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MICROBIAL ECOLOGY OF THE SPOTTED WING FLY *Drosophila suzukii*

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Contents

Abstract

Symbiotic relationships between arthropods and microorganisms are widespread in nature. In the last years these interactions are received considerable attention, as many microorganisms may play relevant roles in the biology and lifecycle of insects (Dale and Moran, 2006; Moran *et al.*, 2008). In this perspective, researchers are directing many efforts to depict the interactions that shape the symbiosis. Furthermore, considering the importance that microorganisms play for their hosts, the modification of the microbiome structure in the insect body could support the development of sustainable strategies, alternative to chemical pesticides. To achieve the development of these methods, the knowledge and the identification of the symbionts associated to the pest of interest, is a mandatory requirement.

Recent studies documented the evidence of stable associations between acetic acid bacteria (AAB) and insects characterized by a sugar-based diet. These include Diptera, Hymenoptera and Hemiptera orders (Crotti *et al*., 2010). It was reported that AAB are essential in the modulation of the immune homeostasis as well as metabolism and larval development. These capacities have been demonstrated in *Drosophila* (Ryu *et al*., 2008; Shin *et al*., 2011), but have been recently confirmed in other models, like *Anopheles* (Chouaia *et al.* 2012, Hughes *et al*., 2014).

Along with bacteria, drosophilid flies establish a mutualistic relationship with yeasts, in particular with those belonging to the Saccharomycetaceae family: these microorganisms represent the main nutritional source for the flies, as they provide proteins, vitamins and other nutrients. Yeasts are vectored by *Drosophila*, from which they are dispersed, favoring the colonization of new habitats (Christiaens *et al.*, 2014). Moreover, they can affect the fly development and fitness in terms of susceptibility to parasitism (Anagnostou *et al.* 2010). Yeasts share the same environments with AAB, supporting the hypothesis of possible microbe-microbe interactions.

The aim of my PhD project was the characterization of the microbiome associated to the spotted wing fly *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), an economically damaging pest of healthy soft summer fruits, rapidly spreading in many countries from South-East Asia (Lee *et al*., 2011). In particular, targets of the research were AAB and yeasts symbionts.

Results revealed that AAB were a major component of *D. suzukii* bacterial community. Members of *Gluconobacter*, *Gluconacetobacter* and *Acetobacter* genera were the main representatives, as shown by culture-dependent (isolation by using specific media, dereplication with ITS-PCR and isolate identification through partial 16S rRNA gene sequencing) and -independent analyses (16S rRNA barcoding and Denaturing Gradient Gel Electrophoresis-PCR). The investigation was performed on specimens of different developmental stages (larvae, pupae and adults), reared on two feeding substrates (fruit or an artificial diet).

The plasmid pHM2(Gfp) was introduced by electroporation in three selected AAB isolates, *Gluconobacter oxydans* DSF1C.9A, *Acetobacter tropicalis* BYea.1.23 and *Acetobacter indonesiensis* BTa1.1.44 to label them with Green fluorescent protein (Gfp). After oral administration to the insects, Gfp-tagged strains were visualized in the host by fluorescence microscopy. The symbionts were able to successfully reach and colonize the epithelium of the insect crop, proventriculus and midgut. Tests performed on bacterial cultures grown in liquid media showed that several AAB isolates are able to produce an extracellular matrix in which the cells are entrapped and that presumably is implicated in the bacterial adhesion to the insect epithelia and maintenance in the digestive system.

By using probes specific for AAB (Texas red-labelled probe AAB455) and Eubacteria (Texas red-labelled universal Eubacterial probe Eub338), fluorescent *in situ* hybridization (FISH) experiments on the host dissected tissues from *D. suzukii*, detected AAB within the peritrophic membrane of the midgut and proventriculus. Probes specific for *Gluconobacter* (Cy5-labelled probes Go615 and Go618) were also designed, and used to confirm the

presence of this species within the intestinal tract of the fly.
Due to the abundance and intimate connection of these symbionts with *D. suzukii* tissues and organs, I predict that AAB have important roles in the lifecycle of the host.

The capacity of *D. suzukii*-selected AAB isolates to emit microbial volatile compounds for flies' specific attraction was subsequently analysed. Microbial volatiles are known to attract or repel insects, inhibit or stimulate the plant growth. For example, acetic acid was described to be an attractant molecule for *Drosophila* flies (Cha *et al*., 2014). With the aim to evaluate the different attraction capabilities of some selected AAB on *D. sukuzii*, a two-choice olfactometer assay was developed and attraction experiments were carried out. After the first evaluation of the bacterial growth to set up the experimental conditions, flies were exposed to volatile organic compounds (VOCs) produced by AAB isolates in comparison to a control, represented by the growth medium without bacteria. Higher attractiveness for flies than the other bacteria was obtained with *Gluconobacter oxydans* DSF1C.9A, *Gluconobacter kanchanaburiensis* L2.1.A.16 and *Gluconacetobacter saccharivorans* DSM1A.65A strains. Since currently traps for flies are composed by vinegar and baker's yeast, the analyses of the best attractive molecules released by specific microorganisms might provide novel tools for *D. suzukii* biocontrol and the assembly of baits specifically targeted for this pest.

Flies at different developmental stages (larvae, pupae and adults) reared on different food sources (fruit or artificial diet) were analyzed through cultivation-independent (DGGE-PCR, 16S rRNA 454-pyrosequencing) and -dependent (isolation trials, and isolate identification) techniques to investigate the yeast community. Most of the analyzed sequences obtained from the excised DGGE bands and pyrosequncng data had close similarity with sequences assigned to Saccharomycetales, in particular *Candida*, *Geotrichum* and *Pichia* genera. These yeasts comprise specialist colonizers of rotten and fermenting fruits, and the skin of intact fruits eaten by *Drosophila*.
A collection of 237 yeast isolates were obtained from the isolation trials, with the purpose to explore the community diversity in individuals of different life stages, and reared on the two-abovementioned food sources. Identification of the yeast species was carried out using RFLP (Restriction Fragment Length Polymorphism) analysis of the ITS1-ITS2 region of the fungal rRNA gene, of all the isolates. Restriction patterns were obtained through the use of HaeIII, HinfI and TaqI endonucleases. After the analysis of the generated digestion patterns, the representative isolates of each RFLP profile were submitted to sequencing analyses of both the D1-D2 region of the large subunit (LSU) of the fungal rRNA gene and the ITS1-ITS2 region of the fungal rRNA gene. Phylogenetic trees completed the analysis. The results strengthened and enlarged the molecular data, and showed that the most abundant species recorded, *i.e*. *Pichia occidentalis*, *Saccharomycopsis craetegensis* and *Arthroascus schoenii*, were the only species isolated from all of the tree life stages (larvae, pupae, and adults). Insects reared on fruits were characterized by a higher diversity in terms of yeast species. In particular, it was also recorded the presence of *Hanseniaspora uvarum*, which was described in previous works as a dominant yeast genus associated to different *Drosophila* species, including *D. suzukii* (Chandler *et al*., 2012, Hamby *et al*., 2012).

Data obtained from the yeast community characterization, as well as from the bacterial community one, were exploited for a screening of the possible interactions among symbionts. In particular, some yeast strains are able to compete for their own ecological niche and nutrients by producing an array of compounds named "killer toxins" (Woods and Bevan, 1968). Since the production of killer toxins could be highly affected by the culture conditions, in terms of pH, temperature and carbon source content, then the optimal ones were developed for the growth of selected yeast isolates, and subsequently antagonistic activity tests were performed. The results highlighted the capacity of a specific yeast isolate, *Candida stellimalicola* AF4.1.P.268, to limit the growth of several yeast and AAB isolates, by creating inhibition haloes. This feature might have a role in a pest management perspective.

In conclusion, this project indicates that AAB and yeast communities establish an intimate association with *D. suzukii*, as they were found stably and abundantly in individuals of different stages and fed on different diets. Recolonization trials performed with AAB strains suggest, in particular, their importance for the biology of this pest. Gathered information might be a basis to develop alternative strategies for a more effective and sustainable biocontrol management of this emerging pest, for whom a successful strategy has not been found yet.

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Riassunto

Le relazioni di tipo simbiotico che si stabiliscono tra artropodi e microrganismi sono molto diffuse in natura. Negli ultimi anni tali interazioni hanno ricevuto una particolare attenzione, in quanto molti microorganismi svolgono ruoli importanti per il ciclo biologico e la vita in generale degli insetti (Dale e Moran, 2006, Moran *et al.,* 2008). Pertanto, numerose ricerche sono state indirizzate verso la caratterizzazione delle interazioni che modellano le simbiosi. Inoltre, nota la rilevanza ricoperta dai microorganismi nei confronti dei propri ospiti, la manipolazione del microbioma all'interno dell'insetto potrebbe contribuire allo sviluppo di strategie sostenibili, sostitutive dei pesticidi. Per conseguire lo sviluppo di tali metodi, un requisito essenziale è la conoscenza e l'identificazione dei simbionti associati all'insetto dannoso di interesse.

Studi recenti hanno evidenziato la costante associazione tra batteri acetici (AAB) e insetti che hanno una dieta zuccherina, appartenenti agli ordini dei Ditteri, Imenotteri ed Emitteri (Crotti *et al*., 2010). È stato riscontrato che i batteri acetici svolgono un ruolo essenziale nella regolazione dell'omeostasi immunitaria, così come nel metabolismo e nello sviluppo larvale. Ciò è stato osservato in *Drosophila* (Ryu *et al*., 2008; Shin *et al*., 2011) e confermato anche in altri modelli, come nelle zanzara del genere *Anopheles* (Chouaia *et al*., 2012, Hughes *et al*., 2014).

Oltre che con i batteri, i moscerini della famiglia Drosophilidae sono in grado di instaurare delle relazioni di tipo mutualistico con i lieviti, in particolare con quelli appartenenti alla famiglia delle Saccharomycetaceae. Tali microrganismi costituiscono la principale fonte alimentare per questi insetti, approvvigionandoli di proteine, vitamine e altri nutrienti. D'altro canto, i lieviti vengono trasportati dagli insetti e conseguentemente dispersi; ciò contribuisce alla colonizzazione di nuovi habitat da parte di questi funghi (Christiaens *et al.*, 2014). In aggiunta, una dieta composta da determinate specie di lieviti può incidere sullo sviluppo e sulla *fitness* dei moscerini dal punto di vista della diversa resistenza al parassitismo.

Scopo di questo progetto di dottorato è stato la caratterizzazione del microbioma associato al moscerino dei piccoli frutti, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), un insetto dannoso che attacca la frutta estiva con buccia sottile, causando un danno economico alle coltivazioni. Quest'insetto si sta diffondendo in diversi paesi a partire da un iniziale areale di distribuzione nel Sud-est asiatico (Lee *et al*., 2011). Obiettivo di questa ricerca sono stati i batteri acetici e i lieviti simbionti di tale insetto.

I risultati ottenuti indicano che i batteri acetici costituiscono la principale componente della comunità batterica associata a *D. suzukii*. Nello specifico, grazie all'utilizzo di tecniche coltura-dipendenti (isolamento su terreni di crescita specifici, riduzione della ridondanza della collezione tramite amplificazione della regione ITS, identificazione degli isolati per mezzo del sequenziamento del gene parziale del 16S rRNA), unitamente ad un approccio indipendente dalla coltivazione (Barcoding del 16S rRNA e tecnica di elettroforesi con gradiente denaturante, denominata DGGE- *Denaturing Gradient Gel Electrophoresis*), si è osservato che i batteri maggiormente rappresentati appartengono ai generi *Gluconobacter*, *Gluconacetobacter* e *Acetobacter*. Tali analisi sono state condotte su individui a diverso stadio di sviluppo (larve, pupe e adulti), allevati su due diverse tipologie di substrati (frutta oppure dieta artificiale).

La tecnica della ibridazione fluorescente *in situ* (FISH - F*luorescent In Situ Hybridization*) è stata condotta su tessuti dissezionati di *D. suzukii*, utilizzando sonde specifiche per i batteri acetici (sonda AAB455, marcata con Texas-red) e per la comunità batterica in generale (sonda universale per gli Eubatteri Eub338, marcata con Texas-red), consentendo di localizzare i batteri acetici all'interno della membrana peritrofica di intestino medio e proventricolo. La presenza di batteri acetici appartenenti al genere *Gluconobacter* all'interno del tratto intestinale dell'insetto è stata similmente confermata, attraverso la costruzione e l'uso di sonde specifiche (sonde Go615 e Go618, marcate con Cy5). Inoltre, al fine di localizzare i batteri acetici all'interno del corpo dell'insetto, il

plasmide pHM2(Gfp) è stato inserito, mediante elettroporazione, in 3 isolati selezionati, quali *Gluconobacter oxydans* DSF1C.9A, *Acetobacter tropicalis* BYea.1.23 e *Acetobacter indonesiensis* BTa1.1.44, per marcarli con la proteina fluorescente Gfp (*Green fluorescent protein*). I ceppi marcati con Gfp sono stati somministrati oralmente agli insetti e poi visualizzati all'interno dell'ospite, attraverso l'utilizzo della microscopia a fluorescenza. In questo modo è stato possibile confermare che i simbionti sono in grado di raggiungere e colonizzare efficientemente l'epitelio di ingluvie, proventricolo ed intestino medio dell'insetto. Saggi di crescita in terreno liquido hanno inoltre permesso di osservare che alcuni isolati producono una matrice extracellulare gelatinosa in cui le cellule batteriche vengono incluse, e che è probabilmente coinvolta nei meccanismi di adesione e mantenimento sull'epitelio del sistema digerente dell'ospite.

Dai dati ottenuti, considerando l'abbondanza e l'intima correlazione dei batteri acetici con i tessuti di *D. suzukii*, si è potuto ipotizzare che questo gruppo di simbionti abbia un ruolo rilevante nel ciclo vitale dell'ospite considerato.

Successivamente è stata analizzata la capacità di alcuni isolati di batteri acetici di emettere nell'ambiente composti volatili (VOC-*Volatile Organic Compunds*), in grado di attrarre specificatamente *D. suzukii*. È noto che le molecole volatili prodotte da batteri possano attrarre o respingere gli insetti e inibire o stimolare la crescita vegetale. Ad esempio, l'acido acetico è stato decritto come molecola attrattiva nei confronti dei moscerini del genere *Drosophila* (Cha *et al*., 2014). È stato quindi sviluppato e condotto un saggio utilizzando un olfattometro, a forma di Y, per valutare le capacità di alcuni batteri acetici, isolati precedentemente da *D. suzukii*, di attrarre il moscerino. In seguito ad un esame della crescita batterica, al fine di impostare le condizioni sperimentali, i moscerini sono stati esposti ai VOC emessi dai batteri acetici, in comparazione ad un controllo rappresentato dal terreno di crescita senza batteri. Il saggio ha mostrato che gli isolati *Gluconobacter oxydans* DSF1C.9A, *Gluconobacter kanchanaburiensis* L2.1.A.16 e *Gluconacetobacter saccharivorans* DSM1A.65A posseggono una significativa capacità attrattiva. Quest'analisi potrebbe avere una implicazione di tipo pratico e costituire uno strumento innovativo per le pratiche di biocontrollo di *D. suzukii*, in particolare per la costruzione di specifiche trappole per questo insetto, le quali al momento sono costituite prevalentemente da aceto e lievito di birra.

L'associazione tra *D. suzukii* e lieviti è stata studiata in insetti a diverso stadio di sviluppo (larve, pupe e adulti), nutriti su diverse fonti di cibo (frutta o dieta alimentare), per mezzo di metodi indipendenti dalla coltivazione (DGGE, pirosequenziamento tramite tecnologia 454 della regione ITS dell' rRNA) e utilizzando tecniche microbiologiche basate su isolamento e conseguente identificazione. La maggior parte delle sequenze ottenute sia dalla riamplifica delle bande DGGE che dai dati di pyrotag hanno evidenziato una stretta similarità con sequenze appartenenti all'ordine dei Saccharomycetales, in particolare con i generi *Candida*, *Geotrichum* e *Pichia*. Questi gruppi includono specie colonizzatrici di frutta marcescente o in stadio di fermentazione, così come specie tipicamente associate alla buccia di frutti intatti, su cui si nutre *D. suzukii*.

Mediante tecniche coltura-dipendenti è stata quindi costituita una collezione di 237 lieviti, a partire da individui a diverso stadio di sviluppo e cresciuti sulle due fonti alimentari sopracitate. I membri di questa collezione sono stati quindi identificati. L'analisi dei polimorfismi di restrizione, denominata RFLP (*Restriction Fragment Length Polymorphism*) è stata condotta sulla regione ITS dell'rRNA dei funghi. I *pattern* di restrizione ottenuti in seguito al taglio con endonucleasi HaeIII, HinfI e TaqI sono stati analizzati e hanno permesso di individuare i rappresentanti di ogni profilo di restrizione; ogni rappresentate è stato quindi sottoposto a sequenziamento della regione D1-D2 della subunità maggiore (LSU) del 26S rRNA e della regione ITS1-ITS2. La costruzione di alberi filogenetici ha completato l'analisi. I risultati desunti da questa caratterizzazione hanno potuto irrobustire ed ampliare i dati ottenuti dalle analisi molecolari, mostrando complessivamente come le specie più abbondanti, ovvero *Pichia occidentalis*, *Saccharomycopsis craetegensis* e *Arthroascus schoenii*, siano anche quelle specie associate

a tutti i tre stadi di età studiati (larve, pupe, adulti). In aggiunta, gli insetti alimentati su frutta sono contraddistinti, rispetto agli individui nutriti su dieta di laboratorio, da una più alta diversità di lieviti in termini di specie. È stata evidenziata in particolare la presenza di *Hanseniaspora uvarum*, già descritto come genere dominante di diverse specie di drosofile, tra cui *D. suzukii* (Chandler *et al*., 2012, Hamby *et al*., 2012).

Le informazioni ottenute dalla caratterizzazione sia della comunità di batteri acetici, che da quella di lieviti, sono state sfruttate per individuare le possibili interazioni tra simbionti. Tra queste, è noto che alcuni ceppi di lievito possano competere per la propria nicchia ecologica e per i nutrienti attraverso la produzione composti denominati "tossine *killer*" (Woods and Bevan, 1968). Dopo aver sviluppato le condizioni ottimali di pH, temperatura e contenuto di fonti di carbonio per la crescita degli isolati selezionati, in quanto la produzione di tali tossine può essere influenzata dalle condizioni di crescita, sono stati condotti dei saggi di attività antagonistica. I risultati di questo screening hanno evidenziato uno specifico isolato, *Candida stellimalicola* AF4.1.P.268, in grado di limitare la crescita di diversi lieviti e batteri acetici, attraverso la formazione di un alone di inibizione circondante la colonia. Anche questa informazione potrebbe avere un interessante risvolto nell'ottica del contenimento di *D. suzukii*.

In conclusione, questo lavoro evidenzia come le comunità di batteri acetici e lieviti possano stabilire una relazione stabile con *D. suzukii*. In particolare, le prove di ricolonizzazione condotte con i batteri acetici, la loro abbondanza e localizzazione nel tratto digerente dell'ospite suggeriscono l'importanza di questo gruppo di microrganismi per la biologia dell'insetto di interesse. I dati complessivi potrebbero costituire la base per lo sviluppo di strategie di biocontrollo alternative, al fine di condurre una gestione più efficace e sostenibile del moscerino dei piccoli frutti, per il quale tuttora non è stato trovato un metodo di contenimento specifico.

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Chapter I

Bacterial and Yeast Microbiome Associated with Drosophilid Flies

Insects are the most abundant and diverse Eukaryotic Class, which have been found to be associated with a huge variety of microorganisms. With many of them, insects established a mutually beneficial symbiotic association, which it started from a pathogenic contingency that became progressively less virulent and provided benefits to the insect host, supporting reciprocal adaptations and genotypic complexity. Symbiosis can be considered a source of evolutionary innovation for the insects, and an example is provided by the impressive metabolic exchanges among some insects and their microbial counterparts (Moran *et al.*, 2008).

Several bacteria live symbiotically in specific organs or special structures, named bacteriomes. On one hand, bacteria find a shelter and nutritive elements from their hosts, on the other they provide nutrients to the insects themselves. In this way, symbiotic bacteria permit insects to keep on using a nutritionally unbalanced diet or, from another point of view, to run away from the constraints that an adequate diet would impose (Akman Gündüz and Douglas, 2009).

Animal symbioses have been categorized depending on both apparent evolutionary age and the codependence between host and microorganisms. Thus, "primary symbiosis" is defined as a relationship in which both partners, hosts and symbionts can live only with one another, and the bacteria live densely packed in the abovementioned specific structures called bacteriomes. For instance, in the aphid–*Buchnera* sp. nutritional mutualism, the host depends on the symbiont for essential amino acids supply for protein synthesis, which cannot be synthesized by the insect from plant sap diet (Douglas, 1998). The evolutionary path of microorganisms and animal is interlocked since a long time that their evolutionary trees are congruent to each other. Estimated ages of these symbioses go from 30 to 270 million years, and this is reflected in the impressive reduction of the microorganisms' genome in some cases.

On the other hand, "secondary symbiosis" refers to the condition in which the presence of those microorganisms that are beneficial to the host is not compulsory for the insect life. They colonize new hosts via horizontal transmission, but also vertically, from females to the offspring, giving a beneficial effect (in terms of survival and protection against diseases) to those females that are able to transmit them to the progeny (Dale and Moran, 2006). Recently, it has been seen that these kind of microorganisms constitute a horizontal gene pool: they act as shuttle of adaptive genes among host lineages; moreover, they can influence the adaptation and the colonization of new ecological niches by their insect hosts (Henry *et al.*, 2014).

Endosymbionts of the genus *Wolbachia* are found in arthropods and nematodes and are transmitted vertically in host eggs. These Alphaproteobacteria symbionts belong, together with other microorganisms of different prokaryotic lineages, to the group of the so-called "reproductive parasites" when present in insects. They have been found in different arthropods hosts (Bandi *et al*., 2001) and, in particular, *Wolbachia pipientis* is believed to infect up to 70% of insect species, from several insect orders (Stouthamer *et al*., 1999, Jeyaprakash and Hoy, 2000). Albeit *Wolbachia* acts as a reproductive manipulator in arthropod hosts, some cases of host dependence on this bacterium have been reported (Saridaki and Bourtzis, 2010). One example is the recently observed nutritional mutualism established between a *Wolbachia* strain and the bedbug *Cimex lecturialus*. This relationship is essential as *Wolbachia* provides B vitamins for the bug's growth and reproduction. The study strongly suggests the presence of biotin synthesis genes in *Wolbachia* strain genome, laterally transferred probably by co-infecting endosymbionts, and sheds a light on the evolutionary transition from facultative symbiosis to obligate mutualism (Nikoh *et al.*, 2014). Not only *Wolbachia*, but also microorganisms within the *Spiroplasma* genus, flavobacteria, gammaproteobacteria, and *Rickettsia* genus (Alphaproteobacteria) have been found and described as male-killing (Hurst and Jiggins, 2000).

Some endosymbionts can also have a detrimental effect on the host in which they live, such as phytoplasmas, bacteria belonging to the Mollicutes Class, characterized by the lack of cell

wall. They are able to multiply not only in the insect body, but also in the phloem cells of the host plants. Among them, there are microorganisms able to cause a group of diseases defined "Grapevine yellows", vectored by the insects themselves. In particular, it has been recorded the spreading of "*Bois Noir*" and the "*Flavescence dorée*" grape yellows, vectored respectively by the planthopper *Hyalesthes obsoletus* (Hemiptera: Cixiidae) and the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae). These insects are characterized by the association with different symbiotic bacteria, both primary and secondary symbionts (Marzorati *et al.,* 2006, Gonella *et al.,* 2011).

The association between *Drosophila* species (Drosophilidae family) and microorganisms have been extensively studied in the past years, and the collected information might be an important contribute for elucidating the nature of the interactions existing among the host, its immune system and the symbionts. One of the data that emerged from the analysis of the bacterial community is that drosophilid flies lack primary symbionts; they usually have secondary symbionts, representing a suitable model to investigate the complex associations among commensals and eukaryotes, including mammals (Smith *et al.,* 2007). In the past years, many screenings for *Wolbachia* have been performed both on long-term laboratory cultures and natural populations of drosophilid flies. The other hereditable symbiont category found in *Drosophila* flies is represented by *Spiroplasma*, together with related bacteria in the phylum Mollicutes (Montenegro *et al,*. 2006). This group is widespread in insect hosts, and some strains are involved in reproductive manipulation, by son killing in infected females or sex ratio bias (Anbutsu and Fukatsu, 2003, Veneti *et al.,* 2005). The presence of other bacterial groups that are known as opportunistic hereditable symbionts of insects, as Gammaproteobacteria (*e.g.* Moran *et al.,* 2005) and members of the phylum Bacteroidetes (Zchori-Fein and Perlman, 2004), has not been registered in Drosophilidflies yet. The reason for the absence of other hereditable symbionts might lie in a robust innate immune response that prevents the settlement of many bacteria (Mateos *et al.,* 2006).

Research on insects has played a relevant role in many areas of biology. For much of the last 100 years, *Drosophila melanogaster* has been a model organism, as it is easily cultured and has a short life cycle, and the comparative behavioral, genetic and genomic experiments have been carried out not only to gain more knowledge about *Drosophila*, but also to find more general biological properties, about diseases, metabolism, hormones pathway, development (Graveley, 2010, Niwa and Niwa, 2011, Pandey and Nichols, 2011, Mirth *et al.,* 2014). In this way, *Drosophila* studies influenced deeply both pure and applied biology (Morgan 1910, Roberts 2006). Many experiments focused on the microbiome associated to flies, their implication for the host health and life *status*, and the formulation of a common effect for this whole insect group have been performed exploiting this successful organism, and the ability to manage it in laboratory conditions (Jennings, 2011). The experimental model *D. melanogaster* owns, indeed, several ideal features that could allow the comprehension of different complex aspects at the base of the symbionts-hosts relationships. A simple microbiota and the availability of tools for manipulating both the hosts and the symbionts permitted and are permitting studies on the host-microbe interactions and the immunity-related responses (Lee *et al.,* 2013, Shin *et al.,* 2011). As instance, in a recent paper researchers show that *Drosophila* distinguishes pathogens from symbionts since the former, by secreting uracil, activates the DUOX-dependent gut immunity through ROS production, while the latter does not initiate ROS production since they do not secrete uracil (Lee *et al.,* 2013).

The present review aims to provide an overview of the current knowledge on the bacterial (with a particular attention to the group of acetic acid bacteria and lactic acid bacteria) and yeast communities associated to *Drosophila* species. The independent and, at the same time, synergistic role played by these two microbial taxa in the biology of the flies will be presented. Moreover, the knowledge of the microbial structure and diversity associated to drosophilid flies could be a helpful tool in the pest control perspective, based on biotechnological applications. Nonetheless, *Drosophila* flies attack rotten and damaged fruits, some species are considered a menace because their preference goes towards undamaged and ripening fruits, like *D. suzukii* and *D. subpulchrella* (Atallah *et al.,* 2014).

D. suzukii (Diptera: Drosophilidae) is a damaging exotic pest that originates from South-East Asia and that it is rapidly invading the countries characterized by a temperate climate, becoming a serious issue for European and American fruit traders. It is also called spotted wing fly, due the black spots that the male individuals have on each wing. Its accidental introduction took place in 2008 in Italy, California and Spain leading to the spread of the pest in the Mediterranean area, on the U.S. East Coast, until recent announcement of its recording in more cold areas, such as Scandinavia, showing adaptations to cold climates and a high thermal tolerance. The damage is caused by the female prominent serrated ovipositor with whom the insect can incise fruits' skin and lay eggs beneath it. Consequently eggs hatch and larvae feed within the fruits (and provide access to pathogens, such as yeasts, filamentous fungi and bacteria, provoking secondary infections), which become soft and rot rapidly. Due to its virulence, short life cycle, the wide variety of attacked fruits and high fecundity (400 eggs during each fly's lifetime, on average), once *D. suzukii* is settled, it is extremely difficult to eradicate it, and crops, mainly high density monoculture ones, permit rapid invasion and population growth.

The knowledge of the insect symbiotic partners is mandatory when it is foreseen to exploit the insect associations with microorganisms in a frame of integrated pest management (IPM): these kinds of relationships can have a beneficial, neutral or harmful effect on the host, thus influencing the population growth (Walsh *et al.,* 2011, Cini *et al*., 2012. Rota-Stabelli *et al.,* 2013).

Bacterial community associated to Drosophila species

454-based sequencing is a powerful method to analyze large-scale data, and to gain an overview of the microbial community associated to many samples. Chandler and coworkers and later Staubach and collaborators, with their research teams (Chandler *et al.*, 2011, Staubach *et al.*, 2013), performed analyses to survey the microbial community associated to several *Drosophila* species, from different geographic localities, in order to assess how food source and host species can influence the bacterial community structure. These are the most comprehensive studies on the bacterial community associated to drosophilid flies performed so far (Wong *et al.,* 2011, Wong *et al.,* 2013). The specimens came from laboratory and natural populations, and bacterial DNA from the whole insect body was analyzed, since bacteria associated with fly surfaces can have an important role, as well as bacteria living inside the insect body, as confirmed by Ren *et al.* (2007). Host species seem not to have a strong effect in shaping the community structure, while food substrate in natural populations and in laboratory reared insects have a consistent effect. In fact, different *Drosophila* species acquired the same kind of microbiome when raised on the same food source, and indifferently from the geographic location. Despite variability can be encountered across different samples among lab-reared flies, the diversity of their bacterial community is minor when compared to natural population (Chandler *et al.,* 2011, Staubach *et al.,* 2013). The environment, in particular fruits and vegetables, on which drosophilid flies feed on, can harbor large and different populations of bacteria, dominated by Enterobacteriaceae family, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria phyla (Leff and Fierer, 2013). Bacteria are acquired from the environment by first-instar larvae, and are maintained through larval development and in external tissues of pupae to adulthood (Ridley *et al*. 2012). In particular, different studies showed that lab-reared and wild-captured individuals were naturally inhabited by representatives of the Acetobacteraceae (Alphaproteobacteria), Enterobacteraceae (Gammaproteobacteria), Enterococcaceae (Firmicutes) families and Bacteroidetes Phylum (Cox and Gilmore, 2007, Corby-Harris *et al.,* 2007, Chandler *et al.,* 2011, Staubach *et al.,*2013).

It is important to note that the high prevalence of bacteria with similar metabolic capabilities and the tolerance to low pH and high ethanol concentrations indicate how the environmental conditions strongly drive the community composition. In wild-caught specimens, bacteria of the *Gluconobacter* genus were found to be the most abundant by Staubach *et al*. (2013). Pathogens of *Providencia* and *Enterococcus* genera were identified too. This result was confirmed also by a preliminary study investigating the bacterial community associated to *D. suzukii*, which showed prevalence of sequences belonging to the *Tatumella* genus

(Enterobacteriaceae) and *Gluconobacter* and *Acetobacter* genera (Chandler *et al.*, 2014). On the contrary, the presence of a core microbiome for *D. melanogaster* is criticized by a study in which it was showed that the prevalence of the same bacterial *taxa* cannot always be recorded in all tested insect guts, from laboratory and wild populations (Wong *et al*., 2013).
454 pyrosequencing of the PCR-generated amplicons of the 16s rRNA gene showed that the gut bacterial diversity of the fruit fly *D. melanogaster* is orders of magnitude lower than the mammalian one (Cox and Gilmore, 2007, Chandler *et al.*, 2011, Wong *et al*., 2011). This is evident at the within-species level, as well as higher taxonomic levels. Guts are habitats characterized by inhospitable conditions, such as the presence of active enzymes (proteases, lysozymes), and low levels of oxygen and pH, in which few organisms can survive. *Drosophila* gut is also a disturbed habitat in temporal scale: the larval gut persists for about 4 days before its metamorphosis, followed by the development of the adult gut that will persist too for 4-5 weeks (Wong *et al*., 2011). The gut microbiota is composed by autochtonous (resident) and allochtonous (non-resident) *taxa*; the latter does not live inside the organ, but just passes through it, after ingestion. The majority of identified sequences belong to *Acetobacter pomorum*, *Acetobacter tropicalis*, *Lactobacillus brevis*, *Lactobacillus fructivorans* and *Lactobacillus plantarum* (Wong *et al*, 2011, Storelli *et al.*, 2011). By taking into account that the composition of the gut microbiota can vary with diet, developmental age and physiological status of the host, it was highlighted the change in the microbial composition with increasing adult age (Wong *et al*, 2011, Wong *et al*, 2013).
Acetic acid bacteria symbionts of Drosophila flies. Despite these studies were focused on the overall flies microbiome, they showed that acetic acid bacteria (AAB) are a significant part of the associated microbial community. AAB are a category of bacteria belonging to the Acetobacteraceae family with whom insects, characterized by having a sugar-rich diet, can establish a secondary symbiotic relationship. In the last years, research is devoting attention to these bacteria, unveiling the relationships existing between this group of microorganisms and the host, and how this kind of association can have a deep impact on the evolutionary success and on the life cycles of many arthropod species (Crotti *et al.*, 2010).
AAB are able to colonize different kinds of insects, that belong to the Diptera, Hymenoptera, Hemiptera and Homoptera orders (Jeyaprakash *et al*., 2003, Kounatidis *et al.*, 2009, Crotti *et al.*, 2009, Favia *et al.*2007, Gonella *et al.*, 2011, Franke *et al*., 2000). They can be transmitted both via horizontal (environmental) and vertical (e.g. transovarian) way (Crotti *et al.*, 2009, Damiani *et al.*, 2008, Gonella *et al.*, 2012). The analysis of AAB genomes unveils ancient pre-adaptation traits to symbiosis that have might favored the strict relationships of these microorganisms to arthropods (Chouaia *et al.*, 2014). AAB distribution in the different arthropod species could depend on the carbon sources provided. For instance, *Gluconobacter* spp. prefer glucose, and indeed they were mostly collected from insects having a honey- and nectar-based diet, while *Acetobacter* spp. use preferentially ethanol as carbon source: they are more likely found in insects like *D. melanogaster*, a species attracted by fermenting fruits.
AAB are obligate aerobic microorganisms, as many of them are not capable to oxidize ethanol, sugars, and polyalcohols completely, and consequently they release their oxidation products into the medium. Inside the insect body, they are preferably associated to the digestive system: this is characterized by particular chemical and physiological conditions, like acidic pH, lack of anoxic conditions and carbohydrates availability, which permit AAB growth and, on the other hand, prevent the proliferation of other kinds of bacteria (Crotti *et al.*, 2010). In addition to this, all AAB are suggested to survive in a micro-oxic environment as they possess in their genomes both operons cytochrome bo_3 and *bd* oxidase, a character already present in the AAB common ancestor (Chouaia *et al.*, 2014).
The fruit fly gastrointestinal gut (GIT) has a similar organization in comparison to the GIT of mosquitoes and bees. In addition to AAB ability to colonize the insect gut, they are also able to successfully colonize other parts of the insect body, like the body surface, gonads, salivary glands and Malpighian tubules. They adhere, and probably interact with the epithelial cells of the abovementioned organs thanks to the polysaccharidic matrices that they are able to produce (*e.g.* cellulose). The cellulosic material could also be a protection against harsh conditions, like pH and osmolarity oscillations (Crotti *et al*, 2010).

It was reported how the bacterial abundance in *D. melanogaster*, in particular referred to AAB (aerobic bacteria) and LAB (anaerobic and aerotolerant anaerobic bacteria) with *Acetobacter* and *Lactobacillus* genera, undergoes an increase correlated to age, both on the surface and in the fly body, without affecting the life span. Interestingly, the cell size of those bacteria inhabiting the surface of the fly body was approximately one fold-smaller than the expected size of bacteria, maybe because of the atypical and harsh environment in which they were found (Ren *et al.*, 2007).

AAB can exert a role in the physiology and life traits of their host, and the model organism *D. melanogaster* was exploited in different cases to verify the correctness of these speculations. The investigation performed by Ridley and colleagues in 2012, using *D. melanogaster*, was executed to gain knowledge of the host-microbial interactions, and in particular to explore the important role played by the resident microbiota in the animal physiology, with a specific attention to the nutrition. These insects were find to bear a microbiota composed mainly by the Alphaproteobacteria *Acetobacter*. The comparison of individuals experimentally deprived of their microbiota by egg dechorionation with untreated animals showed that the axenic flies had a longer larval development time (with no effects on the adult body size, weight and fecundity), a reduced metabolic rate and irregular carbohydrate allocation, like high glucose levels in the female body. One hypothesis for this phenotype could be that bacteria resident in the gut may act as glucose competitors. Additionally, resident AAB and LAB produce acetic acid and lactic acid, respectively, organic compounds that are known to reduce digestibility of starch and other carbohydrates by mammals (Brighenti *et al.*, 1995, Ogawa *et al.*, 2000, Johnston *et al*., 2000, Ostman *et al*., 2002). Recently, AAB are also found to be involved in the promotion of the insulin pathway, with consequent enhancement of the larval developmental rate, body size, energy metabolism and intestinal stem cell activity. In particular, it was demonstrated that *Drosophila* growth promotion requires the periplasmic pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) activity. This acetic acid bacteria enzyme is responsible for acetic acid biosynthesis, one of the necessary components for the insect development. (Shin *et al.*, 2011).

AAB are present in the *D. melanogaster* gut as commensal bacteria and they establish not only a delicate equilibrium with the fly innate immune system, which on the other hand allows the presence of these bacteria in the insect gut, but also they are involved in modulating it. If the equilibrium condition is perturbed, an increasing number of pathogenic bacteria can lead to the gut apoptosis (Ryu *et al*., 2008). In addition to this, the relationships between the host gut immunity and gut commensal bacteria modulating the DUOX (dual oxidase) system can have an effect on the insect health and survival. This system seems to be involved in different host physiological aspects, such as microbial clearance, intestinal epithelial cell renewal (ECR), cross-talk of molecules, and the capacity to discern between bacteria commensals, pathogens and pathobionts (*i.e.* a resident bacterial species which is normally benign, but that could act as a pathogen if the commensal community is deregulated) (Kim and Lee, 2014).

The biological role of a specific acetic acid bacterium, namely *Asaia*, was taken into account. *Asaia* is the main component of *An. stephensi* microbiota, and its removal, performed with antibiotic treatments, confirmed the hypothesis of the importance of *Asaia* for the correct development of mosquito larvae, and for host fitness consequently. Indeed, larvae treated with rifampicin had a delayed development and an asynchrony in the appearance of the late instars (Chouaia *et al.*, 2012). *Asaia* not only affects larval development if removed, but also accelerates it, if present; the evidence was unearthed by another work that analyzed the relationships between the bacterium and another mosquito, *An. gambiae*. After verifying the established symbiosis, which occurs in the insect midgut with bacteria transmitted vertically to the offspring, the development effects were evaluated. The experiments show a direct increase of the developmental rate after administration with *Asaia* (Mitraka *et al*., 2013). *Asaia* sp. owns another capability, which is the crosstalk with the innate immune system of the host. Experiments showed that when the expression of the host gene (AgDscam) involved in the innate immune system was suppressed, *Asaia* sp. was no longer controlled by the innate immune system and it was free to proliferate into the *An. gambiae* haemolymph (Dong *et al.*, 2006).

Lactic acid bacteria symbionts of Drosophila flies.

Together with AAB, attention must be devoted also the group of lactic acid bacteria (LAB), another category of commensal bacteria, that plays a significant role in larval development. *Drosophila* is associated with 5-20 bacterial species and LAB are among the most abundant ones, in particular *L. plantarum*, a *Drosophila* commensal and probiotic species (Tower, 2011).

L. plantarum is one of the most widespread LAB, a commensal species commonly found in metazoan gut, where it colonizes the organ epithelium. In particular, it was reported that *L. plantarum* can favor by itself the larval growth by modulating the hormonal growth signaling pathway, specifically acting on the steroid hormone ecdysone for the growth period and on the insulin-like peptides for the growth rates. Moreover, *L. plantarum* facilitates uptake of dietary proteins and may aid digestion by breaking down yeast proteins in the intestinal lumen and liberating amino acids and peptides (Storelli *et al.*, 2011).

On the other hand, *L. brevis* is a *Drosophila's* gut pathobiont, as it can cause gut cell apoptosis and early host death. The cause of this collapse has to be attributed to the chronic activation of DUOX, probably caused by the release in a constitutive way of uracil by *L. brevis* itself (Lee *et al.*, 2013). For both these two LAB species the genome has been sequenced (Kim *et al.*, 2013a, Kim *et al.*, 2013b).

Symbiotic bacteria, in particular *Lactobacillus* species, were found to influence mating preference (one of the mechanisms for the origin of new species) in *D. melanogaster*, as they influence the levels of cuticular hydrocarbon sex pheromones. In laboratory conditions, mating preference is obtained by rearing separately fly populations for several generations under different environmental conditions (e.g. pH, temperature, diet…). Infection with *Lactobacillus* sp. isolates caused a significant increase in mating preference. The hologenome theory of evolution receives a big support from these data; the hologenome is the sum of the genetic material of the host and its microbiome, and according to this theory they act as a unit of selection in evolutionary path, thus variation can occur by modification in either the host or the microbiota genomes (Zilber-Rosenberg and Rosenberg, 2008). The abundance of *L. plantarum* species under diet change is responsible to mating preference, which in turn influence the variation of the entire holobiont. Nevertheless, how bacteria induce mating preference is still unknown: the hypothesis is the emission of volatile compounds by the holobiont, probably done by bacteria (Sharon *et al.*, 2010).

Like AAB, LAB are acquired by the fruit flies by the environment, as inferred by the data already presented; *L. plantarum* was also found in association with the gastrointestinal tract of *D. simulans* collected from wine; consequently it is vectored to grape must, which it is in turn infected, as the same LAB strain were collected from the fruits (Groenewald *et al.*, 2006).

In addition to this, a recent work focused its attention on the replenishment strategy that insects carry out for the sustainment of the bacterial community (Blum *et al*., 2013). It emerged that *Drosophila* settles and maintains its microbiome thanks to the frequent assumption of bacteria, and consequently the bacterial community in *Drosophila* is modified by fly access to exogenous bacteria. Probably there is an innate or learned mechanism that enables the flies to replenish its microbiome, although the ways by which *Drosophila* is able to recognize the beneficial bacteria is still to be investigated. Moreover, the main symbionts in the insect can keep an association with the host thanks to the repeated assumption of bacteria. If the fruit flies were fed with the LAB *L. plantarum*, they were more protected from intestinal pathogens and infections, confirming the importance of this bacterium for the insect health status. In this way, *Drosophila* and humans might have a similar mode of attraction with the *Lactobacillus* strain, as they both benefit from the interaction with it, but they have to replenish it continuously, because it cannot persist into the host body (Blum *et al*., 2013).

D. melanogaster nutritional traits undergo the impact of each microbial taxon of the gut microbiota and, in particular, the impact derived from the bacteria interspecific interactions with the host. AAB of the genus *Acetobacter* and LAB of the genus *Lactobacillus* possess in this way a synergistic effect on the host, as *Lactobacillus* sp. supports the growth of *Acetobacter* sp., which in turn is negatively correlated with the insect triglyceride content (Newell and Douglas, 2014).

To conclude the overview on the main bacteria constituting the fruit flies microbiome, among gammaproteobacteria the group of bacteria belonging to the Enterobacteriaceae is widely recognized. Bacteria of the *Enterobacter* genus were identified in wild-captured *Drosophila* flies of different species by Chandler and colleagues in 2011, while an exhaustive review regarding culture- based and molecular-based studies regarding Drosophila melanogaster's gut microbe investigation, is provided by Broderick and LeMaitre 2012. Some bacteria belonging to this family are strong entomopathogen, like *Serratia marcescens*; this microorganism is able to penetrate the insect's tissues and it is invulnerable to the host immune response. Enterobacteriaceae members are known to establish tight or loose symbiotic relationships with many of their arthropod hosts, spanning from commensalism to primary symbiosis (Husník, Chrudimský and Hypša, 2011). It is supposed that the association with arthropods could have contributed to the evolution of this family group. To date, aphids are the best studied examples of hereditable symbiosis with Enterobacteriaceae (Sandstrom *et al*,. 2001).

Yeast community associated to Drosophila species

Molecular identification through DNA barcoding for fungi has played in the last 15-20 years a crucial role in fungal ecology research. There is not a universally accepted DNA barcode for fungi (Kiss, 2012, Schoch *et al.*, 2012, Schoch and Seifert, 2012). DNA barcoding uses standardized 500- to 800- base pair sequences to identify species from all eukaryotic kingdoms by using primers that can be applied for the broadest possible taxonomic group. Barcoding gives its best when the chosen primer sequence is unique and constant. The barcode used for animal is a region of the mitochondrial gene that encode the CO1 (cytochrome c oxidase subunit 1), and although fungi also possess this gene, it is difficult to amplify it, as it includes large introns and it is not sufficiently variable. Among the region of ribosomal cistrons, the internal transcribed spacer (ITS) region is the most probable barcode marker to explore and identify the broadest range of fungi (Schoch *et al.*, 2012).

Despite the controversies arose in these last years, regarding the choice of the ITS as the barcode marker for fungi, it is the most suitable one and still it is the official primary barcoding marker for fungi at the Smithsonian's Conservation and Research Centre in 2007. One of the objections refers to the incapability of ITS to discriminate among many closely related fungal species, especially if determined after cloning (Kiss, 2012, Schoch and Seifert, 2012). The ITS region includes the ITS1 and ITS2 regions, separated by the 5.8 gene, and it is situated between the 18S (SSU) and 28S (LSU) genes in the nuclear DNA repeat unit. There are a lot of ITS copies in each cell, therefore it is a candidate target for sequencing, even if the quantity of DNA is low (Bellemain *et al.*, 2010).

A rapid molecular method for the identification of yeast species is the generation of restriction patterns (RFLP- Restriction Pattern Length Polymorphism) from the PCR products of the region spanning the ITS1 and ITS2 and the 5.8S rRNA. Since the ITS regions are less evolutionary conserved than the rRNA coding genes, this is a useful tool for taxonomic purposes as it detects the genetic variability among species (Guillamon *et al.*, 1998). The other region that is often taken in analysis, also in combination with the RFLP on ITS, for the identification of yeasts is the variable domain of the large subunit of the 26s rRNA gene (D1/D2 region); most yeasts can be identified from sequence divergence in this region and therefore it has been included among the molecular tools for yeast taxonomy (Satyanarayana and Kunze, 2009).

Research on fungi has recently highlighted the importance of these organisms for the host health status. Yeasts, in particular, the group of single celled eukaryotic microorganisms comprised into the Kingdom of Fungi, divided into two Phyla, Ascomycota and Basidiomycota, establish a mutualistic relationship with insects, specifically with members of the Coleoptera, Dictyoptera, Diptera, Dermaptera, Rhyncota, Hymenoptera, Homoptera, Isoptera, Lepidoptera and Neuroptera orders. Yeast symbiosis appears to be lineage-specific, as the groups associated to the insects belong to the Pezizomycotina (arbuscular mycorrhizal associates of plant roots) and the Saccharomycotina of the Ascomycota (Gibson and Hunter, 2010, Zacchi and Vaughn-Martini, 2002). The abovementioned *S. titanus*, insect vector of the

"*Flavescence dorée*" yellow disease, was found to carry not only numerous *Cardinium* symbionts, in specific cells similar to bacteriocytes in the ovaries, but also a consistent population of yeast-like symbionts (YLSs). These microorganisms have been identified within specialized cells (mycetocytes) of the fat body and into the ovaries. Thus, both bacteria and yeasts could be transovarially transmitted to the offspring. (Sacchi *et al.*, 2008). YLSs in particular are transmitted vertically when the female smears them onto the eggshells, which are in turn consumed by the hatching larvae (Buchner, 1965).

Presence of YLSs in several homopteran species has already been registered, as they could exert an essential role for the normal development of the host. For instance, in the Asian rice brown planthopper *Nilaparvata lugens* YLSs are involved in nitrogen recycling and uric acid metabolism, and are probably necessary for the correct vitellogenin production (Cheng and Hou, 2005).

In contrast to the distinctive features of the insect-bacterial symbioses described in the previous paragraphs, and apart from the particular case shown by YLSs shown above, the association between fungi and eukaryotic organisms shows a pattern of associations that is primarily horizontally transmitted and facultative. As for bacteria, an ancient pathogenic state or nonpathogenic commensalism is one of the hypotheses for the origin of endosymbiotic yeast-insect association, together with the hypothesis of yeasts as descendants of phytopathogenic or saprophytic fungi. The most probable hypothesis suggesting the origin of the association of yeasts with fruit flies is based on the insects' feeding habits: yeasts were thus initially acquired by chance, as flies fed on fruits, flowers and phylloplane, the surfaces on yeasts are commonly associated, and they were acquired because of the feeding behavior by the insect. Moreover, this association gives the opportunity also for yeasts to be transmitted by inoculation into the plants (Vega and Dowd, 2005). Fungi are, in most cases, hosted extracellularly in co-opted organs, such as the diverticulum, a blind sac situated externally to the gut, or out of the body, such as the mycangia (Hulcr *et al*., 2012). The reason of the scarcity of intracellular, transovarially transmitted fungal symbionts ascribed to the yeast cell size is rejected, as yeast and bacterial range of cell sizes in some cases overlaps.

Several roles have been determined for yeasts and yeast-like fungi (some fungi, derived from the subphylum Pezizomycotina, that have been isolated from insects) associated to insects: firstly, a nutritional role, as yeast can provide enzymes, essential amino acids, vitamins and sterols, detoxifying also toxic plant metabolites in the host's diet. On the other hand, yeasts receive a protected environment and dispersion service (Vega and Dowd, 2005). In particular, some "generalists yeast", such as yeasts belonging to the *Pichia*/*Candida* group, can establish a connection with different insect taxa. These insects receive benefits from the association with yeasts, spanning from response to plant allochemicals, pheromones production, nutrition, and digestive-detoxifying reactions.

Among the different insect groups involved in mutualistic relationships with yeasts, drosophilid flies exert a remarkable role. This kind of association originated as yeasts are the main nutritional source for fruit flies, providing proteins, vitamins and other nutrients for the insect. Some of these microorganisms can pass through the insect gastrointestinal tract scatheless, and in this way they can be dispersed and can colonize new microhabitats. Many drosophilid flies choose substrates, like fruits, fungi and decaying plants, already colonized by fermenting yeasts for mating and oviposition (Becher *et al.*, 2012). As a consequence, *Drosophila* habitat changes according to the physiological characteristics of the associated yeast communities (Starmer, 1981), with consequence coadaptation of *Drosophila* with yeasts in their natural habitat (Starmer and Fogleman, 1986).

The study of the yeast communities associated to different *Drosophila* species from different geographic locations in the world (to enlarge as much as possible the phylogenetic, geographic and ecological diversity) revealed the presence of few dominant *taxa*, belonging to the Saccharomycetaceae family (Ascomycota phylum, Saccharomycetes class, also known as "true yeasts"). In particular, it was recorded the presence of the *Hanseniaspora uvarum* species, which was found to be the dominant yeast genus associated to the *Drosophila* species under scrutiny, followed by representatives of the *Saccharomyces* and *Candida* genera. These groups of yeasts comprise specialist colonizers of rotten and fermenting fruits, and the food

sources *Drosophila* uses to visit. Moreover, as for the bacterial community associated to drosophilid flies, the same yeast lineages are associated with different host species, and the insect diet amenably shapes the yeast community composition, more than host species themselves (Chandler *et al*., 2012).
Specifically, yeast community of *D. suzukii* was investigated with TRFLP (Terminal restriction fragment length polymorphism) and microbiological methods in insects and both infested and uninfested fruits. *H. uvarum* is the yeast species isolated from the majority of *D. suzukii* larvae and adult specimens, followed by *Metschnikowia pulcherrima, Pichia terricola* and *P. kluyveri*, while *Cryptococcus* spp. were isolated from the fruit samples not infested by the insects, and *Metschnikowia* spp. were the dominant yeasts in infested fruits. In particular, infested and uninfested fruits contained a broader diversity of yeasts, but they were still dominated by the same insect-associated species. Information regarding *D. suzukii*'s favorite yeast substrates could be useful for the development of trap attractants in a view of pest management (Hamby *et al.,* 2012).

As already sketched above, among drosophilid flies and yeasts reciprocal effects and benefits have been detected. For instance, several yeast species influence *D. melanogaster* survival and development time, in particular the community competition of the substrates, where larvae feed on, influences the host fitness in term of susceptibility to parasitism (Anagnostou *et al.,* 2010). The same yeast species that are able to grow on fruits are also characterized by the ability to produce an array of volatile compounds. An observed difference of attraction mediated by yeast species, in particular *Saccharomyces* species (Hemiascomycete), depends principally on the variability of aroma compounds made by yeast strains. As said before, vinegar fruit flies that belong to *Drosophila* genus are able to disseminate yeasts, consequently it could be possible that the volatiles produced by yeasts might play an attraction for flies, favoring the dispersal of the microorganisms.
The elicitation of attraction of just some yeast strains could explain the constant association of *Drosophila* with yeast species associated to fruiting plants, mainly *Saccharomyces* sp. In addition to this, Palanca and colleagues (2013) showed that yeasts coming from fruit and vineyard environments, independently from their taxonomic classification, are more attractive than yeasts isolated from non-fruits and non-vineyard sources. Attractive compounds for *D. melanogaster* include alcohols, ethanol and 2-phenylethanol, volatile acids, aldehydes, ethyl esters, and acetate esters; many of them are comprised into the gamma of yeast fruit fermentation volatiles (Goddard, 2008).
Moreover, insects can have several other implications on yeast communities; beside their dispersal, they might be directly involved in yeast niche construction, by reducing yeast species diversity and favoring the expansion of appetizing yeast species. Already Starmer and Fogleman suggested with their experiments that yeast communities have a better stability in the presence of *Drosophila* larvae (Starmer *et al.,* 1986), but recently it was highlighted the positive effect of *D. melanogaster* larvae, in particular, on the densities and the community structures of yeasts growing on bananas. In particular, the presence of larvae encouraged the development of three yeast species, *Candida californica*, *Candida zemplinina* and *P. kluyveri*, reducing the species diversity. The dissemination of specific yeast species by mobile larvae can have a positive effect on the food supplies for the insects themselves, by modifying the micro-biotic environment in which they developed (Stamps *et al*., 2012).

Another positive effect exerted by yeast-flies interdependency is the selective advantage given by passage and the residence into the insect body for Saccharomycetes yeasts. When sugar-rich fruits and protected environments are absent, wild populations of *Saccharomyces cerevisiae* use the queens of the social wasps *Vespa crabro* and *Polistes* sp. as ecological sanctuaries. These organisms are indeed vector and natural refuge of *S. cerevisiae,* in particular during winter. Social wasps are characterized by the overwintering capacity and are able to pass yeast cells to new generations, suggesting the hypothesis that wasps are involved the dissemination of wild *S. cerevisiae* populations in new habitats, in maintenance of their diversity, besides playing a role in their evolutionary path (Stefanini *et al.,* 2012).
The diversity and outbreeding in yeasts is obtained through the break of the *ascus* and release of the tetrad. The tetrad is a structure in which the four spores derived by meiosis of diploid

cells are organized, and it usually occurs when nutrients are limited. This structure is closed by an envelope called *ascus*. Because of this organization, outbreeding is low by default, since it is not simple to break the *ascus* and release the spores, favoring the mating among spores coming from different tetrads. Despite that, in *S. cerevisiae* the dissolution of tetrad ascospores inside the *Drosophila* and wasps gut increases outbreeding, suggesting that association with flies can be an important factor in yeasts evolution. Outbreeding rates were up to ten-fold higher when yeast spores passed through the intestinal tract of the insect vector *D. melanogaster* (Reuter *et al.*, 2007) Thus, insects play an important role in favoring yeast genetic diversity on both a geographic and genomic scale, as recombination helps adaptation to adverse environmental conditions (Stefanini *et al.*, 2012).

Interactions among insects, plants, bacteria and yeasts

In 1965, Paul Buchner was the first scientist to report the variety of both endosymbiotic fungal and bacterial counterparts in arthropod species, suggesting the importance of these associations for some traits of the animal life, such as the diet support.

There are several examples of the positive and negative interaction that can be established among insects and microorganisms, through plant participation ; one case is represented by the grape sour rot.

Sour rot is a disease that causes huge crop losses. Fruit flies (*Drosophila* spp.) play a significant role in vectoring yeasts and AAB that speed up the spread in the entire vineyard. In addition, other opportunistic microorganisms, like fungi, yeasts and other AAB, cause infection in the wounded berries. This disease is mainly induced by a microbial consortium of oxidative, weakly fermentative ascomycetous yeasts (*H. uvarum*, *Candida stellata*, *Pichia membranifaciens*, *Candida krusei*) associated to AAB (*Acetobacter* and *Gluconobacter* spp.). The yeast community of the plants before and after the infection with sour rot is significantly different. The hypothesis is that drosophilid flies are able to induce a chemical change in musts only when the physical barrier posed by the berry skin is broken. Indeed, when the *Drosophila* is absent, the plant defense response mechanisms can be alerted and the fruit skins can be healed while, if the insect is present, a spontaneous fermentative process, initiated by the yeasts and AAB carried by the fly, is activated on the fruits surface. Thus, this disease is caused by a synergic contribute of different factors: the acetic acid produced by AAB seems to be the etiological agent, while flies act as an inoculum source, and a key element, without whom no disease development occurs (Fermaud *et al.*, 2000, Barata *et al*., 2011, Barata *et al.*, 2012).

Studies have shown that fungi and bacteria are able to create in some cases a physically and metabolically interdependent *consortium*, such as the bacterial-fungal biofilm (Morales and Hogan, 2010). The two partners involved in this association can interact and communicate through antibiosis, that is the release of molecules, often deleterious, from one part to the other, or through signaling-based interactions. Communication can also occur via modulation of the physiochemical properties of the environment, like pH modification, metabolites production, and trophic interaction. A mechanical interaction can be obtained through chemotaxis and cellular contact (Nikawa *et al.*, 2001, Deveau *et al*., 2007). If the establishment of this kind of relationship is successful, significant effects can be observed in both partners; and the physiology and development can be influenced, as well as the survival, dispersal and colonization (Vega and Dowd, 2005).

An example of positive effect of fungal-bacterial interaction is represented by the mutualism between “fungus-gardening” (attine) ants and their fungal associates. Ants are strictly dependent on their fungal hosts, as they are the unique food source for larvae and queens; ants defend their fungi by keeping the garden free of microbial pathogens (like the ascomycete *Escovopsis*), by supporting the population of actinomycete bacteria (*Streptomyces* or *Pseudonocardia* species). These bacteria are able to produce an antibiotic that affects *Escovopsis* survival (Currie, 2006, Frey-Klett *et al.*, 2011, McFrederick *et al.*, 2014).

The work presented by Fogleman and Danielson in 2001 is an example of a complex but significative relationships among plants, microorganisms and insects in the Sonoran Desert, in the United States. Endemic *Drosophila* species feed and reproduce in necrotic tissue of

columnar cacti. The metabolic activities of bacteria and yeasts, which live in the tissue necrosis, can influence the substrate chemistry, while the plant affects the drosophilid mating behavior; the substrate, on which larvae are reared on, modifies the adult hydrocarbon epicuticular composition, a determinant of mate choice and premating barrier.
As stated by Broderick and LaMaitre in 2012, the investigation on the interactions between microbiome associated with Drosophila and its host was exclusively directed towards bacteria. Nevertheless, it clear now that much of the attention should be devoted now to the scrutiny of the importance of this group of microorganisms for *Drosophila* species, and the contribute of bacteria has to be integrated in these analyses.

Conclusions and perspectives

The intention of this introduction was to present a wide-ranging overview regarding the complex microscopic world inside the insect body, with a specific attention to the relationships existing among fruit flies of the genus *Drosophila* and those microorganisms that exert a significant role in the biology and lifecycle of their hosts, in particular AAB, LAB and yeasts. The removal of symbiotic AAB and LAB can seriously affects the health status and even the survival of the host (Dong *et al.*, 2006, Ryu *et al*., 2008, Shin *et al.*, 2011, Storelli *et al.*, 2011, Tower, 2011, Chouaia *et al.*, 2012, Lee *et al.*, 2013, Mitraka *et al*., 2013, Kim and Lee, 2014) while, on the other hand, yeasts are involved in a composite ecological picture (Starmer and Fogleman, 1986, Vega and Dowd, 2005, Reuter *et al.* 2007, Goddard 2008, Anagnostou *et al.*2010, Becher *et al.*, 2012, Chandler *et al*., 2012, Stamps *et al*., 2012, Stefanini *et al.*, 2012, Palanca *et al*., 2013). Furthermore, insect body is a congenial habitat for the settlement of these microorganisms, and through their feeding and mating habits insects favor yeasts dispersal and colonization of new ecological niches.

In the last years, a new approach, called Microbial Resource Management (MRM), is taking shape to develop strategies for the control of insect-related problems, through the use of the pest microbial community. From this point of view, many advantages can be obtained from the manipulation of the microbiota associated to insects, even if, first of all, the knowledge of the ecological bases of pest infestation, the role and the effect of microorganisms on the biology and evolution of the host, and the definition of ecological rules, is compulsory (Verstraete 2007, Crotti *et al.*, 2012).
Modern tools of biotechnology might be developed in order to minimize losses from the pest action. One strategy could be the identification of microbial or yeast entomopathogens as biological pesticides, together with the key genes responsible for their ability to cause detrimental effects in insect pests. Their gene products, including toxins, could be exploited for this purpose. Some yeast strains are able to compete for their own ecological niche and nutrients by producing an array of compound named “killer toxins”; this feature might have also a role in a pest management perspective. Yeasts can be classified in three categories, according to the phenotypes they display: “killer”, “sensitive” and “neutral” types. Sensitive cells are killed by the killer ones, while the neutral phenotype do not suffer from the killing properties of the killer strains, neither possess the killer factor affecting the life of the other cell types (Bevan and Makower, 1963). The killer factor is an agent of proteinaceous nature released by the killer strain, lethal to other yeasts, with a specific action spectrum; its production and stability is dependent on pH, temperature and aeration conditions. The production of killer toxins could be highly affected by the culture conditions, and optimal ones might be needed to be found empirically. The similarity with bacteriocins having bactericidal activity is very close. The importance of the killer phenomenon in yeasts is achieving more attention in different fields of biology, as a killer positive property is widespread among yeasts. The killer toxin characterization has been performed in different strains of *S. cerervisiae* (Woods and Bevan, 1968), *Zygosaccharomyces bailii* (Radler *et al.*, 1993), *H. uvarum* (Radler *et al.*, 1990), *P. membranifaciens* (Santos and Marquina, 2004), *Debaryomyces hansenii* (Santos *et al.*, 2002), *Kluyveromyces phaffi* (Ciani and Fatichenti, 2001) and *Scwanniomyces occidentalis* (Chen *et al.*, 2000). They are all mycocins producers, *i.e*. proteins or glycoproteins that bind to polysaccharide structures on the yeasts cell wall. For example, *P. membranifaciens* produces a killer factor of polypeptidic nature that is able to

protect *Vitis vinifera* from the grey mold, caused by the ubiquitous fungus *Botrytis cinerea* (Santos and Marquina, 2004).
Not only yeast strains that produce killer toxins, but also antagonistic species can exert a role in the biological control. Yeasts have been extensively exploited since a long time as biocontrol agents, because of their fast growth and the capacity to keep the flow of pathogens germination down (Rosa-Magri *et al.*, 2011). The mechanism of action does not depend on the production of chemical antagonistic substances, but it is based on ecological interactions, like competition for space and nutrients, mycoparasitism, antibiosis, predation or induction of plant diseases (Janisiewicz and Korsten, 2002). One example is given by the inhibitory of ethanol-producing *Saccharomycetes* on the less alcohol-tolerant yeasts during wine fermentation (Fleet, 2003).
Often, yeast antagonistic action was seen to receive a synergistic help by safe chemicals, including methyl jasmonate (Yao and Tian, 2005), salicylic acid (Farahani and Etebarian, 2011), silicate sodium (Farahani *et al.*, 2012), or sodium bicarbonate. This latter compound was applied in the integrated control of *Penicillium digitatum*, a pathogen causing green mold in oranges, together with the predacious yeast *Saccharomycopsis craetegensis* (Pimenta *et al.*, 2010).
"Predacious yeast" is a term introduced by Lachance and Pang in 1997 to describe those microorganisms that penetrate and kill other yeasts through small appendages called *haustoria*, performing an *haustorium*-mediated predation. Predation among yeasts has been considered a rare phenomenon, even though recent findings indicated that it might be a widespread property of filamentous species belonging to the *Saccharomycopsis* genus and related ones.

The gathered information, and the successful results of pest management practices obtained on other insect groups, might be a basis to develop alternative strategies for a more effective and sustainable biocontrol management of those insects characterized by a pest attitude, and for whom a successful strategy has not been found yet, like the emerging pest *D. suzukii*. Baits currently in use include mixtures of yeasts, sugar, water, ethanol, apple vinegar o cider. High levels of attraction are associated to a combination of vinegar and wine, probably caused by the presence of acetic acid, ethanol and produced volatiles (Landolt *et al.*, 2012). The knowledge of the microbiota associated to *D. suzukii* and infested fruits could be exploited in the development of more attractive baits with more suitable attractive microorganisms or microbe-produced volatiles.

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Rationale and aim of the work

The invasion of new pest species is a menace for biodiversity preservation and a severe issue for growers of the affected areas. The opportunity to fill up free ecological niches with no effective competitors or natural predators allows a quick settlement of the invaded areas by these pests.
One of the last years' most pestiferous insect pest is the spotted wing fly *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), a new exotic pest originating from South-East Asia and rapidly spreading from its native range in Western countries (Lee *et al*., 2011, Cini *et al*., 2012). Current strategies enacted to restrain and control the spread of this pest foresee a ceaseless monitoring of the crop areas coupled with the use of chemicals, traps and insecticides (Walsh *et al*., 2011). Nevertheless, more sustainable and specifically targeted solutions are necessary.
Recent investigations are witnessing the relevant role played by some microorganisms living in association with insects, and affecting different aspects of the host biology and health (Dale and Moran, 2006, Gibson and Hunter 2010). For this reason, the characterization of the microbiome associated to insect pests might provide new insights in a perspective of integrated pest management (Verstraete, 2007).

The **aim** of this PhD doctoral thesis is to present the information collected from the characterization of the microbiome associated to *D. suzukii*, with a particular focus on acetic acid bacteria (AAB) and yeast communities (Crotti *et al*., 2010, Chandler *et al*., 2012, Hamby *et al*., 2012). Moreover, a perspective on the use of microorganisms as a tool for integrated pest management is proposed.
In particular, the **second Chapter** depicts the stable association established between the AAB microbiome and *D. suzukii*. DGGE (Denaturing Gradient Gel Electrophoresis) and pyrosequencing pyrotag molecular analyses, together with the application of cultivation-dependent techniques, revealed the presence of AAB in all the individuals surveyed, of all developmental stages, and reared on two feeding substrates (fruit or artificial diet). Three AAB isolates, labelled with Green fluorescent protein (GFP) were used for recolonization experiments of insect adults' digestive system to visualize the localization of the administered labelled bacteria. It was possible to detect these bacteria on the epithelium of the insect crop, proventriculus and gut. Then, fluorescent *in situ* hybridization (FISH) analyses on tissues confirmed AAB localization on the peritrophic membrane of midgut and proventriculus.
After AAB microbiome characterization, the capacity of some *D. suzukii*-selected AAB isolates to emit microbial volatile compounds for flies' specific attraction was analysed, and the results are discussed in **Chapter III**. Volatile organic compounds (VOCs), such as acetic acid, are known to be attractants or repellent for insects (Cha *et al*., 2014) and, currently, traps for flies are constituted by vinegar and baker's yeast. For this reason, baits specifically targeted for *D. suzukii* could be a successful strategy of integrated pest control. According to this, flies were exposed to attractant molecules produced by AAB (in comparison to a control) in a 2-choice olfactometer assay, and the best fly attractive isolates were identified.
The attention was subsequently addressed to the characterization of the yeast community associated to the spotted wing fly, and the data are exposed in **Chapter IV**. Both cultivation-independent techniques, performed with DGGE and 16S rRNA pyrosequencing, and cultivation-dependent approach (isolation trials, Restriction Fragment Length Polymorphism fingerprinting of the ITS1-ITS2 region of the fungal rRNA gene and sequencing of the D1-D2 region of the large ribosomal subunit) allowed the investigation of the yeast diversity related to *D. suzukii*. The analysis revealed the prevalence of yeasts belonging to the Saccharomycetales order. This group of yeasts comprise genera of specialist colonizers of fruiting plants and *Drosophila* food sources.
The fifth Chapter sheds a light on the possible future applications of spotted wing fly-associated yeasts in a perspective of pest management, by analyzing the capacity of some selected isolates of the obtained collection to produce killer toxins (Woods and Bevan, 1968).

These compounds can be generally used by some strains to compete with other yeasts for space and nutrients. Culture growth conditions suitable for killer toxins' production were realized, and antagonistic activity tests against the same selected isolates of the yeast collection and 12 representative AAB isolates were performed. Data obtained from the screening highlight that the most promising results are related to the capacity of a specific yeast isolate, *Candida stellimalicola* AF4.1.P.268, to create an inhibition zone, preventing the growth of other yeast and AAB isolates.
At last, the **Conclusions Chapter** summarizes the results obtained with the present PhD project.

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Chapter II

The Acetic Acid Bacterial Microbiome of the Spotted Wing Fly, *Drosophila suzukii*

Abstract

Drosophila flies are mainly considered as secondary parasites, due to their behaviour to attack rotten fruits. They harbour an inconsistent microbiome composed of several bacterial taxa, among which the acetic acid bacteria (AAB) are found to be important modulators of insect development through insulin signalling. Conversely, the spotted wing fly *Drosophila suzukii* (Diptera: Drosophilidae) is a highly invasive pest, native of Eastern and South-eastern Asia, rapidly spreading in the many countries, laying eggs in healthy fruits, with a consequent economic damage. With the aim to unravel the microbiome associated to *D. suzukii*, reared on fruits or on artificial diet, cultivation-independent and -dependent techniques have been employed, giving a particular attention to AAB symbionts. By DGGE-PCR on 16S rRNA gene, AAB of the genera *Acetobacter* and *Gluconobacter* have been frequently detected. According to 16S rRNA barcoding, the two groups of insects (reared on fruits or on artificial diet) showed to cluster separately, but in both cases sequences related to Rhodospirillales order, to whom AAB belong, were a predominant group. Isolation data evaluated the extensive presence of cultivable AAB (*Acetobacter, Gluconacetobacter* and *Gluconobacter*) in the fly body, investigating different life stages (larvae, pupae, adults). Recolonization experiments by the use of *green fluorescent protein* (Gfp)-labelled strains and fluorescent *in situ* hybridization indicated the dispersal of AAB in the insect gut. In *D. suzukii* larvae and adults, AAB are mainly localized on the midgut epithelium.

INTRODUCTION

The vinegar fly *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), also known as spotted wing drosophila in the USA, is an exotic pest, endemic to South Asia and recently introduced in the continental USA, Canada and Europe, most probably by the international trade (Cini *et al*., 2012, Hauser 2011, Lee *et al*., 2011). Characterized by a rapid spreading behaviour, this fly is an economically damaging pest, due to the ability, unlike its vinegar fly relatives that attack rotten fruits, to feed on healthy soft summer fruits, laying eggs on them, thanks to the females' large serrated ovipositor (Walsh *et al*., 2011). Once hatched, larvae grow in the fruit, destroying it (Mitsui *et al*., 2006, Walsh *et al*., 2011). *D. suzukii* is able to develop on many host plants either in its native and introduced habitats, with berries being the preferred hosts (Grassi *et al*., 2012, Lee *et al*., 2011, Seljak, 2011, Walsh *et al*., 2011). In most attacked countries *D. suzukii* causes severe economic damage to soft fruits every year (Goodhue *et al*., 2011, Grassi *et al*., 2009). In particular, in Italy the most significant growers' associations reported extensive crop losses (Lee *et al*., 2011).

A recent increased attention has received the study of the bacterial microbiome associated to *Drosophila* flies. Drosophilid flies belonging to different species and with various feeding habits, reared in laboratory conditions or field-captured, have been investigated through deep sampling analysis by 16S barcoding or 16S clonal libraries (Wong *et al.*, 2011, Chandler *et al*., 2011, Wong *et al.*, 2013). A bacterial community dominated by four families as Lactobacillales, Acetobacteraceae, Enterobacteriaceae and Enterococcaceae is commonly associated to these flies, with variations of the bacterial members at the genus level (Chandler *et al*., 2011, Wong *et al.*, 2013). In lab reared and field-sampled flies, acetic acid and lactic bacteria (AAB and LAB, respectively) are dominant symbiotic taxa harboured in the intestinal tract (Wong *et al.*, 2011, Ryu *et al.*, 2008). In particular, AAB establish a delicate balance with the insect innate immune system, being involved in the suppression of the growth of pathogenic bacteria in healthy individuals (i.e. colonization resistance), hence contributing to the host health (Ryu *et al*, 2008, Silvermann and Paquette, 2008). An interesting experiment conducted on the experimental model *Drosophila melanogaster* by the same research team (Shin *et al.*, 2011) demonstrated AAB promotion ability of the insulin pathway, with consequent enhancement of the larval developmental rate, body size, intestinal stem cells activity and energy metabolism.

AAB positive role has been also demonstrated in a different insect model, represented by anopheline mosquitoes: the acetic acid bacterium *Asaia* plays a beneficial role in the development of the mosquitoes and in fact, a delay in the development in *Anopheles stephensi* larvae was observed after antibiotic treatment; the larval development rate compared to the control one could be restored after administration of an antibiotic-resistant *Asaia* strain (Chouaia *et al*., 2012). Another work confirmed that *Asaia* administration boosted the developmental rate of *An. gambiae* larvae, affecting genes involved in cuticle formation (Mitraka *et al*., 2013).
AAB are symbionts of insects mainly localized in the insect gastrointestinal tract (GIT) (Crotti *et al*., 2010). Particularly, the midgut is a sugar and ethanol rich environment, and represents a specific and beneficial habitat for these bacteria (Cox and Gilmore, 2007). They owns different ways of transmission, with the horizontal as the favorable one (Damiani *et al*., 2008, Crotti *et al*., 2010, Gonella *et al*., 2012). The recent comparison of AAB genomes showed several symbiotic traits that could favor the adaptation of AAB as insect symbionts (Chouaia *et al*., 2014). In particular, cytochrome bo3 ubiquinol oxidase might be involved in AAB adaptation to the diverse oxygen levels in the arthropod gut. In fact, AAB cannot be only considered as insect symbionts; they are generally found on sugar- and ethanol-rich substrates, spread in the environment on fruits, vegetables and fermented matrices, niches they share with insects and from which insects can re-acquire them (Crotti *et al*., 2010). Given that strong associations are common between acetic acid bacteria and selected insect orders, in the present study we assessed the presence of this important microbial group in *D. suzukii* individuals, reared on fruits or artificial diet, by means of cultivation-independent and -dependent techniques. We also provided information on tissue localization of these endosymbionts. The knowledge of the bacteria associated to this insect pest could be applied in future biocontrol approaches, as discussed.

MATERIALS AND METHODS

Insects.

Wild specimens of *D. suzukii* were field collected as adults/larvae in Trentino Alto Agide region (Italy) and reared in laboratory condition both on fruits and on a sugar-based artificial diet (composed with 71 g of corn flour, 10 g of soy flour, 5.6 g of agar, 15 g of sucrose, 17 g of brewer's yeast, 4.7 ml of propionic acid, 2.5 g of vitamins mix for each Kg of the preparation) at the Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Turin. Insects were kept in cages at 25 °C with a 14:10 h light-dark photoperiod.

DNA extraction.

Larval, pupal and adult individuals of *D. suzukii* were killed, washed with ethanol 70% and twice with saline and immediately stored at -20°C in ethanol until molecular analysis. Total DNA was individually extracted from larvae, pupae and adults of the laboratory strains by sodium dodecyl sulfate-proteinase K-acethyltrimethyl ammonium bromide treatment, as described in Raddadi *et al.* (2011).

Characterization of the bacterial community associated to D. suzukii by Denaturing Gradient Gel Electrophoresis (DGGE).

A 550 bp fragment of the 16S rRNA gene was amplified from the total DNA extracted from *D. suzukii* individuals, using the forward primer GC357f, containing a 40-bp GC clamp, and the reverse primer 907r, as previously described (Raddadi *et al*., 2011). Gels with a denaturant gradient of 40–60% were prepared with a gradient maker (Bio-Rad, Milan, Italy) following the manufacturer's instructions. Bands were excised and used as template in PCR re-amplification reactions with primers 357f and 907r, as described previously (Raddadi *et al*., 2011). PCR products were sequenced (Macrogen, South Korea), and the resulting sequences were compared, using BLAST (http://www.ncbi.nlm.nih.gov/blast), with deposited sequences in the National Center for Biotechnology Information (NCBI) sequence database (Altschul *et al*., 1990).

Characterization of the bacterial community associated to D. suzukii by 16S barcoding.

DNA previously extracted from fly individuals (namely DS54, DSM, DS41, DS55, Ds159, Ds164, Ds165, Ds167, FP1, FP3, LP1, LP3, MP3 and PP2, Tab. 2) were used in 454 Pyrotag sequencing. The variable regions V1–V3 of the bacterial 16S rRNA gene was amplified by MR DNA (Molecular Research LP, Texas, USA) using the universal bacterial primers 27Fmod (5'-AGR GTT TGA TCM TGG CTC AG-3') and 519Rmodbio (5'-GTN TTA CNG CGG CKG CTG-

3') as described in Montagna *et al.*, 2013. In total, 178,856 raw reads were obtained. Pyrosequencing adaptors, low quality base calls (<30 Phred score) and size-selected (between 350 and 500 bp) were performed by using the QIIME pipeline filtering scripts (Caporaso *et al.*, 2010a). The resulted reads were clustered into operational taxonomic units (OTUs), applying a sequence identity threshold of 97%, using *Uclust* (Edgar, 2010). A representative sequence of each OTU was, then, aligned to Greengenes (http://greengenes.lbl.gov/) using PyNast (Caporaso *et al.*, 2010b). Chimeras were removed using *Chimeraslayer* (Haas *et al.*, 2011). The results of OTUs assignment were then used in the diversity analyses using the various scripts of the QIIME pipeline.

Prevalence of AAB in D. suzukii specimens.

A total of 50 *D. suzukii* adults (25 males and 25 females), reared on fruits, were used for assessing the prevalence of different AAB. After DNA extraction of single flies following Raddadi *et al.* (2011), samples were submitted to AAB-specific amplification of the 16SrRNA gene, followed by digestion with the restriction endonuclease TaqI (Promega, Madison, USA) (Ruiz *et al.*, 2000). Pure cultures of the most representative strains isolated from *D. suzukii* were employed for restriction profile reference.

Localization of D. suzukii symbionts by fluorescent in situ hybridization (FISH).

FISH was carried out on tissues and organs dissected from field-collected *D. suzukii* adults in a sterile saline solution. The dissected organs were fixed for 2 min at 4°C in 4% paraformaldehyde and washed in PBS. All hybridization experiment steps were performed as previously described (Crotti *et al.*, 2009; Gonella *et al.*, 2012), using specific fluorescent probes, specifically designed for the acetic acid bacterial group (AAB455, sequence TGC ACG TAT TAA ATG CAG CT) and for *Gluconobacter* (Go15, sequence AAT GCG TCT CAA ATG CAG TT and Go18, sequence GTC ACG TAT CAA ATG CAG TTCCC). Moreover, the universal eubacterial probe, Eub338 (sequence GCG GGT ACC GTC ATC ATC GTC CCC GCT), was used to detect the localization of the overall bacterial abundance and presence in the organs analysed (Gonella *et al.*, 2011). Probes for AAB and Eubacteria were targeted at the 5' end with the fluorochrome Texas Red (TR; absorption and emission at 595 nm and 620 nm, respectively), whereas probes Go15 and Go18 were labelled with indodicarbocyanine (Cy5; absorption and emission at 650 nm and 670 nm, respectively).

Isolation of AAB.

Insects (5 males, 6 females, a pool of 3 males and a pool of 3 females), reared on fruits, were surface sterilized by rinsing once with ethanol 70% and twice with 0.9% NaCl under sterile conditions, before being homogenized by grinding in 200 µl of 0.9% NaCl. Forty µl of each insect homogenate were inoculated in different enrichment liquid and solid media, selected for AAB growth: enrichment medium I (hereafter indicated as TA1, Yamada *et al*., 1999, Kounatidis *et al.*, 2009), enrichment medium II (hereafter indicated as TA2, Yamada *et al*., 2000), a basal medium (hereafter indicated as TA4, Kadere *et al.* 2008), Hoyer-Frateur medium (De Ley and Frateur, 1974), acid YE medium (yeast extract 2%, ethanol 2%, acetic acid 1%, pH 6). One hunderd µl of serial dilutions of the insect homogenate were spread on plates containing mannitol agar medium (mannitol 2.5%, peptone 0.3%, yeast extract 0.5%, pH 7, agar 15 g/L) or R2A agar (Reasoner *et al.*, 1979), both supplemented with 0.7% $CaCO_3$ and 0.01% cycloheximide. Other 6 insect adults reared on the artificial diet, 6 adults, 3 pupae and 3 larvae reared on fruit diet, werewashed three times with deionized water and the washing water of the last step was plated on MA solid medium. Pupae and larvae were smashed, as previously described, and inoculated in TA1 and TA2 enrichment media. All the enrichment liquid media were incubated at 30°C, in aerobic condition with shaking, until turbidity of the liquid media was reached. Serially dilutions were plated on MA medium, supplemented with $CaCO_3$ (1% D-glucose, 1% glycerol, 1% bactopeptone, 0.5% yeast extract, 0.7% $CaCO_3$, 1% ethanol, 1.5% agar, pH 6.8) and incubated at 30 °C, in aerobic conditions. For the solid media, colonies were picked up and streaked on MA solid medium, with $CaCO_3$. Colonies capable of clearing the calcium carbonate were purified on agarized MA medium, and pure strains were conserved in 15% glycerol at -80 °C. Total DNA was extracted from the isolates by boiling protocol and stored at – 20 °C.

16S rRNA gene-based identification and fingerprinting analysis of the isolates.

Internally transcribed spacer (ITS)-PCR fingerprinting was performed with primers ITSF (5'-GCC AAG GCA TCC AAC-3') and ITSR (5'–GTC GTA ACA AGG TAG CCG TA-3') as previously described (Daffonchio *et al*., 1998). ITS-PCR amplification patterns of all the isolates were visually compared to cluster the isolates into ITS groups or profiles. At least 2 candidates for each ITS profile were selected and 16S rRNA gene was amplified and sequenced for identification by Macrogen (South Korea). 16S rRNA gene was amplified with universal bacteria 16S rRNA gene primers 27F (5'-TCG ACA TCG TTT ACG GCG TG-3') and 1495R (5'-CTA CGG CTA CCT TGT TAC GA-3'). Reaction mixture for 16S amplification was carried out in a final volume of 50 µL , using 1 unit of Taq DNA polymerase, 1X PCR Buffer, 0.12 mM of each dNTP, 0.3 µM of each primer, 1.5 mM $MgCl_2$ and 2 µL of DNA. Reaction was run for 4 min at 94°C, followed by 35 cycles of 1 min at 90°C, 1 min at 55°C, 2 min at 72°C and then a final extension of 10 min at 72°. 16S rRNA gene sequences were compared to the databases at the National Centre for Biotechnology Information (NCBI) using BLASTn (Altshul *et al*., 1990) and aligned with their closest type strain relatives using Clustal W (http://align.genome.jp/).

Transformation of Gluconobacter oxydans DSF1C.9A, Acetobacter tropicalis BYea.1.23 and Acetobacter indonesiensis BTa1.1.44 with the plasmid pHM2-Gfp.

G. oxydans strain DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 were transformed through electroporation introducing the plasmid pHM2-Gfp (Favia *et al.,* 2007). Electrocompetent cells were prepared according to this procedure: exponential phase cells (OD 0.5) grown in GLY medium (2.5% glycerol, 1.0% yeast extract, pH 5) were washed twice with cold 1 mM Hepes, pH 7, and once with cold 10% glycerol. Then, cells were resuspended in cold 10% glycerol to obtain 160-fold concentrated competent cells. Aliquots were stored at -80°C**.** Sixty µl of competent cells were gently mixed with about 0.2 µg of plasmidic DNA, put in a cold 0.1-cm-diameter cuvette, and pulsed at 2000 V with the Electroporator 2510 (Eppendorf, Milan, Italy). After the pulse, 1 ml of GLY medium was added to the cells, which were subsequently incubated at 30°C in aerobic condition with shaking for 4 h. Transformed cells were selected by plating serial dilutions on GLY agarized medium, supplemented with 100 µg ml^{-1} kanamycin, 40 µg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal), and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for Lac^+ phenotype detection. When growth occurred, transformant colonies were chosen and the Gfp expression was checked by fluorescence microscopy. ITS amplification of wild type and transformant strains was performed and compared to ensure the identity of the transformants.

Evaluation of plasmid stability.

To verify plasmid stability in the absence of selection, *G. oxydans* DSF1C.9A(Gfp), *A. tropicalis* BYea.1.23(Gfp) and *A. indonesiensis* BTa1.1.44(Gfp) were grown overnight in GLY medium with 100 µg ml^{-1} kanamycin, with shaking. When growth was visible, suitable dilutions were plated on non-selective GLY agar an incubated at 30°C till the growth of well-separated colonies. Four colonies were then chosen, resuspended in 1.0 ml of GLY medium and vortexed intensely to obtain free cells. Suitable dilutions were plated on selective and non-selective GLY agar. The proportion of kanamycin-resistant bacterial cells was determined through the ratio between the kanamycin-resistant bacterial cells and the total number of cells grown.

Colonization experiments of D. suzukii with G. oxydans DSF1C.9A(Gfp), A. tropicalis BYea.1.23(Gfp) and A. indonesiensis BTa1.1.44(Gfp).

G. oxydans DSF1C.9A(Gfp), *A. tropicalis* BYea.1.23(Gfp) and *A. indonesiensis* BTa1.1.44(Gfp) were grown in GLY medium containing 100 µg ml^{-1} kanamycin up to a concentration of 10^8 cells ml^{-1}. Cells were harvested, then washed and resuspended in sterile water to a final concentration of 2×10^8 cells ml^{-1} or 1×10^9 cells ml^{-1} for colonization experiments of *D. suzukii* adults. Colonization experiments of adults were performed by placing the adults in a small cage. A bacterial suspension (10^7 or 10^8 cells) was added to 0.5 g of adult sterile food and small drops of the obtained mixture were placed inside the cage on parafilm-covered glass slides. Appropriate controls without the addition of bacteria were done. The insects were fed *ad libitum* for 48 h with a sugar solution containing the Gfp-labelled strain, and then they were allowed to feed for 20 h with honey. Organs were then dissected in Ringer solution (0.65% NaCl, 0.014% KCl, 0.02% $NaHCO_3$, 0.012% $CaCl_2$ $2H_2O$, 0.001% NaH_2PO_4 $2H_2O$, pH 6.8) and fixed in 4%

paraformaldehyde at 4°C for 10 min, mounted in glycerol and analyzed by fluorescence (Leica Microsystems, Germany) and confocal laser scanning microscopy, Confocal Laser Scanning Microscopy (CLSM, Leica Microsystems, Germany).

RESULTS

Characterization of the bacterial diversity in D. suzukii by DNA-based analysis.

The bacterial community associated to 32 *D. suzukii* specimens was analysed by 16S rRNA gene DGGE-PCR, using as template the DNA isolated from whole single individuals. In particular, 5 larvae (n. 1-5; Fig 1A), one pupa (n. 6; Fig 1A) and ten adults (n. 7-16; Fig 1B) reared on fruits have been analysed, as well as 4 larvae (n. 29-32), 4 pupae (n. 25-28) and 8 adults (n. 17-24) reared on the artificial diet (Fig.1C). Generally, *Drosophila* flies host low complex bacterial community, with the presence of few abundant bacterial taxa (Chandler *et al.*, 2011; Wong *et al.*, 2013). Here, a lower variability in the community profiles was observed among larvae reared on fruits and among the specimens reared on the artificial diet (Fig. 1A-C): many bands were rather conserved among the samples respectfully to the group to which each specimen belonged. Conversely, only few conserved bands were detected among the adults reared on fruits, which showed to have more complex profiles than larvae reared on fruits or specimens from the artificial diet (Fig. 1A-C). The majority of the bands from specimens reared on fruits showed similarity with species belonging to the Alphaproteobacteria class which comprises AAB, whereas the others indicated closeness with Betaproteobacteria, Gammaproteobacteria, Firmicutes and Bacteroidetes classes (Tab.1). Among larvae reared on fruits, bands A6 and A7 were the most remarkable ones, observed in a frequency of 50% (3 larvae out of 6) and 33% (2 larvae out of 6) of the tested individuals, and with 99% similar to the 16S rRNA gene of *Acetobacter tropicalis* and *Acetobacter persicus*, respectively. In all the larvae and in the sole pupa, a sequence with 98% identity with *Bacillus* sp., corresponding to A3 band in the PCR-DGGE gel, was detected, while band A1, corresponding to a sequence strictly related to *Paracoccus* sp. of the Alphaproteobacteria, was identified only for one individual. For 33% (2 out of 6) of the larvae analysed, sequences with 99% of similarity with the genus *Stenotrophomonas* and with the species *Enterococcus casseliflavus*, which are related to bands A4 and A2 respectively, were found not give clear results (lanes 22, 23, 24; Fig. 1C).

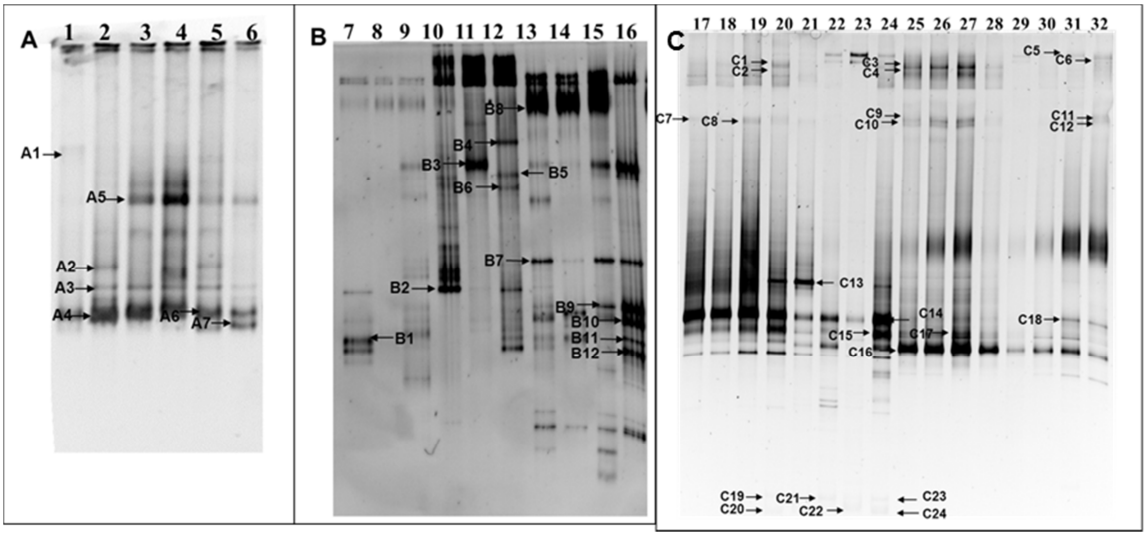

Figure 1*:* Bacterial diversity associated with *D. suzukii* by DGGE-ITS. DGGE profiles, in 7% polyacrylamide gels with 40 to 60% denaturation gradient, of partial 16S rRNA bacterial genes amplified from DNA extracted from whole insects reared on fruit (panels A and B) or artificial diet (panel C). Numbers above the lanes refer to tested individuals. Specimens on fruit: 1-5 larvae; 6, pupa; 7-16 adults; specimens from artificial diet: 17-24 adults; 25-28 pupae; 29-32 larvae. Bands marked with arrows were sequenced; data referred to sequences are given in Table 1.

Table 1: Identification of microorganisms associated to *D. suzukii* according to DGGE profiles in Fig. 1.

Band	Most related species	GenBank Accession no.	% nt similarity (no. of identical bp/total no. of bp[a]	Classification	No. of positive individuals/total no. of individuals[b]
A1	*Paracoccus* sp.	JX515659	98%(551/561)	Alphaproteobacteria; Rhodobacterales	1/6
A2	*Enterococcus casseliflavus*	KC150018	99%(576/580)	Firmicutes; Lactobacillales	2/6
A3	*Bacillus* sp.	AM888231	98%(550/562)	Firmicutes; Bacillales	6/6
A4	*Stenotrophomonas* sp.	KC153268	99%(583/587)	Gammaproteobacteria; Xanthomonadales	2/6
A5	*Wolbachia* sp.	NR_074437	99%(537/542)	Alphaproteobacteria; Rickettsiales	5/6
A6	*Acetobacter tropicalis*	AB681066	99%(542/544)	Alphaproteobacteria; Rhodospirillales	3/6
A7	*Acetobacter persicus*	AB665071	99%(511/512)	Alphaproteobacteria; Rhodospirillales	2/6
B1	*Gluconobacter albidus*	AB178412	100%(507/507)	Alphaproteobacteria; Rhodospirillales	3/10
B2	*Acinetobacter* sp.	HM045831	100%(531/531)	Gammaproteobacteria; Pseudomonadales	4/10
B3	*Chitinophaga* sp.	GQ369124	92%(487/532)	Bacteroidetes; Sphingobacteriales	7/10
B4	*Acetobacter cibinongensis*	JN004206	99%(514/516)	Alphaproteobacteria; Rhodospirillales	1/10
B5	*Gluconobacter albidus*	AB178412	100%(507/507)	Alphaproteobacteria; Rhodospirillales	3/10
B6	*Acetobacter tropicalis*	JF930137	100%(516/516)	Alphaproteobacteria; Rhodospirillales	2/10
B7	*Lampropedia hyalina*	AY291121	98%(526/536)	Betaproteobacteria; Burkholderiales	6/10
B8	*Wolbachia pipientis*	AJ306307	514/519 (99%)	Alphaproteobacteria; Rickettsiales	7/10
B9	*Ochrobactrum* sp.	FJ233847	517/517(100%)	Alphaproteobacteria; Rhizobiales	5/10
B10	*Acetobacter pasteurianus*	AB608081	520/531 (98%)	Alphaproteobacteria; Rhodospirillales	7/10
B11	*Acetobacter aceti*	AJ419840	508/509 (99%)	Alphaproteobacteria; Rhodospirillales	5/10
B12	*Acetobacter senegalensis*	HQ711345	524/535 (98%)	Alphaproteobacteria; Rhodospirillales	6/10
C1	*Ochrobactrum* sp.	JN571744	99%(509/510)	Alphaproteobacteria; Rhizobiales	4/16
C2	*Ochrobactrum* sp.	KF737384	99%(499/505)	Alphaproteobacteria; Rhizobiales	4/16
C3	*Acetobacter* sp.	AB665071	99%(476/480)	Alphaproteobacteria; Rhodospirillales	4/16
C4	*Acetobacter* sp.	AB665071	99%(503/505)	Alphaproteobacteria; Rhodospirillales	5/16
C5	*Lactobacillus* sp.	JX826566	99%(512/518)	Firmicutes; Lactobacillales	1/16
C6	*Lactobacillus plantarum*	KF225698	98%(487/496)	Firmicutes; Lactobacillales	1/16
C7	*Comamonas* sp.	KC853135	99%(526/529)	Proteobacteria; Burkholderiales	5/16
C8	*Comamonas* sp.	KC853135	98%(515/528)	Proteobacteria; Burkholderiales	5/16
C9	*Acetobacter* sp.	AB665071	99%(491/493)	Alphaproteobacteria; Rhodospirillales	4/16

C10	*Acetobacter* sp.	AB665071	99%(486/489)	Alphaproteobacteria; Rhodospirillales	4/16
C11	*Lactobacillus plantarum*	HE646352	96%(493/512)	Firmicutes; Lactobacillales	2/16
C12	*Lactobacillus plantarum*	KF225698	99%(509/516)	Firmicutes; Lactobacillales	3/16
C13	*Lactococcus lactis*	KC293821	100%(464/464)	Firmicutes; Lactobacillales	2/16
C14	*Comamonas* sp.	KC853135	100%(517/517)	Proteobacteria; Burkholderiales	9/16
C15	*Ochrobactrum* sp.	JN853243	93%(350/376)	Proteobacteria; Rhizobiales	1/16
C16	*Acetobacter* sp.	AB680014	99%(452/454)	Alphaproteobacteria; Rhodospirillales	15/16
C17	*Acetobacter* sp	AB680014	99%(508/510)	Alphaproteobacteria; Rhodospirillales	13/16
C18	*Acetobacter* sp.	AB665082	99%(470/477)	Alphaproteobacteria; Rhodospirillales	2/16
C19	*Tsukamurella* sp.	KF499506	100%(438/438)	Actinobacteria; Actinomycetales	1/16
C20	*Streptomyces* sp.	KF889277	100%(429/429)	Actinobacteria; Actinomycetales	1/16
C21	*Propionibacterium* sp.	KF479576	99%(432/433)	Actinobacteria; Actinomycetales	1/16
C22	*Streptomyces* sp.	EU551673	99%(507/509)	Actinobacteria; Actinomycetales	1/16
C23	*Tsukamurella tyrosinosolvens*	AB478957	97%(528/544)	Actinobacteria; Actinomycetales	1/16
C24	*Streptomyces* sp.	HM153793	99%(506/507)	Actinobacteria; Actinomycetales	1/16

[a] nt, nucleotide.
[b] Number of individuals positive for the presence of the specific band in the DGGE analysis compared to the total number of individuals analyzed.

Adults reared on fruits showed a massive presence of AAB-related sequences; in particular, they showed a high sequence similarity with a *Gluconobacter* and several *Acetobacter* species (Fig. 1B and Tab. 1). Bands B1 and B5 indicate both 100% sequence identity with *Gluconobacter albidus*, whereas bands, such as B4, B6, B10, B11, and B12, showed identity with the genus *Acetobacter*. Band B4, observed for only 10% of the tested adults, showed 99% similarity with *A. cibinongensis*; band B6, detected in few (20%) of the tested individuals, showed 100% identity with *A. tropicalis*, whereas band B11, which was repeatedly found (5 out of 10) among the samples had *A. aceti* as the closest relative, with the 98% sequence similarity. Finally, bands B10 and B12 were 98% similar to the 16S rRNA gene of *A. pasteurianus* and *A. senegalensis*, respectively, with a detection frequency of 70 and 60%, respectively. Other sequences matched with *Lampropedia hyaline* (98%), *Acinetobacter* sp. (100%), *Chitinophaga* sp. (92%) and *Ochrobactrum* sp. (100%).

In both larval and adult specimens reared on fruits, a remarkable presence of *Wolbachia pipientis* was documented, particularly in 5 out of 6 larvae and 7 out of 10 adults (bands A5 and B8 for larvae and adults, respectively, Fig. 1 and Tab. 1). Sequences had a 99% identity with *Wolbachia* spp.

Larvae and pupae reared on the artificial diet showed a huge presence of AAB sequences clustering to *Acetobacter* genus (bands C3, C4, C9, C10, C16, C17 and C18 with values of 99% identity), together with sequences related to *Lactobacillus* genus (bands C5, C6, C11 and C12; 96-100% identity). In the case of the adults reared on artificial diet, sequences related to *Ochrobactrum*, *Comomonas*, *Lactobacillus*, *Tsukamurella*, *Streptomyces* and *Propionibacterium* were retrieved (Fig. 1C). No sequences were found to cluster with *Wolbachia*; however, few bands in the upper part of Fig. 1C did To have a wide view of the bacterial community associated to the samples, 16S rRNA barconding, by amplifying the variable regions V1-V3 of the bacterial

16S rRNA gene, was performed on 14 specimens, including both individuals reared on fruits or artificial diet and specimens from different developmental stages (larvae, pupa and adults). Intra-specimen variability among the samples were reported (Tab. 2; Fig. 2). Using the Shannon index to measure α-diversity, it was possible to visualize that all samples reached a plateau; rarefaction curves showed the saturation of the microbial diversity associated to the samples (data not shown). We obtained in total 178, 856 reads after quality evaluation and chimera removing. Singletons and less significative sequences (below the 0.1% threshold) were also deleted from the analysis. Besides the number of OTUs detected for each sample, in table 2 are reported the alpha diversity metrics of 16S barcoding of the 14 samples, at 97% identity level, i.e. Chao-1, Shannon H diversity and Pielou's J evenness indices. On the other hand, the β-diversity related to the samples was evaluated through principal coordinates analysis (PCoA) on the phylogenetic β-diversity matrix obtained by UniFrac (Fig. 2a). The components explain 49.67% of the variation (Fig. 2a). The analysis showed that three clusters could be obtained; the first one, quite separated from the other two, contained the two larvae and the sole pupa reared on the artificial diet, the second one included the adults reared on the artificial diet and the other one was constituted by the specimens reared on fruits (Fig. 2a). By the use of this analysis, adults reared on the artificial diet clustered closer to the specimens reared on fruits than the larvae or pupa reared on the artificial diet.

Table 2: Alpha diversity metrics of 16S barcoding of 14 samples, at 97% identity level.

Sample ID	**Age**	**Rearing environment**	**Barcode sequence**	**N**	**OTUs**	**Chao1**	**H'**	**J**
Ds159	larva	fruit	ACACGACT	12851	68	92.43	1.148	0.272
Ds164	larva	fruit	ACACGAGA	15587	132	148.24	2.151	0.440
Ds165	larva	fruit	ACACGTCA	9835	170	186.73	3.137	0.611
Ds167	pupa	fruit	ACAGAGAC	6638	153	200.57	3.290	0.654
DS41	adult	fruit	AGACGACA	9324	96	109.57	2.651	0.581
DS54	adult	fruit	AAGGTACG	19831	154	166.55	2.848	0.565
DS55	adult	fruit	AGACGAGT	6537	117	136.09	3.014	0.633
DSM	adult	fruit	AAGGCGTA	6189	53	59.0	1.761	0.444
LP1	larva	diet	ACACGTGT	15338	71	122.0	1.975	0.463
LP3	larva	diet	ACACTCTC	20032	84	85.25	1.609	0.363
PP2	pupa	diet	ACAGAGTG	17180	69	74.60	0.907	0.214
FP1	adult	diet	ACACTGTG	7298	89	104.83	2.103	0.468
FP3	adult	diet	ACACTGAC	10321	113	120.58	2.738	0.579
MP3	adult	diet	ACAGACAG	21895	40	41.0	1.162	0.315

N: number of reads for each sample; OTUs: number of OTUs for each sample; Chao-1: Chao-1 values for each sample, H': Shannon H diversity for each sample; J: Pielou's J evenness indices for each sample.

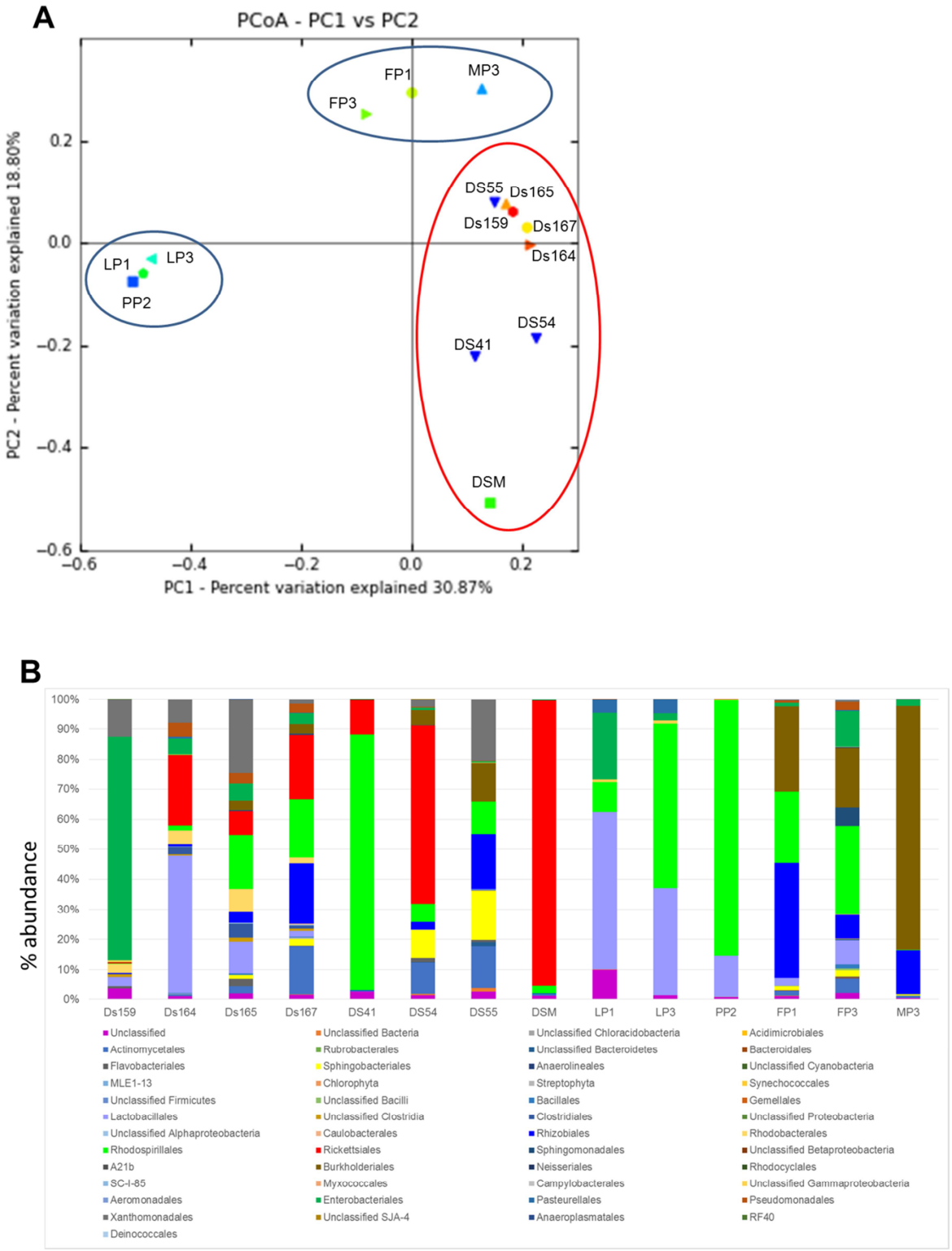

Figure 2: Bacterial diversity associated with *D. suzukii* by 16S rRNA barcoding. (A) Principal coordinate analysis (PCoA) on the phylogenetic β-diversity matrix on *D. suzukii* samples. With red circle are clustered fruit-fed individuals, while with blue circles specimens on the artificial diet. (B) 16S RNA barcoding describing microorganisms, at order level, associated with *D. suzukii*. Names, under histograms, refers to fly specimens submitted to DNA extraction and partial 16S rRNA bacterial genes amplification. In columns, the relative abundances in percentages of the orders identified.

Looking to the sample composition, results showed a high frequency of sequences belonging to the Rhodospirillales order (average percentage of reads was 24.8 per sample), to which AAB belong. Interestingly reads clustering to Rickettsiales, to which *Wolbachia* belongs, were detected only in flies reared on fruits, with an average of 27.5% among the individuals, confirming results obtained by DGGE-PCR (Fig. 2b). In particular, DSM and DS54 showed massive presence of Rickettsiales sequences with percentages of 95.3% and 59.4% out of the total number of reads, respectively. It is noteworthy to underline that all the reads, clustering to the Rickettsiales order, clustered at the genus level with *Wolbachia*. Reads clustering within Rhodospirillales order were present in all the specimens with different loads: the major abundant presences of Rhodospirillales reads were detected in DS41, a specimen reared on fruits, and PP2, a specimen reared on the artificial diet, with percentages of 85.2% and 85.4% out of the total number of sequences for each sample, respectively. Moreover, members of other orders such as Enterobacteriales, Xanthomonadales, Lactobacillales, Rhizobiales, Burkholderiales and Sphingobacteriales constituted the most significative fractions of reads out of the total ones (Fig. 2b).

Prevalence and localization of AAB.

To investigate the prevalence of AAB, at the genus level, in the analysed insects reared on fruits, infection rates of the genera *Gluconobacter*, *Gluconacetobacter* and *Acetobacter* were evaluated in adult flies (Fig. 3). The frequency detected for the genera *Gluconobacter* and *Acetobacter* did not show significant differences, likewise *Gluconobacter* and *Gluconacetobacter* genera that did not present significantly different values ($p<0.05$). Prevalence indicated *Gluconobacter* and *Gluconacetobacter* as the most prevalent genera among the samples with values of 21 and 31%, respectively.

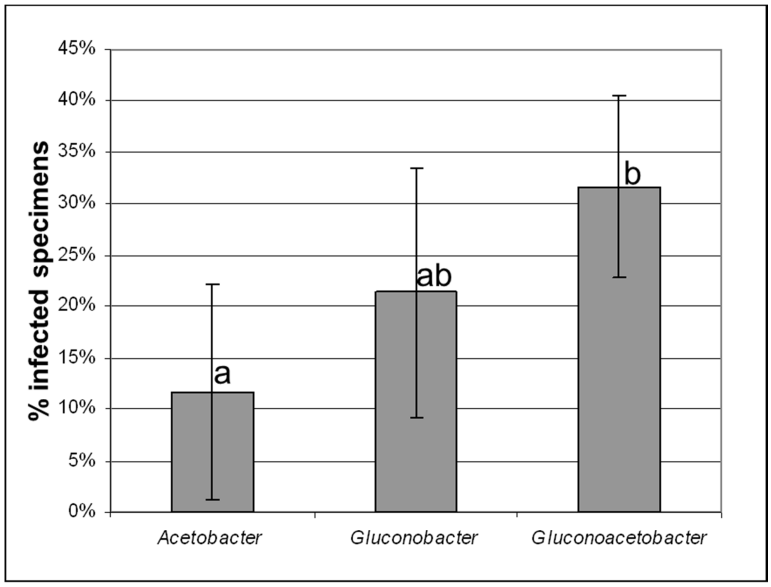

Figure 3: AAB infection rates in adult *D. suzukii*. Columns indicate the percentage of infected individuals within the most common genera found in mass reared flies, i.e. *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter*. Bars represent the standard error. Different letters indicate significantly different values (ANOVA, $P<0.05$)

With the aim to localize AAB, fluorescent *in situ* hybridization (FISH) experiments were carried out using the AAB-specific probe, AAB455, on the insect dissected organs, showing positive signals for proventriculus and gut (Fig. 4). In particular, a strong signal was detected at the level of the proventriculus epithelium, as observable by merging the interferential contrast picture (Fig. 4c) with the FISH micrograph (Fig. 4b) of a midgut section near to the proventriculus. Magnification in fig. 4d allowed to visualise fluorescent AAB microcolonies adhering to the peritrophic membrane. Since *Gluconobacter* was one of the main genera in prevalence assays, the distribution of this genus was observed in the midgut of *D. suzukii*. *Gluconobacter* specific signal was detected in the gut (Fig. 4g) providing the evidence of the distribution of this genus in the inner side of the intestinal lumen. Fig. 4f showed the Texas red-signal for *Eubacteria*, allowing to observe the distribution of *Gluconobacter* in relation to the dispersal of the *Eubacteria* in the same portion of the organ (Fig. 4e-h). *Gluconobacter* is localized in the intestinal tract probably surrounded by other acetic acid bacteria. Attempts to design probes specific for *Gluconacetobacter* or *Acetobacter* genera failed.

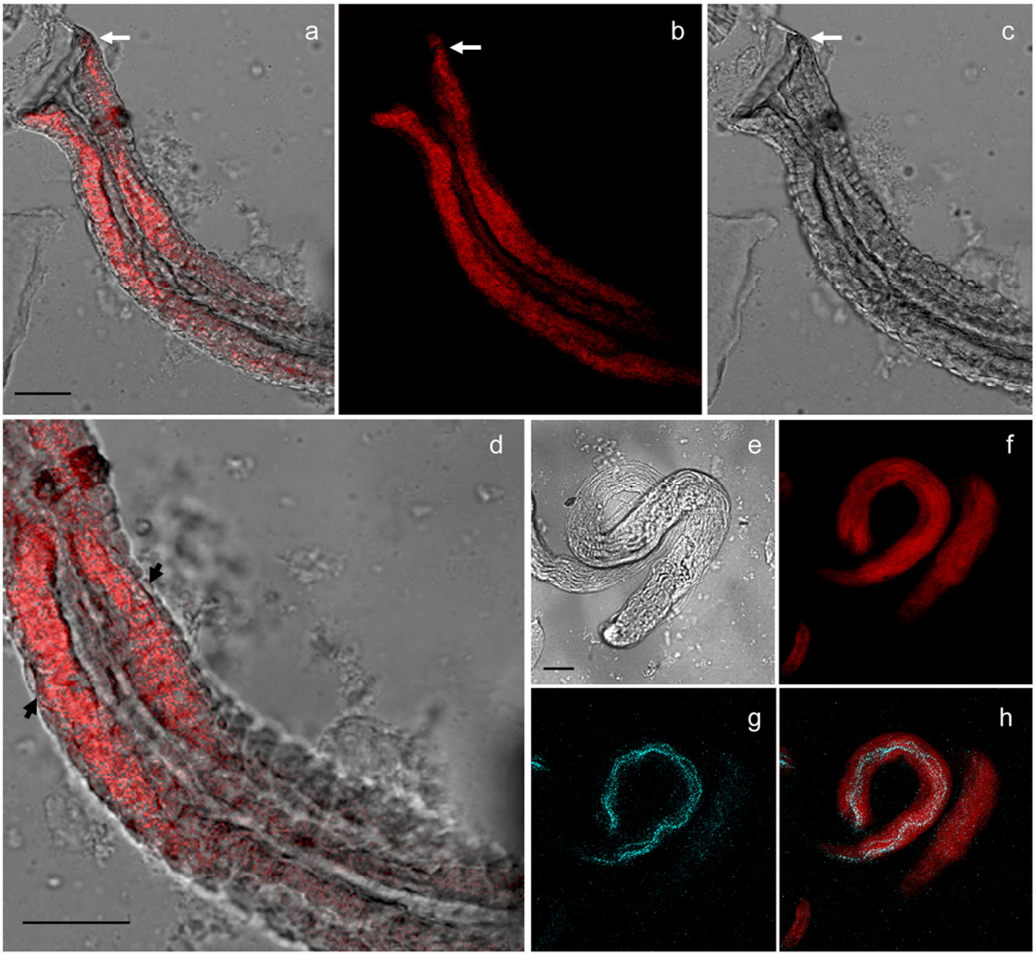

Figure 4: AAB localization in the gut of *D. suzukii*. (a-d) FISH of the insect gut after hybridization with the Texas red-labelled probe AAB455, matching AAB. (a) Superposition of the interferential contrast picture (c) and the FISH image (b) of a midgut part close to the proventriculus (indicated by white arrows). (d) Magnification of the image in b. The massive presence of AAB adherent to the peritrophic membrane (indicated by black arrows) is observed. (e-h) FISH of *D. suzukii* midgut with the Texas red-labelled universal eubacterial probe Eub338 (f) and the Cy5-labelled probe specific for *Gluconobacter*, Go615 and Go618 (g). (e) Intestine portion pictured by interferential contrast. (h) Superposition of hybridization signals of Eubacteria (red) and *Gluconobacter* (blue). Bars = 50 μm.

AAB isolation.

Isolation trials of AAB were performed with different kinds of enrichment and selective media (De Ley and Frateur, 1974, Reasoner *et al.*, 1979, Yamada *et al.*, 1999; Yamada *et al.*, 2000; Kadere, 2008). Since the condition of fruit-rearing was the most close one to the natural habit of the insect, we directed our attention manly on isolation trials from specimens reared on fruits; specimens reared on artificial diet was also included in the analysis in a low extent. After purification, 234 isolates were obtained and subjected to de-replication analysis, clustering them in ITS fingerprinting profiles. 16S rRNA gene sequencing of the candidates chosen for each ITS fingerprinting profile showed a prevalence of bacteria belonging to *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* genera, which constitute the 16.67%, 7.7% and 52.99%, respectively, of the total number of bacteria in the collection (Tab. 3). 22.7% of isolates did not belong to Acetobacteraceae family. Seven *Acetobacter* species were isolated, i.e. *Acetobacter tropicalis*, *A. cibinongensis*, *A. persicus*, *A. peroxydans*, *A. indonesiensis*, *A. orientalis*, *A. orleanensis*, with *A. persicus* being the most abundant one (Tab. 3). *Gluconobacter* genus was found to be present with three species, i.e. *G. kondonii*, *G. oxydans*, and *G. kanchanaburiensis*. The single isolate of *G. kondonii* was collected from an adult fly fed on fruits, while *G. kanchanaburiensis* species were isolated from specimens reared on artificial diet.

Table 3: Identification of cultivable microorganisms associated to *D. suzukii*.

Isolates	**No. of isolates**	**ITS**	**Larval fly**	**Pupal fly**	**AP fly**	**AF fly**
Micrococcus sp.	5	34, 35, 38	0	0	0	5
Microbacterium foliorum	2	39	0	0	0	2
Corynebacterium sp.	1	76	0	0	0	1
Sphingobacterium multivorum	1	74	0	0	1	0
Streptococcus salivarius	1	48	0	0	1	0
Staphylococcus sp.	13	37	0	0	1	12
Paenibacillus sp.	2	62	0	0	0	2
Lactococcus lactis	1	60	0	0	0	1
Lactobacillus plantarum	1	68	0	1	0	0
Lactobacillus brevis	2	69	0	1	1	0
Acetobacter tropicalis	1	46	0	0	0	1
Acetobacter orleanensis/malorum/cerevisiae	5	47, 58	0	1	0	4
Acetobacter peroxydans	1	66	0	0	0	1
Acetobacter indonesiensis	10	49, 50, 55, 59	0	1	1	8
Acetobacter persicus	20	51	2	2	6	10
Acetobacter orientalis	1	54	0	0	0	1
Acetobacter cibinongensis	2	53	0	0	0	2
Gluconacetobacter sp.	26	8, 15, 16, 18, 23, 24, 32, 33, 40, 44	0	0	0	26
Gluconacetobacter hansenii	66	1, 2, 6, 10, 11, 12, 42, 43	0	0	0	66
Gluconacetobacter liquefaciens	4	67	1	3	0	0
Gluconacetobacter europaeus	3	22	0	0	0	3
Gluconacetobacter saccharivorans	14	4, 19, 41	0	0	0	14
Gluconacetobacter intermedius	8	14, 16, 17, 20, 21, 25	0	0	0	8
Gluconacetobacter nataicola	2	7, 31	0	0	0	2
Gluconobacter kondonii	1	52	0	0	0	1
Gluconobacter oxydans	12	5, 9, 26, 27, 28, 29, 45	0	0	0	12
Gluconobacter kanchanaburiensis	5	65	3	1	1	0
Rhodobacter sp.	1	78	0	0	0	1
Pseudomonas geniculata	4	76	0	0	1	3
Serratia sp.	12	80	4	7	0	1
Enterobacter sp.	7	57, 71	1	0	3	3
Total	**234**		**11**	**17**	**16**	**190**

AP: Adults fed with artificial diet; AF: Adults fed with fruit diet

Twelve isolates, collected from the fruit-fed adults, showed high sequence similarity with *G. oxydans* as closest described species, with isolates belonging to different ITS profiles (Tab. 3). One hundred and twenty-three isolates were sequenced and assigned to the genus *Gluconacetobacter*. In particular, 66 *Gluconacetobacter hansenii* (41 coming from females and 25 from fruit–fed males), 14 *Ga. saccharivorans*, and 8 *Ga. intermedius* isolates were isolated from fruit–fed *Drosophila*. The 8 isolates of *Ga. intermedius* derived from the same male, DSM1, but from different media, specifically the enrichment medium I and the basal medium. Three pupae and one larva revealed to harbor *Ga. liquefaciens*, when smashed and plated on enrichment medium I. Twenty-six *Gluconacetobacter* sp. could not be discriminated with the performed analysis, due to the phylogenetic proximity of the species analyzed.

During the isolation procedure, few isolates belonging to the phylum Firmicutes were obtained, i.e. *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactococcus* sp., *Paenibacillus* sp., *Streptococcus salivarius*, and *Staphylococcus* sp. Several isolates of *Sphingobacterium multivorum*, *Corynebacterium* sp., *Micrococcus* sp. and *Microbacterium foliorum* were also found, as well as some representatives of Proteobacteria phylum, i.e. *Rhodobacter* sp., *Pseudomonas geniculata*, *Enterobacter* sp., and *Serratia* sp.

Colonization of D. suzukii with G. oxydans DSF1C.9A(Gfp), A. tropicalis BYea.1.23(Gfp) and A. indonesiensis BTa1.1.44(Gfp).

Isolates *G. oxydans* DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 were selected for inserting a plasmid carrying the Gfp cassette in order to label the bacteria with a fluorescent protein. Plasmid stability into the three different AAB strains was evaluated and data showed that *G. oxydans* DSF1C.9A showed that it was able to retain the plasmid with an high percentage (73.125%, data not shown), while plasmids inserted in *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 were not stably inherited (data not shown). Colonization trials of adult flies were thus performed. Because of the quite rapid loss of the plasmid, the colonization experiments were performed under antibiotic selection by administering 100 $\mu g\ ml^{-1}$ kanamycin in the insect food. After the administration of the Gfp-labelled strains, *Drosophila* specimens were dissected and the gut analyzed by CLSM. Gfp-labelled strains were able to massively recolonize the fly foregut and midgut (Fig. 5-6). In the case of Gfp-labelled *Gluconobacter*, the crop, proventriculus and first part of midgut were successfully colonized by the labelled bacteria (see the magnification views of the crop and the proventriculus in Fig. 5b and 5c). It is noteworthy that the Gfp-labelled cells are clearly restricted to the epithelium side of the proventriculus, embedded in a matrix, probably of polysaccharidic nature close to the peritrophic membrane (Fig. 5c). Likely, the first tract of the intestine, also the central part represented by midgut showed massive colonization pattern (Fig. 5d-e). Since small hernias are visible by interferential contrast (indicated by black arrowheads in Fig. 5e) and since they result Gfp-positive with CLSM, the gelatinous matrix forming the hernias appears like a gel in which the bacterial cells are completely sunk. Black filaments around the organ are the Malpighian tubules, more evident in the confocal laser scanning microscopy picture (Fig. 5d). Also in the case of *A. tropicalis* BYea.1.23, the colonization of the foregut and midgut was successfully performed (Fig. 6A). The labelled bacteria are present in the whole tract and especially they are located close to the gut walls and in the peritrophic membrane (Fig. 6B-E). Images related to *A. indonesiensis* BTa1.1.44(Gfp) were similar and thus not included here.

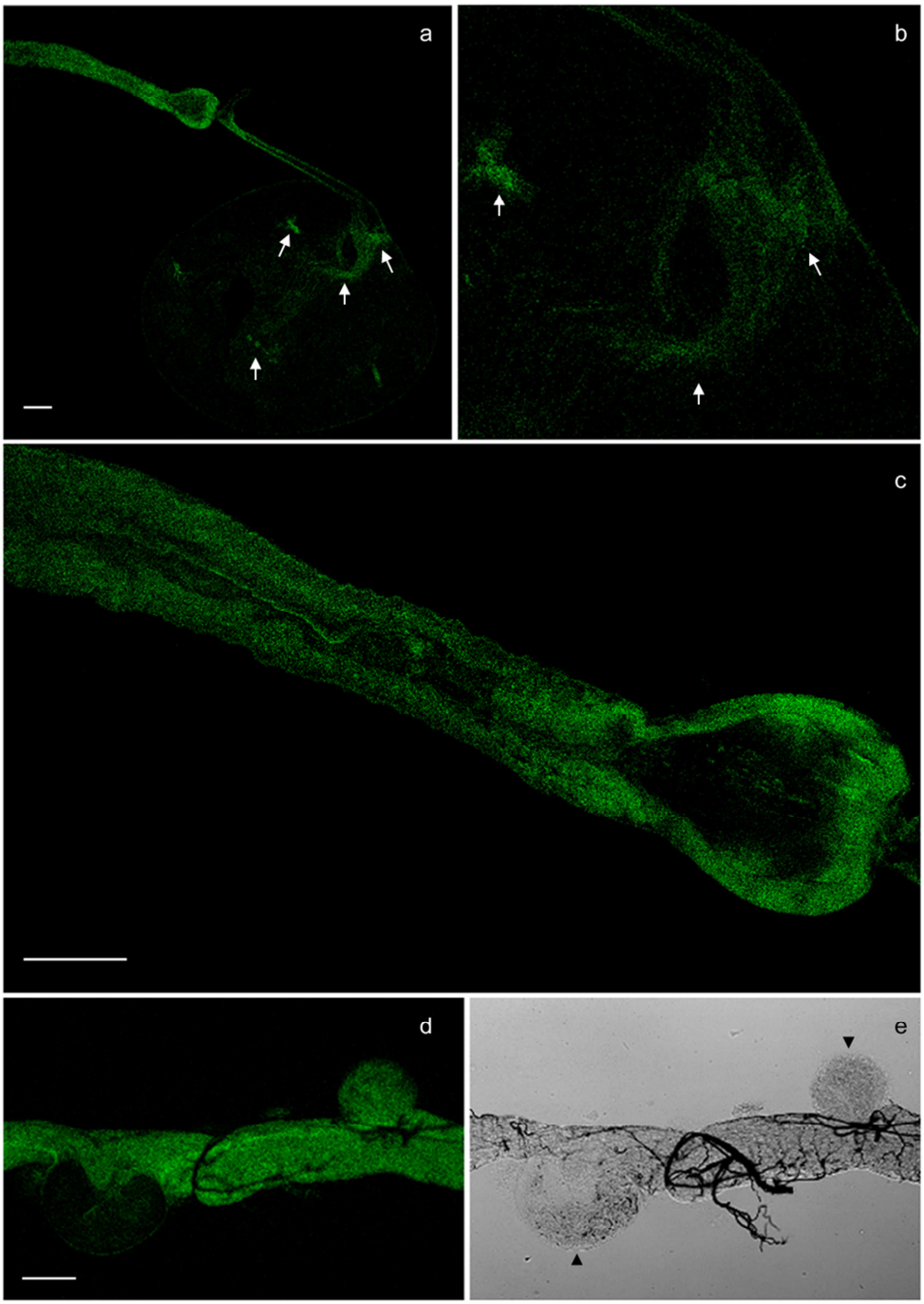

Figure 5: Colonization of *D. suzukii* foregut and midgut by *G. oxidans* DSF1C.9A documented by confocal laser scanning microscopy. (a-c) Intestine portion including the crop, the proventriculus and the first midgut part. (b, c) Magnified views of the crop (b) and the proventriculus (c) showed in a. Masses of fluorescent cells are observed in the crop (arrows); when the marked strain reaches the proventriculus it colonizes the gut part close to peritrophic membrane. (d-e) Interferential contrast (d) and confocal laser scanning (e) pictures of the midgut of *D. suzukii* massively colonized by the *G. oxidans* strain labelled with Gfp. Small hernias (arrowhead) are shown. In some cases, the flow of the gelatinous matrix entering the hernia is composed by fluorescent cells. Bars = 50 µm.

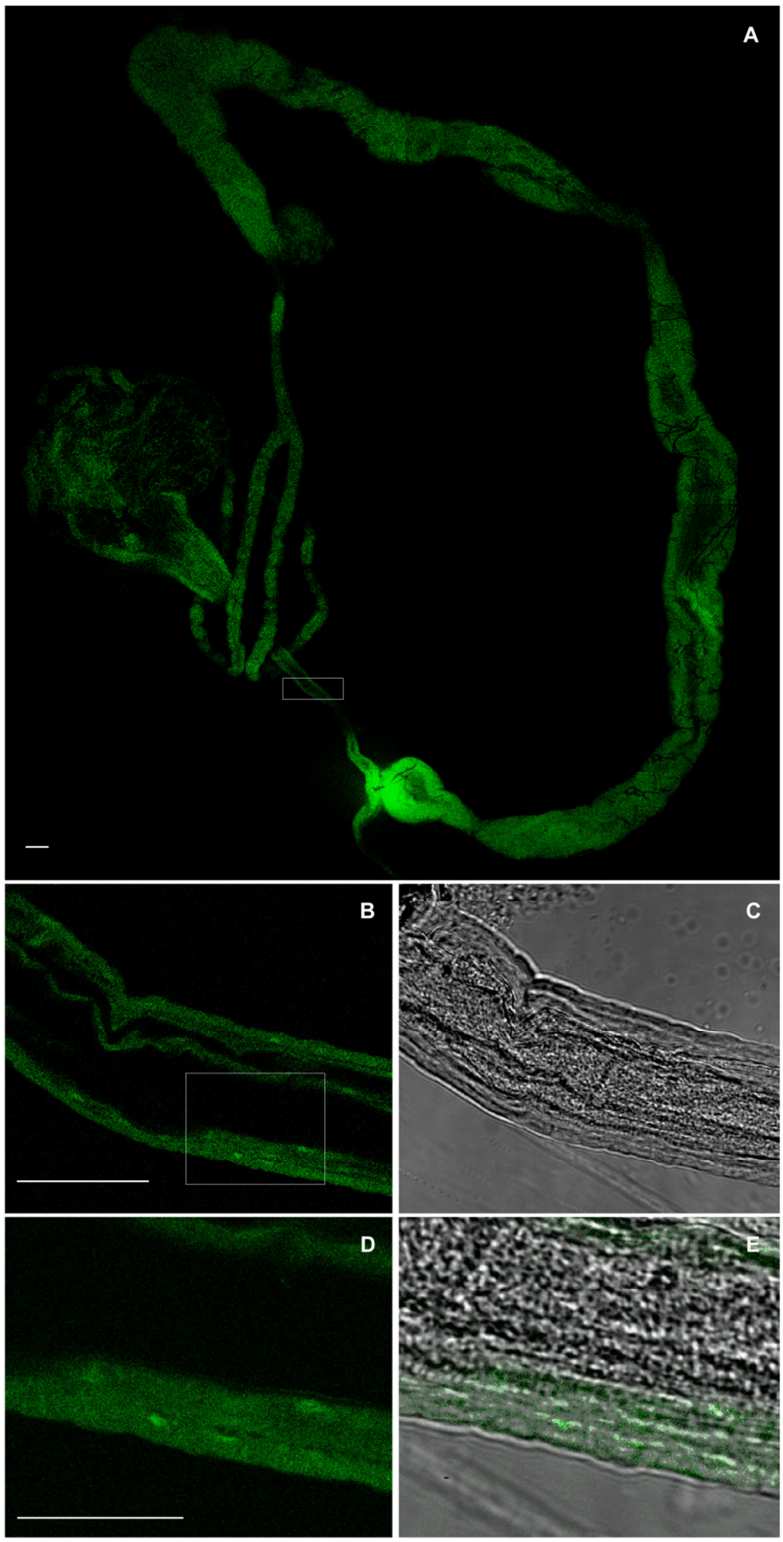

Figure 6: Confocal laser scanning micrographs showing the colonization of *D. suzukii* foregut and midgut by *A. tropicalis* BYea.1.23 (A) Reconstructed image of an intestine obtained by overlapping successive sections. The gut portion includes crop, proventriculus, midgut, and Malpighian tubules. Fluorescent *A. tropicalis* BYea.1.23 cells are visible in the whole tract; the symbiont is especially located close to the gut walls and in the peritrophic membrane. Bar = 50 µm. (B, C) Magnification of the framed crop part in A pictured by CLSM (B) and interferential contrast (C). Masses of fluorescent bacteria are evident in the crop. Bar = 50 µm. (D-E) Interferential contrast (D) and confocal laser scanning (E) magnifications of the framed crop part in B, showing Gfp-marked *A. tropicalis* adhering to the crop wall. Bar = 25 µm.

DISCUSSION

The highly invasive vinegar fly *Drosophila suzukii* is a poliphagous species endemic to the South East Asia and now it is emerging as a dangerous pest in many Mediterranean and North America's countries. The study of its biology, ecology and distribution is in progress, but to develop future management solutions many gaps should be filled. One of these is the characterization of insect microbiome, with particular attention to the analysis of the acetic acid bacteria, important symbionts living in association with this pest. Indeed, in other insect models, these alphaproteobacteria have been described to play important biological roles (Shin *et al.*, 2011, Chouaia *et al.*, 2012, Mitraka *et al.*, 2013). In this work, culture-independent techniques, DGGE-PCR and 16S rRNA pyrotag in particular, gave insights of the overall bacterial community composition of *D. suzukii* and, notably, provided robust evidence of the stable association of AAB, underlining their constant presence in the samples under investigation. Results showed the presence of AAB belonging to the *Acetobacter, Gluconacetobacter* and *Gluconobacter* genera (Fig. 1, Fig. 2).

AAB are a diverse class of organisms, widespread in nature, as a large number of AAB strains have been isolated from a variety of sources (Kersters *et al*., 2006). AAB are recognized by their unique ability to oxidize ethanol to acetic acid in neutral and acidic (pH 4±5) media and to produce polysaccharides that are exploited at industrial level (Kommanee *et al*., 2008). Besides *Drosophila* flies and mosquitoes, AAB have been reported in association with bees, olive fruit flies, parasitic wasps and mealybugs (Ashbolt *et al.*, 1990, Martinson *et al.*, 2011, Kounatidis *et al.*, 2009). For example, *Acetobacter tropicalis*, whose presence was also recorded in *D. suzukii* by the present study, was previously described in association with the olive fruit fly *Bactrocera olae*, with which it establishes a strict association (Kounatidis *et al.*, 2009). The appearance and behaviour of this bacterium were similar to the ones showed by the Gfp-labelled strains used in the actual work: it was indeed observed in contact with the gut epithelium of the insect, entrapped in the polisaccharidic matrix. This peculiar localization in the insect body was also documented in *Asaia* (Favia *et al*, 2007) and it may suggest the importance of the AAB for the insect metabolism and gut functions' maintenance.

16S rRNA barcoding allowed to discriminate three clusters among the samples (Fig. 2a); the first principal component (which explains 30.87% of the variance) segregates the microbiota of two groups of *D. suzukii*, the adults reared on the fruit and the ones on the artificial diet from the larvae and pupa reared on the artificial diet, while the second component allows to discriminate the adults reared on the artificial diet from the fruit-fed ones (Fig 2a).

In both results of cultivation-independent techniques, DGGE-PCR and pyrotag, *Wolbachia* presence was massively recorded in insects reared on fruit. *Wolbachia* is an intracellular reproductive manipulator already described for several insect models, including different *Drosophila* species (Ravikumar *et al* 2011, Verspoor *et al*., 2011, Lee *et al* 2012, Siozios *et al.* 2013). Its finding only in samples reared on fruit and not in samples reared on the artificial diet could be explained by the presence of inhibitory compounds against *Wolbachia* in the artificial diet (Fig. 1, Fig. 2).

However, even if little is known about *Drosophila suzukii* microbiota (Chandler *et al*., 2014), numerous studies have now been conducted in order to assess the microbial community residing in *Drosophila melanogaster* (Brummel *et al.*, 2004, Chandler *et al.*, 2011, Corby-Harris *et al*., 2007, Cox and Gilmore, 2007, Ren *et al.*, 2007, Ridley *et al.*, 2012, Ryu *et al.*, 2008 , Sharon *et al.*, 2010, Storelli *et al.*, 2011, Wong *et al*., 2011), both in the gut and in the whole body. These studies underline the simple bacterial communities in association with *Drosophila*, predominantly made up of Firmicutes phylum, represented by the families Lactobacillaceae and Enterococcaceae, and alpha and gamma classes of Proteobacteria, represented by Acetobacteraceae and Enterobacteriaceae families. Cox and Gilmore (2007), who performed the analysis of the bacterial community of wild and laboratory-reared *D. melanogaster* specimens, revealing the predominant presence of *Acetobacter* genus, and consequent identification of *A. aceti, A. cerevisiae, A. pasteurianus, A. pomorum, Gluconobacter* and *Gluconacetobacter* species. A recent work showed that the differences in the diversity and dominance of bacterial species associated to several *Drosophila* species showed a relationship with food source (Wong *et al*., 2011). Moreover, Chandler and coworkers (2011) observed that all individuals of different

Drosophila species reared on different food sources obtained a similar microbiome when moved to the same medium. Furthermore, Wong *et al.* focused on the microbial composition at different life cycle stages (Wong e*t al*., 2011). Chandler and colleagues (2014) characterized the microbiota of D. suzukii of adult and larval *D. suzukii* collected from cherries, showing a high prevalence of the gamma-proteobacterium *Tatumella*. *Gluconobacter* and *Acetobacter* were found at lower frequency than *Tatumella*. In our case, high prevalence of Rhodospirillales reads was reported with an average of 24.8%, abundance percentages varying from 0.02% to 85.42% (Fig 2). No *Tatumella* sequences were detected among gamma-proteobacteria.

AAB have been shown to be involved in the relationship between the gut microbiota and host health, underlining the importance of the correct microbial balance for the host well-being (Silverman and Paquette, 2008). The normal flora in the fly gut is sufficient to suppress the growth of pathogenic bacteria, and to regulate host immune response (Ryu *et al*, 2008), but also to promote the insulin pathway (Shin e*t al.*, 2011). Consequently, to gain knowledge of the detailed localization of this key group, FISH was performed with AAB-specific probe (Fig.4). The localization of the AAB probe in the wall side of the midgut portion near to the proventriculus showed their distribution, not in the lumen, but in the peripheral side of the organ suggested a role of protective layer between the lumen and the surface epithelium, able to prevent the passage of bacteria. This was already proposed by Kounatidis and colleagues (2009), when observing a similar behaviour in *Bactrocera oleae* gut, colonized by *A. tropicalis*. Several studies reported that another AAB, *Asaia*, is able to colonize the gut and the reproductive organs of different insects, such as the leafhopper *Scaphoideus titanus*, and the mosquitoes *Anopheles stephensi*, *An. gambiae* and *Aedes aegypti* (Favia *et al*., 2007, Crotti *et a*l., 2009, Damiani *et al.*, 2010, Gonella *et al.*, 2012); together with the hypothesis above reported, this supports the evidence that the insect digestive system is a favourable habitat for AAB, in which they establish a strict connection with the epithelial cells (Crotti *et al*., 2010)

Recolonization data strongly supported FISH analysis: several isolates belonging to *Gluconacetobacter*, *Gluconobacter* and *Acetobacter* genera were targeted with a plasmid carrying the Gfp (Fig.5-6). Several efforts were also made to achieve the transformation of *Gluconacetobacter* isolates, but no successful results were obtained. Further experiments will be planned to improve the transformation protocol. Strains *G. oxydans* DSF1C.9A(Gfp), *A. tropicalis* BYea.1.23(Gfp) and *A. indonesiensis* BTa1.1.44(Gfp) were obtained and their dispersal in the fly body was followed by fluorescent microscopy on re-colonized specimens.

The actual control of *D. suzukii* are based on insecticides that however are not very effective (Walsh *et al.*, 2011) and the promising control strategies based on interferences with communication still need more research (Eriksson *et al.*, 2012). Thus a forward-looking concept like the symbiotic control approach, under investigation in the last years (Bextine *et al.*, 2004), should be taken into account. In the light of the development of future control strategies exploiting the remarkable importance of the Acetobacteraceae family for *D. suzukii*, further experiments have to be performed to assess their distribution pattern in the host compartments, their role and involvement in the host homeostasis and possible exploitations of their properties for the host control.

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Chapter III

Evaluation of Volatile Compounds Released by Acetic Acid Bacteria, Symbionts of the Spotted Wing Fly, *Drosophila suzukii*, and their Attractive Effects on the Host

Abstract
The exotic pest D*rosophila suzukii* Matsumura (Diptera: Drosophilidae) establishes, similarly to the other fruit flies of *Drosophila* genus, an association with acetic acid bacteria (AAB), in particular with those belonging to *Gluconobacter*, *Gluconacetobacter* and *Acetobacter* genera. Considering both the importance of these bacteria in the production of chemical compounds and, on the other hand, the attraction of fruit flies for vinegar and acetic acid-based baits, the capacity of some symbiotic AAB strains, already isolated from this insect, to release attractive volatile compounds for spotted wing fly was performed in this work. GC-MS (Gas chromatography–mass spectrometry) analyses, coupled to two-choice olfactometer assays, were used to identify the volatile molecules released by some selected AAB and to estimate their ability to attract *D. suzukii* females. The emission of compounds was evaluated for bacteria after 24 h and 48 h of growth, in comparison to a control. The compounds emitted by the tested strains belonged to the class of alcohols, ketones, carboxylic acids and aldehydes. 2-propanone was released by all the tested strains. The other compounds showed a change in release at 48 hours of bacterial growth. Correspondence analysis clustered the tested bacteria in three groups, according to the volatile emitted: the first group, represented by two *Gluconobacter* strains, was linked to the production of 2-propanol, benzaldehyde and acetic acid; group 2 was related to the production of acetic acid and 2-propanone, and group three was close to butyric acid derivates. Among the tested isolates,, higher attractiveness for flies was obtained with *Gluconobacter oxydans* DSF1C.9A, *Gluconobacter kanchanaburiensis* L2.1.A.16 and *Gluconacetobacter saccharivorans* DSM1A.65A strains. As baits for *D. suzukii* flies are composed by vinegar and baker's yeast, the analyses of the best attractive molecules released by specific AAB symbionts might provide innovative tools for *D. suzukii* biocontrol and for the construction of traps specifically targeted to this pest.

INTRODUCTION
In recent years, several studies were conducted on taxonomy, molecular biology and physiology of acetic acid bacteria (AAB) mainly due to their important roles in commercial food and chemical compound production (Raspor and Goranovič, 2008). AAB are Gram-negative, ellipsoidal to rod-shaped bacteria belonging to the family of Acetobacteraceae within the subclass of α-Proteobacteria. Currently, the sequence analysis of the 16S rRNA gene allowed to classify AAB in 14 genera (Torija *et al.*, 2010, Yamada *et al.*, 2012). However, the most common genera widespread and commercially used are *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (Raspor and Goranovič, 2008, Minenosuke *et al.*, 2011). AAB are mesophilic obligate aerobes that oxidize sugars, sugar alcohols and ethanol in acetic acid through two sequential reactions of membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Saeki *et al.*, 1997). Their oxidative capacity is largely exploited. Besides the vinegar manufacturing, AAB play an important role in the development of desired flavours in cocoa production (Adler *et al.*, 2014). Moreover, bacterial cellulose produced by some *Acetobacter* species, showed excellent physical properties and could be used for many application (Fontana *et al.*, 1990, Vandamme *et al.*, 1998).
Finally, AAB are used for several biotechnological processes such as vitamin C or shikimate production, an intermediate for the synthesis of many antibiotics, herbicides and aromatic amino acids (Adachi *et al.*, 2003, Prust *et al.*, 2005). AAB are widespread in the environment and they are easily isolated from various plants, flowers, fruits and garden soil (Raspor and Goranovič, 2008, Crotti *et al.*, 2011). Strains of *Acetobacter* and *Gluconobacter* are commonly known as spoiler agents on wine and beer and some bacteria caused plant diseases (Rohrbach and Pfeiffer, 1975, van Keer *et al.*, 1981, du Toit and Pretorius, 2000, Bartowsky *et al.*, 2003).

Great attention has recently been addressed to AAB as symbionts in insects. Symbiotic strains are associated with insects that feed on sugar-based diets, in particular those belonging to Diptera, Hymenoptera and Hemiptera orders (Crotti *et al*., 2010). Members of Acetobacteraceae have been isolated at first from *Apis mellifera* L. (Hymenoptera: Apidae). In fact, several *Gluconobacter* spp., that prefer sugar-enriched environments, were isolated from honeybees since the beginning of the twentieth century (White, 1921, Lambert *et al.*, 1981). Moreover, strains of *Gluconobacter*, including the novel acetic acid bacterium *G. sacchari*, were isolated and successfully described by Franke *et al.* (1999, 2000) in the pink sugarcane mealybug *Saccharicoccus sacchari* Cockerell (Hemiptera: Pseudococcidae) and in other mealybugs like *Planococcus* sp. and *Dysmicoccus brevipes* Cockerell (Hemiptera: Pseudococcidae) (Ashbolt and Inkerman, 1990). The order of Diptera hosts a rich AAB microbiome. *Asaia* spp. are the most important AAB symbionts of the pathogen-transmitting mosquitoes of the genus *Anopheles* (Favia *et al.*, 2007, Crotti *et al.*, 2009).
In the olive fruit fly *Bactrocera oleae* Rossi (Diptera: Tephritidae), *Acetobacter* symbionts dominate as well. In particular, a stable association between *A. tropicalis* and the insect was observed (Kounatidis *et al.*, 2009). Different AAB genera were detected in the genus *Drosophila*. *Acetobacter* and *Gluconobacter* represent the most important genera in *Drosophila melanogaster* Meigen and *Drosophila simulans* Sturtevant, and some strains were recently detected also in *D. suzukii* (Chandler *et al.*, 2011, Staubach *et al.*, 2013, Chandler *et al.*, 2014). In some *D. melanogaster* populations, the presence of other AAB genera of symbiotic bacteria, such as some species of *Gluconacetobacter* and *Commensalibacter*, have been characterized (Roh *et al.*, 2008).
AAB establish associations with the insect midgut, which is favourable to their growth due to the availability of a carbohydrate-rich diet, oxygen, and acidic pH. Host enzymes joined with the metabolic activities of the microbial communities permit the degradation of the nutrients in the digestive system supplying important advantages to the host, such as the capability to specialize on nutrient-deficient food or unbalanced diets (*i.e*. insects that feed of plant sap or vertebrate blood) (Crotti *et. al.*, 2010, Crotti *et. al.*, 2011). Besides the nutritional aspects, AAB play important roles in other aspects of insect biology, as in some hosts they are implicated in the immune homeostasis maintenance, or increase lifespan and fitness (Ryu *et al.*, 2008, Shin *et al.*, 2011). Furthermore, AAB could be involved in many other effects such as the defence against other harmful microorganisms, or the interaction with cell-to-cell communication through the production of volatile compounds (Crotti *et al.*, 2010). Concerning the great importance of AAB as symbionts, insects evolved different strategies to transmit bacteria both horizontally and vertically. In *Anopheles stephensi* Liston mosquitoes, the symbiont *Asaia* acquired by females during the mating is successively transmitted to the progeny (Damiani *et al.*, 2008), moreover in *Scaphoideus titanus* Ball leafhoppers *Asaia* is vertically transmitted by egg smearing (Crotti *et al*, 2009), and horizontally transferred by oral and sexual ways (Gonella *et al*., 2012).
The knowledge of the microbial community associated with harmful pests is necessary in order to develop potential control strategies. The symbiotic control approach utilizes naturally occurring or genetically modified bacterial symbionts that once colonized the host are capable to express an antagonistic activities aimed to inhibit insect-vectored disease agents or to interfere with the survival of pest insects (Beard *et al.*, 2001, Crotti *et al.*, 2011). The different and important roles played by AAB make them interesting agents for developing symbiotic control protocols.
Besides this approach, symbiotic bacteria could also be employed for other innovative control strategies. Different studies showed that volatile compounds produced by microorganisms are strongly attractive to insects, as reported for *Pseudomona putida* to the olive fly *B. oleae*, or for a number of bacteria to the Mexican fruit fly *Anastrepha ludens* Loew (Jang and Nishijima, 1990; Robacker *et al.*, 1998, Liscia *et al.*, 2013). AAB could have interesting roles on insect attraction. In fact, the volatile compounds produced by mutualistic microorganisms on plants might be an important way for host-plant interaction (Frago *et al.*, 2012). Beside acetic acid, different other compounds are produced as secondary metabolites (Raspor and Goranovič, 2008) but their attractiveness to the host have still to be evaluated.
Despite the relevance of AAB as insect symbionts including in *Drosophila* spp. (Chandler *et al.*, 2011), their attractiveness has never been investigated. The focus of this study is to detect the volatile compounds produced by different AAB symbionts isolated from *D. suzukii* (Vacchini *et*

al., in preparation), by means of GC-MS (gas chromatography–mass spectrometry) analyses, and to evaluate their attractiveness for female flies by using two-choice olfactometer assays.

MATERIALS AND METHODS

Insect material and bacterial strains

All the experiments were conducted using flies emerged from blueberries, raspberries and blackberries in orchards of Cuneo and Torino provinces, Piedmont (North-West Italy) in summer 2013 and 2014. Emerged insects were reared on fruits (strawberries, blueberries, grapes and kiwi fruits) in plastic cages (24 × 16 × 12 cm) at the DISAFA in growth chamber at 25 ± 1°C, 65 ± 5% RH and 16L:8D photoperiod

Bacteria were previously isolated from *D. suzukii* specimens (Vacchini *et al.*, in preparation) Briefly, they were isolated from the whole body of three males, one female and one larva reared on fruits. After surface sterilization, insects were homogenated in 0.9% NaCl and inoculated in MAN enrichment solid medium (mannitol 2.5%, bactopeptone 3%, yeast extract 5%, agar 1.5%) and in liquid enrichment media: TA1 (enrichment medium I, Yamada *et al*. 1999), TA4 (basal medium, Kadere *et al*. 2008), and Acid YE (yeast extract 2%, ethanol 2%, acetic acid 1%, pH 6). After isolation they were purified on MA solid medium (reference) and identified (Vacchini et al., in preparation). Pure isolates were then conserved at -80 °C.

Volatile profile analysis

Three strains within each genus of AAB isolates, *Acetobacter tropicalis* bYea.1.23 (shortly named 23), *A. persicus* BTa1.3.45 (shortly named 45), *A. cibinongensis* BMan.1.4 (shortly named 44), *Gluconobacter kondonii* BMan.3.1C (shortly named 1C), *G. oxydans* DS1FC.9A (shortly named 9A), *G. kanchanaburiensis* L2.1.A.16 (shortly named 16), *Gluconacetobacter hansenii* DS2MC.114 (shortly named 114), *G. saccharivorans* DS1MA.65A (shortly named 65A), and *G. europaeus* DS1MC.70A (shortly named 70A), were used for the evaluation of volatile profiles. After growing at 30°C in liquid MA medium, cells were adjusted to 10^8 cells/ml and then incubated on Petri dishes containing solid MA at 30 C for 24 or 48 hours.

Volatile profiles of all the bacterial strains were analysed with GC-MS at the Department of Sustainable Organic Chemistry and Technology (SynBioC), of Ghent University, Belgium. A solid phase microextraction (SPME) to examine volatiles was used. A glass vial (20 mL), capped with a Teflon-lined septum, containing 4 g of solid MA + bacteria and 4 g of NaCl was used for SPME sampling. Volatile compounds of sterile solid MA was also analysed as control. The samples were stirred for two min at 50°C to accelerate equilibrium of headspace volatile compounds between the solid matrix and the headspace. Then, volatile compounds extraction was carried out by injecting a Carboxen-polydimethylsiloxane (CAR/PDMS) fiber (black fiber, film thickness 75 µm, needle size 23 Ga, Supelco, Bellefonte, PA, USA) previous conditioned in the GC injector (250°C) for 30 minutes at 50°C. After extraction, samples were desorbed into the injection port of the GC. The analyses of volatile compounds were performed with a HP 6890 Series GC System equipped with a capillary column (DB5-MS, 30 m × 0.250 mm, film thickness 0.25 µm). The carrier gas was helium with a constant flow of 1.2 ml/min. The GC oven temperature was programmed for 29.33 minutes of total running. Since an initial temperature of 35°C, the temperature was increased with a constant rate of 5°C/min to 100°C, 15°C/min until 300°C and held for one minute at that temperature. A HP 5973 Mass Selective Detector (Hewlett-Packard, Wilmington, NC, USA) connected with GC was operated in electron impact mode with electron impact energy of 70 eV. GC-MS data were processed with the MDS-Chemstation software (Agilent Technologies). Volatile compounds were initially identified by comparison of chromatographic retention times and mass spectra with the WILEY6N.L, VITALIB.L and NIST98.I databases. Afterwards, the compounds were identified by comparison with authentic standards in concentration of 1 µL/mL added in glass vials (20 mL), capped with a Teflon-lined septum, containing 10 mL distilled water and analysed with GC-MS at the same condition. Alkanes (C5 to C18) were also run with 4 g of solid MA + 4 g of NaCl to calculate retention indices (RI) for the volatile compounds.

Chemicals

Employed chemicals were: ethanol (CAS No. 64-47-5), acetic acid (CAS No. 64-19-7), acetic acid ethyl ester (CAS No. 141-78-6), 2-propanol (CAS No. 67-63-0), 2-propanone (CAS No. 67-64-1), 2-methylpropanoic acid (CAS No. 79-31-2), 2-methylbutanoic acid (CAS No. 116-53-0), 3-methylbutanoic acid (CAS No. 503-74-2), pentanoic acid (CAS No. 109-52-4), benzaldehyde (CAS No. 100-52-7) and acetaldehyde (CAS No. 75-07-0). Moreover the alkanes: pentane (CAS No. 109-66-0), hexane (CAS No. 110-54-3), heptane (CAS No. 142-82-5), octane (CAS No. 111-65-9), nonane (111-84-2), decane (CAS No. 124-18-5), undecane (CAS No. 1120-21-4), dodecane (CAS No. 112-40-3), tridecane (CAS No. 629-50-5), tetradecane (CAS No. 629-59-4), pentadecane (CAS No. 629-62-9), hexadecane (CAS No. 544-76-3), heptadecane (CAS No. 629-78-7) and octadecane (CAS No. 593-45-3) were used. The putity of all compounds was above 98%.

Two-choice bioassays

Five AAB strains (*A. persicus* 45, *A, cibinongensis* 4, *G. oxydans* 9A, *G. kanchanaburiensis* 16 and *G. saccharivorans* 65A) were tested against the control (sterile MA) with a two-choice assay to evaluate *D. suzukii* preferences based on the analysis of the volatile profiles. A total of 10^8 bacterial cells / mL were obtained as explained above before being plated in plastic flasks containing 20 mL of solid MA and grown at 30°C for 24 or 48 hours. The olfactometer used was similar to that described by Faucher *et al.* (2013). Insects were released in a plastic box (24 × 16 × 12 cm), covered with a fine mesh net on the top, and with a layer of wet cotton on the base to supply humidity. On the bottom of the box, there were two apposite holes (31 mm diameter) closed by silicon plugs. Two glass funnels (46 mm diameter) were fitted in these plugs and inserted each in a 250 mL glass flask placed below the box. A pump (Air 275R, Sera, Heinsberg, Germany) was used to supply the air necessary for the trials. Pumped air was humidified and split in two 5 mm diameter silicon tubes, each entered first in a plastic flask (125 mL) containing the strain or the control to test. The exit air enriched with the volatile compounds was led by another silicon tube (same diameter) into the glass flask through a hole created in the plug close to the funnel. The glass flasks acted as traps, and the flies once entered could not escape.

The strains were tested against the control (solid sterile MA). Olfactometer assays were conducted in a climatic chamber (25 ± 1°C, 65 ± 5% RH and 9 lux). At the beginning of the experiments, illuminance was measured with a lux meter (PCE-172, PCE Group, Lucca, Italy), while the air flow rate of 0.25 L min^{-1} was checked at the downwind end with a digital anemometer (TA-410, PCE Group, Lucca, Italy). For each trial, 70 from 2 to 7 day old *D. suzukii* females were separated and starved on 1.5% agar for 24 hours inside a plastic tube. Then the females were introduced in the centre of the box through a small hole created in the middle of the net and closed with a plug. After 24 hours, the females in the box and in the two flasks were counted. Six replications at 24 and 48 hours for each strain compared with the control were assessed. All the flasks, funnels, plugs and tubes were cleaned with neutral soap and distilled water, and sterilized in autoclave. The box and the net were cleaned with neutral soap, distilled water and ethanol (70%). Responses of *D. suzukii* females were estimated by calculating an olfactory index (OI), defined by Alcorta and Rubio (1988) and Newby and Etges (1998) as OI = no. flies in trap 1/(no. flies in trap 1 + no. flies in trap 2).

Statistical analyses

For the evaluation of volatile compounds, 9 replications were performed for each strain and for the control (3 preliminary replications without considering the bacterial growth time, 3 replications after a 24 hours growth and 3 replications after 48 hours). The frequencies of compounds found for each strain in 9 replications were analysed with correspondence analysis (R i386 3.0.3.Ink software).

For the olfactometer assays, the OI values obtained from the six replications at 24 and 48 hours between the five AAB strain and the control were tested with Student's t single sample test (0.5 as mean). OI values between records obtained after 24 and 48 hours of bacterial growth for each strain were also analysed with Student's t independent samples test. Mean of no choice flies for each strain grown for 24 and 48 hours were analysed with one-way ANOVA followed by TukeyHSD test as post hoc. The statistical analyses of olfactometer results were performed using SPSS version 20 (Chicago, Illinois, USA).

RESULTS

Bacteria materials

AAB bacteria found as symbionts in *D. suzukii* belonged to three main genera: *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*. These bacteria were isolated on solid media, specific for AAB growth.

Chemical analysis

The volatile compounds found in each strains and in the control belonged to the class of alcohols, ketones, carboxylic acids and aldehydes. The first three replications allowed to identify the 11 main substances produced by strains. Unexpectedly, acetic acid was not the main abundant compound produced by bacteria and sometimes it was not recorded. On the contrary, 2-propanone was always registered in all bacteria strains and its presence was always asserted in all replications carried out. Moreover, the first preliminary analysis showed relevant peaks of carboxylic acids (as propanoic acid 2-methyl, butanoic acid 2-methyl, butanoic acid 3-methyl) especially in the three *Gluconacetobacter* strains. In the three AAB-free control replications, ethanol and 2-propanol were always found, whereas in one replication benzaldehyde was recorded.

To elucidate the possible change of volatiles produced by bacteria overtime, three more replications were performed after 24 hours and other three ones after 48 hours. In the first 24 hours all the strains produced 2-propanone and acetic acid with the exception of strain 45, where the acetic acid was never found in the three replications performed. Ethanol was recorded in only four strains: 9A and 16 in the *Gluconobacter* genus, and 65A and 70A within *Gluconacetobacter*, while the butyric acid derivatives (propanoic acid 2-methyl, butanoic acid 2-methyl, butanoic acid 3-methyl) were not produced in the first hours except for strains 45, 114, and 65A. However, in this last strain, only the propanoic acid, 2-methyl was recorded in all replications at 24 hours. Finally, in strains 9A and 16, both related to the genus *Gluconobacter*, a fair production of benzaldehyde was observed.

After 48 hours, 2-propanone was the sole compound continuously released by all the bacteria. In fact, acetic acid, which was present in almost all the strains after a 24 hours growth, was only detected in strains 9A and 16. Moreover, even after 48 hours, benzaldehyde was always found in these bacteria. The presence of butyric acid derivatives considerably increased after 48 hours for many bacteria. As a matter of fact, these acids were identified for strains 45 and 1C, and in the *Gluconacetobacter* isolates. In strains 45 and 4, only butanoic acid, 3-methyl was detected after 48 hours, while for strains 9A and 16 these substances were never emitted. Finally, the analysis of compounds conducted on 24 and 48 hours old sterile media for control confirmed a constant production of ethanol, 2-propanol and benzaldehyde (Table 1).

A correspondence analysis (CA) of the results was performed, in order to visualize grouping tendencies, which could distinguish volatile constituents of the 9 strains of bacteria. The first two principal components explained about 80% of the total variance, indicating that a reduced number of volatile compounds could explain the overall characteristics of samples. CA showed three main groups that include strains with common characteristic. A first group was represented by strains 9A and 16, characterized by the production of substances as 2-propanol, benzaldehyde and acetic acid, ethyl ester. The second group included strains 23, 4 and 1C, related to the production of acetic acid and 2-propanone. *Gluconacetobacter* strains (114, 65A and 70A) and strain 45 completed the third group being especially linked with the production of butyric acid derivatives. Finally, the control, clearly separated from the three groups, was characterized by the production of ethanol and benzaldehyde (Figure 1).

Two-choice bioassays

According to the spatial distribution of the strains along the two component extracted with the CA, together with the results obtained with GC-MS analysis, five bacteria were chosen for the olfactometer bioassays. In the first group strains 9A and 16, two similar bacteria which always produced acetic acid, a substance known for *D. suzukii* attractiveness, were tested. Strain 4 was selected to represent the second group. From the third group strain 65A was examined as a representative of the *Gluconacetobacter* genus, whereas strain 45, which had a profile more similar to *Gluconacetobacter* than to the other two *Acetobacter* strains, was tested as well.

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Table1: Volatile compounds identified with GC-MS analysis in 9 AAB strains and in the control in a preliminary experiment, after 24 and after 48 hours of growth.

Strain	Compound	Identified by[a]	RI[b]		Presence[c]		
			Exp.	Lit.	Preliminary	24 hours	48 hours
A. tropicalis 23	Ethanol	Database; AS	-		+		
	Acetic acid	Database; AS; RI	630	625	+	+	
	Acetic acid, ethyl ester	Database; AS; RI	615	612		+	
	2-Propanol	Database; AS; RI	501	515	+		
	2-Propanone	Database; AS	-		+	+	+
	Propanoic acid, 2-methyl	Database; AS; RI	781	790	+		
	Butanoic acid, 3-methyl	Database; AS; RI	880	875	+		+
	Acetaldehyde	Database; AS	-		+		
A. persicus 45	Acetic acid	Database; AS; RI	620	625	+		
	2-Propanone	Database; AS	-		+	+	+
	Propanoic acid, 2-methyl	Database; AS; RI	792	790		+	+
	Butanoic acid, 3-methyl	Database; AS; RI	878	875		+	+
	Butanoic acid, 2-methyl	Database; AS; RI	875	873		+	+
A. cibinogensis 4	Ethanol	Database; AS	-		+		
	Acetic acid	Database; AS; RI	622	625		+	
	2-Propanol	Database; AS; RI	500	515		+	
	2-Propanone	Database; AS	-		+	+	+
	Butanoic acid, 3-methyl	Database; AS; RI	879	875	+		+
G. kondonii 1C	Acetic acid	Database; AS; RI	628	625		+	
	Acetic acid, ethyl ester	Database; AS; RI	616	612	+		
	2-Propanone	Database; AS	-		+	+	+
	Propanoic acid, 2-methyl	Database; AS; RI	802	790			+
	Butanoic acid, 3-methyl	Database; AS; RI	869	875			+
	Butanoic acid, 2-methyl	Database; AS; RI	874	873			+
G. oxydans 9A	Ethanol	Database; AS	-			+	
	Acetic acid	Database; AS; RI	644	625	+	+	+
	2-Propanol	Database; AS; RI	501	515	+	+	+
	2-Propanone	Database; AS	-		+	+	+
	Benzaldahyde	Database; AS; RI	988	970	+	+	+
G. kanchanaburiensis 16	Ethanol	Database; AS	-		+	+	
	Acetic acid	Database; AS; RI	644	625	+	+	+

	2-Propanol	Database; AS; RI	501	515		+	+
	2-Propanone	Database; AS	-		+	+	+
	Benzaldahyde	Database; AS; RI	990	970	+	+	+
Ga. hansenii 1C	Acetic acid	Database; AS; RI	629	625		+	
	2-Propanone	Database; AS	-		+	+	+
	Propanoic acid, 2-methyl	Database; AS; RI	799	790	+	+	+
	Butanoic acid, 3-methyl	Database; AS; RI	882	875	+	+	+
	Butanoic acid, 2-methyl	Database; AS; RI	896	873	+	+	+
	Pentanoic acid	Database; AS; RI	908	902			+
Ga. saccharivorans 65A	Ethanol	Database; AS	-			+	
	Acetic acid	Database; AS; RI	642	625		+	
	2-Propanone	Database; AS	-		+	+	+
	Propanoic acid, 2-methyl	Database; AS; RI	790	790	+	+	+
	Butanoic acid, 3-methyl	Database; AS; RI	870	875	+		+
	Butanoic acid, 2-methyl	Database; AS; RI	878	873	+		+
	Acetaldehyde	Database; AS	-			+	
Ga. europaeus 70A	Ethanol	Database; AS	-			+	
	Acetic acid	Database; AS; RI	629	625	+	+	
	2-Propanol	Database; AS; RI	501	515	+	+	
	2-Propanone	Database; AS	-		+	+	+
	Propanoic acid, 2-methyl	Database; AS; RI	797	790	+		+
	Butanoic acid, 3-methyl	Database; AS; RI	884	875	+		+
	Butanoic acid, 2-methyl	Database; AS; RI	896	873	+		+
	Pentanoic acid	Database; AS; RI	903	902			+
	Acetaldehyde	Database; AS	-		+		
Control (sterile MA)	Ethanol	Database; AS	-		+	+	+
	2-Propanol	Database; AS; RI	501	515	+	+	+
	Benzaldahyde	Database; AS; RI	1003	970	+	+	+

[a] Identification of the compound through the databases (WILEY6N.L, VITALIB.L or NIST98.I), the application of Authentic Standard (AS) and the Retention Index (RI)
[b] Retention index on DB5-column. Exp.: Retention Index calculated from the experiment. Lit: Retention Index found in literature (http://webbook.nist.gov/chemistry/).
[c] +: presence of the substance in one, two or three replications performed in the preliminary experiments, after 24 hours and after 48 hours.

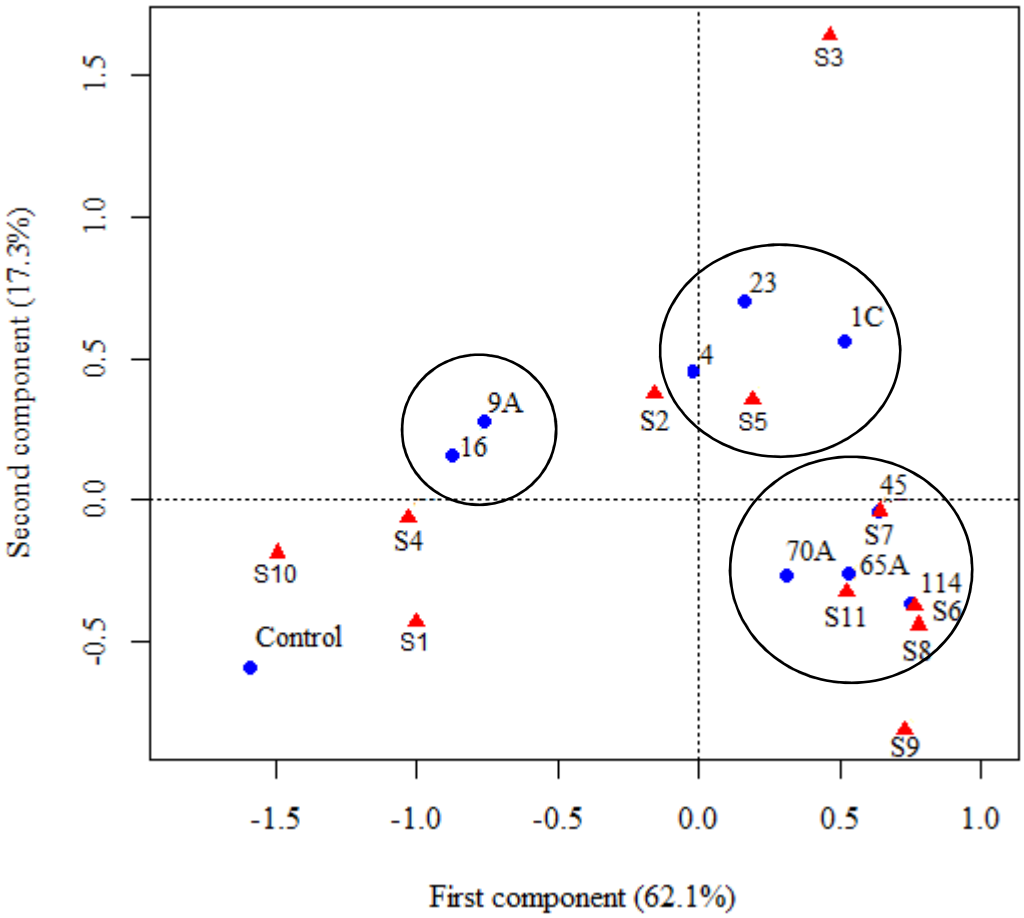

Figure 1: Correspondence analysis of 11 volatile compounds (S1=ethanol; S2=acetic acid; S3=acetic acid, ethyl ester; S4=2-propanol; S5=2-propanone; S6=propanoic acid, 2-methyl; S7=butanoic acid, 3-methyl; S8=butanoic acid, 2-methyl; S9=pentanoic acid; S10=benzaldehyde; S11=acetaldehyde) produced by 9 strains of AAB (Control, 23=*Acetobacter tropicalis*, 45=*Acetobacter persicus*, 4=*Acetobacter cibinogensis*, 9A=*Gluconobacter oxydans*, 1C=*Gluconobacter kondonii*, 16=*Gluconobacter kanchanaburiensis*, 65A=*Gluconacetobacter sacchari-vorans*, 114=*Gluconacetobacter hansenii*, 70A=*Gluconacetobacter europaeus*) in 9 replications.

All the tested bacteria, both after 24 and 48 hours of growth, were significantly more attractive than the control (sterile MA), with the exception of strain 45 (figure 2-3). In fact, no significant difference was found in the comparison between this strain and the control in the first 24 hours of growth (single sample t test: t = 2.008, df = 5, P = 0.101;). However, in the second trial in replicates performed after 48 hours bacterial growth, the control was clearly preferred by flies than the strain 45, with a very significant difference (single sample t test: t = -5.659, df = 5, P = 0.002)

After 24 hours, 4 (single sample t test: t = 6.711, df = 5, P = 0.001), 9A (single sample t test: t = 5.669, df = 5, P = 0.002), and 65A (single sample t test: t = 7.464, df = 5, P = 0.001) strains were always preferred with very significant differences in the comparisons with control, while an extremely significant difference was recorded between the control and strain 16 (single sample t test: t = 15.609, df = 5, P < 0.001). Instead, after 48 hours, extremely significant differences were always observed in all these four strains compared with the control (strain 4: single sample t test: t = 14.613, df = 5, P < 0.001; strain 9A: single sample t test: t = 13.463, df = 5, P < 0.001; strain 16: single sample t test: t = 8.206, df = 5, P < 0.001; strain 65A: single sample t test: t = 12.074, df = 5, P < 0.001).

From the comparison of the OI values between the two growth periods, a very significant difference was observed in strain 45 with a sensible reduction of preference for 48 hours old

bacteria than 24 hours old cells. A significant difference reduction of preference was also detected for strain 4 after a 48 hours growth. Nevertheless, no differences in the choice were recorded between the two growing times for strains 9A, 16, and 65A (Table 2).
The evaluation of *D. suzukii* preference between each AAB strain and the control must also consider the rate of no choice flies. Although *D. suzukii* usually preferred the bacteria than the control, high differences in choice rates were observed among strains. In tests with bacteria grown for 24 hours, low choice rates were detected for *Acetobacter* strains: 77.4±7.2% and 56.5±10.6% of females did not choose in the comparisons between strain 45 *vs* control and strain 4 *vs* control, respectively. A significantly higher rate of choice (about 60%) was recorded in the comparisons of the *Gluconobacter* and *Gluconacetobacter* strains (9A *vs* control, 16 *vs* control and 65A *vs* control) than in the comparison of strain 45 *vs* control (one-way ANOVA: df = 4, 25; F = 6.417; P = 0.001). After 48 hours of bacterial growth, the number of flies that did not choose between strain 4 and the control increased (75.7±7.9%), while for the strain 45 *vs* control comparison the percentage of no choice decreased (59.9±7.9%). An increment of no choice rate was also recorded between 9A and the control (51.3±5.2%). Instead, the number of flies that did not choose in strain 16 *vs* control and 65A *vs* control comparisons was similar even after 48 hours (45.7±6.7% and 35.2±6.1%, respectively) with significant differences among these two comparisons, and that between strain 4 and control (one-way ANOVA: df = 4, 25; F = 5.486; P = 0.003) (Figure 4).

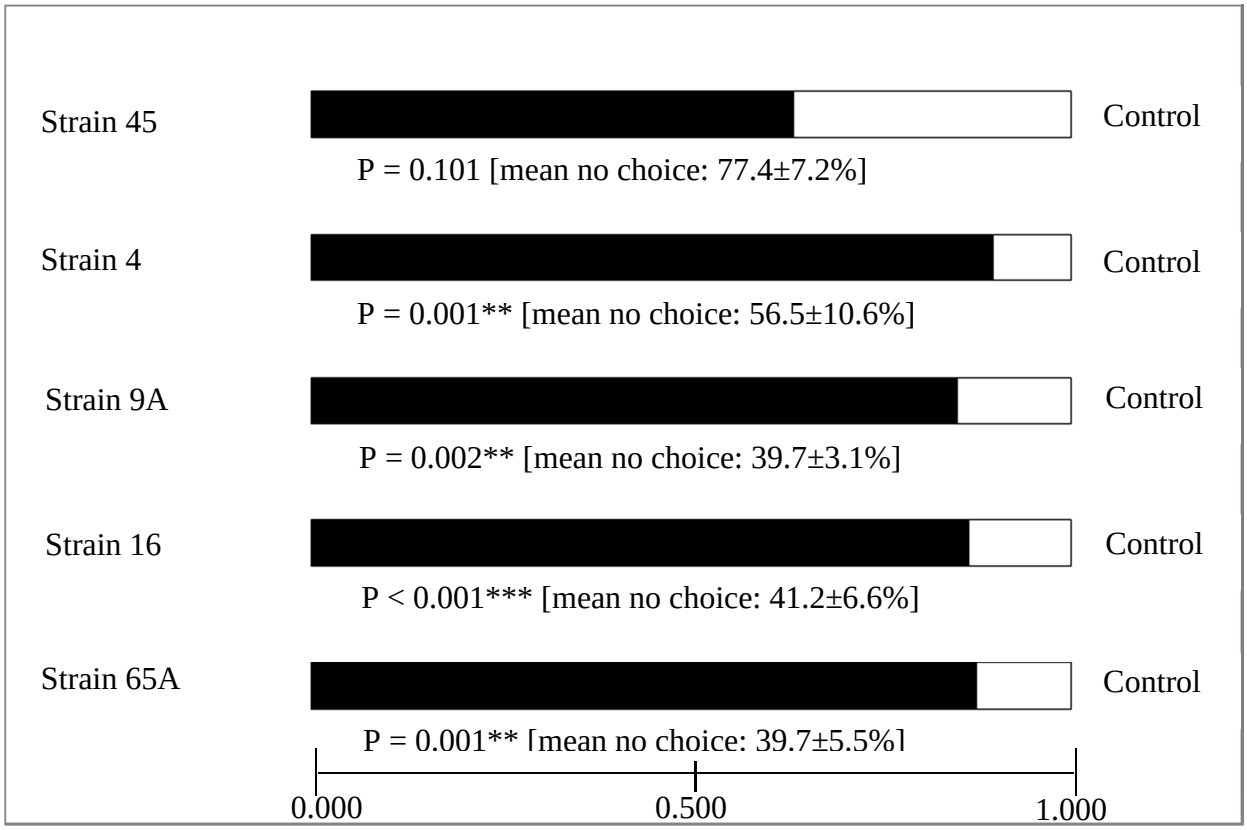

Figure 2: Responses of *Drosophila suzukii* females to the volatile compounds produced by strains *A. persicus* 45, *A. cibinogensis* 4, *G. oxydans* 9A, *G. kanchanaburiensis* 16 and *G. saccharivorans* 65A, tested against sterile medium (control) in six replications carried out in the two-way olfactometer after a 24 hours bacterial growth. Below the bars the mean percentage (±SE) of females that did not choose is reported. Single sample t test (P < 0.05, df = 5), asterisks indicate significant (*), very significant (**) or extremely significant differences (***).

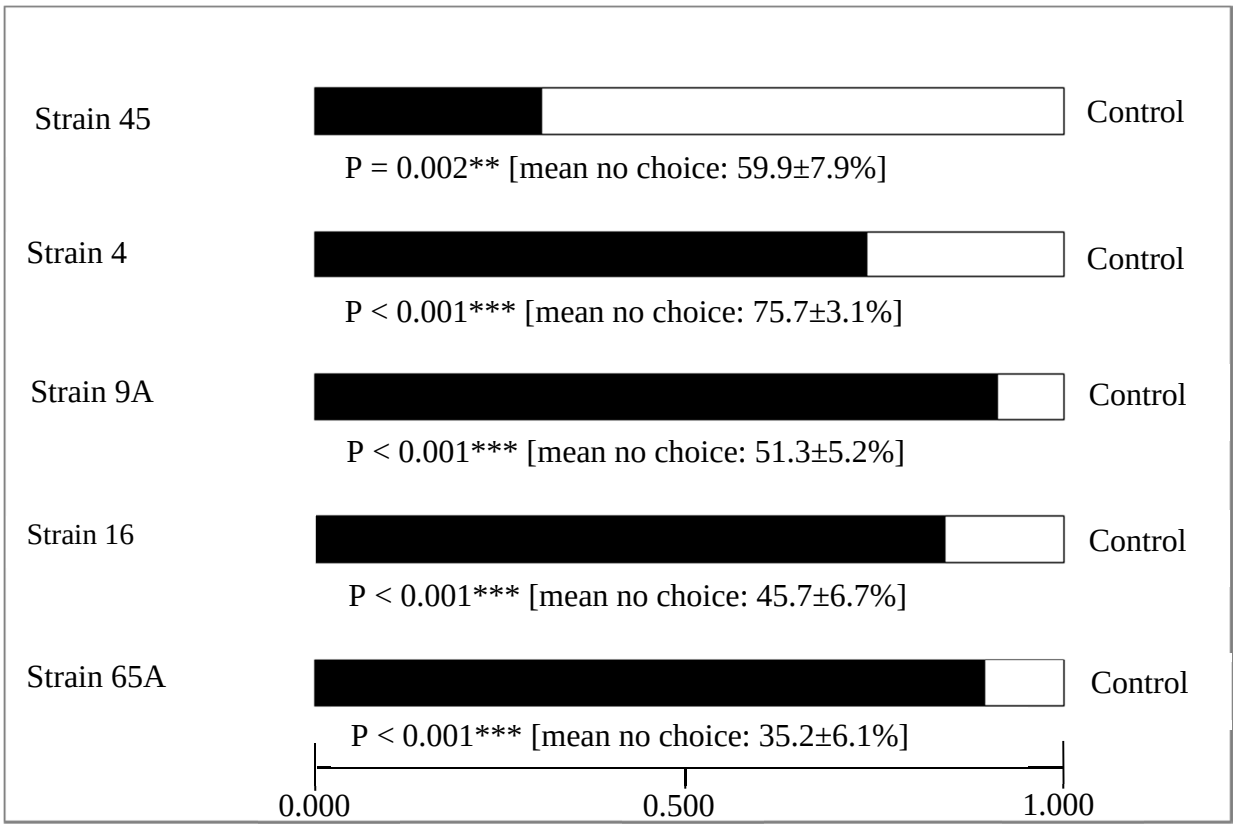

Figure 3: Responses of *Drosophila suzukii* females to the volatile compounds produced by strains *A. persicus* 45, *A. cibinogensis* 4, *G. oxydans* 9A, *G. kanchanaburiensis* 16 and *G. saccharivorans* 65A, tested against sterile medium (control) in the six replications carried out in the two-way olfactometer after a 48 hours bacterial growth. Below the bars the mean percentage (±SE) of females that did not choose is reported, asterisks indicate significant (*), very significant (**) or extremely significant differences (***) (single sample t test; $P < 0.05$, df = 5).

Table2: Mean of olfactory index (OI) after six replications both for 24 and 48 hours old bacteria., Asterisks indicate significant (*) or very significant differences (**) (independent samples t test $P<0.05$, DF = 5).

Strain	Mean OI (24 hours)	Mean OI (48 hours)	t test	P value
A. persicus 45	0.635±0.067	0.302±0.028	-4.393	0.001**
A. cibinogensis 4	0.897±0.059	0.736±0.016	-2.633	0.025*
G. oxydans 9A	0.850±0.062	0.911±0.030	0.873	0.403
G. kanchanaburiensis 16	0.866±0.023	0.841±0.042	-0.514	0.619
Ga. saccharivorans 65A	0.875±0.050	0.894±0.033	0.316	0.758

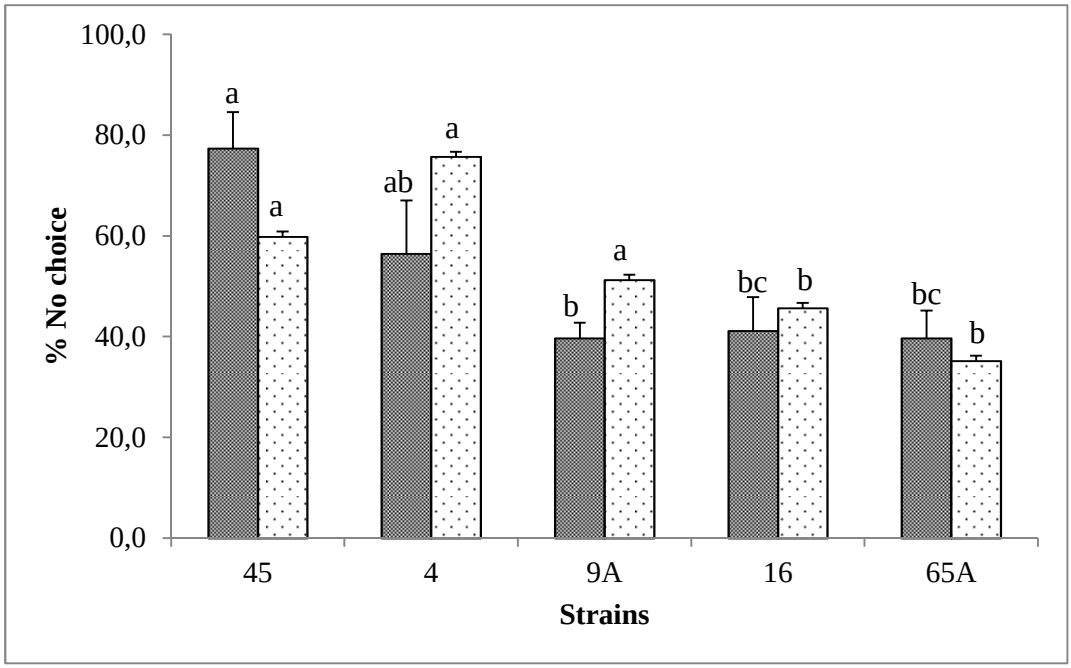

Figure 4: Mean percentage of females that did not choose after 24 hours (black bars) and 48 hours (white bars) bacterial growth during olfactometer bioassays between the control and *A. persicus* 45, *A. cibinogensis* 4, *G. oxydans* 9A, *G. kanchanaburiensis* 16 and *G. saccharivorans* 65A. Different letters above histogram bars indicate significant differences (TukeyHSD post hoc test following ANOVA, $P < 0.05$).

DISCUSSION

The family Acetobacteraceae was confirmed as one of the most important bacterial clades associated to *D. suzukii* (Chandler *et al.*, 2014, Vacchini *et al.*, in preparation) as well as reported in other Drosopihlidae (Chandler *et al*., 2011). In particular, in *D. melanogaster*, which is the majorly studied species, *Acetobacter* is the most representative genus (Staubach *et al.*, 2013, Wong *et al.*, 2011, Wong *et al.*, 2013). From the three strains of *Acetobacter* recorded in our study, *A. tropicalis* and *A. cibinogensis* were isolated and identified in *D. melanogaster* too (Corby-Harris, 2007, Ren *et al.*, 2007, Wong *et al.*, 2011). The other strain isolated from *D. suzukii* (*A. persicus*) was never detected in *Drosophila* spp. On the contrary, strains observed by other authors to be very abundant in *D. melanogaster*, as *A. aceti*, *A. cerevisae*, *A. pomorum* and *A. pasteurianus* (Cox and Gilmore, 2007, Chandler *et al.*, 2011, Wong *et al.*, 2011), were not found in our *D. suzukii* line (Chandler *et al.*, 2014). The remainder of α-Proteobacteria symbionts in *Drosophila* spp. is mainly represented by the genus *Gluconobacter*, however few strains have been reported, and in lower frequencies than *Acetobacter*. *G. cerinus*, *G. frateurii*, *G. oxydans* and *G. morbifer* were the most known strains previously found in *D. melanogaster* (Cox and Gilmore, 2007, Chandler *et al*., 2011, Kim *et al.*, 2012); nevertheless among these species only *G. oxydans* was recorded in our *D. suzukii* samples. This strain was also isolated by Chandler *et al.* (2014) in a recent study conducted on the microbial community in *D. suzukii*. The other two *Gluconobacter* strains isolated from our samples (*G. kanchanaburiensis* and *G. kondonii*) have never been detected in other *Drosophila* spp. The genus *Gluconacetobacter* is less represented as symbiont in fruit flies and in some studies it was not found (Chandler *et al.*, 2011). Only *Ga. europaeus*, detected also in our samples, has been previously detected in flies, with few other strains as *Ga. diazotrophicus* (Corby-Harris, 2007, Cox and Gilmore, 2007). Instead, *Ga. saccharivorans* and *Ga. hansenii* are not reported as symbionts in *Drosophila* spp. in previous studies.

Different volatile molecules were commonly detected in all of AAB strains tested by HS-SPME-GC-MS, although significant changes in the compound profiles obtained for some strains were found between 24 and 48 hours of bacterial growth. As an example, the production of butyric acid derivatives (propanoic acid 2-methyl, butanoic acid 2-methyl, butanoic acid 3-methyl) especially occurred after 48 hours of bacterial growth. Only in *A. persicus* and in *Ga. hansenii* these compounds were detected as soon as in the first 24 hours of growth; moreover, despite these acids

were found in almost all the strains, the highest frequencies and abundance of volatiles were recorded in *Gluconacetobacter* strains and in *A. persicus*. This fact was confirmed by the strain distribution in the two components extracted by correspondence analysis where a major similarity on volatile production was observed between *A. persicus* and *Gluconacetobacter* strains than the other two *Acetobacter* isolates. These strains are close to the butyric acid derivatives compounds. On the contrary, these compounds have been detected less frequently in the other *Acetobacter* members. The production of short-chain fatty acid is not reported in AAB, but it is widely known in Lactic Acid Bacteria (LAB) (Kranenburg *et al.*, 2002). Many typical food flavours derive from compounds produced by the conversion of free amino acids by deaminases, decarboxylases, transaminases and lyases. For example, from branched-chain amino acids it is possible to obtain isobutyrate, isovalerate, 3-methylbutanal, 2-methylbutanal, and 2-methylpropanal, which are found in various cheese types (Kranenburg *et al.*, 2002). The difference of attraction and the peculiarity of *A. persicus* strain is evident also from olfactometer assays, after 24 hours and 48 hours of growth. Females did not choose it in comparison to the control after 24 hours of growth, and its attractiveness for flies lowered at 48 hours, being not significantly attractive. This was the only not-attractive strain in olfactometer tests. The volatile production was stable even after 48 hours of growth in *Gluconobacter* members. *G. oxydans* (9A) and *G. kanchanaburiensis* (16) clustered in correspondence analysis, while the other *Gluconobacter* member (1C) was more related to the production of acetic acid and 2-propanone, in comparison to the other molecules. It showed a profile closer to the group of *Acetobacter* 4 and 23. *G. oxydans* and *G. kanchanaburiensis* are also characterized by the production of benzaldahyde, acetic acid, and ethanol after 24h of growth, coupled with a low rate of no choice for the females flies (40-50%). Seven strains, i.e. *G. kondonii* 1C, all the analysed *Acetobacter* spp. and *Gluconacetobacter* spp. did not release acetic acid after 48 hours of growth and this might be an intriguing information, considering the attractive effect of this compound for *D. suzukii*. Among the tested isolates, higher attractiveness values for flies were recorded for *G. oxydans* 9A, *G. kanchanaburiensis* 16 and *G. saccharivorans* 65A strains.

The five chosen strains were also evaluated by considering the rate of female flies that did not make a choice between bacteria and the control, both after 24 h and 48h of bacterial growth, in the olfactometer assays. In *Acetobacter,* an high percentage of no choice was recorded (between 60 and 80% of females). On the other hand *G. saccharivorans* was always highly preferred in comparison to the control in olfactometer tests, and even the rate of no choice was quite low (35-40%), with no differences between 24 and 48 h of growth.

Despite many compounds exhibit attractiveness to *D. suzukii*, finding the most suitable and efficient alternative strategy to commercially available traps based on vinegar, is not easy, and it might be due to different reasons. For instance, Kleiber and colleagues (2014) found several compounds (methanol, ethanol, propanol, formic acid, acetic acid, ethyl acetate, propyl acetate, phenethyl acetate, phenethyl proprionate, phenethyl butyrate) having an attractive effect in greenhouse trials, but without a significative better attractive effect than apple cider vinegar baits. It was noted that factors influencing the analysis were the compounds' concentrations and their synergistic effects; indeed, concentrations determined in greenhouse tests were much higher than the average concentrations detected in wine and vinegar. It led in some cases to deterrent effects and negative attraction responses of flies. Another factor to be accounted for is the synergistic effect obtained when baits are composed by more than one attractant, and the lack of such a synergistic effect might render the trap ineffective. Ethanol and acetic acid possess a synergistic effect, as spotted wing fly is not attracted by ethanol alone (Landolt *et al.*, 2012a). Baits currently in usefor *D. suzukii* are composed by different fermented food materials (Landolt et al., 2012a, Landolt *et al.*, 2012b), albeit not specific for this insect. A work focused on volatiles produced by overripe mangoes showed that compounds like ethanol, acetic acid, amyl acetate, 2-phenylethanol, and phenylethyl acetate stimulated response from antennae of *D. melanogaster* flies, and a blend of these volatiles were more attractive than the single compounds. Despite that, again, field trials did not provide results as successful as the lab ones (Zhu *et al*., 2003). In addition to this, in 2012 Cha and colleagues were able to determine 13 volatile chemicals from wine and vinegar that, beyond acetic acid and ethanol, are detected by *D. suzukii*, being antennally active towards them. By field trails it was highlighted that, in detail, acetoin, ethyl lactate and

methionine were able to enhance the fly attractiveness of a blend of acetic acid and ethanol (Cha *et al.*, 2012).

To conclude, the work here presented provides an overview of the potential of AAB as living attractors for the emerging pest *D. suzukii*; in particular *G. oxydans* 9A, *G. kanchanaburiensis* 16, *Ga. saccharivorans* 65A isolates are able to elicit *Drosophila* response and a low no-choice rate in comparison to the other bacteria tested was recorded for these strains. Furthermore, by characterizing the array of volatile compounds released by the strains, new data contribute to the current state of the art regarding the molecules involved in fly attractiveness and baits construction. Field trials might be set up to evaluate if this laboratory information could have a potential for the application of innovative strategy of pest management.

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Chapter IV

Characterization of the Yeast Community Associated to the Spotted Wing Fly *Drosophila suzukii*

Abstract
Yeasts can be involved in important mutualistic associations with drosophild flies, with whom they also share the ecological niche. The spotted wing fly, *Drosophila suzukii* (Diptera: Drosophilidae) is an exotic emerging pest and a serious issue for crop losses. To enlarge the knowledge of this pest and to develop strategies for its control, it is compulsory the investigation of different aspects of its biology, among which the fly-associated yeasts. To focus on this issue, the diversity of the yeast community associated to *D. suzukii* individuals of three different developmental stages (larvae, pupae, adults), reared on two different food diets, was assessed by culture-dependent and independent methods. DGGE-PCR and pyrosequencing analyses on the ITS1-5.8S-ITS2 region provided a picture of the yeast community structure and composition, characterized by the prevalence of Ascomycetes. These yeasts, belonging to the Saccharomycetales order, in particular to *Candida*, *Geotrichum* and *Pichia* genera, typically colonize fermenting fruits and food sources on which *Drosophila* feeds. A similar distribution was observed in the collection of 237 isolates, analysed by RFLP fingerprint of the ITS1-5.8S-ITS2 region of the fungal rRNA operon and sequencing. Data showed that the most abundant species, *i.e*. *Pichia occidentalis*, *Saccharomycopsis craetegensis* and *Arthroascus schoenii*, were mainly isolated from the insects reared on the artificial diet, independently from the life stage. On the other hand, insects reared on fruits are characterized by a higher diversity in terms of yeast species. In particular, it was recorded the presence of *Hanseniaspora uvarum*, which was previously described as the dominant yeast genus associated to different *Drosophila* species, including *D. suzukii*.

INTRODUCTION
The spotted wing fly *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) is an insect pest, introduced in the last decade in the many countries from South –East Asia, spreading year by year in new habitats and damaging new host plants (Cini *et al*., 2012; Rota-Stabelli *et al*., 2013). Some unique biological features, including the female serrated ovipositor, which enables the insect to damage and lay eggs in healthy and still ripening soft summer fruits (Walsh *et al*., 2011, Rota-Stabelli *et al.*, 2013), together with the high reproduction rates and short life cycle, make this pest a severe concern for crops (Bolda *et al.*, 2010).
In the last years, the scientific community has witnessed the discovery of a huge and complex variety of microbial associations with animals and with insects, in particular. Different taxa are involved in these associations, spanning from protozoans, fungi, archaea, to bacteria (Dillon and Dillon 2004, Ikeda-Ohtsubo and Brune 2009, Iasur-Kruh *et al.* 2014). The nature of these relationships can be pathogenic, parasitic, mutualistic (Dale and Moran 2006). The association between bacteria and drosophilid flies has been largely investigated (Anbutsu and Fukatsu 2003, Moran et al 2005), especially in the insect models *Drosophila melanogaster* and *Anopheles*. Not only the diversity, but also the positive role played by bacteria in several aspects of the host biology, such as the nutritional complementation, the immunity, the larval development (Dong *et al.*, 2006, Ryu *et al.*, 2008, Crotti *et al.*, 2010, Chouaia *et al.*, 2012) were surveyed. The characterization of the microbial community associated to the spotted wing fly has been recently reported (Chandler *et al.*, 2012, Vacchini *et al.*, in preparation), hypothesising the beneficial contribute that microorganisms, in particular acetic acid bacteria (AAB), might provide to this emerging pest.
Among the different groups involved in mutualistic associations with drosophilid flies, yeasts play a primary role. The association between yeasts and *Drosophila* species with different feeding niches has been recently reported (Chandler *et al.*, 2012). In particular, the community of yeasts inhabiting *D. suzukii* adults and larvae, feeding on cherries and raspberries, has been investigated with microbiological methods (Hamby *et al.*, 2012). This relationship occurs as yeasts are an

important nutritional source for insects: they furnish essential nutrients, like proteins and vitamins, sterols and enzymes for digestion and they detoxify toxic metabolites, introduced with the diet. Thus, yeasts indirectly implement larval growth, body size and survival (Stamps *et al.*, 2012). Consequently, sites colonized by certain species of yeasts, in particular those belonging to the Saccharomycetales order, are chosen preferentially by insects for courting and oviposition (Becher *et al.* 2012); this, in turn, can modify the yeast ecological niche. On the other hand, yeasts can benefit from the association with insects since insects can be yeast vectors, allowing yeasts to colonize new habitats. In addition to this, the capability of yeasts to attract the insects, through the emission of an array of volatile organic compounds (VOCs, Palanca *et al*., 2013), might be a strategy adopted for dispersal. Importantly, when yeast cells are ingested by the animal vector, the dissolution of the *ascus* (the envelope enclosing the tetrads) inside the gut allows the spores to mate with spores from other tetrads, increasing the outbreeding rates and genetic diversity (Reuter *et al*., 2007). Yeasts are able to produce high alcohol concentrations when fermentative processes occur, by giving rise to a nutrient-rich environment for AAB and LAB, and unhospitable conditions for the growth of bacteria less tolerant to ethanol (Barata *et al*., 2011). Therefore, the success of many destructive pests might reside in the mutualistic associations established with microorganisms, both bacteria and yeasts.

In a perspective of "Microbial Resource Management" (MRM) (Crotti *et al*., 2011; Verstraete, 2007) approach, and to develop a biocontrol program targeted for the interest pest, the precise knowledge of the community living inside the host body is necessary.

The characterization of the yeast community associated to *D. suzukii* of different life stages, i.e. larvae, pupae and adults, reared on two different food sources, i.e. fruits and artificial diet, was performed by means of cultivation-independent (ITS barcoding and Denaturing Gradient Gel Electrophoresis-PCR) and -dependent techniques. Our purpose was to enlarge with molecular and consistent microbiological data an already existing picture of the microbial community associated to this pest, by laying the groundwork to a future development of strategies of pest management.

MATERIALS AND METHODS

Insects and DNA extraction.

D. suzukii individuals were reared under controlled laboratory conditions on fruits and on artificial diet (constituted by 71 g of corn flour, 10 g of soy flour, 5.6 g of agar, 15 g of sucrose, 17 g of brewer's yeast, 4.7 ml of propionic acid, 2.5 g of vitamins mix for 1 Kg of preparation) at Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Turin. Insects were kept in cages at 25 °C with a 14:10 h light-dark photoperiod (Vacchini et al., in preparation).

Thirty-three individuals of *D. suzukii* (4 larvae fed on fruits, 4 larvae fed on artificial diet, 3 pupae fed on fruit, 5 pupae fed on artificial diet, 5 female adults fed on fruit, 4 female adults fed on artificial diet, 4 male adults fed on fruit, 4 male adults fed on artificial diet) were surface sterilized and stored at -20°C in ethanol until molecular analyses. Total DNA of whole specimens was individually extracted according to a method described by Polo and colleagues (2010). Twenty-one specimens (4 larvae reared on artificial diet, 3 pupae reared on artificial diet, 8 adults fed on fruit and 6 adults fed on artificial diet) were employed in yeast isolation trials.

Characterization of the yeast community associated to D. suzukii by Denaturing Gradient Gel Electrophoresis (DGGE)-PCR.

The length-variable internal transcribed spacer (ITS1-5.8S-ITS2) region of the fungal rRNA operon was subjected to amplification, prior to DGGE analysis, using a semi-nested PCR approach with primer pairs NS5/ITS4 (NS5 primer sequence: 5'-AAC TTA AAG GAA TTG ACG GAA G-3', ITS4 primer sequence: 5'-TCC TCC GCT TAT TGA TAT GC-3'), followed by a second PCR of the amplified PCR product with the primers ITS1F-GC/ITS4 (GC clamp: 5'-CCG GCG CCG CGG CGG GCG GGG CGG GGG CAC GGG-3', ITS1F primer sequence: 5'-CTT GGT CAT TTA GAG GAA GTA A-3').(Giacomucci *et al.*, 2011).

The reaction mixture for the first PCR (25 µL) was composed of 2 µL of template DNA, 1X Buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primers, and 0.625 U Taq polymerase (Invitrogen). Initial denaturation at 95°C for 3 min was followed by 30 cycles of 95°C for 45s, 52°C for 45 s, 72°C for 2 min, and a final extension step at 72°C for 10 min. The PCR product (2 µL) was used as template for the second PCR. The reaction mixture for the second PCR (50 µL)

differed from the mixture of the first one for the concentration of dNTPs (0.12mM) and primers (0.3 µM) used. The PCR conditions show some differences: the denaturation step at 94°C for 5 min was followed by 35 cycles of 94°C for 45s, 58°C for 45 s, 72°C for 2 min, and a final elongation step at 72°C for 10 min (Giacomucci *et al.,* 2011).
The amplicons were then analysed by DGGE. Polyacrylamide gels constituted by 7% of a [37:1] acrylamide:bisacrylamide mixture in 1X Tris-acetate-EDTA (TAE) 1X buffer with a urea-formamide linear denaturing gradient of 30–45% (100% denaturing polyacrylamide was defined as 7 M urea and 40% [w/v] formamide, Muyzer *et al.,* 1993, Raddadi *et al.,* 2011) were cast with a gradient maker (Bio-Rad, Milan, Italy) according to the manufacturer's guidelineS. For polymerization, 10% ammonium persulfate solution (APS) and TEMED (N,N,N',N'–tetramethylenediamine) were added. Approximatively 100 ng of PCR products were loaded using a syringe and electrophoresis was conducted in 1X Tris-acetate-EDTA buffer using a D-code electrophoresis system (Bio-Rad) to separate PCR products. A pre-run at 70 V for about 60 min was performed to warm up the buffer and reach the running temperature of 60°C for 16 hours with a constant voltage of 90 V. The gels were stained for 30 min in 1X TAE buffer containing 1X SYBR Green (Invitrogen, Milan, Italy) and then washed in distilled water for 30 min. DGGE gels were digitally visualized and capture by GelDoc 2000 apparatus (Bio-Rad, Milan, Italy) using the Quantity one software (Bio-Rad). Selected and excised bands with a sterile scalpel were eluted in 50 µL milli-Q water by incubation at 37°C for 3 h and used as a template in PCR re-amplification reactions with primers ITS1F and ITS4, as described in Giacomucci *et al.* (2011). PCR products were sequenced (Macrogen, South Korea) and the resulting sequences were compared, using BLASTn (http://www.ncbi.nlm.nih.gov/blast), with those in the National Center for Biotechnology Information (NCBI) sequence database (Altschul *et al*., 1990) and in the Centraalbureau voor schimmelcultures (CBS-KNAW) yeast nucleotide database (http://www.cbs.knaw.nl/Collections/Biolomics.aspx?Table=CBS+strain+database).

Multivariate analysis of community structures and diversity detected by DGGE

DGGE band patterns were converted to a binary dataset and a Bray–Curtis similarity matrix (Bray and Curtis, 1957) was calculated based on the dataset. Tests of the multivariate null hypotheses of no differences among a priori defined groups were examined using the nonparametric statistical test PERMANOVA (Anderson, 2001). Statistical analysis was performed with the factors of stage (fixed, orthogonal and three levels, larvae, pupae and adults) and diet (fixed, orthogonal and two levels, fruit and artificial food). PERMANOVA analyses were conducted with 999 permutations and run with software PERMANOVA + for PRIMER 6. All the multivariate statistical tests performed in this study were considered significant using a threshold of $p<0.05$ unless indicated otherwise.

Pyrosequencing on the internal transcribed spacer (ITS1-5.8S-ITS2).

DNA extracted from 3 individuals (one larva, one pupa and one female adult), all reared on fruits, was selected for 454 Pyrosequencing sequencing (Ronaghi *et al.,* 1998). The hypervariable fragment of the ITS region of the fungal rRNA operon was amplified from each sample using the universal fungal primers (ITS1F: 5'-CTT GGT CAT TTA GAG GAA GTA A-3', ITS4R: 5'-TCC TCC GCT TAT TGA TAT GC-3'). The analyses were performed by MR DNA (molecular research LP, Texas, USA). Analyses were performed using the QIIME pipeline (Caporaso *et al.,* 2010).

Yeast isolation.

Insects (4 larvae reared on artificial diet, 3 pupae reared on artificial diet, 10 adults reared on fruits and 6 adults reared on artificial diet) were selected for isolation trials. Larval specimens were picked up, rinsed with distilled water, then put on YM solid plates (yeast extract 3 g/L, glucose 10 g/L, peptone 5 g/L, malt extract 3 g/L, agar 20 g/L; Yarrow, 1998), supplemented with chloramphenicol (100 µg/mL) and allowed to walk for about 15 minutes, to permit the isolation of yeasts from the body surface. Pupae, which are not able to move, were placed on the medium and trundled manually in sterile conditions. The specimens were subsequently washed once with ethanol and then twice with deionized water. Serially dilutions were plated on the following solid media, specific for yeasts and fungi isolation, and incubated at 30°C, in aerobic conditions: YM agar (Kreisel and Schauer, 1987), Rose Bengal Chloramphenicol Agar (RBCA, Hamby *et al.,*

2012), GYP agar (glucose 20 g/L, yeast extract 5 g/L, peptone 5 g/L, agar 20 g/L; Barata *et al.*, 2011), Potato Dextrose Agar (PDA, Atlas, 1997). Once growth was visible, colonies were purified on solid PDA for three times. Four of the 10 adults fed on fruits were surface sterilized and vortexed three times under sterile conditions, before homogenization by grinding in 200 µl of 0.9% NaCl. Forty µl of each insect homogenate were inoculated in enrichment liquid TA2 medium, supplemented with chloramphenicol (100 µg/mL) enrichment medium II, Yamada *et al.*, 2000). Flasks were incubated at 30°C, in aerobic condition with shaking, until turbidity of the liquid media was reached. Serially dilutions were plated on MA medium, (1% D-glucose, 1% glycerol, 1% bactopeptone, 0.5% yeast extract, 1% ethanol, 1.5% agar, pH 6.8) and incubated at 30 °C, in aerobic conditions. Colonies were picked up and streaked on MA solid medium. Pure isolates were then collected and conserved at -80°C. Total DNA was extracted from the isolates with boiling lysis (Marasco *et al.*, 2013) and cetyltrimethylammonium bromide (CTAB) method (Polo *et al.*, 2010) and stored at – 20 °C.

Yeast collection analysis by Restriction Fragment Length Polymorphism (RFLP).

The 5.8rRNA gene and two sideward regions ITS1 and ITS2 of the ribosomal RNA-encoding DNA region of the 237 isolates was amplified using primers ITS1F and ITS4 (Manter and Vivanco, 2007). The amplification reaction was conducted in 50 µL volume containing 1 µL of DNA, 1X buffer (Invitrogen), 1.8 mM MgCl2, 200 µM dNTPs, 0.5 µM of primers, 2 U Taq DNA Polymerase (Invitrogen) in a PCR Thermocycler (BioRad, Milano). The thermal protocol was constituted by an initial denaturation of 94^{o}C for 7 min, 30 cycles constituted by a denaturation step of 94^{o}C for 45 s, followed by an annealing step of 55^{o}C for 45 s, 72^{o}C for 1 min extension, and a final extension step at 72^{o}C for 10 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis at 100V in 0.5X TBE buffer (Tris 1M, boric acid 13.7 g, EDTA 0.5 M pH 8). The ITS1-5.8S-ITS2 fragment (50 ng) was digested with 10 U of HaeIII (SibEnzyme Ltd.) and TaqI (Fermentas, Life sciences), and 20 U of HinfI (SibEnzyme Ltd.) in a 20 µL reaction volume at 37^{o}C as indicated by the manufacturer. The restriction patterns of the rRNA digests were analyzed by electrophoresis on 2.0% (w/v) agarose gel in 1X TBE at 100V and fragment sizes were determined by comparison to a DNA molecular marker 50 bp (O' Range Ruler, Fermentas). The gels were stained in ethidium bromide 0.5 mM and then washed in distilled water. Gel images were captured with GelDoc 2000 apparatus (Bio-Rad, Milan, Italy) using the Quantity one software (Bio-Rad).

Sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA

A selection of isolates representing each PCR-RFLP profile was chosen for sequencing analyses. Amplification of the D1/D2 loop of the 26S rRNA region was performed using NL1 and NL4 primers according to Kurtzman and Robnett (1998). The amplified fragments were delivered to Macrogen (South Korea) for purification and sequencing. The obtained sequences were compared to the databases at the NCBI (http://www.ncbi.nlm.nih.gov/) using BLASTn (Altshul *et al.*, 1990) and at CBS-KNAW database (http://www.cbs.knaw.nl/Collections/Biolomics.aspx?Table=CBS+strain+database).

Phylogenetic tree

ITS1-5.8S-ITS2 and D1/D2 sequences of the representative isolates of each RFLP profile were subjected to alignment, together with the relative species sequences retrieved from GenBank, with BioEdit Sequence Alignment Editor, version 7.0.0 (Hall T.A., 1999). Portions with uncertain alignment were removed. A maximum likelihood criterion for ITS and 26S multiple sequences alignment was conducted with MEGA6 phylogenetic software (Tamura *et al.*, 2013) and phylogenetic relatedness among taxa was constructed by using the Jukes-Cantor evolutionary model. Bootstrap support values >50% for 1000 replications were indicated at the node of each branch.

RESULTS

Denaturing Gradient Gel Electrophoresis and Pyrosequencing analyses of D. suzukii-associated yeast community. The yeast community diversity in *D. suzukii* was surveyed by investigating the diversity of ITS1-5.8S-ITS2 region. The DNA extracted from 33 *D. suzukii*

individuals of different ages (larvae, pupae and adults), fed on two different food sources (fruit and artificial diet), was submitted to analysis of the yeast community by DGGE-PCR, amplifing the ITS1-5.8S-ITS2 region of the rRNA fungal operon (Schoch *et al.*, 2012). An example of DGGE profile gel, in 7% polyacrylamide gels with 30% to 45% denaturation gradient, is represented in Fig. 1. The sequences obtained from the excised bands are presented in table 1, along with their closest relatives found in the NCBI database. A composite and variable community profile, together with the lack of conserved bands among samples of the same type, can be observed. In addition to this, many sequences showed a low percentage of similarity with sequences in Genbank database, which enable to assign them just to the family level (Dipodascadaceae and Saccharomycetae families) or to Saccharomycetale order. Fig. 2 gives an overview of DGGE total results: for both the samples reared on fruits and on artificial diet, the majority of sequences showed close similarity with *Pichia cecembensis* and *Candida inconspicua* (BLAST analysis provides the same score for these two species, preventing the unambiguous attribution of the species to the bands of interest). Samples reared on fruits are also characterized by the abundance of sequences belonging to the *Geotrichum candidum* and Dipodascadaceae family, which is a result shared only partially with the specimens reared on the artificial diet: they indeed show an abundance of sequences close to the Dipodascadaceae family and Saccharomycetales order. In addition, data in Fig. 2 show the greater diversity in larval individuals, reared on both food sources, in comparison to the other two life stages (pupae and adults). In particular, larvae from artificial diet showed the presence of bands assigned to species *Pichia occidentalis*, *Meyerozyma caribbica* and *Fusarium solani*, not detected in the other samples.

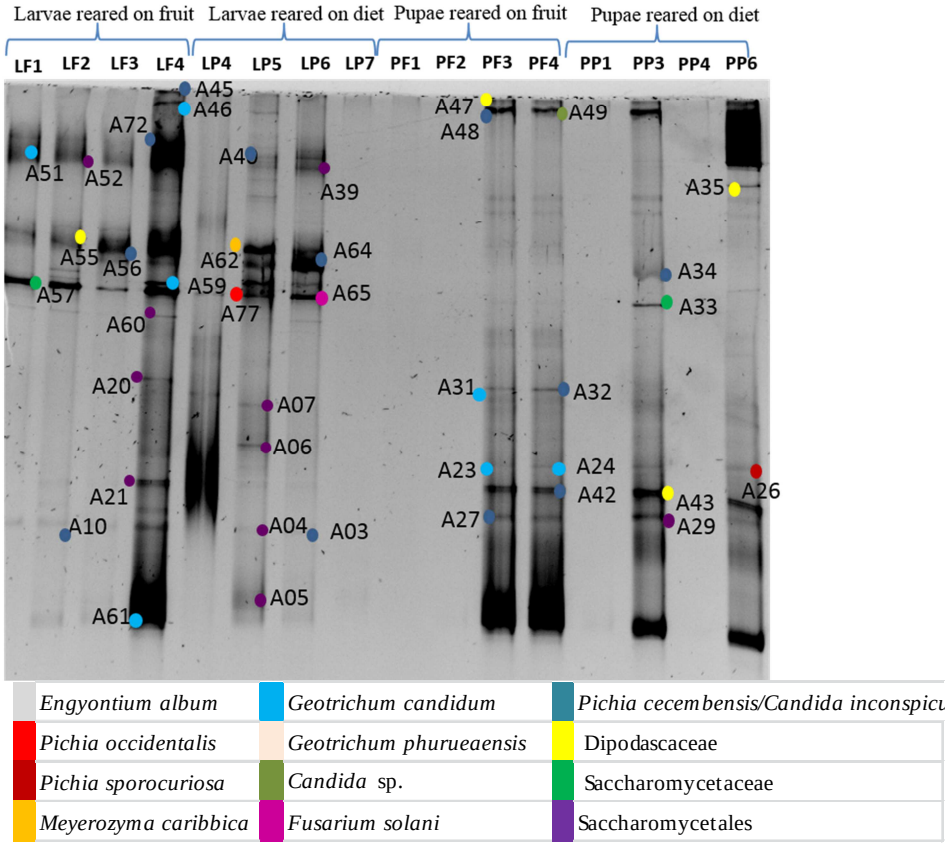

***Figure 1*:** Yeast diversity associated with *D. suzukii*. Representative DGGE gel, in 7% polyacrylamide gels along a denaturing gradient, of the ITS1-5.8S-ITS2 fungal rRNA fragment. Numbers above the lanes refer to individuals: LF, larvae reared on fruit; LP, larvae reared on artificial diet; PF, pupae reared on fruit; PP pupae reared on artificial diet. Bands marked with coloured dots were sequenced; data referring to the sequences are given in **Table 1.**

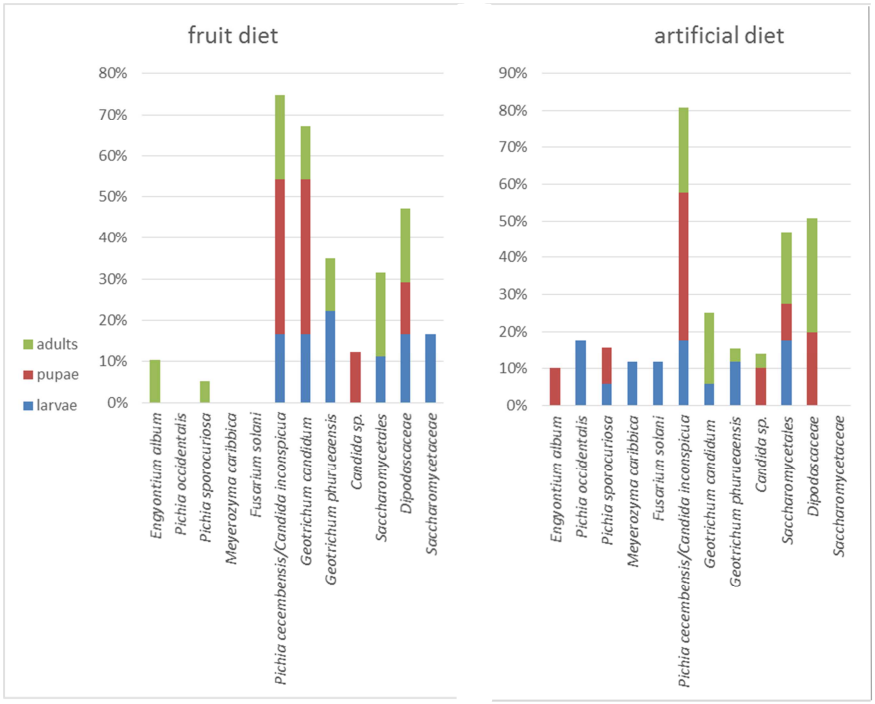

Figure 2: Identification and distribution of DNA fragments excised from DGGE, according to the developmental stage (larva, pupa and adult) and diet (fruits or artificial diet).

The comparison of the yeast community composition in individuals of different age, fed on two different aliments, was performed by PERMANOVA statistical analysis. The analysis did not show a significant statistical difference in the distribution of the yeast community, considering the effect of the diet ($p>0.05$). On the other hand, the developmental stage was identified to be a factor having an influence in the community composition ($p=0.01$). Furthermore, the effects of the diet type on the different stages confirmed the non-statistically difference among sample groups ($p>0.05$).

Table 1: Identification of the excised and sequenced bands in the PCR-DGGE fingerprint profiles (marked in table1

Band	Closest described relative	GenBank Accession no.	% nt identity*	Most related species	Putative classification	Lane**
A03	Saccharomycetes sp.	EU315761	99%(332/333)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LP6
A04	Saccharomycetes sp.	EU315762	92%(207/225)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LP5
A05	*Geotrichum phurueaensis*	HE663403	81%(278/344)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LP5
A06	*Geotrichum candidum*	KF112070	89%(311/351)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LP5
A07	*Pichia fermentans*	KC510080	100%(23/23)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LP5
A10	Saccharomycetes sp.	EU315761	99%(430/432)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LF2
A32	Saccharomycetes sp.	EU315761	97%(418/432)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	PF4
A20	*Geotrichum phurueaensis*	HE663403	84%(280/333)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LF4
A21	*Pichia misumaiensis*	FR774550	90%(73/81)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LF4
A23	*Geotrichum candidum*	KF112070	97%(335/347)	*Geotrichum candidum*	Saccharomycetes, Dipodascaceae	PF3
A24	*Geotrichum candidum*	KF112070	99%(304/305)	*Geotrichum candidum*	Saccharomycetes, Dipodascaceae	PF4
A27	Saccharomycetes sp.	EU315761	99%(423/425)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	PF3
A29	*Schizoblastosporion starkeyihenricii*	HF558658	76%(305/400)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	PP2
A31	*Geotrichum candidum*	KC816559	96%(358/371)	*Geotrichum candidum*	Saccharomycetes, Dipodascaceae	PF3

A33	*Candida californica*	JX188104	93%(375/404)	Saccharomycetaceae family	Saccharomycetes, Saccharomycetacae	PP2
A34	Saccharomycetes sp.	KJ535099	95%(365/383)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	PP2
A39	*Galactomyces geotrichum*	JQ668739	88%(314/358)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LP6
A40	Saccharomycetes sp.	KJ535099	100%(238/238)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LP5
A42	Saccharomycete sp.	EU315761	99%(423/426)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	PF4
A43	*Galactomyces* sp.	JQ437602	95%(321/338)	Dipodascaceae family	Saccharomycetes, Dipodascaceae	PP2
A45	Saccharomycetes sp.	KJ535099	98%(370/376)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LF4
A46	*Geotrichum candidum*	KF112070	99%(369/371)	*Geotrichum candidum*	Saccharomycetes, Dipodascaceae	LF4
A47	*Galactomyces* sp.	JQ437602	91%(287/317)	Dipodascaceae family	Saccharomycetes, Dipodascaceae	PF3
A48	Saccharomycetes sp.	KJ535099	100%(251/251)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	PF3
A49	*Candida californica*	JX188104	95%(398/418)	*Candida* sp.	Saccharomycetes, Saccharomycetacae	PF4
A26	*Pichia occidentalis*	KJ535099	100%(270/274)	*Pichia occidentalis*	Saccharomycetes, Saccharomycetacae	PP6
A51	*Geotrichum candidum*	KF975700	97%(300/308)	*Geotrichum candidum*	Saccharomycetes, Dipodascaceae	LF1
A52	*Galactomyces* sp.	JQ437602	80%(273/340)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LF2
A35	*Galactomyces* sp.	KF768298	99%(339/341)	Dipodascaceae family	Saccharomycetes, Dipodascaceae	PP6
A55	*Galactomyces* sp. *Geotrichum candidum*	KF112070	100%(347/347)	Dipodascaceae family	Saccharomycetes, Dipodascaceae	LF2

A56	Saccharomycetes sp.	KJ535099	100%(237/237)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LF3
A57	*Candida diversa*	FR819717	94%(347/370)	Saccharomycetaceae family	Saccharomycetes, Saccharomycetacae	LF1
A59	Uncultured *Galactomyces* sp.	KF768298	97%(347/359)	*Geotrichum candidum*	Saccharomycetes, Dipodascaceae	LF4
A60	*Geotrichum phurueaensis*	HE663403	82%(286/347)	Saccharomycetales order	Saccharomycetes, Saccharomycetacae	LF4
A62	*Meyerozyma caribbica*	KF728801	99%(524/528)	*Meyerozyma caribbica*	Saccharomycetes, Debaryomycetaceae	LP5
A64	Saccharomycetes sp.	EU315761	99%(399/402)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LP6
A65	*Fusarium solani*	EU315761	99%(546/549)	*Fusarium solani*	Sordaryomycetes, Nectriaceae	LP6
A72	Saccharomycetes sp.	EU315761	99%(408/413)	*Pichia cecembensis*/ *Candida inconspicua*	Saccharomycetes, Saccharomycetacae	LF4
A61	*Geotrichum candidum*	KF112070	100%(360/360)	*Geotrichum candidum*	Saccharomycetes, Dipodascaceae	LF4
A77	Saccharomycetes sp.	EU315761	99%(332/335)	*Pichia occidentalis*	Saccharomycetes, Saccharomycetacae	LP5

* In brackets: no. of identical bp/total no. of bp.** Sample names refer to the tested individuals: LF, larvae reared on fruit; LP, larvae reared on artificial diet; PF, pupae reared on fruit; PP pupae reared on artificial diet.

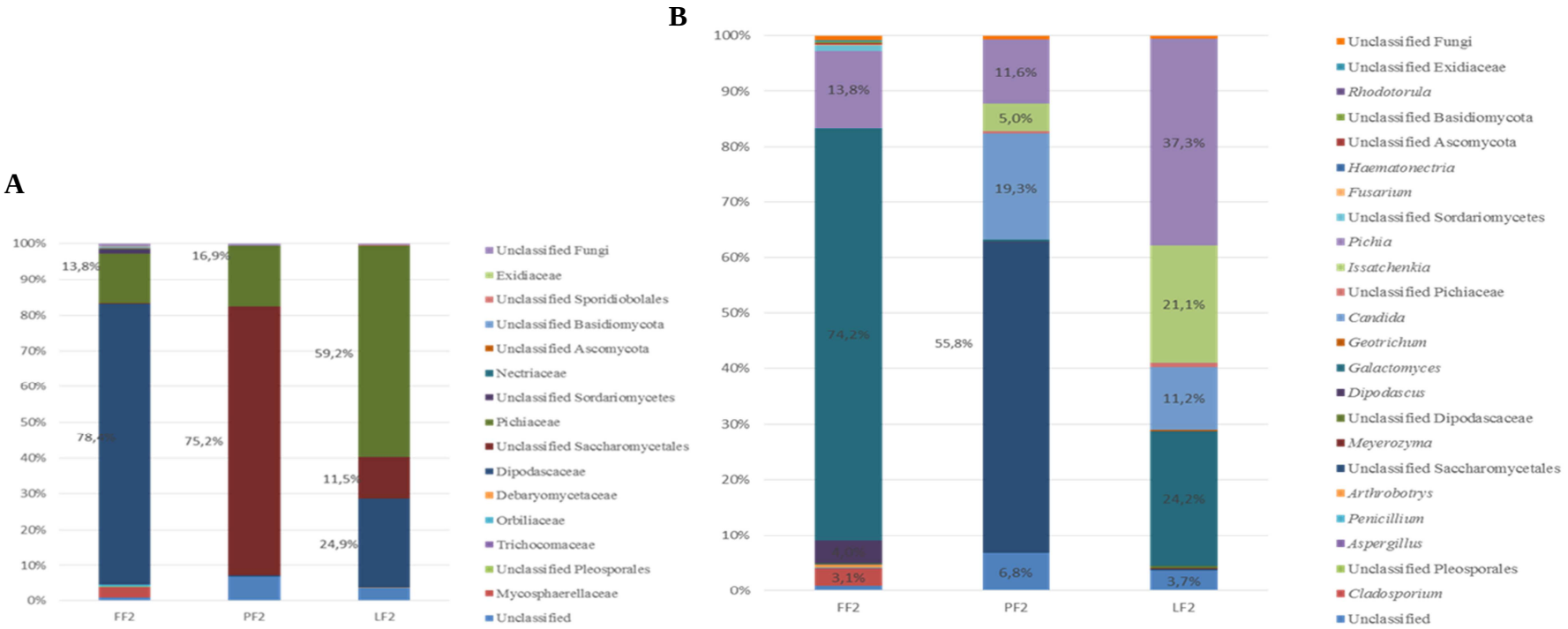

Figure 3: Identification of yeasts, at family (A) and genus (B) level, associated with *D. suzukii*, according to pyrosequencing data. Names refer to fly specimens submitted to DNA extraction and ITS1-5.8S-ITS2 fungal rRNA amplification. In ordinate, the relative abundances in percentages of the families and genera identified.

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Table 2: Relative sequence percentages at family (A) and genus (B) levels, retrieved from barcoding analyses, with the colour key for **figure 3** results.

A.

	FF2	PF2	LF2
Unclassified	0,9%	6,8%	3,7%
Mycosphaerellaceae	3,1%	0,0%	0,0%
Unclassified Pleosporales	0,0%	0,0%	0,0%
Trichocomaceae	0,1%	0,0%	0,0%
Orbiliaceae	0,5%	0,0%	0,0%
Debaryomycetaceae	0,1%	0,0%	0,1%
Dipodascaceae	78,4%	0,4%	24,9%
Unclassified Saccharomycetales	0,3%	75,2%	11,5%
Pichiaceae	13,8%	16,9%	59,2%
Unclassified Sordariomycetes	1,0%	0,0%	0,0%
Nectriaceae	0,2%	0,0%	0,0%
Unclassified Ascomycota	0,2%	0,0%	0,1%
Unclassified Basidiomycota	0,3%	0,0%	0,0%
Unclassified Sporidiobolales	0,1%	0,0%	0,0%
Exidiaceae	0,2%	0,0%	0,0%
Unclassified Fungi	0,8%	0,7%	0,5%
	100%	100%	100%

B.

	FF2	PF2	LF2
Unclassified	0,9%	6,8%	3,7%
Cladosporium	3,1%	0,0%	0,0%
Unclassified Pleosporales	0,0%	0,0%	0,0%
Aspergillus	0,0%	0,0%	0,0%
Penicillium	0,0%	0,0%	0,0%
Arthrobotrys	0,5%	0,0%	0,0%
Unclassified Saccharomycetales	0,1%	**55,8%**	0,3%
Meyerozyma	0,1%	0,0%	0,1%
Unclassified Dipodascaceae	0,2%	0,0%	0,4%
Dipodascus	4,0%	0,0%	0,0%
Galactomyces	**74,2%**	0,4%	**24,2%**
Geotrichum	0,0%	0,0%	0,3%
Candida	0,1%	**19,3%**	**11,2%**
Unclassified Pichiaceae	0,0%	0,3%	0,8%
Issatchenkia	0,0%	**5,0%**	**21,1%**
Pichia	**13,8%**	**11,6%**	**37,3%**
Unclassified Sordariomycetes	1,0%	0,0%	0,0%
Fusarium	0,2%	0,0%	0,0%
Haematonectria	0,1%	0,0%	0,0%
Unclassified Ascomycota	0,2%	0,0%	0,1%
Unclassified Basidiomycota	0,3%	0,0%	0,0%
Rhodotorula	0,1%	0,0%	0,0%
Unclassified Exidiaceae	0,2%	0,0%	0,0%
Unclassified Fungi	0,8%	0,7%	0,5%
	100%	100%	100%

Since no statistical differences were retrieved according to the diet administered to the insects, three samples of DNA, extracted from a larva, a pupa and an adult, respectively, and reared on fruits, named LF2, PF2 and FF2, were submitted to barcoding analyses, by choosing as target of amplification the variable region ITS1-5.8S-ITS2 of the fungal rRNA operon. In table 2 the sequences obtained from the analyses, at family and genus level are listed, respectively, whereas figure 3 shows a representation of those genera and families with high similarity with sequences obtained from each sample.

Overall, ITS1-5.8S-ITS2 barcoding showed that Ascomycota is the predominant yeast phylum (97.7%, Basidiomycota). Among this phylum, family Dipodascaceae accounted for 78.4% of the total abundance in the adult sample (FF2), together with Pichiaceae (13.8%). Pichiaceae family was the most abundant component (59.2%) in the larval individual (LF2), which was also dominated by Dipodascaceae (24.9%) and by a group of yeasts, whose sequences were classified at the order level (Saccharomycetales, *Incertae sedis* 11.5%). Pupa sample (PF2) was massively dominated by this group (75.2%) and by Pichiaceae (16.9%), while for the remaining sequences the assignment to a taxonomic category was not possible. Thus, the overall yeast community of the individual was dominated by sequences belonging to the Pichiaceae and Dipodascaceae families, and the Saccharomycetales order.

By looking at the genus level, the adult sample was characterized by the prevalence of *Galactomyces* sequences (74.2%) which constituted, together with 4.0% of *Dipodascus*, the two genera of the Dipodascaceae family present in the community of this sample. *Pichia* genus, which constituted the 13.8% out of the total reads, represented the only genus of the Pichiaceae family in this individual, which included also some reads belonging to *Cladosporium* genus (3.1%). Figure 3B shows for PF2 the distribution of the yeast community: *Pichia* and *Issatchenkia* (11.6% and 5.0%, respectively of the total number of sequences) were the representative genera for the Pichiaceae family, while *Candida* (19.3%) and the abundant fraction of not-classified Saccharomycetales (55.8%) constituted the majority of sequences. Finally, the sample LF2 is characterized by the following distribution of genera: 24.2% out of the total sequences belonged to *Galactomyces*, 11.2% to *Candida*, 37.3% to *Pichia* and 21.1% to *Issatchenkia*.

Yeast isolates classification

In addition to the screening of *D. suzukii*-associated yeast community with molecular methods, yeast biodiversity was surveyed with cultivation-dependent techniques. As assumed in previous studies (Staubach *et al.*, 2013), the total microorganisms associated with the whole fly body were taken into account, not focusing just on the intestinal tract, because the community associated to the fly surface might have an important function in the host biology. One enrichment liquid medium (enrichment medium II, Yamada *et al.* 2000), and four different and selected solid media, specific for yeasts and fungi isolation (YM agar, Kreisel and Schauer 1987; RBCA, Hamby *et al.*, 2012; GYP agar, Barata *et al.*, 2011 and PDA, Atlas, 1997) permitted to perform isolation trials. Select yeast colonies with different morphologies were then purified. Two-hundreds and thirty-seven yeast isolates was submitted to DNA extraction with boiling lysis, or otherwise with cetyltrimethylammonium bromide (CTAB) method when the fast approach (boiling lysis) did not succeed. This was probably due to the fact that some yeast isolates are characterized by a thick cell wall that requires a more sophisticated extraction approach, or the presence of some inhibition products after the extraction with boiling lysis (Polo *et al.*, 2010). After this step, DNA was stored at –20 °C.

Molecular characterization was accomplished using a combination of different techniques for the identification of the isolates. To reduce the genotypic redundancy of the collection, analyses were performed by using RFLP on the ITS1-5.8S-ITS2 region, through HaeIII, HinfI and TaqI endonucleases.

Eighteen different profiles, named from A to R were generated (Table 3). To confirm the profiles, full-length ITS1-5.8S-ITS2 sequences of the identified species in the collection were recovered from NCBI (http://www.ncbi.nlm.nih.gov/) and Using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) *in silico* restriction digestion of the ITS1-5.8S-ITS2 sequences was performed with HaeIII, HinfI for all the species, and in addition with TaqI for *Meyerozyma* group. Predicted band sizes, obtained from available sequence data, were comparable to band sizes observed from gel runs, except from restricted bands smaller than 20 bp.

Table 3: Restriction analysis profiles of 237 yeasts according to the different fragment sizes determined by restrictions with endonucleases HaeIII, HinfI, TaqI. Sequences information obtained by amplification of the ITS1/ITS2 and D1/D2 domain of representative strains are reported (sequence identity and closest relative species in the NCBI GenBank database).

Group	PCR sizes	*HaeIII*	*HinfI*	TaqI	Isolate No.	Acc. No. 26S*	Acc. No. ITS*	Species
1(A)	500 bp	350+90+60	250+140+110	-	93	EU315761 (99%)	AB847526 (100%)	*Pichia occidentalis*
2(B)	480 bp	340+140	240+240	-	2	FJ153178 (99%)	JN377463 (100%)	*Pichia sporocuriosa*
3(C)	500 bp	360+90+50	260+240	-	4	DQ198954 (99%)	DQ198955 (99%)	*Pichia membranifaciens*
4(D)	450 bp	450	300+150	-	4	FJ713025 (100%)	FJ527046 (100%)	*Candidaapicola*
5(E)	500 bp	500	260+240	-	5	KC544509 (100%)	HE657235 (100%)	*Starmerella bacillaris*
6(F)	690bp	650+40	350+220+120	-	13	HM450996 (100%)	AB719452 (100%)	*Zygoascus meyerae*
7(G)	720 bp	720	350+370	-	22	AF411061 (100%)	KF738162 (100%)	*Saccharomycopsis craetegensis*
8(H)	750 bp	650+100	350+400	-	29	FN868151 (100%)	FN868151 (100%)	*Arthroascus schoenii*
9(I)	460 bp	460	260+110+90	-	1	JN226397 (95%)	KF052126 (100%)	*Galactomyces* sp.
10(J)	650 bp	300+220+130	300+250+100	-	12	AF411061 (100%)	KF738162 (100%)	*S. craetegensis*
11(K)	600bp	600	310+290	-	1	FM199968 (98%)	JF749211 (100%)	*Candida stellimalicola*
12(L)	650 bp	400+150+100	330+320	250+180+130+50	14	KC111450 (99%)	AB863536 (99%)	*Meyerozyma caribbica*
13(M)	750 bp	750	350+230+170	-	6	FJ515178 (99%)/ FM199954 (99%)	JX188159 (99%) FM199954 (100%)	*Hanseniaspora uvarum/ opuntiae*
14(N)	800 bp	600+140+60	350+240+160+50	-	21	DQ872858 (98%)	JX458121 (100%)	*Zygosaccharomyces bailii*
15(O)	650 bp	650	350+300	-	1	KF738162 (100%)	AF411061 (87%)	*Saccharomycopsis* sp.
16(P)	700 bp	550+120+30	370+330	-	2	FN868151 (100%)	FN868151 (100%)	*A. schoenii*
17(Q)	650 bp	570+80	350+300	-	1	AF411061(100%)	AF411061 (100%)	*S. craetegensis*
18(R)	650 bp	400+150+100	330+320	250+180+120+50+50	4	KF619551 (99%)	AB568370 (100%)	*M. guilliermondii*

*In brackets are reported the percentage of sequence similarities.

After the analysis of the generated restriction patterns, selected isolates representing each RFLP profile group were chosen for sequencing analyses of the D1/D2 domain of the 26S rRNA gene and of the polymorphic ITS1/ITS2 region. Table 3 showed the identification of the selected strains; corresponding GenBank accession numbers are provided. Ninety-three isolates showed the restriction profile named A, corresponding to the profile of *Pichia occidentalis* (type strain), and were assigned to this species. The restriction profile B generated for 2 isolates was applied the *Pichia sporocuriosa* species group, while the 4 isolates showing the RFLP profile C had close sequence similarity with *Pichia membranifaciens*. *Candida apicola*, *Starmerella bacillaris* and *Zygoascus meyerae* species were assigned to D (4 isolates), E (5 isolates) and F (13 isolates) profiles, respectively. The restriction profiles G, J, O, Q, to whom 22, 12, 1, 1 isolates were attributed, were assigned to *Saccharomycopsis craetegensis* species. Similarly, profiles H (29

Table 4: Summary of total yeasts isolated from each individual of *D. suzukii* larvae, pupae and adults.

Species identification	No. of isolates		Larvae			Pupae			Adult artificial diet (AP)						Adult fruit fed (AF)							
			L1	L2	L3	P1	P2	P3	AP1	AP2	AP3	AP4	AP5	AP6	AF1	AF2	AF3	AF4	AF5	AF6	DS2M	DS2F	DS3F
Pichia occidentalis	93	39%	1	11	13	11	10	6	6	4	3	4	11	0	5	4	0	1	1	2	0	0	0
Pichia membranifaciens	4	2%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	1	0	0	0
Pichia sporocuriosa	2	1%	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
Candida apicola	4	2%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	0	0	0
Candida stellimalicola	3	1%	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Saccharomycopsis craetegensis	36	15%	1	3	1	3	2	7	0	1	0	1	0	1	3	2	2	3	5	1	0	0	0
Arthroascus schoenii	31	13%	0	0	1	3	5	0	3	5	1	7	3	1	1	1	0	0	0	0	0	0	0
Zygoascus meyerae	13	5%	0	0	0	0	1	0	0	0	0	0	0	0	1	2	3	2	4	0	0	0	0
Galactomyces sp.	1	0%	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Meyerozyma caribbica	14	6%	1	1	0	0	0	1	0	0	0	0	0	0	2	4	3	1	1	0	0	0	0
Meyerozyma guilliermondii	4	2%	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0
Hanseniaspora uvarum	3	1%	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
Hanseniaspora opuntiae	3	1%	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0
Starmerella bacillaris	5	2%	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	0	0	0
Zygosaccharomyces bailii	21	9%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	2	11
	237	100%	4	16	15	17	18	14	9	10	4	14	15	2	17	15	15	9	15	7	8	2	11
			35			49			54						99								

isolates), and P (2 isolates) corresponded to *Arthorascus schoenii* and were assigned to this species. The only isolate with RFLP profile I showed close sequence similarity with *Galactomyces* sp. species, and was assigned to it, while the sole isolates identified as *Candida stellimalicola* belonged to the K profile group. *Zygosaccharomyces bailii* is the species attributed to the isolates having a RFLP pattern categorized as N. 6 isolates showed the restriction profile named M, which was identical for the species *Hanseniaspora uvarum* and *H. opuntiae*. Distinction was made possible with the sequencing of the ITS region. Then, the differentiation of the profiles for the *Meyerozima caribbica/guilliermondi* group, otherwise identical, was permitted when the digestion with TaqI endonuclease of the amplified fragments under investigation was done (Romi *et al.*, 2014). The generated RFLP profiles, named L (14 isolates) and R (4 isolates), were assigned to *M. caribbica* and *M. guilliermondii*, respectively. Identified species belonging to the *Candida* genus and *Hanseniaspora uvarum*/*opuntiae* complex are characterized to lack restriction sites with HaeIII enzyme, as already reported in literature (Pham *et al.*, 2011).

The phylogenetic identification of the yeast isolates underlined the diversity of the community structure of the different categories of samples, which are represented in table 4 and in figure 4. They showed the predominance (39% out of the total number of the strains) of *P. occidentalis* (Tab. 4); this species was surveyed in all larval and pupal samples, representing the 71 % and 55% of the total collection, respectively, in 5 of the 6 adults reared on artificial food (52%), and 5 of 9 adults fed on fruits (13%). Isolates belonging to *S. craetegensis* and *A. schoenii* species constituted the 15% and 13% of the collection, respectively (Tab. 4). These three species were the only species present in all the four categories of samples, showing to be ubiquitous across all sample types (Fig. 4).

Larvae harbored low percentages of isolates affiliated to *C. stellimalicola*

(3%), *M. caribbica* and *M. guilliermondii* (6% and 3% respectively; Fig. 4-1). Pupae showed, besides the above mentioned most abundant species (*P. occidentalis, S. craetegensis* and *A. schoenii*), the presence of *M. caribbica* and *Z. meyerae* in small proportion (2% each; Fig. 4-2).
Adults fed on artificial diet exhibited a yeast community profile constituted mainly by *P. occidentalis* and *A. schoenii* (52% and 37%). The latter species was isolated from all the individuals. All other species were found in small amount, and were assigned to *Galactomyces* sp. (the only isolate of the collection), *C. stellimalicola* and *M. guilliermondii* (2% each). *S. craetegensis* constituted 5% of the isolates obtained from adult fed on the artificial diet (Fig. 4-3).
S. craetegensis was the second most prevalent yeast isolated from adults reared on fruits (16%). In these individuals, a wider diversity of species was detected. *Z. bailii* was the only species detected from three flies that were isolated on enrichment liquid TA2 medium, and constitutes the 21% of the isolates from fruit-fed adults. Several species were characterized and found to be present in similar proportions; in particular, 12% of the isolates were sequenced and assigned to the genus *Z. meyerae*, followed by *P. occidentalis* (13%) and *M. caribbica* (11%). Flies reared on fruits were also characterized to harbor species showing affiliation with several Saccharomycetaceae, i.e. *P. membranifaciens* (4%), *P. sporocuriosa* (2%), *H. uvarum* and *H. opuntiae* (3% each), *C. apicola* (4%) and *C. stellimalicola* (1%)
while, among Debaryomycetaceae, the species complex of the already mentioned *M. caribbica* and *M. guilliermondii* (2%) can be identified (Fig. 4-4).

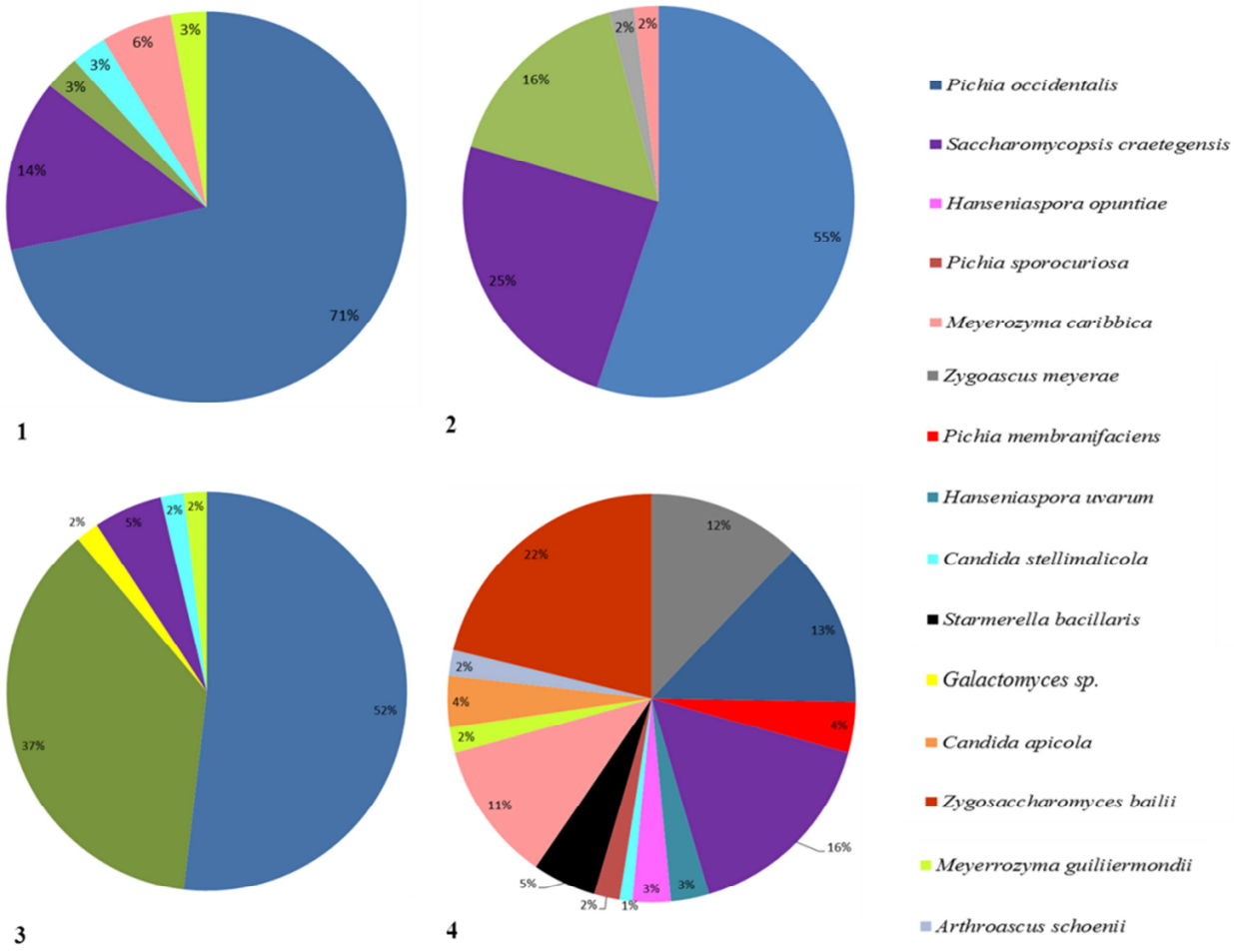
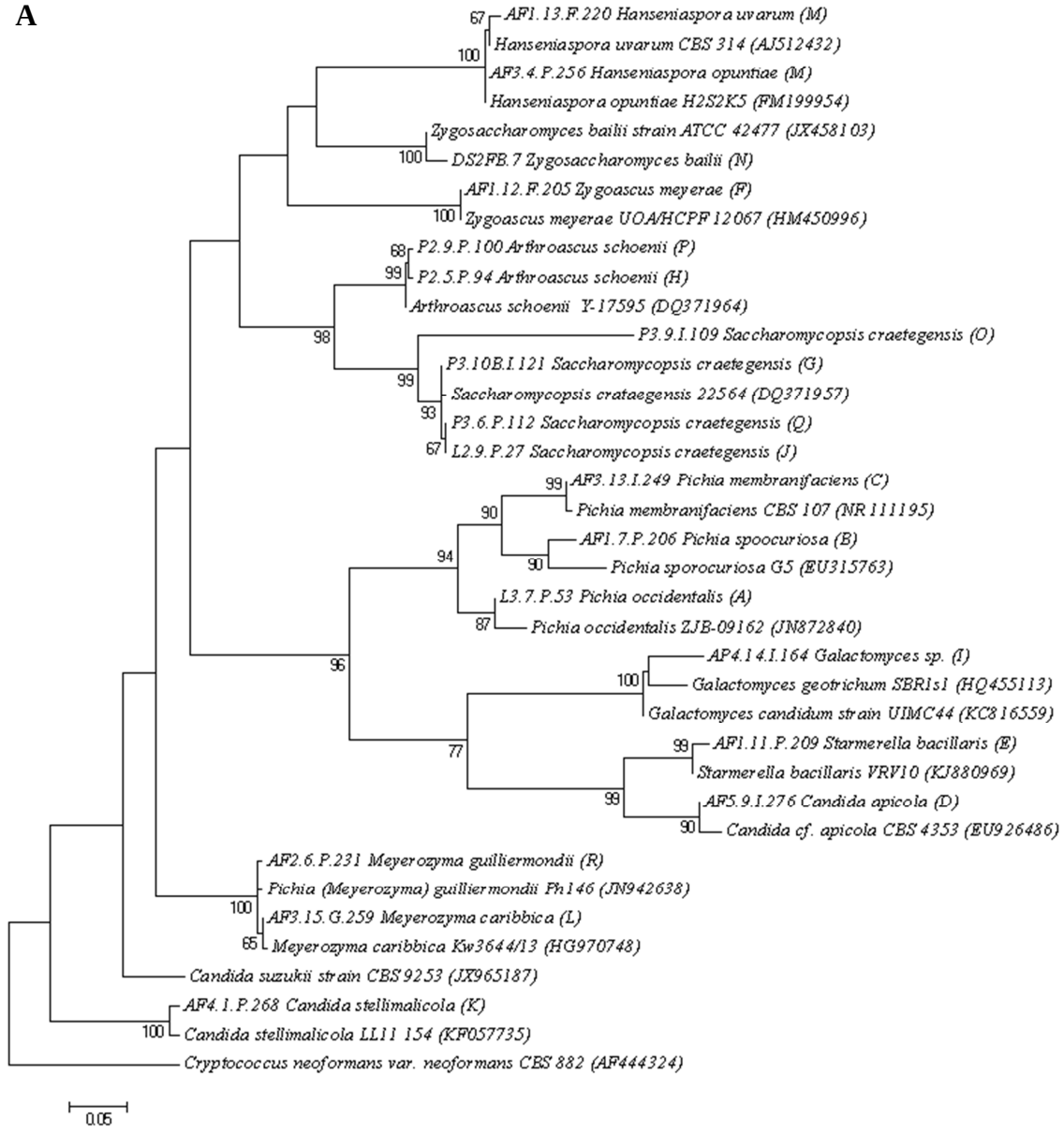


Figure 4: Graphs representing the distribution and diversity of the yeast collection according the sample origin. (1) Larvae, (2) pupae and (3) adults reared on artificial diet, (4) adults reared on fruits.

Phylogenetic analyses

Phylogenetic trees obtained from the analyses on the ITS region and D1/D2 domain of LSU rRNA gene are shown in figure 5 (A and B, respectively). Separation both by ITS and 26S sequences succeeded with high bootstrap replication, and LSU gene and the ITS gene sequences give a similar resolution taxa, as the dendogram topologies derived from the two sets of sequences are highly similar. In comparison to the 26S tree, ITS sequences were more informative and allowed a better separation of the *Hanseniaspora* species group. The long branch observed for P3.9.I.109 *Saccharomycopsis craetegensis* (RFLP profile: O) was probably due to the short ITS sequence of the isolate, the sole representative of the RFLP profile "O".

A



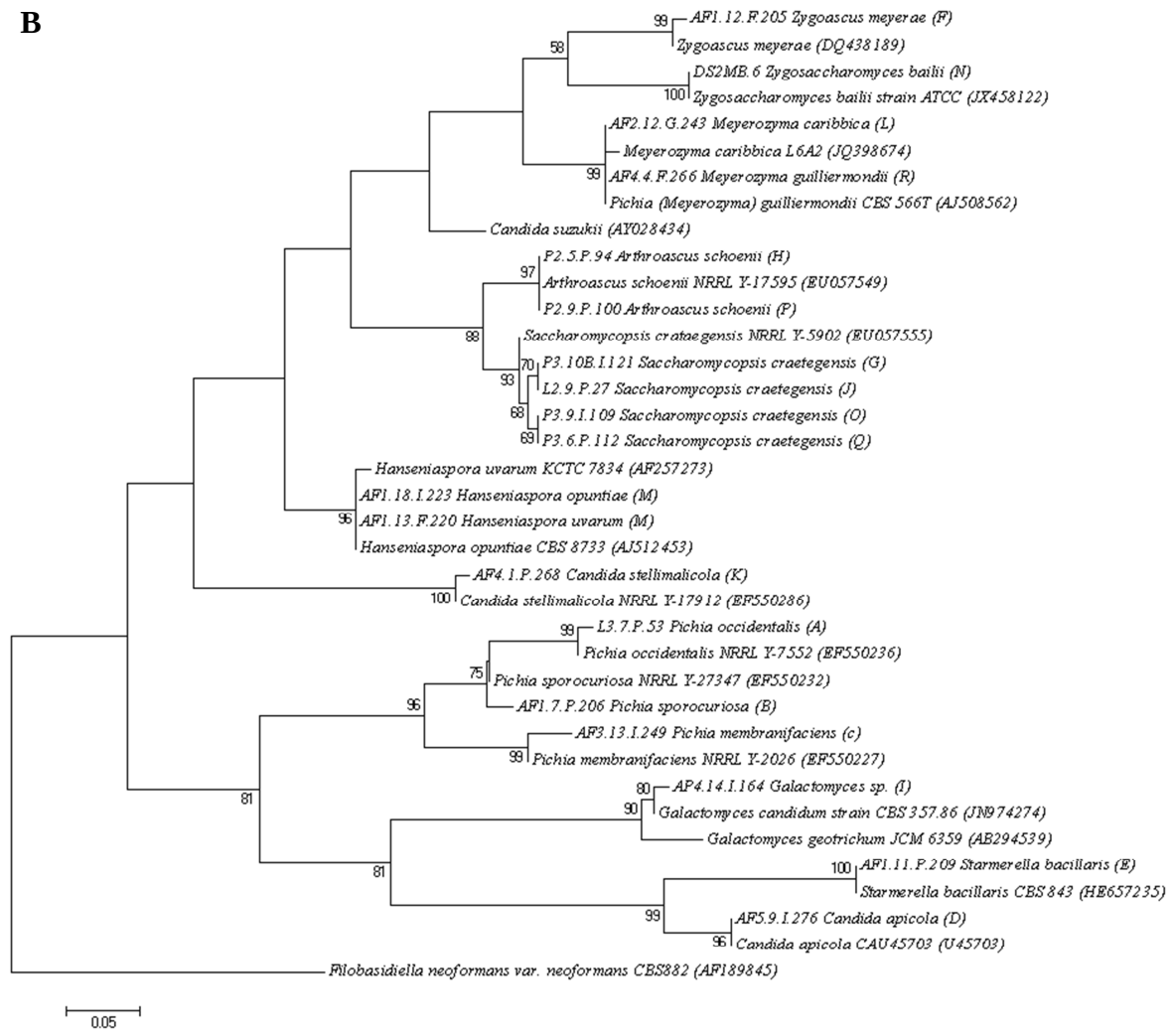


Figure 5: Maximum likelihood phylogenetic trees inferred from ITS (A) and D1/D2 domain (B) sequences of the representative isolates for each RFLP profile. Numbers at nodes represent bootstrap values determined for 1000 iterations and are indicated when values were >50%. Reference species names are followed by the GenBank accession numbers, in parentheses. Collection species names are preceded by the isolate code and followed by the letter referred to the RFLP profile (in parentheses). The scale bar shows the degree of sequence divergence (0.05 substitutions per nucleotide position). The species used as outgroup in each tree belong to Basidiomycota phylum.

DISCUSSION

Yeast microbiome analysis here presented provides an overview of the community associated to the insect pest *D. suzukii* by means of culture-dependent and -independent methods, with the aim to enlarge the background information regarding this pest. Yeasts of the Ascomycota phylum constitute a monophyletic lineage. The shared character of this group is the *ascus*, the structure in which the nuclear fusion and meiosis take place. These yeasts play an important role for industrial and biotechnological processes (baking, brewing, and synthesis of recombinant proteins). They are ecologically different in comparison to basidiomycetes, their sister group, as they are often found in specialized niches, characterized by the presence of a liquid interface and high carbon content. The possibility to live in these environments characteristics require the interactions with superior organisms, *i.e.* plants and animals and in particular invertebrates, upon which they rely for dispersal (Suh *et al.*, 2006). They can act as mutualists, providing reciprocal advantages (Becher *et al.*, 2012). For instance, fruit flies of the genus *Drosophila* are able to disseminate non-motile yeasts, in particular during courting and mating between males and females, and then on fruits during oviposition (Becher *et al.*, 2012).

In this work, culture-independent techniques, DGGE and ITS barcoding, were applied to describe the overall yeast community associated with *D. suzukii*. These two molecular approaches provide the evidence of the stable association of flies with yeasts belonging, in particular, to the Saccharomycetales order: in fact, the microorganisms of this taxonomic group were constantly found in the different categories of specimens. Limitation of the techniques adopted, e.g. the impossibility of species quantification in PCR-DGGE technique, or the number of samples submitted to the barcoding analyses, did not allow to have a complete overview of the community composition. Moreover, PCR-based results can suffer in some cases from underestimation, due to the preferential amplification of some groups (Suzuki and Giovannoni, 1996). In detail, PCR-DGGE analyses provided picture of a yeast community slightly different among insects of different developmental stages, but not among insects reared on the two different diets, as confirmed by statistical analyses.

By using culture-dependent methods, a collection of yeasts was obtained. Many of the species found in the isolation trials were not detected by DGGE analysis: we have to mention that for DGGE it was not possible to assign many sequences at the species or genus level and thus they were characterized at the family and order taxonomic range. One of the difficulties in analysing the systematics and the phylogenetic relationships of this Kingdom is the identification of closely related species, due to the necessity to find the best barcode marker or the best molecular tool to succeed. ITS was accepted as universal gene marker for fungi barcoding (Schoch *et al.*, 2012), despite the controversy of some studies suggesting the utilization of a portion of the D1-D2 region of the large subunit of the fungal rRNA gene (LSU), which is amplified by NL1 and NL4 primers (Kurtzman 2014), to reach species discrimination. Actually, since the advantage to use both D1/D2 and ITS sequences, which have a comparable species resolution, is due to a taxon-specific variation, the use of both sequences is recommended (Kurtzman, 2014). Restriction analyses of the rRNA region composed by ITS1, 5.8s rRNA gene and ITS2 in a complementary molecular method is used in support to the traditional microbiological work. Indeed, RFLP can be used in combination with ITS-PCR to differentiate closely-related species. The advantages of this method is that it can go over the limitations of the usual identification techniques and it is reproducible and easy to do. Nevertheless, it might happen that some profiles can be genus-specific: in this way, those profiles have to be verified with other approaches (Guillamon *et al.*, 1997). In the specific case of this work, almost all the isolates were distinctly differentiated by using HaeIII and Hinf endonucleases. HinfI is characterized by a higher polymorphism than HaeIII and it helped the differentiation. It was necessary to use a third enzyme, Taq I, to differentiate the isolates of the *Meyerozyma* species complex into two genotypes groups (Romi *et al.*, 2014). The yeast *M. caribbica* was indicated as a biocontrol yeast, as it competes for nutrients (sucrose and fructose mainly) that are otherwise used by the pathogen for its development; it also produces hydrolytic enzymes, and biofilm through quorum sensing (Bautista-Rosales *et al.*, 2013). Its close relative, *M. guilliermondii*, a Crabtree-negative species, was isolated from hummingbirds and nectivirous bats (Belisle *et al.*, 2014) and it was found to act as a killer yeast against *Colletotrichum gloeosporioides* (deLima *et al.*, 2013). These two species have been often found in fermented food (Romi *et al.*, 2014) and, as said above, might have a potential in the field of biological control of those fungi that spoil fruits and vegetables in postharvest stage. Moreover, phylogenetic analyses showed that ITS is an informative region for phylogenic trees construction and for differentiation among related taxa, allowing to distinguish among closely related species. In fact, data of the phylogenetic 26S tree show that *Pichia* genus is a polyphyletic group, as it is not clustered in a single branch, but it is distributed among the ascomycetous yeasts. This is in agreement with the affirmation of Kurtzman and Robnett (1998). It is interesting to note that in the phylogenetic tree constructed with the 26S sequences the *Starmerella* clade branch is longer than the branches of the other clades: the reason might be ascribed to high speciation rates of this genus, as hypothesized by Lachance and collaborators (2009).

Barcoding data showed that members of *Galactomyces*, *Candida*, *Pichia* and *Issatchenkia* genera dominate the yeast microbiome of *D. suzukii* individuals of different life stages. *Galactomyces* genus belongs to Dipodascaceae family: many sequences affiliated to this taxonomic level were recorded in DGGE, together with bands assigned to *Geotrichum candidum* and *Geotrichum phurueaensis*, from larvae, pupae and (in lower proportion) from adults. The anamorph of

Galactomyces candidus, *Geotrichum candidum*, is a common soil-borne fungus that causes sour-rot in tomatoes, citrus fruits and vegetables (Thornton *et al.*, 2010). It was isolated also from earthworms from tropical habitat (Parthasarathi *et al.*, 2007, Lachance *et al*., 2010). Interestingly, Pimenta *et al*., in 2005, isolated and characterized a close relative of the *Galactomyces* genus, *Geotrichum silvicola*, from *Drosophila* flies and oak tasar (an oriental moth that produces brownish silk) and silkworm larvae. The species *Ga. candidus* was introduced to adjust the *Ge. candidum* complex, a more common and randomly distributed group.
Other species, retrieved from barcoding analysis, are part of the Saccharomicetaceae and Pichiaceae families, which comprise well-known yeasts. These families constitute the larger part of the isolates in the collection. For instance, *C. apicola* is an asexual species (Lachance *et al.*, 2010), isolated from 2 out of 9 adults reared on fruits. It lives in association with floricolous insects, like stingless bees and endoparasitoid wasps (Lachance *et al*., 2010, Rosa *et al.*, 2003), the European hornet (*Vespa crabro*) and paper wasps (*Polistes* sp.; Stefanini *et al.*, 2012). Members of this genus contribute to the optimal development of the insect, due to their nutritional role. Starmer reported in 1982 that *Candida ingens* is able to metabolize toxic fatty acids in cactus tissues, providing a beneficial effect for *Drosophila mojavensis*, while *Candida sonorensis* and *Criptococcus cereanus* are able to process 2-propanol in decaying cactus tissues, with an advantage for *Drosophila* larvae and adults. *P. membranifaciens*, recorded in 2 out of 9 adults reared on fruits, was isolated from the gut of D*rosophila* adults (Fermaud *et al.*, 2000), from rove beetle species (Klimaszewski *et al.*, 2013) and wine environments (Saez *et al.*, 2011). Cactophilic *Drosophila* spp. receive an advantage from the association with this species and other yeasts (Heed *et al.*, 1976). *P. sporocuriosa*, isolated as well from 2 out of 9 adults reared on fruits, was recorded the first time from food sources and invertebrates; it was isolated from the rambutan tree (*Nephelium lappaceum*; Peter *et al.*, 2000) and from the frass of the litchi fruit borer *Conomorpha cramerella* (Thanh *et al.*, 2003).
Isolates from larvae, pupae and adults fed on the artificial diet included *S. craetegensis*, *A. schoenii* and *P. occidentalis*, which were also found in the fruit-fed adults. *S. craetegensis* was isolated from orange juice (Arias *et al.*, 2002). It is a predacious yeast since it produce appendages, or *haustoria* to kill other yeast and fungal cells, by penetrating them (Pimenta *et al.*, 2010, Lachance and Pang, 1997). Isolates clustering to the *Saccharomycopsis* genus were found to be associated to the black turpentine beetle *Dendroctonus valens* (Lou *et al.*, 2014). *P. occidentalis* was found to be preferably associated with fruits (Baffi *et al.*, 2011), also affected by grape sour rot (Guerzoni-Marchetti 1987).
Adults reared on fruits contained a broader diversity of yeasts; the most frequently identified yeast species from adults reared on fruit was *Z. bailii*, a food-spoilage and an acid-resistant yeast (Loeffler *et al.*, 2014), as it is able to metabolize acetic acid in the presence of a high sugar environment. This capacity is reinforced by the protective role played by ethanol when present in the medium, against the possible deleterious effects of acetic acid, by inhibiting the transport and accumulation of this organic acid. Consequently, the intracellular concentration of the acetic acid can be kept at low level (Sousa *et al.*, 1996). In addition to this, a strain of *Z. bailii* was found to have killer properties, exerting an antagonistic effect against *S. cerevisiae* and *C. glabrata* strains (Radler *et al.*, 1993).
The species *H. uvarum* is an apiculate yeast with a lemon-shape, present on the grape skin. It was isolated from 3 adults reared on fruits. It is known to be a colonizer of decaying fruits, commonly isolated from vineyard environments (Bourret *et al.*, 2012, Barata *et al.*, 2011): its contribution to *Drosophila* diet was detected in the past (Vacek *et al.*, 1979). Its presence was recorded in healthy vineyard and in those damaged by honeydew, sour rot (Barata *et al.*, 2008; Barata *et al.*, 2012) and Botrytis-affected ones (Nisiotou *et al.*, 2007). The detection on the body surface and gut of adult *Drosophila* individuals of the anamorph of *H. uvarum*, *Kloeckera apiculata*, by electron microscopy was reported in previous studies (Fermaud *et al.*, 2000). *Kloeckera apiculata* was also isolated from adult flies belonging to the *Drosophila fasciola* subgroup, a group of drosophilid flies living in tropical rainforests, thus characterized by having a narrow feeding niche. Yeasts isolated from *Drosophila* habitats in temperate and tropical regions usually belong to the group of good fermenters, as they are able to assimilate a broad range of polysaccharides, disaccharides and alcohols (Morais *et al.*, 1995). *H. uvarum* was previously detected in a work focused on the

characterization of the yeast communities of different *Drosophila* species, colonizing different feeding niches (Chandler *et al.*, 2012). It is the dominant yeast genus found in association with *Drosophila* species, in particular with fruit and flower feeders. It was not detected in the cactus feeding species surveyed, *D. mojavensis*, and it is rare in the two mushroom feeders species. It belongs to a species complex of colonizers of *Drosophila* food sources constituted by *H. lachancei*, *H. guillermondii*, *H. pseudoguillermondii*, *H. opuntiae*, *H. meyeri*, *H. clermontiae*. *Hanseniaspora opuntiae*. In the yeast collection we found three representatives, as it was isolated from two adults feeding on fruits. It was described the first time by Cadez and coworkers in 2003, isolated from prickly pear cactus in Hawaii. Furthermore, the first work which provided an overview of the yeast community associated to *D. suzukii* showed that *H. uvarum* is the yeast species identified in all specimens, e.g. larvae, adults, infested and uninfested fruits (cherries and raspberries), by cultivation methods and TRFLP analyses (Hamby *et al.*, 2012).

Many species of flies of *Drosophila* genus choose fruits and other vegetable materials as sites for finding mates and oviposition. Sites are preferably chosen by flies when are colonized by *Saccharomyces* and yeast species associated to fruiting plants, like those affiliated to *Pichia* and *Hanseniapora* genera. Yeast growth and metabolism contribute to suitable conditions for larvae and for adult mating and oviposition (Fogleman *et al.*, 1990). It was also demonstrated that and array of both fruit and yeast odors, released by microbes in early stages of fruit fermentations, are necessary for the flies attraction and oviposition stimulation (Fermaud *et al.*, 2000, Palanca *et al.*, 2013, Christiaens *et al.*, 2014); as an indirect consequence, flies seed yeasts in new habitats.

In conclusion, the characterization of the yeast community presented in this work outlines a microbiome constituted by ascomycete yeasts belonging to the Saccharomycetales order. Different approaches, both dependent and independent from cultivation, contribute with different extent, due to the possibility of these methods to characterize the community at different taxonomic levels. Despite that, the work here presented confirms that *D. suzukii* is associated with yeasts species that were already detected in association with this pest (Hamby *et al.*, 2012) and other *Drosophila* species (Morais *et al.*, 1995, Chandler *et al.*, 2012). In particular, not only these species are commonly found in orchard and vineyard environments (Arias *et al.*, 2002, Saez *et al.*, 2011, Barata *et al.*, 2011), but also they are involved in the early stages of fermentation and are recovered when the stage of fruit ripening occurs. *D. suzukii* attacks fruits and oviposits during this step of fruit maturation and, as a consequences, it will be associated by the yeast species characterizing mostly the early stages of fermentation. Indeed, before *véraison*, the microbial community of grape berries is similar to the plant leaves, dominated by basidiomycetous yeasts (*e.g. Cryptococcus* spp., *Rhodotorula* spp.); on the contrary, during ripening, berry skin starts to softer, availability of nutrients increases and then a prevalence of ascomycetous species, characterized by fast growth and an oxidative or weakly fermentative metabolism, is registered (*Candida* spp., *H. uvarum/K. apiculata*, *Pichia* spp.). When grape cuticle is severely damaged, high sugar amount leads to the dominance of species with higher fermentative metabolism (*Pichia* spp., *Zygoascus* spp.), including species that are known to be wine spoilers (*Zygosaccharomyces* sp., *Torulaspora* spp.), together with AAB of the *Gluconobacter* and *Acetobacter* genera, mostly (Barata *et al.*, 2012). For example, since the sugar fermenting yeast *Saccharomyces cerevisiae* is not commonly found on undamaged fruits (Barata *et al.*, 2012), is consequently not detected among yeasts associated to *D. suzukii*. Yeasts share the same environment, several ecological niches and metabolic pathways with AAB, symbionts living in association with drosophilid flies: AAB were reported to establish symbiotic relationships with *D. suzukii* too (Chandler *et al.*, 2011, Chandler *et al.*, 2014, Vacchini *et al.*, in preparation). Therefore, future efforts might be directed towards the investigation of the interactions existing between these two groups of microorganisms and the possible effects on the pest health and well-being. The spotted wing fly *D. suzukii* is an economically damaging pest of healthy soft and still ripening summer fruits (Lee *et al.*, 2011). The sustainable management of this pest should exploit the knowledge gathered on the flies' microbiome as an integration of the currently adopted strategies for the restraint of this invading species.

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Chapter V

Evaluation of the Antagonistic Properties of Different Yeasts Isolated from the Spotted-Wing Fly, *Drosophila suzukii*

INTRODUCTION

Among the different insect groups involved in mutualistic relationships with yeasts, Drosophilid flies, including the spotted wing fly *Drosophila suzukii,* establish symbiotic relationships with different microorganisms, among which yeasts of the Saccharomycetales order (Hamby *et al,* 2012, Chandler *et al*., 2012). The reasons of this association are various, ranging from nutritional, mating and oviposition benefits for flies (Becher *et al.,* 2012), dispersion in new habitats, chance for overwintering inside the host body, to increase outbreeding rates for yeasts (Vega and Dowd, 2005, Reuter *et al*. 2007, Stefanini *et al.* 2012).
The ecological niches colonized by yeasts, mainly fruits and plant, such as grape berries, are characterized by high species diversity (Barata *et al*., 2012); this leads to the competition among microorganisms for their own niche construction and nutrient supply. To gain these purposes, some yeast strains are able to synthesize and release in the environment an array of compounds named "killer toxins", molecules of proteinaceous nature, lethal to other microorganisms, with a specific action *spectrum*. The similarity with bacteriocins, strain-specific molecules having bactericidal activity released by bacteria (Riley and Wertz, 2002), is very close. According to this property and the phenotypes they display, yeasts can be classified in three categories: "killer", "sensitive" and "neutral" types. Sensitive cells are the target of the molecules released by the killer ones, while the neutral types are not killed by the killer strains and neither possess the killer factor (Bevan and Makower 1963, Woods and Bevan, 1968). Killer property is a widespread phenomenon in yeasts; indeed, the killer toxin characterization was conducted in several strains of *Saccharomyces cerevisiae* (Woods and Bevan, 1968), *Zygosaccharomyces bailii* (Radler *et al.,* 1993), *Hanseniaspora uvarum* (Radler *et al.,* 1990), *Pichia membranifaciens* (Santos and Marquina, 2004), *Debaryomyces hansenii* (Santos *et al.,* 2002), *Kluyveromyces phaffi* (Ciani and Fatichenti, 2001) and *Scwanniomyces occidentalis* (Chen *et al.,* 2000). This capacity was exploited in biological control field, not only for the production of killer toxins, but also for the physical competition for space and nutrients, mycoparasitism, antibiosis, predation or induction of plant diseases (Janisiewicz and Korsten, 2002). Some yeasts are known to be predacious yeasts, like *Saccharomycopsis craetegensis* (Pimenta *et al.,* 2010). Lachance and Pang used in 1997 the term "predacious yeast" first (Lachance and Pang, 1997), for those species that perform their antagonistic behavior by penetrating other yeasts through small appendages, named *haustoria,* and kill them.

This work aims to find putative strains having killer or antagonistic properties in a perspective of possible future applications for pest control. With this regard, the evaluation of the antagonistic properties of some selected yeast isolates, against yeasts and acetic acid bacteria strains, was conducted. These microorganisms were previously isolated and collected from the exotic pest *D. suzukii*. Since the production, activity and stability of the killer factor is dependent on pH, temperature and aeration, and it could be highly affected by the culture conditions, the optimal ones in terms of pH, temperature and carbon source content were used.

MATERIALS AND METHODS

Eleven strains isolated from *D. suzukii* and maintained at DeFENS (Department of Food, Environmental and Nutritional Sciences) were selected for antagonistic activity assays (Tab. 1).

Table 1: Yeast isolates, obtained from *D. suzukii,* used in this study

Abbreviation	Strain name
27	*Saccharomycopsis craetegensis* L2.9.P.27
60	*Saccharomycopsis craetegensis* P1.1B.G.60
71	*Arthroascus schoenii* P1.10.I.71

137	*Pichia occidentalis* AP1.2.P.137
164	*Galactomyces* sp. AP4.14.I.164
206	*Pichia sporocuriosa* AF1.7.P.206
220	*Hanseniaspora uvarum* AF1.13.F.22*;*
249	*Pichia membranfaciens* AF3.13.I.249
268	*Candida stellimalicola* AF4.1.P.268;
276	*Candida apicola* AF5.9.I.276
279	*Zygoascus meyerae* AF5.1.P.279

The yeast strain *Wickeramomyces anomalus Wa*F17.12, isolated from *Anopheles stephensi* mosquitoes (Ricci *et al.*, 2011), was chosen due its killer properties (Cappelli *et al.*, 2014) and used as reference strain. Twelve acetic acid bacteria (AAB) isolates were selected from the bacterial collection previously obtained from *D. suzukii* (Vacchini *et al.*, in preparation), for the screening of the antimicrobial properties exerted by the abovementioned yeast isolates (Table 2).

Table 2: Bacterial isolates, obtained from *D. suzukii*, used in this work.

Abbreviation	**Strain name**
23	*Acetobacter tropicalis* bYea.1.23
46	*Acetobacter indonesiensis* BTa1.1.46
44	*Acetobacter indonesiensis* BTa1.1.44
16	*Acetobacter peroxydans* L2.1.A.16
4	*Acetobacter cibinongensis* BMan.1.4
9A	*Gluconobacter oxydans* DS1FC.9A
1c	*Gluconobacter kondonii* BMan.3.1C
15	*Gluconobacter kanchanaburiensis* L2.2.A.15
65A	*Gluconacetobcter saccharivorans* DS1MA.65A
114	*Gluconacetobacter hansenii* DS2MC.114
34	*Gluconacetobacter liquefaciens* L3.2A.A.34

In addition, strain *Asaia* sp. SF2.1, which was isolated from *A. stephensi* (Favia *et al.*, 2007), was tested too.

A modification of the protocol adopted by Cappelli and collaborators (2014) was developed for the growth of the strains to be tested, both yeasts and AAB. Yeast cells were grown for 36 h at 26°C in 100mL flasks containing 20 mL of liquid YPD, a growth medium suitable for the stimulation of the soluble toxin production (Cappelli *et al.*, 2014). After 36 h, growth phase was verified to be 10^8 cell/mL and subsequently 100 µL of growth culture of each yeast species were seeded by spreading on YPD solid medium (agar 20 g/L). On each plate, the other yeast isolates were cross-examined by spotting 5 µL of the same culture growth in stimulating conditions. The plates were incubated for 72 h at 26°C and then the presence of inhibition haloes was observed.

For antimicrobial activity assay, the abovementioned AAB strains were allowed to growth till concentration reached 10^8 cell/mL on GLY liquid medium (Cappelli *et al.*, 2014), and then plated on GLY solid medium (agar 20g/L). As for yeast *versus* yeast test, on each plate 5 µL of every yeast was spotted. The plates were incubated for 72 h at 26°C and inhibition haloes were checked.

Table 3: Summary of the antagonistic activity tests performed by cross-examination on yeast strains, and on AAB isolates. In columns, the yeast isolates tested for the production of killer toxins are listed; in rows are reported the strains, the growth inhibition of which was observed. In the table, the numbers in rows and columns refer to the abbreviations reported in tables 1 and 2.

		Antagonistic yeast isolates											
		27	60	71	137	164	206	220	249	268	276	279	F17.12
Yeast isolates	27	-	-	-	-	-	-	-	-	+	-	-	-
	60	-	-	-	-	+	-	-	-	+	-	+	+
	71	-	-	-	-	-	-	-	-	+	-	-	-
	137	-	-	-	-	-	-	-	-	-	-	-	-
	164	-	-	-	-	-	-	-	-	-	-	-	-
	206	-	-	-	-	+	-	-	-	-	-	-	-
	220	-	-	-	-	-	-	-	-	-	-	-	-
	249	-	-	-	-	-	-	-	-	-	-	-	-
	268	-	-	-	-	-	-	-	-	-	-	-	-
	276	-	-	-	-	-	-	-	-	+	-	-	-
	279	-	-	-	-	-	-	-	-	-	-	-	-
	F17.12	-	-	-	-	-	-	-	-	-	-	-	-
AAB isolates	23	-	-	-	-	-	-	-	-	-	-	-	-
	46	-	-	-	-	-	-	-	-	-	-	-	-
	44	-	-	-	-	-	-	-	-	-	-	-	-
	16	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
	9A	-	-	-	-	-	-	-	-	-	-	-	-
	1c	-	-	-	-	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-	+	-	-	+
	65A	-	-	-	-	-	-	-	-	+	-	-	+
	114	-	-	-	-	-	-	-	-	-	-	-	-
	34	-	-	-	-	-	-	-	-	-	-	-	-
	SF2.1	-	-	-	-	-	-	-	-	+	-	-	-

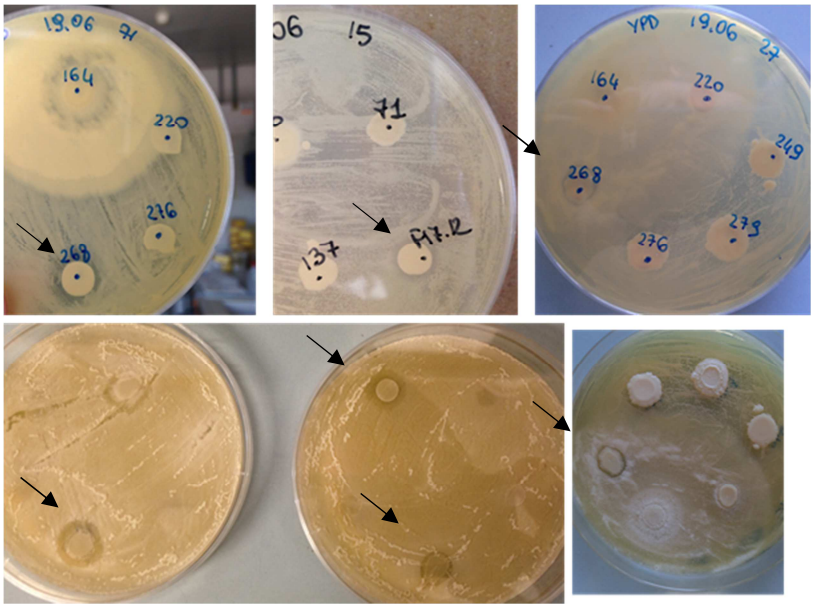

Figure 1: Examples of the inhibition zones created by the killer-producing strains. From left top, the pictures represent the halo of *C. stellimalicola* AF4.1.P.268 on the seeded culture of *A. schoenii* P1.10.I.71 (first picture) and the antagonistic activity of *W. anomalus* WaF17.12 against *G. kanchanaburiensis* L2.2.A.15 (picture in the middle). The inhibition zone of *C. stellimalicola* AF4.1.P.268 against *Saccharomycopsis craetegensis* L2.9.P.27 is visible in the photo on the right top. Below, some exemplificative pictures are reported. Black arrows indicate the inhibition halo surrounding the killer strains.

RESULTS

Selected isolates from the yeast and bacterial community collection of the spotted wing fly were screened to evaluate the presence of antagonistic yeast strains. The production of killer toxins depends on the culture conditions used. A protocol for the optimization of the killer toxins production, modified from the one used by Cappelli and co-workers (2014), was applied, and subsequently antagonistic activity tests were performed. The results of this assay are presented in Table 3, while in Figure1 some pictures representing the inhibition results are given. In Table 3 it is possible to observe that a specific yeast isolate, *C. stellimalicola* AF4.1.P.268, was able to create an inhibition zone around 7 isolates, 4 yeasts and 3 AAB. *W. anomalus* inhbited the growth of one yeast and three bacteria, while *Galactomyces* sp. AP4.14.I.164 succedeed in limiting the growth of two yeast strains. It is interesting to note that the yeast *S. craetegensis* P1.1B.G.60 was inhibited by four different yeasts, among which *C. stellimalicola* AF4.1.P.268.

DISCUSSION

The work here presented provides a fast and ready-to use method for the optimization of culture growth conditions for the production of killer factors. These compounds can be generally used by some yeast strains to compete with other microorganisms for space and nutrients (Bevan and Makower, 1963). In addition, the results highlighted the capacity of some selected isolates, in particular of *C. stellimalicola* AF4.1.P.268, to limit the growth of several yeast and AAB isolates, by creating inhibition haloes. Future analyses might be devoted in completing the screening of the yeast and bacterial collection, by using the most promising strains here presented. Evaluation of the chemical characteristics and properties of the killer toxins, identifying the nature of the toxins released by these yeasts, would be an interesting advancement of the current knowledge. The present data and the future investigations might contribute to enlarge the state of the art and to develop a strategy in the perspective of *D. suzukii* management.

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Conclusions and Future perspectives

Many insect orders are involved in symbiotic interactions with microorganisms, which may have a relevant role for the biology, physiology and health status maintenance of the host species (Dale and Moran, 2006; Moran *et al.*, 2008). Remarkably, insects of the Diptera, Hymenoptera and Hemiptera orders, including fruit flies of the *Drosophila* genus, and in particular the insect pest *Drosophila suzukii* (Diptera: Drosophilidae) (Cini *et al.*, 2012), establish a tight symbiotic relationship with the Alpha-Proteobacteria group of acetic acid bacteria (AAB). These symbionts influence different aspects of the host life, such as the modulation of immunity, the larval developmental rate, body size and energy metabolism (Ryu *et al*., 2008, Crotti *et al*., 2010, Mitraka *et al*., 2013). Furthermore, *Drosophila* species are involved in mutualistic associations with yeasts, mostly of the Saccharomycetales order (Hamby *et al.*, 2012, Chandler *et al*., 2012). Both flies and microorganisms benefit from these associations: on one hand, yeasts are the main nutritional source for flies and substrates colonized by these microorganisms are preferentially chosen by the insects for mating and oviposition (Becher *et al.*, 2012 Christiaens *et al.*, 2014). On the other hand, yeasts' mobility and colonization of new substrates are favored by flies' dispersion service, the overwintering can be done inside the insect body, and the outbreeding rates are increased (Vega and Dowd, 2005, Reuter *et al*., 2007, Stefanini *et al.*, 2012). The analysis of the composition and structure of the microbial community poses the basis for future speculations about the roles of these microorganisms within their host.

The first part of the thesis, here presented, was focused on the characterization of AAB diversity in *D. suzukii*. Molecular analyses (16S rRNA barcoding and Denaturing Gradient Gel Electrophoresis-PCR) were coupled with culture-dependent techniques (isolation trials, dereplication with ITS-PCR and isolate identification through partial 16S rRNA gene sequencing), and allowed to indicate that AAB, in particular *Gluconobacter*, *Gluconacetobacter* and *Acetobacter* genera, are an abundant component of the spotted wing fly microbiome. It was shown that this association is maintained in individuals at different stages of their life cycle (larvae, pupae and adults), independently from the diet administered (fruits or an artificial preparation). These three genera are naturally associated with the fruit fly *Drosophila melanogaster*, in particular with the insect's digestive system (Cox and Gilmore, 2007, Ren *et al.*, 2007, Corby-Harris *et al.*, 2007). Fluorescent *in situ* hybridization (FISH) experiments showed that AAB, and *Gluconobacter* genus in detail, localize within the peritrophic membrane of the midgut and proventriculus. Moreover, to evaluate AAB capacity to colonize the fly's body, selected strains, *Gluconobacter oxydans* DSF1C.9A, *Acetobacter tropicalis* BYea.1.23 and *Acetobacter indonesiensis* BTa1.1.44, labelled with Green fluorescent protein (Gfp), were used for recolonization experiments of *D. suzukii*. Visualization by fluorescence microscopy revealed the symbionts localization in the epithelium of the insect crop, proventriculus and midgut. It was observed, indeed, that several AAB isolates, under liquid growth conditions, were able to produce a gelatinous matrix, in which the cells are entrapped, and which it probably contributes to the bacterial adhesion to the epithelia of the digestive system. Overall, the results of this work suggest the relevance of this bacterial group also for this insect species' biology. Future perspectives of this work foresee the investigation of the role that AAB play for their host. Indeed, their prevalence in different specimens, their abundance and localization in the digestive system, both in fruit-fed individuals and in specimens reared on the artificial diet, suggest that an important contribution could be exerted by these symbionts.

AAB isolated in the first part of this thesis were then used to perform attractive assays of *D. suzukii*. Since volatile organic compounds (VOCs) can be insects' attractants or repellents, it was shown that selected AAB isolates were able to emit VOCs with attractive capabilities for *D. sukuzii* (Cha *et al*., 2014). *D. suzukii* adults were then submitted to two-choice olfactometer assays in which AAB were used as VOCs producers. *Gluconobacter oxydans* DSF1C.9A, *Gluconobacter kanchanaburiensis* L2.1.A.16 and

Gluconacetobacter saccharivorans DSM1A.65A strains showed the higher attractiveness for flies than other bacteria. It was also demonstrated that the best attractive molecules released by these bacteria belonged to the class of alcohols, ketones, carboxylic acids and aldehydes. The data obtained by this work shed a light on the possible future strategies for baits specifically constructed for *D. suzukii* as currently, traps for this pest are composed by generic compounds, like vinegar and baker's yeast.

The association of spotted wing drosophila of three developmental stages (larvae, pupae and adults) reared both on fruit and artificial diet, with yeasts, was proved too. Cultivation-independent approaches (DGGE-PCR, 16S rRNA -pyrosequencing) indicated that Saccharomycetales yeasts constitute the major fraction of the analyzed community. These groups of yeasts comprise specialist colonizers of rotten and fermenting fruits, and the food sources *Drosophila* is used to visit.

Identification of the 237 yeast isolates confirmed and extended the molecular data and showed that the most abundant species collected (*Pichia occidentalis*, *Saccharomycopsis craetegensis* and *Arthroascus schoenii*) were also those species found in all flies stages. Furthermore, this characterization showed that the samples reared on fruit diet harbored a higher number of species that those fed on lab diet. Phylogenetic trees confirmed the relationships of the yeasts identified. Importantly, these are species associated to fruit substrates at different stages of fermentation. They shared the ecological niche with drosophilid flies and AAB (Barata *et al*., 2012). It was further confirmed the association with *Hanseniaspora uvarum*, previously described as the dominant yeast associated to different *Drosophila* species, including *D. suzukii* (Chandler *et al*., 2012, Hamby *et al*., 2012).

The last part of the thesis was dedicated to the analysis of selected isolates of the yeast collection in order to find putative "killer toxin" producers. Some strains compete with other species for space and nutrients through the releasing, under specific pH, temperature and carbon source parameters, of killer toxins compounds (Woods and Bevan, 1968). It was thus observed that some yeast isolates, particularly *Candida stellimalicola* AF4.1.P.268, were able to create inhibition zones that limited the growth of different yeast and AAB strains.

The results presented in this work might be a starting point for the development of new concept and sustainable biocontrol strategies, alternative to the use of insecticides, and based on the use of symbionts, their attractive properties and antagonistic molecules. Future perspectives of this work foresee the characterization of the interactions existing between the microbial symbionts and the host, including also the ones established between yeasts and bacteria.

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ACTIVITIES PERFORMED DURING THE PHD

Training courses and congresses:

- 26th -27th January 2012, Annual PhD International Workshop: VII Workshop of the CBEA School, University of Milan.

- 3rd-5th 2012, National Meeting: Cortona Procarioti, organized by Scuola Genetica di Cortona, Società di Microbiologia Generale e Biotecnologie Microbiche (SIMGBM), Associazione Genetica Italiana (AGI), Cortona (AR).

- October 2012, Short Course: "Tecniche molecolari applicate alle analisi ecologiche" - Dott. D. Fontaneto (CNR, Italy), University of Milan.

- 21st January 2013, Annual PhD International Workshop: VIII Workshop of the CBEA School, University of Milan.

- 27th February 2013: Workshop "Nuove associazioni tra parassotoidi indigeni e insetti esotici" – M.L. Dindo, S. Francati, E. Marchetti, F. Santi , University of Milan.

- 11th-13th March 2013, Winter School "Symbiomes: systems metagenomics of host-microbe interactions" - Fondazione E. Mach, San Michele all'Adige, Trento.

- 1st-5th April 2013, Winter School: DNA barcoding course "Integrative taxonomy and taxonomic expertise in the framework of the DNA-barcoding initiative"- Muséum national d'Histoire naturelle, Paris, France

- 27th- 28th January 2014, Annual PhD International Workshop: IX Workshop of the CBEA School, University of Milan

- 4th – 8th August 2014, Summer School: 5th IRSAE Summer School 2014 "Methods to evaluate the effects of climate change on ecosystems and populations" Telemark University College, Department of Environmental and Health Studies, Norway

Oral dissertations in national or international meetings:

- 21st January 2013, Annual PhD International Workshop: VIII Workshop of the CBEA School, University of Milan

- 13th March 2013, Winter School "Symbiomes: systems metagenomics of host-microbe interactions" - Fondazione E. Mach, San Michele all'Adige, Trento

- 27th- 28th January 2013, Annual PhD International Workshop: IX Workshop of the CBEA School, University of Milan

- 4th – 8th August 2014, Summer School: 5th IRSAE Summer School 2014 "Methods to evaluate the effects of climate change on ecosystems and populations" Telemark University College, Department of Environmental and Health Studies, Norway

Posters:

- 19th-24th August 2012. "Microbial community associated with the red palm weevil, *Rhynchophorus ferrugineus*".
Chouaia B., Montagna M., Mazza G., Epis S., Crotti E., Prosdocimi E., **Vacchini V.**, Daffonchio

D., Cervo R. and Bandi C.
24th International Congress of Entomology (ICE), Daegu, South Korea.

- 5th–7th November 2012. "Acetic acid bacteria and the factors driving their roles as insect symbionts".
Crotti E., Chouaia B., **Vacchini V.**, Prosdocimi E.M., Sansonno L. and Daffonchio D.
EU US Environmental Biotechnology Workshop, St. Louis, Missouri, USA.

- 16th-19th December 2012. "Acetic acid microbiome associated to the spotted wing fly *Drosophila suzukii*".
Crotti E., Gonella E., **Vacchini V.**, Prosdocimi E. M., Mazzetto F., Chouaia B., Mandrioli M., Sansonno L., Daffonchio D. and Alma A.
Proceedings of the BIODESERT International Conference on Microbial Resource Management for Agriculture in Arid Lands, Hammamet, Tunisia.

- 22nd-25th June 2014. "Microbiota of the maize leafhopper *Dalbulus maidis* (Delong&Wolcott) (Hemiptera: Cicadellidae) associated with the infection by maize bushy stunt phytoplasma (MBSP)".
García González J., Crotti E., **Vacchini V.**, Gonella E., Alma A., Daffonchio D., Tanaka F., Spotti Lopes J. R.
2014 Hemipteran-Plant Interactions Symposium (HPIS)", University of California – Riverside, USA.

- 4th – 8th August 2014. "Ecology and interactions of microbial symbionts in the spotted-wing fly *Drosophila suzukii*".
Vacchini V., Gonella E., Mazzetto F., Prosdocimi E. M., Chouaia B., Mandrioli M., Crotti E., Alma A. and Daffonchio D.
5th IRSAE Summer School 2014 "Methods to evaluate the effects of climate change on ecosystems and populations", Telemark University College, Department of Environmental and Health Studies, Norway.

Full-papers in international conferences:

December 16th – 19th 2012, Co- author in a Selected Lecture: "Investigation of the microbial symbionts of the red palm weevil, *Rhynchophorus ferrugineus*".
Chouaia B., Montagna M., Mazza G., Crotti E., Epis S., Prosdocimi E. M., **Vacchini V.**, Cervo R., Longo S., Bandi C., Daffonchio D.
BIODESERT International Conference on Microbial Resource Management for Agriculture in Arid Lands. Tunis.

Papers published in international journals:

-"Microbial symbionts of honeybees: a promising tool to improve honeybee health"
Crotti E., Sansonno L., Prosdocimi E. M., **Vacchini V.**, Hamdi C., Cherif A., Gonella E., Marzorati M., Balloi A.
New Biotechnology, 2013, 30(6),716-722.

Extra activities:

- 12th – 25th July 2014, Among the organizers of the FACILIS 2014 Summer School, University of Milan