

1    **Olfactory attraction of *Drosophila suzukii* by symbiotic acetic acid bacteria**

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18    **Author contributions**

19    FM, EG, EC, SM and AA conceived of and designed the research. FM, VV, MS and MP conducted  
20    the experiments. SM and DD contributed materials and tools and FM analyzed the data. FM and EG  
21    wrote the manuscript. All authors read and approved the manuscript.

22

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25

## 26    **Abstract**

27    Some species of acetic acid bacteria (AAB) play relevant roles in the metabolism and physiology of  
28    *Drosophila* spp. and in some cases convey benefits to their hosts. The pest *Drosophila suzukii*  
29    harbors a set of AAB similar to those of other *Drosophila* species. Here, we investigate the potential  
30    to exploit the ability of AAB to produce volatile substances that attract female *D. suzukii*. Using a  
31    two-way olfactometer bioassay, we investigate the preference of *D. suzukii* for strains of AAB, and  
32    using gas solid phase microextraction chromatography-mass spectrometry we specifically  
33    characterize their volatile profiles to identify attractive and non-attractive components produced by  
34    strains from the genera *Acetobacter*, *Gluconobacter* and *Komagataeibacter*. Flies had a preference  
35    for one strain of *Komagataeibacter* and two strains of *Gluconobacter*. Analyses of the volatile  
36    profiles from the preferred *Gluconobacter* isolates found that acetic acid is distinctively emitted  
37    even after two days of bacterial growth, confirming the relevance of this volatile in the profile of  
38    this isolate for attracting flies. Analyses of the volatile profile from the preferred *Komagataeibacter*  
39    isolate showed that a different volatile in its profile could be responsible for attracting *D. suzukii*.  
40    Moreover, variation in the concentration of butyric acid derivatives found in some strains may  
41    influence the preference of *D. suzukii*. Our results indicate that *Gluconobacter* and  
42    *Komagataeibacter* strains isolated from *D. suzukii* have the potential to provide substances that  
43    could be exploited to develop sustainable mass-trapping-based control approaches.

## 45    **Keywords**

46    Spotted wing drosophila, Insect symbionts, Olfactometer bioassays, Volatile profile analysis,  
47    *Gluconobacter*, *Komagataeibacter*

## 49    **Key Message**

- Environmentally friendly strategies for the management of *D. suzukii*, like mass trapping, could benefit from identifying new efficient and specific lures to improve traps designed to control this pest.
- This work demonstrated that different acetic acid bacteria isolated from *D. suzukii* have attractive effects on female flies which may be exploited for bait development.
- Many of volatile substances produced by these strains appear to have essential roles in modulating *D. suzukii* preference as well.

## 58    **Introduction**

59    Acetic acid bacteria (AAB) are Gram-negative bacteria belonging to the family Acetobacteraceae  
60    within the class Alphaproteobacteria. Their taxonomy, molecular biology and physiology have been  
61    scrutinized because of their importance in commercial food and chemical compound production  
62    (Raspor and Goranovič 2008). AAB are pervasive in the environment and easy to isolate from  
63    various plants, flowers, fruits and garden soil (Raspor and Goranovič 2008; Crotti et al. 2010).  
64    Although some strains are spoilage agents of wine and beer and others cause plant diseases  
65    (Rohrbach and Pfeiffer 1975; van Keer et al. 1981; du Toit and Pretorius 2000; Bartowsky et al.  
66    2003), numerous studies have also established symbiotic associations between AAB and insects that  
67    feed on sugar-based diets, specifically those belonging to the orders Diptera, Hymenoptera and  
68    Hemiptera (Crotti et al. 2010). Model species from the genus *Drosophila*, *Drosophila melanogaster*  
69    Meigen and *Drosophila simulans* Sturtevant, host several AAB strains, but predominantly those  
70    belonging to the genera *Acetobacter* and *Gluconobacter* (Cox and Gilmore 2007; Ren et al. 2007;  
71    Chandler et al. 2011; Wong et al. 2011; Kim et al. 2012; Staubach et al. 2013; Wong et al. 2013);  
72    strains belonging to the genera *Gluconacetobacter* and *Commensalibacter* have also been isolated  
73    from some *D. melanogaster* populations (Roh et al. 2008).  
74    The insect midgut is a favorable niche for growth of AAB because of the availability of  
75    carbohydrate-rich food in an aerobic, acidic environment. Meanwhile, AAB can convey numerous  
76    advantages to their hosts, such as improving their digestive opportunities or by positively  
77    influencing larval development (Crotti et al. 2010; 2011; Chouaia et al. 2012). Some AAB are  
78    implicated in maintaining the immune homeostasis or increasing the lifespan and fitness of their  
79    hosts (Ryu et al. 2008; Shin et al. 2011), while others could be involved in defense against other  
80    harmful microorganisms or may participate in cell-to-cell communication (Crotti et al. 2010).  
81    Therefore, the relationship between AAB bacteria and their hosts is considered to be mutually  
82    symbiotic.

83 AAB produce a number of volatile compounds as secondary metabolites in addition to acetic acid  
84 (Raspor and Goranovič 2008), some of which may attract host insects and facilitate the ingestion of  
85 bacteria, as reported for other symbionts (Davis et al. 2013). *Pseudomonas putida* has been shown  
86 to produce volatiles that attract the olive fly, *Bactrocera oleae* Gmelin (Liscia et al. 2013), and  
87 numerous bacteria have been shown to produce volatiles that attract the Oriental fruit fly,  
88 *Bactrocera dorsalis* (Hendel), and the Mexican fruit fly, *Anastrepha ludens* (Loew) (Jang and  
89 Nishijima 1990; Robacker et al. 1998). Similarly, a recent study on yeasts isolated from the larval  
90 frass and adult midguts of spotted-wing drosophila, *Drosophila suzukii* Matsumura, and their fruit  
91 food source, found a specific association between the flies and some yeasts with a preference for  
92 *Hanseniaspora uvarum* (Scheidler et al. 2015). The volatile compounds produced by mutualistic  
93 microorganisms living inside host insects that have a symbiotic relationship with plants might  
94 trigger their trophic interaction (Frago et al. 2012). In the case of pest insects, exploiting the  
95 relationships between bacteria, their hosts and plants may be a useful tool for developing  
96 sustainable control strategies.

97 *Drosophila suzukii*, recently introduced from Asia (Asplen et al. 2015), is currently one of the most  
98 serious threats to fruit production in Europe and North America. It was found to host several strains  
99 of AAB (Chandler et al. 2014) and their presence were also detected in a recent companion study  
100 conducted by Vacchini et al. (submitted). However, the attractiveness of the bacterial volatiles has  
101 not yet been investigated. Here, we use a two-way olfactometer to assess the preference of flies for  
102 AAB symbionts versus the control. We then characterize their volatile profiles by gas  
103 chromatography-mass spectrometry analysis. This survey could allow to evaluate differences in fly  
104 preference among strains and species of symbiotic AAB; along with identifying volatiles emitted by  
105 attractive bacteria. These compounds may be very useful for sustainable mass-trapping *D. suzukii*  
106 management programs.

107

## 108 **Materials and methods**

## 109 **Insect material and bacterial strains**

110 In the summer of 2014, we collected *D. suzukii* larvae on blueberries, raspberries and blackberries  
111 in orchards of the Cuneo and Torino provinces of Piedmont (NW Italy). Emerged insects were  
112 reared in plastic cages (24×16×12 cm) containing different types of fruit (strawberries, blueberries,  
113 grapes, bananas and kiwi fruits) at the Dipartimento di Scienze Agrarie, Forestali e Alimentari  
114 (DISAFA) in a growth chamber at 25±1°C, 65±5% RH and a 16 h:8 h L:D photoperiod.

115 We selected AAB strains based on previous characterizations of the isolates by Vacchini et al.  
116 (submitted), including the most commonly found genera in Italian populations of *D. suzukii*: two  
117 isolates from the genus *Acetobacter* (*A. persici* DS4MR.45 and *A. cibinongensis* DS5FR.4), two  
118 isolates from the genus *Gluconobacter* (*G. oxydans* DS1FC.9A and *G. kanchanaburiensis*  
119 L2.2.A.15) and two isolates from the genus *Komagataeibacter* (DS2MC.114 and DS1MA.65A).  
120 Universal bacterial 16S rRNA gene primers 27F (5'-TCG ACA TCG TTT ACG GCG TG-3') and  
121 1495R (5'-CTA CGG CTA CCT TGT TAC GA-3') were used to amplify the 16S rRNA gene from  
122 the 2 strains *K. hansenii* and *K. saccharivorans*, as previously described (Mapelli et al. 2013). Near-  
123 full-length sequencing 16S rRNA was performed and consensus sequences were compared to the  
124 public databases at the National Center for Biotechnology Information using BLASTn (Altschul et  
125 al. 1990); near-full-length 16S rRNA sequences were deposited in the European Nucleotide  
126 Archive's database under the accession numbers LN901337 and LN901338.

## 127 **Two-way olfactometer bioassays**

128 Selected AAB strains were tested against the control (sterile growth medium) with a two-way  
129 olfactometer assay to evaluate the preferences of *D. suzukii*. Isolates were cultured on liquid MA  
130 medium (10.0 g/l glucose, 10.0 g/l glycerol, 10.0 g/l meat peptone, 5.0 g/l yeast extract and 1%  
131 ethanol) for 24 hours at 30°C. Cells were harvested following centrifugation (10 min, 3000 g) and  
132 adjusted to a concentration of 10<sup>8</sup> cells/ml; 100 µl of the bacterial suspensions were then plated in  
133 plastic flasks containing 20 ml of solid MA (obtained by adding 1.5% agar) and grown at 30°C for  
134 24 or 48 h. The comparison between two sterile MAs serving as a blank was also performed.

135 Olfactometer assays were conducted following Mazzetto et al. (2015). The olfactometer consisted  
136 of a plastic box (24×16×12 cm) covered with a fine mesh net on the top, and a layer of wet cotton  
137 on the base to promote humidity. On the bottom of the box, there were two holes (31 mm diameter)  
138 closed by silicon plugs. Two glass funnels (46 mm diameter) were fitted in these plugs and each  
139 was inserted into a 250 ml glass flask placed below the box. An air pump (Air 275R, Sera,  
140 Heinsberg, Germany) was used to supply the air necessary for the trials. Pumped air was humidified  
141 and split into two 5 mm diameter silicon tubes, each entered first into a plastic flask (125 ml)  
142 containing the strain or the sterile MA. The exit air, which was enriched with the volatile  
143 compounds, was provided by another silicon tube (same diameter) into the glass flask through a  
144 separate hole created in the plug, close to the funnel. The glass flasks acted as traps, and the flies  
145 could not escape once they had entered.

146 The experiment was conducted in a climatic chamber (25±1°C, 65±5% RH). At the beginning of the  
147 experiments, illuminance (9 lux) was measured with a luxmeter (PCE-172, PCE Group, Lucca,  
148 Italy) and the rate of airflow (0.25 l/min) was measured with a digital anemometer (TA-410, PCE  
149 Group, Lucca, Italy) at the downwind end. For each trial, 2–10-d old *D. suzukii* females were  
150 separated from males according to the external genitalia (Hauser 2011) and females were starved on  
151 1.5% agar (15 ml) for 24 h inside into a plastic tube (30 diameter, height 114 mm). Seventy females  
152 were then introduced to the center of the olfactometer box through a small hole created in the  
153 middle of the net and closed with a plug. After 24 h, we counted the number of flies in the box (no  
154 choice) and in each of the two flasks (one containing the volatiles of AAB strain and the other  
155 containing the volatiles of the sterile MA). Nine replicates at 24 and 48 h of bacterial growth were  
156 carried out; nine replicates of comparisons between two MA controls were assessed too. All flasks,  
157 funnels, plugs and tubes were cleaned with neutral soap and distilled water and sterilized in an  
158 autoclave; the box and the net were cleaned with neutral soap, distilled water and ethanol (70%  
159 v/v). The numbers of flies in the test trap, control trap and those remaining alive in the cage (about  
160 90%) were compared by a Friedman-ANOVA followed by Wilcoxon signed-rank tests with a



161 Bonferroni correction factor ( $P < 0.05$ ). Statistical analyses were performed through SPSS Statistics  
162 22 (IBM Corp. Released 2013, Armonk, NY).

163

#### 164 **Volatile profile analysis**

165 After testing the preference of flies for cultured AAB strains versus the control, volatile profiles  
166 produced by these isolates were studied. Before the analysis of volatiles, bacteria were grown at  
167 30°C in liquid MA medium. The cells were adjusted to  $10^8$  cells/ml as explained above and then  
168 incubated on Petri dishes containing solid MA at 30°C for 24 or 48 hours.

169 Prior to analyses, 20-ml glass vials (Supelco Inc., Bellefonte, USA) were filled with 4 g of solid  
170 MA + bacteria and 4 g of NaCl and crushed with a spoon. The vials were closed with PTFE septa  
171 and open-top polypropylene (Supelco) caps. Sterile solid MA and 4 g of NaCl, crushed in the vial  
172 was also used as control. The samples were shaken for 2 min at 50°C to accelerate equilibrium of  
173 headspace volatile compounds between the solid matrix and the headspace. Volatile compounds  
174 were extracted by solid-phase microextraction (SPME) by inserting a carboxen-  
175 polydimethylsiloxane fiber (black, 75- $\mu$ m-thick film, 23-Ga needle, Supelco, Bellefonte, PA, USA)  
176 for 30 min at 50°C. After extraction, samples were desorbed into a CIS-4 programmed temperature  
177 vaporization injector (Gerstel, Mülheim an der Ruhr, Germany). The volatile compounds were  
178 analyzed using an HP 6890 Series gas chromatography (GC) system equipped with a capillary  
179 column (DB-5MS, 30 m  $\times$  0.250 mm, 0.25- $\mu$ m-thick film). Helium gas was used as the carrier gas at  
180 a constant flow of 1.2 ml/min. Oven temperature of the GC was programmed for a 29.33 min total  
181 running time. From an initial temperature of 35°C, the temperature was increased at a constant rate  
182 of 5°C/min up to 100°C and then 15°C/min up to 300°C where it was held constant for 1 min. A HP  
183 5973 Mass Selective Detector (Hewlett-Packard, Wilmington, NC, USA) connected with the GC  
184 system was operated in electron impact mode with an electron impact energy of 70 eV. GC-MS data  
185 were processed with the MSD-Chemstation software (Agilent Technologies). Volatile compounds  
186 were initially identified by comparison of chromatographic retention times and mass spectra with

187 the WILEY6N.L and NIST98.I databases and only those showing match quality higher than 75%  
188 were considered for analyses. Next the volatiles were identified by comparison with authentic  
189 standards (1 µl/ml concentrated) in 10 ml of distilled water in glass vials (20 ml) capped with a  
190 Teflon-lined septum and analyzed with GC-MS. Alkanes (C<sub>5</sub> to C<sub>18</sub>) were also run with 4 g of solid  
191 MA + 4 g of NaCl to calculate retention indices (RI) for the volatiles.

192 Six replicates were performed for each strain and for the control (three replicates after 24 h growth  
193 and three replicates after 48 h) and the mean percentage of each compound found according to the  
194 total peak area integrated by the analysis program in the three replicates of each strain was  
195 calculated.

196

## 197 **Chemicals**

198 Ethanol (Chem-Lab, ≥99.8%) acetic acid (Acros Organics, 99.5%), 2-propanol (Acros Organics,  
199 99.5+%), 2-propanone (Acros Organics, 99.8%), 2-methylpropanoic acid (Acros Organics, 99+%),  
200 2-methylbutanoic acid (Acros Organics, 98%), 3-methylbutanoic acid (Acros Organics, 99%),  
201 benzaldehyde (Acros Organics, 98+%) and acetaldehyde (Acros Organics, 99.5%). The alkanes:  
202 pentane (Acros Organics, 99+%), hexane (Sigma-Aldrich, ≥97.0%), heptane (Sigma-Aldrich,  
203 ≥99%), octane (Sigma-Aldrich, 98%), nonane (Sigma-Aldrich, 99%), decane (Sigma-Aldrich,  
204 ≥99%), undecane (Sigma-Aldrich, ≥99%), dodecane (Sigma-Aldrich, ≥99%), tridecane (Acros  
205 Organics, 99+%), tetradecane (Sigma-Aldrich, ≥99%), pentadecane (Sigma-Aldrich, ≥99%),  
206 hexadecane (Acros Organics, 99%), heptadecane (Sigma-Aldrich, 99%) and octadecane (Sigma-  
207 Aldrich, ~99%).

208

## 209 **Results**

### 210 **Identification of volatile-producing AAB strains**

211 To perform the two-way olfactometer bioassays experiments, we selected six AAB strains, two  
212 isolates from the genus *Acetobacter* (*A. persici* DS4MR.45 and *A. cibinongensis* DS5FR.4), two

213 from the genus *Gluconobacter* (*G. oxydans* DS1FC.9A and *G. kanchanaburiensis* L2.2.A.15) and  
214 two from the genus *Komagataeibacter* (DS2MC.114 and DS1MA.65A). Sequencing of near-full-  
215 length 16S rRNA gene of the two isolates from *Komagataeibacter* genus was performed to obtain  
216 more information on their taxonomic identification. Results indicated that DS2MC.114 and  
217 DS1MA.65A showed 99% identity with *Komagataeibacter hansenii* and 100% identity with  
218 *Komagataeibacter saccharivorans*, respectively.

219

## 220 **Two-way olfactometer bioassays**

221 Results of the two-way olfactometer bioassays, statistical analyses with significant differences and  
222 the rate of no choice are reported in Table 1 and Figures 1-2.

223 First, we tested the response of *D. suzukii* to two identical stimuli (sterile MA) and found a high  
224 percentage of no choice in nine replicates and no difference between the two flasks. Thereafter, each  
225 AAB isolate was compared with the control. Flies showed a significant preference for *G. oxydans*,  
226 *G. kanchanaburiensis* and *K. saccharivorans* strains over the control (sterile MA) after both 24 and  
227 48 h of growth (Figs. 1-2). Moreover, response to these strains had the lowest percentages of no  
228 choice after 24 and 48 h of bacterial growth; the strain of *K. saccharivorans* had the lowest rate of  
229 no choice (Table 1). No significant difference was found in the comparison between *A. persici* strain  
230 and the control in the first 24 h of growth, accompanied by a high percentage of no choice;  
231 however, after 48 h of bacterial growth, flies significantly preferred the control and the rate of no  
232 choice decreased. No preference was found for the two remaining strains after 24 or 48 h, with a  
233 high percentage of no choice: the rate of no choice was around 50% for *K. hansenii* strain and over  
234 60% for *A. cibirnogensis* strain (Figs. 1-2).

235

## 236 **Volatile profile analysis**

237 The volatile profiles of the bacterial strains and the control included alcohols, ketones, carboxylic  
238 acids and aldehydes (Table 2). The analysis of compounds conducted on 24- and 48-h-old sterile

media for the control confirmed a constant emission of ethanol (66-70%), 2-propanol (~ 30%) and benzaldehyde (0.2%) (Table 2).

Concerning AAB, in the first 24 h, all strains produced 2-propanone and acetic acid with the exception of *A. persici*, where acetic acid was not found in any of the three replicates. The relative amount of 2-propanone produced was similar within genera: *Acetobacter* strains (above 65%), *Komagataeibacter* strains (approximately 50%) and *Gluconobacter* strains (less than 20%). Ethanol was still detectable in *G. oxydans*, *G. kanchanaburiensis* and *K. saccharivorans* strains and always below 3%. Both *Gluconobacter* and *A. cibinogensis* were the only strains where 2-propanol was found. All butyric acid derivatives identified in this work (2-methylpropanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid) were produced in the first 24 h by *A. persici* and *K. hansenii*, while *K. saccharivorans* produced only one derivative (2-methylpropanoic acid). Moreover, the cumulative relative presence of butyric acid derivatives of *A. persici* (~ 28%) was double that of *K. hansenii* and quadruple that of *K. saccharivorans* strain. Finally, the *Gluconobacter* strains were the only where benzaldehyde was present, although only below 2%, and *K. saccharivorans* was the only strain to produce acetaldehyde, although only at about 7%.

After 48 h of bacterial growth, 2-propanone was the sole compound continuing to be released by all strains of bacteria (Table 2), and although a decrease in percentage was observed for all strains with the exception of *K. saccharivorans* (66%), *Acetobacter* strains still had high relative abundance (> 50%) of 2-propanone in the volatile profiles. Even though acetic acid was present in all strains after 24 h of growth, with the exception of *A. persici*, it was only detected in *G. oxydans* and *G. kanchanaburiensis* after 48 h, although in increased relative abundance. In these two strains the presence of 2-propanol was still recorded. After 48 h, the cumulative production of the butyric acid derivatives increased for all strains that expressed them at 24 h with the addition of *A. cibinogensis* strain (~ 37% relative production); neither *Gluconobacter* strain produced these derivatives. After 48 h, no remnant of ethanol or acetaldehyde was present but for *G. kanchanaburiensis* an emission of less than 1% benzaldehyde was still detected.

## 265 Discussion

266 The Acetobacteraceae family was confirmed to include some of the most important bacteria  
267 associated with *D. suzukii*, similar to those found for other species from the Drosophilidae family  
268 (Chandler et al. 2011). Olfactometer bioassays of six AAB strains among those isolated from *D.*  
269 *suzukii* in our companion study (Vacchini et al. submitted) showed that female flies have a  
270 significant attraction for half of the tested isolates with percentage of choice comparable to those  
271 obtained by other authors, either on fruits (Abraham et al. 2015) and on apple cider vinegar  
272 (Mazzetto et al., 2015). Flies always showed an attraction for *G. oxydans*, *G. kanchanaburiensis* and  
273 *K. saccharivorans* strains, no attraction for *A. cibinogensis* and *K. hansenii* and no attraction for *A.*  
274 *persici* at 24 hours but rejection at 48 hours.

275 We performed SPME/GC-MS to characterize the attractive and non-attractive profiles of volatiles  
276 of each strain. Qualitative and quantitative differences were identified in the volatile profiles of the  
277 six bacterial strains, including high variability between 24 and 48 hours of bacterial growth.

278 Considerable differences were evident from the volatile profiles of the preferred strains, which  
279 included both *Gluconobacter* isolates and *K. saccharivorans*. This last strain was the most highly  
280 preferred for the duration of the experiment and had the lowest average rate of no choice both after  
281 24 and 48 hours bacterial growth (Figs. 1-2). *Gluconobacter oxydans* and *G. kanchanaburiensis*  
282 produced the highest percentage of acetic acid and from both strains ethanol was emitted in the first  
283 24 hours. Similarly, for *K. saccharivorans* ethanol emission and acetic acid production was present  
284 in the first 24 hours. These compounds are known to be attractive substances to *Drosophila*,  
285 including *D. suzukii* (West 1961; Reed 1938; Cha et al. 2012; Landolt et al. 2012), and their  
286 combined presence could be one of the keys of fly attraction. Moreover, although 2-propanone was  
287 the only compound released by all bacteria throughout the experiment, its relative production was  
288 the lowest in *Gluconobacter* strains (Table 2). Instead, the most attractive strain, *K. saccharivorans*,  
289 produced relatively high levels of 2-propanone after 24 hours and even higher levels after 48 hours.  
290 The role of 2-propanone is quite controversial: although this compound is reported to be a repellant

291 for vinegar flies (Elamrani et al. 2001), Riveron et al. (2009) showed that 2-propanone can be  
 292 repellent or attractive to *D. melanogaster* according to its concentration. Moreover, Newby and  
 293 Etges (1998) reported that 2-propanone is a general attractant to *D. mojavensis* and can engender  
 294 increased adult longevity. These evidences combined with our results suggest that 2-propanone  
 295 could exhibit different effects according to its concentration and the fly species; its role for *D.*  
 296 *suzukii* attraction must be further investigated.

297 *Komagataeibacter saccharivorans* continued to be the preferred strain despite the absence of acetic  
 298 acid and ethanol after 48 hours. Perhaps having the highest production of butyric acid derivatives  
 299 after 48 hours can explain why this strain was most preferred. Lactic acid bacteria are known to  
 300 produce short-chain fatty acids like butyric acid derivatives that are precursors of many food flavor  
 301 compounds (van Kranenburg et al. 2002); the production of butyric acid derivatives is also known  
 302 in AAB. For example, 2-methylbutanoic acid, an aromatic compound found in fruits and used in the  
 303 food industry, is produced by *Gluconobacter* from 2-methylbutanol (Saichana et al. 2015). Butyric  
 304 acid derivatives produced by AAB may be an attractive volatile for *D. suzukii*. However, no clear  
 305 conclusion can be drawn because while *K. saccharivorans* strain produced the greatest amount of 2-  
 306 methylbutanoic acid, one strain that was not preferred (*K. hansenii*) and one strain that was not  
 307 preferred and later rejected (*A. persici*) also produced this volatile. Thus, the volatile profiles from  
 308 *K. saccharivorans*, *K. hansenii* and *A. persici* do not appear to explain the variety in response by *D.*  
 309 *suzukii*. Potentially an interaction among volatiles could be involved in fly attraction. This might  
 310 explain also why *A. cibirnogensis* strain was not preferred by flies and fluctuated considerably for *D.*  
 311 *suzukii* choice, even producing 3-methylbutanoic acid.

312 To rule out an effect from the volatiles produced by the medium, we also analyzed its profile and  
 313 found that benzaldehyde, ethanol and 2-propanol were always present in the sterile medium.  
 314 Previous research reported that benzaldehyde is attractive to *D. melanogaster* larvae (Larkin et al.  
 315 2010; Lavagnino et al. 2013) but has a repellent effect on adult flies (Rodrigues and Siddiqi 1978;  
 316 Hoffmann 1983). Hoffmann (1985) found that four species of *Drosophila* were not attracted by 2-

317 propanol; on the other hand ethanol is known to be an attractant to *Drosophila* flies (Devineni and  
318 Hebrlein 2009). In our bioassays, a high percentage of no choice and an equal distribution between  
319 the two control flasks was recorded (Figs. 1-2), indicating that this blend has little effect on  
320 attraction of *D. suzukii* females. Moreover, some of the compounds detected in the medium could  
321 be involved in bacterial metabolism, as suggested by absence of the medium-related volatiles in the  
322 control in half of the strains.

323 To better explain *D. suzukii* preference for some isolates, further studies should investigate the fly  
324 responses to the single volatile (tested at different concentrations) produced by all symbiont strains.  
325 Although we could not provide an overall attraction pattern to the volatile profiles of each bacterial  
326 strain, we did show that *Gluconobacter* and *K. saccharivorans* produced the most attractive  
327 volatiles. Thus, a combination of the most effective substances could be used for the optimizing the  
328 traps used in Integrated Pest Management of *D. suzukii*. Despite intensive research on specific  
329 chemical substances (Landolt et al. 2012; Cha et al. 2013; 2014; 2015; Burrack et al. 2015), a clear  
330 direction to resolve the current problem of *D. suzukii* as a pest has not yet been established. Traps  
331 developed in the last few years have been unable to prevent crop damage while killing many non-  
332 target insects (Iglesias et al. 2014; Asplen et al. 2015). Because the biology of AAB is already well  
333 known and because they are currently used extensively in biotechnological applications (Saichana  
334 et al. 2015), the exploitation of these *D. suzukii* symbionts has potential for the development of  
335 attractive and selective traps for their management.

336

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478

479 **Captions of Tables and Figure**

480 **Table 1** Results of the statistical analysis of the two-way olfactometer bioassays performed after 24  
481 and 48 h of bacterial growth.  $\chi^2$  values from the Friedman-ANOVA, performed to evaluate the  
482 differences between the number of flies that chose each of the volatiles, the control, or did not  
483 choose, are reported with their significance (df=2 in all tests). Significance of Wilcoxon signed rank  
484 tests with a Bonferroni correction factor between each strain and the control are indicated, whereas  
485 differences with no choice rate were not considered.

486 **Table 2** Volatile compounds identified by GC-MS analysis from six strains of AAB and in the  
487 control (sterile MA medium) after 24 and 48 h of bacterial growth.

488 **Fig. 1** Results of the two-way olfactometer bioassays performed after 24 h of bacterial growth.  
489 Responses of *D. suzukii* females to volatile compounds produced by a control (sterile medium) and  
490 several strains of AAB: *Acetobacter persici* DS4MR.45, *Acetobacter cibinongensis* DS5FR.4,  
491 *Gluconobacter oxydans* DS1FC.9A, *Gluconobacter kanchanaburiensis* L2.2.A.15,  
492 *Komagataeibacter* sp. DS2MC.114 and *Komagataeibacter* sp. DS1MA.65A. Nine replicates were  
493 performed for each strain. Mean percentages ( $\pm$ SE) on the right report the flies that did not choose  
494 either the control or the volatile. Asterisks (\*) indicate significant differences between the number  
495 of flies that chose the control and the volatile according to the Friedman-ANOVA and Wilcoxon  
496 signed-rank tests with a Bonferroni correction ( $P < 0.05$ ) factor.

497 **Fig. 2** Results of two-way olfactometer bioassays performed after 48 h of bacterial growth.  
498 Responses of *D. suzukii* flies to volatile compounds produced by a control (sterile medium) and  
499 several strains of AAB: *Acetobacter persici* DS4MR.45, *Acetobacter cibinongensis* DS5FR.4,  
500 *Gluconobacter oxydans* DS1FC.9A, *Gluconobacter kanchanaburiensis* L2.2.A.15,  
501 *Komagataeibacter* sp. DS2MC.114 and *Komagataeibacter* sp. DS1MA.65A. Nine replicates were  
502 performed for each strain. Mean percentages ( $\pm$ SE) on the right report flies that did not choose  
503 either the control or the volatile. Asterisks (\*) indicate significant differences between the number

504 of flies that chose the control and the volatile according to the Friedman-ANOVA and Wilcoxon  
505 signed-rank tests with a Bonferroni correction ( $P < 0.05$ ) factor.

Figure 1

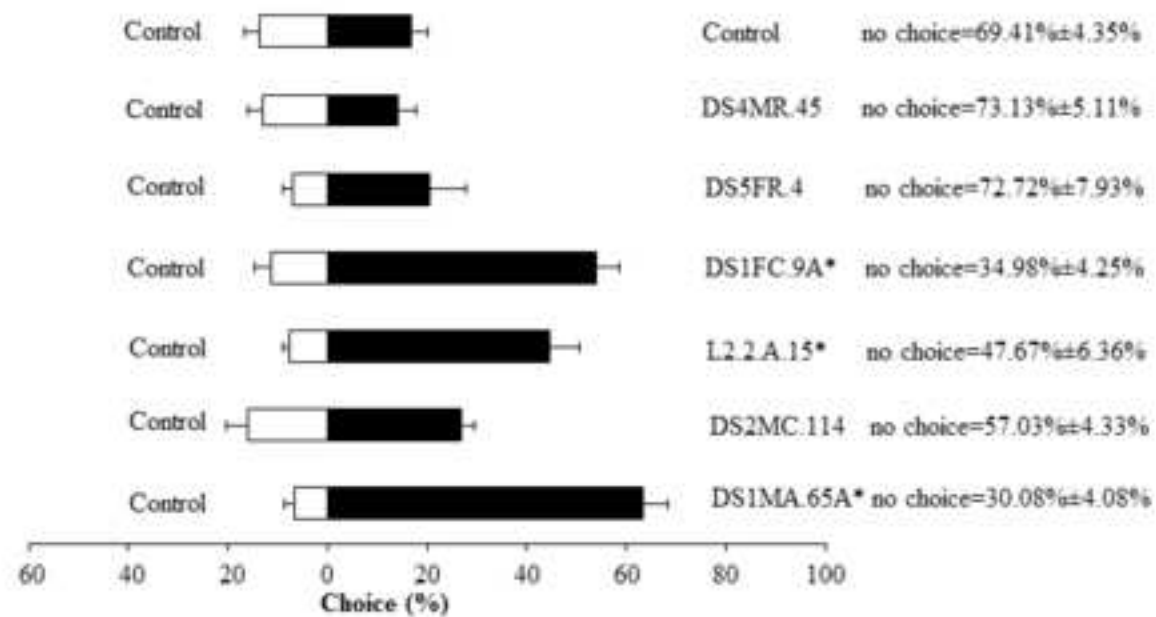
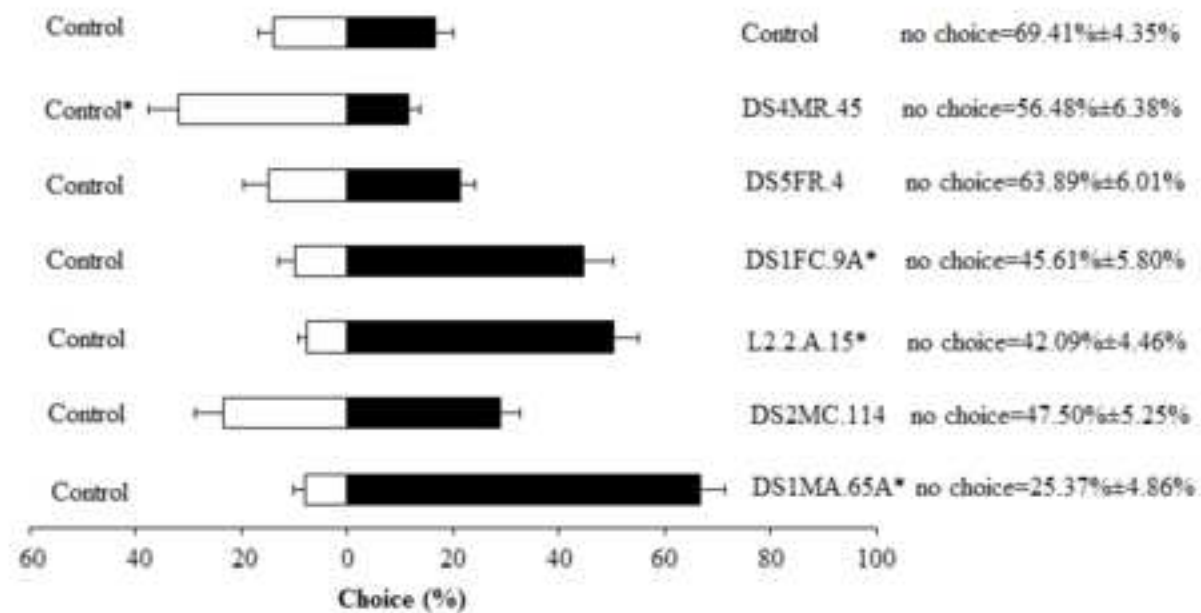




Figure 2



**Table 1** Results of the statistical analysis of the two-way olfactometer bioassays performed after 24 and 48 h of bacterial growth.  $\chi^2$  values from the Friedman-ANOVA, performed to evaluate the differences between the number of flies that chose each of the volatiles, the control, or did not choose, are reported with their significance (df=2 in all tests). Significance of Wilcoxon signed rank tests with a Bonferroni correction factor between each strain and the control are indicated, whereas differences with no choice rate were not considered.

Strain	$\chi^2$ ; significance (Friedman-ANOVA)		Significance (Wilcoxon signed rank test) VS control	
	24h	48h	24h	48h
Control	14.000; 0.001	-	ns	-
<i>A. persici</i>	13.886; 0.001	14.114; 0.001	ns	0.012 <sup>a</sup>
<i>A. cibinogensis</i>	9.556; 0.008	11.556; 0.003	ns	ns
<i>G. oxydans</i>	12.667; 0.002	10.889; 0.004	0.008 <sup>b</sup>	0.008 <sup>b</sup>
<i>G. kanchanaburiensis</i>	13.556; 0.001	13.556; 0.001	0.008 <sup>b</sup>	0.008 <sup>b</sup>
<i>K. hansenii</i>	10.889; 0.004	3.556; ns	ns	ns
<i>K. saccharivorans</i>	16.222; <0.001	13.771; 0.001	0.008 <sup>b</sup>	0.008 <sup>b</sup>

ns = not significant (P>0.05)  
<sup>a</sup> = insect preference for the control versus a specific strain  
<sup>b</sup> = insect preference for a specific strain versus the control

**Table 2** Volatile compounds identified by GC-MS analysis from six strains of AAB and in the control (sterile MA medium) after 24 and 48 h of bacterial growth.

Strain	Compound	Identified by <sup>a</sup>	RI <sup>b</sup>		Presence <sup>c</sup>	
			Exp.	Lit.	24 hours	48 hours
<i>A. persici</i>	2-Propanone	Database; AS	ND	-	70.6%±1.0%	55.0%±5.4%
	2-Methylpropanoic acid	Database; AS; RI	792	790	7.7%±1.3%	26.5%±1.6%
	3-Methylbutanoic acid	Database; AS; RI	878	876-882	14.4%±2.6%	12.4%±1.9%
	2-Methylbutanoic acid	Database; AS; RI	875	876	7.3%±0.1%	6.1%±2.6%
<i>A. cibinogensis</i>	Acetic acid	Database; AS; RI	622	600-646	13.0%±3.3%	
	2-Propanol	Database; AS; RI	500	515	18.4%±1.0%	
	2-Propanone	Database; AS	ND	-	68.6%±2.5%	63.3%±4.9%
	3-Methylbutanoic acid	Database; AS; RI	879	876-882		36.7%±4.9%
<i>G. oxydans</i>	Ethanol	Database; AS	ND	-	1.2%±0.4%	
	Acetic acid	Database; AS; RI	644	600-646	65.4%±3.6%	69.4%±9.3%
	2-Propanol	Database; AS; RI	501	515	17.4%±1.2%	17.8%±5.1%
	2-Propanone	Database; AS	ND	-	14.2%±2.6%	12.8%±4.4%
	Benzaldehyde	Database; AS; RI	988	980	1.8%±0.3%	
<i>G. kanchanaburiensis</i>	Ethanol	Database; AS	ND	-	0.9%±0.3%	
	Acetic acid	Database; AS; RI	644	600-646	62.8%±3.7%	80.4%±0.7%
	2-Propanol	Database; AS; RI	501	515	24.5%±2.3%	13.9%±0.7%
	2-Propanone	Database; AS	ND	-	10.9%±0.7%	5.1%±0.3%
	Benzaldehyde	Database; AS; RI	990	980	0.9%±0.3%	0.6%±0.2%
<i>K. hansenii</i>	Acetic acid	Database; AS; RI	629	600-646	38.1%±9.7%	
	2-Propanone	Database; AS	ND	-	48.3%±8.1%	11.3%±2.3%
	2-Methylpropanoic acid	Database; AS; RI	799	790	9.8%±1.0%	17.2%±1.3%
	3-Methylbutanoic acid	Database; AS; RI	882	876-882	3.5%±0.5%	51.3%±0.9%
	2-Methylbutanoic acid	Database; AS; RI	896	876	0.3%±0.1%	20.2%±2.5%
<i>K. saccharivorans</i>	Ethanol	Database; AS	ND	-	2.7%±0.3%	
	Acetic acid	Database; AS; RI	642	600-646	33.6%±4.5%	
	2-Propanone	Database; AS	ND	-	49.7%±4.4%	66.0%±2.4%
	2-Methylpropanoic acid	Database; AS; RI	790	790	6.7%±1.6%	2.4%±0.5%
	3-Methylbutanoic acid	Database; AS; RI	870	876-882		8.0%±0.1%
	2-Methylbutanoic acid	Database; AS; RI	878	876		23.6%±2.7%
	Acetaldehyde	Database; AS	ND	-	7.3%±0.8%	
Control (sterile MA)	Ethanol	Database; AS	ND	-	66.4%±0.8%	69.9%±1.1%
	2-Propanol	Database; AS; RI	501	515	33.4%±0.8%	29.9%±1.1%
	Benzaldehyde	Database; AS; RI	1003	980	0.2%±0.1%	0.2%±0.1%

<sup>a</sup> Compound identified through the databases WILEY6N.L or NIST98; application of the Authentic Standard (AS) or the Retention Index (RI).

<sup>b</sup> Retention index on the DB-5MS column, Exp.: RI calculated from the experiment, Lit: RI found in the literature (NIST, 2015) and ND: Not determined.

<sup>c</sup> Mean percentage ± SE of the substance from the first, second and third replicates performed after 24 and 48 h of bacterial growth.