International Journal of Zoology Studies
www.zoologyjournals.com
ISSN: 2455-7269
Received: 10-06-2021, Accepted: 26-06-2021, Published: 10-07-2021
Volume 6, Issue 4, 2021, Page No. 09-12

Paraquat effects on gut microbial diversity in *Drosophila melanogaster*

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Abstract
In insects gut microbial diversity play important role in host physiology, functions and fitness. Many factors known to affects the gut microbial diversity such as diet, age and environmental factors. In addition to this environmental stressors such as pollutant ie., pesticide and herbicide can also alter the gut microbiota of hosts, which in turn can affect their health and immune systems. Paraquat is one of the chemical pollutant wieldy used in agricultural fields to kill the pest therefore present study has been undertaken to study the effects of paraquaton Gut Microbial Diversity in *Drosophila melanogaster*. Axon-specific 16S rRNA gene primers for *Acetobacter pomorum, Acetobacter tropicalis, Lactobacillus brevis, Lactobacillus fructivorans* and *Lactobacillus plantarum* were used to identify major gut microbial diversity of control and paraquat treated *D. melanogaster* using primer3 software and unique regions identified from alignments of full 16S rRNA gene sequences. It was noticed that analysis of gut microbial diversity of control and paraquat treated flies revealed two species *Acetobacter* species group namely *Acetobacter pomorum* and *Acetobacter tropicalis* and three species belong to *Lactobacillus* species namely *Lactobacillus brevis, Lactobacillus fructivorans* and *Lactobacillus plantarum*. However their relative abundance varies in control and paraquat treated flies. The density of *Lactobacillus* species such as *L. brevis, L. fructivorans* and *L. plantarum* were found to be lowest in control flies. While these species were highest inparaqut treated flies. Further *A. pomorum* and *A. tropicalis* were found to be lowest abundance in control and they were found to be greater abundance inparaquat treated flies. Thus, these studies suggests that significant influence of paraquat treatment on gut microbial density in *D. melanogaster*.

Keywords: paraquat, microbiota, 16S RRNA, Pyro sequencing, *D. melanogaster*

Introduction

Majority of chemical compounds used in the field were in the form of pesticides they have serious effects on host intestinal microflora (Evariste *et. al.,* 2019) [9]. Insects have been exposed to toxic compounds such as plant toxins, artificial pesticides in the field and they try to adapt these factors by neutralization of toxins, changes in the target site, the mechanism of detoxification (Evariste *et.al.,* 2019) [9] It is believed that gut microbiota may also involve in insect's resistance to phytotoxins and pesticides because chemicals, interrupting the functions of the intestinal microflora, lead to changes in homeostasis of animals (Defois *et.al.*,2018; Itoh *et.al.*,2018) [5, 16]. Thus the toxicological significance of the interaction of intestinal microbiota with chemical pollutants is a serious concern,

In animals such as in green turtles Kittle *et al*., (2018) [17] have showed effects of glyphosate pesticide of different concentrations on the intestinal micobes and decrease in density and inhibition of gut bacterial growth were observed. Whereas in Chinese mitten crab Yang*et.al.*, (2019) [34] have noticed decreased antioxidant ability of the intestine due to the effect of the pesticide glyphosate when they performed sequenceing analysis for gut microbe have shown that glyphosate reduced the diversity of the intestinal microbiota of the Chinese mitten crab. Now a day's research directions are currently being involved in changes in gut micro biota with various pollutants, including pesticides.

Paraquat (PQ, 11, 10-dimethyl-4, 40-bipyridinium dichloride) is an herbicide commonly used in the laboratory to generate oxidative stress in animal models (Bus and Gibson, 1984) [3]. Here PQ undergoes an *in vivo*, NADPH-dependent reduction, yielding a stable PQ radical that reacts with oxygen to generate superoxide anion, a reactive oxygen species (ROS).The accumulation of ROS, and subsequent depletion of reducing agents, creates an environment of oxidative stress (OS), where ROS can cause damage to lipids, proteins, and DNA. Oxidative damage in response to PQ exposure has been demonstrated in a wide variety of organisms including *D.melanogaster* (Chen *et al.,* 2010 ; Magwere *et al*., 2006; Strub *et al.,* 2008; Legan *et al*., 2008) [4, 20, 28, 19]. However its effects on gut microbial diversity has not been studied. Therefore present study has been undertaken to understand effect of PQ on gut microbial diversity in *Drosophila melanogaster.*

Drosophila surve has a good model organism to study gut microbial diversity due to its short life cycle and human-like metabolic traits (Erkosar *et.al.,* 2013; Wong *et.al*., 2016; Erkosar and Leulier, 2014) [7, 32, 6]. Further in *D.melanogaster* using 16s amplicon on microbial diversity have shown presence of simple bacterial diversity consisting of four species *Lactobacillaceae, Acetobactereace, Enterobactereaceae* and *Enterococcaceae*. Despite variation in the density and composition of these species *Lactobacillus* and *Acetobacter* are more common (Storelli *et al,* 2011; Wong *et al,* 2011) [27, 32]. Therefore, present study has been undertaken to understand effects of Paraquat on gut microbial diversity *D.melanogaster*.

Material and Methods

Experimental stock Oregon-K strain of *D. melanogaster* obtained from the Drosophila Stock Centre, University of Mysore, Mysore was used. Twenty flies per culture bottle

(150ml) containing 30 ml of standard wheat cream agar medium with yeast. These culture bottles were maintained at 22±1 °C and 70–80% relative humidity.

PQ treatment

The wheat cream agar media (WCA) diet is a high quality diet that closely resembles the nutritional content of the diet to which *D. melanogaster* is adapted in the wild. For the WCA method, food was allowed to cool to 35 C and poured into standard Drosophila rearing vials with a PQ concentration of 10, 20, or 30 mm. A total of 15 flies were placed in each vial. Control flies were maintained on WCA diet. Flies raised on control and pqtreated diet were subjected for analyzing gutmicrobial diversity as follows.

Collection of Gut Microbe and Isolation of DNA

Twenty midguts were separately isolated from control and paraquat treated flies using 705 ethonol to extract DNA using QIA amp DNA mini kit (Qiagen, 51304). Midguts were externally sterilized with 70% ethanol and using homogenized in 180 µL ATL buffer, containing 0.5% reagent DX for foam minimization using an electric pestle (Kimble™ Kontes™ Pellet Pestle, 749540-0000). For additional lysis, 20 µL proteinase K SOLUTION was added to samples and incubated for 30 min at 56°C with shaking at 650 rpm. The samples were further lysed by homogenization using glass beads (425-600µm, sigma Aldrich, G8772-100G) in a fast prep FP120 machine (Bio 101 Savant) and afterward incubated for another 60 min at 56°C. For RNA digestion, RNASE A was added (Qiagen, 19101) and incubated the samples for 2 min at room temperature. After cool down, 200 µL of ethanol was added and the samples were transferred to the spin column as per manufacture instructions the washing and elution steps were performed. The samples were afterward further concentrated by sodium acetate precipitation.

Pyro Sequencing of 16s RRNA for Identification of Bacterial Species

Axon-specific 16S RRNA gene primers were used to identify major gut microbial diversity of D. melanogaster such as *Acetobacterpomorum*, *Acetobactertropicalis*, *Lactobacillus brevis*, *Lactobacillus fructivorans* and *Lactobacillus plantarum* using primer3 software and unique regions identified from alignments of full 16S RRNA gene sequences. Preliminary experiments confirmed that the primers generated to detectable cross-amplification between species. PCRs were performed as above with 65°C annealing temperature and 35 cycles. PCR products were separated by gel electrophoresis using 1% agarose gel and visualized with SYBR (Invitrogen), and their identities were confirmed by Sanger sequencing.

Measurement of Bacterial Loads

Quantification of gut microbe found in gut of control and paraquat treated flies of *D. melanogaster*. MR Sagar was used for quantifying all microbes except for the strains, which were *Acetobacterpomorum* quantified on mannitol plates. Guts were placed on either MRS or mannitol agar plates to record the microbe growth and viable bacterial load were calculated on the basis of colony forming units (CFU's) a colony –forming unit is a unit/mi (used to estimate the number of viable bacteria in a sample). viable is defined as the ability to multiply via binary fission under the controlled condition. Counting with colony forming units requires culturing the microbes and counting only viable cells, in contrast with microscopic examination. Abundance is calculated using colony forming units' which were expressed using logarithmic notation.

Result and Discussion

Most compelling studies on gut microbial diversity have shown that host diet and environment play important role in changing gut microbial diversity (Hasegawa *et al,* 2015; Hill-Burns *et.al.,* 2017; Scheperjana *et al.,* 2015) [14, 15, 25]. In recent decades, an increase in the use of pesticides to protect plants from pests, diseases, weeds, etc. has been observed (Montesion, 2003; Mahmood *et. al.,* 2016) [22, 21]. Numerous authors have studied various aspects of pesticide toxicity for non-target organisms (Sanchez-Bayo, 2012; Ware, 1980; Stanley and Preetha, 2016; Goulson, 2013) [24, 30]. In recent years, interest in the study of animal microbiome has sharply increased, as well as of its change under the influence of various physicochemical factors (Fraune and Bosch, 2011; Hamdi *et.al*., 2011;Ezenwa *et al.,* 2012; Bahrndorff *et al*., 2016; Apprill, 2017; Esser *et al.,* 2019; Turner, 2018; Reese, 2018) [11, 13, 10, 1, 8, 29, 23]. The toxicological significance of the interaction of intestinal microbiota with pollutants is a serious concern, since chemicals, interrupting the functions of the intestinal microflora, lead to changes in homeostasis of animals (Defois *et al.,* 2018) [5]. The number of publications on the effect of various pesticides on the microbiological composition of the intestines of animals, both model and agricultural, has increased.

In the present study the control and paraquat treated flies were subjected for gut microbial diversity to understand the effect of chemical pollutants on gut microbiota. It was noticed from the experiment that, a total of five microbial species were identified with the help of diagnostic primers listed in the Table 1. There were two species gut microbe belong to *Acetobacter* species group namely *Acetobacter pomorum* and *Acetobacter tropicalis* and three species of gut microbe belong to *Lactobacillus* species group namely *Lactobacilius brevis, Lactobacillus fructivorans* and *Lactobacillus plantarum*. However, the relative abundance of each species varies in relation to paraquat treatment Figure 1. The density of lactobacillus species such as *Lactobacilius brevis, Lactobacillus fructivorans* were found to be more or less same density in control and10mMparaquat treated flies. Highest density of these species found in 20mM paraquat treated flies. (Figure 1). In *L.plantanum* relative abundance was significantly greater in 20mMparaquat treated flies. In *L. plantanum* abundance was significantly greater in 20mm paraquat treatment onwards whereas control and 10mM paraquat treated flies had lowest abundance. In Acetobacter species *A. pomaram* and *A.tropicales* abundance was lowest in control and 10mM paraquat treated flies. However the density of this species increased with increasing concentration of paraquat treatment (Figure 1). This result suggests that significant influence chemical pollutant paraquat on gut microbial diversity in *D.melanogaster*. Present study also confirms the work of Kittle *et al*, (2018) [17] who while studying in green turtles have also noticed effect of pesticide glyphosate on intestinal microflora. Even in Chinese mitten crab it has been shown that pesticide glyphosate has significant influence on antioxidant ability of intestine there by affecting intestinal microbiota (Yang *et al.,* 2019) [34].

Further in the present study the reads obtained by pyro sequencing each sample were assigned to their respective OTUs and then analyzed for microbiota richness and evenness through determination of their respective indices suggesting that gut microbial diversity varied with control and paraquat treated flies (Table 2). Further the observed results can be explained that gut microbial diversity in the present study could be attributed to deleterious effect of paraquat treatment on host physiology. Further the treatment of paraquat had significant effect on the gut microbiota which in turn had affected drosophila performance control and paraquat treated flies suggesting that the association has a nutritional basis. It was also noticed that the processes contributing to interaction between the microbiota and host metabolism are likely multiple and interactive. It was also suggested that the host signaling pathways regulating the metabolism of males and females may respond differently to microbial products and their absence, and the metabolic traits of the microbiota may be influenced by many metabolic and other physiological differences between the sexes, especially the nutritional demands in females for egg production. Thus these studies suggested that paraquat treatment had significant influence on gut microbial diversity in *D. melanogaster*.

Acknowledgments

I am grateful to the University of Mysore for sanctioning minor research project and providing financial help. I am also grateful to Chairperson, Department of Studies in Zoology, University of Mysore, for providing facilities to carry out this work.

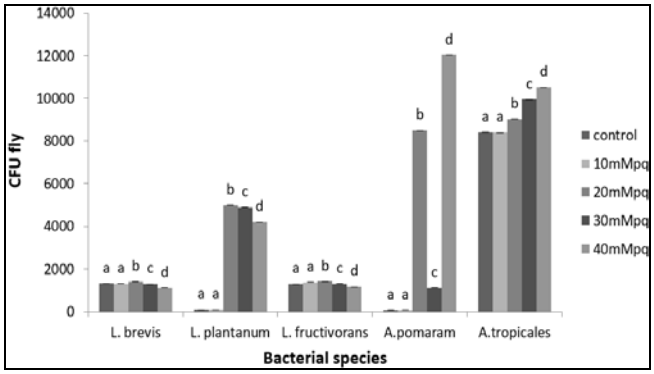

Fig 1: Effect of paraquat on five major gut microbial species of *D. melanogaster*

[*L. brevis*; f=20373.066, df=4,27, P< 0.001: *L. plantarum*; f= 24073.060, df= 4,27, P< 0.001: *L. fructivorans*; f= 13073.062, df= 4,27, P <0.001: *A.pomaram*; f=34073.051, df=4,27, P< 0.001: *A. tropicales*; f=173.061, df= 4,27, P<0.001] Different letters in superscript of bar graph indicate significant at 0.05 level by Tukey's post hoc test

Table 1: Diagnostic primers used for identification of bacteria

Bacterial species	End point PCR		QRT-PCR	
	Forward	Reverse	Forward	Reverse
Acetobacter pomorum	5'-TGGGTGGGGGATAACACTGGGA-3'	5'-AGAGGTCCCTTGCGGGAAACA-3'	5'-TGTTTCCCGCAAGGGACCTCT -3'	5'-AGAGTGCCCAGCCAACCTGA-3'
Acetobacter tropicalis	5'-AGGGCTTGTATGGGTAGGCT-3'	5'-CAGAGTGCAATCCGAACTGA-3'	5'-TAGCTAACGCGATAAGCACA -3'	5'-ACAGCCTACCCATACAAGCC-3'
Lactobacillus brevis	5'-ACGTAGCCGACCTGAGAGGGT-3'	5'-AGCTTAGCCTCACGACTTCGCA-3'		
Lactobacillus fructivorans	5'-TGGATCCGCGGCGCATTAGC-3'	5'GCCCCCGAAGGGGACACCTA-3'	5'-AACCTGCCCAGAAGAAGGGGA -3'	5'-GCGCCGCGGATCCATCCAAA-3'
Lactobacillus plantarum	5'-TCCATGTCCCCGAAGGGAACG-3'	5'-TGGATGGTCCCGCGGCGTAT-3'	5'-TGTCTCAGTCCCAATGTGGCCG -3'	5'-GGCTATCACTTTTGGATGGTCCCGC-3'

Table 2: Diversity estimations were obtained following normalization of OUT's

Diet	OTUs	Chao1	Shannon	Evenness
Control	53	66	2.26	0.71
10mMPQ	67	69	3.61	0.82
20mMPQ	65	77	3.76	0.91
30MmMPQ	71	73	3.78	0.92
40mMPQ	73	75	3.82	0.93

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