International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 08-08-2022, Accepted: 24-08-2022, Published: 10-09-2022
Volume 7, Issue 9, 2022, Page No. 98-107

Altitudinal and seasonal variation on gut microbial diversity of three sympatric *Drosophila* species in Chamundi hills

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Abstract
The microbial communities that live inside of them have an impact on many elements of animal ecology and physiology, which in turn has an impact on the health, survival, and reproductive fitness of the organisms. Gut bacterial diversity within an organism is influenced by both biotic and abiotic causes. In nature, seasonal and altitudinal change are significant forces that also influence the variety of the bacteria in the gut. Determining the effects of altitude and seasonal fluctuation on gut microbial diversity in three sympatric species of *Drosophila*, including *D. malerkotliana*, *D. bipectinata*, and *D. nasuta* in Chamundi Hill, is the goal of the current study. Here, using 16S rRNA gene sequencing, we examined the quantitative changes in intestinal microbiota in three sympatric species of *Drosophila* collected at various altitudes and seasons. Two species from the *Acetobacter* species group, *Acetobacter pomorum* and *Acetobacter tropicalis*, three species, *Lactobacillus brevis*, *Lactobacillus fructivorans*, and *Lactobacillus plantarum*, found to belong to the *Lactobacillus* species were found in all three altitudes and seasons examined in the current study. The relative abundance of each of the aforementioned microbial species fluctuates significantly depending on height and season, according to further investigation. Further relative abundance of gut bacteria also varied between sympatric Drosophila species in different altitudes and seasons. Except *A. pomorum* all other gut bacteria were found to be significantly greater at low altitude compared to middle and high altitudes. Among the seasons, the density of *L. fructivorans, A. tropicalis* and *A. pomorum* were found to be greatest in monsoon season followed by premonsoon and post monsoon seasons. Thus theseresults imply that there is a significant effect of altitude and season on the abundance of gut bacteria in three sympatric *Drosophila* species of Chamundi hills.

Keywords: gut, bacteria, season, altitude, *Drosophila*

Introduction

An organism's gut microbiota is crucial for food digestion, absorptionand assimilation, which has an impact on the physiology, health, survival, and reproduction of the organism (Pi *et. al.*, 2017) [30]. Additionally, it has an impact on animal ecology and evolution (McFall-Ngai *et.al.*, 2013) [23]. Studies have also shown that the diversity of gut microbes is impacted by altitudinal variation, which also affects digestion and absorption. For example, acute hypoxia exposure showed that air pressure is a significant exogenous factor that significantly affects the composition of intestinal microbiota (Maity *et. al.*, 2013; Han *et. al.*, 2016) [24, 14]. Altitude has an impact on the composition of the gut microbiota in humans and pikas living at various altitudes, according to comparative research (Li and Zhao, 2015; Li, *et. al.*, 2016) [21, 20].

According to Moronidis (2014) [26], the study of seasonal dynamics in insect populations focuses primarily on population density, structure, regularities of distribution, and spatial dynamic changes. It can quantify the relationship between various influencing elements and the population change rule. These variables are split into two categories, biotic and abiotic, respectively (Orsted *et. al.*, 2017) [29]. Conspecifics, food supplies, and natural enemies make up the majority of the biotic variables. Temperature, precipitation, wind, humidity, light, and pesticides are examples of the abiotic variables, often known as physical elements. Temperature comes out as one of the most important abiotic elements in insect seasonal dynamics (Mironidis, 2014; Sala *et.al.*, 2000) [26, 32]. Seasonal variations can have an impact on the diversity of gut bacteria and the availability of food (Hahn and Denlinger, 2007; Oslen and Duman, 1997) [13, 27].

Insects that overwinter in temperate climates experience significant seasonal changes in their eating (Hahn and Denlinger, 2007) [13], gut contents (Olsen and Duman, 1997) [27], immunology (Ferguson and Sinclair, 2017) [10], and physiology (Denlinger and Lee, 2010) [6]. Given that the host's physiological condition determines the microbiome's composition (Douglas, 2015) [7], these seasonal changes in host physiology are probably also going to have an impact on the microbiome's makeup. Additionally, because diet may affect the gut microbe (Franzini *et. al.*, 2016) [11], seasonal changes in food may affect the insect gut microbe (Ludvigsen *et. al.*, 2015) [23]. However, in the context of cold temperatures and overwintering, nothing is known about the seasonality of insect gut microbiomes.

Both culture-dependent and culture-independent techniques have been used to examine the microbiota in the insect gut (McFall-Ngai *et. al.*, 2013) [25]. However, depending on the circumstances and the methodologies employed, culture-dependent procedures frequently yield skewed results. A clearer and more complete image of bacterial communities has been produced by culture-independent molecular ecology methods based on study of the 16S rRNA gene, which has significantly improved our understanding of the microbes that inhabit the bellies of insects.

Drosophila's neuroendocrine architecture resembles that of higher mammals, particularly humans, making it a useful animal model for studying the diversity of microbes

(Harshavardhana and Krishna, 2019) [15]. Due to its brief life cycle and metabolic characteristics that are similar to those of humans, it is also an easy model system to comprehend the relationship between the host microbiota and organism health (Harshavardhana and Krishna, 2019) [15]. Due to the low diversity of its gut microbiota, which is frequently dominated by as few as five species from the easily cultivable genera *Acetobacter and Lactobacillus* (Wong *et al.,* 2011; Chandler *et. al.,* 2011) [38, 5], the genus Drosophila serves as an important model for the study of microbiomes.

Research on the diversity of microorganisms in Drosophila using the 16s amplicon has revealed the presence of four bacterial taxa, including *Lactobacillaceae, Acetobactereace, Enterobactereaceae,* and *Enterococcaceae.* Despite differences in these organisms' density and makeup, *Lactobacillus* and *Acetobacter* are more prevalent (Harshavardhana and Krishna, 2019) [15]. Gut bacteria in *Drosophila* have the potential to influence their dietary needs, sexual behaviour, development, longevity, and gut architecture (Wong *et. al.,* 2011; Anitha and Krishna, 2021) [38, 2]

The variety of gut microbes in *Drosophila* has demonstrated that there is fluctuation in the abundance of some microbial species between different strains and also depending on immunological activity (Wong *et. al.,* 2011) [38]. These investigations demonstrated that pathogenic bacteria suppression is related to the preservation of innate immune homeostasis. Additionally, as people age, alterations in innate immune homeostasis may potentially be linked to microbiome changes. Additional *Drosophila* research revealed that age and diet are two significant factors that are known to alter the variety of gut microbes in this species (Harshavardhana and Krishna, 2019; Anitha and Krishna, 2021) [2, 15]. They discovered five different types of gut bacteria, including *A. Pomorum A. tropicalis L. brevis* Although *L. fructivorans, L. plantarum,* are found in *Drosophila,* the relative abundance of each of these species varies depending on the host's age and diet. Further they also noticed that the density of *L. plantarum* was found to be highest in old aged flies and to be lowest in young aged flies. However, the effects of altitude and season on gut bacterial diversity in *Drosophila* species have not been researched. In nature, altitude and season are two important factors that are known to affect biodiversity, population density, population fluctuations, intra and inter species competitions, temperature, precipitation, humidity, photoperiod, etc. Determining how altitude and season affect gut microbial diversity in three sympatric species of Drosophila in the Chamundi Hills is the goal of the current study.

Materials and methods

From January 2021 to December 2021, three sympatric species of *Drosophila,* including *D. bipectinata, D. malerkotliana,* and *D. nasuta,* were captured from Chamundi hills primarily using bottle trapping and net sweeping techniques at various altitudes (650m, 800m, 950m), seasons (premonsoon, monsoon, postmonsoon), and locations (wild locality of Chamundi hills). The locations of the chosen collection points were 25° 11' N latitude and 94° 55' E longitude.

Vegetation collection site at 650 metre: *Mango orchards, Acacia catechu, Anacardiumoccidentale,* and other trees encircled the base of the hill. *Celastruspaniculata, Cipadessabaccifera, Clematis trifolia, Dalbergiapaniculata, Dioscoreapentaphylla, Bombaxceiba, Breynearestusa, Cassia spectabilis, Ficusbengalensis, Ficusreligiosa, species of Glyrecidia, Exercise sylvestres, Ichnocarpusfrutescens, Lantana camera, Hibiscus malvaTectonagrandis, Sidaretusa, Phyllanthus species, Tamarindusindica, Thunbergia species, Pongamiaglabra, and several shrubs, including cacti.*

Vegetation found at 800 metres: There were a number of significant plants discovered in these areas, including *Albizzia amara, Andrographis serpellifolia, Argyria* species, *Bignonia species, Breynearestusa, Bridalia species, Cassia fistula, Cassineglauca, Eucalyptus grandis, Garcinia species, Lantana camera, Phyllanthus microphylla, Sida rhombifolia, Terminal Acacia catechu, Anacardium occidentale, Autocarpus integrifolia,*

Vegetation found at 950 metres: There were a number of significant plants discovered in these areas, including *Albizzia amara, Andrographis serpellifolia, Argyria* species, *Bignonia species, Breynearestusa, Bridalia species, Cassia fistula, Cassineglauca, Eucalyptus grandis, Garcinia species, Lantana camera, Phyllanthus microphylla, Sida rhombifolia, Terminal Acacia catechu, Anacardium occidentale, Autocarpus integrifolia,* species of jasmine, *Camera of lantana, Murraya paniculata, Tamarindus indica, Zizipusjujuba, Leusaspera, Mallotus philippensis,* and *Tamarindus indica.*

To explore the quantitative changes in gut microbes at various altitudes and seasons, progeny of three sympatric species of *Drosophila,* including *D. malerkotliana, D. bipectinata,* and *D. nasuta,* were obtained from isofemale lines was used.

DNA Isolation and Gut Microbe Gathering

Using 70% ethanol, the midguts of *D. malerkotliana, D. bipectinata,* and *D. nasuta* were removed. Using the QIA amp DNA micro kit, DNA was extracted from twenty mid guts taken from each species (Qiagen, 51304). These midguts were homogenised in 180 L of ATL buffer with 0.5 percent reagent DX for foam minimization after being externally disinfected with 70 percent ethanol (Kimble TMK ontesTM Pellet Pestle, 749540-0000). For the next step, samples were treated with 20 L proteinase K SOLUTION and incubated for 30 min. at 56°C with 650 rpm of shaking. Additional samples were homogenised with glass beads (425-600 m, Sigma Aldrich, G8772-100G) to lyse them in a rapid prep FP120 machine (Bio 101 Savant), which was then incubated for an additional 60 min at 56 °C. RNase A (Qiagen, 19101) was added for RNA digestion in order to extract RNA, and the sample was then incubated for 2 minutes at room temperature. Following the addition of 200 L of ethanol and the spinning of the samples, the washing and elution procedures were carried out in accordance with the manufacturer's instructions. Additional samples were concentrated by precipitation of sodium acetate.

Pyro Sequencing of 16s rRNA for Bacterial Species Identification

Axon-specific 16S rRNA gene primers (Table 1) and distinctive areas discovered from alignments of entire 16S rRNA gene were used to determine the primary gut microbial diversity of *D. malerkotliana, /D. bipectinate /D. nasuta.*

Primer3 software was used to detect the rRNA gene sequences *of Acetobacter pomorum, Acetobacter tropicalis, Lactobacillus brevis, Lactobacillus fructivorans*, and *Lactobacillus plantarum*. Initial research has shown that the primers produced measurable cross-species amplification. 35 cycles of PCR at 65°C annealing temperature were carried out as before. Using 1 percent agarose gel electrophoresis, PCR products were separated, detected with SYBR (Invitrogen), and their identities were verified through Sanger sequencing.

Calculating Bacterial Loads

With the exception of the gut bacteria, *Acetobacter pomorum* was measured on mannitol plates, all remaining four gut bacteria from sympatric species such *D. malerkotliana*, *D. bipectinata*, *D. nasuta* collected from various elevations and seasons of Chamundi hills were quantified using MRS agar. Guts were placed on either MRS or mannitol agar plates to measure microbial growth. Colony forming units (CFUs), which are used to quantify the number of live bacteria in a sample and are defined as having the capacity to reproduce by binary fission under controlled conditions, were used to calculate the viable bacterial load. In contrast to microscopic examination, counting using colony forming units necessitates cultivating the bacteria and counting only viable cells. Colony forming units, which were written in logarithmic notation, are used to compute abundance.

Table 1: Diagnostic primers used for identification of gut 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 gut bacteria in sympatric Drosophila species. Diversity estimations were obtained following normalization of OTU'S

	Sympatric species		
	D. malerkotliana	***D. bipectinata***	***D. nasuta***
OTU'S	54	61	65
Chaol	61	66	67
Shannon	2.05	2.75	3.10
Evennes	0.81	0.79	0.81

Table 3: Two ways ANOVA on Altitudinal variation in gut bacterial specie in sympatric Drosophila species at Chamundi hills

Gut Bacterial species	Source	Mean sum of square	df	Sum of square	F-value
Lactobacillus brevis	Species	45661722.222	2	22830861.111	18.707^{***}
	Altitude	17008977388.889	2	8504488694.444	6968.266^{***}
	Species*altitude	18108111.111	4	4527027.778	3.709^{*}
	Error	98857250.000	81	1220459.877	
	Total	40040722500.000	90		
Lactobacillus plantarum	Species	71661.667	2	35830.833	1.297^{NS}
	Altitude	946681.667	2	473340.833	17.134^{***}
	Species*altitude	157516.667	4	39379.167	1.425^{NS}
	Error	2237650.000	81	27625.309	
	Total	202597200.000	90		
Lactobacillus fructivorans	Species	6589555.556	2	3294777.778	5.169^{*}
	Altitude	1017212722.222	2	508606361.111	797.922^{***}
	Species*altitude	4290111.111	4	1072527.778	1.683^{NS}
	Error	51630500.000	81	637413.580	
	Total	5326070000.000	90		
Acetobacter pomorum	Species	111205555.556	2	55602777.778	5.872^{*}

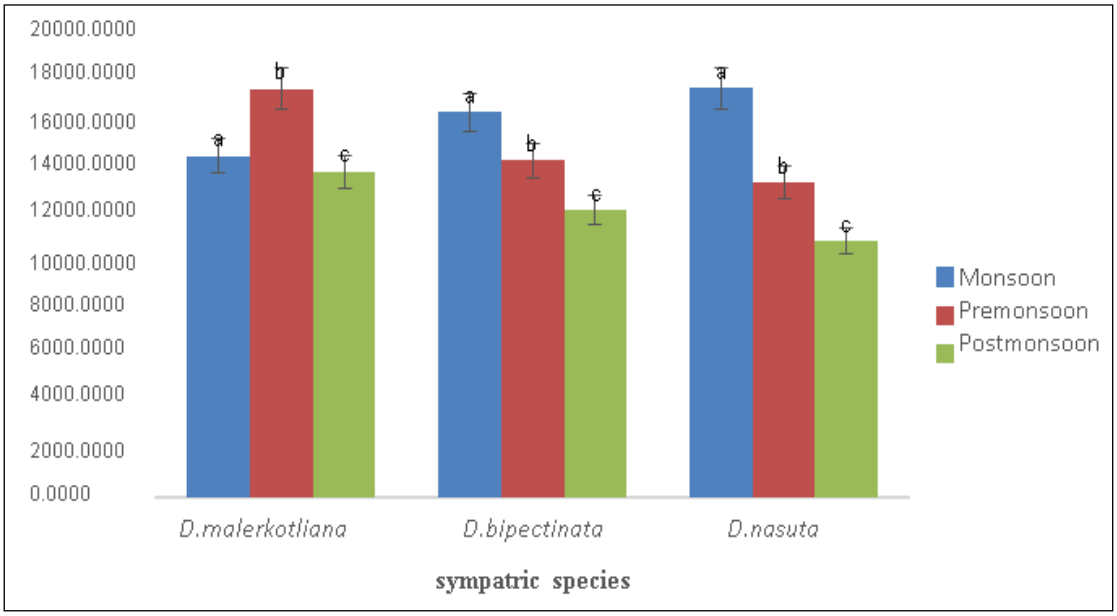
	Altitude	33901422222.222	2	16950711111.111	1790.160***
	Species*altitude	350011111.111	4	87502777.778	9.241**
	Error	766975000.000	81	9468827.160	
	Total	190049125000.000	90		
Acetobacter tropicalis	Species	29930055.556	2	14965027.778	19.391***
	Altitude	180473388.889	2	90236694.444	116.926***
	Species*altitude	7931777.778	4	1982944.444	2.569NS
	Error	62511000.000	81	771740.741	
	Total	10612785000.000	90		

***significant at 0.0001 level; **significant at 0.001; *significant at 0.05 level; NS=non-significant

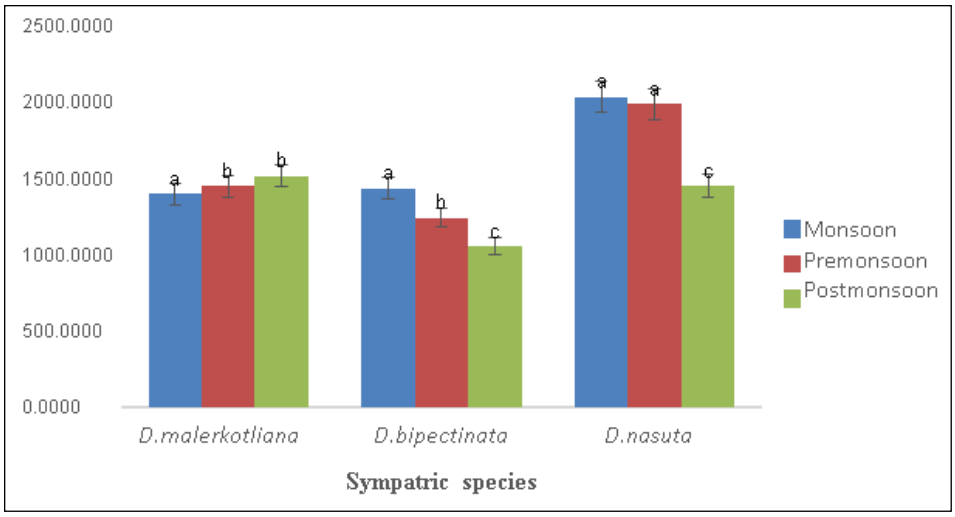
Table 4: Two ways ANOVA on Seasonal variation in gut bacterial specie in sympatric Drosophila species at Chamundi hills

Lactobacillus brevis	Species	4356172287.142	2	228861.111	16.73***
	Altitude	15008972388.889	2	565042388694.444	5958.266***
	Species*altitude	145108111.111	4	4527027.778	3.24*
	Error	88353250.000	81	1430459.877	
	Total	40245622500.000	90		
Lactobacillus plantarum	Species	61341.67	2	34330.833	1.297NS
	Altitude	867681.667	2	4745440.833	17.134***
	Species*altitude	257516.667	4	34279.167	1.425 NS
	Error	214650.000	81	23425.309	
	Total	2024537200.000	90		
Lactobacillus fructivorans	Species	5534555.556	2	22453777.778	5.169*
	Altitude	1017352722.222	2	4086065641.111	797.922***
	Species*altitude	42954631.111	4	1074427.778	1.683NS
	Error	41686720.000	81	536313.580	
	Total	434636000.000	90		
Acetobacter pomorum	Species	103425555.556	2	55602777.778	6.564**
	Altitude	33901422222.222	2	16950711111.111	1532.160***
	Species*altitude	350011111.111	4	87502777.778	9.132**
	Error	766975000.000	81	9468827.160	
	Total				
Acetobacter tropicalis	Species	2932555.556	2	14965027.778	17.179***
	Altitude	18043378.89	2	90236694.444	114.29***
	Species*altitude	8933327.778	4	1982944.444	2.22NS
	Error	62511000.000	81	771740.741	
	Total	1061453000.000	90		

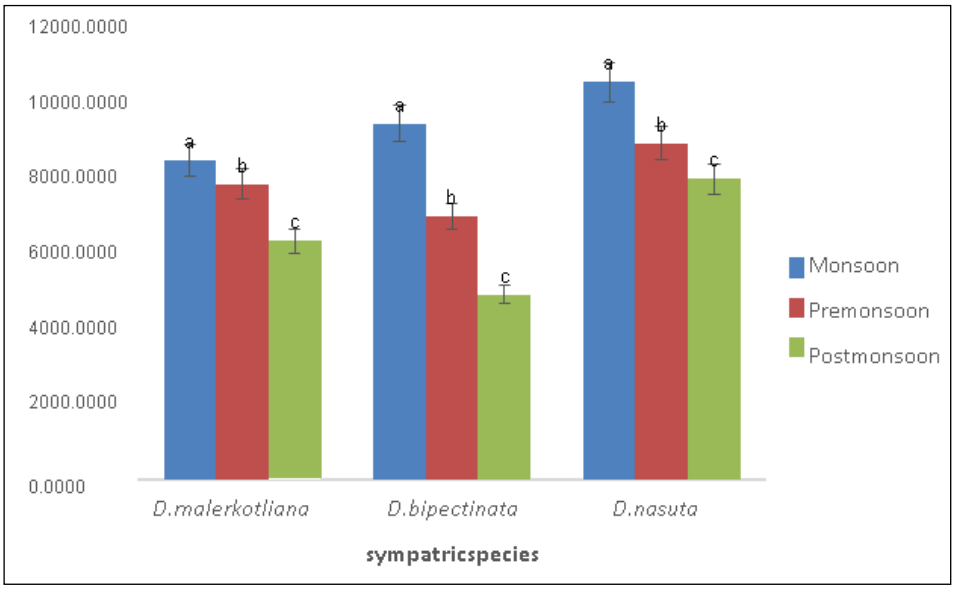
***significant at 0.0001 level; **significant at 0.001; *significant at 0.05 level; NS=non-significant



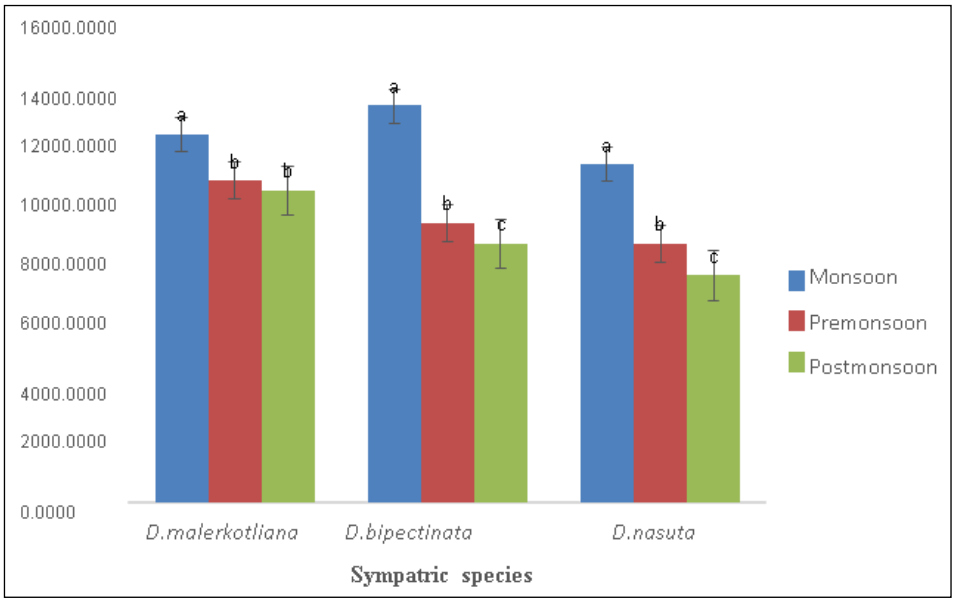
a. *Lactobacillus brevis*



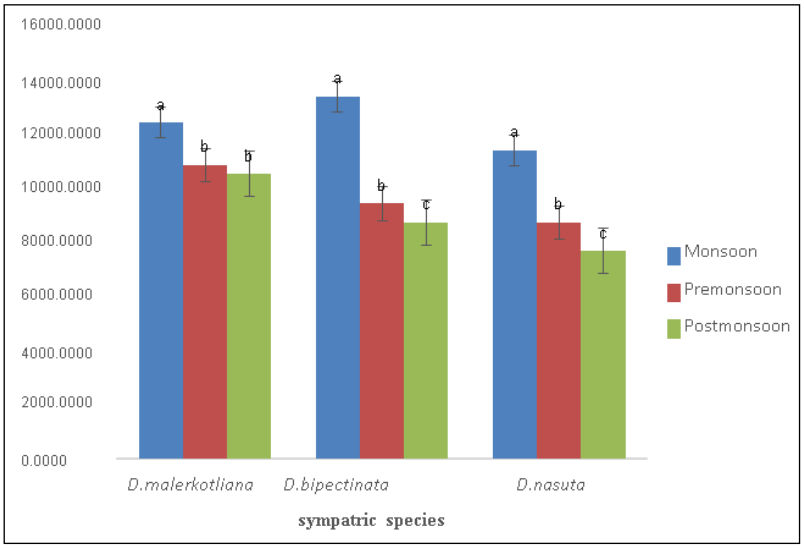
b. *Lactobacillus planktarum*



c. *Lactobacillus fructivorans*



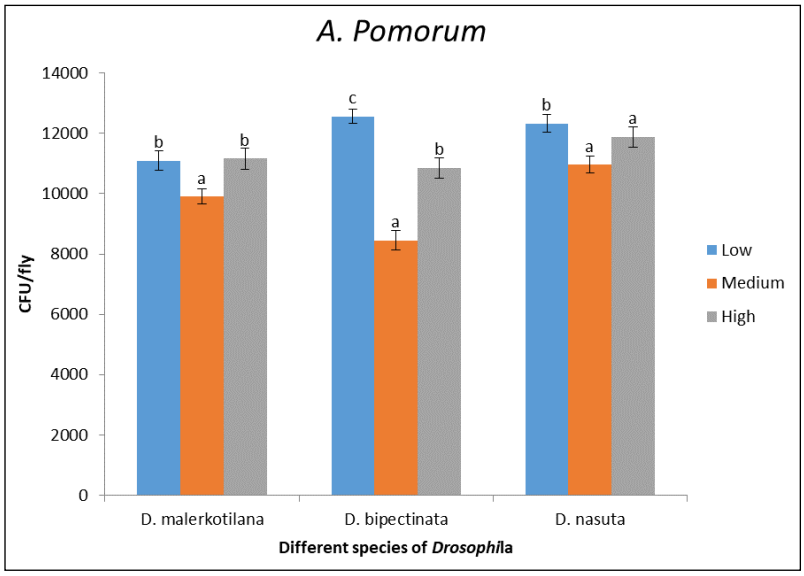
d. *Acetobacter pomorum*



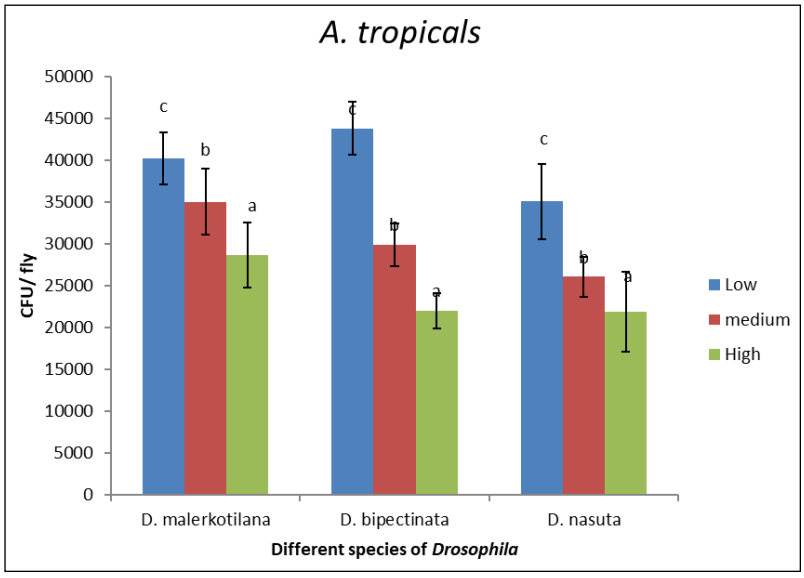
e. *Acetobacter tropicalis*

Fig-1a-e: Seasonal variation on density of gut bacteria in sympatric Drosophila species at Chamundi hills

Different letters on the bar graph indicates significance difference at 0.05 level by Tukey's post hoc test



a



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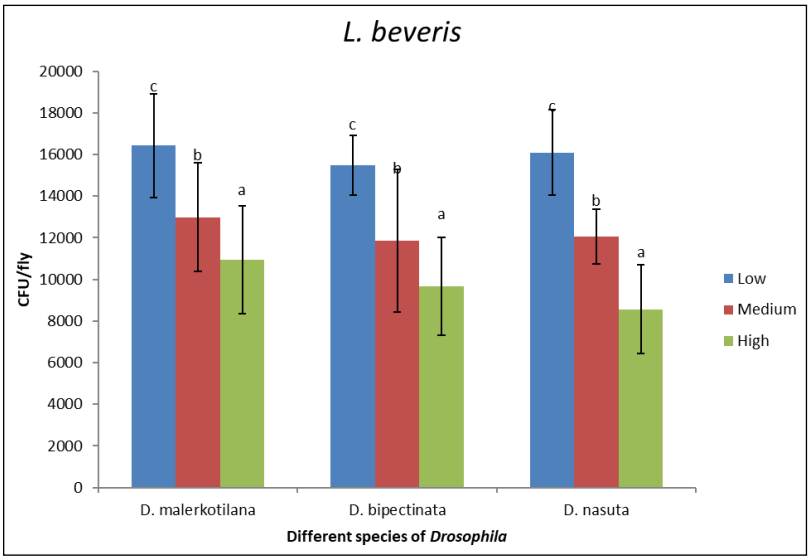
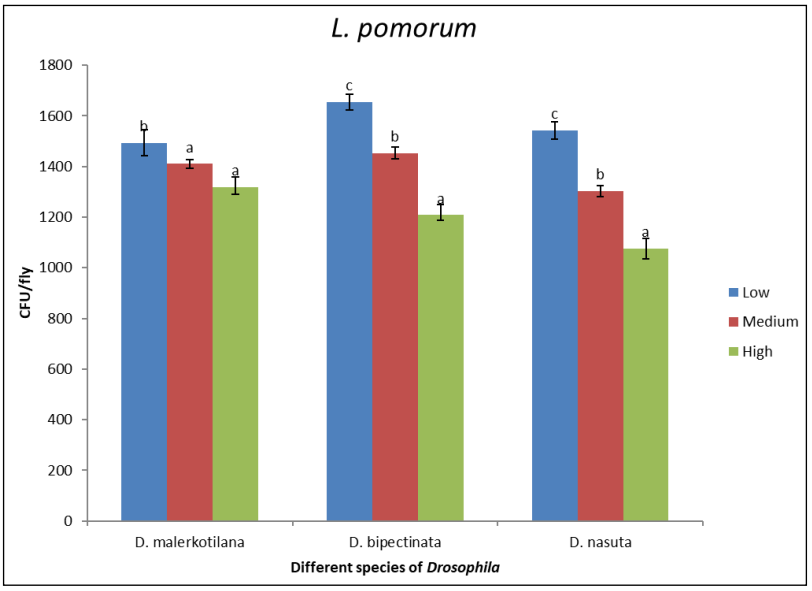
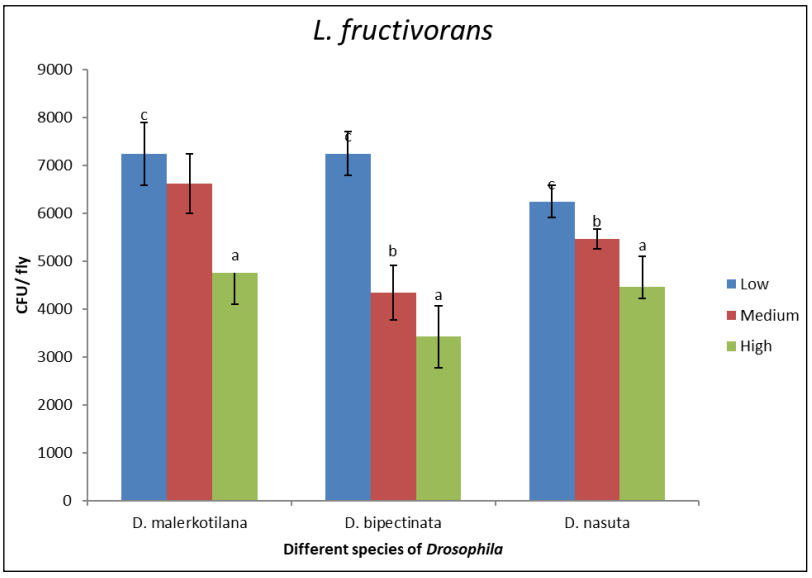


c



d.



e

Fig-1a-e: Altitudinal variation [Low-650m: Medium-800m: High-950m] on density of gut bacteria in sympatric Drosophila species at Chamundi hills.

Results and Discussion

The most convincing research on gut microbial diversity has demonstrated how host nutrition and environmental variables can impact the gut microbial diversity in *Drosophila* species (Hasegawa *et.al.,* 2015; Hill-Burns *et.al.,* 2017; Scheperjans *et. al.,* 2015)[16,17, 33] . To understand the impact of various altitudes and seasons on physiology and, in turn, its impact on resident microbiota, flies collected from different altitudes and seasons of the Chamundi hills were exposed to quantitative variation in gut microbial analysis in the current study. The experiment also revealed that a total of five microbial species were identified using the diagnostic primers indicated in Table 1, and each of the discovered species was quantified using CFUs (Table 2; Figure 1a-e and Figure 2a-e). Five different gut microbes have been found in all three sympatric species of Drosophila, regardless of altitude or season. *Acetobacter pomorum* and *Acetobacter tropicalis*, two of the five gut bacteria, and *Lactobacillus brevis*, *Lactobacillus fructivorans*, and *Lactobacillus plantarum*, the other three species, all belong to the *Lactobacillus* species group. Except *A. pomorum* all other gut bacteria were found to be significantly greater at low altitude compared to middle and high altitudes. Among the seasons, the density of *L. fructivorans*, *A. tropicalis* and *A. pomorum* were found to be highest in monsoon season followed by premonsoon and post monsoon seasons. Mean data subjected to two way Anova followed by Tuckey's posthoc test showed significant variation in between seasons, between altitudes, between sympatric Drosophila species and interaction between season and sympatric species, between season and sympatric species (Figure 1a-e and Figure 2a-e and Table 3 and 4). These findings imply a major impact of altitudinal and seasonal variation on the gut microbial diversity in three closely related *Drosophila* species. Changes in temperature, precipitation, partial pressure of atmospheric gases, atmospheric turbulence, wind speed, and radiation input, including short-wave ultra-violet radiation at various wavelengths, that take place as one ascends an altitudinal transect, can all be attributed to the observed phenomenon. Additionally, changes in territory and climate warming may be the cause of variations in gut microbial abundance at different elevations. Other aspects to take into account include greater competitive pressure, increased physiological stress, and a decrease in food supply (Alwyn *et. al.,* 2019)[1]. Additionally, there is an abundance of flora, food in the form of rotting fruits, and a nice environment that is beneficial to the growth of flies at lower elevations. Further explanations for the reported results include the possibility that altitudinal and seasonal cycles on host physiology are responsible for the gut microbial diversity in the current study. At various periods of the year, there were noticeable variations in the relative gut bacterial density of three sympatric *Drosophila* species of Chamundi hill. At all altitudes, the pre-monsoon season had the lowest density of gut microbes while the monsoon season had the highest. The increased density of flies during the monsoon season may be due to the availability of suitable food in the form of rotting fruits and the favourable climate for fly reproduction. This theory is supported by the fact that a lot of the local flora has fruiting seasons that coincide with the monsoon season. The monsoon season is characterised by heavy downpours, chilly air, and increased humidity. As the monsoon season comes to an end, rainfall and humidity drop, creating a dry climate. Population density starts to drop during the post-monsoon season and reaches a minimum during the pre-monsoon season. As a result, variations in the size of the *Drosophila* population may be connected to the rainy and dry seasons.

Additionally, our finding supports past laboratory investigations of host diet-related changes in gut bacteria in *Drosophila* (Hasegawa *et. al.,* 2015; Hill-Burns *et.al*., 2017; Scheperjans *et. al.,* 2015)[16,17, 33]. Diet-related variations in the diversity of gut bacteria also indicate alterations in the host organism's metabolism. An essential role for the resident microbiota in animal nutrition has been discovered by earlier investigations on the diversity of bacteria in the gut (Le chatelier *et. al*., 2013; Ursell *et. al.,* 2012; Lloyd-price *et. al.,* 2017; Swann *et. al.,* 2011)[18, 35, 22, 34]. This is due to the fact that the gut bacteria involved in acquiring and distributing animal nutrients had a significant impact on the nutritional makeup of an animal. Additionally, these gut microbes may consume ingested nutrients or give the host additional nutrients, allowing them to monitor feeding and nutrient absorption rates. Anitha and Krishna (2021) who while studying in *D.melanogaster* have also found that quantity of carbohydrate and protein in the diet also had significant influence on the relative density of five gut bacteria in *D.melanogaster*. They also pointed out that the ratio of carbohydrate and protein in the diet too affects gut bacterial density. Studies of Harshavardana and Krishna (2021) in *D.melanogaster* also showed significant influence of sucrose concentration in the diet on gut bacterial density .Thus these studies suggests that significant influence of quantity of nutrients available in the food affects gut bacterial density in species of Drosophila.

The results of the present study were attributed to two factors: first, the host and microbiota do not compete for dietary nutrients available at different altitudes and seasons, which could be a sign of having a lower density of gut microbes. This suggests that the various dietary nutrients in different altitudes and seasons are either not utilised by both host and microbiota, or they are abundant enough that their consumption by microbiota does not limit host Further evidence that the link has a nutritional basis comes from the influence of altitude and seasonal variation on the gut microbiota, which in turn affected how well drosophila performed on diets with low or imbalanced nutrient content. Additionally, it was shown that there are probably a number of interconnected pathways that affect how the microbiota and host metabolism interact. Additionally, it was suggested that the host signalling pathways controlling the metabolism of males and females may react differently to microbial products and their absence, and that many metabolic and other physiological differences between the sexes, particularly the nutritional requirements in females for egg production, may influence the metabolic traits of the microbiota. Thus, our investigations revealed that among three sympatric species of drosophila in the Chamundi hills, altitudinal and seasonal variation had a major impact on gut microbial diversity. The relative abundance of each of the five gut bacteria fluctuates depending on height and season in Chamundi hill. Further, abundance of these gut bacteria also varied significantly between three sympatric Drosophila species studied.These results therefore imply that there is a significant influence of altitude and season on the abundance of gut bacteria in three sympatric *Drosophila* species.

Acknowledgements
The authors are grateful to the Chairman, Department of Studies in Zoology, University of Mysore and Drosophila Stock Center, University of Mysore for providing the facilities to carry out the research work.

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