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Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria

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Running title: Acetic Acid Bacteria of *Drosophila suzukii*

Abstract

The pivotal role of diet in shaping gut microbiota has been evaluated in different animal models, including insects. *Drosophila* flies harbour an inconstant microbiota among which acetic acid bacteria (AAB) are important components. Here, we investigated the bacterial and AAB components of the invasive pest *Drosophila suzukii* microbiota, by studying the same insect population separately grown on fruit-based or non-fruit artificial diet. AAB were highly prevalent in the gut under both diets (90 and 92% infection rates with fruits and artificial diet, respectively). Fluorescent *in situ* hybridization and recolonization experiments with green fluorescent protein (Gfp)-labelled strains showed AAB capability to massively colonize insect gut. High-throughput sequencing on 16S rRNA gene indicated that the bacterial microbiota of guts fed with the two diets clustered separately. By excluding AAB-related OTUs from the analysis, insect bacterial communities did not cluster separately according to the diet, suggesting that diet-based diversification of the community is primarily reflected on the AAB component of the community. Diet influenced also AAB alpha-diversity, with separate OTU distributions based on diets. High prevalence, localization and massive recolonization, together with AAB clustering behaviour in relation to diet, suggest an AAB role in the *D. suzukii* gut response to diet modification.

Keywords

16S rRNA gene pyrosequencing, cultivation-dependent approach, fluorescent *in situ* hybridization (FISH), symbionts, green fluorescent protein

INTRODUCTION

The insect gut microbiota plays very critical and essential roles for the host biology, physiology and immunity (Hamdi *et al.,* 2011). Diet, together with other factors, such as environmental habitat, host developmental stage and phylogeny, profoundly affect its diversity and structure, consequently influencing insect functionality (Colman *et al.,* 2012; Yun *et al.,* 2014).

In last years, increased attention has been focused on the study of the bacterial microbiota associated to different species of drosophilid flies. *Drosophila* represents a powerful insect model for a vast array of studies, including the defence mechanism-based investigations and the exploration of host-commensal interactions (Erkosar *et al*., 2013; Lee and Lee, 2014). With the aim to unravel host-microbiome interactions beyond laboratory boundaries, researchers have been prompted to investigate the gut microbiota diversity of different natural species of drosophilid flies (Chandler *et al.,* 2011; Wong *et al.*, 2013; Cox and Gilmore, 2007). By using molecular techniques four bacterial families have been found to be commonly associated to field-captured or laboratory-reared flies, namely Enterobacteriaceae, Acetobacteraceae, Lactobacillaceae and Enterococcaceae (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; Wong *et al.*, 2013). In particular, Acetobacteraceae (acetic acid bacteria, AAB) are among the dominant taxa in laboratory-reared *D. melanogaster* (Ryu *et al.*, 2008; Wong *et al.*, 2011). Conversely, field-captured *Drosophila* flies show an inconstant bacterial community, where AAB are, however, frequently associated (Wong *et al.*, 2013).

AAB are a bacterial group widespread in sugar- and ethanol-rich matrices, such as flowers' nectar, fruits, vegetables and fermented matrices, all niches shared by drosophilid flies and from which they can pass to the *Drosophila* gut, a sugar- and ethanol-rich environment (Blum *et al.,* 2013; Cox and Gilmore, 2007 Crotti *et al.*, 2010). AAB establish a delicate interaction with the insect innate immune system, being involved in the suppression of the growth of pathogenic bacteria in healthy individuals (Ryu *et al*, 2008), but also the modulation of the insulin pathway and the enhancement of the larval developmental rate, body size, intestinal stem cells activity and energy metabolism (Shin *et al.*, 2011). A beneficial role of AAB has been also demonstrated for mosquito larval development (Chouaia *et al*., 2012; Mitraka *et al*., 2013).

The spotted wing fly *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), an endemic pest in South-East Asia, has been accidentally introduced in USA, Canada and Europe (Cini *et al*., 2012; Hauser, 2011; Lee *et al*., 2011). Unlike its relatives that attack rotten fruits, *D. suzukii* lays eggs on healthy soft summer fruits where the larvae grow (Walsh *et al*., 2011; Mitsui *et al*., 2006). So far, little information is available on the bacterial community associated to *D. suzukii* specimens collected in USA (Chandler *et al.*, 2014), while just few other publications studied *Wolbachia* infection (Mazzetto *et al.*, 2015; Cattel *et al.*, 2016; Siozios *et al.*, 2013).

Considering AAB abundance and importance in drosophilid flies, we aimed to assess the effect of two different diets (i.e. based or not on fruit) on the diversity of bacterial and AAB microbiota of *D. suzukii*. Specifically, we evaluated the possibility that AAB are involved in the gut microbiota diversification when insects are exposed to two different alimentary regimes. For studying the effect of diets on the bacterial microbiota diversity, we first confirmed the significance of AAB in the *D. suzukii* gut. We determined their prevalence, the gut localization through fluorescent *in situ* hybridization (FISH) and the ability to recolonize the insect gut by using green fluorescent protein (Gfp)-tagged derivatives of a series of strains from a *D. suzukii* isolate collection. As a second step of the study we assessed the changes of the bacterial microbiota structure and diversity by means of cultivation-independent techniques.

RESULTS

Prevalence of* Wolbachia *and AAB. Since *Wolbachia* is a frequent symbiont of drosophilid flies, the prevalence of this bacterium has been evaluated on adults obtained both from fruit and artificial diet rearings. In flies reared on fruit *Wolbachia* showed an infection rate of 66% (33 out of 50 positive specimens). *Wolbachia* prevalence was significantly lower (GLM, $p < 0.05$) in individuals maintained on the artificial diet (infection rate of 28%, 14/50 positives). Conversely, AAB occurred in almost all of the analysed individuals reared on both food sources, with 90 and 92% infection rates in flies maintained on fruits and artificial diet, respectively (45 and 46 out of 50 individuals) with no significant difference in infection incidence (GLM, $p=0.727$).

AAB isolation. Since the condition of fruit-based rearing is the closest to the diet of *D. suzukii* in field conditions, we concentrated our efforts on individuals reared on this diet; however, specimens reared on artificial diet have been also included in the analysis. The final collection included 234 isolates that were de-replicated according to the ITS fingerprinting profiles. 16S rRNA gene sequencing of representatives of each ITS profile identified the isolates as belonging to *Komagataeibacter*, *Gluconacetobacter*, *Acetobacter* and *Gluconobacter* genera (Yamada *et al.*, 2012a; 2012b), while only 16.3% of the isolates did not belong to Acetobacteraceae family (Tab. 1). Twenty-eight isolates have been affiliated to the *Acetobacter* genus, including the species *A. cibinongensis*, *A. indonesiensis*, *A. orientalis*, *A. orleanensis*, *A. peroxydans*, *A. persici* and *A. tropicalis*. *A. persici* and *A. indonesiensis* were the most represented species. Eighteen *Gluconobacter* isolates have been affiliated to three species, *G. kanchanaburiensis*, *G. kondonii* and *G. oxydans*. The unique isolate of *G. kondonii* in the collection has been collected from an adult fly fed on fruits, while *G. kanchanaburiensis* isolates have been obtained from specimens reared on artificial diet. Twelve isolates collected from adults fed on fruit showed high sequence similarity with *G. oxydans*. One hundred and twenty-three isolates have been assigned to *Gluconacetobacter* and *Komagataeibacter* genera. In particular, 118 *Komagataeibacter* isolates have been obtained from fruit–fed *Drosophila.* Due to the phylogenetic proximity of the species of this genus, discrimination at the species level was not possible with the actual 16S rRNA sequencing. *Ga. liquefaciens* isolates (no. 4) have been obtained from three pupae and one larva using the TA1 medium.

Finally, the attribution to either *Gluconacetobacter* or *Komagataeibacter* genera could not be discriminated according to the actual 16S rRNA sequence (Tab. 1).

Localization of AAB in the* D. suzukii *gut and colonization by Gfp-labelled strains. Fluorescent *in situ* hybridization (FISH) on the insect dissected organs using the AAB-specific probe AAB455, gave positive signals in the proventriculus and the gut (Fig. 1), whereas no fluorescence was detected in the absence of probe. The proventriculus epithelium gave a strong signal, observable by merging the interferential contrast (Fig. 1c) with the fluorescent (Fig. 1b) images. Magnification in fig. 1d allowed the visualisation of fluorescent AAB microcolonies adhering to the peritrophic matrix.

Gluconobacter cells have been observed in the midgut (Fig. 1g) suggesting the distribution of this genus in the inner side of the intestinal lumen. Fig. 1e-h show *Gluconobacter* distribution (Fig. 1g) in relation to the dispersal of *Eubacteria* (Fig. 1f), indicating that it is surrounded by other bacteria, presumably AAB (Fig. 1d). However, we could not ascertain such hypothesis because all the attempts to design specific probes effective for *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter* genera, failed.

Strains *G. oxydans* DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 have been successfully transformed with a plasmid carrying the Gfp cassette. Plasmid stability experiments showed that *G. oxydans* DSF1C.9A retained the plasmid with a relatively high percentage (73.1%), while this was not the case for strains BYea.1.23 and BTa1.1.44. Thus, colonization experiments of adult flies have been performed under antibiotic (kanamycin) administration in the insect food. The Gfp-labelled strains massively recolonized the fly foregut and midgut (Fig. 2); no auto-fluorescence has been observed in control flies. *G. oxydans* DSF1C.9A successfully colonized the crop, the proventriculus and the first part of the midgut (see the magnifications in Fig. 2b and 2c). The Gfp-labelled cells are clearly restricted to the epithelium side of the proventriculus, embedded in the peritrophic matrix (Fig. 2c). Likely, the midgut showed the same massive colonization pattern as the foregut (Fig. 2d-e). In this tract, small hernias are also visible by interferential contrast (indicated by black arrowheads in Fig. 2e), probably due to microscopic damages produced during the dissection. These hernias appeared full of a gelatinous matrix that resulted Gfp-positive by CLSM, showing that Gfp-labelled cells are completely sunk in the gel and suggesting that the bacterial cells are actually contained by the peritrophic matrix. The black filaments around the organ are the Malpighian tubules, more evident in the CLSM picture (Fig. 2d). Also *A. tropicalis* BYea.1.23(Gfp), and *A. indonesiensis* BTa1.1.44(Gfp) strains successfully colonized the foregut and midgut (Fig. S1): since they showed an identical colonization pattern, only strain BYea.1.23(Gfp) images are shown. The labelled bacteria were present in the whole tract and they have been especially located close to the gut walls and within the peritrophic matrix (Fig. S1).

Characterization of* D. suzukii *bacterial diversity by DNA-based analysis. At first, to have a general view of the bacterial community associated to *D. suzukii*, DNA extracted from 32 specimens has been used, as template, in PCR-DGGE assays (targeting a fragment of the 16S rRNA gene, Tab. S1). In particular, five larvae (n. 1-5), one pupa (n. 6) and ten adults (n. 7-16; Fig. S2a-b) reared on fruits have been analysed, as well as four larvae (n. 29-32), four pupae (n. 25-28) and eight adults (n. 17-24) reared on the artificial diet (Fig. S2c). Consistent with previous data reported for other drosophilid flies (Chandler *et al.*, 2011; Wong *et al.*, 2013), *D. suzukii* specimens showed relatively simple bacterial communities with the presence of few prevalent bacterial taxa. The lowest variability in the community profiles has been observed among larvae reared on fruits and on the artificial diet: many PCR-DGGE bands were conserved among the samples belonging to the same diet. Conversely, only few conserved bands were detected among adults reared on fruits, which showed more complex profiles than larval ones either reared on fruits or on the artificial diet (Fig. S2a-c). PCR-DGGE profiles allowed observing the influence of diet on the insect bacterial community structure and composition (Fig. S2): the bacterial community of adults reared on fruit diet was clearly more complex than the one of adults reared on artificial diet. Moreover, PCR-DGGE sequencing results revealed high prevalence of AAB in insects reared on both diet substrates (Tab. S2).

Thus, to sturdily investigate the diet influence on the insect bacterial community, 16S rRNA gene pyrosequencing was performed on 14 specimens, including eight individuals reared on fruits and six on the artificial diet and considering different developmental stages (five larvae, two pupae and seven adults). Variability among the samples has been reported (Tab. S3; Fig. 3a). Using the Shannon Index to measure α-diversity in each sample and plotting it on a rarefaction curve, we confirmed the saturation of the bacterial diversity associated to the samples (Fig. S3). We obtained in total 178,856 reads after quality evaluation and chimera removal. The different ecological estimators showed that, on average, the bacterial communities associated with the specimens reared on fruits exhibited a greater diversity than those from individuals reared on artificial diet (118 ± 42 and 78 ± 24 OTUs, respectively; Tab. S3). As a matter of fact, the microbiota of *D. suzukii* specimens reared on fruit showed on average a greater richness (Chao1 = 137.4 ± 48.3), a higher diversity (H' = 2.5 ± 0.75) and a higher evenness (J = 0.52 ± 0.13), when compared to the microbiota of flies reared on artificial diet (Chao1 = 91.4 ± 31.1; H' = 1.75 ± 0.67; J = 0.4 ± 0.13).

β-diversity has been evaluated through principal coordinates analysis (PCoA) on the similarity matrix obtained by UniFrac. The two principal components explain 49.67% of the variation (Fig. 3b). PCoA showed three clusters of samples ($p<0.05$): the first one encompasses the two larvae and the sole pupa reared on the artificial diet; the second one includes all the adults reared on the artificial diet, while the third is constituted by all the specimens reared on fruits (Fig. 3b). Interestingly, the exclusion of AAB OTUs from the analysis showed a loss of the clustering pattern observed before (Fig. 3c). Specifically, the three abovementioned clusters were not significantly different one to each other ($p>0.05$), highlighting that AAB could be more responsive than other bacterial groups following diet modification. Thus, we evaluated the distribution of AAB at OTU level among the specimens exploring the 16S rRNA gene pyrosequencing dataset: a clustering tendency of the samples in relation to the different diets has been further observed (Fig. 3d).

Looking to the bacterial community's composition, the results showed that the average percentage of reads belonging to Acetobacteraceae family was 24.8% per specimen (18% in case of fruit-reared insects and 33.9% for specimens fed with artificial diet; Fig. 3a). At genus level, 16S rRNA gene pyrosequencing revealed that in *D. suzukii* specimens, reared on fruit and on the artificial diet, Acetobacteraceae family was composed mainly by the genera *Acetobacter* and *Gluconobacter* (average 20% of 3.9% out of the total reads respectively, Fig. S4; Tab. S4).

Interestingly, reads affiliated to Rickettsiales, to which *Wolbachia* genus belongs, have been detected only in flies reared on fruits, with an average of 27.5%, confirming results obtained by PCR-DGGE (Fig. 3a; Fig. S2). *Wolbachia* was the only representative of Rickettsiales order in the dataset. Reads clustering within Rhodospirillales order (the order to which Acetobacteraceae belongs) were present in all the specimens with different abundance; in some cases it reached percentages of 85.2 and 85.4 out of the total number of sequences per sample (MF1 and PP2, respectively). Members of other orders such as Enterobacteriales, Xanthomonadales, Lactobacillales, Rhizobiales, Burkholderiales and Sphingobacteriales constituted relevant fractions of the remaining bacterial communities (Fig. 3a).

DISCUSSION

Prevalence, FISH and 16S rRNA gene PCR-DGGE and pyrosequencing analyses confirmed that AAB are invariably present in *D. suzukii* gut in our experimental conditions. In *D. melanogaster* and other insects, AAB have been demonstrated as prevalent symbionts with important biological roles (Shin *et al.*, 2011; Chouaia *et al.*, 2012; Mitraka *et al.*, 2013). For instance, *Acetobacter tropicalis*, a species that we found in *D. suzukii*, was previously described in association with the olive fruit fly *Bactrocera oleae* (Kounatidis *et al.*, 2009).

Localization and intimate association of AAB with *D. suzukii*, revealed by FISH (Fig. 1), support the hypothesis that these bacteria may indeed influence the gut functionality. In the midgut, AAB localization along with the peritrophic matrix suggests a bacterial interaction with the host gut epithelium. Moreover, recolonization experiments with Gfp-labelled strains (i.e. *G. oxydans* DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44) strongly supported the capability of AAB to colonize the gut (Fig. 2 and Fig. S1). As indicated elsewhere (Favia *et al.*, 2007), recolonization experiments have been performed under the antibiotic pressure of kanamycin, a required procedure when Gfp cassette is encoded on a plasmid to avoid the loss of the plasmid itself. Certainly, the use of antibiotic could have a negative side effect on the insect host and other gut symbionts. Further investigations could help in verifying if the used concentration of antibiotic might have detrimental effects for the host and/or the gut microbiota. However, such investigation was beyond the purpose of the experiments that were designed to assess which gut portions were recolonized by the strains. For *A. tropicalis* a very similar gut localization pattern to that of *D. suzukii* has been already observed in the olive fruit fly *B. oleae* (Kounatidis *et al.*, 2009), where the bacterium was observed in contact with the gut epithelium of the insect, entrapped in a polysaccharidic matrix. Similarly, in other insects, such as the leafhopper *Scaphoideus titanus*, and *Anopheles* and *Aedes* mosquitoes, other AAB of the genus *Asaia* massively colonize the epithelia of the gut and the reproductive organs (Crotti *et al.*, 2009; Damiani *et al.*, 2010; Favia *et al.*, 2007; Gonella *et al.*, 2012). The AAB localization observed in the gut of *D. suzukii* confirmed that guts of sugar-feeding insects are primary habitat for AAB, in which they establish strict topological and presumably functional connections with the epithelial cells (Crotti *et al.*, 2010; Chouaia *et al.*, 2014).

D. suzukii microbiota diversity has been investigated at little extent and just one paper has been published describing the insect bacterial community (Chandler *et al.*, 2014). By the use of a next generation sequencing (NGS) technique, authors analyzed pools of specimens collected from cherries sampled at different developmental stages, showing an high frequency of the gamma-Proteobacterium *Tatumella*, while the two AAB *Gluconobacter* and *Acetobacter* genera were found at lower abundance (Chandler *et al.*, 2014). Conversely, in our study, sequences related to *Tatumella* genus have not been retrieved in any of the analysed samples, but a high prevalence of AAB have been found (average of

24.8%). Insects in Chandler and colleagues' work (2014) have been collected in USA, while our populations derive from Italian field-collected individuals. Moreover, different variable regions on 16S rRNA gene have been amplified in the two studies. Such environmental and methodological differences may explain the differences between our and the Chandler *et al.* work (2014). However, further investigations are needed to determine *Tatumella* prevalence in different *D. suzukii* populations, considering with special attention insects collected in different locations, as already mentioned by Chandler *et al.* (2014).

It is widely recognized the importance of diet in shaping the insect bacterial community (Montagna *et al.*, 2015; Colman *et al.,* 2012; Yun *et al.,* 2014). Particularly, in *D. melanogaster* the establishment and maintenance of the microbiota are determined by bacterial intake from external sources (Blum *et al.*, 2013). Differences in the diversity and dominance of bacterial species associated to several *Drosophila* species are thus related to food source (Wong *et al.*, 2011). This has been substantiated by Chandler and coworkers (2011) who observed that individuals of different *Drosophila* species reared on different food sources enriched a similar microbiota when moved to the same medium. With the present study, we confirmed that also in case of *D. suzukii* there are differences in the bacterial communities between animals reared on fruits and on artificial diet (Fig. 3). Specifically, the fruit-based diet determined a higher diversity in the bacterial community rather than the artificial diet, confirming what already reported in literature about the reduction of the insect microbial community complexity in case of artificial diet-fed animals in comparison to natural diet-fed ones (Lehman *et al.*, 2009). In our study, the fruit-based diet can be considered similar to the natural one *D. suzukii* is exposed to in orchards. The diet appeared as a more important factor than the life stage in discriminating the insect associated microbiota, since discrimination at the life stage was possible only between juvenile stages and adults reared on the artificial diet ($p<0.05$; Fig. 3b). Chandler *et al.* (2011), analyzing clone libraries of the bacterial community associated to different species of *Drosophila* flies, field-collected or reared in the laboratory, found AAB in both types of individuals: sequences related to *Commensalibacter* and

Acetobacter have been retrieved, while the authors reported the nearly complete lack of *Gluconobacter* sequences and the complete lack of *Gluconacetobacter* ones within their samples. In our 16S rRNA gene-based survey of the *D. suzukii* microbiota, *Acetobacter* and *Gluconobacter* have been detected while *Gluconacetobacter* and *Komagataeibacter* have not, although isolates of these two genera have been obtained. The 16S rRNA sequence phylogenetic proximity of AAB genera and the small region, targeting the bacterial 16S rRNA gene used in our PCR amplifications (about 500 bp), could have masked the discrimination of *Gluconacetobacter* and *Komagataeibacter* sequences (Fig. S4). In this perspective, the use of multiple primer pairs and the choice of longer regions (however taking into account limitations of the current NGS techniques) could lead to a more representative view of the structure of the host bacterial community. Another factor that might have introduced biases in the microbiota analysis is the DNA extraction method. Even though in our work, DNA has been extracted through one of the most widely used, cost-effective and efficient methods available for DNA extraction, i.e. the using sodium dodecyl sulfate-proteinase K-CTAB treatment, the parallel use of alternative methods on the same set of samples might help to better evaluate the reliability of the obtained data.

Our results indicated that AAB may play a role in structuring the gut community. In the AAB OTUs distribution in relation to the specimens, a clustering pattern based on the food source was recognized (Fig. 3d), further strengthening the results of the clustering already observed in fig. 3b. Such findings indicate that AAB are primarily involved in the response to the diet, and suggest that they may be directly or indirectly involved in the bacterial community shift following a different diet exposition. We have evaluated the impact of the diet on the bacterial community, without considering the AAB contribution: by excluding AAB OTUs from the analyzed dataset, we found the loss of the previously observed clustering pattern ($p>0.05$; compare Figs. 3b and 3c). Taken together, these data highlight not only the differentiation of the AAB community in response to the diet type, but also indicate that AAB are crucial in determining samples' grouping along with diet variation. It is also noteworthy that the insects reared on the artificial diet originated from the same field population of the fruit-fed insects.

Another variable that could be associated with the distinction of the samples between fruit-fed and artificial diet-fed animals is the presence of *Wolbachia*, but we concluded that it cannot be considered as a driver of the bacterial community modification in this case. Although *Wolbachia* was detected by PCR-DGGE and 16S rRNA barcoding just in fruit-fed samples, the complementary PCR analysis performed for determining *Wolbachia* in the two diet groups, demonstrated its presence in the artificial diet-fed animals. *Wolbachia* is generally considered as intracellular reproductive manipulator, described in many insect species, including different *Drosophila* spp. (Werren *et al*., 2008; McGraw and O'Neill, 2004). The different incidence in samples reared on fruits respect to the artificial diet could be explained by the presence of inhibitory compounds in the artificial diet, hindering or somehow temporarily influencing *Wolbachia* growth. Lack of *Wolbachia* by high throughput sequencing in flies reared on artificial diet could be the result of the number of analyzed insects (n. = 6), since the *Wolbachia* prevalence rate in our *D. suzukii* population has been verified to be 28%. On the other hand, the *Wolbachia* strain associated to *D. suzukii* has been reported to be imperfectly maternally transmitted, showing polymorphic infection (Hamm *et al*., 2014). Moreover, the results could indicate a diversification of infection rates linked to the diet source; indeed, prevalence analysis pointed out a lower infection rate than previously reported in a similar population (Mazzetto *et al*., 2015).

A competition phenomenon between *Asaia* and *Wolbachia* has been described to occur at the level of mosquito gonads (Rossi *et al*., 2015) and *Asaia* has been indicated as responsible for inhibiting *Wolbachia* transmission in mosquitoes (Hughes *et al*., 2014). In this study, we could not observe competition phenomena between AAB and *Wolbachia*. However, no specific investigations have been performed at gonad level. It should be underlined that so far competition has been described only for *Asaia*, a symbiont that has never been described in *D. suzukii* or other *Drosophila* flies.

In conclusion, AAB’s high prevalence in individuals fed on both diet types, their localization and ability to massively recolonize the insect gut indicate that AAB are major components of the *D. suzukii* microbiota and, similarly to *D. melanogaster*, they might play important roles in the physiology and behaviour of the host. The AAB diversity shifts and their weight in determining the clustering behaviour of the bacterial microbiota in relation to diet might indicate their crucial role in determining the microbiota response to diet in *D. suzukii* gut.

EXPERIMENTAL PROCEDURES

Insects. Field-captured larvae of *D. suzukii* emerging from blueberries, raspberries and blackberries in orchards of the Cuneo province, (Piedmont, North-West Italy) in summer 2013 have been reared for at least eight generations in laboratory condition both on fruits (strawberries, blueberries, grapes and kiwi 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 Torino. Insects have been kept in plastic cages (24 × 16 × 12 cm) in a growth chamber at 25 ± 1 °C, 65 ± 5% RH and 16L:8D photoperiod, until collected for analyses (Tab. S1). Bacterial community evaluation was carried out on 2nd-3rd instar larvae, pupae, and 7-20 day-old adults.

Prevalence of AAB and* Wolbachia *and AAB isolation. Prevalences of *Wolbachia* and AAB have been evaluated as described in Method S1. The strategy of isolation was to collect as many AAB isolated colonies as possible according to diversity of colony morphology obtained from different sources (the insect specimens) and different media. A bacterial collection has been obtained and identified as indicated in Method S2. 16S rRNA sequences of representative isolates have been deposited in the ENA database under the accession numbers LN884027-LN884133.

Localization of D. suzukii AAB by fluorescent in situ hybridization (FISH) and colonization experiments with Gfp labelled strains. FISH has been carried out on tissues and organs dissected from mass-reared *D. suzukii* adults in a sterile saline solution. The dissected organs have been fixed for two minutes at 4°C in 4% paraformaldehyde and washed in Phosphate-Buffered Saline (PBS). All hybridization experiment steps have been performed as previously described (Crotti *et al.*, 2009; Gonella *et al.*, 2012), using fluorescent probes, specifically designed for the acetic acid bacterial group (AAB455, sequence GCGGGTACCGTCATCATCGTCCCCGCT) and for *Gluconobacter* (Go15, sequence AATGCGTCTCAAATGCAGTT and Go18, sequence GTCACGTATCAAATGCAGTTCCC). The universal eubacterial probe, Eub338 (sequence GCTGCCTCCCGTAGGAGT), has been used to detect the localization of the overall bacterial abundance and presence in the organs analysed (Gonella *et al.*, 2012). Probes for AAB and Eubacteria have been labelled 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 have been labelled with indodicarbocyanine (Cy5; absorption and emission at 650 nm and 670 nm, respectively). After hybridization, the samples have been mounted in anti-fading medium and then observed in a laser scanning confocal microscope SP2- AOBS (Leica). Hybridization experiments in the absence of probes have been performed as negative controls.

G. oxydans strain DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 have been transformed through electroporation introducing the plasmid pHM2-Gfp (Favia *et al.,* 2007) as described in Method S3. Plasmid stability has been verified for the transformants as reported in Method S4. Recolonization experiments using *G. oxydans* DSF1C.9A(Gfp), *A. tropicalis* BYea.1.23(Gfp) and *A. indonesiensis* BTa1.1.44(Gfp) have been performed as indicated in Method S5.

Characterization of the* D. suzukii *bacterial community through molecular ecology approaches. Immediately after collection larval, pupal and adult individuals of *D. suzukii* have been washed once with ethanol 70% and twice with saline and immediately stored at -20°C in ethanol until molecular analyses. Total DNA has been individually extracted from larvae, pupae and adults by sodium dodecyl sulfate-proteinase K-cethyltrimethyl ammonium bromide (CTAB) treatment, as described in Raddadi *et al.* (2011).

PCR-DGGE has been performed as described in Method S6. The obtained sequences have been deposited in the EMBL database under the accession numbers LN884134-LN884176.

Genomic DNA previously extracted from designated individuals (codes: LF1, LF2, LF3, PF1, MF1, FF2, FF3, MF4, LP1, LP3, PP2, FP1, FP3, and MP3, Tab. S1, Tab. S3) were used in 16S rRNA gene pyrosequencing as described in Method S7. 16S rRNA gene sequences obtained from 16S rRNA gene pyrosequencing analysis have been deposited in European Nucleotide Archive with accession numbers PRJEB10109. The OTU table obtained from 16S rRNA gene pyrosequencing analysis has been filtered and only OTU sequences of AAB have been kept. Statistical significance ($p<0.05$) of sample distribution in different clusters along Axis 1 of PCoA analysis has been examined by t-test using the software GraphPad Prism version 5.03. Heatmap based on the distribution of AAB OTUs has been prepared as described in Method S8.

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TABLE

Table 1. Identification of cultivable bacteria associated to *D. suzukii*. All the isolates showed a percentage of identity >97% in relation to the indicated species.

Isolates	**No. isolates**	**LP**	**PP**	**AP fly**	**AF fly**
Acetobacter tropicalis	1	0	0	0	1
Acetobacter orleanensis/malorum/cerevisiae	4	0	0	0	4
Acetobacter peroxydans	1	0	0	0	1
Acetobacter indonesiensis	10	0	1	1	8
Acetobacter persici	10	0	1	1	8
Acetobacter orientalis	1	0	0	0	1
Acetobacter cibinongensis	1	0	0	0	1
Gluconacetobacter liquefaciens	4	1	3	0	0
Komagataeibacter sp	118	0	0	0	118
Gluconacetobacter/Komagataeibacter sp.	1	0	0	0	1
Gluconobacter kondonii	1	0	0	0	1
Gluconobacter oxydans	12	0	0	0	12
Gluconobacter kanchanaburiensis	5	3	1	1	0
Pseudomonas geniculata	1	0	0	1	0
Serratia sp.	8	2	6	0	0
Micrococcus sp.	5	0	0	0	5
Microbacterium foliorum	2	0	0	0	2
Streptococcus salivarius	1	0	0	1	0
Staphylococcus sp.	12	0	0	0	12
Paenibacillus sp.	2	0	0	0	2
Lactococcus lactis	1	0	0	0	1
Lactobacillus plantarum	1	0	1	0	0
Total	**202**	**6**	**13**	**5**	**178**

LP: larvae fed with artificial diet; PP: pupae fed with artificial diet; AP: Adults fed with artificial diet; AF: Adults fed with fruit diet

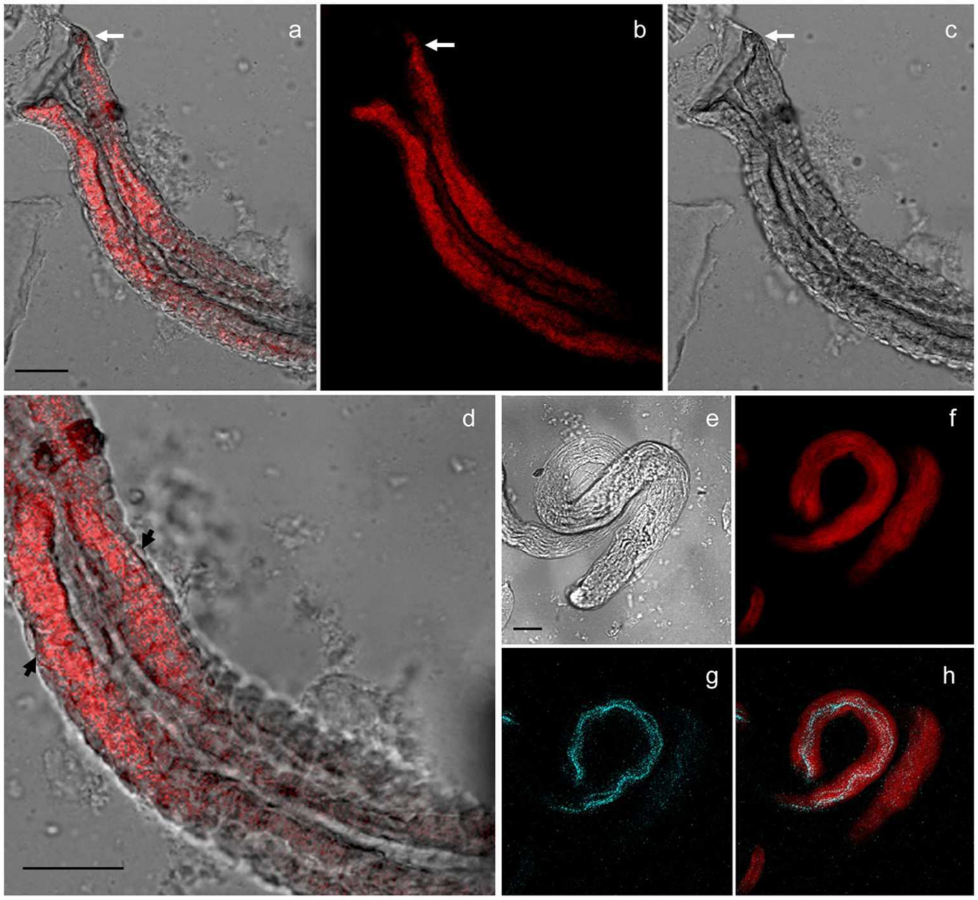
FIGURES

Figure 1. 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 (c) and the FISH (b) pictures of the midgut close to the proventriculus that is indicated by white arrows [for a scheme of the morphology of the initial part of the midgut and the upstream region refer to panel (a) of Figure 3]. (d) Magnification of the image in (b). The massive presence of AAB adherent to the peritrophic matrix (the black line below the first layer of cells indicated by black arrows) is observed. (e-h) FISH of posterior 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.

Figure 2. Colonization of *D. suzukii* foregut and midgut by Gfp-labelled *G. oxydans* DSF1C.9A1 documented by confocal laser scanning microscopy. (a) The scheme represents the first tract of the digestive system and shows the different gut portions highlighted in the next panels. (b-d) Digestive tract portions including the crop, the proventriculus and the first part of the midgut. (c, d) Magnified views of the crop (c) and the proventriculus (d) showed in (b). Masses of fluorescent cells are observed in the crop (arrows). When the fluorescent strain cells reach the proventriculus (d), they colonize the gut part close to peritrophic matrix. (e-f) Interferential contrast (f) and confocal laser scanning (e) pictures of the posterior midgut of *D. suzukii* massively colonized by the *G. oxydans* strain labelled with Gfp. Small hernias (arrowhead) are shown. In some cases, the gelatinous matrix in the hernias present fluorescent cells. Bars = 50 µm.

Figure 3. Bacterial diversity associated with *D. suzukii* by 16S rRNA gene pyrosequencing. (a) 16S RNA gene pyrosequencing describing bacterial communities, at order level, associated with *D. suzukii*.

Names, under histograms, refer to fly specimens; in columns, the relative abundances in percentages of the identified orders are showed. Sequences that did not match with anything in the database are indicated as "Unclassified sequences"; bacterial sequences that have not been assigned to any taxonomical group are indicated as "Bacteria_unclassified"; bacterial orders under 3% representation per sample have been grouped and indicated as "Class. Bac. Orders under 3%". (b) Principal coordinate analysis (PCoA) on the phylogenetic β-diversity matrix on *D. suzukii* samples, considering all the bacterial OTUs. (c) Principal coordinate analysis (PCoA) on the phylogenetic β-diversity matrix on *D. suzukii* samples, considering all the bacterial OTUs, except for the ones belonging to AAB group. Red circle indicates fruit-fed individuals, while blue circles mark specimens fed on the artificial diet. (d) Distribution of AAB in *D. suzukii* hosts. The relative abundance of AAB OTUs, determined at 97% identity, is showed in the heatmap. Coloured scale represents OTUs abundance for each sample (indicated on the vertical axis). In bold are indicated samples from fruit-rearing; the remaining samples are related to artificial diet-fed animals. First letter of codes refers to the fly stage (M: male adult; F: female adult; L: larva; P: pupa); second letter of codes refers to feeding system (F: fruit-based diet; P: artificial diet); third letter of codes is related to subsequent number of samples.



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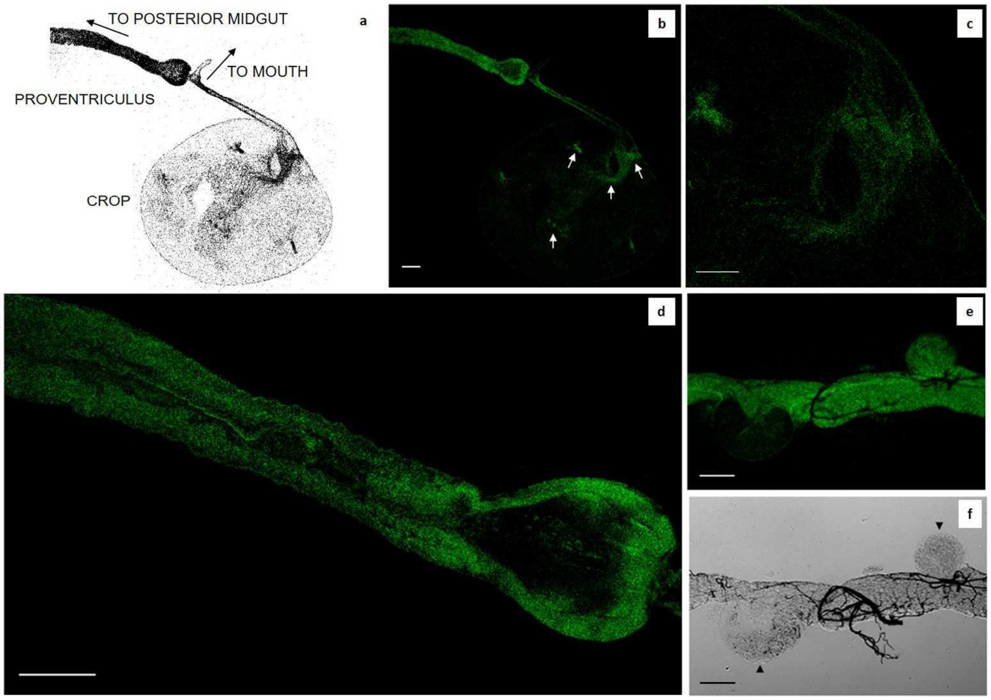

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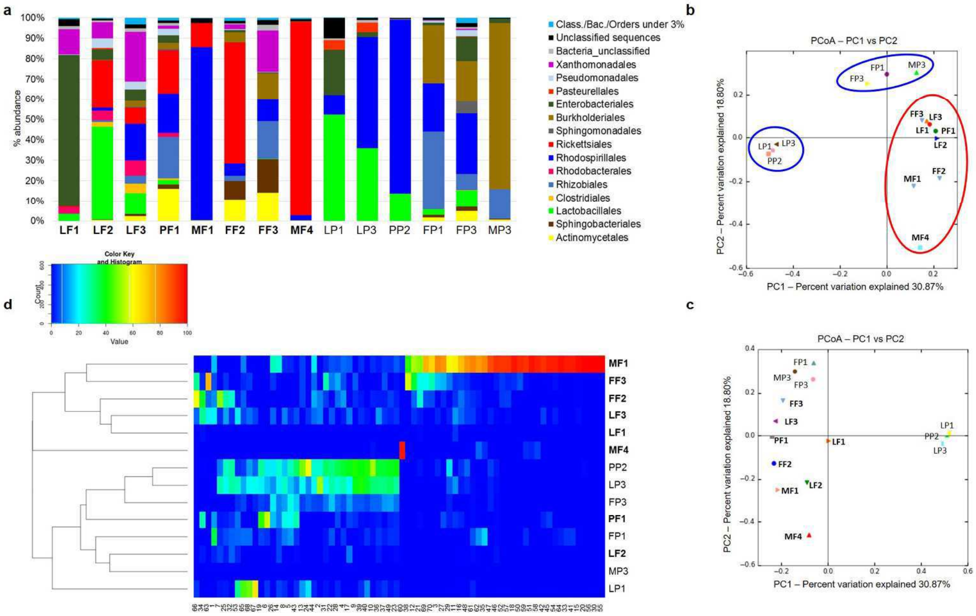

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