Research Article

High sugar induced changes in gut microflora in *Drosophila melanogaster*: Protective role of *Gymnema sylvestre*

Hassan Rangegowda Harshavardhana, Mysore Siddaiah Krishna*

Drosophila Stock Centre, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore 570006, India

Received: 3 March 2019 Revised: 18 May 2019 Accepted: 24 May 2019

Abstract

Objective: Animal nutrition and gut microbiota are co influenced by each other in modulating host physiology. Present investigation has been undertaken to understand the effect of age and high sucrose diet on gut microflora in *D. melanogaster* and protective role of *Gymnema sylvestre (GS)* on toxic effects of high sucrose and altered gutmicrobial diversity. **Material and methods:** Flies of *Drosophila melanogaster* (w^{118}) was raised in control diet, 30% high sugar diet and treatment diet and further subjected for analysis of gut microbial diversity. **Results and conclusion:** It was noticed in *D. melanogaster* that gutmicrobiota vary with age and high sucrose diet. The investigation revealed that gut microbial flora in relation to host age corresponds to *Acetobacter* and *Lactobacillus* species. *A. pomorum, A. tropicalis, L. brevis, L. fructivorans*, and *L. plantarum*. The relative abundance of each of the above species varies in relation to host age, Density of *L. fructivorans* and *L. plantarum* was found to be lowest in young aged flies and it was increased with increasing fly age and highest density was noticed in old aged flies. Further *A. tropicalis* and *A. pomorum* were strongly represented in middle-aged flies. The present study also revealed that the species of *Lactobacillus* and *Acetobacter* showed diversity in relation to dietary sucrose. The density of *Lactobacillus brevis* and *L. plantarum* was high in flies fed with 30% sucrose compared to flies fed with control diet. Further flies fed with 30% sucrose along with leaf extract of *Gymnema sylvestre* have a significant influence on the gut microbiota. Thus these studies clearly suggest that host physiology changes with age and diet, in turn, it has a significant influence on resident gut microbial diversity in *D. melanogaster*. Further, *Gymnema sylvestre* feeding had an influence on bringing back to normal gut microbial diversity.

Keywords: Gut microflora, *Drosophila melanogaster*, *Gymnema sylvestre,* high sugar diet

Introduction

Phenotype of an organism is due to the cumulative effect of various factors such as genetic system, immediate environment and also the parental environment. The microbial communities found in an organism are known to influence on the host nutrition and energy balance which is also an important factor in shaping up the phenotype of an organism (Hooper et al., 2002). These gut microbes are involved in acquisition and allocation of nutrients in animal system through multiple ways a] they can consume ingested nutrients or provide supplementary nutrients to the host b] they can modify feeding and nutrients to the host c] they can change nutrient allocation patterns of the host by changing the nutrient sensing and signaling pathways in an organism (Backhed et al., 2004; Carcilli and sad, 2013; Goodman et al., 2009; Vijayakumar et al., 2010).

Earlier Studies in the understanding of the nutritional significance of microbiota in animals comes from untreated animals and germ-free animals (Gordon and Pesti, 1971; Smith et al., 2007; Yi and li, 2012). These studies have suggested that inclusive information to the nutritional significance of microorganisms to their animal host can only be obtained from comparative analysis using diets showing varied composition (Adams et al., 2010).

The microbial community found in an organism provides the host with a wide variety of metabolites which also includes vitamin and aminoacids (Chaston et al., 2016; Broderick et.al., 2014). Metazoan gut inhabits diverse microbial communities which contributes to the nutritional homeostasis of the host

Address for Corresponding Author:
Prof. Mysore Siddaiah Krishna
Drosophila Stock Centre, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore 570006
Email: drosokrish@gmail.com

DOI: https://doi.org/10.31024/ajpp.2019.5.6.4

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(Han et al., 2017; Hooper et al., 2002). Most of the disorders resulted due to metabolic irregularities such as obesity, type 2 diabetes, cardiovascular diseases, and bowel syndromes are also associated with the imbalance in the interactions between host and its microbiota (Blumberg et al., 2012; Khan et al., 2014). Today in the context of growing interest in unraveling the potential causes for the metabolic diseases nessacitates a model system to study the importance of gut microbiota and its interaction with the host for maintaining metabolic homeostasis. Presently there are only a few studies focusing on elucidating the effect of gut microbiome on host health. Understanding the microbial dynamics nowadays is made possible in the fruit fly *D. melanogaster* due to its similarity in neuroendocrine architecture with that of higher mammals especially humans (Erkosar et al., 2013; Wong et al., 2016).

Drosophila forms a simple model system to understand the host-microbiota relationship and organismal health because of its short life cycle and human-like metabolic traits (Erkosar and Leuier, 2014; Erkosar et al., 2013; Wong et al., 2016). Most of the earlier studies conducted on *Drosophila* for analyzing the composition and changes in gut microbiome under different situation have used the 16s amplicon method. Results obtained during these analyses showed that though *Drosophila* supports very simple bacterial colonies major contribution to the microbiome of *Drosophila* is contributed by four species, *Lactobacillaceae, Acetobactereace, Enterobactereaceae,* and *Enterococcaceae.* Despite variation in the density and composition of these species lactobacillus and acetobacter are more common (Ryu et al., 2008; Storelli et al., 2011; Wong et al., 2011). Most of the earlier studies conducted on *D. melanogaster* regarding gut microbiome concentrated on analyzing bacterial densities across various strains and also on the immune activity inside the fly and its associated commensal bacteria (Lhocine et al., 2008; Ren et al., 2007; Broderick et al., 2012). These studies have shown 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 be seen which accompanies changes in the microbiota, as a result, excessive proliferation of intestinal stem cells and intestinal dysplasia can be noticed. On the other hand, the germ-free flies showed stunted larval growth and took a long time for development and showed decreased insulin signaling which is promoted by commensal gut bacteria i.e., *Lactobacillus plantarum* and *Accetobacter pomorum*. These studies suggest that *D. melanogaster* forms an ideal model system to understand the interrelation between the host and its microbiota.

Therefore Metagenome analysis regarding changes in microbiota composition of the fly reared under altered nutritional condition especially under high sucrose diet was not analyzed. As there is growing awareness regarding diet composition and their associated problems. The study involving the alteration in gut microbiome as an indicator of altered diet and the treatment strategies involved are in much need. Present experiment also carries out a testing procedure of an ayurvedic plant *Gymnema sylvestre* leaf extract which is a potent ayurvedic formulation in counteracting toxic effect of high sucrose diet and altered gut microbial environment in *D. melanogaster*.

Material and methods

Fly stock and rearing

Experimental stock used in the present experiment was W^{118} strain flies expressing a t-GPH protein which were obtained from *Drosophila* stock center Bloomington, Indiana University. This strain was earlier used for understanding insulin resistance in many experiments (Coogan et al., 2013; Morris et al*.,* 2012). These flies were used to collect the eggs using Delcour's procedure (Delcour, 1969). Eggs (100) were transferred to each of the *Drosophila* culture bottle (250 ml) containing wheat cream agar medium and were maintained at 22± 1^0C and 70% RH with a 12:12 L: D photoperiod. Unmated males and virgin females were isolated within 3 hrs of their eclosion. These flies were used in the present experiment.

Effects of age on gut microbial diversity

Assignment of age classes

Longevity of unmated male and females of above strain was isolated within 3 hrs of their eclosion and 10 males/females were transferred separately into *Drosophila* culture vial (3x1cm) containing wheat cream agar. These flies were transferred into new vial once in a week and maintained them in the same laboratory condition until their death. Fifty replicates were made and this data obtained was used to calculate the mean longevity (number of days lived by each fly from the time of its eclosion until their death). Mean longevity of *D. melanogaster* flies was found to be 52±3 days. Therefore young, middle and old aged flies were selected as follows .1^{st} week flies were considered as young, 3^{rd}-week flies as middle-aged and 7^{th} week flies as old.

Twenty flies collected above (20 males and females together/and also separately) were transferred to quarter-pint bottles (250 ml) containing 5 ml of control diet (wheat cream agar media- Following this, flies were subjected to gut microbial analysis as mentioned below. Experiment was carried out separately for 1^{st} week,3^{rd} week and 7^{th}week flies.

Effect of high sucrose diet on gut microbiota

Collection of Plant Material

Gymnema sylvestre leaves were collected from Government Ayurvedic plant collection center, Mysuru,

India and authenticated by the Institute personnel. The plant leaves were dried (5% humidity, room temperature) in shade and powdered for further extraction.

Leaf extract preparation

The shade dried leaf powder of *GS* was subjected to the extraction process as follows. Exactly 50 g of the plant material was extracted with various organic solvents successively in the ascending order of polarity (hexane, dichloromethane, ethyl acetate, and methanol) in Soxhlet apparatus. In brief, 50 g of the plant material was initially extracted with 1L hexane at 60°C for 24 h, the residue obtained was completely dried and extracted with 1 L of dichloromethane for 24 h, and subsequently, the residue obtained was extracted with 1 L of ethyl acetate and followed by 1 L of methanol. Extracts were concentrated by Rotar evaporator under reduced pressure at room temperature, and 5mg of dried extract was used for the treatment**.**

Twenty flies (20 males and females together/and also separately) were transferred to quarter-pint bottles (250 ml) containing 5 ml of control diet (wheat cream agar media), 30% High sugar diet (HSD-wheat cream agar media+30% sucrose), treatment diet (30% HSD+*GS* extract, prepared by adding 5 mg of *GS* extract/litre of 30% HSD) for 10 days. Following this flies were subjected to gut microbial analysis**.**

Collection of Gut and Isolation of DNA

Midguts of experimental flies [1st week, 3rd week and 7th week flies as in first experiment and guts of flies reared in control diet (wheat cream agar media), 30% High sugar diet (HSD-wheat cream agar media+30% sucrose), treatment diet (30% HSD+GS extract, prepared by adding 5 mg of *GS* extract/litre of 30% HSD)as in experiment-2]were removed by dissecting the flies with 70% ethanol. Approximately 20 midguts were isolated per each experimental classes. Genomic DNA from midguts of experimental flies were extracted separately using the QIAamp DNA Mini Kit (Qiagen, 51304). For this, experimental flies midgutwere 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, 200µL ethanol was added and the 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

For sequencing of the *Drosophila melanogaster* gut microbiome and identification of major 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. Preliminary experiments confirmed that the primers generated no 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®Safe (Invitrogen), and their identities were confirmed by Sanger sequencing.

Measurement of bacterial loads

Microbial seed amount was quantified for experimental flies by either O.D. measures or dilution plating, and the microbial load was quantified by serial dilution plating of isolated guts. MRS agar was used for quantifying all microbes except for the *A. pomorum* strains, which were 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)/ml (a colony-forming unit is a unit 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.

Statistical analysis

One way ANNOVA followed by tukeys post hoc test carried out on the above data showed significant variation in gut microbial diversity between age classes in all the gut microbial species identified. Tukeys post hoc test showed that old aged flies had significantly greater number of CFU's of *L. brevis, L. fructivorans* and *L. plantarum* compared to middle and young aged flies. Whereas greater number of CFU's of *A. tropicalis* and *A. pomorum*were noted in middle aged flies than young and old flies.

Results and discussion

Host diet and age are two important factors known to affect the resident microbiota of an organism. In the present investigation host age-related changes in gut microbial diversity has been studied in w^{118} strain of *Drosophila*

melanogaster. Prior to experiment as these flies have been standardized by in rearing under wheat cream agar media and further these flies were used for the analysis.

Further, all the flies were reared under same condition and food, flies of different age classes (1st week, 3rdweek, and 7th week) were subjected for gut microbial diversity analysis to understand how host physiology changing with age has an effect on resident microbiota.

In the present experiment, five microbial species were identified using diagnostic primers listed in table 1, and further each of the identified species were quantified through CFU's. It was noticed from the figure 1 that gut microbial flora in relation to hostage corresponds to *Acetobacter* and *Lactobacillus* species. *A. pomorum, A. tropicalis, L.brevis, L. fructivorans*, and *L. plantarum*. The relative abundance of each of the above species varies in relation to host age. The density of *L. brevis, L. fructivorans* and *L. plantarum* was found to be lowest in young aged flies and it was increased with increasing flies age and highest density was noticed in old aged flies (7th-week fly). Further *A. tropicalis* and *A. pomorum* were strongly represented in middle-aged flies (3rdweek). This result clearly suggests that host physiology (developmental physiology) changes with age which has a significant influence on resident gut microbial diversity.

Our result also confirms the earlier studies of host age-related changes in gut microbial diversity in *D. melanogaster* (Han et al., 2017). In the present study, flies were reared under the same condition and media therefore observed variation in gut microbial diversity resulted in physiological changes noticed with increasing host age. These changes in gut microbial diversity with age also indicate metabolic changes in the host organism.

It was widely recognized that the resident microbiota plays an important role in animal nutrition (Flint et al., 2012; Karasov and Douglas, 2013). Microbiota involved in the acquisition and allocation of animal nutrients play a key role in shaping the nutritional status of an animal. These microorganisms either consume ingested nutrient or provide supplementary nutrient to host thereby they can alter feeding and nutrient assimilation rate.

In the present study toxic effects of high sucrose on gut microbial diversity in *D. melanogster* and potential benefit of leaf extract of *Gymnema sylvestre* fed with high sucrose diet in *D. melanogaster* have been investigated. Figure 2 revealed that species of *Lactobacillus* showed diversity in relation to dietary sucrose.The density of *Lactobacillus brevis* and *Lactobacillus fructivorans* was high in flies fed with 30% sucrose compared to flies fed with control diet. Further flies fed with 30% sucrose along with leaf extract of *Gymnema sylvestre* have a significant influence on the gutmicrobiota which caused a moderate reversal of increased bacterial species. This suggests that gut microbial diversity has profoundly affected by host diet.

The reads obtained by pyrosequencing each sample were assigned to their respective OTUs and then analyzed for microbiota richness and evenness via determination of their respective indices, which showed that microbial communities varied along with age and diet (Table 2).

One way ANNOVA followed by tukey's post hoc test carried out on the above data showed significant variation in gut microbial diversity of *Lactobacilus* between flies fed with experimental diets (Control, 30% HSD and 30%

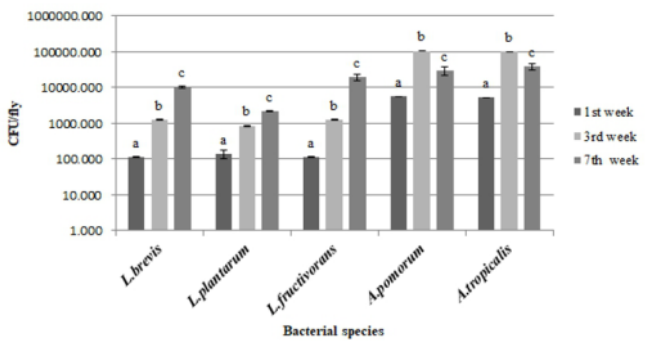

Figure 1. Age related changes in five major gut microbial species of *Drosophila melanogaster* [*L. brevis*; f=10855.054,df=2,27, P<0.0001: *L. plantarum*; f=327.727, df=2,27, P<0.0001: *L. fructivorans*; f=4.312, df=2,27, P<0.0001, *A. pomorum*; f=13816.457,df=2,27, P<0.0001: *A. tropicalis*; f=966900.627, df=2,27, P<0.0001]

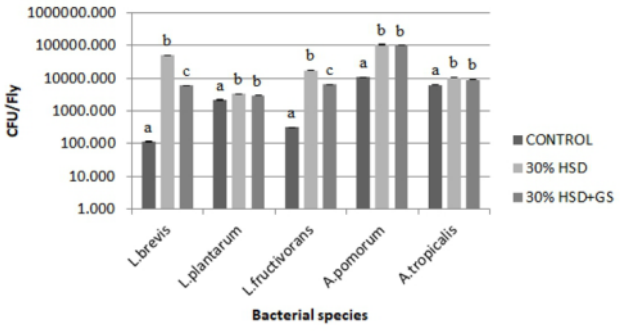

Figure 2. Effect of *Gymnema sylvestre* on in five major gut microbial species of *Drosophila melanogaster* under high sugar diet. [*L. brevis*; f=13073.060, df=2,27, P<0.0001: *L. plantarum*; f=20065.891, df=2,27, P<0.0001: *L. fructivorans*; f=6.012, df=2,27, P<0.05: *A. pomorum*; f=11613.325, df=2,27, P<0.0001: *A. tropicalis*; f=54722.984, df=2,27, P<0.0001]

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'
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. Richness and evenness estimation of the microbiota in each of the fly samples. Diversity estimations were obtained following normalization of OTU's.

Strain	Age/Nutrient Condition	OTU's	Chao1	Shannon	Evenness
W^{118} (Across Age)	1st week	67	59	2.68	0.66
	3rd week	63	74	3.26	0.76
	7 thweek	72	69	3.17	0.80
W^{118} HSD	Normal	58	65	2.12	0.77
	Hyperglycemic	61	63	3.12	0.71
	HSD+GS	53	74	3.36	0.78

HSD+*GS*). All other bacterial phyla showed no significant variation under *Gymnema sylvestre* treatment. Tukey's test showed that HSD flies had significantly greater number of CFU's of *Lactobacillus brevis, Lactobacillus fructivorans* compared to control and HSD+*GS* treated flies.

Further the observed results can be explained that the gut microbiota is either beneficial (promotes host performance) or being (no desirable effect on host performance) but not deleterious to *Drosophila* reared on high sucrose diet. The implications are two-fold first; host and microbiota do not compete for dietary nutrients which would be an indication of having a lower density of microbiota, 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. Second *Drosophila* is not dependent on its microbiota for the normal physiological function which would be revealed as the superior performance of conventional *Drosophila* on all diets. Instead, the microbiota particularly promoted *Drosophila* performance on diets of low or unbalanced nutrient content, indicating that the association has a nutritional basis.

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. Further feeding of *Gymnema sylvestre* leaf extract has a profound beneficial effect in treating the toxic effects of high sucrose diet in *D. melanogaster*.

Acknowledgments

The authors are grateful to the Chairman of the Department of Studies in Zoology, University of Mysore, and the *Drosophila* Stock Center, University of Mysore for providing facilities to carry out this work. We also thank to UGC-SAP CAS Phase 1 for financial support.

Conflicts of interest

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

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