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RESEARCH ARTICLE

Gut microbiota affects development and olfactory behavior in *Drosophila melanogaster*

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ABSTRACT

It has been shown that gut microbes are very important for the behavior and development of *Drosophila*, as the beneficial microbes are involved in the identification of suitable feeding and egg-laying locations. However, in what way these associated gut microbes influence the fitness-related behaviors of *Drosophila melanogaster* remains unclear. Here, we show that *D. melanogaster* exhibits different behavioral preferences towards gut microbes. Both adults and larvae were attracted by the volatile compounds of *Saccharomyces cerevisiae* and *Lactobacillus plantarum*, but were repelled by *Acetobacter malorum* in behavioral assays, indicating that an olfactory mechanism is involved in these preference behaviors. While the attraction to yeast was governed by olfactory sensory neurons expressing the odorant co-receptor Orco, the observed behaviors towards the other microbes were retained in flies lacking this co-receptor. By experimentally manipulating the microbiota of the flies, we found that flies did not strive for a diverse microbiome by increasing their preference towards gut microbes that they had not experienced previously. Instead, in some cases, the flies even increased preference for the microbes on which they were reared. Furthermore, exposing *Drosophila* larvae to all three microbes promoted *Drosophila* development, while exposure to only *S. cerevisiae* and *A. malorum* resulted in the development of larger ovaries and in increased egg numbers in an oviposition assay. Thus, our study provides a better understanding of how gut microbes affect insect behavior and development, and offers an ecological rationale for preferences of flies for different microbes in their natural environment.

KEY WORDS: Host–microbe interaction, Olfaction, Gut bacteria, Yeast, Behavior

INTRODUCTION

Gut microbiomes play important roles in different physiological processes of their hosts, such as nutrition (Wong et al., 2014; Newell and Douglas, 2014; Tefit and Leulier, 2017; Leitão-Gonçalves et al., 2017), development (Ridley et al., 2012; Shin et al., 2011; Storelli et al., 2011; Tefit and Leulier, 2017), longevity (Guo et al., 2014; Clark et al., 2015), immunity (Sansone et al., 2015) and disease avoidance (Van Nood et al., 2013; Zhang et al., 2015). The fruit fly, *Drosophila melanogaster*, has been largely used to study host–microbe interactions related to innate immunity and pathogenic association (Lemaitre and Hoffmann, 2007; Keesey et al., 2017). Recently several independent studies analyzing the diversity of gut microbes in *D. melanogaster* showed that the *Drosophila* microbiome mainly consists of yeasts, and two genera of bacteria, *Acetobacter* and *Lactobacillus* (Chandler et al., 2011; Broderick and Lemaitre, 2012; Staubach et al., 2013; Wong et al., 2011, 2017).

The environmental microbes that flies have been exposed to as larvae and adults not only drive the composition of the flies' gut microbiome (Chandler et al., 2011), but can also affect the flies' behaviors, such as oviposition (Tefit and Leulier, 2017) or foraging (Wong et al., 2017; Leitão-Gonçalves et al., 2017; Keesey et al., 2017). Furthermore, *Drosophila* larvae and adults can be attracted by odors emanating from food patches that have been previously used by larvae (Durisko and Dukas, 2013; Durisko et al., 2014) and a study performed with axenic *Drosophila* revealed that at least some of these attractants are produced by the larval gut bacteria (Venu et al., 2014). These results suggest that *Drosophila* adults may rely on microbe-derived volatiles for long-distance attraction to suitable feeding and egg-laying sites. Recent studies have demonstrated that *Drosophila* prefer a microbe co-culture, due to the metabolite exchange of the different microbes when grown together (Fisher et al., 2017), and that gut microbe composition can modify microbial and nutritional preferences of *D. melanogaster*, suggesting that microbiota can affect host chemosensory responses, preferences and behavior (Wong et al., 2017). However, we have limited understanding of how gut microbes, such as yeast and bacteria, affect *Drosophila* behaviors.

Several innate dedicated olfactory circuits in *Drosophila* have been described for detecting attractive yeast volatiles for oviposition (Dweck et al., 2015a), as well as circuits for aversive volatiles for detecting danger by fungal mold or parasitoids (Stensmyr et al., 2012; Ebrahim et al., 2015). However, it remains unclear whether similar circuits exist that help *Drosophila* to identify food containing healthy or preferred gut microbes. Here, we investigate whether fly health is affected by a diet containing primarily one microbe species, and whether flies raised on such a diet change their food preferences (i.e. they prefer food with microbes that could not be accessed previously, and were missing from their dietary intake). To do so, instead of treating flies with antibiotics and/or sterilizing eggs by dechorionation to produce axenic flies (Sabat et al., 2015; Koyle et al., 2016), we raised flies on diets enriched with *Saccharomyces cerevisiae*, *Lactobacillus plantarum* or *Acetobacter malorum*. We found that *Drosophila* raised on different microbes later differed regarding their olfactory behavioral preference, developmental time and fecundity.

MATERIALS AND METHODS

Drosophila stocks

All experiments were carried out with wild type (WT) or $Orco^{-/-}$ transgenic *Drosophila melanogaster* of the strain Canton-S, which

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were obtained from the Bloomington *Drosophila* Stock Center (https://bdsc.indiana.edu/). *D. melanogaster* were raised on standard diet at 25°C with 70% humidity, and a 12 h light:12 h dark cycle. For behavioral experiments, 3- to 5-day-old flies of both sexes or 3rd-instar larvae were used.

Microbe strains

Saccharomyces cerevisiae (DSM1333), *Lactobacillus plantarum* (DSM-20174), *Acetobacter malorum* (DSM-14337) were purchased from Leibniz Institute DSMZ-German collection of microorganisms and cell cultures. Microbes were kept at −80°C in 50% glycerol for long-term storage. Fresh cultures were generated daily and grown at 30°C and 250 r.p.m. in YM medium (*S. cerevisiae*) or MRS medium (*L. plantarum* and *A. malorum*). To expose flies to specific microbes, 1 ml of stationary phase microbe culture pellet (OD=1) was washed and resuspended in 100 µl PBS; this was then inoculated on the surface of antibiotic-free fly food in a 1.5-cm-diameter fly vial, as previous described (Tefit and Leulier, 2017). One- or 2-day-old flies were distributed on fly food associated with the corresponding microbe, and transferred to a new vial twice per day. In order to test for short- and long-term effects of exposure to specific microbes, flies were either exposed to the corresponding microbes for 2 days, or were continuously bred for several generations under these conditions. For the latter, flies were allowed to lay eggs on medium with one of the microbes for 1 day and were discarded afterwards. The medium was not changed until the next generation of flies eclosed. Newly hatched flies were transferred to fresh medium with the same added microbes and their offspring were raised as before. The flies of the fifth generation raised under these conditions were transferred to fresh medium twice per day and were tested at an age of 3–5 days.

Trap assays

Trap assays were performed as previously described (Keesey et al., 2017). Briefly, 35 flies (30 female and 5 male flies, 3–5 days old, starved for 24 h) were introduced into a transparent plastic cup (length, 10×8×10 cm) with holes in the lid, that contained two smaller containers (height, 4.5 cm; diameter, 3 cm) with a cut pipette tip (tip opening, 2 mm). Experiments were always started at the same time of day and carried out in a climate chamber with the same conditions of fly breeding. Containers were equipped with a disc of filter paper (diameter, 5 mm) that was loaded either with 50 µl growth medium containing the equivalent of the microbe pellet or with 50 µl of growth medium only. By the use of a hemocytometer we estimated the numbers of cells per pellet as roughly 10^6 for *S. cerevisiae* and each 10^8 for *L. plantarum* and *A. malorum*. The number of flies inside and outside the traps was counted after 24 h. The attraction index (AI) was calculated as $AI=(O-C)/T$, where O is the number of flies that entered the microbe trap, C is the number of flies that entered the growth medium trap and T is the sum of all flies tested. The resulting index ranges from −1 (complete avoidance) to 1 (complete attraction). A value of zero characterizes a neutral or non-responsive treatment. Each experiment was repeated 9–10 times.

Oviposition assays

Oviposition assays were carried out in a small container (10×10×20 cm) equipped with two Petri dishes (diameter, 5 cm) containing 0.5% agarose, of which one was loaded with 50 µl growth medium containing the equivalent of the microbe pellet or with 50 µl growth medium only. Twenty female flies, 4–5 days old, were placed in each container. Experiments were carried out in a climate chamber at the same conditions as the fly breeding. The number of eggs was counted after 24 h. The oviposition index was calculated as $(O-C)/(O+C)$, where O is the number of eggs on microbe treatment plate, and C is the number of eggs on the growth medium plate. Each experiment was repeated 10 times.

Feeding assays

Twenty-five flies (20 female and 5 male flies) were collected and tested at age 3–5 days. Flies were starved beforehand for 24 h with constant access to water. Flies were cooled for 3 min at −20°C and then transferred to the behavioral arena. The capillary feeder (CAFÉ) assays utilized glass micropipettes with liquid medium that were filled by capillary action and then inserted through pipette tips into the container holding the adult flies as previously described (Keesey et al., 2016, 2017). One capillary contained the control growth medium, while the other contained the microbe culture. The volume consumed from each side was measured after feeding for 4 h. Feeding index were calculated as $(O-C)/(O+C)$, where O is the amount of food consumed from the microbe solution and C is the amount of food consumed from the control solution. Each experiment was repeated 10 times.

Larval two-choice assays

The larval olfactory choice assays were performed as previously described (Ebrahim et al., 2015). The 50 3rd-instar larvae were placed in the center of a Petri dish which was filled with 0.5% agarose. The Petri dish contained two lids of an Eppendorf cap which were placed at opposite positions at the periphery of the Petri dish; 30 µl growth medium containing the equivalent of the microbe pellet or with 30 µl of growth medium only were loaded in each cap lid. Larvae were allowed to crawl for 5 min before their position on the Petri dish was determined. Attraction index was calculated as $(O-C)/T$, where O is the number of larvae on the side of the dish loaded with microbe, C is the number of larvae on the medium control side and T is the total number of larvae. Each experiment was repeated 9–10 times.

Single pair courtship and mating assays

Newly emerged virgin flies were collected; males were kept individually in separate vials, and females were reared in groups of 20–30 flies. All courtship experiments were performed with 4- to 5-day-old virgin flies and the behavioral experiments were conducted within a circular courtship arena (height, 0.5 cm; diameter, 1 cm). Mating and courtship behaviors were documented for 60 min and then analyzed. Copulation latency was measured as the time that the male and female took until successful copulation. Copulation success was calculated as the percentage of all pairs that mated within the 60 min time span. For each combination of flies, the experiments were repeated 20–24 times.

Adult body weight, body size and ovary size measurement

Male and female adults were collected and exposed to each microbe for 2 days in a similar fashion as for the oviposition assays. Fifteen individuals for each treatment were weighed on a Sartorious analytical balance ME235P (Sartorius Weighing Technology GmbH, Goettingen, Germany). The images of 6 male and female adults for each treatment were taken under a stereo microscope (Axio Zoom V16, Zeiss, Germany), where the areas of head, thorax and abdomen were measured with ImageJ software. The ovaries from 10 female flies of each group were dissected in 1× PBS; images were recorded and area measured as above.

Fecundity assessment

Ten female and 5 male virgin wild-type or *Orco*$^{-/-}$ flies were collected directly after emergence and raised continuously on control diet or on diet enriched with one of the three microbes. The diet was changed every 24 h and egg numbers were recorded every day for 1 week. Each experiment was repeated 10 times.

Larvae developmental timing

Fifty 1st-instar larvae were transferred to control diet or diet enriched with one of the three microbes. The number of pupae appearing was counted twice per day until the last larvae of the population reached the pupae state. Each experiment was repeated 10 times.

Chemical analysis

To analyze the volatiles emitted by the different microbes, a 500 ml laboratory glass bottle was filled with 400 ml fresh culture of the microbes and closed with a custom-made polyether ether ketone (PEEK) stopper. The headspace was collected for 24 h on a Super-Q filter (50 mg, Analytical Research Systems; www.ars-fla.com) according to standard procedures. Airflow at 0.5 l min^{-1} was drawn through the bottle by a pressure pump. The filter was eluted with 1 ml hexane, and samples were stored at −20°C until analysis. The fly bodywash extracts were obtained by washing 1 fly in 30 µl of methanol for 6 h as previously described (Keesey et al., 2017; Dweck et al., 2015b), 8-10 individual flies for each treatment were extracted. GC-MS (HP5 and HP-innowax) analyses were performed on all volatiles and insect body wash collections. Microbe volatiles as well as fly odors were analyzed via GC-MS. The GC was equipped with a HP5 (for fly bodywash) or HP-innowax (for microbe headspace) MS column (30 m long, 0.25 mm id, 25 µm film thickness; Agilent Technologies) with helium used as carrier gas (1.1 ml min^{-1} constant flow). The inlet temperature was set to 250°C. The temperature of the GC oven was held at 50°C for 2 min and then increased by 15°C min^{-1} to 280°C. The final temperature was held for 15 min. The MS transfer line was held at 280°C, the MS source at 230°C, and the MS quad at 150°C. Mass spectra were taken in EI mode (at 70 eV) in the range from 33 $m\,z^{-1}$ to 350 $m\,z^{-1}$ with a scanning rate of 4.42 scan s^{-1}. GC-MS data were processed with the MDS-Chemstation software (Agilent Technologies). Compounds were identified with the NIST 2.0 mass spectra database using the NIST algorithm. Identification was confirmed by comparison of Kovats retention indices with published data. Several compounds were also confirmed by comparison with synthetic standards (spectrum and retention time), obtained from Sigma-Aldrich (http://www.sigma-aldrich.com) at highest available purity. The internal standard bromodecane was used for quantification and statistical comparisons between analyzed samples.

Statistics analysis

Statistical analysis were performed using Prism 5, figures were prepared using Prism 5, Microsoft Excel and Adobe Illustrator CS5. Data were tested for a normal distribution and afterwards analyzed using two-tailed, paired *t*-tests or one-way ANOVA with Tukey's multiple comparison tests.

RESULTS

Drosophila preference for gut microbes

We first performed trap, oviposition and feeding assays (Fig. 1A) to analyze innate preferences of the flies for each of the different gut microbes. We next compared these preferences with those of flies that were either briefly exposed to one of the three species of gut microbes (*S. cerevisiae*, *L. plantarum* or *A. malorum*) or were reared on one of these microbes for several generations.

Flies raised on control diet without microbes were attracted by the headspaces of *S. cerevisiae* and *L. plantarum*, but were repelled by *A. malorum* (Fig. 1B). When we repeated the experiments with *Orco*$^{-/-}$ flies lacking functional odorant receptors (ORs), the preference for *S. cerevisiae* was abolished, while the preference for *L. plantarum* and the avoidance of *A. malorum* were not affected (Fig. 1C). We conclude that flies can detect the headspace of all tested microbes, and that the preference for yeast is governed by Orco-dependent ORs. We next gave the flies the opportunity to choose between oviposition sites with or without microbes (Fig. 1A). In contrast to the pure attraction assay, both wild-type flies (Fig. 1D) and *Orco*$^{-/-}$ flies (Fig. 1E) preferred to lay eggs on the plates with any of the three species of microbe. Hence, flies consider the presence of microbes during oviposition, and as this preference is conserved in *Orco*$^{-/-}$ flies, oviposition preference seems to be governed by ionotropic receptors (IRs) or gustatory receptors (GRs) that do not depend on the co-receptor Orco for the detection of environmental chemical cues.

In addition, we tested the flies' feeding preference for the same set of microbes by performing a CAFÉ assay. In this assay, flies can choose between a solution with or without the microbe (Fig. 1A). As the liquids are presented in tiny glass capillaries, any preference should be based mainly on cues detected by the labellum and palps of the flies (although we cannot fully exclude the evaporation of volatile compounds from the capillaries and thereby the involvement of antenna in any kind of choice). However, we did not observe any preference for microbes versus the control liquid, and we assert that the labellum and palps do not seem to be involved in the flies' preference or avoidance of *S. cerevisiae*, *L. plantarum* or *A. malorum* (Fig. 1F,G).

We next asked whether the adult preferences are conserved in larvae using a larval attraction assay (Fig. 1A, bottom). Larvae showed the same preference trend as displayed by adult flies in the trap assay, i.e. larvae were attracted to *S. cerevisiae* and *L. plantarum*, but repelled by *A. malorum*, indicating that both larvae and adults share the same olfactory mechanisms involved in these consistent preference behaviors (Fig. 1H).

Flies detect cues of high ecological relevance, like harmful microbes or parasitoids via highly specialized neuronal circuits that are dedicated to detection of signature odors (Stensmyr et al., 2012; Ebrahim et al., 2015). In order to investigate whether the behavior towards any of the gut microbes of this study was governed by such a labeled line, we analyzed the headspaces of the different microbe cultures via gas chromatography-coupled mass spectrometry (GC-MS) (Fig. S1). In our samples, we also identified compounds like 3-methyl-1-butanol and 2-phenylethanol that are described as attractants (Knaden et al., 2012; Becher et al., 2012) and benzaldehyde which has been shown to be aversive (Knaden et al., 2012). However, all of these compounds are detected by rather widely tuned receptors at the concentrations that have been tested (Hallem and Carlson, 2006). Although we cannot exclude that we overlooked novel ligands that might be detected by a dedicated pathway, the presence of a wide range of general odors that are well known to attract flies, makes the involvement of a labeled line unlikely in *Drosophila melanogaster* response towards these microbes.

The effect of gut microbiome on behavioral preference of flies

After showing that the flies become attracted to two of the microbe types and that all microbes positively affect the flies' oviposition

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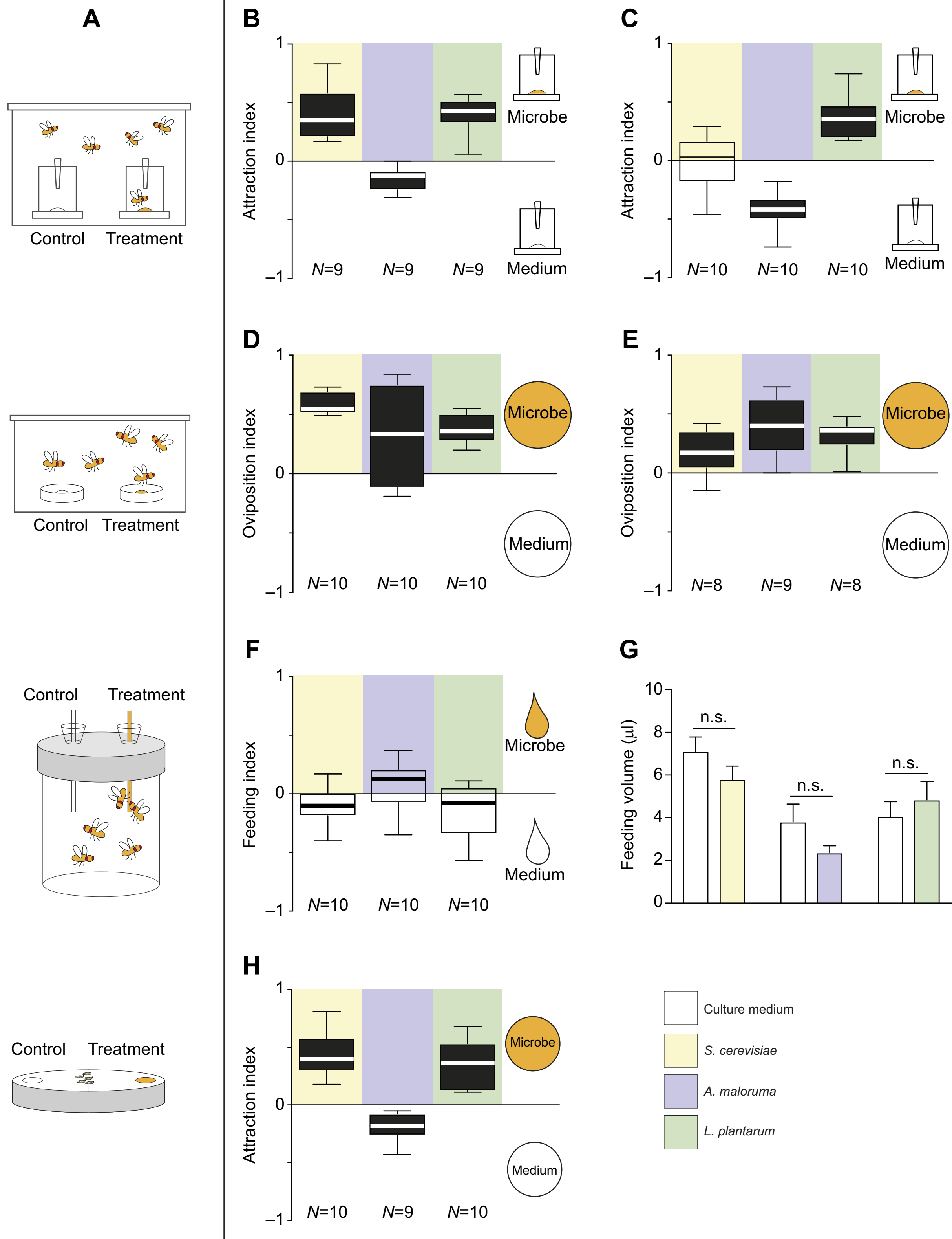

Fig. 1. Attraction assays of *Drosophila* toward different microbes. (A) Experimental designs used for attraction, oviposition and feeding assays. (B,C) Attraction index of naïve wild-type (B) or *Orco*$^{-/-}$ (C) flies towards the olfactory cues from each microbe culture and medium control. (D,E) Oviposition preference index of wild-type (D) or *Orco*$^{-/-}$ (E) flies towards the olfactory cues from each microbe culture and medium control. (F,G) Feeding index of wild-type flies towards each microbe culture and medium control (F) and the feeding volume (G) after 4 h. (H) Attraction index of the 3rd-instar larvae towards the olfactory cues from each microbe culture and medium control. Error bars represent s.e.m. Significance from zero is denoted by filled boxes in B–F,H (P<0.05, two-tailed, paired t-test); no significant differences are denoted by n.s. above bars in G (one-way ANOVA, Tukey's multiple comparison test). In box plots, bar indicates median, box indicates upper and lower quartile, whiskers indicate maximum and minimum values.

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choices, we next asked whether the flies' behavior would change after they have been exposed to one of the microbes for a prolonged time. We hypothesized that flies after exposure to only one microbe should switch their preference to the other microbes in order to keep a diverse and healthy gut microbiome. As an alternative hypothesis, flies could instead prefer those microbes they are familiar with. *Drosophila* were manipulated by raising them on fly food enriched with one of the microbe species for either 2 days or for several generations. With these manipulated flies, we performed the same behavioral assays as before. Irrespective of their pre-experimental exposure to one of the microbes, the flies still became attracted by the headspaces of *S. cerevisiae* and *L. plantarum* and repelled by that of *A. malorum* (Fig. 2A–C, Fig. S2). However, pre-exposure to *S. cerevisiae* significantly increased the preference to this microbe and the avoidance of *A. malorum*, suggesting that exposure to these microbes may generate a learned response that accentuates the behavioral decisions towards these microbes. We therefore conclude that flies do not increase their preference towards gut microbes that they had not been in contact with previously. We furthermore did not find effects of pre-exposure on the oviposition preference of these flies (Fig. 2D–F). Interestingly, exposing flies to *S. cerevisiae* and *A. malorum* significantly increased the total egg numbers that the flies laid during the oviposition assay (Fig. 2G–I). It remains unclear why flies are repelled by the headspace of *A. malorum*, which has a positive effect on the flies' fecundity.

We next tested whether the oviposition behaviors were correlated with the flies' courtship and mating behaviors. The behavioral performance of individual pairs of flies was analyzed in courtship and mating assays. To do so, we paired flies reared on the different diets in all possible combinations. The copulation success rates of *L. plantarum*-treated flies was lower than that of all other flies (Fig. 3A) and their copulation latency was significantly higher (Fig. 3B). Interestingly, a strong increase in latency was observed only when both sexes were reared on *L. plantarum* (Fig. 3B). We hypothesized that the decline in courtship and mating performance

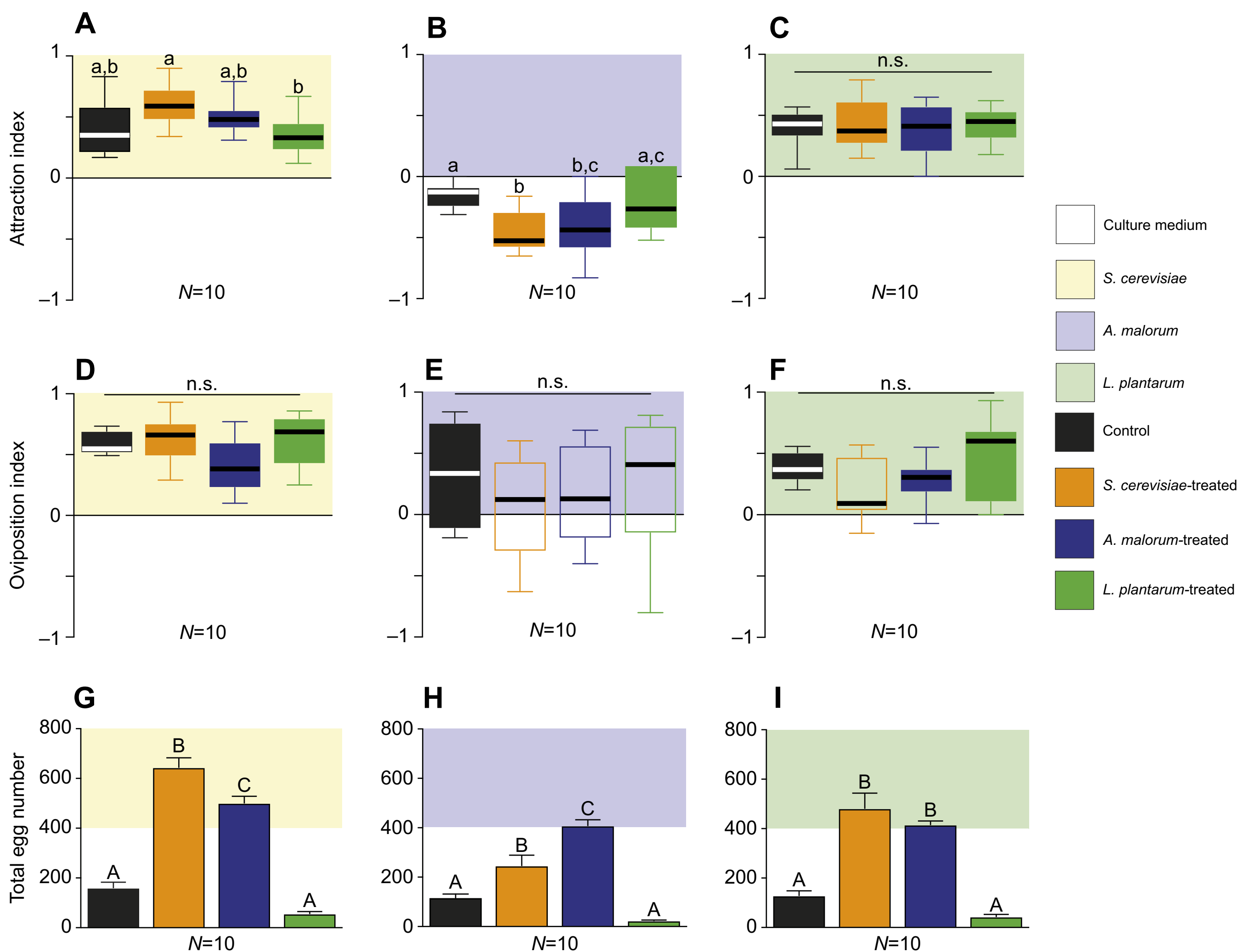

Fig. 2. Attraction and oviposition assays of differently pre-treated *Drosophila* toward microbes. (A–C) Attraction index of adult *Drosophila* pre-exposed to different microbes for 2 days toward the olfactory cues from *S. cerevisiae* (A), *A. malorum* (B) and *L. plantarum* (C) (top) compared with the cues from growth medium control (bottom). Filled box plots are different from 0 (Wilcoxon rank sum test, $P<0.05$). (D–F) Oviposition preference index of adult *Drosophila* manipulated with different microbes for 2 days toward the olfactory cues from *S. cerevisiae* (D), *A. malorum* (E) and *L. plantarum* (F) compared with the cues from growth medium control. Filled box plots are different from 0 ($P<0.05$, two-tailed paired *t*-test). (G–I) Total egg numbers laid in experiments D–F. Error bars represent s.e.m. Significance from zero is denoted by filled boxes in A–F ($P<0.05$, two-tailed, paired *t*-test); significant differences are denoted by different letters in G–I ($P<0.01$; one-way ANOVA, Tukey's multiple comparison test).

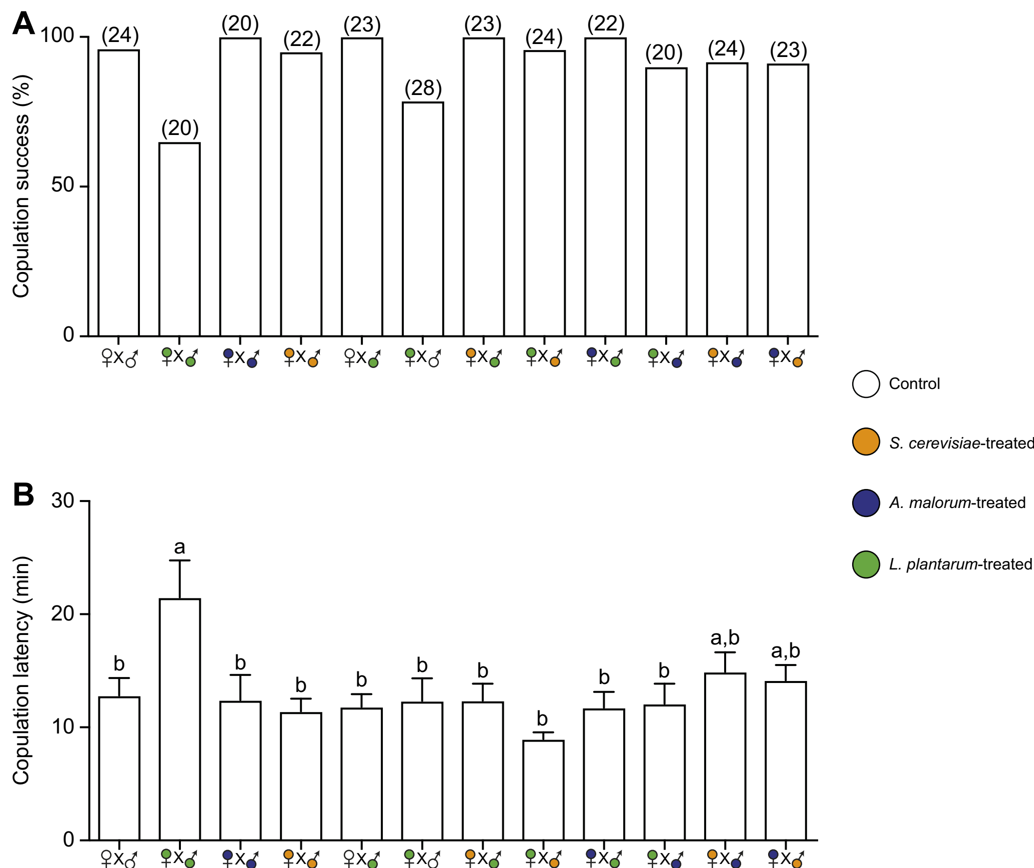

Fig. 3. Copulation success and latency in control and pre-exposed *Drosophila*. Percentage of copulation success (A) and copulation latency (B) for control and for *Drosophila* pre-exposed to microbes for 2 days. Error bars represent s.e.m. Significant differences are denoted by different letters (*P*<0.05, one-way ANOVA, Tukey's multiple comparison test). Sample sizes are given in brackets above bars in A.

of *L. plantarum*-treated flies could be one of the reasons for their subsequently lower number of eggs in the oviposition assays. To answer what affected the courtship behavior, we prepared the bodywash of both male and female flies of all treatments and analyzed the resulting compounds by GC-MS. When testing for effects of the microbes on the amount of sex- and aggregation-related pheromones like methyl laurate (ML), methyl myristate, methyl palmitate (MP) (Dweck et al., 2015b), cis-vaccenyl acetate (cVA) (Bartelt et al., 1985) and the male-specific (Z)-7-tricosene (Lacaille et al., 2007), we found only minor (in most cases non-significant) differences depending on the treatment (Fig. S3A–D). Furthermore, two female-specific cuticular hydrocarbons, 7(Z),11(Z)-heptacosadiene (7,11-HD) and 7(Z),11(Z)-nonacosadiene (7,11-ND), which play important roles in *Drosophila* courtship (Ferveur, 1997; Toda et al., 2012) were significantly increased in female flies after treatment with *S. cerevisiae* and *A. malorum* (Fig. 4). However, as *L. plantarum*-treated flies did not differ from flies reared on standard diet regarding these compounds, the increased courtship latency found in *L. plantarum*-treated flies (and specifically associated with females), remains unclear.

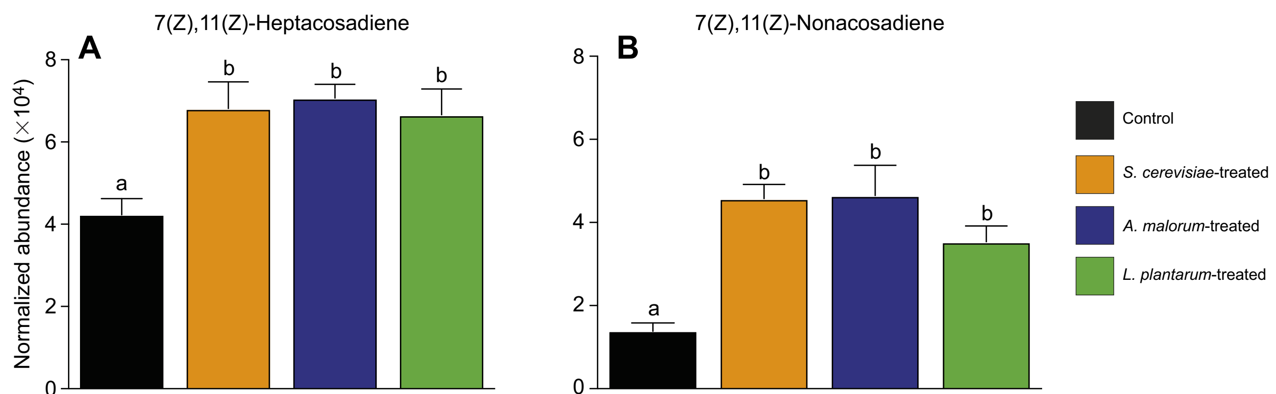

Fig. 4. GC-MS analysis of body wash from differently pre-exposed *Drosophila*. Significantly different cuticular hydrocarbons 7(Z),11(Z)-heptacosadiene (A) and 7(Z),11(Z)-nonacosadiene (B) found in females (bromodecane as internal standard). Error bars represent s.e.m. Significant differences are denoted by different letters (*N*=8–10 replicates; *P*<0.05, one-way ANOVA, Tukey's multiple comparison test).

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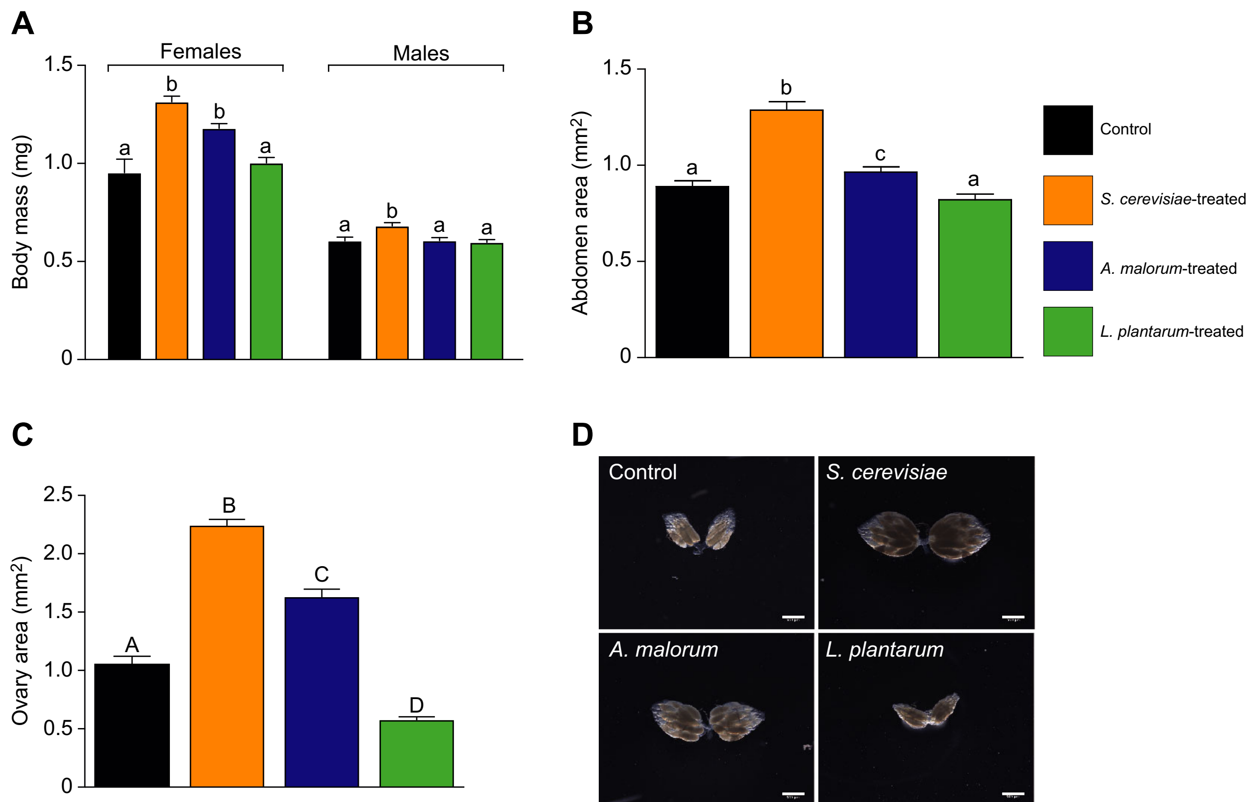

Fig. 5. Anatomical measurements from differently pre-exposed *Drosophila*. Single fly body weight (A) (*N*=15 females and males), female abdomen size (B) (*N*=6 females) and ovary size (C) (*N*=10 females) measurement and ovary images (D) of *Drosophila* that were either reared on control diet or pre-exposed to microbes for 2 days. Error bars represent s.e.m. Significant differences are denoted by letters ($P<0.05$ with lowercase letters in A,B; $P<0.01$ with capital letters in C, one-way ANOVA and Tukey's multiple comparison test). Scale bars: 500 µm.

Gut microbes affected fly ovary development

To find out how the microbes affect the flies' egg laying behavior, we tested the effect of the microbes on single fly body weight, body size and ovary size. Consistent with the observed increase in egg numbers for flies treated with *S. cerevisiae* and *A. malorum*, females in these treatment groups were heavier than control flies and heavier than *L. plantarum*-treated flies. For male flies, only *S. cerevisiae*-treated flies were slightly (but significantly) heavier than the others (Fig. 5A). We next took images of the different treated flies and measured the size of the abdomen. Interestingly, there was no difference in abdomen size in any of the male flies, while females treated with *S. cerevisiae* exhibited a bigger abdomen than *A. malorum*-treated flies, and both of these were bigger than those in *L. plantarum*-treated and control flies (Fig. 5B, Fig. S4). As the difference of egg number fitted well with the female abdomen size, we next dissected and measured female ovaries for each microbial treatment. Again, ovaries of *S. cerevisiae*-treated females were bigger than those of *A. malorum*-treated flies, and the ovaries of both of these two groups were significantly bigger than those of control flies and flies kept on *L. plantarum* (Fig. 5C,D).

The effect of gut microbes on fly fecundity

Having shown that the treatment with *S. cerevisiae* or *A. malorum* resulted in more eggs in the oviposition assay and increased abdomen and ovary sizes in female flies, we next asked whether this would also result in an overall increased fecundity of these flies. When we kept the flies for several days on the different diets and counted the number of eggs on a daily basis, all flies started to lay eggs after 2 days of reproductive maturation, with increasing egg numbers per day until the fourth day. Afterwards, the egg number kept steady per day until the end of the experiment. As expected from the previous results of our study, flies treated with *S. cerevisiae* laid the highest number of eggs (higher during each day and also in total during the full week), while *A. malorum*-treated flies still laid more eggs than *L. plantarum*-treated flies and control flies. We next asked whether a temporary exposure to *S. cerevisiae* would be sufficient to increase a female fly's fecundity over its entire lifetime. However, when we transferred the flies back to a control diet after 4 days on a diet with *S. cerevisiae*, the daily egg count decreased dramatically and reached that of flies fed on control diet or *L. plantarum* after 2 days (Fig. 6A,B). This indicates that increased fecundity needs a constant supply of *S. cerevisiae* and that the change in fecundity is temporary. Interestingly, although we showed that *Orco*$^{-/-}$ flies became less attracted to the *S. cerevisiae* (Fig. 1C), the lack of functional OSNs expressing the co-receptor Orco did not diminish the positive effect of the microbes on the flies' fecundity (Fig. 6C,D). Obviously, although *Orco*$^{-/-}$ flies become less attracted by the headspace of *S. cerevisiae*, they still consume this microbe when being reared on them.

Gut microbe mediated larval growth acceleration

Finally, we also tested whether the exposure of flies to the microbes affected the larval development. It became apparent, that when testing 50 larvae per treatment type (e.g. *S. cerevisiae*), the number of larvae that succeeded in pupation was similar for all diets (Fig. 7A). However, the treatment affected the average timespan until larvae reached the pupal stage, thus the developmental rate was affected by microbe exposure. While

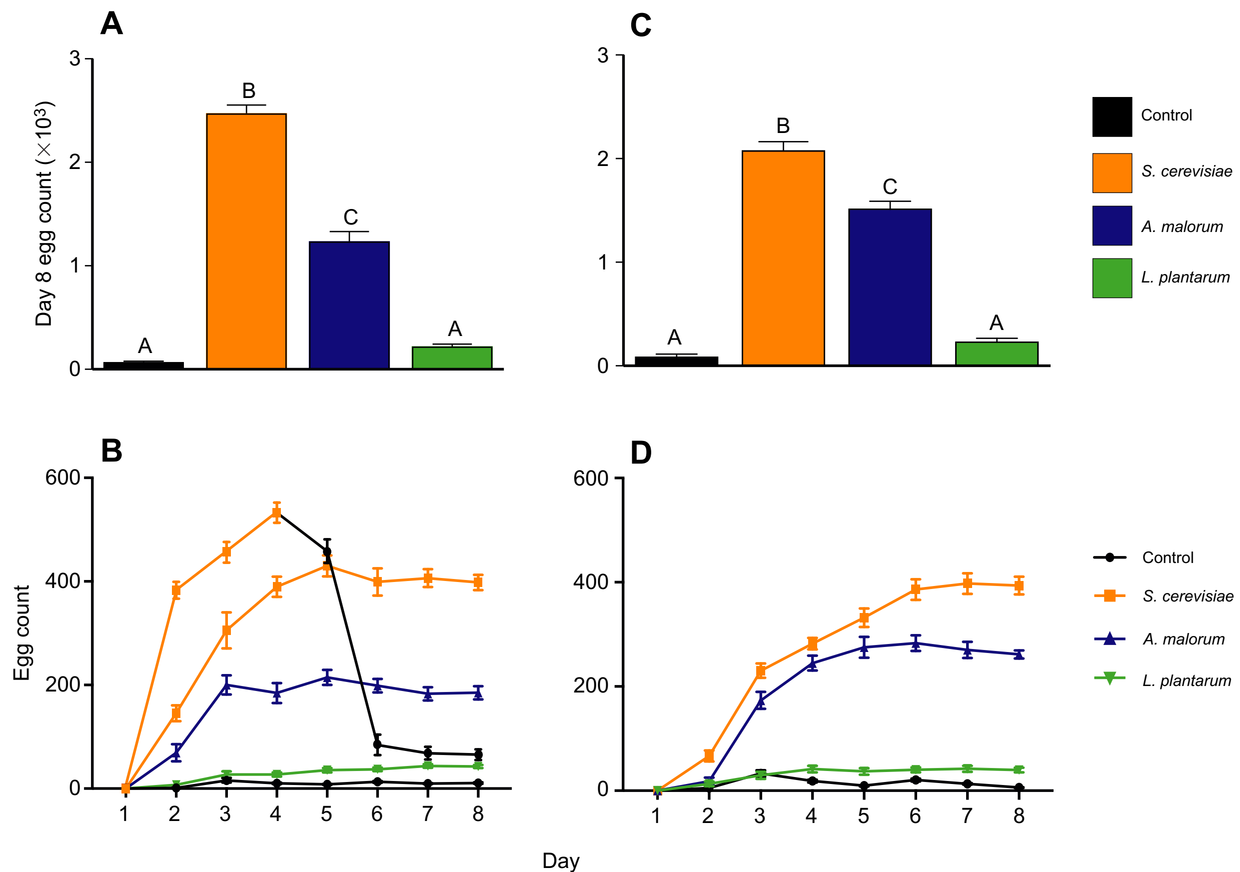

Fig. 6. Fecundity of *Drosophila* fed on control diet or on diet containing different microbes. (A,C) Comparison of total egg count after 8 days in *Drosophila* wild type (A) and *Orco*$^{-/-}$ (C) fed on different diets. (B,D) Comparison of daily egg number in *Drosophila* wild type (B) and *Orco*$^{-/-}$ (D) fed on different diets. Error bars represent s.e.m. of N=10 samples for each group. Significant differences are denoted by different letters (P<0.01, one-way ANOVA and Tukey's multiple comparison test). Orange/black line in B, data from flies that were kept for 4 days on *S. cerevisiae* and were moved to control diet afterwards.

most larvae that were reared on *S. cerevisiae* reached pupation stage after 3.5 days, all other treatments resulted in durations of 4–4.5 days (Fig. 7B), giving flies reared on *S. cerevisiae* a 30% faster development time.

DISCUSSION

In this study, we found that *D. melanogaster* showed different behavioral preferences to gut microbes. It is known that *Drosophila* are highly attracted to volatiles associated with yeast (Becher et al., 2012) and fermenting fruit (Becher et al., 2010; Keesey et al., 2015). The same attractive compounds, such as 3-methyl-1-butanol and 2-phenylethanol, were also identified in *S. cerevisiae* culture in our experiments. Previous studies have shown that *D. melanogaster* flies display positional avoidance towards carboxylic acids (i.e. one major compound produced by lactic and acetic acid bacteria), while the flies show a preference to lay eggs on sites containing these acids (Joseph et al., 2009; Chen and Amrein, 2017). When, however, not presenting the acids alone, but the full headspace of the bacteria, we

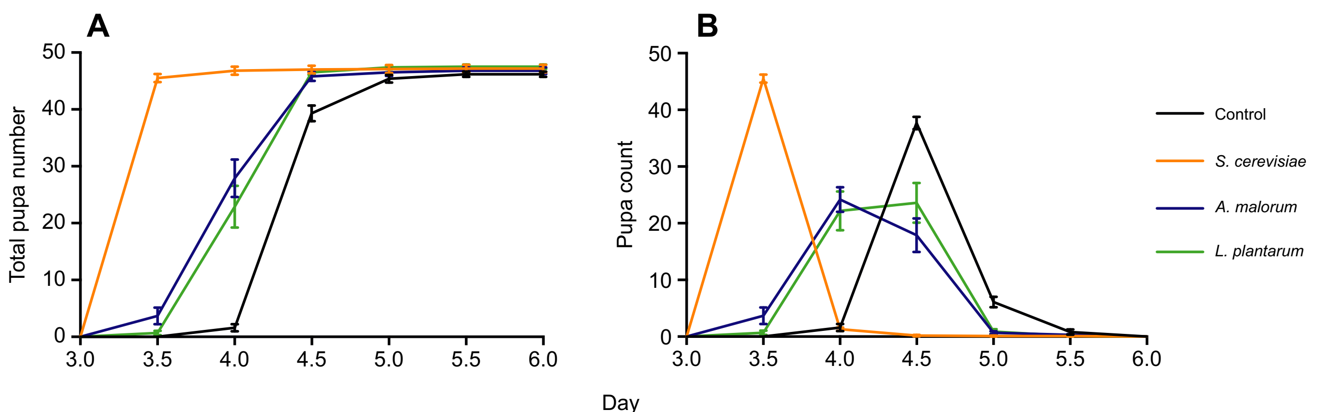

Fig. 7. Larval developmental time on control diet or diet with different microbes. (A) Accumulation curve of pupae appearing on different diets. (B) Number of pupae visible every day on different diets. N=10 samples for each.

found that both larvae and adults were attracted by the lactic acid bacteria *L. plantarum* and repelled by the acetic acid bacteria *A. malorum* in choice assays. Obviously attraction towards lactic acid bacteria is not driven by the acids themselves but rather by accompanying compounds in the blend. As the preference for *S. cerevisiae* was absent in *Orco*$^{-/-}$ flies and the flies did not prefer any microbes in a CAFÉ assay (that tests for gustatory preference while basically excluding the impact of olfactory stimuli), olfaction is at least partly involved in the flies' attraction to gut microbes for feeding. However, as *Orco*$^{-/-}$ flies still targeted *L. plantarum* in the trap assay and preferred all microbes in the oviposition assay, our data suggest that ionotropic receptors (IRs) (Benton et al., 2009) and/or gustatory receptors (GRs) are also involved in this behavioral preference. Sensory neurons expressing IRs have been reported to mainly detect acids (Ai et al., 2010; Grosjean et al., 2011; Min et al., 2013; Hussain et al., 2016) and have been shown to mediate *Drosophila* oviposition preference on acid-containing medium (Chen and Amrein, 2017). Hence, the attraction towards and the oviposition preference for the different microbes might be governed by several different receptor types, or sensory modalities.

Compared with *Drosophila* raised on standard diet, raising the flies on diets enriched with one of the microbes in some cases resulted in different preferences in the trap assays (Fig. 2A,B), which is in agreement with Wong et al. (2017) who found different foraging preferences in flies that were either axenic or those flies that were mono-associated with *L. plantarum* or *Acetobacter pomorum*. The Wong et al. (2017) study used axenic flies, whose guts are basically free of microorganisms or contain only a single microbe species due to a specific treatment. This comes with the benefit that the microbiome of the studied flies is under full control as compared with our treatment where the flies' gut microbiome was manipulated just by exposing them to single microbe types over a specific time. Despite this potential drawback, we assume that our flies had a more conventional gut microbiome like the one expected in natural conditions. However, our data suggest that – even in flies with a 'normal' gut microbiome – an exposure to one microbe species can alter the flies' behavioral preferences.

We also found that exposure to the different microbes not only affected the flies' behaviors, but also promoted their growth and development. It is known that yeasts are vital for larval development and survival (Anagnostou et al., 2010; Becher et al., 2012) as they seem to provide proteins as well as most other non-caloric nutritional requirements in *Drosophila* (Piper et al., 2013). *A. pomorum*, which is a close relative of the microbe *A. malorum* that was used in our study, is able to influence the systemic larval development of *Drosophila* by affecting both growth rate and body size via the insulin signaling pathway (Shin et al., 2011). Furthermore, *L. plantarum* can promote larval growth and increase the growth rate of the flies on yeast-poor medium, without affecting the size of flies, by regulating hormonal signals through TOR-dependent nutrient sensing (Storelli et al., 2011). Finally, it is shown that deprivation of essential amino acids (eAAs) can induce increased yeast intake and decreased reproduction of *Drosophila*, but both changes can be rescued by the introduction of healthy gut bacteria, *A. pomorum* and *Lactobacillus* sp. (Leitão-Gonçalves et al., 2017).

In agreement with the aforementioned studies of the microbial impact on the flies' growth and development, we found that the gut microbes have a different impact on female *Drosophila* ovary development and fecundity. More specifically the total egg number of control flies and flies reared on *L. plantarum* in oviposition assays was similar and significantly lower when compared with flies from the other two microbes (Fig. 2G–I). In addition, there was only a slight impact on total fecundity (Fig. 6A,B) and on larval development (Fig. 7) when flies were treated with *L. plantarum*. However, we found clear positive effects when flies were reared on *S. cerevisiae* and *A. malorum*, such as faster larval development times and larger ovaries that accompanied increased fecundity. Thus, these results demonstrate that *S. cerevisiae*, *L. plantarum* and *A. malorum* can be beneficial partners for *D. melanogaster*, and our results help to explain the common and natural association of these microbes with this fly. It remains, however, open as to why flies become attracted by the former two microbes but avoid the headspace of *A. malorum*. In conclusion, our study demonstrates the importance of preference among microbial associations for the ecological advantage of *Drosophila* in their natural environment, where some microbes promote either fecundity or developmental speed, the latter of which aids these insects in avoiding predation and parasitism during their most vulnerable larval stages.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.Q., I.K., B.S.H., M.K.; Methodology: H.Q., I.K., M.K.; Validation: M.K.; Formal analysis: H.Q., I.K.; Investigation: H.Q.; Resources: B.S.H.; Writing - original draft: H.Q.; Writing - review & editing: I.K., B.S.H., M.K.; Supervision: I.K., M.K.; Funding acquisition: H.Q.

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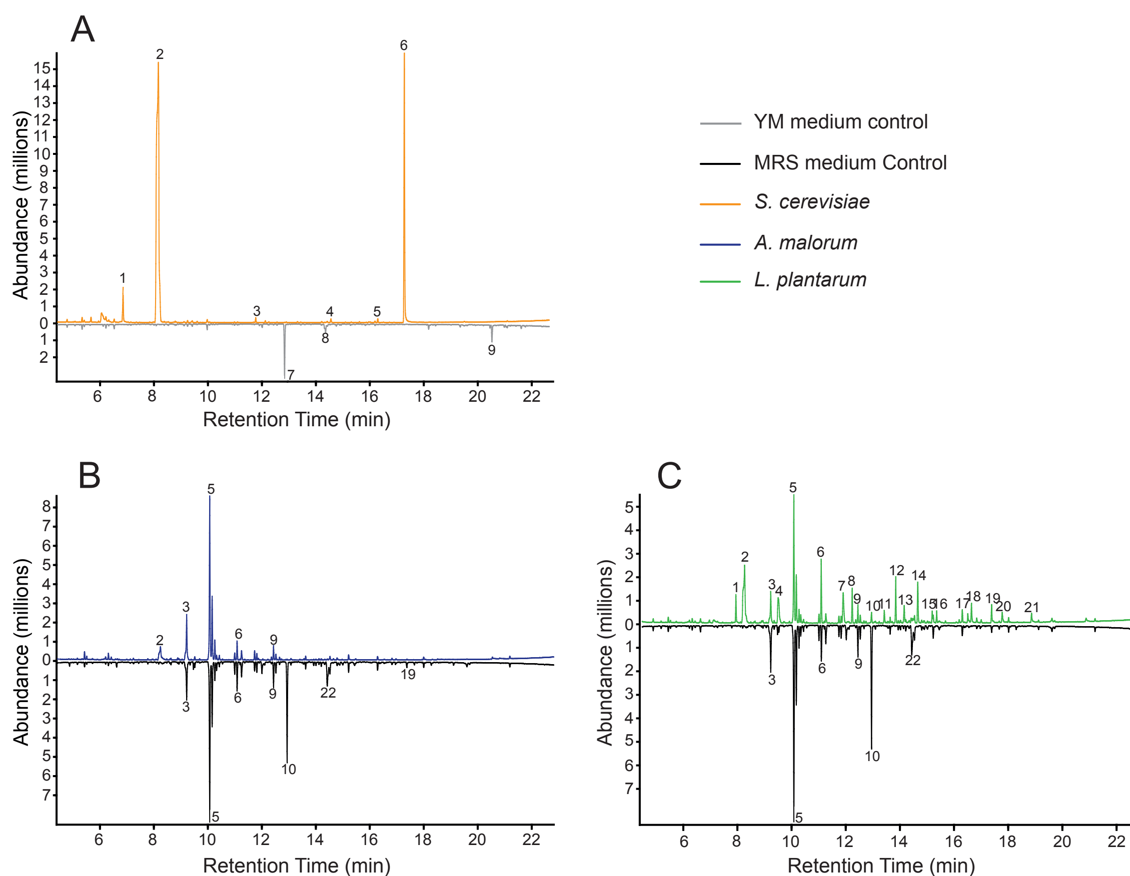

Fig. S1. GC-MS profile of headspace odors from different microbe culture and its medium control. Numbers from GC-MS refer to peaks in **A**: (1) isopentyl acetate; (2) 3-methyl-1-butanol; (3) isovaleric anhydride; (4) 2-methylhexanoic acid; (5) 2-phenethyl acetate; (6) 2-phenylethanol; (7) benzaldehyde; (8) phenylacetaldehyde; (9) (E)-1-(6,10-Dimethylundeca- 5,9-dien-2-yl)-4-methylbenzene. Numbers from GC-MS refer to peaks in **B and C**: (1) 2-heptanone; (2) 3-methyl-1-butanol; (3) methylpyrazine; (4) acetoin; (5) 2,5-dimethylpyrazine; (6) 2-nonanone; (7) acetic acid; (8) 2,6-dimethyl-4-heptanol; (9) 2-ethenyl-6-methyl pyrazine; (10) benzaldehyde; (11) isobutyric acid; (12) 2-undecanone; (13) butanoic acid; (14) isovaleric acid; (15) 2-Undecanol; (16) 1-methoxynonane; (17) 2-acetylphenol; (18) hexanoic acid; (19) phenylethyl alcohol; (20) (Z)-4-decen-1-ol, methyl ether; (21) octanoic acid; (22) benzeneacetaldehyde.

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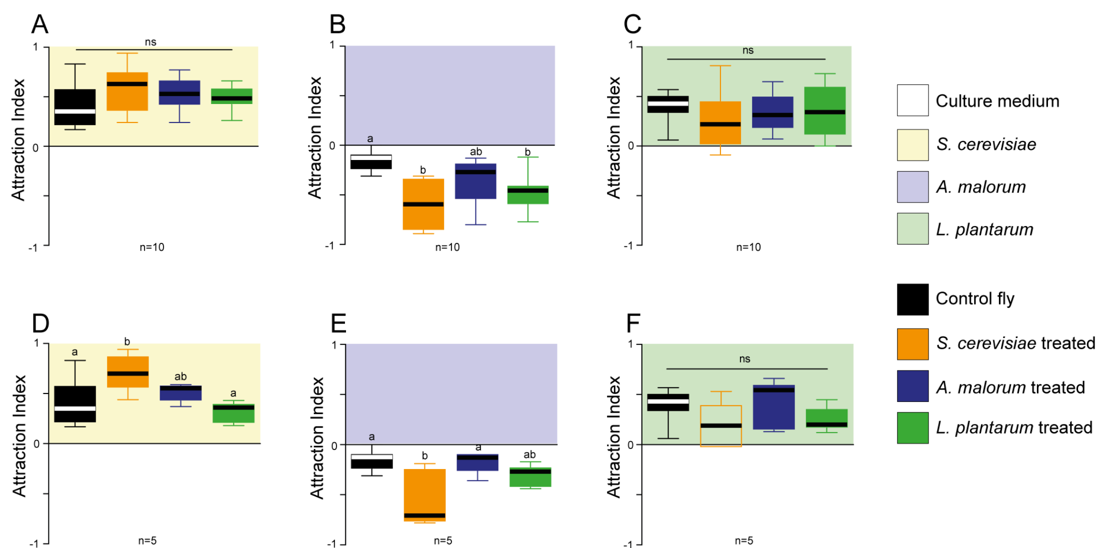

Fig. S2. Attraction assays of *Drosophila* manipulated for 1 generation (A-C) and 5 generations (D-F) toward different microbes. Error bars represent SE. Significance from zero are denoted by filled boxes ($p<0.05$, Two tailed paired t test), significant differences between each group are denoted by letters above ($p<0.05$ with lower letter, One way ANOVA, Tukey's multiple comparison test). Filled boxplots are different from 0 (Wilcoxon rank sum test, $p<0.05$).

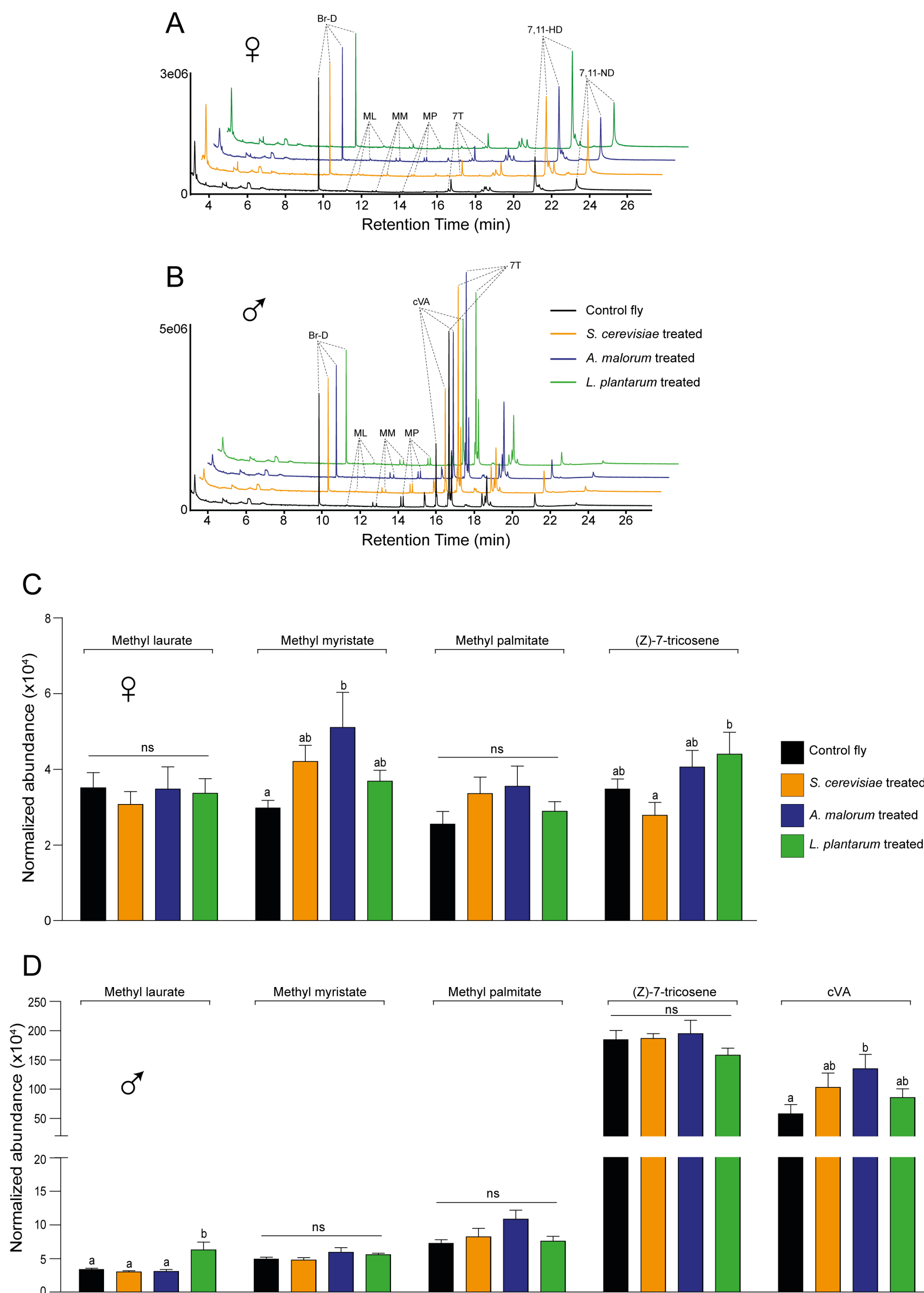

Fig. S3. GC-MS profiles of body wash from female (A) and male (B), and quantitative analysis of compounds from female (C) and male (D) *Drosophila* control and manipulated for 2 days with different microbes. Br-D, bromodecane (internal standard); ML, methyl laurate; MM, methyl myristate; MP, methyl palmitate; 7T, (Z)-7-tricosene; cVA, cis-vaccenyl acetate; 7,11-HD, 7(Z),11(Z)-heptacosadiene; 7,11-ND, 7(Z),11(Z)-nonacosadiene. (N=8-10 replicates, $p<0.05$ with lower letter, One way ANOVA, Tukey's multiple comparison test).

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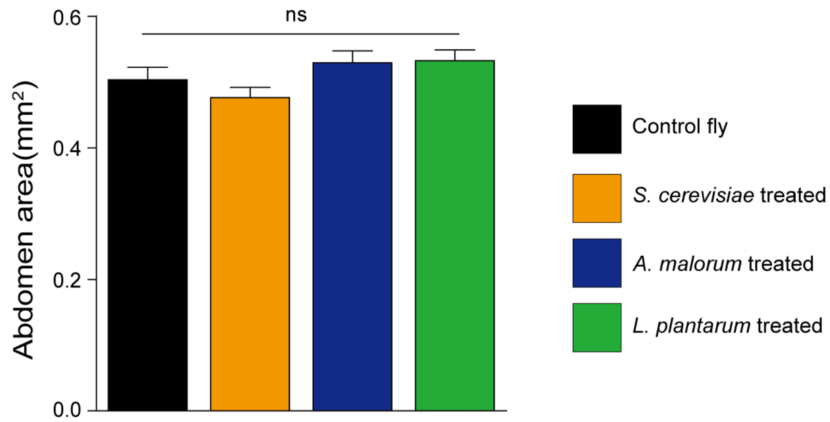

Fig. S4. Male abdomen size of control and 2 days manipulated *Drosophila*. Error bars represent SE. Significant differences are denoted by letters (One way ANOVA, Tukey's multiple comparison test).

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