of DWV infection on gut community was even more marked in our experiment involving artificially infected bees. Indeed, in artificially infected bees a significant reduction in α-diversity is observed, indicating a potential destabilizing effect of the viral infection on the gut microbial diversity. The loss of microbial diversity has been already described by studies on virus-associated gut dysbiosis in mammals *(Bernard-Raichon et al., 2022; Vujkovic-Cvijin et al., 2020)*, and in honey bees is reported as the effect of different

stressors *(Raymann et al., 2017; Ye et al., 2021)*, suggesting that the decrease in α-diversity is a common signature of gut dysbiosis.

In conclusion, our study provides novel insights into the intricate relationship between DWV infection, host immunocompetence, and honey bee gut microbiota composition. We demonstrated that DWV infection negatively correlates with host immunocompetence and is associated with specific shifts in gut microbial community composition. The identified microbial taxa that respond to DWV infection could potentially serve as biomarkers for honey bee health assessment and management. Further research is needed to unravel the underlying mechanisms driving these interactions and to determine their impact on honey bee colony health and resilience in the face of viral infections. An unanswered question is: Is DWV-induced dysbiosis the result of a systemic infection in which gut immunity is compromised? Or other routes, such as brain-gut axes, are involved? Understanding how viral infections shape the honey bee gut microbiota will have broad implications for honey bee health and colony survival, with potential applications in the development of strategies to mitigate the impacts of viral infections on pollinator population.

CHAPTER 3

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF DWV PROTEIN INTERACTORS

3.1 INTRODUCTION

Pathogenic viruses affecting honey bees are among the most intensively studied pathogens due to their great impact on beekeeping, being one of the main causes of honey bee colony losses *(Allen & Ball, 1996; Kielmanowicz et al., 2015)*. However these viruses may represent a big threat at ecosystem scale, due to their capacity to be transmitted from honey bee to other bees, via shared flowers *(Yañez et al., 2020)*.

Among the many viruses infecting honey bees, the most studied is Deformed Wing Virus (DWV), an endemic RNA positive single-stranded virus *(de Miranda & Genersch, 2010),* which is vectored by the parasitic mite *Varroa destructor* *(Van Der Zee et al., 2012; Wilfert et al., 2016)*. At low viral loads DWV is asymptomatic and occurs in the nearly totality of hives *(Brutscher et al., 2015; Nazzi & Pennacchio, 2014)*. But, when honey bee colonies are heavily infested by *V. destructor*, DWV rapidly spreads and the transition from common asymptomatic infections to devastating manifest infections occurs *(de Miranda & Genersch, 2010; Mockel et al., 2011)*.

The increasing viral infection is a self-boosted process, as the growing DWV titer generates an escalating immunosuppression, by targeting NF-kB signalling through an as yet unknown mechanism, which has devastating consequences on bee health *(Di Prisco et al., 2013; Nazzi*

et al., 2012; Nazzi & Pennacchio, 2014). Understanding the molecular mechanisms underlying DWV infection, such as protein-protein interaction at the host-pathogen interface or co-infection dynamics with other pathogens *(Farooq et al., 2021)*, may offer the opportunity to develop new management strategies and therapeutic approaches based, for example, on RNA interference via dsRNA administration to honey bee colonies.
Here, in collaboration with Dr. Anna Valenti group of the Institute of Biosciences and BioResources - CNR, Naples, we used a proteomic approach based on co-immunoprecipitation experiments coupled to mass spectrometry to identify the honey bee proteins interacting with DWV. This analysis led to the selection of the target protein Arginine kinase, which was subsequently characterized from a functional point of view.

3.2 MATERIALS AND METHODS

3.2.1 Biological material

A. mellifera colonies were maintained at the experimental apiaries of the Department of Agricultural Sciences (University of Napoli Federico II), based in Portici (Napoli, Italy). Adult bees and brood frames were collected between June and August and stored at 32 ± 1°C, 60 ± 2% relative humidity, under dark conditions.

3.2.2 Protein isolation

To obtain samples to be processed for studying protein interactors involved in DWV infection, symptomatic and asymptomatic adult honey bees were collected from highly infested and *Varroa*-free apiaries, respectively, and stored at -80 °C until protein extraction. Head and rest of the body of each sampled honey bee were separated and individually collected in different microcentrifuge tubes containing 250 and 500 uL 1X PBS + protease inhibitor cocktail (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma Aldrich), respectively. Tissues were grinded using plastic pestles, centrifuged for 5 minutes at 13,000 g at 4 °C and the aqueous part was then transferred to fresh tubes, leaving tissue debris pellet behind. Protein extracts were stored in 20% glycerol at – 80°. Before

storage, total protein concentration of each sample was measured as described by Bradford *(1976)*, using the reagent Coomassie Protein Assay Reagent (Thermo Scientific) and bovine serum albumin (BSA) as standard.
RNA was isolated from the pelleted debris using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), to distinguish DWV-infected from DWV-free samples, as described below.

3.2.3 Western blot analysis

In collaboration with Dr. Anna Valenti group of the Institute of Biosciences and BioResources - CNR, Naples, honey bee protein extracts obtained were used to perform immunoprecipitation experiments. Total protein extracts, obtained by mixing head and body protein extracts, prepared as described above, were analyzed by Western blot (WB), to check the presence of viral proteins in symptomatic and asymptomatic honeybees. Samples were run in 8% polyacrylamide gels in 1x Tris-Glycine SDS Running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3). After electrophoresis, proteins were transferred onto PVDF filters (Bio-Rad) using the Trans-Blot1 TurboTM Blotting System (Bio-Rad). Subsequently, the PVDF membrane was stained with Ponceau-S and rinsed with water. The data were saved using a scanner. To remove Ponceau-S from stained proteins, the PVDF membrane was washed with PBS with 0.1% Tween® 20 Detergent (PBST; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM $KH2PO_4$, 0.05% Tween 20, pH 7.4) several times for 5 min under shaking. Subsequently, the PVDF membrane was incubated with the PVDF blocking reagent for 60 min at room temperature and washed thrice with TBST for 5 min with shaking. After blocking, the PVDF membrane was incubated with a policlonal antibody (DWV-Ab) generated against DWV particles and purified in the laboratory of Brenda Ball at Rothamsted Research (Harpenden, UK). The primary antibody was used at dilution of 1:2,000 for 1 hour and with a secondary anti Rabbit antibody (PIERCE) at dilution of 1:50,000. Chemiluminescent bands were revealed after incubation with Biosciences ECL Plus solution using a VersaDoc apparatus (Bio-Rad).

3.2.4 Immunoprecipitation

Immunoprecipitation (IP) experiments were performed using the DWV-Ab and protein extracts from symptomatic or asymptomatic honeybees. Control IP experiments were performed using an antibody produced in rabbit (C-Ab). All IP steps were performed on ice or at 4 °C using freshly thawed protein extracts and Buffers, accordingly to manufacturer's instructions (ROCHE Protein A Agarose Code: 11134515001) and adding protease inhibitors at all Buffers used (Roche). In order to reduce unspecific protein binding to the beads, protein extracts (0,5 mg) were incubated with protein A agarose (60 uL) for 2 hours at 4°C with gentle agitation, afterward each sample was centrifugated and a half of the soluble fraction was incubated with DWV-Ab or with C-Ab for 1 hour at 4°C. After incubation, 30 uL of Protein A agarose were added to each sample and incubated O/N at 4°C. Finally, co-immunoprecipitated proteins were analyzed by SDS-PAGE and western blot. For mass spectrometry analysis, four independent coIP experiments (both for sample and control) were performed and an aliquot was analyzed by western blot to check the presence of virion proteins. Co-immunoprecipitated proteins were run on SDS-PAGE and subjected to mass spectrometry analysis as described below.

3.2.5 LC-MS/MS

All immunoprecipitated proteins were opportunely separated by SDS-PAGE, gel extracted, digested, and identified by Mass Spectrometry (in collaboration with the Core Facilities at Istituto Superiore di Sanità (ISS) of Rome).
Immunoprecipitated samples in quadruplicate were separated in a precast polyacrylamide gradient (4-12%) gel (Invitrogen Carlsbad, CA, USA), run in MES buffer (Invitrogen Carlsbad, CA, USA) for 25 min at 200 V and the proteins were stained with the Imperial Coomassie stain (Invitrogen). Each lane was divided into 6 bands, 48 bands in total, which where destained with 50mM ammonium bicarbonate and acetonitrile (ACN) 1:1 (v/v), treated for cysteine reduction (10mM dithiothreitol at 56°C for 45min) and alkylation (55mM iodoacetamide at RT for 30min in the dark), and for enzymatic digestion with 12.5ng/µl trypsin (Promega Corporation, WI, United States) at 37°C overnight. The resulting peptide mixture was analyzed by LC-MS/MS using an Ultimate 3000 HPLC coupled with an Orbitrap Fusion Tribrid (Thermo Fisher Scientific, CA, United States). Peptides were desalted

on a trap column (Acclaim PepMap 100 C18, Thermo Fisher Scientific) and separated on a 19 cm long silica capillary (MS WIL, Aarle-Rixtel the Netherlands), packed in-house with a C18, 1.9um, 100 A resin (Michrom BioResources, CA, United States). The analytical column was encased by a column oven (Sonation; 40 °C during data acquisition) and attached to a nanospray flex ion source (Thermo Fisher Scientific, CA, United States). Peptides were separated on the analytical column by running a 95 min gradient of buffer A (5% ACN, and 0.1% formic acid) and buffer B (95% acetonitrile and 0.1% formic acid), at a flow rate of 250 nl/min. The mass spectrometer operated in positive ion mode and full MS was acquired in the Orbitrap in the m/z 350–1550 scan range at 120K resolution. Data-dependent acquisition was performed in top-speed mode (3 s long maximum total cycle): the most intense precursors were selected through a monoisotopic precursor selection (MIPS) filter and with charge >1, quadrupole isolated (1.6 m/z width) and fragmented by 30% higher-energy collision dissociation. Product ion spectra were acquired in the ion trap with a rapid scan rate.
Peptide spectra were analyzed with Proteome Discoverer 2.4 (Thermo Fisher Scientific) by the search engine Sequest HT using a mixed database containing *Apis mellifera* (reviewed and unreviewed 20818 sequences; release 26/01/2022), *homo sapiens* (42253 sequences, v2017-10-25) and *Oryctolagus_cuniculus* (892 sequences, release 07/03/2022), and *Deformed wing virus* (1067 sequences, release 26/01/2022), all downloaded from Uniprot (https://www.uniprot.org/).
Precursor and fragment mass tolerance were set to 15 ppm and 0.6 Da, respectively. Cysteine carbamydomethylation was set as static modification, while methionine oxidation and N-acetylation on protein terminus were set as variable modifications. Specific trypsin cleavages with maximum two miscleavages were admitted. Spectral matches were filtered at 1% false discovery rate (FDR), based on q values, and target-decoy approach, using the Percolator node. Only master proteins were taken into account.
Quantification was based on precursor intensity of unique and razor peptides.

3.2.6 Bioinformatic analysis

The Perseus software (1.6.15) was used to perform statistical analyses and evaluate differential protein expression.
After contaminant removal (rabbit and human proteins), the abundance values were log2 transformed and filtered for containing at

least four valid values in at least one group. Missing values were denoted with the constant value 13. A Student's t-test was performed applying 250 randomizations, setting S0=2 and 0.01 FDR, and used to draw a Volcano plot to evaluate significantly different IP/IgG ratios.
MS spectra were obtained using Orbitrap instrument and analysed by software Proteome Discoverer software identifying 405 proteins of which 372 were also quantified. The quantitative analysis was performed by evaluating the ratio between IP and its relative IgG negative control using the ANOVA test and calculating the abundance of each protein as the sum of the intensities of all the peptides that derive from it. A PCA (Principal Component Analysis) analysis was performed using the Perseus software, showing a clear distinction between the samples of the control and IP IgG groups.

3.2.7 *ArgK* expression and DWV load in field-collected bees

To localize *ArgK* expression, honey bee pupae and newly emerged adults were dissected using microforceps, under a stereomicroscope (SteREO Discovery V8, Zeiss, Jena, Germany). Brain, hemolymph and gut samples were collected in microcentrifuge tubes containing TRIzol reagent (Thermo Fisher Scientific). To study the relationship between DWV infection and *ArgK* expression in field conditions we sampled mature larvae (4-5 days from hatching), nurse bees and foragers from two hives. Nurse bees were identified by observing their activity of nursing immatures on the frame. The two hives were in similar conditions of strength and provisions. Sampled bees and tissues were stored at -80 °C until RNA extraction.

3.2.8 Controlled DWV infection in vivo

To assess the effect of DWV infection on *ArgK* expression, we artificially infected newly emerged bees and maintained them under controlled conditions. Newly emerged bees were injected with a DWV lysate obtained as described elsewhere *(De Miranda et al., 2013)*. Briefly, adult bees with symptomatic DWV infection were homogenized in 500 µL 1X PBS in 1.5 mL microcentrifuge tubes. Tubes were centrifuged at 14,000 g for 10 minutes at 4°C and supernatant was clarified by adding an equal volume of chloroform. Samples were vortexed for 30 seconds, centrifuged at 10,000 g for 10 minutes at 4°C and supernatants were filtered through 0.22 µm sterile syringe filters (VWR, Radnor, Pennsylvania, USA). Lysates were

stored in 40% glycerol at -80° until use. RNA isolated from a subsample of the viral lysates were evaluated for DWV load by qRT-PCR, as described below, and for the presence of other common bee viruses by PCR, as described elsewhere (Chen et al., 2006b). Newly emerged bees were cold-anesthetized at -20° for 3 minutes and then either injected (or not) with 2.5 µL of 1X PBS containing 10^4 DWV copies, under a stereomicroscope (SteREO Discovery V8, Zeiss, Jena, Germany). Viral lysates were injected between 4th and 5^{ft} abdominal segments by using a beveled 33G NanoFil Needle, (NF33BV-2) mounted on a 10 µL NanoFil syringe (World Precision Instruments, Berlin, Germany). After injection each bee was manually fed with 5 µL of honey bee gut homogenate, using a micropipette, to establish the characteristic gut community, as described elsewhere (*Dosch et al., 2021; Kwong et al., 2017a; Powell et al., 2014)*. Briefly, to obtain freshly prepared gut homogenates, whole guts, dissected from four nurse bees, were homogenized in 500 µL 1X PBS and diluted 1:1 with 50% filtered sucrose/water solution. DWV-injected and non-injected controls were confined in separated disposable plastic cages (28x11x11 cm) and provided with pollen paste (90% w/w fresh corbicular pollen with water) and 50% filtered sucrose/water solution (w/v) *ad libitum*, inside an incubator at the abovementioned conditions. After 3 days, experimental bees were sampled and stored at -80°C until dissection.

3.2.9 dsRNA synthesis

Gene-specific primers flanked by T7 promoter sequences (F: TAATACGACTCACTATAGGGAGAATTTGCTGACCTCTTCGACCC; R: TAATACGACTCACTATAGGGAGAAAAACCTGTCCAAGGTCACCG) were designed to amplify a 496 bp fragment of *ArgK* (AF023619.1), which is not targeted by the qRT-PCR primers used to evaluate the level of ArgK expression.

cDNA from adult females of *V. destructor* was obtained as described above and used as template (2 µL) for PCR reactions containing the following components: 25 µL Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), the two primers at 500 nM final concentration and nuclease-free water to a total volume of 50 µL. The cycle conditions were: 10 s at 98°C; 1s at 98°C, 5 s at 66°C and 15 s at 72°C for 5 cycles; 1s at 98°C and 15 s at 72°C for 30 cycles. Four samples were assembled to produce at least 1 µg of the amplicon, the minimum required for subsequent dsRNA synthesis. 5 µL of the

amplified products were run on a 1% agarose gel to verify that the PCR reaction produced a single band of the expected size. The amplified products were then purified with PureLink PCR Purification Kit (Thermo Fisher Scientific), eluted in nuclease-free water, and quantified measuring the absorbance with Varioskan Flash (Thermo Fisher Scientific). Following the manufacturer's instructions (MEGAscript RNAi kit, Thermo Fisher Scientific), the transcription reactions were assembled using 1.2 µg of purified PCR product. After DNA and ssRNA digestion, the dsRNA was purified and eluted in 50 µL of a 0.9% NaCl solution. The dsRNA concentration was determined spectrophotometrically, and the quality checked on a 1% agarose gel. A GFP dsRNA, exploited in control experiments, was similarly produced starting from the cloning vector pcDNA 3.1/CT-GFP TOPO (Thermo Fisher Scientific), which was used as template for a PCR reaction, performed as described elsewhere (Caccia et al., 2020).

3.2.10 Honey bee cell culture

To investigate the role of *ArgK* in DWV infection process, we used an *in vitro* system based on culturing primary honey bee pupal cells, as described elsewhere *(McMenamin et al., 2021; Watanabe et al., 2021)*. To collect pupal cells, white eye stage pupae were carefully removed from comb cells using soft tip forceps and surface-sterilized in a sterile polystyrene petri dish (100 mm × 15 mm, Fisher), in which pupae were swirled in 0.6% hypochlorite solution (diluted bleach 1:5) for 3 min, 70% ethanol for 3 min, and briefly rinsed in autoclaved water. In groups of two, four pupae were placed in in a 47 mm dish containing 4 mL of supplemented Grace's insect medium (Gibco, Thermo Fisher Scientific) with 10% heat-treated fetal bovine serum (FBS, Corning, NY, USA) and 2.5% Antibiotic/antimycotic mix (A5955, Sigma Aldrich). Thorax and abdomen of pupae were opened dorsally using sterile forceps to vigorously disturb tissues and release cells into the medium. The medium containing haemolymph cells was filtered using a cell strainer (Corning Cell Strainer, 40 µm Nylon, Corning) to remove fat bodies and other debris, and transferred to a 50 mL conical tube, where cells were pooled. A total of 15 mL of media (containing cells from 15 pupae) was centrifuged at 500 g (swing arms) at room temperature for 15 min to collect cells. Cells were then suspended in 1 ml of culture medium and centrifuged at 300 g for 5 min (swing arms). This washing step was repeated once more, and the cells were suspended in 1 mL of culture medium. Resuspended

cells (100 µL) were dispensed into a well of a 96-well tissue culture plate (Costar 3595, Corning), which was sealed with parafilm tape and incubated at 32 ± 1°C, 60 ± 2% relative humidity, under dark conditions.

3.2.11 *ArgK* silencing and DWV infection in vitro.

To investigate the effect of *ArgK* silencing on DWV infection, dsRNA (1 µg/well) was applied to day 0 cells (the start of the ex vivo procedure). On day 3 cells were inoculated with 1×10^5 total-DWV copies and harvested on day 5.
Cells viability was assessed using the trypan blue exclusion assay as previously described *(Becchimanzi et al., 2017)* with slight modifications. Briefly, 50 µL of 0.4% trypan blue stain solution (Gibco, Thermo Fisher Scientific) were added to cells directly into a well of the plate. After 5 minutes of incubation, non-adherent cells and medium were aspirated using a micropipette and adherent cells were washed twice with 200 µL of 1X PBS. Cells adherent to the well bottom were submerged with 100 µL of 1X PBS and observed under an inverted microscope (Axiovert 135, Zeiss).
To isolate RNA from cultured adherent (alive) cells, wells were gently washed twice with 200 µL of 1X PBS to remove viral particles and non-adherent cells. Then, 100 µL of TRIzol reagent (Thermo Fisher Scientific) was added to each well, mixed by pipetting and the plate was incubated for 10 min at -80 °C, to promote cells lysis by freeze-thaw. Plates were incubated for 5 min at RT and the content was transferred in plastic tubes to perform RNA isolation.

3.2.12 RNA isolation and RT-qPCR

RNA was isolated using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. The quantity and the quality of DNA and total RNA were assessed using Varioskan Flash spectrophotometer (Thermo Fisher Scientific).
DWV genome and *ArgK* copies was performed using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems) as described elsewhere *(Annoscia et al., 2020)*. All primers used are shown in **Table 5**. Primers for *ArgK* were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Due to the co-occurrence of both DWV-A and DWV-B variants in the sampled bees, we used primers targeting a conserved section for multiple DWV variants (total-DWV) *(M. M. Bradford, 1976)*. The absolute

quantification of total-DWV and *ArgK* copies was performed by relating the Ct values of unknown samples to established standard curves. Each standard curve was established by plotting the logarithm of 10-fold dilutions of a starting solution containing 0.1 ng of purified PCR product (PureLink PCR Purification Kit, Thermo Fisher Scientific), against the corresponding Ct value as the average of three repetitions. The PCR efficiency was calculated based on the slope and coefficient of correlation (R^2) of the standard curve, according to the following formula: $E = 10^{(-1/\text{slope})} - 1$ (**Table 6**). Loads of total-DWV and *ArgK* were expressed as copies per nanogram of total RNA.
Differential relative expression was measured by one-step RT-qPCR, using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. Each reaction was prepared in 20 µL and contained 10 µL qRT-PCR mix 2X, 100 nM of forward and reverse primers, 0.16 µL of 125X RT enzyme mix, DEPC treated water and 50 ng of total RNA. All samples were analyzed in duplicate on a Step One Real Time PCR System (Applied Biosystems). *β- actin* was used as endogenous control for RNA loading. Relative gene expression data were analyzed using the ΔΔCt method (*Livak & Schmittgen, 2001*).

Table 5 - Primers used for quantification of DWV load and gene expression by qPCR

Primer Name	Sequence	Target organism	Target gene	Amplicon size (bp)	Tm (C°)	Reference
Pan DWV_f	ACGCAACCCCAGGAATC	DWV variants	DWV genome	168	65.5	(Bradford et al., 2017) Modified
Pan DWV_r	TACCCAATCTTTAAATTGT TTCGGT				65.8	
qARGk_f	CGGCTTCTTTTCTCACAC AATG	*Apis mellifera*	*ArgK*	60	64.2	This work
qARGk_r	CCAAGTTAGTCGGGCAGA AAGT				64.5	
qACTf	TTGTATGCCAACACTGTC CTTT	*Apis mellifera*	*β-actin*	120	65.2	(Simone et al., 2009)
qACTr	TGGCGCGATGATCTTAAT TT				66.8	

Table 6 - Standard curves used for absolute quantification by qPCR

Target	Equation	R^2	Efficiency %
panDWV	y = -3.3744x + 40.818	0.97	104.72
ArgK	y = -3.2138x + 37.221	0.99	97.85

3.2.13 Statistical analyses

To analyze correlation between DWV load and *ArgK* expression we used Spearman's rank correlation. Kruskall-Wallis followed by Dunn's post hoc test were used to analyze *ArgK* expression in different honey bee tissues, DWV load and *ArgK* expression following controlled DWV infection. Student's t-test was used to analyze the effect of gene silencing on the expression of *ArgK* and DWV relative abundance. The assumption of normal distribution of data was tested and met via Shapiro-Wilk test. Each dataset was checked for homoscedasticity using Levene's test. All statistical analysis were performed using GraphPad Prism 7.

3.3 RESULTS

3.3.1 Identification of honey-bee proteins interacting with DWV particles

The quantitative proteomics analysis identified 405 proteins of which 142 were honey bee proteins associated with DWV capsid proteins. Data analysis of mass spectrometry results showed that the majority of honey bee proteins associated with the viral proteins are involved in fundamental biological processes, included translation, catabolic functions, cytoskeleton organization and protein transport. Among these, Arginine kinase (*ArgK*) is a member of the phosphagen kinase family and plays an important role in cellular energy metabolism in invertebrates *(Alonso et al., 2001)*, by catalyzing the reversible phosphorylation between phosphoarginine and ADP. Moreover, *ArgK* is an essential component of the RNA-induced silencing complex *(Alonso et al., 2001; Barik & Banerjee, 1992)*. The ability of many viruses to use viral and cellular kinases for their replication *(Ma et al., 2014; J. Wang et al., 2013)* and the involvement of *ArgK* in antiviral immunity, due to its significant upregulation in the shrimp *Litopenaeus*

vannamei infected with white spot syndrome virus (WSSV) (Ma et al., 2014), suggested its potential role in the DWV infection.

3.3.2 Functional characterization of ArgK

The expression profile in different host tissues and developmental stages assessed by qRT-PCR analysis indicated that *ArgK* gene is highly transcribed in the brain of newly emerged adult bees (Figure 27A), more than 10-fold compared to adult hemolymph samples. In pupal stage a completely different pattern was observed, with a reduced expression in brain and no statistical differences between sampled tissues. Spearman's rank correlation test revealed that *ArgK* expression and DWV load were positively correlated in field-collected larvae and nurse bees (Figure 27B and Figure 27C), while no correlation was observed in foragers (Figure 27D).

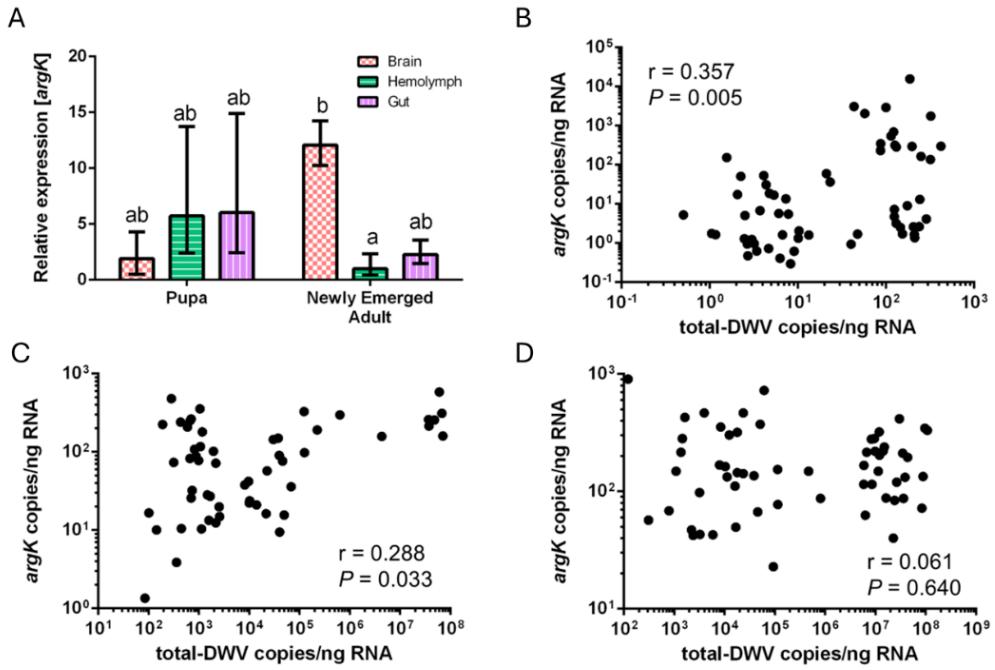


Figure 27 – Transcription profile of *ArgK* in field-collected honey bees. a) *ArgK* transcript levels in pupal and adult tissues. *ArgK* gene expression was significantly higher in the adult brain, compared with the other tissues analyzed (Kruskall-Wallis test: $H(6,24) = 14.73$, $P < 0.05$, n=4). Error bars indicate standard deviation; statistically significant differences are denoted with different letters ($P < 0.05$). b-d)

Transcription of *ArgK* is positively correlated with total-DWV load (Spearman's rank correlation, r > 0, P < 0.05, n = 55) in larvae (b) and nurse bees (c), but not in foragers (d). DWV and ArgK copies were normalized on the nanograms of total RNA. Values on X- and Y-axis are reported in Log10 scale. Exact Spearman'r rank correlation coefficient (r) and P-values (P) are reported in the figures.

To assess the effect of DWV infection on *ArgK* expression, we performed RT-qPCR experiments on adult bees injected with a known dose of DWV particles (10^4 copies/bee). At three days from the artificial infection, DWV load was significantly higher in DWV-injected (DWV), compared to PBS- (PBS) and non-injected (NI) controls (Figure 28A). DWV load of DWV-injected bees ranged between 10^6 and 10^7, while PBS-injected bees showed the largest range, with values lying between 10^2 and 10^5.
In artificially infected bees, high DWV loads resulted associated with an enhanced transcription of *ArgK*, which was approximately two-fold compared with non-injected (NI) and PBS-injected (PBS) bees. However, Kruskall-Wallis test showed that *ArgK* expression was significantly higher in DWV bees, compared to NI, while no differences were observed between PBS and the other groups (Figure 28B).

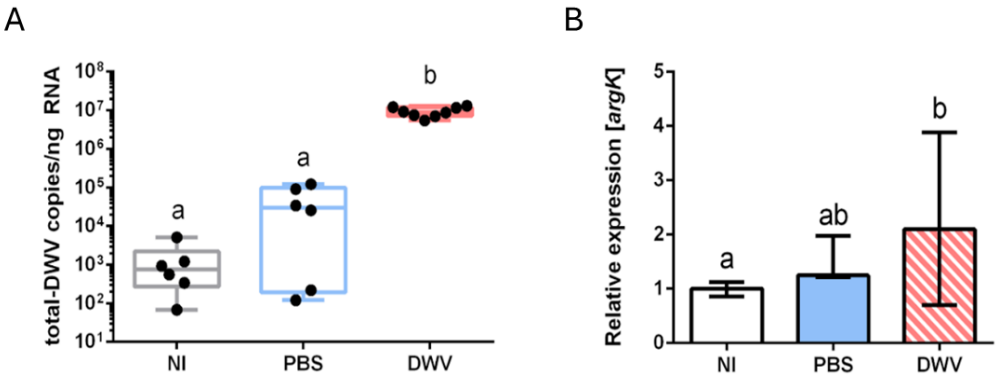


Figure 28 - Effect of controlled DWV infection on *ArgK* expression. a) DWV load was significantly higher in DWV-injected (DWV), compared to PBS- (PBS) and non-injected (NI) control bees (Kruskall-Wallis test: H(3,18) = 14.32, P < 0.0001, n=6). Boxes denote inter-quartile range, bar denotes median, and whiskers denote range. b) *ArgK* expression was significantly higher in DWV bees, compared to NI, while no differences were observed between PBS and the other groups (Kruskall-Wallis test: H(3,18) = 8.643, P = 0.0017, n=6). Error bars indicate standard deviation.

Statistically significant differences are denoted with different letters (Dunn's post-hoc test, $P < 0.05$).

To assess *ArgK* involvement in DWV infection, we performed gene knockdown and artificial infection *in vitro* using primary cultures of honey bee pupal cells. After 5 days from dsRNA administration, relative expression of *ArgK* was significantly downregulated (5-fold) in dsRNA-treated cells (Figure 29A), a condition which was associated with a three-fold reduction of DWV load, compared to cells treated with control dsRNA (Figure 29B).

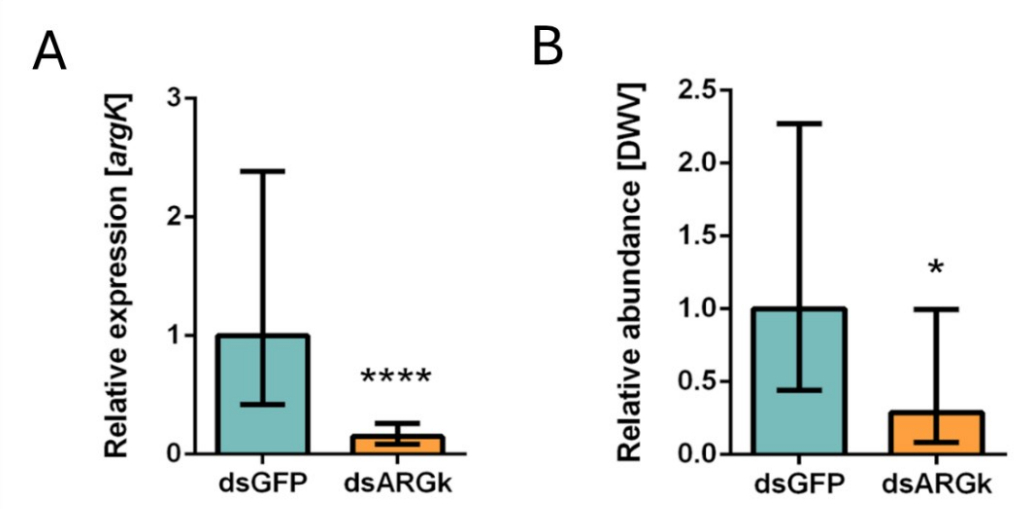


Figure 29 - Effect of ArgK knockdown on DWV load in honey bee cells. a) *ArgK* transcription level was significantly lower in honey bee cells treated with dsRNA targeting *ArgK* (dsARGk), compared to cells treated with a control dsRNA (dsGFP) (Student's t-test: $t(14) = 5.364$). b) In *ArgK*-silenced honey bee cells (dsARGk), DWV relative abundance was significantly lower, compared with control cells (dsGFP) (Student's t-test: $t(14) = 2.284$). * $P < 0.05$, ****$P < 0.0001$.

3.4 DISCUSSION

Due to its causal association with honeybee colony losses, DWV has been the subject of intensive investigation *(Martin & Brettell, 2019)*. Nonetheless, knowledge on the molecular strategies adopted by this major honey-bee pathogen to bind, enter and replicate in the host's cells is still limited *(Škubník et al., 2017)*.

As obligate intracellular parasites, viruses rely on the exploitation of host factors to establish a successful infection. Therefore, elucidation of the molecular mechanisms underlying host-virus interactions is essential for our understanding of viral pathogenesis and the identification of useful targets for the development of efficient antiviral strategies.

Accumulating evidence, mostly deriving from investigation on mammalian RNA viruses, indicated that viral structural proteins forming the capsid that encloses the viral genome play a key-role at all stages of the viral life cycle, and can regulate distinct cellular processes by interacting with host factors *(Ding et al., 2016)*. This prompted us to employ an immunoprecipitation approach coupled with mass-spectrometry to identify host cellular proteins interacting with the structural proteins of DWV.

By using this approach, we identified 142 honey bee proteins which interacts with DWV capsid proteins. Close inspection of this protein list revealed the presence of several enzymes that are well-known components of the main pathways of central carbon metabolism: glycolysis (glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, ATP-dependent 6-phosphofructokinase), tricarboxylic acid cycle (citrate synthase, isocitrate dehydrogenase, oxoglutarate dehydrogenase) and pentose phosphate pathway (6-phosphogluconate dehydrogenase). This finding correlates with previous reports on the active recruitment of glycolytic and fermentation enzymes by plants infective viruses *(Chuang et al., 2017; W. Lin et al., 2019; Prasanth et al., 2017)*. In particular, it was demonstrated that tomato bushy stunt virus (TBSV) co-opted these enzymes within specialized viral replication organelles, to support robust viral RNA synthesis *(Molho et al., 2021)*. Analogous replication compartments were found in viruses infecting animals and were considered a hallmark of positive strand RNA viruses *(Wolff et al., 2020)*. In addition, it was shown that hepatitis viruses actively manipulate the host metabolism through molecular interactions with specific enzymes such as glucokinase, the first rate-limiting enzyme of

glycolysis *(Perrin-Cocon et al., 2022; Ramière et al., 2014)*. We speculate that association of DWV capsid proteins with cellular enzymes responsible for the conversion of sugars into energy and metabolic precursors may target the host metabolism at critical nodes.
On the basis of literature data reporting the involvement of *ArgK* in viral infections of diverse shrimps species, we selected *ArgK* as a promising candidate for further functional assays aimed at characterizing this putative interactor having a key-role in host infection by DWV. This association has been previously reported for a DNA virus, the white spot syndrome virus (WSSV), which infects several shrimp species *(Ma et al., 2014; Xu et al., 2017)*.
By interacting with WSSV envelope proteins, shrimp *ArgK* enhanced viral replication in either *Litopenaeus vannamei* or *Marsupenaeus japonicus* *(Ma et al., 2014; Xu et al., 2017)*. Notably, *ArgK* enzymatic activity was necessary for successful WSSV infection in the latter system, as WSSV proliferation decreased when a mutant form of *ArgK* carrying a deletion in the active site was injected in shrimps, or a selective inhibitor was used to suppress the kinase activity of the enzyme *(Xu et al., 2017)*.
Exploitation of *ArgK* by both DWV and WSSV is a novel example of evolutive convergence in the strategies used by phylogenetically unrelated viruses to hijack host cellular functions. In this context, it is interesting to note that also another *phosphagen kinase*, *creatine kinase B* (CKB), was previously implicated in virus pathogenicity. CKB plays in vertebrates the same role as *ArgK* in invertebrates, that is coupling energy production with cellular function *(Uda et al., 2006)*. To perform this task, CKB catalyses the reversible transfer of the phosphate group of phosphocreatine to ADP, generating creatine and ATP. During human hepatitis C virus infection (HCV), CKB binds to the NS3-4A complex formed by two viral non-structural proteins and upregulates both the viral RNA and DNA unwinding and the replicase activity of this complex, ensuring efficient replication and propagation of the virus *(Hara et al., 2009)*. In addition, contribution of a CKB enzyme to viral replication was reported also in grouper fish infected by the nervous necrosis virus (NNV) (Huang et al., 2020).
Active recruitment of *ArgK* might similarly delineate as a molecular strategy adopted by DWV to acquire a readily available source of ATP. *ArgK* is an evolutionarily conserved enzyme that plays a critical role in energy homeostasis in invertebrates, by catalysing the transfer of a phosphate group between ATP and arginine *(Uda et al., 2006)*. The reversible nature of this reaction allows the synthesis of a metabolically inert pool of phosphorylated arginine during normal

metabolic conditions and the usage of this pool to quickly replenish ATP stores during periods of high metabolic activity. Therefore, *ArgK* function is particularly relevant in tissues with high and fluctuating energy demands, such as muscles and nervous system *(Ellington, 2001; Newsholme et al., 1978; Wallimann et al., 1992)*. This is consistent with our finding that the gene encoding for *ArgK* is highly transcribed in the brains of adult bees, major sensory and behavioral centers where DWV actively replicates *(Shah et al., 2009)*.

As a first step in the functional characterization of the DWV-*ArgK* interaction in honeybees, we performed a population survey aimed at evaluating the relationship between DWV infection and *ArgK* transcript accumulation. The obtained results indicated that higher DWV load associated with higher levels of *ArgK* transcript in honeybee larvae and nurse bees. The same pattern is not observed in foragers likely due to other factors affecting *ArgK* expression, such as aging or exposure to environmental stressors. The positive correlation between *ArgK* and DWV levels was also confirmed by artificial *in vivo* infection experiments, indicating that DWV inoculation triggers *Argk* gene transcription in honey bees.

Since *ArgK* is a phosphagen kinase affecting ATP production and utilization, increased expression of the *ArgK* gene, along with association of the *ArgK* enzyme with DWV capsid proteins, may have a strong impact on one or more steps of DWV infection. As a major source of energy in the cell, ATP is in fact required in different processes occurring during the life cycle of viral pathogens *(Ando et al., 2012)*. For instance, it has been demonstrated that transcriptional initiation and replication of RNA viruses are ATP-dependent processes *(Klumpp et al., 1998; Nomaguchi et al., 2003; Vreede et al., 2008)*. ATP is also required for the unwinding of viral genomes by helicase enzymes *(Frick & Lam, 2006)*. Finally, it has been shown that ATP is involved in the assembly and/or disassembly of viral particles *(Gurer et al., 2005; P. P. Li et al., 2009)*.

Therefore, interaction with *ArgK* may be a strategy developed by DWV to regulate local ATP levels, thus acquiring the energy resources necessary for progression of viral infection. Our data showed that downregulation of *Argk* expression by RNAi reduced viral load under experimental *in vitro* infection, thus demonstrating that *ArgK* is involved in DWV replication. These findings shed light on the molecular mechanism underlying the recently observed reduction of DWV load in cultured honey bee head tissues treated with quercetin *(Wu et al., 2021)*. Indeed, quercetin is a known antiviral drug with strong inhibitory activity towards *ArgK* of *Locusta migratoria*

manilensis by binding to Trp residues in the active site *(H.-R. Wang et al., 2011)*. Our results suggest that honey bee *ArgK* is one of those host's proteins to which quercetin binds, resulting in the inhibition of DWV replication *in vitro* *(Wu et al., 2021)*.
Altogether our results suggested that gene knockdown tools targeting DWV interactors in honey bees can be implemented in novel strategies aimed at limiting viral replication, as recently proposed *(Sun et al., 2023)*, contributing to preserve pollinators health against pathogens.

CONCLUSIONS

In the first part of this PhD project, we discovered a hidden impact of DWV infection on honey bee health, consisting of a mild gut dysbiosis characterized by a depletion of Bacilli and an increase of *Alphaproteobacteria* and *Clostridia*. This condition has a negative impact on honey bee immunity, especially in adult bees, in which high DWV levels are associated with a reduced expression of *dorsal-1A* immune gene. We demonstrated that DWV infection can be limited by supplementing honey bees with *Lactobacillus* species, a taxon which is significantly reduced by DWV infection. These kinds of studies are a key-step in the development of alternative strategies based on the use of probiotics and immune-stimulating diets aimed at preserving pollinators health against pathogens. In the second part of this project, we identified honey bee proteins which interact with DWV and are promising candidates for developing new therapeutic strategies based on gene knockdown. The expression of one of these targets, *Arginine Kinase*, correlates positively with DWV load, and its knockdown in honey bee cells reduces DWV levels by three-fold. These results suggest a possible use of dsRNA as a powerful tool to limit viral replication when directed against viral interactors in the host's genome.

The present work represents a novel contribution to the fast-growing research area of probiotics and therapeutics aimed at preserving pollinators health against pathogens.

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APPENDIX

Abroad experience

During my PhD project, I was able to visit for 6 months (June 2023-November 2023) the laboratory of Professor Giles Budge, at Newcastle University to further expand the ongoing studies on virus-microbiota interaction in honey bees and to deepen studies on the composition of the intestinal microbiota.
At Newcastle University, I performed an artificial infection of emerged honey bee with the viral lysate by following the protocol previously described in order to analyze the microbial community both from the whole gut and specific sections (midgut, ileum, and rectum) at different time points.

Papers and communications

- Giorgia Giordani; Giulia Cattabriga; Andrea Becchimanzi; Ilaria Di Lelio; Giovanna De Leva; Silvia Gigliotti; Francesco Pennacchio; Giuseppe Gargiulo; Valeria Cavaliere "**Role of neuronal and non-neuronal acetylcholine signalling in drosophila humoral immunity**". Insect Biochemistry and Molecular Biology (2023). Journal article, 0965-1748; DOI: 10.1016/j.ibmb.2022.103899
- Giovanna De Leva, Andrea Becchimanzi, Giovanni Jesu, Alfonso Cacace, Ilaria Di Lelio, C. Damiani, Emilio Caprio, G. Favia, Francesco Nazzi, Francesco Pennacchio. "***Deformed Wing Virus* infection induces gut dysbiosis in honey bees**". *Manuscript in progress.*
- Andrea Becchimanzi, Giovanna De Leva, Rosanna Mattossovich, Anna Valenti, Emilio Caprio, Silvia Gigliotti, Francesco Pennacchio. "**Honey bee arginine kinase is involved in Deformed Wing Virus infection**". *Manuscript in progress.*

Poster And Meeting Abstract Phd

- Andrea Becchimanzi, Giovanna De Leva, Giovanni Jesu, Alfonso Cacace, Ilaria Di Lelio, C. Damiani, Emilio Caprio, G. Favia, Francesco Nazzi, Francesco Pennacchio. **DWV infection induces gut dysbiosis in honey bees**. XXVII Congresso

Nazionale Italiano di Entomologia (CNIE), 2-16 September 2023 in Palermo.

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