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Gut Microbial Diversity in Rotenone Induced and Transgenically Created PD (Parkinson Disease) Flies of *Drosophila melanogaster*

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Abstract In the present study of gut microbial diversity in control and experimental treated *D. melanogaster* flies showed that there were two species belong to *Acetobacter* species group namely *Acetobacter pomorum* and *Acetobacter tropicalis* and three species belong to *Lactobacillus* species namely *Lactobacillus brevis, Lactobacillus fructivorans* and *Lactobacillus plantarum.* Further analysis revealed that the relative abundance of each of the above microbial species varies in control and experimental flies. The density of *lactobacillus* species such as *L. brevis, L. fructivorans* and *L. plantarum* were found to be lowest in control and creatine supplemented flies. While these species were highest in rotenone induced and transgenically created PD flies. Further *A. pomorum* and *A. tropicalis* were found to be lowest abundance in control and creatine treated flies whereas they were found to be greater abundance in rotenone induced and transgenically created PD flies of *D. melanogaster*. Thus, these studies suggest that significant influence of host diet related changes in gut microbial density in *Drosophila.*

Keywords Parkinson's Disease, Microbiota, 16S rRNA, Pyro Sequencing, *D.melanogaster* Transgenic Flies

1. Introduction

The term microbiome of an organism comprises of bacteria, archaeabacteria, fungi, protozoa and viruses. Nowadays the role of microbiota on human physiology has received much attention to understand its effect on health and variations on gut microbe have been linked to numerous human illnesses including neurodegenerative diseases [1]. Realizing this importance in 2007 the national institutes of health (NIH) launched the human microbiome project to identifying and understand the role of microbial species in human health and diseases [1]. Studies of such analysis have revealed that total microbiome present in an organism was more than 100 times the number of genes found in the human genome [2-3].

Changes in the intestine due to gut microbe can influence brain function [4]. Studies on gut brain axis have shown that it is a bidirectional communication system that uses neural, endocrine and immunological signals. In such an axis the neural information is thought to travel along vagal or spinal innervation between the CNS and the gut [4]. The changes in gut microbial diversity could send the signal directly through CNS and indirectly through the synthesis of neurotransmitters or neurochemical-like precursors. Example GABA synthesis by lactobacillus and bifidobacterium species; noradrenaline by Escherichia, bacillus and saccharomyces species; serotonin by candida, streptococcus, Escherichia and enterococcus species; dopamine by bacillus species and ultimately, acetylcholine by lactobacillus [5].

Studies on human PD had shown changes in the microbial resident population had its effect on its metabolites when comparing the human microbiome. Studies carried out on healthy control versus PD patient on human have found significant dysbiosis in PD patient which is correlated to the changes in the relative abundance of gut microbiota. The changes may include appearance and disappearance of particular microbial species and changes in their relative abundance [6-7]. Studies on animal models of PD have shown a link between microbial dysbiosis and neurodegeneration [8]. Such studies revealed that healthy intestinal development requires microbiota colonization and robust antibiotic treatment can also influence the host physiology [9-10]. Studies using pharmacologically –induced PD models

have shown exposure to rotenone showed variations in abundance reported such as increases in *Bifidobacterium* and *Lactobacillus* genus and a decrease in *Prevotellaceae* after rotenone treatment or the increase in *Enterobacteriaceae* in MPTP –treated mice were similar to what has been observed on human PD cases [6-7,10-15]. Further these studies have also showed changes in the intestinal phenotype such as gut inflammation and increased intestinal permeability leading to constipation suggesting changes in the gut microbial diversity directly or indirectly was responsible for these changes [9, 16-17].

Drosophila becomes a good animal model to understand the microbial diversity due to its similarity in neuroendocrine architecture with that of higher mammals especially humans [18-19]. Further it is a simple model system to understand the host microbiota relationship and organismal health because of its short life cycle and human-like metabolic traits [18-20]. Studies on microbial diversity in *Drosophila* have shown that fly has under different situation have used the 16s amplicon simple bacterial colonies comprising of four species *Lactobacillaceae, Acetobactereace, Enterobactereaceae and Enterococcaceae*. Despite variation in the density and composition of these species *Lactobacillus* and *Acetobacter* are more common [21-22]. Gut microbial diversity in *Drosophila* have shown variation in abundance of certain microbial species do occur across various strains and also immune activity. Such studies revealed that maintenance of innate immune homeostasis is associated with the suppression of pathogenic bacteria. Further, as the age increased changes in the innate immune homeostasis can also related to changes in the microbiota [23-25]. Further using germfree flies showed decreased insulin signaling which is promoted by commensal gut bacteria i.e. *Lactobacillus Plantarum* and *Acetobacter Pomorum*. Thus, these studies suggest *Drosophila* forms a very good model to understand changes in health of an organism due to changes associated with its gut microbial diversity. Therefore, present study has been undertaken to understand gut microbial diversity in rotenone induced and transgenically created PD flies of *Drosophila*.

2. Materials and Methods

Details procedure used to establish and maintenance of control and experimental flies [Creatine treated, rotenone treated (rotenone-induced PD flies), co-exposure of rotenone and creatine, transgenic created PD flies] of *D.melanogaster* were presented in our earlier paper [26]. These flies were subjected to gut microbial analysis.

2.1. Collection of Gut Microbe and Isolation of DNA

Midguts of control and experimental flies were removed by using 70% ethanol. Twenty midguts per experimental flies were isolated 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.

2.2. 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 Acetobacter pomorum, Acetobacter tropicalis, 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.

2.3. Measurement of Bacterial Loads

Quantification of gut microbe found in gut of control and experimental flies of *D. melanogaster.* MRSagar was used for quantifying all microbes except for the strains, which were *Acetobacter pomorum* quantified on mannitol plates. To measure microbe growth guts were placed on either MRS or mannitol agar plates. Viable bacterial load was 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.

3. Statistical Analysis

One-way ANOVA followed by Tukey's post hoc test carried out on the above data showed significant variation in gut microbial diversity between control and experimental flies in all the gut microbial species identified.

4. Results and Discussion

Most compelling studies on gut microbial diversity have shown that host diet is an important environmental factor known to affect the gut microbial diversity [6, 27-28]. In the present study two compounds such as rotenone, a known antioxidant inducer and in chronic doses it is known to produce Parkinson disease in *D.melanogaster* [26, 29]. Creatine, on the other hand, known potent antioxidant [26, 29]. In the present study, effect of rotenone and creatine supplementation on gut microbial diversity of *D.melanogaster* was made prior to the experiment the experimental flies were cultured in a wheat cream agar medium and maintained them in the same conditions. In experimental flies rotenone and creatine were treated separately and also in one of experimental flies co exposed with these two compounds. Further PD flies were also created using transgenic flies. The control and above experimental flies were subjected for gut microbial diversity to understand the effect of host diet on physiology in turn its effect on resident microbiota. It was also noticed from the experiment, a total of five microbial species were identified with the help of diagnostic primers listed in the Table 1 and it was also quantified each of the identified species using CFU's (Table 2).

There were two species gut microbe belong to *acetobacter* species group namely *Acetobacter pomorum* and *Acetobacter tropicalis* and three species 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 host diet. The density of *lactobacillus* species such as *Lactobacilius brevis, Lactobacillus fructivorans* and *Lactobacillus plantarum* were found to be lowest in control and creatine supplemented flies (Figure 1). On the other hand, they were highest in rotenone induced and transgenically created PD flies.

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'-AGAGTGCCCAGCCCAACCTGA-3'
Acetobacter tropicalis	5'-AGGGCTTGTATGGGTAGGCT-3'	5'-CAGAGTGCAATCCGAACTGA-3'	5'-TAGCTAACGCGATAAGCACA-3'	5'-ACAGCCTACCCATACAAGCC-3'
Lactobacilius 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'-TGGAATGGTCCCGCGGCGTAT-3'	5'-TGTCTCAGTCCCAATGTGGCCG-3'	5'-GGCTATCACTTTTGGATGGTCCCGC-3'

Table 2. Richness and evenness estimation of the microbiota in each of control and experimental flies of *D. melanogaster*. Diversity estimations were obtained following normalization of OUT'S

	Control flies	Experimental flies				
		Creatine supplemented flies	Rotenone induced PD	Rotenone induced PD +Creatine	Transgenically Created PD flies	Transgenically created PD flies +Creatine
OUT'S	55	60	63	65	64	62
Chaol	63	64	65	64	67	65
Shannon	2.10	3.11	3.14	3.18	3.36	3.10
Evenness	0.72	0.74	0.78	0.79	0.80	0.74

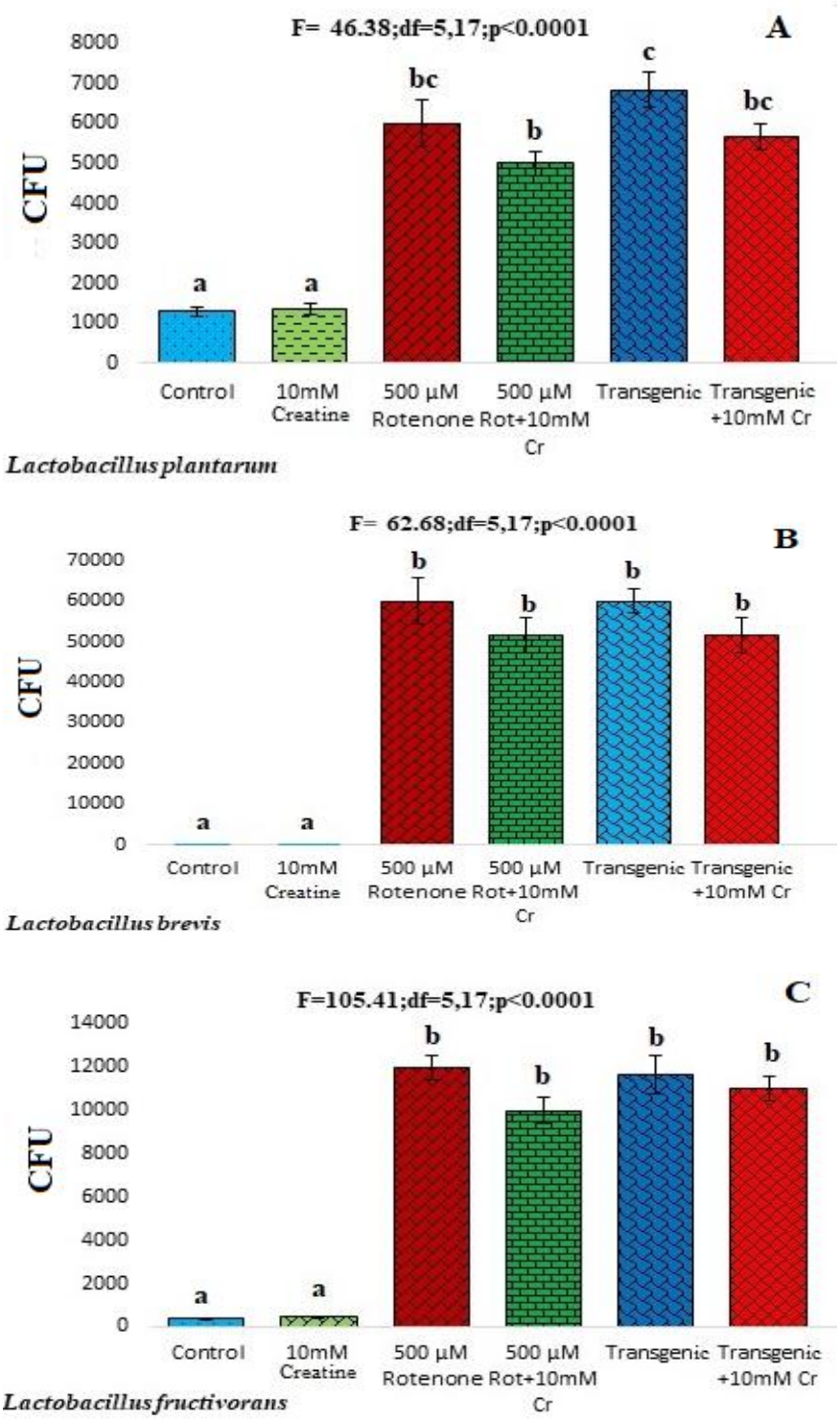

Figure 1. CFU's of *Lactobacillus plantarum (A), Lactobacilius brevis (B) and Lactobacillus fructivorans (C)* in control and experimental flies of *D.melanogaster.* Values are mean ± SE (three replicates). Different letter on the superscript of bar graph indicate significance at $p<0.05$ level by Tukey's post hoc test

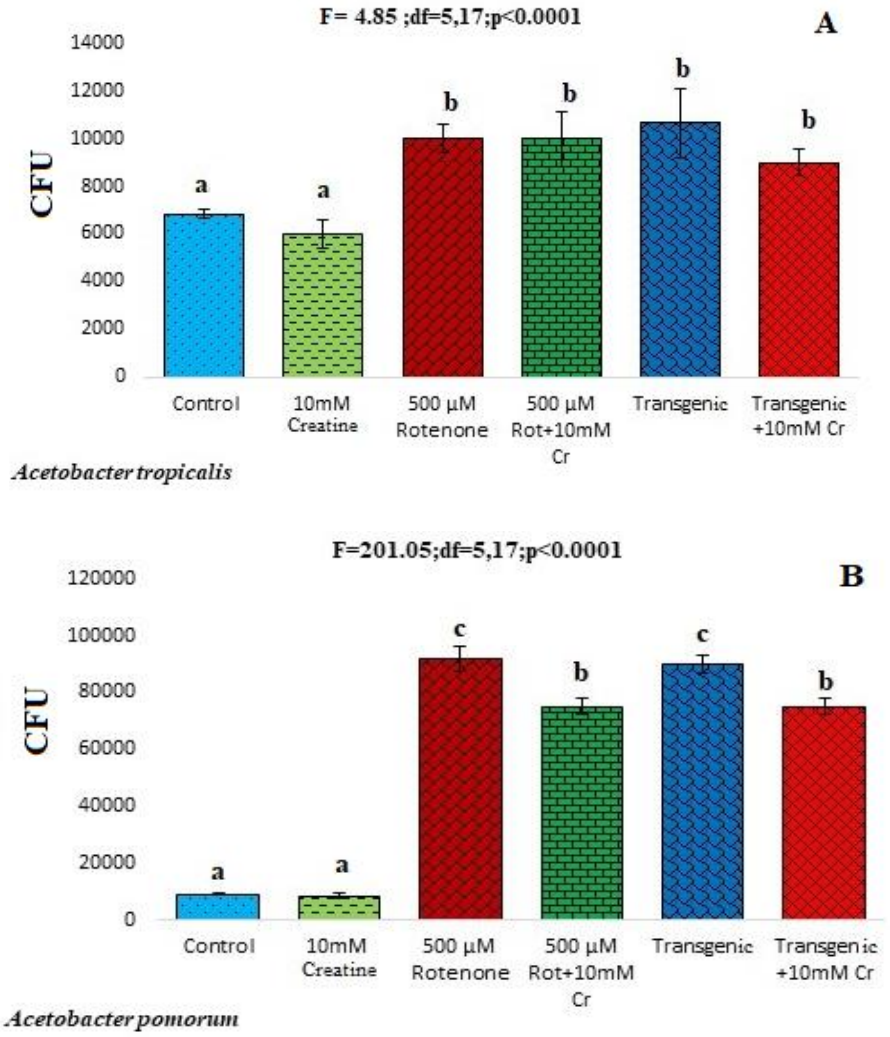

Figure 2. CFU's of *Acetobacter tropicalis* (A) and *Acetobacter pomorum (B)* in control and experimental flies of *D.melanogaster.* Values are mean ± SE (three replicates). Different letter on the superscript of bar graph indicate significance at $p<0.05$ level by Tukey's post hoc test

Acetobacter pomorum and *Acetobacter tropicalis* were found to be lowest abundance in control and creatine treated flies, whereas they were found to be greater abundance in rotenone induced and transgenically created PD flies of *D.melanogaster* (Figure 2). These results suggest that significant influence of host diet on gut microbial diversity in *D.melanogaster.* Our result also confirms the earlier studies of host diet related changes in gut microbial in *Drosophila* [6, 27- 28]. This change in gut microbial diversity with diet also shows metabolic changes in the host organism. Earlier studies on gut microbial diversity have recognized that the resident microbiota play important role in animal nutrition [30-33]. This is because the gut microbiota involved in acquisition and allocation of animal nutrients had played key role in shaping the nutritional statues of an animal. Further this gut microbe either consumed ingested nutrient or provide supplementary nutrient to the host thereby they can alert feeding and nutrient assimilation rate.

In the present study, toxic effects of rotenone on microbial diversity in *D.melanogaster* and potential benefit of creatine supplement to rotenone induced PD and transgenically created PD on gut microbial diversity has been studied. It was noticed from the Figure 1 that species of *lactobacillus* showed diversity in relation to rotenone treatment. Further the density of *lactobacillus* species was significantly greater in rotenone and transgenically created PD flies. The density of *lactobacillus brevis* and *Lactobacillus fructivorans* were found to be greater (Figure 1). Further creatine supplementation to rotenone induced and transgenically created PD flies had influence in the gut microbiota which caused a moderate reverse of increased bacterial species. This clearly suggests that host diet has significant effect on gut microbial diversity in *D.melanogaster*. 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 host

diet (Table 2). One-way ANOVA followed by Tukey's post hoc test applied on alone data showed significant variation in gut microbial diversity of lactobacillus and acetobacter species in control and experimental flies of *D. melanogaster* (Figure 1 and Figure 2). Further the observed results can be explained that gut microbial diversity in the present study could be attributed to deleterious effect of rotenone treatment on host physiology. Whereas the creatine supplement had either beneficial (promote host performance) or no desirable effect. The result obtained in the present study was attributed to two folds, host and microbiota do not compete for dietary nutrients which could be an indication of having a lower density of gut microbe, which suggests that the various diet derived nutrients are either not utilized by both host and microbiota or they are in sufficient abundance that their consumption by microbiota does not limit host performance. Further the treatment of rotenone in diet had effect on the gut microbiota which in turn had effect on drosophila performance on diet of low or unbalanced nutrient content, 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 rotenone treatment and creatine supplement had significant influence on gut microbial diversity in *D.melanogaster.* Tukey's post hoc test showed that control and creatine treated flies had significantly lower number of CFU's in *Acetobacter pomorum, Acetobacter tropicalis, Lactobacillus brevis, Lactobacillus fructivorans and Lactobacillus plantarum* whereas rotenone induced, transgenically created PD, rotenone induced +creatine and transgenically created PD +creatine had significantly greater number of CFU's in *Acetobacter pomorum, Acetobacter tropicalis, Lactobacillus brevis, Lactobacillus fructivorans and Lactobacillus plantarum.*

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