Ratio Between Lactobacillus Plantarum and Acetobacter Pomorum on the Surface of Drosophila Melanogaster Adult Flies Depends on Cuticle Melanisation

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Research note

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Ratio between *Lactobacillus plantarum* and *Acetobacter pomorum* on the surface of *Drosophila melanogaster* adult flies depends on cuticle melanisation

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Abstract

Objectives

As in most organisms, the surface of the fruit fly *Drosophila melanogaster* is associated with bacteria. In order to study the genetic parameters of this association, we developed a simple protocol for surface bacteria isolation and quantification.

Results

On wild-type flies maintained in the laboratory, we identified two persistently culturable species as *Lactobacillus plantarum* and *Acetobacter pomorum* by 16S rDNA sequencing. For quantification, we showered single flies for DNA extraction avoiding the rectum to prevent contamination from the gut. Using specific primers for quantitative PCR analyses, we determined the relative abundance of these two species in surface wash samples. Repeatedly, we found 20% more *L. plantarum* than *A. pomorum*. To tentatively study the importance of the cuticle for the interaction of the surface with these bacteria, applying Crispr/Cas9 gene editing in the initial wild-type flies, we generated flies mutant for the *ebony* gene needed for cuticle melanisation and determined the *L. plantarum* to *A. pomorum* ratio on these flies. We found that the relative abundance of *L. plantarum* increased substantially on *ebony* flies. We conclude

that the cuticle chemistry is crucial for surface bacteria composition. This finding may inspire future studies on cuticle-microbiome interactions.

Keywords
Microbiome, bacteria, insect, *Drosophila*, cuticle

Introduction

Bacteria populate the surface of many organisms. While skin bacteria are well analysed in vertebrates including humans [1, 2], surface bacteria-insect interactions have been largely neglected to date. Most of data on surface bacteria come from studies in ants such as *Camponotus femoratus* and *Crematogaster levior*. In ant colonies, surface bacteria are considered to be involved in protection against fungal infection [3, 4]. A few data are available on bacteria on the surface of the fruit fly *Drosophila melanogaster*. The most common surface bacteria in this species belong to the genera *Lactobacillus* and *Acetobacter* [5]. The role of surface bacteria in *D. melanogaster* has not been studied.

The parameters on the insect defining bacteria-insect surface association are largely unknown. It is conceivable that microorganisms interact with components of the cuticle that is a stratified extracellular matrix composed of chitin, proteins, catecholamines and lipids [6]. Especially, the components of the surface called envelope including waxes and cuticular hydrocarbons (CHCs) [7] may be used as a substrate for bacterial attachment and/or for nutrition. In addition, this interaction may also depend on the inner-cuticle chemical environment including water content that in turn, at least partly, depends on the hardening and melanisation degree of the cuticle that involves a well-studied cascade of reactions catalised by cytoplasmic and extracellular enzymes [8].

In the present work, we have designed a protocol for surface bacteria isolation and relative quantification in *D. melanogaster*. In a pilot experiment, we show that the bacterial composition depends on cuticle melanisation.

Main text

Materials and Methods

Fly work

Wild-type Tübingen 2018 flies were kept under laboratory conditions (22°C, 50-70% air humidity) in vials with artificial diet that consists of corn meal, agar, beet sugar, propionic acid, dry yeast and Nipagin M. For fluorescein feeding, fluorescein sodium salt (Sigma Aldrich) was mixed with fresh baker's yeast added to the vials.

Isolation of surface bacteria

Flies were individually rubbed against the surface of a sterile agar plate (China Blue, ECI, EMB, LB, BHI and MRS, ingredients from Sigma-Aldrich) inside laminar conditions using sterile forceps forming a short lane and incubated for 1-5 days aerobically. Cultivation condition for each media were chosen depending on used media. We obtained mixed populations of different microorganisms. Individual colonies were isolated and sub-cultured twice to ensure purity. Isolated bacteria were characterized morphologically using a light microscopy and identified using a 16S rDNA analysis.

Molecular biology

The DNA template was prepared by picking an individual colony of each bacterial strain. Amplification of the 16S rDNA gene was carried out according to a standard protocol by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [9]. PCR products were purified before sequencing using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich). To identify the species, the sequences were aligned to sequences of the NCBI database using BlastN.

For species-specific quantitative PCR (qPCR) experiments, single flies were immobilised with forceps and spilled with Tris-EDTA (pH8.0) containing 200ng/µl Proteinase K avoiding the rectum. The wash solutions of 20 flies were combined, incubated at 65°C for 30 minutes and

frozen at -20°C. After thawing and centrifugation, 5µl of this solution was used in a 10µl reaction solution containing 1µl of each species-specific primer. For species-specific qPCR, the primers pREV (5'-TCGGGATTACCAAACATCAC-3') and pLanF (5'-CCGTTTCTGCGGAACACCTA-3') to amplify *recA* (318 bp) in *Lactobacillus plantarum* [10] and PASTEU-F (5'-TCAAGTCCTCATGGCCCTTATG-3') and PASTEU-R (5'-TCGAGTTGCAGAGTGCAATCC-3') to amplify 130 bp of the 16S rDNA loci of *Acetobacter* species including *A. pomorum* and *pasteurianus* were used [11].

To mutate the *ebony* locus in Tübingen 2018 flies, gene editing according to the Crispr/cas9 method was applied. We used the published *ebony* gDNA (oligos: 5'-GCGTTTAGTCGCAAAGAAGAA-3' and 5'-TACTGCCCGAGGTGTAGAGC-3') directed against the *ebony* gene sub-cloned in the pCDF3 vector [12]. This construct (550ng/µl TE buffer) was injected into pre-blastoderm embryos together with 250 ng/µl of Cas9 protein (New England Biolabs). To identify mutant *ebony* alleles, the respective flies were crossed to flies segregating the TM6B balancer that carries the $ebony^{1}$ allele. Stocks of dark flies were established. Homozygous *ebony* mutant flies ($ebony^{cc1\ or\ 3}$) were sequenced to identify the mutation.

Microscopy

Bacterial colonies were observed and imaged on a Leica EZ4 stereomicroscope with in-built camera using the software LAX. Bacterial cells were viewed on a Nikon Ti2 microscope using phase contrast microscopy with a S Plan Fluor ELWD 40x Ph2 ADM objective.

To visualize fluorescein traces on the fly surface, flies were anesthetized with CO_2 and viewed with the Nikon AZ100 using fluorescence microscopy mode with a LED light source and a F36-525 HC-set EGFP filter.

Results and discussion

Isolation and quantification of D. melanogaster surface bacteria

We have developed a simple protocol to isolate and quantify surface bacteria from adult *D. melanogaster* by single fly showering for DNA extraction and subsequent qPCR. During the wash procedure, we avoided the contact of the wash solution with the rectum thereby preventing contamination with gut microbes. This protocol differs substantially from a protocol published recently on ant surface bacteria identification [13]. In this case, whole ants were stirred in microtubes for DNA extraction. In a strict sense, this protocol cannot exclude gut microbe contamination in the wash solution. We assume that our simple protocol is applicable on any insect species.

Lactobacillus plantarum and Acetobacter pomorum are the major culturable bacteria on the fly surface

To isolate and characterise fly surface bacteria, we streaked the dorsum of living wild-type flies from Tübingen on different media including MRS (DeMan, Rogosa, Sharpe). To exclude gut-derived bacteria, we avoided contacting the rectum with the medium. Persistently, in independent experiments, we observed two types of colonies on MRS plates (Fig. 1). The colonies were round and white or yellowish. Under the light microscope, bacteria from both colonies showed a rode shape (Fig. 1). To determine the species, we amplified the 16S rDNA locus using universal primers and sequenced the amplicon. Alignment of the amplified sequences with sequences from the NCBI nucleotide database revealed that the 16S rDNA sequence from bacteria forming white colonies was highly similar to the respective sequence from *Lactobacillus plantarum* (Table 1), while the 16S rDNA sequence from bacteria forming yellow colonies was highly similar to the respective sequence from *Acetobacter* species including *pomorum* and *pasteurianus* (Table 1). To distinguish between these two species, we determined the sequence of the *groEL* gene. The *groEL* sequence amplified from our bacteria

was more similar to the *groEL* sequence of *A. pomorum* than to the respective sequence of *A. pasteurianus*.

These two species had been found to be present in the *D. melanogaster* gut [14]. In the gut, *L. plantarum* was reported to promote growth by interfering with the insulin and ecdysone signalling pathways on poor-condition medium [15]. In another work, it was found that, by contrast, intestinal *L. plantarum* had a negative effect on *D. melanogaster* life span [16]. We presume that *L. plantarum* on the cuticle surface does not contribute to any of these effects as the gut and surface micro-environments are fundamentally different. Indeed, while in the gut these bacteria live under anaerobic conditions, on the fly surface, they rather face aerobic conditions. Supposedly, their physiology and, by consequence, their role changes accordingly. For instance, aerobic but not anaerobic cultures of *L. plantarum* produce H_2O_2 [17]. At the cuticle surface, H_2O_2 might oxidise cuticular hydrocarbons (CHCs) and thereby modify the barrier function of this layer. A second possible function of *L. plantarum* on the cuticle surface is attraction of partners and to promote crowding. Indeed, *D. melanogaster* has been shown to be attracted by yet unidentified volatile compounds of *L. plantarum* [18].

The fly surface is not soiled by faeces

L. plantarum and *A. pomorum* are also present in the gut suggesting that their presence on the fly surface may originate from faeces. To verify whether the surface of flies contains excreted material, we fed adult flies with fluorescein and imaged their surface by fluorescence microscopy (Fig. S1). Only very little fluorescence signal was detected on the fly surface. We conclude that, overall, contamination of the surface by faeces is negligible.

Relative quantification of bacteria by qPCR

Isolation and cultivation of bacteria from the fly surface on media plates does not allow relative quantification as standardisation of bacterial transfer from flies to the plate is not possible. Therefore, we determined the ratio between *L. plantarum* and *A. pomorum* indirectly using species-specific primers in quantitative PCR (qPCR) experiments. We first compared *L.*

150 *plantarum* and *A. pomorum* abundance on Tübingen 2018 flies (Fig. 2). Approximately, there
151 were 20% more *L. plantarum* than *A. pomorum* on the surface of Tübingen 2018 flies.
152 In order to test the influence of the cuticle on the load of *L. plantarum* and *A. pomorum*, by
153 gene editing, we introduced mutations in the *ebony* gene of Tübingen 2018 flies that codes for
154 β-alanyl-dopamine (NBAD) synthase involved in the cuticle melanisation pathway [8]. Three
155 independent mutations in the *ebony* gene ($ebony^{cc1}$, $ebony^{cc3}$ and $ebony^{cc4}$) were recovered. The
156 respective homozygous mutant flies that are darker than wild-type flies are viable. We
157 determined the ratio between *L. plantarum* and *A. pomorum* on the surface of $ebony^{cc1}$ and
158 $ebony^{cc3}$ flies by qPCR (Fig. 2). We found that compared to Tübingen 2018 control flies, the
159 ratio between *L. plantarum* and *A. pomorum* on the surface of *ebony* flies was enhanced. We
160 conclude that the *L. plantarum* and *A. pomorum* load depends on Ebony and probably on
161 melanisation. It remains to be shown whether Ebony and melanisation either promote *L.*
162 *plantarum* or inhibit *A. pomorum* growth. Classically, according to the melanism-desiccation
163 hypothesis, enhanced melanisation has been considered as a response to dry environment to
164 prevent desiccation [7]. For instance, in the melanic drosophilid *D. kikkawai* higher abdominal
165 melanisation correlates with enhanced desiccation resistance [19]. However, there are cases
166 reported that contradict this hypothesis [20, 21]. Desiccation resistance, for example, did not
167 correlate with the body colour intensity in *D. melanogaster* wild populations in India [21]. We
168 conclude that melanisation is probably a trade-off trait not only dictated by humidity conditions.
169 Based on this assumption, we speculate that Ebony-driven melanisation may also be involved
170 in controlling the interaction between the fly body and bacteria conferring a yet unknown
171 advantage. Alternatively, Ebony may have a direct or indirect function in the differentiation of
172 the envelope and the surface CHCs. Indeed, in a recent work, it was found that longer chain
173 CHCs prevailed in *ebony* mutant females [22]. This suggests that *L. plantarum* and *A. pomorum*
174 differ in their preference on CHC environment. In summary, these data support the view that
175 the insect cuticle surface is not an inert substrate for bacteria.

Limitations

The ratio between *L. plantarum* and *A. pomorum* on the surface of *D. melanogaster* changes depending on the genetic background suggesting that the insect-bacteria interaction may be under genetic control. The significance of this interaction is unclear as our conclusion relies only on the impact of a single gene i.e. *ebony* on the insect-bacteria interaction. More work is needed in this direction.

We should also point out that the flies used in this work were kept under laboratory conditions. Hence, it is unclear whether our work reflects the situation in the field.

A major uncertainty in this work concerns the bacterial species. The amplified *A. pomorum* 16S rDNA sequence is 100% identical to the respective sequence in *A. pasteurianus* [11]. The provisional identification *A. pomorum* is based on the *groEL* sequence. The *groEL* sequence determined in this work is, however, not identical to the *A. pomorum groEL* sequence from the NCBI database. Thus, it is well possible that the *Acetobacter* species isolated in this work is neither *pomorum* nor *pasteurianus* but a third yet unknown species not present in the sequence databases. Additional analyses are needed to clarify this issue.

Declarations

List of abbreviations

Apom: *Acetobacter pomorum*

bp: base pairs

CHC: Cuticular hydrocarbon

Crispr/Cas9: Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9

gDNA: guide DNA

groEL: 60 kDa chaperonin

Lpla: *Lactobacillus planatrum*

qPCR: quantitative polymerase chain reaction

16S rDNA: 16S ribosomal DNA

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

All data are presented in the manuscript. Upon request, fly stocks will be shared by the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

VM, NG and BM carried out the molecular biology. NG and BM generated CRISPR/Cas9-edited fly strains. VM and BM executed the analysis. SJM wrote the initial draft of the manuscript. VM. YW and BM interpreted the data. BM finalized the manuscript. All authors read and approved the final manuscript.

Corresponding author

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Tables

Table 1. Identification of the bacterial species.

locus	Sequence length	identity	species
16S rDNA	853bp	98,25% 98,02%	*Lactobacillus plantarum* *Lactobacillus pentosus*
16S rDNA	982bp	99,80% 99,69%	*Lactobacillus plantarum* *Lactobacillus pentosus*
16S rDNA	940bp	99,25%	*Acetobacter pomorum*

		99,25%	*Acetobacter pasteurianus*
16S rDNA	1050bp	100% 100%	*Acetobacter pomorum* *Acetobacter pasteurianus*
groEL	560bp	98,57% 97,96%	*Acetobacter pomorum* *Acetobacter pasteurianus*

The sequence of the 16S rDNA locus suggests that the white colonies shown in Figure 1 are *L. plantarum*. This sequence is not sufficient to determine the species of the beige colonies. Based on the *groEL* sequence, we assume that these bacteria are *A. pomorum*.

Figure legends

Figure 1. *L. plantarum* and *A. pomorum* are present on the surface of *D. melanogaster*.
Upon streaking *D. melanogaster* on a plate, two types of heaps of bacteria were observed (A). We isolated single colonies that were white or beige (B). After sequencing the 16S rDNA, the white colonies were identified as *L. plantarum* (Lpla) and the beige colonies as *A. pomorum* (Apom). Under the microscope, both bacteria are a rode shaped (*L. plantarum*, C, *A. pomorum*, D).

Figure 2. The ratio between *L. plantarum* and *A. pomorum* depends on the fly genotype.
Applying qPCR, we detected *L. plantarum* and *A. pomorum* in the wash solution of fly surfaces in three independent experiments (1-3). In *ebony* mutant flies ($ebony^{cc1\ \&\ 3}$) that derive from Tübingen 2018 flies (Tü 2018) by gene editing (Crispr/Cas9), the *L. plantarum* to *A. pomorum* ratio is higher than in the original flies. The expression levels on Tübingen 2018 flies are set to one. Statistical analyses are not useful as the expression changes in the different experiments are enormous, although a clear trend is obvious.

Supplementary information

Additional file

Figure S1. There are only little faeces on the fly surface.

315 The surface of flies fed with yeast supplemented with fluorescein did not show abundant
316 fluorescence signal (arrows).

Figure 1

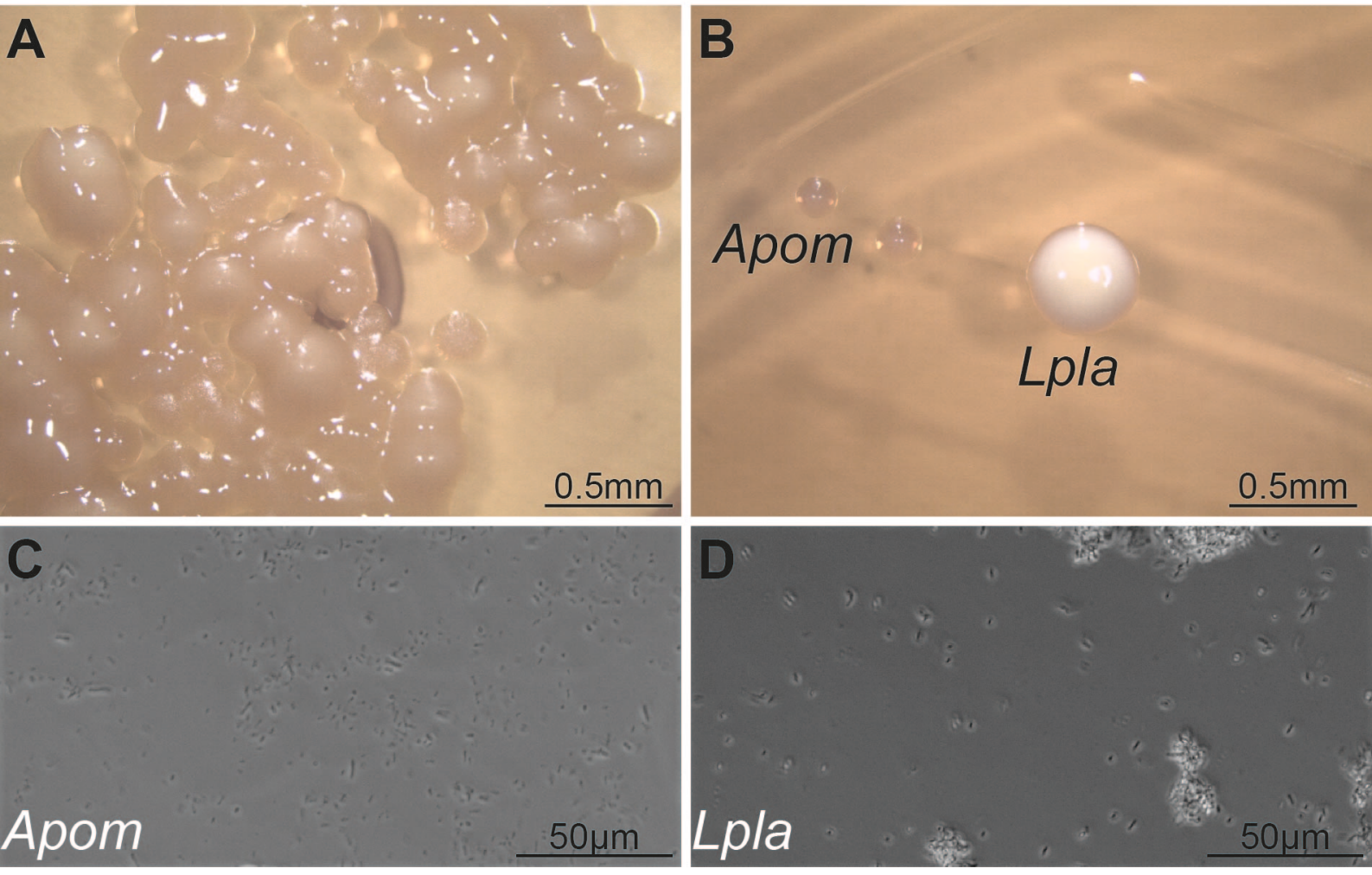
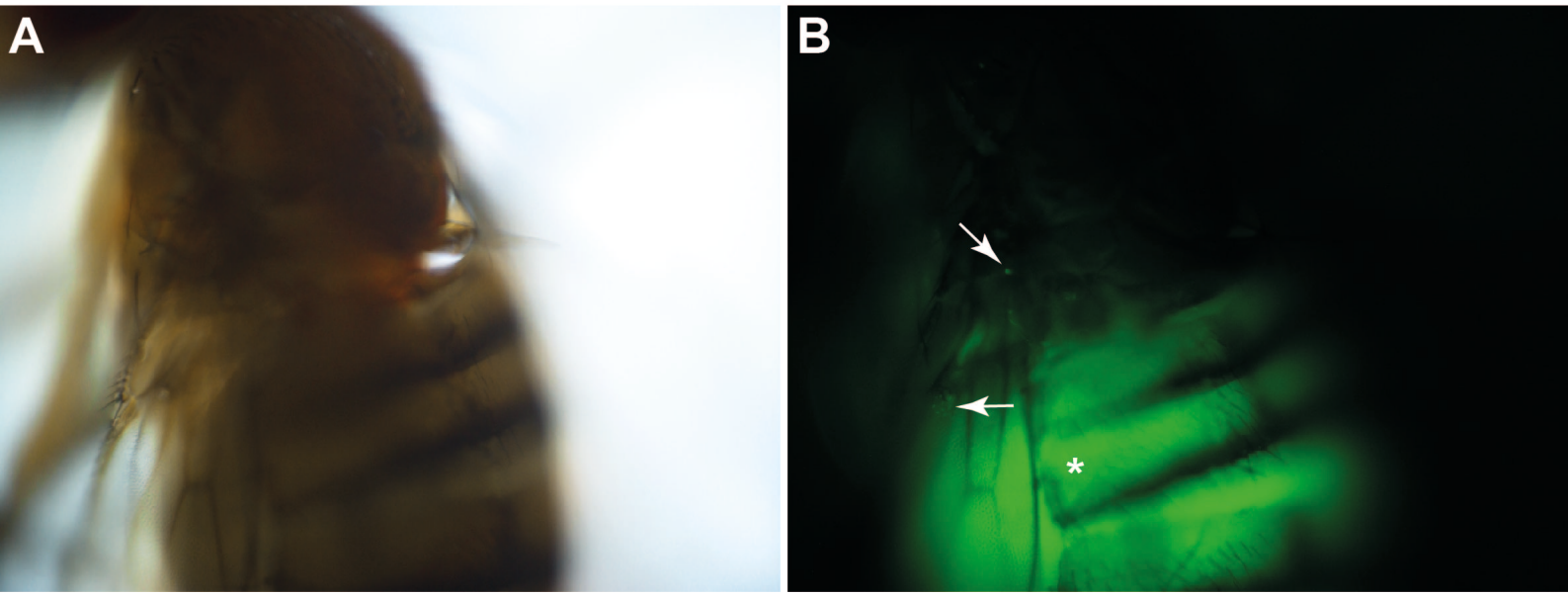


Figure 2



Figures

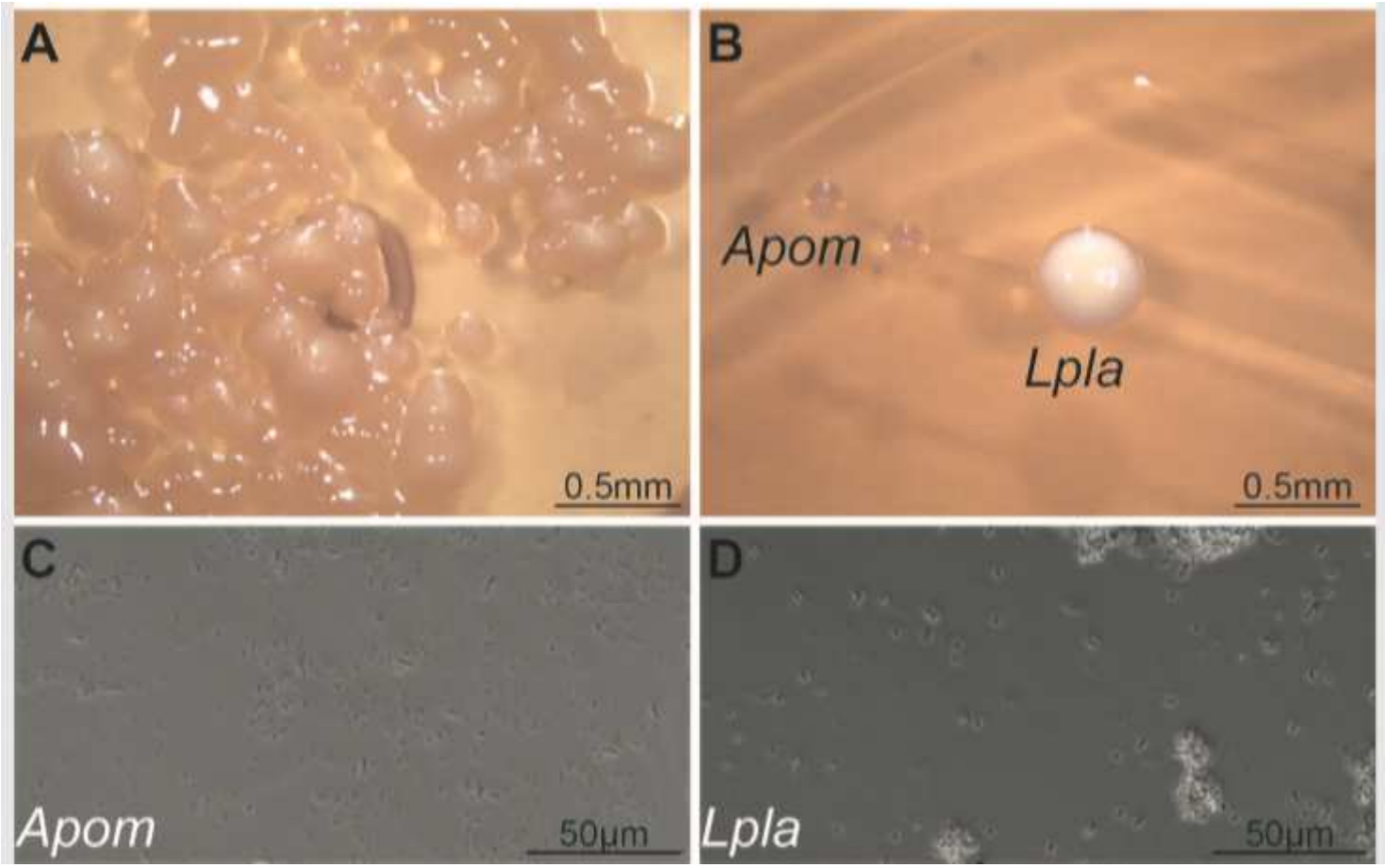


Figure 1

L. plantarum and A. pomorum are present on the surface of D. melanogaster. Upon streaking D. melanogaster on a plate, two types of heaps of bacteria were observed (A). We isolated single colonies that were white or beige (B). After sequencing the 16S rDNA, the white colonies were identified as L. plantarum (Lpla) and the beige colonies as A. pomorum Apom). Under the microscope, both bacteria are a rode shaped (L. plantarum, C, A. pomorum, D).

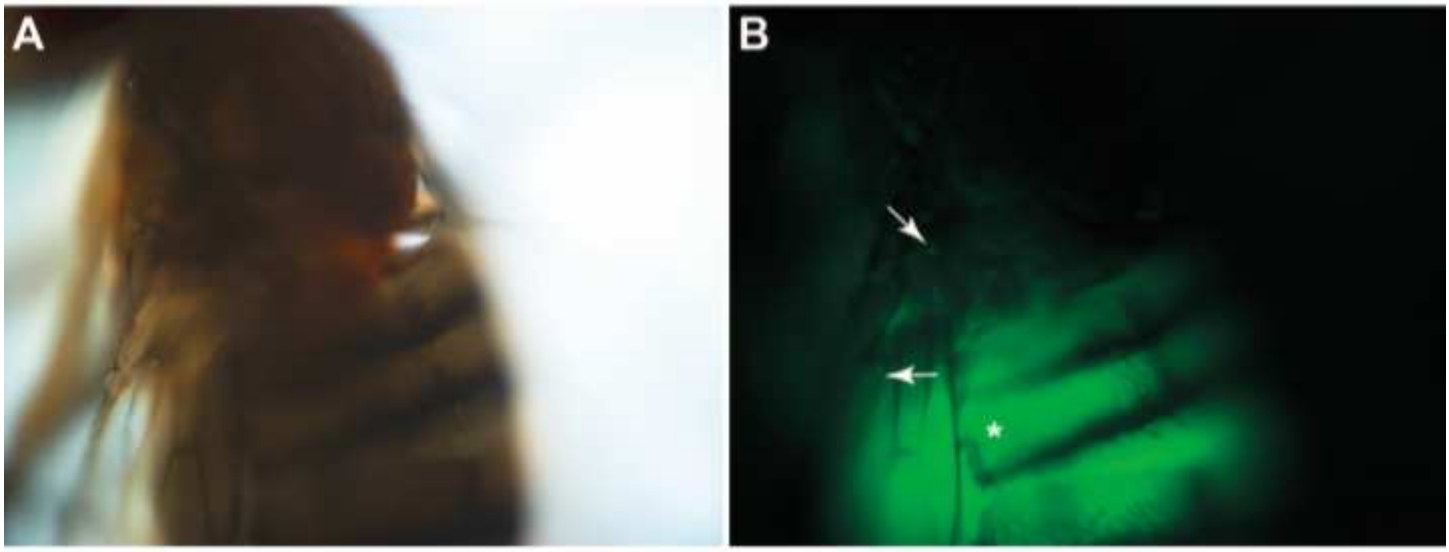


Figure 2

The ratio between L. plantarum and A. pomorum depends on the fly genotype. Applying qPCR, we detected L. plantarum and A. pomorum in the wash solution of fly surfaces in three independent experiments (1-3). In ebony mutant flies (ebonycc1 & 3) that derive from Tübingen 2018 flies (Tü 2018) by gene editing (Crispr/Cas9), the L. plantarum to A. pomorum ratio is higher than in the original flies. The expression levels on Tübingen 2018 flies are set to one. Statistical analyses are not useful as the expression changes in the different experiments are enormous, although a clear trend is obvious.

Supplementary Files

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- FigureS1.jpg