Olfactory attraction of Drosophila suzukii by symbiotic acetic acid bacteria

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

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Author contributions

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

Acknowledgements

The authors are grateful to Enrico Busato and Marco Pansa for insect rearing and technical support.

Abstract

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

Keywords

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

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.

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Introduction

Acetic acid bacteria (AAB) are Gram-negative bacteria belonging to the family Acetobacteraceae within the class Alphaproteobacteria. Their taxonomy, molecular biology and physiology have been scrutinized because of their importance in commercial food and chemical compound production (Raspor and Goranovič 2008). AAB are pervasive in the environment and easy to isolate from various plants, flowers, fruits and garden soil (Raspor and Goranovič 2008; Crotti et al. 2010). Although some strains are spoilage agents of wine and beer and others cause plant diseases (Rohrbach and Pfeiffer 1975; van Keer et al. 1981; du Toit and Pretorius 2000; Bartowsky et al. 2003), numerous studies have also established symbiotic associations between AAB and insects that feed on sugar-based diets, specifically those belonging to the orders Diptera, Hymenoptera and Hemiptera (Crotti et al. 2010). Model species from the genus *Drosophila*, *Drosophila melanogaster* Meigen and *Drosophila simulans* Sturtevant, host several AAB strains, but predominantly those belonging to the genera *Acetobacter* and *Gluconobacter* (Cox and Gilmore 2007; Ren et al. 2007; Chandler et al. 2011; Wong et al. 2011; Kim et al. 2012; Staubach et al. 2013; Wong et al. 2013); strains belonging to the genera *Gluconacetobacter* and *Commensalibacter* have also been isolated from some *D. melanogaster* populations (Roh et al. 2008).

The insect midgut is a favorable niche for growth of AAB because of the availability of carbohydrate-rich food in an aerobic, acidic environment. Meanwhile, AAB can convey numerous advantages to their hosts, such as improving their digestive opportunities or by positively influencing larval development (Crotti et al. 2010; 2011; Chouaia et al. 2012). Some AAB are implicated in maintaining the immune homeostasis or increasing the lifespan and fitness of their hosts (Ryu et al. 2008; Shin et al. 2011), while others could be involved in defense against other harmful microorganisms or may participate in cell-to-cell communication (Crotti et al. 2010). Therefore, the relationship between AAB bacteria and their hosts is considered to be mutually symbiotic.

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

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

Materials and methods

Insect material and bacterial strains

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

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

Two-way olfactometer bioassays

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

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

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

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

Volatile profile analysis

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

Prior to analyses, 20-ml glass vials (Supelco Inc., Bellefonte, USA) were filled with 4 g of solid MA + bacteria and 4 g of NaCl and crushed with a spoon. The vials were closed with PTFE septa and open-top polypropylene (Supelco) caps. Sterile solid MA and 4 g of NaCl, crushed in the vial was also used as control. The samples were shacked for 2 min at 50°C to accelerate equilibrium of headspace volatile compounds between the solid matrix and the headspace. Volatile compounds were extracted by solid-phase microextraction (SPME) by inserting a carboxen-polydimethylsiloxane fiber (black, 75-µm-thick film, 23-Ga needle, Supelco, Bellefonte, PA, USA) for 30 min at 50°C. After extraction, samples were desorbed into a CIS-4 programed temperature vaporization injector (Gerstel, Mülheim an der Ruhr, Germany). The volatile compounds were analyzed using an HP 6890 Series gas chromatography (GC) system equipped with a capillary column (DB-5MS, 30 m ×0.250 mm, 0.25-µm-thick film). Helium gas was used as the carrier gas at a constant flow of 1.2 ml/min. Oven temperature of the GC was programmed for a 29.33 min total running time. From an initial temperature of 35°C, the temperature was increased at a constant rate 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 5973 Mass Selective Detector (Hewlett-Packard, Wilmington, NC, USA) connected with the GC system was operated in electron impact mode with an electron impact energy of 70 eV. GC-MS data were processed with the MSD-Chemstation software (Agilent Technologies). Volatile compounds 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_5 to C_{18}) 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. cibinogensis* 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

239 media for the control confirmed a constant emission of ethanol (66-70%), 2-propanol (~ 30%) and
240 benzaldehyde (0.2%) (Table 2).
241 Concerning AAB, in the first 24 h, all strains produced 2-propanone and acetic acid with the
242 exception of *A. persici*, where acetic acid was not found in any of the three replicates. The relative
243 amount of 2-propanone produced was similar within genera: *Acetobacter* strains (above 65%),
244 *Komagataeibacter* strains (approximately 50%) and *Gluconobacter* strains (less than 20%). Ethanol
245 was still detectable in *G. oxydans*, *G. kanchanaburiensis* and *K. saccharivorans* strains and always
246 below 3%. Both *Gluconobacter* and *A. cibinogensis* were the only strains where 2-propanol was
247 found. All butyric acid derivatives identified in this work (2-methylpropanoic acid, 2-
248 methylbutanoic acid and 3-methylbutanoic acid) were produced in the first 24 h by *A. persici* and *K.*
249 *hansenii*, while *K. saccharivorans* produced only one derivative (2-methylpropanoic acid).
250 Moreover, the cumulative relative presence of butyric acid derivatives of *A. persici* (~ 28%) was
251 double that of *K. hansenii* and quadruple that of *K. saccharivorans* strain. Finally, the
252 *Gluconobacter* strains were the only where benzaldehyde was present, although only below 2%,
253 and *K. saccharivorans* was the only strain to produce acetaldehyde, although only at about 7%.
254 After 48 h of bacterial growth, 2-propanone was the sole compound continuing to be released by all
255 strains of bacteria (Table 2), and although a decrease in percentage was observed for all strains with
256 the exception of *K. saccharivorans* (66%), *Acetobacter* strains still had high relative abundance (>
257 50%) of 2-propanone in the volatile profiles. Even though acetic acid was present in all strains after
258 24 h of growth, with the exception of *A. persici*, it was only detected in *G. oxydans* and *G.*
259 *kanchanaburiensis* after 48 h, although in increased relative abundance. In these two strains the
260 presence of 2-propanol was still recorded. After 48 h, the cumulative production of the butyric acid
261 derivatives increased for all strains that expressed them at 24 h with the addition of *A. cibinogensis*
262 strain (~ 37% relative production); neither *Gluconobacter* strain produced these derivatives. After
263 48 h, no remnant of ethanol or acetaldehyde was present but for *G. kanchanaburiensis* an emission
264 of less than 1% benzaldehyde was still detected.

Discussion

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

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

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

337 ## References

338 Abraham J, Zhang A, Angeli S, Abubeker S, Michel C, Feng Y, Rodriguez-Saona C (2015)
339 Behavioral and antennal responses of *Drosophila suzukii* (Diptera: Drosopohilidae) to
340 volatiles from fruit extracts. Environ Entomol 44:356–367
341 Altschul SF, Gish W, Miller W, Myers EW, Lipman (1990) Basic local alignment search tool. J
342 Mol Biol 215(3):403–410

343 Asplen MK, Anfora G, Biondi A, Choi D-S, Chu D, Daane KM, Gibert P, Gutierrez AP, Hoelmer
344 KA, Hutchison WD, Isaacs R, Jiang Z-L, Kárpáti Z, Kimura MT, Pascual M, Philips CR,
345 Plantamp C, Ponti L, Vétek G, Vogt H, Walton VM, Yu Y, Zappalà L, Desneux N (2015)
346 Invasion biology of spotted wing Drosophila (*Drosophila suzukii*): a global perspective and
347 future priorities. J Pest Sci 88:469–494
348 Bartowsky EJ, Xia D, Gibson RL, Fleet GH, Henschke PA (2003) Spoilage of bottled red wine by
349 acetic acid bacteria. Lett Appl Microbiol 36:307–314
350 Burrack HJ, Asplen M, Bahder L, Collins J, Drummond FA, Guédot C, Isaacs R, Johnson D,
351 Blanton A, Lee JC, Loeb G, Rodriguez-Saona C, van Timmeren S, Walsh D, McPhie DR
352 (2015) Multistate comparison of attractants for monitoring *Drosophila suzukii* (Diptera:
353 Drosophilidae) in blueberries and caneberries. Environ Entomol 44(3):704–712
354 Cha DH, Adams T, Rogg H, Landolt PJ (2012) Identification and field evaluation of fermentation
355 volatiles from wine and vinegar that mediate attraction of Spotted Wing Drosophila,
356 *Drosophila suzukii*. J Chem Ecol 38:1419–1431
357 Cha DH, Hesler SP, Cowles RS, Voght H, Loeb GM, Landolt PJ (2013) Comparison of a synthetic
358 chemical lure and standard fermented baits for trapping *Drosophila suzukii* (Diptera:
359 Drosophilidae). Environ Entomol 42:1052–1060
360 Cha DH, Adams T, Werle CT, Sampson BJ, Adamczyk JJ, Rogg H, Landolt PJ (2014) A four-
361 component synthetic attractant for *Drosophila suzukii* (Diptera: Drosophilidae) isolated from
362 fermented bait headspace. Pest Manag Sci 70:324–331
363 Cha DH, Hesler SP, Park S, Adams TB, Zack RS, Rogg H, Loeb GM, Landolt PJ (2015) Simpler is
364 better: fewer non-target insects trapped with a four-component chemical lure vs. a
365 chemically more complex food-type bait for *Drosophila suzukii*. Entomol Exp Appl
366 154:251–260
367 Chandler JA, James PM, Jospin G, Lang JM (2014) The bacterial communities of *Drosophila*
368 *suzukii* collected from undamaged cherries. PeerJ 2:e474. doi 10.7717/peerj.474

369 Chandler JA, Lang JM, Bhatnagar S, Eisen JA, Kopp A (2011) Bacterial communities of diverse
370 *Drosophila* species: ecological context of a host–microbe model system. PLOS GENET
371 7:e1002272. doi:10.1371/journal.pgen.1002272
372 Chouaia B, Rossi P, Epis S, Mosca M, Ricci I, Damiani C, Ulissi U, Crotti E, Daffonchio D, Bandi
373 C, Favia G (2012) Delayed larval development in *Anopheles* mosquitos deprived of *Asaia*
374 bacterial symbionts. BMC Microbiol 12:S2
375 Cox CR, Gilmore MS (2007) Native microbial colonization of *Drosophila melanogaster* and its use
376 as a model of *Enterococcus faecalis* pathogenesis. Infect Immun 75:1565–1576
377 Crotti E, Gonella E, Ricci I, Clementi E, Mandrioli M, Sacchi L, Favia G, Alma A, Bourtzis K,
378 Cherif A, Bandi C, Daffonchio D (2011) Secondary symbionts of insects: acetic acid
379 bacteria. In: Manipolative tennants: bacteria associated with arthropods (E. Zcori-Fein and
380 K. Bourtzis eds.), CRC Press Taylor & Francis Group, Boca Raton, pp 45–72
381 Crotti E, Rizzi A, Chouaia B, Ricci I, Favia G, Alma A, Sacchi L, Bourtzis K, Mandrioli M, Cherif
382 A (2010) Acetic acid bacteria, newly emerging symbionts of insects. Appl Environ
383 Microbiol 76:6963-6970
384 Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK (2013) Microbial volatile emissions as insect
385 semiochemicals. J Chem Ecol 39:840–859
386 Devineni AV, Heberlein U (2009) Preferential ethanol consumption in *Drosophila* models features
387 of addiction. Curr Biol 19:2126–2132
388 du Toit M, Pretorius IS (2000) Microbial spoilage and preservation of wine: using weapons from
389 nature's own arsenal – a review. S Afr J Enol Vitic 41:48–56
390 Elamrani A, David JR, Idaomar M (2001) Parallel changes in enzyme activity and oviposition
391 behavior in adults of *Drosophila melanogaster* submitted to alcohols, acetaldehyde or
392 acetone. Invertebr Reprod Dev 40:17–25
393 Frago E, Dicke M, Godfray HCJ (2012) Insects symbionts as hidden players in insect-plant
394 interactions. Trends Ecol Evol 27:705–711

395 Hauser M (2011) A historic account of the invasion of *Drosophila suzukii* (Matsumura) (Diptera:
396 Drosophilidae) in the continental United States, with remarks on their identification. Pest
397 Manag Sci 67:1352–1357
398 Hoffmann AA (1983) Bidirectional selection for olfactory response to acetaldehyde and ethanol in
399 *Drosophila melanogaster*. Genet Sel Evol 15:501–518
400 Hoffmann AA (1985) Interspecific variation in the response of *Drosophila* to chemicals and fruit
401 odours in a wind tunnel. Aust J Zool 33:451–460
402 Iglesias LE, Nyoike TW, Liburd OE (2014) Effect of trap design, bait type, and age on captures of
403 *Drosophila suzukii* (Diptera: Drosophilidae) in berry crops. J Econ Entomol 107:1508–1518
404 Jang EB, Nishijima KA (1990) Identification and attractancy of bacteria associated with *Dacus*
405 *dorsalis* (Diptera: Tephritidae). Environ Entomol 19:1726–1731
406 Kim E-K, Kim S-H, Nam H-J, Choi MK, Lee K-A, Choi S-H, Seo YY, You H, Kim B, Lee W-J
407 (2012) Draft genome sequence of *Gluconobacter morbifer* G707^{T}, a pathogenic gut
408 bacterium isolated from *Drosophila melanogaster* intestine. J Bacteriol 194:1245
409 Landolt PJ, Adams T, Rogg H (2012) Trapping spotted wing drosophila, *Drosophila suzukii*
410 (Matsumura) (Diptera: Drosophilidae), with combinations of vinegar and wine, and acetic
411 acid and ethanol. J Appl Entomol 136:148–154
412 Larkin A, Karak S, Priya S, Das A, Ayyub C, Ito K, Rodrigues V, Ramaswami M (2010) Central
413 synaptic mechanisms underlie short-term olfactory habituation in *Drosophila* larvae. Learn
414 Memory 17:645–653.
415 Lavagnino NJ, Arya GH, Korovaichuk A, Fanara JJ (2013) Genetic architecture of olfactory
416 behavior in *Drosophila melanogaster*: differences and similarities across development.
417 Behav Genet 43:348–359
418 Liscia A, Angioni P, Sacchetti P, Poddighe S, Granchietti A, Setzu MD, Belcari A (2013)
419 Characterization of olfactory sensilla of the olive fly: behavioral and electrophysiological

responses to volatile organic compounds from the host plant and bacterial filtrate. J Insect Physiol 59:705–716

Mapelli F, Marasco R, Rolli E, Barbato M, Cherif H, Guesmi A, Ouzari I, Daffonchio D, Borin S (2013) Potential for plant growth promotion of rhizobacteria associated with *Salicornia* growing in Tunisian hypersaline soils. Bio Med Res Int 2013:248078. http//dx.doi.org/10.1155/2013/248078

Mazzetto F, Pansa MG, Ingegno BL, Tavella L, Alma A (2015) Monitoring of the exotic fly *Drosophila suzukii* in stone, pome and soft fruit orchards in NW Italy. J Asia Pac Entomol 18:321–329

Newby BD, Etges WJ (1998) Host preference among populations of *Drosophila mojavensis* (Diptera: Drosophilidae) that use different host cacti. J Insect Behav 11:691–712

NIST (2015) http://webbook.nist.gov/chemistry/. Accessed 21 September 2015.

Raspor P, Goranovič D (2008) Biotechological applications of Acetic Acid Bacteria. Crit Rev Biotechnol 28:101–124

Reed MR (1938) The olfactory reactions of *Drosophila melanogaster* Meigen to the products of fermenting banana. Physiol Zool 11:317–325

Ren C, Webster P, Finkel SE, Tower J (2007) Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. Cell Metab 6:144–152

Riveron J, Boto T, Alcorta E (2009) The effect of environmental temperature on olfactory perception in *Drosophila melanogaster*. J Insect Physiol 55:943–951

Robacker DC, Martinez AJ, Garcia JA, Bartelt RJ (1998) Volatiles attractive to the Mexican Fruit Fly (Diptera: Tephritidae) from eleven bacteria taxa. Fla Entomol 81:497–508

Rodrigues V, Siddiqi O (1978) Genetic analysis of chemosensory pathway. P Indian Acad Sci B 87:147–160

444 Roh SW, Nam Y-D, Chang H-W, Kim K-H, Kim M-S, Ryu J-H, Kim S-H, Lee W-J, Bae J-W
445 (2008) Phylogenetic characterization of two novel commensal bacteria involved with innate
446 immune homeostasis in *Drosophila melanogaster*. Appl Environ Microbiol 74:6171–6177
447 Rohrbach KG, Pfeiffer JB (1975) The interaction of four bacteria causing pink disease of pineapple
448 with several pineapple cultivars. Phytopathol 66:396–399
449 Ryu J-H, Kim S-H, Lee H-Y, Bai J-Y, Nam Y-D, Bae J-W, Lee DG, Shin SC, Ha E-M, Lee W-J
450 (2008) Innate immune homeostasis by the homeobox gene Caudal and commensal-gut
451 mutualism in *Drosophila*. Science 319:777–782
452 Saichana N, Matsushita K, Adachi O, Frébort I, Frebortova J (2015) Acetic acid bacteria: a group of
453 bacteria with versatile biotechnological applications. Biotechnol Adv 33(6)2:1260–1271
454 Scheidler NH, Liu C, Hamby KA, Zalom FG, Syed Z (2015) Volatile codes: correlation of olfactory
455 signals and reception in *Drosophila*-yeast chemical communication. Sci Rep 5:14059. doi:
456 10.1038/srep14059
457 Shin SC, Kim S-H, You H, Kim B, Kim AC, Lee K-A, Yoon J-H, Ryu J-H, Lee W-J (2011)
458 *Drosophila* microbiome modulates host developmental and metabolic homeostasis via
459 insulin signaling. Science 334:670–674
460 Staubach F, Baines JF, Künzel F, Bik EM, Petrov DA (2013) Host species and environmental effects
461 on bacterial communities associated with *Drosophila* in the laboratory and in the natural
462 environment. PLOS ONE: e70749.doi: 10.1371/journal.pone.0070749
463 Vacchini V, Gonella E, Crotti E, Prosdocimi EM, Mazzetto F, Chouaia B, Callegari M, Mapelli F,
464 Mandrioli M, Alma A, Daffonchio D Diversity and gut localization of the acetic acid
465 bacterial microbiome in the spotted wing fly, *Drosophila suzukii.* Submitted to Appl Environ
466 Microbiol
467 van Keer C, Vanden Abeele P, Swings J, Gossele F, De Lay J (1981) Acetic acid bacteria as causal
468 agents of browning and rot of apples and pears. Zentralblatt für Bakteriologie: I.
469 Abt.Originale C: Allgemeine, angewandte und ökologische Mikrobiologie 2:197–204

van Kranenburg R, Kleerebezem M, van Hylckama Vlieg J, Ursing BM, Boekhorst J, Smit BA, Ayad EHE, Smit G, Siezen RJ (2002) Flavour formation from amino acids by lactic acid bacteria: predictions from genome sequence analysis. Int Dairy J 12:111–121

West AS (1961) Chemical attractants for adult *Drosophila* species. J Econ Entomol 54:677–681

Wong AC-N, Chaston JM, Douglas AE (2013) The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. ISME J 7:1922–1932

Wong AC-N, Ng P, Douglas AE (2011) Low diversity bacterial community in the gut of the fruit fly *Drosophila melanogaster*. Environ Microbiol 13:1889–1900

Captions of Tables and Figure

Table 1 Results of the statistical analysis of the two-way olfactometer bioassays performed after 24 and 48 h of bacterial growth. χ^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.

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.

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

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

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Figure 1

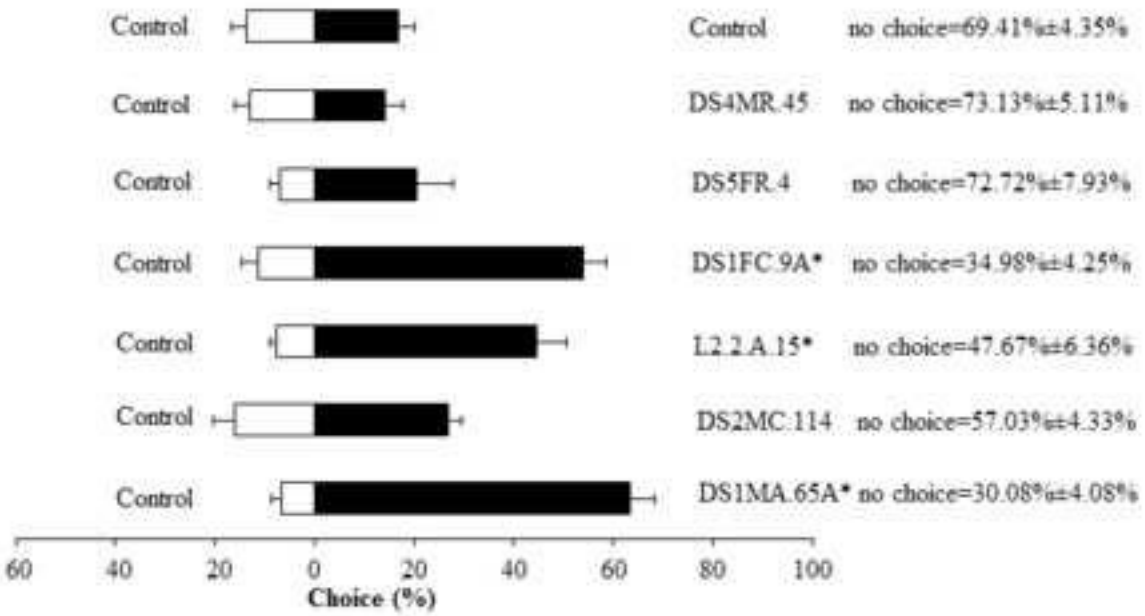

Figure 2

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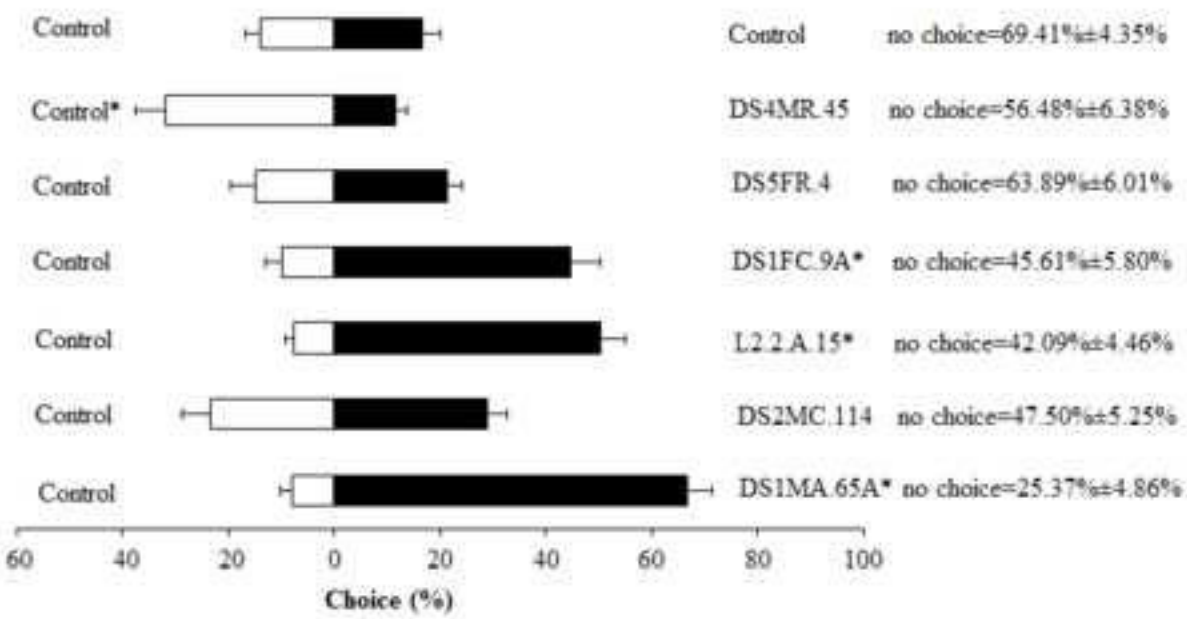

Table 1 Results of the statistical analysis of the two-way olfactometer bioassays performed after 24 and 48 h of bacterial growth. χ^2 values from the Friedman-ANOVA, performed to evaluate the differences between the number of flies that chose each of the volatiles, the control, or did not choose, are 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	**χ^2; significance (Friedman-ANOVA)**		**Significance (Wilcoxon signed rank test) VS control**	
	24h	**48h**	**24h**	**48h**
Control	14.000; 0.001	-	ns	-
A. persici	13.886; 0.001	14.114; 0.001	ns	0.012[a]
A. cibinogensis	9.556; 0.008	11.556; 0.003	ns	ns
G. oxydans	12.667; 0.002	10.889; 0.004	0.008[b]	0.008 [b]
G. kanchanaburiensis	13.556; 0.001	13.556; 0.001	0.008 [b]	0.008 [b]
K. hansenii	10.889; 0.004	3.556; ns	ns	ns
K. saccharivorans	16.222; <0.001	13.771; 0.001	0.008 [b]	0.008 [b]

ns = not significant (P>0.05)
[a] = insect preference for the control versus a specific strain
[b] = 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[a]	RI[b]		Presence[c]	
			Exp.	Lit.	24 hours	48 hours
A. persici	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%
A. cibinogensis	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%
G. oxydans	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%	
G. kanchanaburiensis	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%
K. hansenii	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%
K. saccharivorans	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%

[a] Compound identified through the databases WILEY6N.L or NIST98; application of the Authentic Standard (AS) or the Retention Index (RI).

[b] 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.

[c] Mean percentage ± SE of the substance from the first, second and third replicates performed after 24 and 48 h of bacterial growth.