Response to Reviewers' comments

Dear Dr. Bonfante,

We are grateful to you and the reviewers for taking the time to review our manuscript. We appreciate the reviewer's comments and advice. They have helped us to improve the content, clarity and impact of the manuscript.
We have restructured the manuscript taking into account the Reviewers suggestions. Such modifications included also the title of the manuscript that has been now modified as "Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria". We have also modified the sequence of presentation of the data and Figure 1 is now Figure 3 in the revised manuscript.
We have addressed reviewers' comments and concerns. In the response to the reviewers' comments, each of the comments are reported in black, followed by our answers in blue.

Reviewer 1:
This manuscript provides a description of the bacteria in the gut of *Drosophila suzukii* of different ages and on different foods. Multiple methods are adopted, including DGGE, 16S pyrosequencing, cultivation and FISH.
The title of the paper suggests that acetic acid bacteria (AAB) may play an important role in structuring the gut community, but this interesting hypothesis is not stated explicitly in the text and is certainly not tested. Consequently, the purpose of this study is uncertain. This uncertainty is compounded by a weak Discussion section that re-iterates results and offers little interpretation. For example, the authors refer to the discrepancy between their results and the results of Chandler et al, but give no explanation for this interesting difference.
Response: We thank the reviewer for the comment that helped us to prepare a more focused manuscript. We acknowledge that the hypothesis indicated by the reviewer is partially tested, so we have modified the manuscript to present what we have actually evaluated, i.e. the impact of the diet on the AAB diversity in the gut. For improving the manuscript to better reflect the experimental results, we have made several substantial modifications. 1) We have changed the title that now reads "Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria". 2) We have better explained in the introduction the aim of the study and the steps of the investigation. 3) To strengthen the discussion, we have cited and discussed papers describing the link between the modification of the diet and the modification of the bacterial community in *Drosophila* spp. (Chandler *et al.*, 2011) and insect models other than drosophilid flies (i.e. Montagna *et al.*, 2014; Colman *et al.*, 2012; Yun *et al.*, 2014). 4) We presented and discussed the results showed by Chandler and colleagues (2014) on the bacterial community of *Drosophila suzukii*, comparing their results to ours and presented possible explanations for differences.

The authors are recommended to pay close attention to the quality of the English. Although the text is intelligible, it includes multiple grammatical errors or poor phraseology. For example, the opening sentence of the Introduction should start with "The" gut microbiome (line 49), "were" should read "have been" at line 52 and so on throughout the text.
Response: We carefully checked the quality of English and modifications and ameliorations have been included in the manuscript.

The authors are also advised to attend to their referencing For example, Chandler 2011 (an original article) and Erkosar 2013 (one of multiple review articles published in recent years) are cited repeatedly, and not always appropriately, in the Introduction; and Chandler 2014 on *D. suzukii* isn¹t mentioned in the Introduction.

Response: We followed the Reviewer comment and checked the enclosed references. Appropriate references are now included. The paper Chandler *et al* (2014) has been cited in the introduction and results have been compared to ours (please, refer to the discussion section).

The figures are unsatisfactory. Figure 1 comprises multiple units that are poorly structured on the page and require considerable zooming in to read. Figures 2 & 3 are micrographs taken at low magnification and without any counterstaining of insect structures (e.g. DAPI for insect cell nuclei) for identification of host structures. The main purpose of these figures is to show the localization of bacteria of interest relative to gut region (crop, proventriculus, anterior midgut ¬ but, curiously, not distal midgut or hindgut) and the peritrophic envelope of the midgut. Unfortunately, many of the detailed interpretations of localization provided in the text cannot be deduced from the figures, requiring the reader to take the authors conclusions on trust.
Response: Figures have been modified as requested; in particular:

- Figure 1 has been modified (please, refer to the response to comment n° 1 of Reviewer 2) and is now marked as Fig. 3.
- The original Figures 2 and 3 are now marked as Figures 1 and 2. In these two figures we actually focused on the midgut, as it has been demonstrated to be a key organ for AAB colonization (Ryu et al., 2008). Our pictures are not only on the anterior part. We show the entire anterior gut (Figure S1a) including the crop (Figures 2b,c and S1a-e), proventriculus (Figures 1a-d, 2b,d and S1a), and the anterior (Figures 1d, 2d and S1a) and posterior (Figures 1e-h, 2e-f and S1) midgut. We realized that it was not appropriately specified in the figure captions. So we added details to explain that we aim to provide a full overview of the midgut, including in the novel Figures 2 and S1 explanations of the different sections of the gut. Concerning the picture quality, they were meant to allow to distinguish the bacterial distribution along the midgut, more than focusing on single tissue structures. For the purpose of identifying the different regions of the anterior gut to the end of the midgut, we show in the figures the interferential contrast images (Figures 1 and 2). We believe that the details provided, along with the superposition of fluorescence results and interferential contrast micrographs, adequately show the organ positions and localization of AAB. In the case of recolonization experiments, we did not attempt a DAPI staining because it could have hampered the vitality of Gfp-expressing bacterial cells.

Line 154 The difference in prevalence of *Wolbachia* in flies of different provenance should be tested by chi-squared. A t-test is not an appropriate test.
Response: We are grateful for this comment. We realized that the t-test was actually not the most suitable in this case. As we are not testing our results against an expected prevalence, we decided to perform a binomial GLM test, which is considered an appropriate one to compare infection rates (e.g. García-Munguía et al., 2011).

Reviewer 2:
The paper by Vacchini et al. provides results of a thorough analysis of the microbiome of *Drosophila suzukii* with an emphasis on acetic acid bacteria. It has a good hypothesis, uses sound scientific tools, reports strong and new results, provides a reasonable discussion and is generally well written and clear. However, it could be improved if the following points would be addressed.

1. The presentation of the deep sequencing results need to be improved. First, the description of Figure 1 states that "Names, under histograms, refer to fly specimens". The fact that the meaning of these names is mentioned only in the supplementary material makes it hard to follow. I suggest giving the samples meaningful names along the line of AF, AP used in Table 1 and make the connection to sample description in the sup. table. Second, Figure 1a is not very useful, as it is hard to read and understand. For example, the figure description says "identified orders", but in the graph

legends there are names such as "Proteobacteria" and "Bacteroidetes" which are phyla. Obviously "unclassified betaProteobacteria" should have been under Proteobacteria etc.. The fact that unclassified bacteria are separated under different categories (with "unclassified bacteria" as a different color from "unclassified") is also confusing. Finally, in the Results part (page 6, lines 127-132) the distribution of bacterial genera and families is discussed, but the data are not shown. Consider producing a separate graph of genera of AAB.

Response: According to the comments of Reviewer #1 we have modified the sequence of data presentation so that Figure 1 is now Figure 3 in the revised manuscript. We improved the presentation of mass sequencing data as suggested by the Reviewer (Fig. 3a). We checked and corrected the graph legend; the modifications can be found in the caption of Fig. 3. To simplify the graph and obtain a better visualization of the results, bacterial orders under 3% reads per sample have been grouped and indicated as "Class./Bac./Orders under 3%" and the appropriate explanation has been included in the figure legend. Sequences that did not match with anything in the used database are indicated as "Unclassified sequences", while bacterial sequences that have not been assigned to any taxonomic group are indicated as "Bacteria unclassified".

According to the suggestion, we modified the sample names along the line of AF, AP. New codes are explained in the captions of Fig. 3 and Supporting Fig. S3.

We prepared and included into supplementary material a figure related to the prevalence of AAB genera by 16S rRNA pyrosequencing (Supporting information S3).

2. The localization experiments with at least two of the Gfp-transformed bacteria (*A. tropicalis* and *A. indonesiensis*) were conducted on bacteria-free insects (Page 9, lines 199-203; to my understanding ALL experiments were done under antibiotic treatment). I think it strongly reduces the power of the results, and suggest that if the experiments with *G. oxydans* where done without kanamycin, only these results will be reported. Otherwise the Gfp results should be taken very carefully and that point should be mentioned in the discussion.

Response: We performed all the colonization experiments under the antibiotic pressure of kanamycin, as indicated at lines 205-206. This procedure is necessary in case of Gfp-labelled strains obtained by the insertion in the bacterium of a plasmid carrying the Gfp. The maintenance of strains under antibiotic selection prevents the loss of plasmid from the bacterial cells, as indicated in the paper by Favia *et al.* (2007). In other publications, appropriate antibiotics have been administered in recolonization trials of a variety of different insect hosts (in case of fluorescent proteins carried on plasmids) to avoid the plasmid loss. Here, few recent examples are indicated: The et al, 2016; Battisti et al., 2015; Chavshin et al. 2015; Capone et al., 2013; Chavshin et al., 2013.

With this kind of experiments, we aimed to investigate the capability of the indigenous strains to efficiently recolonize the insects and what portions of the gut they are capable to colonize after ingestion. We were not aimed at measuring their abundance after colonization trials or their relationship with the other components of the microbiota. Indeed, we observed the capability of the strains to move into the digestive tract after oral administration. Furthermore, the Gfp fluorescence data are confirmed by the FISH data (as in the case of the proventriculus). Gfp data, taken together with results of fluorescent *in situ* hybridization, strengthen the concept that AAB are inhabitants of different portions of the insect digestive system.

However, we acknowledge the usefulness of the comment of reviewer #2 on the use of antibiotics. In the discussion section, we added a specific part with comments on the issue that the use of antibiotic could have a negative side effect on the insect host and on the other gut symbionts. We specify that further studies have to be performed to understand if the used concentration of antibiotic could be detrimental for the host and/or the whole bacterial community and to verify if bacteria-free insects could derive following this treatment. Up to now, many bacteria recolonization studies in the literature used our procedure, i.e. to administer labelled bacteria under antibiotic pressure, unless plasmid stability has been previously verified and guaranteed for an appropriate number of bacterial generations or when strains are labelled on the chromosome.

Additionally, the choice of these specific bacterial isolates need to be explained, as they are not necessarily the most abundant or influential ones.
Response: For the insect colonization experiments, we decided to label AAB strains with a plasmid carrying the Gfp-cassette. Thus, among the isolates in the collection we selected some representatives of *Acetobacter*, *Gluconobacter* and *Komagataeibacter* genera to be used in electroporation assays with appropriate competent cells. Since they are environmental strains, transformation procedure was not always successful: we succeeded in the transformation of strains *G. oxydans* DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44, whereas no *Komagataeibacter* strains labelled with Gfp-plasmid have been obtained in spite of the several attempts that have been made.

3. All confocal studies should be accompanied with the negative controls.
For the very least it should be mentioned in the "Results" that no auto-fluorescence was seen when probes and GFP labeled bacteria were not used.
Response: Negative controls were included in all confocal studies. We added a sentence in the Results specifying the absence of auto-fluorescence signals.

4. In the Material part More details are needed in the part where the characterization of the fly bacterial community is discussed. It says there (page 14, line 314) that insects were collected, but details such as how long the colonies were kept in the lab before the analysis are missing.
Response: We added more details on the flies used for our study.

5. Language editing is recommended.
Among other things I think "e.g." is erroneously used.
Response: We carefully checked the quality of English as also suggested by Reviewer 1. We checked the use of “e.g.”

Reviewer 3:
Vacchini and colleagues report on the gut bacterial diversity, and its shifts, of the spotted wing fly, *Drosophila suzukii* in response to diet (natural fruit-based vs artificial non-fruit) using 16S rRNA gene next generation sequencing approaches. The data suggest diet affects the structure of the communities and this is probably due to acetic acid bacteria (AAB) which present high prevalence in insects reared in both diets. The authors also used cultivation-dependent approaches to isolate AAB as well as FISH to localize them at different developmental stages. Recolonization experiments confirmed previous studies in other insect species that AAB are able to massive colonize insect gut. Overall this is an interesting study.

The manuscript can be significantly improved if it’s edited by a native English speaker. In addition, it can be shortened to become a more concise manuscript.
Response: We carefully checked the quality of English. A native English speaker has revised the manuscript and the manuscript has been shortened where possible.

1. The authors used a single pair of primers in the 16S rRNA gene pyrosequencing analysis. There are several studies suggesting that the use of a single pair may not provide a representative view of the structure of a bacterial community. It would be nice if the authors discuss the potential limitations of the use of the pair 27Fmod and 519Rmodbio, if any, taking into consideration of previous studies on insect associated bacterial communities. It would also be nice if they discuss the possibility that their DNA extraction method (CTAB) might have introduced a bias in their results?

Response: We have now included in the manuscript comments about the use of a single pair of primers in 16S rRNA gene pyrosequencing and about the possibility that DNA extraction method might have introduced biases.
In order to analyze the bacterial community of *Drosophila* flies by the use of next generation sequencing techniques, different regions on 16S rRNA gene could be selected. In case of *D. suzukii*, there is just one report in literature about the characterization of bacterial DNA using an Illumina platform (Chandler *et al.*, 2014). In this paper, the target of the analysis was the V4 region of 16S rRNA gene with primers 515F and 806R (Chandler *et al*., 2014). In our work, we decided to target the V1-V3 region by 16S rRNA gene pyrosequencing. It is well known that the selection of primers may influence the profile of a bacterial community analyzed by sequencing, but the extent of the bias on mass sequencing methodologies has not been completely elucidated (Kumar *et al*., 2011). Kumar and coworkers performed a study on the bacterial community associated to a specific host tissue, i.e. the subgingival plaque of smokers, to investigate the bias introduced by target region selection and by primer degeneracy in comparison to accurate Sanger sequencing. They found that the use of specific region for pyrosequencing allowed the detection of greater numbers of species than by Sanger sequencing and that did not led a significant difference in the number of rare and abundant taxa detected. They verified that concatenated data from community fingerprints obtaining by the use of V1–V3 and V7–V9 primers gave results similar to the ones obtained with Sanger sequencing (Kumar *et al.*, 2011). In our work one of the two abovementioned regions has been used for 16S rRNA gene pyrosequencing, i.e. V1–V3 region; this region has been used also to examine the bacterial community from other insects (Montagna *et al.*, 2015).
To notice, it is that in our work two different cultivation-independent methods, i.e. PCR-DGGE and 16S rRNA gene pyrosequencing, characterized by different analytical power, have been employed; while the former targeted V3-V5 region, the latter targeted V1-V3 region. In both cases, AAB resulted an abundant group.

In relation to DNA extraction method, samples used for deep sequencing analysis have been extracted by sodium dodecyl sulfate-proteinase K-CTAB treatment, as described in Raddadi *et al.* (2011). CTAB method is a widely used and efficient method for DNA extraction. Indeed, it has been used for DNA extraction from a huge variety of samples, including insect samples (Chen *et al.*, 2010) or "difficult" samples such as recalcitrant plant (Healey *et al*., 2014). It is a cost-effective method with a high performance in terms of results. However, the use of different DNA extraction methods on the same set of samples might help to better evaluate the reliability of the obtained data.
We want also to underline that, before the application of 16S rRNA gene pyrosequencing technique, we checked the presence and quality of bacterial DNA in the extraction product by direct PCR amplification of the 16S rRNA gene using universal bacterial primers, and not only by measuring DNA concentration spectrophotometrically and by gel electrophoresis. To mention it is that high DNA yield from the extraction would not correspond to a high amount of bacterial DNA as, of course, the insect genome is always co-extracted (Prosdocimi *et al.*, 2015).

2. What's the gender of the adults used in the bacterial diversity experiments? Did you notice any differences associated with gender? What was their age?
Response: Please, find below a table describing the gender of adults used in 16S rRNA gene pyrosequencing studies.

Sample name	gender
MF1 (DS41)	male
FF2 (DS54)	female
FF3 (DS55)	male
MF4 (DSM)	female
FP1	female

FP3	female
MP3	male

We did not notice any difference linked to insect gender both considering AAB or *Wolbachia*. Concerning the age, we decided to use mature adults instead of newly emerged flies: adults used in the experiments were about 7-20 days old. Preliminary experiments carried out with younger and older adults within this range showed no apparent difference in the microbial community composition, hence we pooled the results.

3. Lines 80-83: rephrase.
Response: We modified the text.

4. Line 102: introduce the DGGE-PCR before you start about bands.
Response: We modified the text as suggested.

5. Lines 109-110: use "16S rRNA gene next generation sequencing or 16S rRNA gene pyrosequencing" throughout the text, tables and figures.
Response: We modified the text and used 16S rRNA gene pyrosequencing.

6. Lines 133-135 and throughout the next with reports to *Wolbachia*: (a) what's the gender of the adults tested? (b) density might play an important role particularly if there are competitive exclusion phenomena between *Wolbachia* and AAB. If DNA still exists in the samples used, I would suggest the authors to evaluate the *Wolbachia* density and see if the presence / density of this symbiont affects the structure of the bacterial communities.
Response:

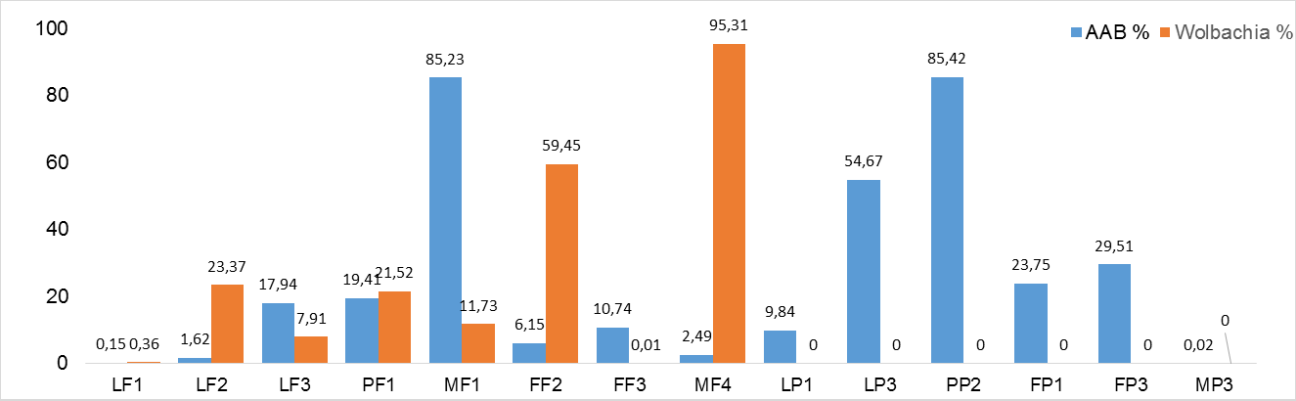
a) In order to know the gender of the insect adults, please check the response number 2. In the manuscript the gender of the insect individuals examined can be deduced from the letter code of the sample that is explained in the caption of Figure 3.
b) Regarding densities of *Wolbachia* and AAB, unfortunately the DNA from the samples have been already consumed in the different experiments and it is not possible to carry out quantitative PCR for establishing the densities of the two bacterial groups. In the absence of this possibility, we further checked the pyrosequencing data to compute the relative number of reads of the two groups in the different samples. We included below a table summarizing the abundance of the abovementioned taxonomical groups in the different insects by 16S rRNA gene pyrosequencing.

Tab. R1. Abundance in percentage of AAB or *Wolbachia* (Wol) for each specimen.

	LF1	LF2	LF3	PF1	MF1	FF2	FF3	MF4	LP1	LP3	PP2	FP1	FP3	MP3
AAB %	0.15	1.62	17.94	19.41	85.23	6.15	10.74	2.49	9.84	54.67	85.42	23.75	29.51	0.02
Wol %	0.36	23.37	7.91	21.52	11.73	59.45	0.01	95.31	0.00	0.00	0.00	0.00	0.00	0.00

Please, find also below Fig. R1, a graph corresponding to the values showed in the above table to better visualize a possible correlation between *Wolbachia* and AAB abundance.

Fig. R1. Abundance in percentage of AAB or *Wolbachia* for each specimen.



Above each histogram the abundance (in percentage) of AAB (in blue) and *Wolbachia* (in orange) is indicated. Y axis: abundance in %.

According to this figure, in general we could not observe any evident correlation between *Wolbachia* and AAB densities, as otherwise reported in case of mosquitoes (Rossi *et al*., 2015). In *Anopheles* and other mosquitoes, it was indeed demonstrated a competition phenomenon that occurs in the gonad niche where a negative interference between the acetic acid bacterium *Asaia* and *Wolbachia* has been found. Moreover, it has been found that the native bacterial community of mosquitoes inhibits *Wolbachia* transmission (Hughes *et al*., 2014): in particular, Hughes and co-workers (2014) demonstrated that the acetic acid bacterium *Asaia* is responsible for inhibiting *Wolbachia* transmission.

In our case, in relation to the analysed samples, we could not observe any competition phenomenon between AAB and the reproductive manipulator *Wolbachia*, but no investigations have been performed at gonad level. To notice is that, up to now, the competition phenomena that involve *Wolbachia* have been described only for the acetic acid bacterium *Asaia* that has never been described as a symbiont of *Drosophila suzukii* or *Drosophila* spp., in general. Also in our work *Asaia* has not been detected in *D. sukuzii* specimens.

7. Line 173: not four, seven species.
Response: We modified the text.

8. Line 174: it should be A. indonesiensis.
Response: We corrected as suggested.

9. Line 184: remove “.”after sequencing
Response: We changed it.

10. Line 275: there are many other more appropriate references for the role of Wolbachia as reproductive manipulator in Drosophila species.
Response: We modified the references included in the manuscript. We included Werren *et al*., 2008 and McGraw and O’Neil, 2004.

11. Lines 304-311: the last paragraph is weak, not really relevant to the main topic of the manuscript; so, I would suggest the authors remove it because it doesn¹t really provide much.
Response: We removed it.

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