TOJSR
The Oxford Journal of Science and Research

ISSN No.: 2581-9380
TOJSR, 2020, *3* (1), 45-52

Antagonistic Activity of *Lactobacillus* isolated from the gut of *Drosophila melanogaster*

Raji Sukumar*, Vindhya M. Hullur, Tathagata Pal, Vikas Choudhary, Tushar Biswas, Swetha M.Menon, Varra Swetha,Yuvashree B.

Department of Zoology and Genetics, The Oxford College of Science, HSR layout, Bangalore 560 102, India

Abstract: The gastrointestinal tract is a complex ecosystem which associates a resident microbiota and cells of various phenotypes lining the epithelial wall expressing complex metabolic activities. The resident microbiota in the digestive tract is a heterogeneous microbial ecosystem. The intestinal microbiota plays an important role in normal gut function and maintaining host health. The host is protected from attack by potentially harmful microorganisms by the physical and chemical barriers created by the gastrointestinal epithelium. The microbiome has tremendous potential to impact host physiology and metabolism Gut bacteria in particular have been linked to diverse functions and specific diseases. Mechanistic studies remain challenging in part due to the complexity of the mammalian gut microbiome, which can vary greatly between individuals and is composed of approximately 1,000 species of microorganisms. Invertebrate systems are fruitful models for dissecting complex host–microbe interactions. In particular, *Drosophila melanogaster*, the fruit fly, is one of the most powerful models for animal genetics and has a simple microbiome composed of 5 to 20 microbial species. There is a growing interest of using *Drosophila melanogaster* as a model system to study the host microbe interaction. The present study aims to understand, compare and identify the biodiversity of microorganisms present in the gut of *Drosophila melanogaster* depending on the substrate provided and also to evaluate the antagonistic activity of *Lactobacillus* isolated from the gut of *Drosophila melanogaster* against some of the pathogenic strains of bacteria.

Keywords: *Lactobacillus, Drosophila gut microbiome, Antagonistic activity, Probiotics, Well diffusion method.*

INTRODUCTION

Drosophila is a genus of flies, belonging to the family Drosophilidae, whose members are often called "small fruit flies"a reference to the characteristic of many species to appear around overripe fruits. One species of *Drosophila* in particular, *D. melanogaster*, has been heavily used in research in genetics and is a common model organism.

The *Drosophila* life cycle is short, and therefore it is easy to raise a large number of individuals for genetic, biochemical, and molecular analysis. In the laboratory, *Drosophila melanogaster* is usually cultured at 25°C, except where specifically indicated[8]. The generation time is roughly 10 days from fertilized egg to a grown adult, and the maximum life span ranges from 60 to 80 days depending on the culture conditions. *Drosophila* is a holometabolous insect, and its life cycle can be divided into four stages: embryo, larva, pupa, and adult. Females lay roughly 100 embryos per day, and embryogenesis lasts only 24hours. The first instar larva begins to feed immediately on the surface of the medium and passes through two molts. Second instar larvae burrow into the medium, and when the third instar larva is mature, it leaves the culture medium and

* *rajishibu12@gmail.com*

wanders up the walls of the flask, searching for a place to pupariate for 24–48 hours and followed by the morphogenesis of adult structures[7]. Finally, the adult emerges between 9 and 10 days after egg fertilization.

The metazoan gut is associated with a number of benign or beneficial microorganisms. Current studies have revealed that the influence of these resident microorganisms is profound, altering many aspects of host physiology, especially digestive and immune functions[1].Studieswith gnotobiotic animals, associated with genomic tools focused to capture the full extent of microbial diversity and function within the gut, have greatly modified our vision of host-microbe interactions. These interactions are now accepted as essential elements of host health and the conditioning of host immune defenses. Over the last few decades *Drosophila melanogaster*, the common fruit fly, has been largely used to decode the mechanisms of host-microbe interactions in the context of innate immunity and pathogenic associations [16]. More recently, studies have suggested the utility of this model to elucidate mechanisms underlying more benign or beneficial host-gut microbiota interactions due to its accountability to genetic study, lower microbiota complexity, and the ease in raising axenic flies[2].

The studies of microbes connected with Drosophila are almost are age old as the genetic model itself (TH Morgan 1909). Thus, the impact of microbes collaborated with Drosophila on the host was to some extent valued even before they were subject of undeviating study. These preliminary studies were focused with decreasing variability in experiments by calibrating the impact of nutrition, as it was essential to discriminate environmental from genetic influences on phenotypic characters. Research into the 1960s by Sang and others proved the fine chemical composition of fly medium indispensable for normal growth of axenic flies[6]. This pathfinder work revealed the composition, persistence, and transmission of gut-associated bacteria of laboratory grown wild-type flies[9,15]. One of her key conclusions was the observation that microbes were pass on to offspring by contamination of the eggshells, which are gobbled by young instar larvae. Her experiments also revealed the view that the endurance of bacteria during the Drosophila life cycle is non-fortuitous [3]. This unique study still furnishes many fascinating observations for today's scientists and establish a foundation for contemporary studies, which by expanding these concepts and amalgamating current technologies can launch to decipher the mechanistic basis of these associations.

Several independent studies analyzing *Drosophila*-associated microbiota have provided a good deal of insight into the diversity of bacteria in laboratory stocks of *Drosophila melanogaster*[4]. These studies have analyzed samples from either whole flies or dissected guts of surface-sterilized flies [5]. Bacteria were identified by either characterization of cultivable species or through culture-independent assessment of microbial diversity by direct PCR amplification and sequencing of 16S rRNA genes from extracted DNA. More recently, a study using high throughput sequencing of a large number of 16S rRNA amplicons allowed identification of almost all detectable bacterial species at unprecedented depth and across the different life stages [17]. These studies indicate that laboratory stocks are associated with a relatively low number of taxa, corresponding to 1–13 OTUs (defined as 97–99% identity, depending on the study) and are most frequently associated with bacteria from two genera, *Acetobacter* and *Lactobacillus* [12]. Some species are clearly laboratory specific, such as *Enterococcus faecalis*, *Gluconobacter morbifer*, and Enterobacteriaceae Group Orbus, while *Lactobacillus plantarum* and *Acetobacter pomorum/pasteurianus* have been found in most laboratory stocks [14]

Altogether, these studies demonstrate that *Drosophila*, as has been studied for most insects species, is related to with a much lower diversity of bacterial species than observed in mammals[10]. Altogether, this reduced diversity of microbes suggests that the niches provided by the Drosophila and mammalian gut are not similar. The cause for this difference is not well known, but a number of factors associated with host have been put forwarded. It has been hypothetical that the acquired immune system of higher multicellular organism has facilitated the association with a greater diversity of microorganisms[11]. Alternatively, the more frequent confusion of the insect gut niche has been suggested as a circumscribe to higher diversity. Insect guts tend to be transient, given the very short life span of many insects, and confront frequent episodes of variations [13]. Zones of the gut (foregut and hindgut) are shed during molting and in holometabolous insects, including flies, the entire larval gut is restored by a new adult gut during metamorphosis. Thus, the perplexed and temporary nature of the guts of holometabolous insects such as Drosophila may be contradictory with the development of a highly diverse microbial flora.

Research indicates that, though there are habitual dwellers, gut-associated bacteria of Drosophila stocks can differ greatly between laboratories and even between stocks within the same laboratory. These differences between stocks are maintained even on an identical laboratory defined food source since fly stocks are essentially kept isolated as separate entities. Analyzing how different environmental and host factors shape the Drosophila gut microbial flora is an important question in the area of research. Recently, a few studies have begun to provide some insight.

Diet has been shown to influence the bacterial composition of mammals and several insects and is proposed to be an important factor in shaping community composition. Similarly, Chandler et al. and Sharon et al. have demonstrated that host diet plays a substantial role in shaping bacterial microbiome composition in Drosophila as well. First, when comparing diverse, wild populations of Drosophila spp, Chandler et al. found that microbiota composition of species feeding on the same type of substrate were more similar to each other than to more closely related species that were feeding on different substrates. In addition, they found that switching a large pool of isogenic *D. melanogaster* onto different sterile diets led to changes in gut microbiota composition over time.

The present study aims to understand, compare and identify the biodiversity of microorganisms present in the gut of *Drosophila melanogaster* depending on the substrate provided and also to evaluate the antagonistic activity of *Lactobacillus* isolated from the gut of *Drosophila melanogaster* against some of the pathogenic strains of bacteria.

MATERIALS AND METHODS

In the present study, wild-type *Drosophila melanogaster* collected from stock culture procured from GKVK, Bangalore were subculture in different types of solid culture media All the materials (for control media rava) except propanoic acid and yeast slurry was mixed in 100ml distilled water and allowed to boil till a thick slurry was formed. The media was allowed to cool and 0.68ml 0f propanoic acid was added. After this the media was transferred into 4 vials and kept for 3-4hours for solidification. A thick yeast slurry was made and around three drops were added to each vial after media solidification. The vials were then cotton plugged tightly. For the other media rava was replaced with Proteinpowder, Groundnut powder Celluloseand alcohol.

INTRODUCTION OF FLIES

After the prescribed substrate *Drosophila* culture media (control media, protein media, lipid media, cellulose media, alcohol media) were prepared, flies were introduced in the different medias. (set of male and female flies). Thus, the life cycle continues and we wait till the F2 generation 3rd instar larva appears respectively in each culture bottle. Within the course of time the male and female flies population was monitored in each bottle.

DISSECTION OF LARVAE:

3rd instar larvae were isolated from each culture bottle separately on different glass slides each containing a drop of Ringers solution. The larvae gut was dissected by using a pair of needles under a dissection microscope in each case separately. The dissected gut was then put in a watch glass containing a drop of Ringer's solution and then macerated using a glass rod. Ringer's solution was a solution of several salts dissolved in water for the purpose of creating an isotonic solution relative to the body fluids of an animal. The grinding was done separately for different samples and different glass rods were used for each grinding. The remaining debris were then removed.

Composition of Ringer's solution:

Substance	Composition(mg/100ml)
NaCl	7.29
KCl	0.39
$CaCl_2$	0.17
pH	7.3

PLATING OF SAMPLE:

The media used for the master plate cultures was Nutrient agar media. Once the media gets solidified, the gut extract sample prepared were inoculated using a sterile inoculation loop by continuous streak method. Once all the samples were inoculated on different plates, they were labelled by the name of the *Drosophila* culture media from which the sample of the larvae was taken. Then the plates were incubated at 37°C overnight.

BACTERIAL IDENTIFICATION:

GRAM STAINING

For the confirmation of morphology bacterial growth gram staining was done separately for each plate. Smear of culture was made on a clean grease free slide using an inoculation loop and then the smear was flooded with crystal violet and kept for 1 minute then wash with tap water, then gram's iodine was added to the smear and was kept for 1min and then rinsed, then smear was flooded with 70% alcohol and then finally safranin was added for 45 seconds and washed again with tap water and air dried which was observed under microscope.

STARCH HYDROLYSIS

For the confirmation of starch hydrolysis (amylase +ve activity) by the bacteria starch agar media was prepared. This media contains starch as carbon source for the bacterial growth. If the bacteria were capable of utilizing the starch from the media it produces a clear zone around the colony when it comes in contact of iodine. Here 3.75 gm of agar was dissolved in 250ml of distilled water. It was stirred vigorously and dissolved by boiling the media on Bunsen burner. Once the agar gets dissolved rest of the contents were added and dissolved. After which it was sterilized in autoclave for 15 minutes at 121°C. It was then allowed to cool and poured in 5 sterile petri plates and allowed to solidify inside a laminar air flow chamber.

Once the media gets solidified, it was inoculated using a capillary tube by continuous streak method from each of the master plate samples. Once all the solutions were inoculated on different plates, they were labelled. All the inoculation process was done inside a laminar air flow chamber near to a Bunsen burner to avoid contamination. Then the plates were incubated at 37°C overnight.

CATALYSE TEST:

This experiment was used to decipher whether the given bacterial colony was positive for catalyse activity or not. 5 glass sheets were

taken and labelled according to the smear made on them from respective master plates. The smear was diluted with a drop of distilled water. Now drops of H_2O_2 solution was added to each of the smear and mixed well with different applicator stick or toothpicks. The sheets were kept in room temperature for few seconds or a minute. Now the smears were checked for vigorous bubble formation.

LACTOBACILLUS BROTH:

For the confirmation of the bacteria, *Lactobacillus* broth was prepared. *Lactobacillus* broth was a special media that allows the growth of only *Lactobacillus* bacteria.. Now from each of the master plate inoculum was added and incubated at 37^{o}C for 24hrs.

BACTERIAL ANTAGONISM*:*

This phenomenon was called "bacterial antagonism," "bacterial interference," or "colonization resistance" was a characteristic of some of the bacterial types which inhabits the growth of other bacteria in the same colony. This was achieved by releasing certain toxic chemical compound which restricts the growth of other bacteria. These toxic chemical compounds were collectively known as "bacteriocin". For the confirmation of bacterial antagonism nutrient agar media was prepared.

Once the media gets solidified the whole media was inoculated with different organisms (*E.coli,Staphylococcus sps, Streptococcus ,Salmonella*) using different ear buds each time. Now using a gel puncture a hole was created at the center and a 100 microliter of *Lactobacillus* suspension from each culture was put in the center hole to the respective plates. It was then incubated at 37℃ for 24 – 48 hrs.

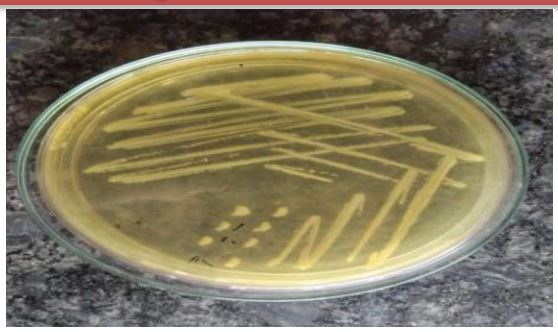

FIG 1-Pure culture of Lactobacillus

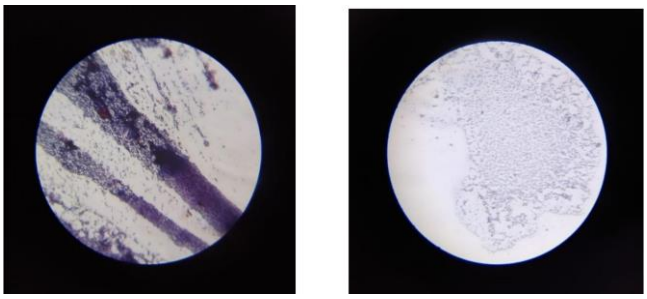

FIG 2 - Gram positive *Lactobacillus*

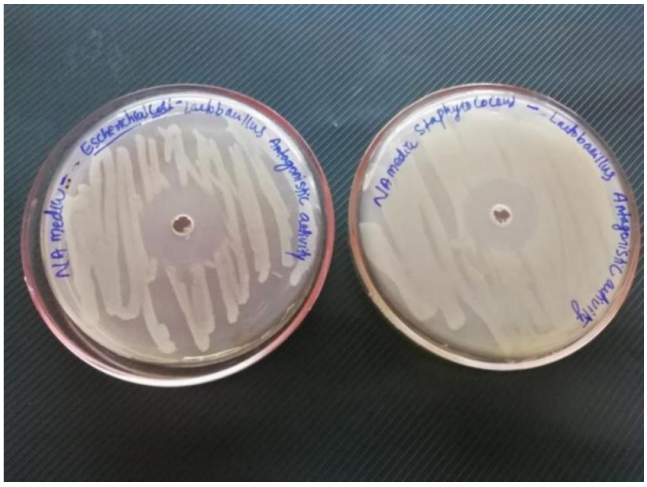

FIG 3 - *Lactobacillus* culture showing antagonistic activity to ***E. coli*** and *Staphylococcus*

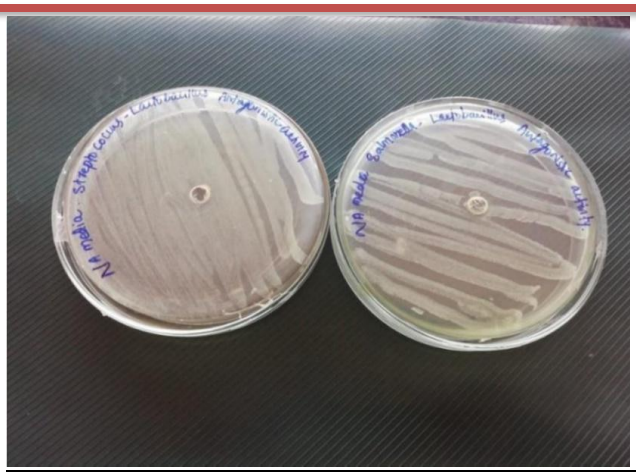

FIG 4- No antagonistic activity to *Streptococci* and *Salmonella*

RESULTS AND DISCUSSION

The *Drosophila melanogaster* samples that were used throughout the project was collected from Gandhi Krishi Vignan Kendra, Bangalore. The point of separation of flies form control media and growing them in different media was to check the influence of diet on the gut microbial flora of *Drosophila melanogaster*.The bacteria were isolated by dissecting the gut and making an extract out of it which was cultured in nutrient agar media separately. The isolated colonies were purified by quadrant streak method (Fig 1) One of the isolated colonies was grown on de Man Rogosa and Sharpe(MRS) agar medium at pH 6.5. The colonies appeared shiny small irregular and rod shaped. Few of the isolated bacteria were confirmed with laboratory test.

Gram's Staining:

The isolated bacteria were found to be short, rod shaped (Bacillus) gram positive in nature(Fig - 2). They were also non-spore forming which indicates that they belong to Lactobacillus sp.and was confirmed by biochemical tests.

Catalase Test:

The isolated bacteria were found to be catalase negative because no reaction was observed when the bacterial smear was allowed to react with H2O2 solution. This indicates that these isolates belong to Lactobacillus sp.

Starch Hydrolysis Test:

The isolated bacteria were checked for the starch hydrolysis test and it was found that most of the organisms grown on de Man Rogosa and Sharpe(MRS) agar media were found to have starch hydrolysis negative.

Antagonistic Activity of Lactobacillus Broth:

The Lactobacillus sp. have the characteristic activity of bacterial antagonism which does not allow the growth of other bacterial species which invade the insect gut. To prove this, test was conducted with four different bacterial species which were E. coli, Streptococcus, Staphylococcus and Salmonella. A clear zone was observed for E. coli and Streptococcus which indicates the antagonistic activity of Lactobacillus. The diameter of inhibitory zone was larger for E. coli when compared with the inhibitory zone of Streptococcus(Fig 3). Thus, by this observation it was concluded that *Lactobacillus* possesses bactericidal activity for *E. coli* and *Streptococcus* where as it was incapable of its antagonistic activity against *Salmonella* and *Staphylococcus.* (Fig 4)

DISCUSSION:

In this study, the wild *Drosophila melanogaster* gut was dissected and with thorough experiments the *Lactobacillus sp.* were isolated and identified. For the identification of bacteria various biochemical and microbial tests were performed. The antagonistic activity of *Lactobacillus* was tested against for four human pathogenic strains of bacteria and it was satisfactorily concluded through observations that it was found to be positive for *E. coli* and *Streptococcus.* This is because of the fact that an antimicrobial chemical was released by *Lactobacillus sp*. called "Bacteriocin". Therefore, the *Lactobacillus sp*. of *Drosophila*

melanogaster gut are considered as potential probiotics.

ACKNOWLEDGMENT

We thank the management,Principal and Head Department of Zoology and Genetics ,The Oxford College of Science for providing all the facilities to conduct this project.

REFERENCES

1. Adam C.-N. Wong, Adam J. Dobson, Angela E. Douglas,(1894-1901), Gut microbiota dictates the metabolic response of *Drosophila* to diet, Journal of Experimental Biology 2014, 217, doi: 10.1242/jeb.101725.

2. Alison L. Gould, Vivian Zhang, Lisa Lamberti, Eric W. Jones, Benjamin Obadia, Nikolaos Korasidis, Alex Gavryushkin, Jean M. Carlson, Niko Beerenwinkel, and William B. Ludington(2018, December 18), Microbiome interactions shape host fitness, Proceedings of the National Academy of Science, 115(51),doi: 10.1073/pnas.1809349115.

3. Angela E Douglas (2018, June), The *Drosophila* model for microbiome research, Nature, 157-164, doi: 10.1038/s41684-018-0065-0.

4. Benjamin Obadia, Erin S. Keebaugh, Ryuichi Yamada, William B. Ludington, and William W. Ja(2018, may 15),Diet influences host–microbiota associations in ***Drosophila,*** Proceedings of the National Academy of Science, 115(20), doi: 10.073/pnas.1804948115

5. HuiliQiao, Ian W. Keesey, Bill S. Hansson, Markus Knaden (2019, march 01), Gut microbiota affects development and olfactory behaviour in *Drosophila melanogaster,* Journal of experimental bology , 225(5), doi: 10.1242/jeb.192500.

6. Erin S.Keebaugh , RyuichiYamada ,BenjaminObadia , William B.Ludington, William W.Ja, (June 29, 2018) Microbial Quantity Impacts ***Drosophila*** Nutrition, Development, and Lifespan, iscience, 247-259 , https://doi.org/10.1016/j.isci.2018.06.004.

7. Fernández, M. P. & Kravitz, E. A, (2013), Aggression and courtship in *Drosophila*: pheromonal communication and sex recognition. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 199, 1065–1076.

8. Florence Capo,Alexa Wilson and Francesca Di Cara, (2019), The Intestine of ***Drosophila melanogaster***: An Emerging Versatile Model System to Study Intestinal Epithelial Homeostasis and Host-Microbial Interactions inHumans,*microorganisms* 2019, ***7***(9),336, https://doi.org/10.3390/microorganisms7090336.

9. Leftwich, P. T., Clarke, N. V. E., Hutchings, M. I. & Chapman, T. (2017), Gut microbiomes and reproductive isolation in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 114, 12767–12772.

10. LuisA.Ramírez-Camejo Génesis Maldonado-Morales, and Paul Bayman,(2017), Differential Microbial Diversity in *Drosophila melanogaster*: Are fruit flies potential vectors of opportunistic pathogens?, International journal of microbiology 2017, 6 pages , doi: 10.1155/2017/8526385.

11. Jessika Consuegra , Théodore Grenier , Patrice Baa-Puyoulet, Isabelle Rahioui, Houssam Akherraz, Hugo Gervais, Nicolas Parisot, Pedro da Silva, Hubert Charles, Federica Calevro, François Leulier (March 20, 2020) *Drosophila*-associated bacteria differentially shape the nutritional requirements of their host during juvenile growth, American Society for Microbiology, https://doi.org/10.1371/journal.pbio.3000681

12. Nichole Broderick and Bruno Lemaitre(2012 jul 1),Gut-associated microbes of *Drosophila melanogaster*, lands bioscience, 307-321, doi: 10.4161/gmic.19896.

13. Peter D. Newell, Angela E. Douglas, (November 2013),Interspecies Interactions Determine the Impact of the Gut Microbiota on Nutrient Allocation in *Drosophila*

melanogaster, American Society For Microbiology DOI**:** 10.1128/AEM.02742-13.
14. Rama K. Simhadri, Eva M. Fast, Rong Guo, Michaela J. Schultz, Natalie Vaisman, Luis Ortiz, Joanna Bybee, Barton E. Slatko, Horacio M. Frydman (2017), The gut commensal microbiome of *Drosophila melanogaster* is modified by the endosymbiont wolbachia, mSphere , 2(5), doi: 10.1128/mSphere.00287-17
15. Rooks, M. G. & Garrett, W. S, (2016), Gut microbiota, metabolites and host immunity. *Nature. Rev. Immunol.* 16, 341–352.
16. Seung Chul Shin Sung-Hee Kim Hyejin You, Boram Kim, Aeri C. Kim, Kyung-Ah Lee, Joo-Heon Yoon, Ji-Hwan Ryu, Won-Jae Lee,(2011 November 04) , ***Drosophila*** Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling, Science, 670-674, doi: 10.1126/science.1212782.
17. Wong, A. C.-N., Chaston, J. M. & Douglas, A. E, (2013), the inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. *ISME J.* **7**, 1922–1932.