Chapter 5

5.1. Endosymbiosis in insects

Insects are the most diverse group on earth, and living in the variety of habitats adds to their abundance and species richness. The successful exploitation of diverse habitats by insects in different ecological niches is often facilitated by their association with commensal microbiota. It has been estimated that nearly 15-20% of them live in symbiotic relationship with bacteria. These endosymbiotic bacteria have been known to occur from the arthropod lineage of 385 million years (MY) ago and become rapidly diversified along with their arthropod hosts. Therefore, in the beginning (300 MY ago), the establishment of symbiotic association between the hosts and bacteria together with the nutritional enrichment could be the key factor for the evolutionary success of insects (Gil et al., 2004; Moran and Bauman, 2000).

Endosymbiotic bacteria live in a secure environment; inside the specialized cells of host called bacteriocytes forming an organ like structure (bacteriome) in the insect’s body cavity. Its association is found to be either obligate (the host needs the bacteria for its normal growth and reproduction) or facultative (involved in the defence or host fitness). Bacteriocyte associated microbiota are vertically transmitted from mother to offspring often exhibiting complex developmental events that occur through the development of egg or embryo (Gil et al., 2004; Buchner, 1965). The association between the aphids and *Buchnera aphidicola* is the best example for bacteriocyte related symbiotic relationship in insects (Munson et al., 1991). Similar type of associations were also reported in termites ((Breznak 2000), ants (Currie et al., 2003), white flies (Marubayashi et al., 2014), tephritids; *B. dorsalis* (Kamala Jayanthes et al., 2016), *B. zonata* (Naaz et al., 2016), *B. cucurbitae* (Hadapat 2017) and *Actrocera oleae* (Ras et al., 2017). In some cases like mealy bugs, the intercellular bacterial symbiosis has been reported in the form of bacteriocytes that contain b-proteobacterial endosymbionts.
which are colonized themselves by intracellular g-proteobacteria (von Dohlen et al., 2001). Further, 16S based molecular phylogenetic studies of insects revealed the phylogenetic congruence of primary endosymbionts that have association with their hosts (Fromont et al., 2016).

5.1.1. Role of bacterial symbionts in insects

Endosymbiob bacteria occur in a diverse array of insects exhibiting vertical transmission from mother to offspring (primary symbionts). In addition to primary symbionts, plant sap sucking insects also harbour diverse secondary symbionts that aid in digestion of food or nutrition supplements to host. Recent reports revealed that they are involved in host defence towards pathogen/parasites, influences insect-plant interactions, adaptation to environment and also impact host population dynamics. This gives a clue to understand the role of microbes in insects and the restructuring of these subtle relationships often offer insights into their pest invasiveness or impact (Su et al., 2013).

5.1.1.1. Host defence against pathogens or parasites

Several insects endure various attacks from natural enemies, viz., pathogens, parasites, predators and parasitoid wasps. Regulation of secondary endosymbionts in a number of insects showed increased resistance towards parasites and pathogens. Recently, an obligate symbiont, Buchnera in addition to other facultative symbionts such as Arsenophonous, Serratia symbiotica, Spiroplasma, Regiella insecticola, Rickettsia and Rickettsiella have been reported from aphid species (Oliver et al., 2010).

Generally aphids are commonly infected by fungal diseases but presence of bacterial symbionts protects its host from the infectious fungal agents, for an example Regiella insecticola, a facultative bacterial symbiont protects the pea aphids from fungal disease
caused by *Pandora* (Erynia) *neoaphidis* (Lukasik et al., 2013). Similar type of symbiont mediated protection against pathogens has also been observed in beetles on pine trees and attine ants (Scott et al., 2008; Currie et al., 2003). A streptomycetes symbiont isolated from Southern pine beetle, *Dendroctonus frontalis*, Zimmermann synthesizes an antifungal molecule that found to interfere with its host antagonistic fungi activity (Scott et al., 2008).

5.1.1.2. Influences insect-plant interaction

Insects have evolved different strategies to feed on their host plants with the support of endosymbiotic bacteria. They are the important mediators involved in the interactions between the herbivore insects and their host plants. The most important character of bacterial symbionts in insects is supplementing the essential nutrition to their hosts.

The manipulated host symbionts association of stink bugs provide a novel system for the better understanding of insect–microbe mutualism. For example presence of gut flora found in saliva and regurgitate of chewing insects found to have the potential to modulate plant defence by involving in the synthesis of N-acyl amino acids (Spiteller et al., 2000). Another example is that the presence of endosymbiont *Wolbachia* in apple leaf- mining moth helps in the production of cytokinins that inhibit senescence, maintains chlorophyll content and control nutrient transport (Giron et al., 2007).

5.1.1.3. Impact on host population dynamics

Endosymbiotic bacteria highly influence the host population dynamics through different ways such as parthenogenesis induction, cytoplasmic incompatibility, feminization and male- killing. The symbiotic bacteria such as *Arsenophonus, Cardinium, Spiroplasma* and *Wolbachia* manipulate host reproduction among insects by vertical transmission
Parthenogenesis induction is nothing but the conversion of haploid host eggs into viable diploid female offspring resulting in rapid reduction of genetic diversity. The symbiotic bacteria found in the ovaries of two species of *Encarsia* wasps (Hymenoptera: Aphelinidae) involved in the parthenogenesis induction in *Encarsia hispida* De Santis similar to *Rickettsia* which involved in parthenogenesis induction in leaf miner, *Liriomyza trifoli* Burgess (Hagimori et al., 2006). In cytoplasmic incompatibility, an uninfected female mated with infected male leads to sterile crosses producing few or no offspring and the gene flow between these strains was decreased due to reduction in efficient migration rates. The Wolbachia bacteria not only reported to induce cytoplasmic incompatibility in parasitoid wasps, *Encarsia hispida* De Santis but further it causes feminization in mite, *Brevipalpus phoenicis* Geijskes leading to reduction in genetic diversity and also drive the small population to extinction (Su et al., 2013).

### 5.1.1.4. Pesticide detoxification

Chemical insecticides are widely used for controlling agricultural pest insects and continuous applications of pesticides results in the development of insecticide resistance in various insect pests which have evolutionary changes in pest genome viz., increased levels of degrading esterases, alteration of pesticide target locus and enhanced pesticide excretion (Denholm et al., 1992). Recent report explained that the repertoire bacteria in the genus *Burkholeria* impart protection against organo-phosphorous pesticides in stinkbugs (Kikuchi et al., 2012). This general detoxification ability of microbes could plausibly provide a potential as well as rapid acquisition of pesticide resistance in their hosts.

### 5.1.1.5. Behavioral manipulation
The phenomenon of endosymbiotic bacteria modifying host behaviour which provides adaptation to predators or parasites is commonly called as manipulation by parasite. Perhaps, the symbiotic bacteria can enhance specialized communication signals for different types of behavioural process in insects, viz., mate selection, super parasitism, reproduction, dispersal and enhanced wandering (Varalda et al., 2003; Kenyon and Hunter 2007; Goodacre 2009; Hosokawa et al., 2008; Kamala Jayanthi et al., 2016).

5.1.1.6. Host adaptation

In addition to direct effects on insects, temperature causes indirect effects by means of changing the diversity of the symbionts within the host or their effective transmission of symbionts to offsprings. Arakaki et al., 2001 revealed that cytoplasmic incompatibility and parthenogenesis caused by symbiont wolbachia losses its effect when insects exposed to high temperature. Similarly Buchnera symbionts level found to be reduced in the host pea aphid, Acyrthosiphon pisum when exposed to a heat shock treatment.

Phenotypic plasticity which is a major ecological trait in pea aphids that influences their susceptibility to parasites and predators, is also reported to be greatly influenced by symbiotic bacteria. Generally aphids exhibit colour polymorphism which increase their ability of resistance to natural enemies. Ecological study report revealed that red aphids were likely to be preyed by predators, lady bird beetles. Whereas green aphids endured higher rates of parasitoid wasps attack. Presence of an endosymbiotic Rickettsiella in pea aphids increased the blue-green pigment made up of polycyclic quinines which changes host’s body colour from red to green (Tsuchida et al., 2010).

5.1.2. Bacterial symbionts confers host fitness against heat stress in Tephritids
Tephritid fruit flies are small bodied ectotherms that are constantly confronted with a wide range of temperatures with varying intensity. Subtle changes in its surrounding environment can drastically affect its survival and fitness. Response of fruit flies to temperature stress may involve an array of behavioral, physiological and cellular changes. Many studies reported that the insects harbor endosymbionts not only for nutrition supplements but also for enhancing their fitness. The relationship of such symbionts ranges from obligate to facultative in protecting their hosts from different types of stresses. Specifically the bacterial endosymbionts are interdependent in nature and their mutualism with the host insect may be fragile in the face of a changing environment. However, there are limited studies focused on tephritid fruit flies in relation to their bacterial symbionts acquired tolerance to temperature stress. Further, the influence of temperature stress on bacterial symbionts diversity as well as activity remains unclear. Thus, understanding how these tephritids successfully cope with temperature related stress present a serious issue to ecologists.

Hence this study was conducted to explain the variations in bacterial endosymbionts diversity that aid adaptation and survival in fruit fly, *B. dorsalis* against heat or cold stress.

5.2. Methodology followed

5.2.1 Sample collection

The fruit fly, *B. dorsalis* infested mango fruits were collected from the field and the maggots were reared as per procedure described earlier (Kamala Jayanthi and Verghese, 2001) at Division of Entomology and Nematology, Indian Institute of Horticultural Research, Bangalore-560089, Karnataka (13.13°N; 77.49°E). The emerging adult flies from these
cultures were used in this study. The flies were maintained under laboratory conditions (27 ± 1ºC, 75 ± 2 % RH with a photoperiod 14L:10D) using a banana (Musa spp.) fruit as a laboratory host by following the procedures as described by Kamala Jayanthi and Verghese, 2001). The oviposited fruits (by gravid females of B. dorsalis) were collected and used to initiate all experiments.

5.2.2. Effect of temperature on B. dorsalis cultured at different temperature conditions

To test the effect of temperature on B. dorsalis in Generation 1(G1), infested banana fruits by Oriental fruit flies were placed individually in a sterile plastic container containing sterile sand with mesh covers under controlled temperature chambers that were maintained consistently at 18°C (n = 20) for providing cold stress and 35°C (n=20) for providing heat stress. All the cultures were maintained under 14L and 10D photoperiod with 75% RH. Infested fruits kept at room conditions (RC: 27 ± 1ºC; 75 ± 2 % RH; 14L: 10D) served as control (RC). Observations were recorded daily on the larval development and mortality. Adult surveillance rates in all the three treatments were also observed and recorded. Based on the above described procedure, oviposited banana fruits from these respective cages (18°C, 35°C, RC) were collected and maintained further in similar conditions to establish the consecutive following generations viz., second (G2) and third (G3).

5.2.3. Identification of bacterial symbionts from B. dorsalis using 16S rRNA gene clone libraries

Both male and female (15 days old) fruit flies reared under different treatments (18°C, 35°C and RC) were used for this study. All procedures involved in isolation and culturing of endosymbiotic bacteria associated with B. dorsalis were carried out under sterile conditions of laminar air flow chamber (Scientific Instruments, India). The surface sterilization of flies
was done by rinsing with 10 volumes of 70% ethanol, followed by 5 volumes of 1% sodium hypochloride and finally with distilled water (thrice) through vigorous shaking to remove external surface contaminants. Surface sterilized flies were dissected individually in a petridish filled with phosphate- buffer saline [PBS; 137mM NaCl, 8.1 mM Na₂HPO₄, 2.7mM KCl, 1.5mM KH₂PO₄ (pH 7.4)] using fine sterile forceps under a dissection microscope (Leica, Germany). The dissected gut and reproductive tracts of flies were isolated and crushed in 1mL PBS buffer using a hand held homogenizer. For culturing, 50 µl of the homogenate was spread on the sterile Luria Bertani (LB: 10g peptone; 5g yeast extract; 5g sodium chloride; 12g agar was made up to a litre using distilled water) (Himedia, India) agar plates and the inoculated plates were placed in the incubator (27ºC). After 48 h of incubation, morphologically different colonies were picked and streaked on LB agar plates and the process was repeated thrice to ensure that the isolated cultures are pure.

The isolated pure cultured bacterial colonies were grown over 24 hrs to 48 hrs in the liquid LB (containing 10g peptone; 5g yeast extract; 5g sodium chloride; made up to a litre using distilled water) broth at 27ºC and the genomic DNA was extracted separately using Nucleospin tissue extraction kit (Macherey-Nagal, Germany), following the manufacturer’s instructions. The extracted genomic DNA samples were amplified using 16S rDNA primers 8F and U1492R (Turner et al., 1999) under specified reaction conditions with a final volume of 25 µl containing 25ng of DNA template, 300nM of each primers (8F and U1492R, synthesized from Bioserve, India), 0.5µl of 10mM dNTPs, 2.5mM of Taq buffer and 0.03 µl of Taq DNA polymerase with PCR water (Merck Genei, India). The reaction conditions were as follows, 94ºC, 5 min for initial denaturation, 94ºC, 45 sec for denaturation, 51.5ºC, 45 sec for annealing, 72ºC, 1 min for extension followed by a final extension at 72ºC, for 8 min with a negative control (PCR mixture without template) was kept every time for comparison. The amplified PCR products (nearly 1500bp) were run on agarose gel (1.5%)
electrophoresis stained with EtBr, visualized using a geldoc system (UV Tech, India) and products were eluted and purified using Nucleospin Gel and PCR clean up kit (Macherey-Nagal, Germany). The purified PCR products were cloned into pTOPO 2.1 vector (Invitrogen, USA) according to supplier’s instruction and a total of 32 isolated clones were subjected to complete sequencing at Bioserve sequence service, Bengaluru, India.

The obtained 16S rDNA bacterial gene sequences were assembled and edited with Bioedit (Hall 1999) and the remaining vector sequence was removed from the cloned sequences. Chimeric sequences were eliminated with Bellerophon (version 3) on the Greengenes websites (De Santis 2006) and were further checked manually. The retained sequences with 99% identity were grouped using DOTUR and were subjected to clustal analysis. The clustal sequences were then used in nucleotide BLAST (www.ncbi.nlm.nih.gov/BLAST/) analysis to characterize the sequences and then submitted to GenBank.

A final dataset comprising 1870 positions in the clustal aligned sequences of bacterial symbionts of *B. dorsalis* were subjected to phylogenetic analysis with *Methanosphaerula palustris* (EU156000.1, retrieved from NCBI) as an out group followed by additional basic sequence parameter calculations (viz., variable sites, parsimony- informative sites, base composition biases and nucleotide frequencies), codon evolution model testing and, inter and intra population divergence evaluations were also done using MEGA 6 software (Tamura et al., 2011). The most frequently used algorithms such as Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor Joining (NJ) were applied to reconstruct the phylogenetic trees in MEGA 6 software using Kimura2-parameter (K2P) distance model (17). Bayesian Inference (BI) was performed using MRBAYES v.3.1 software (Tamura et al., 2011; Kimura 1980). The best fitting models of gene evolution out of 24 possible codon
evolution were determined using a model test module and implemented in MEGA 6 using Maximum Likelihood value (lnL), the Bayesian Information Criterion (BIC) and the corrected Akaike Information Criterion (AICc). The robustness of the tree was assessed further using a bootstrap procedure with 1000 replications.

5.2.4. Microbiota supplementation assay

To study the influence of temperature on B. dorsalis symbionts, microbiota supplementation assay was performed. Starved flies (3 h) were introduced into sterile vials containing 5mL BD medium (1g yeast extract, 1g peptone and 1mL honey in 5mL of water) supplemented with 100 µg/mL of ampicilin, 30 µg/mL of tetracyclin and 100 µg/mL streptomycin (Kamala Jayanthi et al., 2016). The influence of temperature on commensal bacterial symbionts of B. dorsalis was studied using antibiotic treated flies as treatment while the flies fed with BD medium without antibiotics served as control.

A ten sets of each containing 50 antibiotic treated fruit flies (25 ♂ and 25 ♀) were allowed to feed on autoclaved BD medium supplemented with the respective treatment flies microbiota (1 X 10^6/mL cells, isolated from B. dorsalis reared at 18°C, 27°C and 35°C) separately served as antibiotic treated infected (Ab-F (Inf)). Similarly another ten sets of each antibiotic treated 50 fruit flies (25♂ and 25 ♀) were fed with only BD medium without microbiota, served as antibiotic treated uninfected (Ab-F (Uninf)). For each experimental setup, flies without antibiotic and microbiota feeding, served as a control. Thereby a control and the test flies of each set were transferred and maintained in separate cages under corresponding controlled conditions (18°C, 35°C and RC). The specific bacteria that are involved in the thermal adaptation were further determined using ten sets of 20 antibiotic treated flies (10 ♂ and 10 ♀) and allowed them to feed on autoclaved BD medium supplemented with all the three microbiota isolated from the different conditions (18°C, 35°C and RC). Another ten sets of
flies were allowed feed on autoclaved BD medium without microbiota served as control. Survival rate of flies was noted at alternate days for a period of 30 days and subjected for statistical analyses.

5.2.5. Statistical analyses

The mean duration (days) of different stages (larval development, pupal emergence and adult survival) at different temperatures (18°C, 35°C and RC) for three generations was subjected to 2-way ANOVA. Further, the mean number of flies survived after different antibiotic assays in each treatment was also subjected to ANOVA independently. In each of these tests, difference at $P \leq 0.05$ was considered as significant criterion to separate the treatment effect. Further, the multiple comparisons were performed with the Tukey’s test. All the statistical analyses were conducted in SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

5.3. Results

5.3.1. Effect of temperature on the survival of B. dorsalis

The flies showed varied response to different treatments across the developmental stages viz., larvae, pupae and adults. The results of two way ANOVA revealed that the temperature do influence significantly the phenological stages of B. dorsalis cultured at different conditions (18°C, 35°C and RC). Larval development ($F= 2042.12; df=2; P>0.001$) (Fig. 11a), pupal emergence ($F= 420.82; df=2; P> 0.001$) (Fig. 11b) and adult survival ($F= 119.79; df =2; P> 0.001$) (Fig. 11c) differed significantly across the treatments and generations. Further the experiment clearly revealed that flies reared at higher temperature (35 °C) could finish larval stages faster and emerged rapidly compared to lower (18°C). Nevertheless, the adult flies survived longer at lower temperature than others.
Fig 11: ANOVA revealed that the temperature significantly (P>0.001) influences the phenological stages of *B. dorsalis* cultured at different conditions (18ºC, 35ºC and RC) (a) larval development (b) pupal emergence and (c) adult survival.

5.3.2. Effect of temperature on bacterial symbionts of *B. dorsalis*

A total of 54 bacterial colonies were screened, of which 31 distinct clones were identified and submitted to Genbank (Plate 10; Table 11).

**Table 11: Molecular characterization of bacterial symbionts from *B. dorsalis* reared at different temperatures using 16S rRNA gene clone libraries**

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Prevalence of bacterial symbionts associated with <em>B. dorsalis</em> reared at different temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCBI Bankit Accession No.</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>KX161728</td>
</tr>
<tr>
<td>Acinetobacter radioresistens</td>
<td>KX161731</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>KP322624</td>
</tr>
<tr>
<td>B. cereus</td>
<td>KP322621</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>KX161732</td>
</tr>
<tr>
<td>B. methylotrophicus</td>
<td>KY196530</td>
</tr>
<tr>
<td>B. pocheonensis</td>
<td>KX161733</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>KX161730</td>
</tr>
<tr>
<td>B. tequilensis</td>
<td>KP322621</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>KX161727</td>
</tr>
<tr>
<td>Brevibacillus invocatus</td>
<td>KX161723</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
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</tr>
<tr>
<td>Cedecea davisiae</td>
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</tr>
<tr>
<td>Entrobacter asburiae</td>
<td>KP322628</td>
</tr>
<tr>
<td>E. hormaechei</td>
<td>KP322622</td>
</tr>
<tr>
<td>E. ludwigii</td>
<td>KP322618</td>
</tr>
<tr>
<td>Strain</td>
<td>Accession</td>
</tr>
<tr>
<td>--------------------------------</td>
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</tr>
<tr>
<td>Enterococcus faecalis</td>
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</tr>
<tr>
<td>Klebsiella oxytoca</td>
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<tr>
<td>Klebsiella sp.</td>
<td>KP322611</td>
</tr>
<tr>
<td>Kocuria rosea</td>
<td>KX161722</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>KX161729</td>
</tr>
<tr>
<td>Micrococcus indicus</td>
<td>KP322609</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td>KP322613</td>
</tr>
<tr>
<td>Proteus penneri</td>
<td>KP322626</td>
</tr>
<tr>
<td>Providencia rettigera</td>
<td>KP322615</td>
</tr>
<tr>
<td>P. burhodogranariae</td>
<td>KP322610</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>KX161734</td>
</tr>
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<td>KP322623</td>
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<td>KX161721</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>KP322633</td>
</tr>
</tbody>
</table>

From the 54 isolates, 25 isolates were obtained from flies reared under RC (20 isolates were from ♀ and 5 isolates were from ♂); 15 isolates obtained from flies reared at 18°C reared

Plate 10: 16s rRNA amplification of *B. dorsalis* cultured under different temperature stress conditions. Lane M: 250bp DNA ladder; Lane 1 to Lane 13: 16s PCR amplified products.
flies (10 isolates from ♀ and 5 isolates from ♂); 14 isolates were from flies reared at 35°C (9 isolates from ♀ and 5 isolates from ♂). Of these, the identified distinct isolates (n=31) (based on 16S rRNA) across the treatments exhibited closest match forming into three distinct phyla such as Proteobacteria (Pr), Firmicutes (Fi), Actinobacter (Ac). The percent composition of of bacterial isolates (Pr:Fi:Ac) obtained from *B. dorsalis* reared under different temperatures were found to be 55:20:10 in female flies and 80:20:0 in male flies reared under RC; the ratio of 50:50:0 were in female flies and 20:80:0 in male flies reared under 18°C and the ratio of 67:33:0 in female flies and 33:67:0 in male flies reared under 35°C.
From the total isolates, *Bacillus cereus* (Phylum: Firmicutes; Gram +ve) and *Morganella morganii* (Phylum: Proteobacteria; Gram -ve) were found commonly in all the three treatments irrespective of the sexes. The genus that found commonly only in female flies across the treatments is *Klebsiella oxytoca* and *Klebsiella sp* (Phylum: Proteobacteria; Gram -ve). Results revealed that the bacterial symbionts found to be more abundant in *B. dorsalis* flies reared at RC when compared to that of flies reared at 18°C and 35°C. The prevalence of bacterial symbiont communities also varied greatly among temperatures. The bacterial symbiont species viz., *Bacillus methylotrophicus*, *Enterococcus faecalis*, *Lactobacillus lactis*, *Acinetobacter radiotolerans* and *Staphylococcus epidermis* were found only in 18°C reared females whereas *B. pumilus* and *S. aureus* were found in males. However, the *Bacillus sp.* was found commonly in both sexes of flies reared at 18°C. Similarly the bacterial symbiont species such as *A. baumannii* was found only in female flies reared at 35°C, whereas in male flies the abundant species were found to be *B. licheniformis*, *B. methylotrophicus* and *B. pochoensis*. Nevertheless, *Bacillus sp.* was found commonly across the sexes when flies reared at 35°C.

### 5.3.3. Phylogenetic analysis of bacterial symbionts

16s rDNA sequence homology of ML analysis clearly depicted three different clusters of phyla such as proteobacter, actinobacter and fermicutes (Plate 10 & 11). The null hypothesis of equal evolutionary rate throughout the ML tree topology was rejected (at 5% significance). According to the sequence analysis of 16S rDNA from 31 distinct isolates, a monophyletic clade was formed with the genera *M. palustris*. There were three major branches in the phylogenetic tree based on 31 bacterial isolates (~1500bp per clone) obtained from all three treatments (Plate 11). The first branch cluster constituted Phyla, Proteobacteria containing 16 isolates viz., *Acinetobacter radiotolerans*, *A. baumannii*, *Pseudomonas stutzeri*, *Klebsiella*
sp, *K. oxytoca*, *Citrobacter freundii*, *Pantoea agglomerans*, *Enterobacter asburiae*, *E. hormaechei*, *Serratia marcescens*, *Cedecea davisae*, *E. ludwigi*, *Morganella morganii*, *Proteus penneri*, *Providencia rettgeri* and *P. burhodogranariea*. The second branch cluster constituted Phyla Actinobacter containing two isolates viz., *Micrococcus indicus* and *Kocuria rosea*. The third branch cluster constituted Phyla Fermicutes containing 13 isolates such as *Brevibacillus invocatus*, *Enterococcus faecalis*, *Bacillus sp.*, *B. methylotrophicus*, *B. pumilus*, *B. cereus*, *B. anthracis*, *B. tequilensis*, *B. licheniformis*, *Staphylococcus epidermis* and *S. aureus*. The estimated value of the shape parameter for the discrete Gamma Distribution is 2.0479. Mean evolutionary rates in these categories were 0.26, 0.55, 0.85, 1.23, 2.11 substitutions per site. The maximum Log likelihood for this computation was -27789.006.

5.3.4. Microbiota supplementation assay

The assay results revealed that both antibiotic treated flies and microbiota supplemented flies (cultured at 18°C, 35°C and RC) showed a varying level of temperature tolerance. The mean
Fig. 12: The survival of AB-F (Inf) and AB-F (Uninf) fruit flies (both sexes) cultured under different conditions showed significant survival in relation to different temperature regimes; where AB-F (Inf) represented antibiotic fed flies treated with microbiota and AB-F (Uninf) were antibiotic fed flies treated without microbiota.

survival of AB-F (Inf) and AB-F (Uninf) fruit flies (both sexes) cultured under different conditions were given in the Fig. 11. The results of one way ANOVA of all the treatments viz., 18°C ($F=3.893; df=30; P<0.0001$ for ♀; $F=50.13; df=30; P<0.0001$ for ♀), 35°C ($F=62.43; df=27; P<0.0001$ for ♀; $F=43.15; df=27; P<0.0001$ for ♀) and RC, 27°C ($F=41.88; df=30; P<0.0001$ for ♀; $F=41.09; df=30; P<0.0001$ for ♀) showed significant variation in host survival (Fig. 12). Further, the supplementation assay using specific microbiota (isolated from flies reared at specific temperature) that are involved in host adaptation with relation to temperature stress also showed significant variation in flies survival in different treatments. The mean survival of AB-F (Inf) and AB-F (Uninf) fruit flies (both sexes) fed with all three specific microbiota under (18°C, 35°C and RC) were given in the Fig. 12. It was found that the ABIF flies (both sexes) reared at 18°C showed increased survival when fed with specific microbiota isolated at 18°C compared to 35°C and RC flies ($F=25.93; df=2; P<0.0001$ for ♀; $F=47.44; df=2; P<0.0001$ for ♀). Similarly ABIF flies (both sexes) reared at 35°C showed increased survival when fed with specific microbiota isolated at 35°C compared to 18°C and RC flies ($F=30.35; df=2; P<0.0001$ for ♀; $F=30.35; df=2; P<0.0001$ for ♀) and RC, 27°C ($F=25.91 df=2; P<0.0001$) also showed significantly increased survival only with 35°C and 27°C isolates respectively. Whereas the AB-F (Inf) flies fed with other than specific bacterial isolates showed less survival across the treatments (Fig. 13).
Fig 13: The mean survival of AB-F (inf) and AB-F (Unf) fruit flies (both sexes) fed with all three specific microbiota under different temperatures (18°C, 35°C and RC)

5.4. Discussion:
Being ectotherms, insects are constantly confronts with a wide range of temperatures with varying intensity and even a small change in the environment can rapidly affect their fitness. The survival response of insects in relation to abiotic stress is of particular interest, yet meagrely understood. As mentioned earlier reviews, host bacterial symbionts were considered as suitable models to understand the plasticity that facilitates the host fitness or adaptation to changing environment and their occurrence was already reported in aphids (Montllor et al., 2002; Russell et al., 2006). The present study revealed the role of commensal microbiota in aiding host survival using Oriental fruit fly, \textit{B. dorsalis} and its associated bacterial symbionts as a case study. The fruit fly, \textit{B. dorsalis} culture was reared at two different temperature regimes namely 18°C, 35°C and made comparisons of fly survival in relation to associated bacterial symbionts with the flies that are reared at ambient conditions (RC, 27°C). Temperature dependent development was noticed in all phenological stages of \textit{B. dorsalis}, characterized with faster development and rapid emergence at higher temperature compared to lower. Similarly results were also reported in other tephritids viz., \textit{Bactrocera carambola}, \textit{Bactrocera papaya}, \textit{Bactrocera invadens}, \textit{Bactrocera dorsalis}, \textit{Bactrocera zonata} and \textit{Bactrocera correcta} where temperature found to influence the developmental stages (Rwomushana et al., 2008; Vargas et al., 1996; Duyck et al., 2004; Liu and Ye 2009).

The abundance and prevalence of bacterial symbionts varied greatly across the temperatures and was more in RC reared flies (20 isolates) compared to the flies reared at low temperature (18°C, 15 isolates) or high temperature (35°C, 14 isolates) conditions. This study supports the Wernegreen et al. (2012) findings that revealed highly dependent mutualisms often meltdown with extreme temperature. Other reports cited distinct loss in host symbionts as observed in pentatomid stinkbugs and green stink bug, \textit{Nezara viridula} Linnaeus with marginal increase in temperature (Prado et al., 2010 and Kikuchi et al., 2016).
The three major phyla such as Proteobacteria (Pr), Firmicutes (Fi), Actinobacter (Ac) were found to be associated with *B. dorsalis* under different treatment temperature regimes studied. However, the phyla of Ac disappeared under temperature stress implying that they are basically involved in the host defence mechanism (provide innate immune responses) directed against pathogens (Kikuchi et al., 2016). Endosymbiotic bacterial diversity found to be varied in *B. dorsalis* with relation to temperature regimes revealing that there is an intricate dynamic relationship exists between the host and its symbionts under altering abiotic stress situations.

Recent reports revealed that the prevalence of secondary symbionts of bacteria confer conditional adaptive advantages to their host, such as *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, *Rickettsia* and *Acinetobacter* in whiteflies and *Salmonella symbiotica* and *Hamiltonella defensa* in aphids found to involve in their host defence mechanism (Montllor et al., 2002; Russell and Moran 2006; Singh et al., 2012; Cheil et al., 2007). Other symbionts like *Pseudomonas* sp., *Acinetobacter* sp., *Stenptrophomonas* sp. and *Serratia marcescens* in the gut of diamondback moth reported to be involved in the production of antifungal siderophore compounds like pyoverdine that may contribute to host antagonism against entomopathogens and also in the production of chitinase for host nutrition (Indiragandhi et al., 2007). Particularly the genus *Acinetobacter* have been reported from larval mid-gut of different insect species such as white butterflies, saturniid moths, mosquitoes (Robinson et al., 2010; Pinto-Tomas et al., 2011). The metagenomic analysis of gut microbial community from cabbage root fly larvae showed the presence of *Acinetobacter*, *Serratia*, *Providencia* and *Pectobacterium* and found to be involved in the detoxification of isothiocyanate compound thereby benefiting host fitness (Winde and Wittstock 2011). The symbiont *M. morganii* is also known to have commensal relationship within the intestinal tracts of insects.
such as housefly (Zhijian Su et al., 2010) but their potential role in their host system is not clear.

Previous studies explored the bacterial microbiome diversity in *B. dorsalis* gut that revealed presence of *Klebsiella, Serratia, Citrobacter, Proteus,* and *Bacillus* as common isolates across the tephritid fruit flies such as *B. tau*, *B. tryoni* and *C. capitata* (Kuzina et al., 2001; Behar et al., 2005; Pramanik et al., 2014; Wang et al., 2011; Khan et al., 2014). Presence of bacterial microbiota similarity across several studies explains their association with the tephritid flies, supporting the concept of holobiont theory where every species either plant or animal, live in symbiosis with their associated microbiota (Khan et al, 2014; Michael Ben et al., 2008). Thus, these associated microbes have an enormous influence on the physiology, anatomy, behavior, reproduction, and fitness of their hosts (Bordenstein and Theis 2015; McFall- Nagai 2002; Archie and Theis 2011; Ezenwa et al., 2012). However, there is no information on symbiotic bacterial community that conferred host adaptation against temperature in *B. dorsalis*.

The present study showed that the intestinal and reproductive tract of the *B. dorsalis* contains a diverse bacterial community with respect to their growth temperatures. Of these 12% of microbiota viz., *Klebsiella oxytoca, Providencia Rettgeri, Morganella morganii, Bacillus cereus* were stable and the remaining 88% differed with temperature. We found that certain genera of *A. baumanii, Brevibacillus inovocatus, B. licheniformis, B. pochoenensis* and *P. stutzeri* were found consistently associated with *B. dorsalis* under high temperature stress, may be involved in host adaptation under high temperature stress. Similarly, the genera viz., *A. radioresistens, B. methylorophicus, E. faecalis, L. lactis, S. aureus* and *S. epidermis* were found associated with *B. dorsalis* at lower temperatures. Therefore, here it is quite opt to mention 'Holobiont' concept which explains mutualism between the host and its
symbionts that contributes to holobiont (Host+symbiont) fitness in toto. Variation in
temperatures gradually affects arthropods, as they have to sustain cellular metabolism at both
higher and lower temperature extremes. Studies reported that microbiota can support the host
to overcome temperature stress by providing enzymes that are optimized for temperature
regimes (Kuzmina and Pervushina, 2003). Likewise the acquisition of certain symbiotic
algae increased the temperature tolerance of corals supporting their survival under current
climate change scenarios (Berkelmans and van Oppen 2006).

This study showed the presence of varied bacterial symbionts among the female and
male flies across the treatments, indicating the possibility of sex-specific symbiont
interactions as reported in crickets on dietary selection (Schmid 2014). Interestingly, the
isolates viz., *K. oxytoca*, *Klebsiella sp* were found associated constantly with females and
completely absent in males. Earlier studies also reported *Klebsiella* as a 'trail of female scent'
to male flies particularly in *B. dorsalis* as the released volatile metabolites of *K. oxytoca*
found to have profound influence on the mate attractive behavior (Kamala Jayanthi et al.,
2016; Davis et al., 2014).

Remarkable differences among bacterial isolates were found between the flies reared
at both temperature extremes (18°C and 35°C), as mutualism among symbionts and its host
may collapse at temperature extremes and the host insect may depend on specific symbionts
for its survival (Kuzmina and Peruvushina 2003; Kikuchi et al., 2012). According to
Wernegreen (2013) thermal adaptation in insects is a complex phenomenon involving
behavioural, physiological, and cellular responses. Nevertheless, he opined that insects are
prone to establish vital associations with symbiotic bacteria which influence their adaptation
to changing environment. Thus, certain symbionts play crucial role against environmental
stresses and thereby contributing to the host insect adaptation. In the present study also
feeding of specific microbiota supported the flies to overcome the given temperature stress. Therefore, further studies are required to elucidate the associated metabolic changes and biological functions of the specific endosymbionts that are associated with *B. dorsalis* under temperature stress.