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Comparative Analysis of the Native Microbiota in *gammaCop* Mutant *versus* Wild-Type Genetic Background of *Drosophila melanogaster*

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ILDA CZOBOR BARBU[A], ATTILA CRISTIAN RAȚIU[B,*], ANA MARIA FLORESCU[B], MARIANA CARMEN CHIFIRIUC[A,C], ALEXANDRU AL. ECOVOIU[B]

[a]*Research Institute of the University Bucharest, ICUB, Earth, Environmental and Life Sciences Division, Spl. Independentei 91-95, Bucharest, Romania*
[b]*University of Bucharest, Faculty of Biology, Department of Genetics, Intrarea Portocalelor 1-3, 60101 Bucharest, Romania*
[c]*University of Bucharest, Faculty of Biology, Department of Botany and Microbiology, Intrarea Portocalelor 1-3, 60101 Bucharest, Romania*
***Corresponding author:** Attila Cristian Ratiu; **Email:** attila.ratiu@bio.unibuc.ro*

Abstract

Drosophila melanogaster represents a genetically tractable model for studying the mechanisms used by the infectious microorganisms to colonize healthy individuals. Since native microbiota plays an important role in host resistance to colonization, our aim was to study the influence of the genetic background on *D. melanogaster* microbiota diversity, as a prerequisite for using different lines in experimental infection challenge assays. In this purpose, we have assessed the microbiota of *D. melanogaster* wild-type *Oregon* and *gammaCop* (*γCop*) mutants males, by genetic and culture dependent methods. The cultivable fly-derived microbiota was estimated by viable cell counts. Each morphological type of bacterium was considered for identification through biochemical tests. The diversity of the microbial communities was assessed also by amplified ribosomal DNA restriction analysis (ARDRA). Our study emphasizes that both wild-type and mutant *D. melanogaster* males harbor a low-diversity bacterial community**,** highlighting the potential of this system to dissect the complex cellular and molecular interactions that occur between a eukaryotic host and its microbiota. The commonly found taxa were primarily represented by *Enterobacteriaceae* family (*Providencia* sp., *Serratia* sp. and *Escherichia* sp.) and the *Bacilli* class. The ARDRA approach highlighted a prevalence of 75% of the *Enterobacteriaceae*, especially *P. rettgeri* and *E. coli*, followed by *Lactobacillaceae* and *Enterococcaceae*. The amount of the cultivable bacteria in *D. melanogaster* varied quantitatively among different genetic backgrounds, gradually decreasing from *Oregon* to heterozygous and homozygous γCop^{14a} males. Our results suggest that *γCop* gene is involved in configuring the profile of native microbiota of *D. melanogaster*, an aspect that should be considered when wild-type versus specific mutant *D. melanogaster* lines are used as models in infectious pathogenesis experiments.

Key words: bacterial low-diversity, *Drosophila melanogaster*, experimental model, *gammaCop*, mutant allele, native microbiota

1. Introduction

Both vertebrate and invertebrate animals are in continuous contact with a diverse array of resident microorganisms termed microbiota (QIN & al. [1]). Over the past two decades, *D. melanogaster* has emerged as an ideal animal model to study the pathological interactions between host and infectious bacteria, with a special focus on the study of innate immune responses (LEULIER & ROYET [2]). Apidianakis and Rahme (2011), anticipate that, in the following years, the fly model will contribute to translational research investigating microbial and host genetic components by providing the complex cellular composition of a real organism

and bringing the advantage of relatively inexpensive experiments (PANDEY & NICHOLS [3]). Such experimental approaches could generate biological findings that are broadly applicable to human health and disease (APIDIANAKIS & RAHME [4]). Host-microbe interactions often begin with colonization of mucosal surfaces. These relationships are highly specific, as certain microbial species are found only in particular microenvironments. The mucosal immune system and resident microbiota form a cooperative system efficient for developing specific resistance against pathogens colonization (COW & al., MANDRIOLI, RYU & al. [5-7]). Microbiota could be related to ecological immunity by acting as a sort of extended immune system, which is able to modulate the competence of the host immune system and to limit the accumulation of pathobionts (OTTAVIANI & al. [8]). Recent studies reporting the interactions between pathogenic bacteria and *D. melanogaster* were primarily performed on wild-type flies and in the absence of a preliminary assessment of their commensal microbiota. The purpose of this study was to determine the diversity of the whole microbiota of wild-type and strains of *D. melanogaster* harboring mutant alleles of *γCop* gene. In order to accomplish this, we used both culture dependent and genetic (ARDRA) methods. Our results are indicative for the expected complexity of the interaction and communication between the colonizing bacteria. To our best knowledge, recent studies were focused primarily on studying the microbial consortia of either laboratory-reared or natural wild-type stocks (COX & GILMORE [9]), therefore this is the first study attempting to describe the commensal microbiota of *D. melanogaster* individuals with wild-type and, respectively, mutant genetic backgrounds. Therefore, we consider that our results are filling a gap of prerequisite information for future experimental infections with different virulent bacterial strains.

2. Material and Methods

The present study focused on describing the cultivable commensal microbiota in wild-type *Oregon* versus homozygous and heterozygous γCop^{14a} adult males. A revertible insertion of *P{lacW}* artificial transposon in 5'UTR of *γCop* defines the null-equivalent $\gamma Cop^{S057302}$ allele which is a recessive, embryo lethal allele in homozygous condition (DEAK & al. [10]). By inducing transposon excision (ECOVOIU & al. [11]) we obtained a hypomorphic allele symbolized γCop^{14a}, which is unable to complement $\gamma Cop^{S057302}$ but is viable in homozygous condition. Since *γCop* is a haplosufficient locus, $\gamma Cop^{14a}/+$ and $\gamma Cop^{14a}/\gamma Cop^{14a}$ adults are viable and males from these two types of mutants were used in experimental infections along with males from an *Oregon* wild-type strain. We have chosen to employ γCop^{14a} heterozygous as well as homozygous male mutants as their respective genetic background might affect their microbiota profiles.

2.1. Assessment of cultivable commensal microbiota of D. melanogaster

The *D. melanogaster* individuals were reared on standard, sterilized, Bloomington semi-defined medium (Nutry-Fly "German food", Genesee Scientific). We performed two independent experiments for assessing the cultivable commensal microbiota. In the first one, the evaluation of every considered genotype was performed in biological triplicates, each consisting in three adult males. The male flies were maintained for several days at 25°C consecutive to collection from the source culture. Homogenization and the ten-fold subsequent dilutions of the selected males were performed in 1 ml sterile saline solution. From 10^{-3} diluted whole-body homogenates, three gelose-agar plates were inoculated by incorporation (300 μL of homogenate/plate in 20 ml gelose-agar) for each biological replicate and were incubated at 37°C overnight under aerobic conditions. Consecutive to the incubation step, the morphological characteristics of the bacterial colonies and the number of colony-forming unit (CFU)/plate were recorded. For comparisons, we estimated the number of CFU/fly (n) as revealed by the

readings of each inoculated plate and obtained nine values per genotype (technical replicates). Logarithmic values ($log_{10}n$) were used for statistical analysis using 1way ANOVA with Bartlett's test for equal variances and Tukey's multiple comparison test. Colonies representing each morphological type were streaked for isolation on gelose, blood and MacConkey agar culture media. Following incubation, single colonies were examined for catalase, oxidase and Gram staining and identified by biochemical galleries for enteric bacteria (API 20E, Biomérieux, Marcy-l'Étoile, France), non-fermentative Gram-negative bacilli (API 20NE), Gram-negative cocci (GN2 MicroPlate, Biolog, Inc. Hayward, CA, USA) and Gram-positive bacilli (conventional biochemical tests).

A second experiment, intended to confirm the bacterial loads revealed by the first one, was performed only on *Oregon* and $\gamma Cop^{14a}/\gamma Cop^{14a}$ flies, using two biological replicates, each consisting in ten adult males. Prior to this experiment, the males were maintained for a maximum of four days at 18°C, in order to test if the temperature decrease could have quantifiable effects. Following the previously mentioned homogenization, dilution and inoculation steps, we have obtained six CFU values per each genotype (technical replicates). For statistical analysis, we used the unpaired t test with Welch's correction employing the logarithm of CFU values. The statistical significance of the differences in the bacterial loads among *D. melanogaster* genetic backgrounds was analyzed using *GraphPad Prism* version *5.04* for Windows, GraphPad Software, San Diego California USA (www.graphpad.com).

2.2. Evaluation of the diversity of the microbial communities by ARDRA technique

Complementary to the culture based methods, we evaluated the diversity of the microbial communities associated with laboratory reared *D. melanogaster* strains by using ARDRA technique. A total of 26 microbial colonies recovered from the nutritionally rich Luria Bertani (LB) agar medium plates inoculated with mechanically disrupted *D. melanogaster* tissues were simultaneously identified by conventional microbiological methods using selective/differential growth media (Eosin-Methylene-Blue - EMB, De Man, Rogosa and Sharpe agar - MRS, Baird-Parker, Chapman and Hoyle) and processed for ARDRA analysis. To this purpose, DNA cellular lysates were obtained by boiling the microbial suspensions at 100ºC for 10 minutes, followed by immediate freezing at minus 80ºC for at least two hours. In parallel, employing the same protocol allowed us to obtain the DNA lysates from some clinical and reference strains, such as *Serratia marcescens* (clinical strain), *Providencia rettgeri* (clinical strain), *Lactobacillus acidophilus* ATCC 4356, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212.

ARDRA technique required the amplification of *16S rDNA* gene followed by the digestion of the PCR amplicons using AluI, MspI and HinfI restriction enzymes. The universal *16S rDNA* primers, the forward primer 8F (AGAGTTTGATCCTGGCTCAG) and the reverse primer 1492R (GGTTACCTTGTTACGACTT), were used for PCR. Amplification of *16S rDNA* gene was performed for each considered cellular lysate. We used 10 µL of genomic DNA and 0.5 µM of each primer in a final reaction volume of 50 µL. The PCR mixture also contained 1X enzyme buffer (Promega), 1U of Taq DNA polymerase (Promega), 200 µM dNTP's (Promega), 15 µg BSA (Promega), 1.5 mM $MgCl_2$ (Promega) and was adjusted with nuclease-free water (Promega) up to the final volume. PCR amplifications were run on a Corbett Research PCR machine. The reactions consisted of an initial denaturation step at 95ºC for 10 minutes, followed by 30 cycles, each comprising of denaturation at 94ºC for 1 minute, annealing at 50ºC for 2 minutes, and elongation at 72ºC for 3 minutes. A final extension step at 72ºC for 10 minutes completed the reaction. Prior to enzymatic digestion, the DNA amplicons standing for *16S rDNA* gene from the reference bacterial strains were purified using *ISOLATE PCR and Gel Kit* (Bioline). The PCR products were digested using AluI, HinfI and, respectively, MspI

restriction enzymes (Promega). From each restriction enzyme, 5 U were added to the 10 µL amplification product, together with 2 µg BSA, 1X enzyme buffer and nuclease-free water, up to a 20 µL total reaction volume. The mixtures were incubated at 37ºC for 2 hours, followed by 20 minutes at 65ºC and a final cooling step at 4ºC. The digestion products were run in a 1.5% agarose gel in 1X TAE buffer, against both 100 bp DNA Step Ladder (Promega) and Gene Ruler 100 bp Step Ladder (Thermo Scientific) and visualized using ethidium bromide staining. Digital images of the electrophoresis gel were captured with a Sony Cyber-shot DSC-H10 camera and were normalized with the molecular weight markers that ran concomitantly with the target probes. The images were analyzed with PyElph 1.4 software (BRANDUSA & VASILE [12]). Considering the outcome of enzymatic digestions, specific genetic distance matrices and corresponding phylograms connecting each experimental strain with the reference strains were computed with PyElph 1.4 software using the Neighbor-Joining (NJ) method (SAITOU & NEI [13]). An overall phylogenetic characterization was achieved by manually averaging the genetic distances previously calculated followed by analyzing the average values using MEGA5 (TAMURA & al. [14]).

3. Results

The main purpose of this study was to investigate the whole microbiota in adult males of similar age belonging to both wild-type and mutant *D. melanogaster* genetic backgrounds. In order to minimize the diversity of allochthonous taxa, the *D. melanogaster* individuals were raised on culture medium with constant composition. Consecutive to collection, we have established the total diversity of cultivable microbial species and the influence of genetic background.

We found that the cultivable commensal microbiota is of a relatively low diversity, which is in agreement with other studies showing that *D. melanogaster* has a simpler microbiota (1-30 taxa) compared with the complex diversity of vertebrates associated one (> 500 taxa). The results of the conventional microbiological tests have shown that the most commonly found taxa were primarily represented by *Enterobacteriaceae* (*Providencia* sp., *Enterobacter* sp., *Hafnia* sp., *Serratia* sp.), *Bacillaceae* (*Bacillus* sp.) and, occasionally, *Enterococcaceae* families. Moreover, this approach indicated that the commensal bacterial species did not varied significantly among males with different genetic backgrounds, i.e. *Oregon*, γCop^{14a}/+ and γCop^{14a}/ γCop^{14a}.

In the first experiment, we assessed the cultivable commensal microbiota of both γCop^{14a}/+ and $\gamma Cop^{14a}/\gamma Cop^{14a}$ mutant males, harboring one and two mutant alleles, respectively. As expected, the qualitative genetic differences determined a certain quantitative variation of the commensal microbiota, as revealed by the statistically significant differences found among the compared groups ($p < 0.0001$, one-way ANOVA with Bartlett's test for equal variances and Tukey's multiple comparison test). Specifically, the cultivable microbiota of $\gamma Cop^{14a}/\gamma Cop^{14a}$ males (1.58 X 10^5 CFU/fly) proved to be significantly different when compared to the cultivable microbiota harbored by γCop^{14a}/+ males (2.76 X 10^5 CFU/fly) and *Oregon* males (5.19 X 10^5 CFU/fly) (Figure 1A).

A second, independent experiment was performed to confirm the quantitative differences between the cultivable commensal microbiota particular to $\gamma Cop^{14a}/\gamma Cop^{14a}$ and *Oregon* male adults, respectively (Figure 1B). Similar to the first experimental setup, the flies were kept on fresh standard medium for a few days, but at 18°C, in order to assess the influence of the rearing temperature over the commensal microbiota. The bacterial load in *Oregon* males (1.25 X 10^5 CFU/fly) was significantly higher than in $\gamma Cop^{14a}/\gamma Cop^{14a}$ mutants (0.59 X 10^5 CFU/fly) ($p < 0.0001$, unpaired t test with Welch's correction). Notably, the lower temperature induced four times decrease of the log 10 of CFU/mL in the case of *Oregon* males and respectively three times decrease in case of $\gamma Cop^{14a}/\gamma Cop^{14a}$ males.

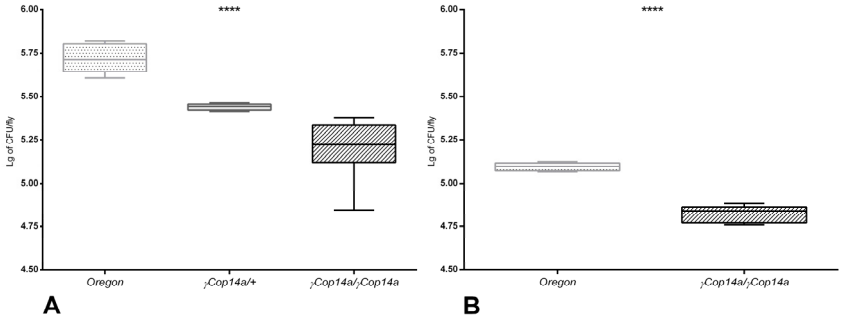

Fig. 1. The comparative bacterial loads among the tested *D. melanogaster* genotypes. In **figure 1A**, the lowest average load of 1.58 X 10^5 CFU/fly is observed for $\gamma Cop^{14a}/\gamma Cop^{14a}$ males. A similar result is evident from **figure 1B**, where the comparative bacterial loads between $\gamma Cop^{14a}/\gamma Cop^{14a}$ and *Oregon* males were scored by a second, independent experiment. It is plausible that lowering the temperature at which the males were maintained prior to bacterial loads assessment negatively influenced the bacterial loads found by the second experiment. For each comparison, the employed statistical tests, 1way ANOVA (figure 1A) and unpaired t test (figure 1B), indicated that the differences among mean bacterial loads are statistically significant, with p values lower than 0.0001 (****).

ARDRA is a very powerful technique for differentiating bacterial phyla and is conditioned by the number of restriction enzymes used in the analysis. The NJ dendrograms obtained by ARDRA analysis (Figure 2) demonstrated that some of the bacterial strains identified in both laboratory reared *Oregon* and $\gamma Cop^{14a}/\gamma Cop^{14a}$ males might pertain only to a small subset of four distinct bacteria. ARDRA analysis revealed that the majority of the bacterial strains identified in our experiment (75%) are presumably represented by the *Enterobacteriaceae* family (i.e. *E. coli* and *P. rettgeri*) and by the order *Lactobacillales* (most notable *L. acidophilus* and *E. faecalis*). We also found that *P. rettgeri* and *E. coli* are the most abundant bacterial species indicated by our analysis as particular to *Oregon* males and, respectively, $\gamma Cop^{14a}/\gamma Cop^{14a}$ males. Overall, *L. acidophilus* seems to be the most abundant Gram-positive bacterial species.

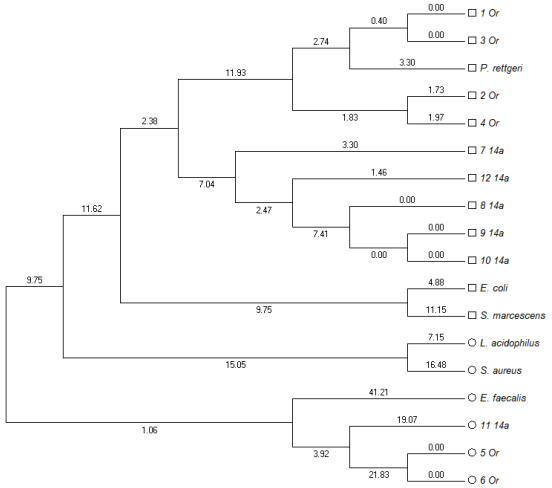

Fig. 2. The figure illustrates the NJ dendrogram of phylogenetic relationships of 18 bacterial cultures, including six bacterial reference strains (indicated with complete names). The optimal tree with the sum of branch length = 220.87 is shown. The phylogenetic distances were inferred by averaging specific distances computed with PyElph 1.4 software from electrophoretic images of *16S rDNA* specific amplicons digested by MspI, AluI and, respectively, HinfI restriction enzymes. Evolutionary analyses were conducted in MEGA5. With square symbols are indicated the reference Gram-negative bacteria strains and the possible related bacteria identified in *D. melanogaster* homogenates. With circles are highlighted the reference Gram-positive bacteria strains and the possible related bacterial taxa identified in *D. melanogaster* homogenates. The ***Or*** abbreviation stands for bacterial strains identified in *Oregon* males, while ***14a*** stands for the specific bacterial strains identified in $\gamma Cop^{14a}/\gamma Cop^{14a}$ males.

4. Discussion

D. melanogaster is a powerful and versatile experimental model for studying the mechanisms of bacterial infection (APIDIANAKIS & RAHME [15]). In the -omics era, the high-throughput methods are a key player in the process of data generation and they are able to offer a genome level picture of the variations of genic expression profiles induced by experimental infections. Nevertheless, the results should be carefully interpreted by using rigorous statistical approaches as well as various host related data, such as mutant or disease status. The experimental infection studies should always take into consideration the complex influences exerted by the host commensal microorganisms. The species that make up these communities vary among hosts and are influenced by diet, genotype, geographical origin and colonization history (DETHLEFSEN & al. [16]. The involvement of commensal microbiota in the host protection can vary during insect life, since the composition of the bacterial community that populates the gut can change during lifespan due to variation in the nutritional composition of the food and to the aging process (DE VEALE & al. [17]).

Our experiments revealed few types of commensal bacteria, mostly strains of *Enterobacteriaceae*, *Bacillus* sp. and, occasionally, enterococci. In general, a low diversity community could be generated by the restrictive habitats of wild *D. melanogaster,* which can grow and feed in very inhospitable conditions (WONG & al. [18]). Previous research has identified various taxa associated with *D. melanogaster*, including different species of *Lactobacillus*, *Enterococcus*, *Acetobacter* and *Staphylococcus* (BRODERICK & LEMAITRE, CORBY-HARRIS & al., CROTTI & al., REN & al. [19-22]). The prevalence of *Enterococcus* in the microbiota of insects was examined in 37 insect phylotypes belonging to eight orders, including the *Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Isoptera, Lepidoptera,* and *Orthoptera* (MARTIN & MUNDT [23]). Enterococci were observed in approximately 53% of 403 insect homogenates examined, and the bacterial loads ranged from 10^3 to 3×10^7 bacteria/g. The largest fraction of enterococcal isolates (43.5%) was identified as *E. casseliflavus*, *E. faecalis* and *E. faecium* (MARTIN & MUNDT [23]).

The *γCop* gene is a component of COPI coatomer, which is involved in the vesicle cargo transport of proteins between Golgi and endoplasmic reticulum (ER), and therefore in protein secretion. Primary bioinformatics annotation of *γCop* gene's role in *D. melanogaster* was confirmed *in vivo* by extensive experimental data. Specific RNAi mediated depletion in S2 cells from *D. melanogaster* induces fusion of Golgi membranes with the ER, ranking *γCop* as a class A gene required for constitutive protein secretion (BARD & al. [24]). Other pieces of evidence are represented by localization of *γCop* protein in ER and cis-Golgi units in S2 cells and by Golgi and ER disruption in specific mutant embryos (JAYARAM & al. [25]). Different genome-wide RNAi screenings performed on *D. melanogaster* S2 cells revealed that *γCop* is involved in: cytokinesis (ECHARD & al. [26]); host-pathogen interaction between *Listeria monocytogenes* and S2 cells (CHENG & al. [27]); phagocytosis of *Candida albicans* by S2 cells (STROSCHEIN-STEVENSON & al. [28]) and in *Drosophila* C virus replication in S2 cells (CHERRY & al. [29]). In the case of *L. monocytogenes*, RNAi studies performed on S2 cells revealed that *γCop* protein, which is involved in *D. melanogaster*'s vesicular protein trafficking, affects both entering and escape pathways of the intracellular parasite. Mutants of *D. melanogaster* described by others (GRIEDER & al. [30]) revealed that *γCop* is involved in luminal secretion of important proteins affecting morphogenesis of the epithelial tubes of the tracheal system, a reliable tubular organ model.

The relatively low abundance of commensal microbiota in γCop^{14a} homozygous mutants may be explained by the fact that a normal bacterial colonization could have been impaired by the lack of a proper processing of some extracellular receptors involved in the microbial

recognition. It is tempting to consider that *γCop* is required for a proper localization of apical and basal proteins in the epithelial cells of the *D. melanogaster*'s gut (a main entrance gate for the invasive bacteria), in a manner similar to the one previously described for the tracheal system (GRIEDER & al. [30]). Therefore, we could speculate that the colonizing bacteria do not find the most favorable epithelial phenotype in $\gamma Cop^{14a}/\gamma Cop^{14a}$ mutant individuals, a condition that impairs the density of the commensal bacteria relative to flies harboring at least a wild-type copy of *γCop.* The normal conformation and/or distribution of some trans-membrane ligands interacting with the bacterial adhesins responsible for the host-microbial cell interactions may also be disturbed in the $\gamma Cop^{14a}/\gamma Cop^{14a}$ mutants, affecting the ability of bacteria to flourish in such a genetic background. Previous studies have demonstrated that germ-free insects are more susceptible to infection than normal insects and died prematurely due to infection of pathogens, such as *Pseudomonas aeruginosa, Penicillium* sp. and *Bacillus subtilis* (OTTAVIANI & al. [8]). By having a low abundance of commensal microbiota, *D. melanogaster* males with different *γCop* genetic backgrounds could exhibit different degrees of vulnerability to pathogens, making them useful models for investigation of the relationship between the composition of host microbiota, infection susceptibility and intercellular communication dynamics.

ARDRA analysis performed with three restriction enzymes revealed that the bacteria from *Enterobacteriaceae* family and *Lactobacillales* order are the most common taxa encountered in the analyzed *D. melanogaster* males, where *P. rettgeri* and *E. coli* are associated with the majority of the cultures derived from *Oregon* and $\gamma Cop^{14a}/\gamma Cop^{14a}$ males. Previous experiments showed that a consistent discrimination is achieved by ARDRA technique when using four to five distinct restriction enzymes (DE BAERE & al., AQUILANTI & al. [31, 32]), but successful phylogenetic affiliations were also recorded using only three restriction enzymes (MOYER & al. [33]). In the latter case, it was possible to allocate unknown bacterial strains to Gram-positive phylum and *Enterobacteriaceae* with a rate of success ranging from 50% to 81% and 74% to 83%, respectively. Thus, even when using only three restriction enzymes, ARDRA could still be considered a feasible and reliable technique for studying the bacterial phyla content in insects, including *D. melanogaster* (MOYER & al., CHANDLER & al. [33, 34]).

Our results are similar to those obtained in a sequencing-based experiment focused on identifying bacterial species in wild and wild-type laboratory reared *D. melanogaster* strains, showing that the most common bacterial families were *Enterobacteriaceae* (60%), followed by the order *Lactobacillales* (21%) (CHANDLER & al. [34]). More intriguing, *Providencia* was discovered to be the most common genus found within laboratory flies, which also contained around five operational taxonomic units (OTUs), far less than the ones gathered from the wild. *P. rettgeri* can induce mild infections, responsible for moderate mortality rates (GALAC & LAZZARO [35]), and it also has one of the few known mobile quorum-sensing systems (WEI & al. [36]). Previous studies performed on *Drosophila* also identified several species of enterococci, including *E. faecalis* (COX & GILMORE [37]).

5. Conclusion

The phenotypic and genetic analysis of cultivable native microbiota revealed that the laboratory breed *D. melanogaster* males harbor a low-diversity bacterial community, which varies qualitatively and quantitatively among strains with different genetic backgrounds. From this perspective, it is necessary to examine the resident microbiota of a certain *D. melanogaster* genetic background before using it as a potential candidate for the *in vivo* investigation of microbial infection and pathology. In addition, specific allelic conditions should be taken into account when commensal microbiota is assessed, at least for genes potentially involved in pathogen-host interactions, as revealed herein for *γCop*.

Conflict of interest
The authors confirm that this article content has no conflicts of interest.

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