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DEVELOPMENTAL SHIFTS IN SYMBIOTIC BACTERIAL COMPOSITION AND ABUNDANCE IN *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTIDAE)

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ABSTRACT

In the current investigation, 16S rRNA sequencing showed the Microbial abundance of *Spodopteralitura* was higher (20 numbers) than *Spodopterafrugiperda* (15numbers) and the microbiome composition underwent significant changes across developmental stages in each species. Fifth-instar larvae exhibited greater bacterial diversity than pupae and adults in both species. Phylum, Pseudomonadota was dominant (73.34%) in *Spodopterafrugiperda*, followed by Bacillota (26.66%). In *Spodopteralitura*, Bacillota was dominated (55%), followed by Pseudomonadota (45%). Enterobacteriaceae was the dominant family, accounting 33.34% in *Spodopterafrugiperda* and 40% in *Spodopteralitura*. Genera, *Klebsiella*, *Acinetobacter*, *Mammaliicoccus* and *Enterococcus* were found dominant across developmental stages in both species. The study revealed the microbiome in haemolymph of *Spodopterafrugiperda* and *Spodopteralitura*, along with comparison of endosymbionts diversity between two species.

Key words: Haemolymph, Endosymbionts, Diversity, 16S r RNA

Introduction

The lepidopteran insect Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) and Tobacco cutworm, *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidaeare) are highly destructive agricultural pests. Larvae is the damaging stage that attacks various crops which are significant commercially and cause serious economic losses each year due to their polyphagous behaviour (He *et al.*, 2021). Hence, to mitigate against losses attributed to the pest, farmers intensively applied chemical pesticides. However, this chemical spray continues to pose risks to the quality of yield, leads to environmental contamination and is believed to contribute to selective pressure on S. frugiperda and S. litura in developing resistance against synthetic pesticides (Ingber *et al*., 2018; Gutiérrez-Moreno *et al*., 2019). Thus, alternative control methods are urgently needed against this resistance development.

During the evolution, these insects have harbor diverse microorganisms in gut, providing their host with physiological and ecological advantages (Philipp and Nancy, 2013; Jang and Kikuchi, 2020). It is one of the factors responsible for the successful infestation by these pests (Blow and Douglas, 2019), as these endosymbionts play a crucial role in the survival, development, and selection of their host.

In recent years, there has been an increasing number of studies on the gut microbial diversity *of S. frugiperda* and *S. litura* (Li *et al*., 2022; Devi *et al*., 2022). But work are still need to be done on the haemolymph endosymbionts of these insects. Hence, this study focused on the abundance and diversity of haemolymph endosymbionts across developmental stages (larvae, pupae and adults) of laboratory-reared S. frugiperda and S. litura using 16S rRNA sequencing. The community structure of haemolymph endosymbionts is altered during the different life stages of host insects (Chen *et al*., 2016). We also compared the composition and diversity of

Table 1: Culturable bacteria in the haemolymph from different developmental stages of *Spodoptera frugiperda* and *Spodoptera litura.*

Spodoptera frugiperda			***Spodoptera litura***		
S. no	**Bacterial species 5th Instar Larva**	**Accession ID**	**S. no**	**Bacterial species 5th Instar Larva**	**Accession ID**
1	*Klebsiella pneumoniae*	OR342318	1	*Klebsiella variicola*	OR088578
2	Acinetobacter junii	OR342327	2	Enterococcus casseliflavus	OR074488
3	*Mammaliicoccussciuri*	OR342328	3	*Mammaliicoccussciuri*	OR073651
4	Acinetobacter haemolyticus	OR342328	4	Enterococcus mundtii	OR073759
5	Acinetobacter baumannii	OR366523	5	Enterobacter cloacae	OR074442
6	*Enterococcus mundtii*	OR122030	6	*Staphylococcus gallinarum*	OR073816
7	Klebsiella variicola	OR342319	7	*Bacillus paramycoides*	OR074135
	Pupa		8	*Atlantibactersubterranea*	OR098641
8	*Klebsiella pneumoniae*	OR414380	9	*Acinetobacter rhizosphaerae*	OR074179
9	Serratia marcescens	OR342318	10	*Klebsiella pneumoniae*	OR074744
10	*Mammaliicoccussciuri*	OR342330	11	*Staphylococcus saprophyticus*	OR074916
11	Providencia rettgeri	OR121929	12	*Enterobacter bugandensis*	OR098503
	Adult			**Pupa**	
12	Klebsiella pneumoniae	OR342336	13	*Klebsiella variicola*	OR414380
13	*Acinetobacter baylyi*	OR910105	14	*Enterococcus faecium*	OR342318
14	*Mammaliicoccussciuri*	OR342337	15	*Mammaliicoccus sciuri*	OR342330
15	Kluyveraascorbata	OR394103	16	*Lysinibacillus mangiferihumi*	OR121929
				Adult	
			17	*Klebsiella variicola*	OR342336
			18	*Enterococcus mundtii*	OR910105
			19	*Mammaliicoccussciuri*	OR342337
			20	*Klebsiella pneumoniae*	OR394103

haemolymph endosymbionts between Spodoptera frugiperda and Spodoptera litura.

Materials and Methods

Collection and rearing of experimental insects

A laboratory population of fall armyworm and tobacco cutworm originally collected from infested maize and castor fields respectively in GKVK, UAS, Bangalore (13.0781° N, 77.5792° E), was established and maintained in our laboratory. The collected egg masses were placed in plastic containers (size - 25 cm in diameter) with the natural hosts. After hatching from egg larvae of *S. frugiperda* were reared in groups on natural host at room temperature (26 ± 1°C, 70 ± 10% RH and 14L: 10D h photoperiod) till the larva reach the third instar, after that they were reared separately in individual vials (4 × 3 × 3 cm) to avoid cannibalism until adult moth emergence. Whereas *S.litura* larvae were reared in groups throughout the larval period where, cannibalism is not a problem. Pupa of both species were collected and placed in a container. Emerged moths were released into the oviposition cage (35×35×35 cm). The walls of the cage were provided with white paper as a supporting platform for egg laying by the moths and maize seedlings and castor leaves were also placed inside the cage as substrate for oviposition. A piece of cotton soaked with 10% honey solution was provided as a source of food for the adults. First generation of the laboratory population was used for the experimental study.

Experimental design

From the laboratory population, 15 individuals from each developmental stageof *S. frugiperda* and *S. litura* were selected. Each treatment group consisted of 3 biological replicates. Before the experiment, 5th instar larvae and adults were starved for 2 hours and they were immobilized by freezing at -20°C for 5 minutes further, the surface of 5th instarlarvae, Pupae and adults were washed with 0.5% NaOCl for 2 min, 75% ethanol for 1 min and rinsed three times with sterilized-deionized water (Chen *et al.*, 2018).

Collection of haemolymph sample and bacterial DNA extraction by CTAB method

Haemolymph was sampled from the dorsal prothoracic region (5th instar larvae, pupae and adults) using a sterile ice-chilled Hamilton needle and haemolymph was drained into sterile 1.5 mL tubes.

The total nucleic acid was extracted using the CTAB

method (Suganthi *et al*., 2023). The sterile PBS without insect tissue was used as a negative control both in DNA extraction and PCR amplification to detect reagents and environmental contamination. The integrity and quality of the extracted DNA were evaluated on 1% agarose gel electrophoresis. The 16S rRNA fragment was amplified using a thermocycler (Eppendorf- vapo. Protect, Germany) with the primers, forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-ACGGCTACCTTGTTACGACTT-3'). PCR cycling conditions were as follows: 95°C for 5 min, 30 cycles of 95°C for 30 s, 56°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. All samples were amplified in triplicate. The PCR amplified products were subjected to agarose gel electrophoresis (Lee *et al*., 2012) on 1 per cent agarose gel and documented in a software gel documentation system. The nucleotide sequencing was performed at Eurofins Genomics India Pvt. Ltd. Bangalore. The obtained DNA sequences corresponding to the 16S rRNA gene were confirmed using the basic local alignment search tool (BLAST) and the raw reads with maximum coverage were deposited into the NCBI, GenBank and accession numbers were obtained for all the bacterial strains Neighbor-Joining phylogenetic tree of the 16S rRNA sequences was constructed, with 1000 bootstrap replications, using MEGA 11 software (version 11.0.13).

Results and Discussion

Sequencing Data of 16S rRNA

Data sequencing and analysis of 15 samples (*S. frugiperda*) and 20 samples (*S.litura*) for studying diversity were completed. The observed haemolymph microorganisms were classified into 2 phyla, 2 classes, 4 orders, 6 families and 7 genera in *S. frugiperda* and 2 phyla, 2 classes, 4 orders, 6 families and 9 genera in case of *S. litura.* Sequencing data statistics of all samples are shown on Table 1. Phylogenetic trees for each stage were constructed from the obtained bacterial sequences (Fig. 1 and 2).

Diversity in the microbial composition in the haemolymph at different developmental stages

Results showed that a total of 15 bacteria were isolated from haemolymph of *S. frugipeda*. Phylum Pseudomonadota was found to be dominant in all three developmental stages with 73.34 % followed by Bacillota with 26.66 %. Where as in the case of *Spodoptera litura* total of 20 haemolymph bacteria were isolated, Phylum Bacillota was the dominant with 55 per cent followed by Pseudomonadota with 45% (Fig. 3). At the genus level, in case of *S. frugiperda* Klebsiella and Acinetobacter

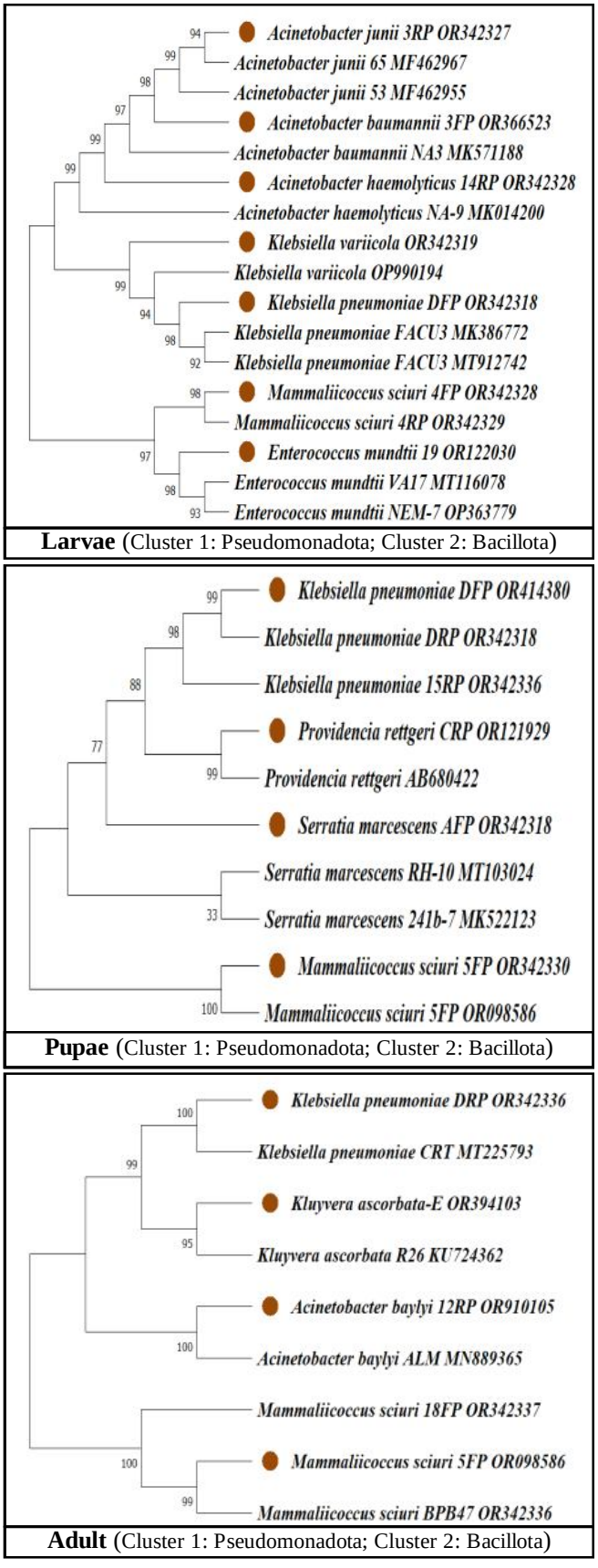
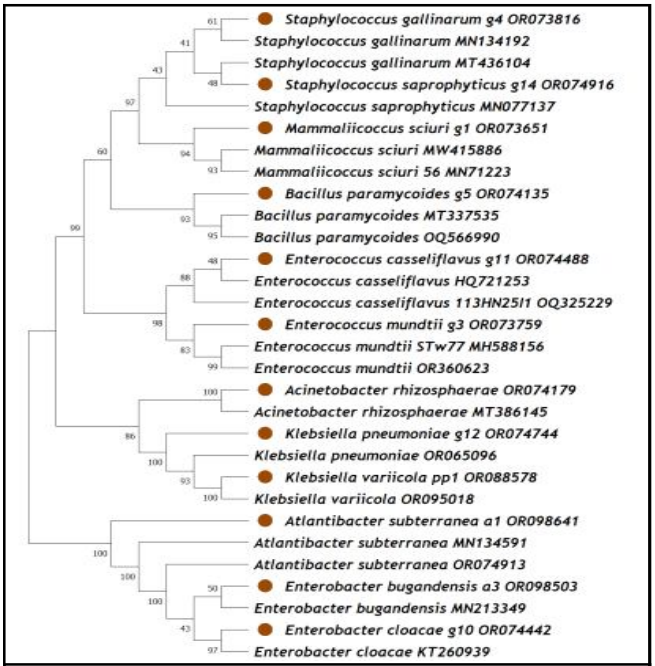
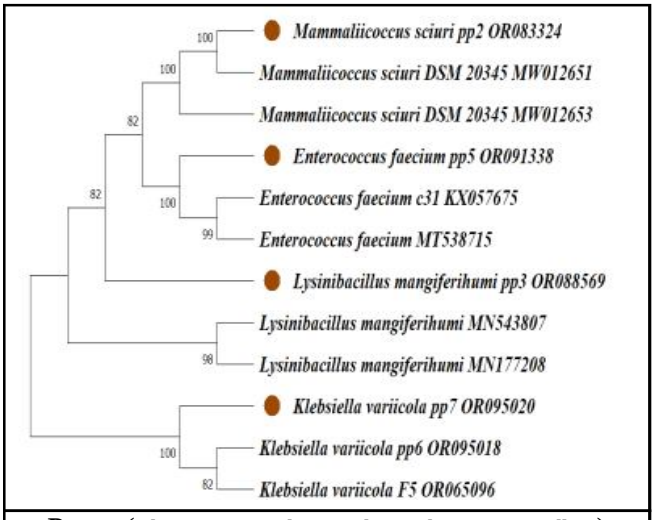


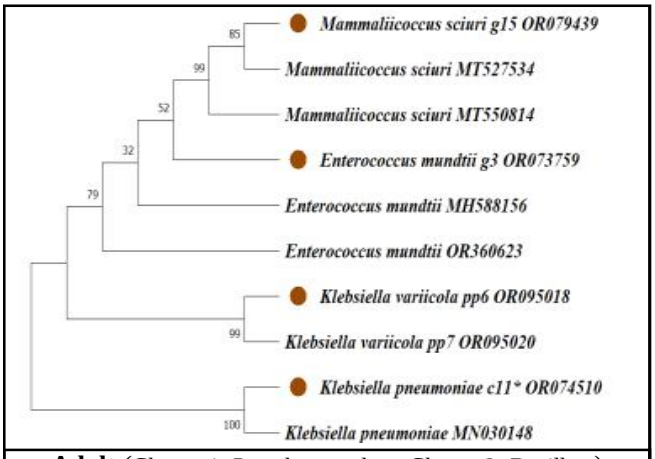
Fig. 1: Phylogenetic analysis of bacterial isolates based on 16S r RNA gene sequencing from *Spodoptera frugiperda.*



Larvae (Cluster 1: Pseudomonadota; Cluster 2: Bacillota)



Pupae (Cluster 1: Pseudomonadota; Cluster 2: Bacillota)



Adult (Cluster 1: Pseudomonadota; Cluster 2: Bacillota)

Fig. 2: Phylogenetic analysis of bacterial isolates based on 16S r RNA gene sequencing from *Spodoptera litura*.

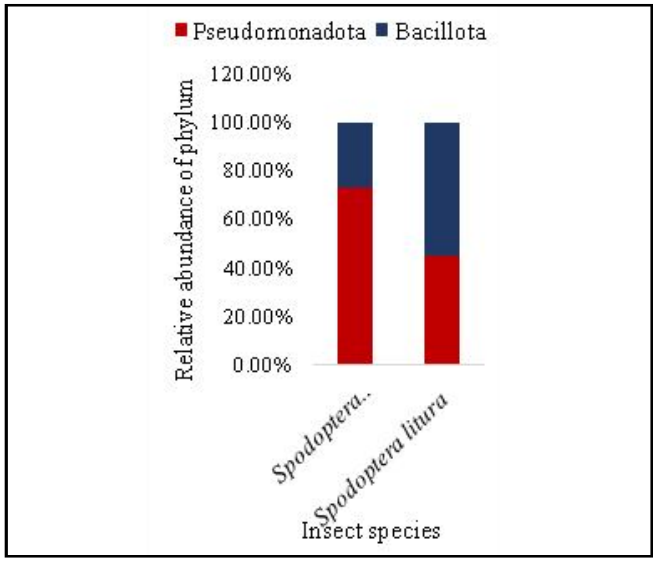


Fig. 3: Relative abundance of bacterial phylum in both the species.

were most abundant with 27.08 per cent each followed by Mammaliicoccus (20.83%) and genera Enterococcus, Providencia, Kluyvera, Serratia each showed 6.25 per cent of abundance and in *S. litura* genera Klebsiella was abundant (25%) followed by Enterococcus (20%), Mammaliicoccus (15%), Enterobacter and Staphylococcus contributing 10% each and Bacillus, Atlantibacter, Acinetobacter and Lysinibacillus contributed 5% each (Fig.4).

Comparison of the microbial diversity in the haemolymph at different developmental stages

Seven bacterial species identified from larvae of *S.*

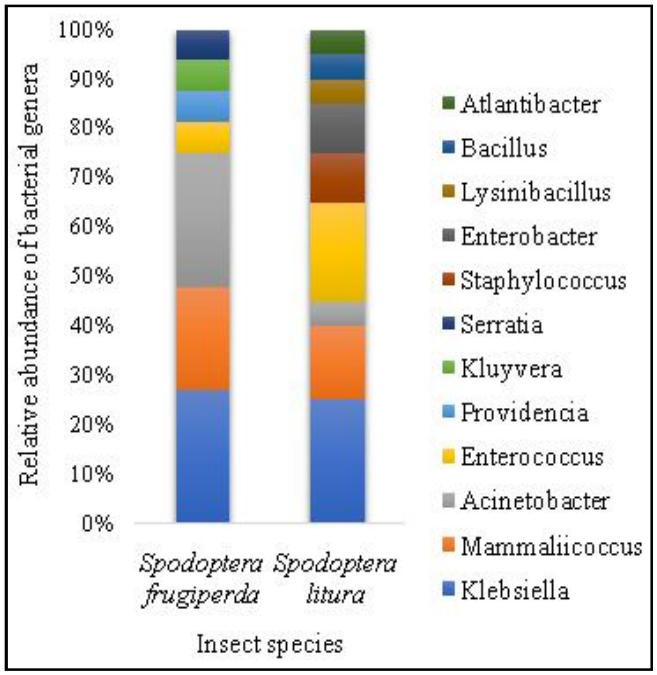


Fig. 4: Relative abundance of bacterial genera in both the species.

frugiperda belong to four genera *Mammaliicoccus, Acinetobacter, Klebsiella and Enterococcus* each accounting for 14.28 percent, 42.85 per cent, 28.55 per cent and 14.18 percent respectively. Genera *Acinetobacter and Klebsiella* were abundantly found in 5th instar larval stage than compared to other stages and genera Enterococcus was exclusively detected in the 5th instar larva. Pupa of *S. frugiperda* was found to inhabit four bacterial species belonging to four genera *Klebsiella, Serratia, Providencia* and *Mammaliicoccus* contributing 25 percent each. Where genera *Providencia and Serratia* were exclusively detected in pupal stage. Adults stage inhabit four bacterial species belonging to four genera *Kluyvera, Acinetobacter, Mammaliicoccus* and *Klebsiella*c on tributing 25 per cent each. Genera Kluyvera was found only in the adult stage. The bacterial diversity was abundant in the larval stage with 46.66 percent and decreased in the pupal and adult stages with 26.66 percent each. The larvae of *Spodoptera litura* were found to inhabit twelve bacterial species belonging to eight genera *Klebsiella, Staphylococcus, Enterococcus* and *Enterobacter* accounting for 16.6 per cent each and *Bacillus, Atlantibacter, Acinetobacter, Mammaliicoccus* contributed 8.33 per cent each. The genera *E. mundtii* and *E. cloacae* were abundantly found in larval and adult stages and the genera *Lysinibacillus* was exclusively found in pupal stage. Pupa inhabit four bacterial species belonging to four genera *Klebsiella, Mammaliicoccus, Enterococcus and Lysinibacillus* contributing 25 per cent each. Adult inhabit four bacterial species belonging to three genera *Klebsiella* (50%), *Mammaliicoccus and Enterococcus* 25 per cent each. The bacterial diversity was found to be abundant in the larval stage with 60 per cent and decreased in the pupal and adult stages with 20 per cent each (Fig. 5). The variations in microbiome diversity are witnessed across the developmental stages (larval, pupal, and adult) of the species. Where, bacterial diversity was abundant in the larval stage and decreased in the pupal and adult stages,

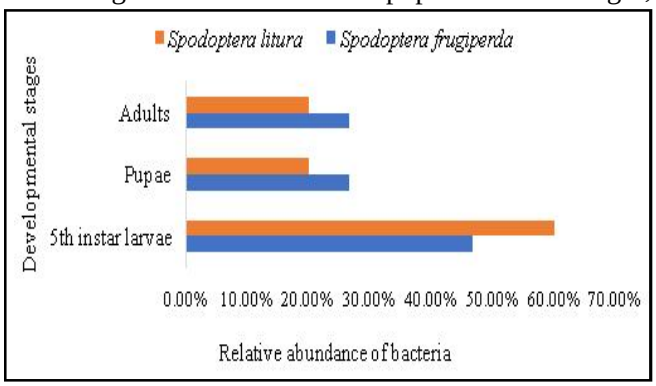


Fig. 5: Relative abundance of Bacterial endosymbionts in different developmental stages.

which strongly implies the potential loss of certain prominent larval bacterial groups during metamorphosis. Nevertheless, the presence of several bacterial groups in both larval and adult stages provides evidence for their persistence and transmission throughout different developmental stages (Gichuhi *et al.*, 2020).

Systematically analyzing the diversity of microbial communities is challenging due to the high complexity of sampling volume, sampling method, and sampling stage (Li *et al.*, 2022). For example, due to the less haemolymph content in pupal and adult stages, a large number of samples is required for sequencing. In this study, we found support for our hypotheses that *S. frugiperda* and *S. litura* both harbor endosymbionts in their haemolymph and there is difference in the bacterial communities at different developmental stages. However, we also found that some of the endosymbiotic bacterial taxa acting as capable of inhibiting the growth of important haemolymph endosymbionts, it will affect the biology of insect which can be used in pest management strategy.

In this study, we found that Proteobacteria were the dominant phylum at larval, pupal and adult stages of *S. frugiperda* followed by Firmicutes. The possible reason is that bacteria belonged to the phylum pseudomonadota involved in the degradation of insecticides and plant secondary substances and host immunity. These findings are in parallel with the results of Ugwu *et al.*, (2020), they identified Proteobacteria (Pseudomonadota) as the predominant phylum, followed by Firmicutes (Bacillota), in the gut of *S. frugiperda*. Firmicutes and Proteobacteria have been reported to play key roles in the nutritional supplementation, energy absorption, preservation of gut homeostasis and host immunity (Colston and Jackson, 2016; Wang *et al.*, 2020). Our results were in congruence with other studies on *S. frugiperda* and other Lepidopterans whereby Proteobacteria and Firmicutes were the most dominant bacterial phyla in the gut (Chen *et al.*, 2016; Xia *et al.*, 2018; Gichuhi *et al.*, 2020; Li *et al.*, 2022). This finding is also in accordance with Gichuhi *et al.*, (2020), who identified that the most prevalent bacterial phyla in fall armyworm gut samples were Pseudomonadota, Bacillota and Bacteroidetes, along with very low proportion of Actinobacteria. Acinetobacter was abundantly found in the 5th instar larvae the possible reason is that they are involved in the metabolic degradation of plant secondary metabolite and also involved in the degradation of hemicellulose. This observation is consistent with the research conducted by (Palacio *et al.*, 2021; Carreto *et al.*, 1996) in the gut of *S. frugiperda*. Similarly, *Klebsiella* was dominant in the gut of the 5th instar larva and adult of *S. frugiperda*

which is involved in digestion and they also reported that the relative abundance of *Klebsiella* increased with an increase in food intake (Liu *et al.*, 2022).

In contrast, *S. litura* had Bacillota as the dominant phylum followed by Pseudomonadota. This finding is consistent with the results of Devi *et al.*, (2022), Xiang *et al.*, (2006), Xia *et al.*, (2013), Chen *et al.*, (2016), Snyman *et al.*, (2016) who found that Firmicutes (Bacillota) was the dominant phylum followed by Proteobacteria (Pseudomonadota) and Actinobacteria in the gut of *S. litura*. Interestingly, *Enterococcus* was exclusively detected in the 5th instar larva the possible reason is that it has a major role in detoxification and modulation of host immune response, *S. litura* this finding is consistent with the results of (Broderick *et al.*, 2004; Vilanova *et al.*, 2016). The genera *E. mundtii* and *E. cloacae* were abundantly found in larval and adult stages of the the possible reason is that these two bacteria are involved in the defence against pathogens in lepidopteran insects, this finding is consistent with the results of Acevedo *et al.*, (2017). Previous studies have shown that *Enterococcus* is able to degrade alkaloids and latex, and has a putative role in detoxifying plant toxins (Brinkmann *et al.*, 2008; Yun *et al.*, 2014; Gao *et al.*, 2019; Gomes *et al.*, 2020; Liu *et al.*, 2020). Additionally, *Enterobacter* contributes to the synthesis of vitamins and pheromones, the degradation of plant compounds and the process of nitrogen fixation (Lilburn *et al.*, 2001; Morales-Jiménez *et al.*, 2012). The higher abundances of *Enterococcus and Enterobacter* at the larval and adult stages implies that they may contribute to *S. litura* nutrient absorption.

Conclusion

This study reveals significant differences in the haemolymph microbiomes of *S.frugiperda* and *S. litura* across developmental stages. *S. frugiperda* predominantly harbored Proteobacteria, while *S. litura* was dominated by Firmicutes. Both species showed increased bacterial diversity in the larval stage, which declined in pupal and adult stages, suggesting a potential loss of key bacterial groups during metamorphosis. Key genera such as *Klebsiella, Acinetobacter, and Enterococcus* were found across developmental stages, indicating their potential role in insect physiology and interactions with their host. These findings provide insights into the microbiome dynamics of these pests and could inform pest management strategies.

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