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Alloxan induced changes in gut microflora in *Drosophila melanogaster*: Protective role of creatine

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Abstract

Objective: Food consumed by the organism modifies the gut microbe thereby modulating host physiology which in turn affects health and fitness of an organism. Current research was conducted to understand the effects of Alloxan on gut microbial diversity using *D.melanogaster* as model organism and also to evaluate the effects of creatine on gut microbial diversity. Pyro sequencing of 16S rRNA isolated from the gut of *D.melanogaster* raised in control, 3% Alloxan, 5% Creatine, 3% Alloxan + Creatine were used for identification of bacterial species. It was noticed that the species of *Acetobacter tropicalis, Acetobacter pomorum* and *Lactobacillus brevis, Lactobacillus fructivorans* and *Lactobacillus plantarum* showed diversity in terms of Alloxan diet treatment. The density of *Lactobacillus* and *Acetobacter* species was high in flies fed with alloxan compared to flies fed with control diet. Further flies fed with alloxan along with creatine showed a significant decrease in the density of all the species of *Lactobacillus* and one species of *Acetobacter*. Thus these studies clearly suggest Alloxan treatment has an effect on host physiology which in turn affects density of gut microbe in *D. melanogaster*. Further, creatine feeding along with Alloxan was responsible in bringing back to normal gut microbial condition in *D. melanogaster*.

Keywords: gut microflora, alloxan, *Drosophila melanogaster*, creatine

Introduction

Gut microbiota is one of the influential factors in determining the phenotype of an organism because it is involved in the absorption and distribution of nutrients in animal systems in various ways, they can consume the nutrients they ingest and provide additional nutrients to the host, also they can change the host's feeding and nutrients, in turn they can change the nutrient distribution pattern of the host by altering the nutrient sensing and signaling pathways of the organism. (Hooper *et al*., 2002; Backhed *et al*., 2004; Carcilli and Saad, 2013; Goodman *et al*., 2009; Vijayakumar *et al*., 2010) [1, 2, 3, 4, 5]. Now a day the gut microbiota has been utilized to understand the animal physiology and illness of an organism (Turnbough *et al*., 2007) [6]. Literature survey on gut microbial diversity revealed that the composition and density of the intestinal microbiome depend on host-specific factors such as age, sex and genotype, as well as external factors such as habitat and diet. (Anitha and Krishna, 2021; Ali and Krishna, 2020; Harsha and Krishna, 2019) [7, 8, 9]. These studies have shown that as the host age increases their physiology also change thereby one can see the changes in the composition and density of gut microbes. Further, they also found that physiology of male and female also vary which in turn also effects the gut microbial composition and diversity. In addition to this different genotype of the same species they vary in their development in different environment which may also change the composition and density of gut microbe. The addition of certain compounds to the diet favorably alters the gut microflora by promoting the growth of beneficial bacteria or counteracts microbial community imbalances with the aim of supporting a healthy host. (Harsha and Krishna, 2019) [9].

Several methods have been used to induce diabetes in experimental animals with varying success and many difficulties. Although surgical removal of the pancreas is effective, at least 90-95% of the pancreas must be removed to cause diabetes. (Rastellini *et.al*., 1997) [10]. Most studies published in the field of ethnopharmacology between 1996 and 2006 used chemically induced models. Streptozotocin (STZ) 69% and alloxan 31% are the most commonly used drugs to date, and this model has helped to study multiple aspects of the disease. (Rydgren *et.al*., 2007) [11]. Both drugs have diabetes-inducing effects when given parenterally (intravenously, intraperitoneally, or subcutaneously). The dose of these drugs required to induce diabetes depends on the animal species, route of administration and nutritional status. (Balmurugan *et.al*., 2003) [12]. Further, in the animal system Alloxan induces oxidative stress by the interaction of Alloxan and its reduction product dialuric acid establish a redox cycle with the formation of superoxide radicals (Viana *et.al*., 2004) [13]. Now a day, Alloxan is used in bakery to produce bakery products which are consumed by the people. Therefore, present study has been undertaken to study the toxic effects of Alloxan on the gut microbial diversity in *D.melanogaster*.

Drosophila becomes a good animal model to understand the microbial diversity because the neuroendocrine functioning resembles that of higher mammals, especially humans, (Erkosar *et.al*., 2013; Wong *et.al.*, 2016) [14, 15]. Moreover, due to its short life cycle and human-like metabolic properties, it is a simple model system for understanding the health relationships of host microorganisms and organisms. (Erkosar *et.al*., 2013; Wong *et.al*., 2016; Erkosar *et.al*., 2014) [14, 15, 16]. Studies on microbial diversity in Drosophila have shown that fly has under different situation have used the 16s amplicon simple bacterial colonies consisting of four species *Lactobacillaceae, Enterobactereaceae*, *Enterococcaceae* and *Acetobactereace*. Despite differences in density and composition of these species, Lactobacillus and Acetobacter are more common. (Storelli *et.al*., 2011; Wong *et.al.,* 2011) [17, 18]. Gut microbial diversity in Drosophila has shown variation in abundance of certain microbial species does occur across various strains and also immune activity. These studies have shown that maintenance of innate immune homeostasis is associated with suppression of pathogenic bacteria. Additionally, with age, changes in innate immune homeostasis may also be associated with changes in the microbiome. (Ren *et.al*., 2007; Lhocine *et.al*., 2008; Broderick *et.al*., 2012) [19, 20, 21]. Further, use of germfree flies showed a decrease in insulin signaling with the help of symbiotic enterobacteria, namely *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.

Creatine (Cr) is a naturally occurring chemical in the human body that is partially generated endogenously and eaten exogenously through diet, particularly meat and fish. It is responsible for supplying fast energy during muscular contraction by transferring the N-phosphoryl group from phosphorylcreatine (PCr) to ADP to produce ATP via a reversible mechanism catalysed by phosphorylcreatine kinase (PCK) (Gualano *et.al.,* 2010) [22]. Cr is primarily responsible for transferring energy from mitochondria to the cytosol. In tissues with high energy demands, such as the brain and muscle, the PCr-PCK system acts as an energy buffer by linking the mitochondrial sites of energy generation with the cytosolic sites of energy consumption (Wyss *et.al*., 2000) [23]. One of the most important physiological activities given to the PCr-PCk system *in vivo* is the avoidance of oxidative stress through direct and indirect antioxidant activity (Greenhalf., 2001) [24].

Cr is currently regularly utilized by athletes as an ergogenic dietary supplement to boost muscle performance (Froiland *et.al*., 2004) [25]. The benefits of Cr supplementation in individuals with atrophy, muscular weakness, and metabolic dysfunctions have been demonstrated in several investigations. Recent research has found that Cr supplementation may have therapeutic advantages, such as better brain function in both young and old persons (Watanabe *et.al*., 2002) [26], diminution of mental tiredness and reduction of stress-induced cognitive impairment (Mc Moris *et al*., 2007) [27]. Several studies have found that Cr has unique protective benefits in animal models of neurodegenerative disorders including ischemic stroke (Baker *et.al*., 2003; Bender *et.al*., 2006; 2008) [28, 29, 30]. In cell and animal models, the neuroprotective effects of Cr have been attributed to the buffering capacity of cellular ATP levels along with mitochondrial focused antioxidant characteristics (Andres *et.al*., 2005; Lensman *et.al*., 2006; Sestili *et.al*., 2006) [31, 32, 33]. Therefore present study has been undertaken to study effects of Alloxan on gut microbial diversity and the reversal effects of creatine in *D.melanogaster*.

Materials and Methods

Flies of *D.melanogaster* obtained from *Drosophila* stock center, University of Mysore was used. Eggs were collected from these flies using Delcour's procedure (Delcour., 1969) [34]. Eggs (100) were placed to each of the culture bottle (250 ml) containing wheat cream agar medium. These culture bottles were maintained at 22º ± 1 C and 70% RH with a 12:12 L: D photoperiod. Unmated males and virgin females were isolated within 3 hrs of their eclosion. Twenty flies were transferred to quarter pint bottles (250 ml) containing 5 ml of control diet (wheat cream agar media), 3% Alloxan diet, 5% Creatine diet, Alloxan diet with 5% creatine diet(wheat cream agar media+3% Alloxan)for 10 days. Following this, flies were used for gut microbial analysis as follows:

Collection of Gut and Isolation of DNA

Midguts of control and experimental flies [control diet (wheat cream agar media), 3% Alloxan diet (wheat cream agar media+3% Alloxan), 5% Creatine diet, Alloxan diet with 5% creatinine diet] were removed by dissecting the flies with 70% ethanol. Separate experiment was carried out for each of control and experimental flies used. Approximately 20 midguts [control and experimental] were used to extract Genomic DNA separately using the QIAampDNA Mini Kit (Qiagen, 51304). For this, midgut obtained were externally sterilized with 70% ethanol and homogenized in 180µL ATL buffer, containing 0.5% Reagent DX for foam minimization using an electric pestle (Kimble™ Kontes™ Pellet Pestle, 749540-0000). For additional lysis, 20µL Proteinase K solution was added to the samples and incubated for 30 minat 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 (Bio101 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 was added and the ethanol samples were transferred to the spin column. The washing and elution steps were performed according to the manufacturer's instructions. 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 designed for *A. tropicalis, A. pomorum, L. brevis, L. fructivorans* and *L. plantarum* using Primer3 software and unique regions identified from alignments of full 16S rRNA gene

sequences (Table 1) for sequencing of the gut *D. melanogaster* microbiome and identification of major bacterial species. Before using, preliminary experiments were run to confirm the primers generated have no detectable cross-amplification between speciesand then these primers were used. The annealing temperature 65ºC and 35 cycles were used during PCRs performed. PCR products weres separated by gel electrophoresis using 1% agarose gel and visualized with SYBR®Safe (Invitrogen), and their identities were confirmed by Sanger sequencing.

Measurement of Bacterial Loads

Dilution plating or O.D. measures was used to quantify microbial seed of experimental and serial dilution plating of isolated guts was used to quantify the microbial load. MRS agar was used for quantifying all microbes except for the strains, which were *A. pomorum* quantified on mannitol plates. To measure microbe growth guts were placed on either MRS or mannitol agar plates. Viable bacterial loads were calculated on the basis of colony forming units (CFU's) a colony-forming unit is a unit /ml (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 conditions. 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.

Results and Discussion

Analysis of control and experimental flies [control diet (wheat cream agar media), 3% Alloxan diet (wheat cream agar media+3% Alloxan), 5% Creatine diet, Alloxan diet with 5% creatine diet] using Axon-specific 16S rRNA gene primers in PCR revealed that two *Acetobacter* species and three *Lactobacillus* species were noticed. In genus *lactobacillus* species found were. *L.brevis*, *L.fructivorans* and *L.plantarum* whereas in *Acetobaccter genus*, species *A.pomorum and A.tropicalis* were identified. Densities of all these bacterial species were least in control flies and highest in Alloxan treated flies. One way ANOVA followed by Tuckey's post hoc test (Fig 1-5) carried out on density of these bacterial species showed significant difference between control and experimental flies used. control flies had significantly least density of these bacterial species compared to Alloxan treated and coexposure of Alloxan with 5% creatine.

The toxic effects of alloxan on the intestinal microbial diversity in *D.melanogaster* and the potential benefit of alloxan-supplied creatine in *D.melanogaster* were investigated. Density of all these bacterial species was least in control flies and highest in Alloxan treated flies. One way ANOVA followed by Tuckey's post hoc test (Fig 1-5) carried out on density of these bacterial species showed significant difference between control and experimental flies used. Control flies had significantly least density of these bacterial species compared to Alloxan treated and coexposure of Alloxan with 5% creatine. Further, co-exposure of flies to Alloxan along with creatine had a significant effect on the gut microbiota, moderately reversing the increased bacterial population to normal density. This suggests that Alloxan has significant influence on gut microbial diversity. Readings obtained by pyrosequencing each sample were assigned to their respective OTUs, and then their respective indices were determined to analyze the abundance and uniformity of the microbial community, which showed that the microbial community varied with diet (Table 2). One-way ANOVA followed by Tukey's post hoc tests performed on the above data showed significant differences in *Lactobacillus* gut microbial diversity between flies fed the experimental diet. Many factors such as host genotype, age, diet, immediate environment and immunity are known to affects gut microbial diversity (Anitha and Krishna, 2021; Ali and Krishna, 2020; Harsha and Krishna, 2019) [7, 8, 9]. In the present study same flies having same age class were used. These flies were also raised in the same environmental condition, further only difference was use of Alloxan in treated flies therefore the observed result is Alloxan and with other factors.

The processes that facilitate the interaction between the microbiome and host metabolism are numerous and likely to interact. Host signaling pathways that regulate metabolism in males and females may respond differently to microbial products and the absence of microorganisms; and The metabolic properties of the microbiome can be influenced by many metabolic and physiological differences between the sexes, especially the nutrients females need for egg production (Blatch *et al.*, 2010; Piper *et al.*, 2011) [35, 36] showed that on the most unbalanced diet tested (25 g yeast and 200 g glucose l−1), the microbiota spared the Drosophila requirement for the B vitamin riboflavin. Changes in bacterial phyla proportions during obesity have captured science attention worldwide, especially because of their effects on metabolism. Further, the processes contributing to interactions between the microbiota and host metabolism are likely multiple and interactive. 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 demand in females for egg production. Thus this study suggests that host physiology varies with age and diet, in turn, it affects gut microbial diversity.

The observed results can also be explained by the fact that the gut microbiome is either beneficial (promoting host productivity) or not detrimental to Drosophila grown on an alloxan diet (does not affect host productivity). First, the result is twofold. Host and microbiota do not compete for dietary nutrients, which may result in lower microbiota densities, suggesting that various dietary-derived nutrients are either not utilized by both the host and microbiota or are abundant enough for the microbiota to consume and does not limit host

performance. Second fruit fly does not depend on the microbiome for its normal physiological function, which will be demonstrated by the superior performance of the normal fruit fly on any diet. Instead, the microbiome contributed to productivity of fruit flies, particularly inlow-nutrient orunbalanced diets, indicating that the association has a nutritional basis. Creatine is well-known for itsusage as an adaptogen to increase human performance and as a therapy for oxidative stress-related illnesses (Ravikumar and Ramesh, 2010) [37]. Creatine is known for its use as an ergogenic supplement to improve human performance and as a treatment for oxidative stress-mediated disorders. Study on transgenic model of Parkinson's disease and rotenone induced Parkinson's disease (PD) provided with creatine supplement showed significant reduction in the endogenous levels of oxidative markers such as malondehyde and hydro peroxide suggesting that anti-oxidative property of creatine (F5, F6, F7, F8, F9) (Ravikumar and Ramesh, 2010) [37] have also noticed similar results with reference to creatine supplement against rotenone induced PD in *D.melanogaster* (Ali and Krishna, 2019) [8]. Current research confirmed the antioxidative property of creatine and was effective in reverting the change in gut microbiome caused by alloxan treatment.

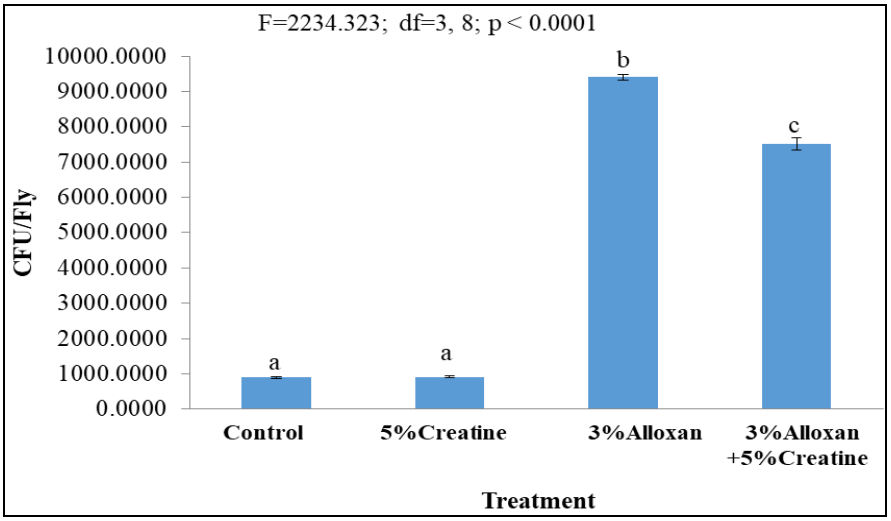
Finally, analysis of the microbiome relationships obtained from various fly samples showed that the survival and health of host flies are highly influential factors for the formation of unique bacterial communities in the gut microbiome of *D. melanogaster*. Thus present study suggests Alloxan treatment has an effect on the host physiology which in turn affected the gut microbial diversity. Creatine treatment along with Alloxan was responsible in bringing back the normal gut microbial density in *D.melanogaster*

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'AGGGCTTGTATGGGTAGGC T-3'	5'CAGAGTGCAATCCGAACTGA -3'	5'TAGCTAACGCGATAAGCACA -3'	5'ACAGCCTACCCATACAAGCC-3'
Lactobacillus brevis	5'ACGTAGCCGACCTGAGAGGGT-3'	5'AGCTTAGCCTCACGACTTCGCA-3'	---	---
Lactobacillus fructivorans	5'TGGATCCGCGGCGCATTAGC-3'	5'GCCCCCGAAGGGGACACCT A-3'	5'AACCTGCCCAGAAGAAGGGGA -3'	5'GCGCCGCGGATCCATCCAAA-3'
Lactobacillus plantarum	5'TCCATGTCCCCGAAGGGAACG-3'	5'TGGATGGTCCCGCGGCGTAT-3'	5'TGTCTCAGTCCCAAGTGGCCG -3'	5'GGCTATCACTTTTGGATGGTCCCGC-3'

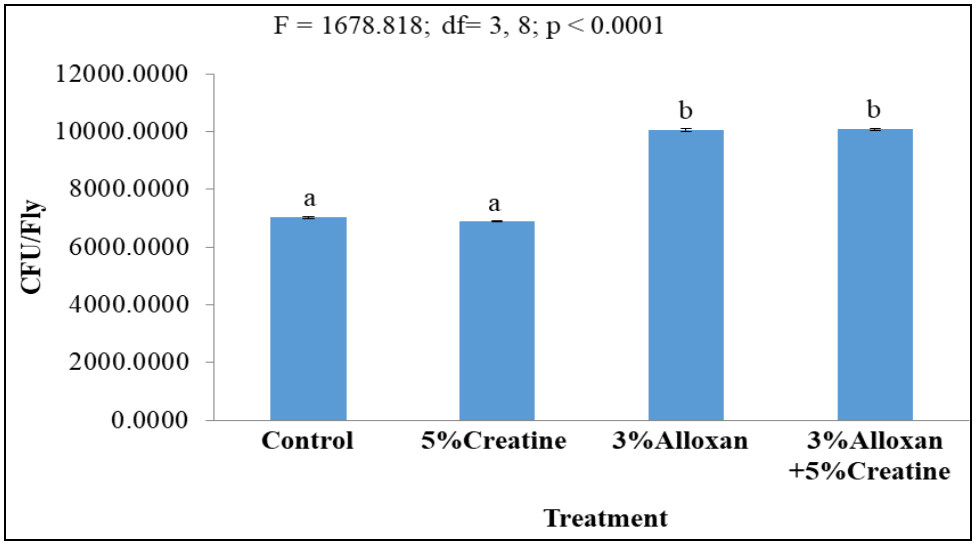
Table 2: Richness and evenness estimation of the microbiota in each of control and experimental flies of *D. melanogaster*. Diversity estimations were obtained following normalization of OUT'S

	Control flies	5% Creatine	3% Alloxan treated flies	3% Alloxan + 5% Creatine treated flies
OTU's	52	55	60	57
Chaol	60	62	66	63
Shannon	2.16	2.36	3.05	2.42
Evenness	0.78	0.79	0.82	0.78



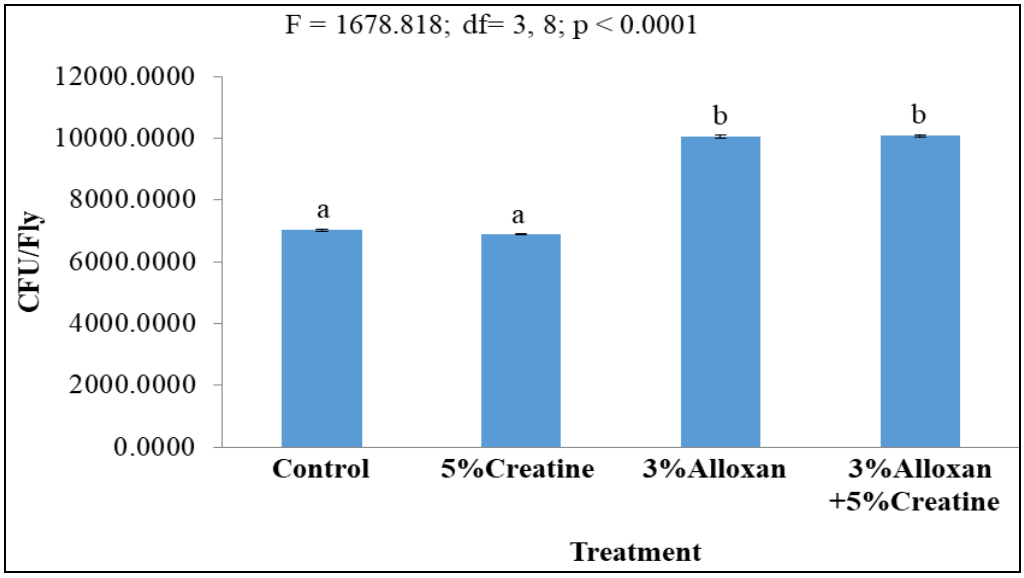
[Different alphabet on the superscript of bar graph indicates significance at p<0.05].

Fig 1: Density of *Acetobacter pomorum* in control and Alloxan treated flies of *D.melanogaster*.



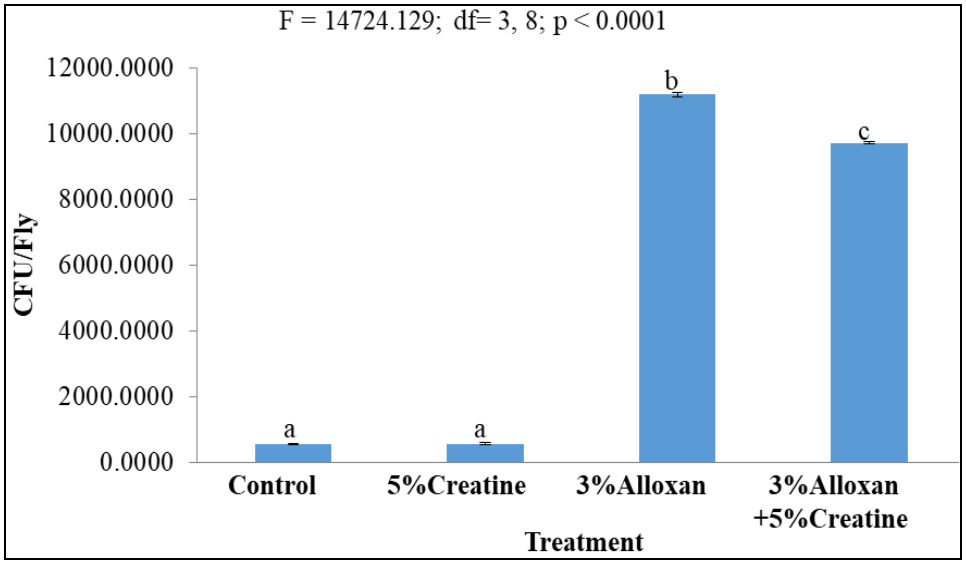
[Different alphabet on the superscript of bar graph indicates significance at p<0.05].

Fig 2: Density of *Acetobacter tropicalis* in control and Alloxan treated flies of *D.melanogaster*.



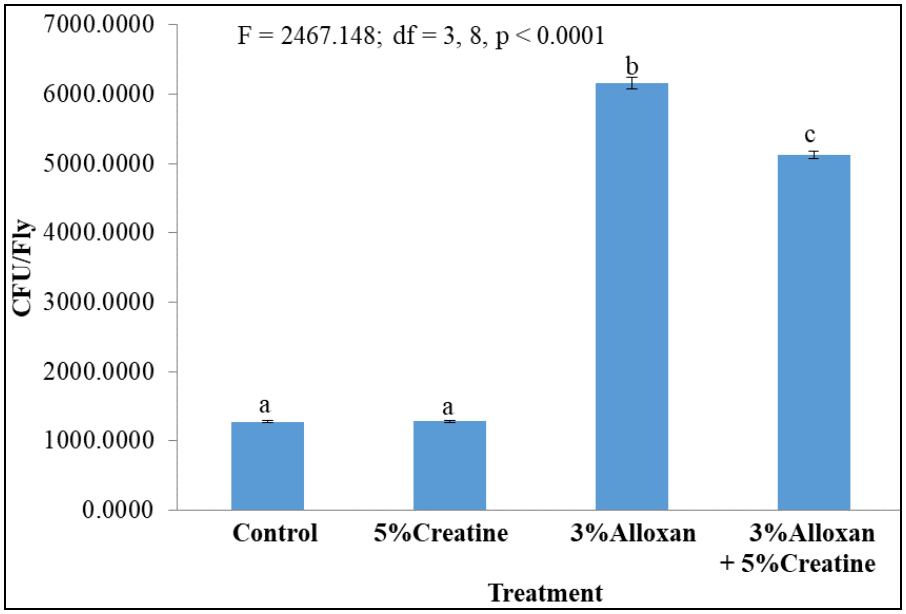
[Different alphabet on the superscript of bar graph indicates significance at p<0.05].

Fig 3: Density of *Lactobacillus brevis* in control and Alloxan treated flies of *D.melanogaster*.



[Different alphabet on the superscript of bar graph indicates significance at p<0.05].

Fig 4: Density of *Lactobacillus fructivorans* in control and Alloxan treated flies of *D.melanogaster*.



[Different alphabet on the superscript of bar graph indicates significance at p<0.05].

Fig 5: Density of *Lactobacillus plantarum* in control and Alloxan treated flies of *D.melanogaster*.

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Conflicts of interest

The authors do not have any conflict of interest regarding the publication of this manuscript.

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