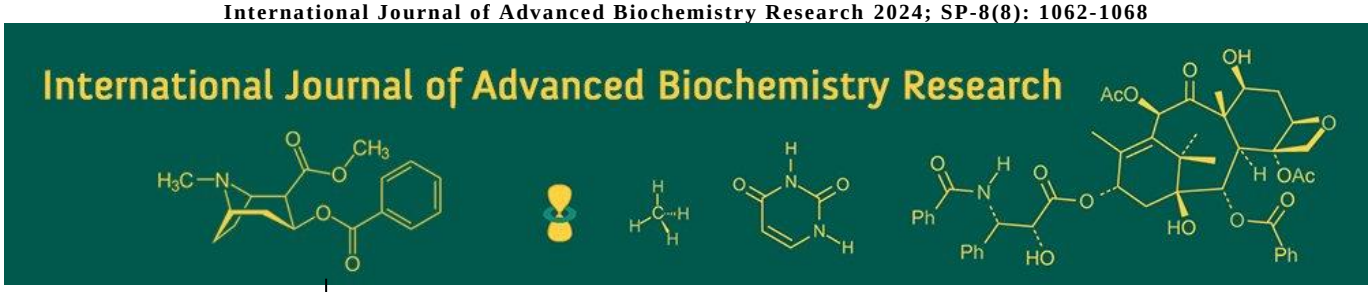

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Symbiotic microbial diversity in the hemolymph of *Spodoptera litura* (Lepidoptera: Noctuidae)

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Abstract
The tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae), is a significant global agricultural pest with a host range of over 120 plant species. While previous studies have focused on the gut microbiota of *S. litura*, this study explores the diversity of the hemolymph microbiota across its developmental stages—larvae, pupae, and adults—using 16S rRNA sequencing. The results reveal a dominance of *Bacillota* and *Pseudomonadota* phyla, with the genera *Klebsiella* and *Mammaliicoccus* consistently present across all stages, suggesting trans-stage transmission. This study highlights the potential role of hemolymph microbiota in the physiology and development of *S. litura* and provides insights into bacterial transmission throughout the insect's life cycle.

Keywords: *Spodoptera litura*, hemolymph microbiota, 16S rRNA sequencing, *Bacillota*, *Pseudomonadota*, trans-stage transmission, insect-microbe interaction, developmental stages

Introduction
The tobacco cutworm, *Spodoptera litura* (Fabr.) (Lepidoptera: Noctuidae), is a global agricultural pest, widespread in countries like Bangladesh, Hong Kong, India, Japan, Malaysia, Philippines, Burma, etc. (Feakin, 1973; Kranz *et al*., 1977) [15, 24]. It is highly polyphagous pest, host range includes around 120 species of vegetable crops as well as other ornamental, weeds and wild plants (Brown and Dewhurst, 1975; Holloway, 1989; Moussa *et al*., 1960) [6, 20, 33], able to feed on many families of plants, including important crops such as cotton, beans, tobacco, vegetables, and rice (Dhir *et al*., 1992; Qin *et al*., 2004; Zhou, 2009; Ahmad *et al*., 2013) [10, 37, 49, 2]. Most of the plant damage is caused due to voracious feeding by caterpillars. Thus, various control measures have been adopted till date to control this pest. Initially, several insecticides were used to control *S. litura* population. This strategy proved successful, but later the pest resisted most of the insecticides including cypermethrin, fenvalerate and quinalphos [Ramakrishnan *et al*., 1984] [38]. Biological practices such as the release of egg and larval parasitoids in agricultural fields have achieved only partial success in the past (Patel *et al*., 1971) [35]. Modern pest management strategies employ microorganisms to control pest populations.

Therefore, there is an urgent need for alternative control methods for *S. litura*. Reports are saying that throughout evolution, insects have hosted a variety of microorganisms in their gut, fat body, exoskeletons, and hemocytes, which offer physiological and ecological benefits to their hosts (Philipp and Nancy, 2013; Jang and Kikuchi, 2020) [36, 22]. Previous studies have begun to explore gut microbial diversity and the function of *S. litura*. Gut microbes have been recognized as a virtual "organ," which substantially impacts on the nutrition, development, life span, reproductive capacity, defense and immune responses (Spor *et al*., 2011; Engel and Moran, 2013; Douglas, 2015; Schwab *et al*., 2016; Akami *et al*., 2019) [41, 14, 12, 39, 3]. For instance, Pantoea agglomerans in the gut of the locust Schistocerca gregaria produces aggregation pheromone by breaking down dietary ingredients to affect the locust aggregation (Dillon *et al*., 2002) [11]. Lacto *Bacillus* plantarum contributes to the systemic growth of Drosophila melanogaster (Storelli *et al*., 2011) [43]. Removal of the gut bacteria represses oogenesis and expedites maternal-to-zygotic-transition in the offsprings of *D. melanogaster* (Elgart *et al*., 2016) [13]. Microbial diversity of *S. litura* across various developmental stages (Li *et al*., 2022; Gichuhi *et al*., 2020; Chen *et al*., 2020; Lu *et al*., 2023) [27, 18, 8, 30].

It has been shown that the diversity of gut microbiota in insects is influenced by factors such as diet (Franzini *et al*., 2016) [16], host taxonomy (Kolasa *et al*., 2019; Huang *et al*., 2021) [23, 21], environmental conditions (Ng *et al*., 2018), and social interactions (Martinson *et al*., 2012) [31].

But, the microbiome of the insect hemolymph has not been reviewed previously. Many entomologists consider hemolymph to be microbe-free, or nearly so, in the healthy insect (Steinhaus, 1947; Lemaitre and Hoffmann, 2007) [42, 26]. This general expectation is based on the prediction that a microbe that gains access to the hemolymph is either eliminated by immunological attack or proliferates without check, leading to septicemia and insect death. There is, however, unambiguous evidence that not all microbial colonists of hemolymph overt pathogens. Some hemolymph microorganisms appear to be resident for long periods up to the full lifespan of the insect host, while others are transient. Although there have been some studies on the gut microbes of *S. litura*, but there is lack of systematic studies on the diversity of haemolymph bacteria. Hence studies need to be conducted on haemolymph endosymbionts of *S. litura*.

This study examined the abundance and diversity of hemolymph microbiota across different developmental stages (Larvae, pupae, and adults) of laboratory-reared *S. litura* using 16S rRNA sequencing. The research offers valuable insights into the hemolymph microbiome throughout the life cycle of *S. litura* and sheds light on the transmission of endosymbionts across developmental stages.

Material and methods

Collection and rearing of experimental insects

The egg masses and larvae of cutworm were collected from infested castor field in GKVK, UAS, Bangalore. The collected egg masses were placed in plastic containers (Size - 25 cm in diameter) with the natural hosts i.e., maize and castor. Once egg hatching occurs larvae started to feed on leaves. Larvae-containing containers were kept at room temperature (26±1 °C, 70±10% RH and 14L: 10D h photoperiod) are reared in groups. Emerged moths were released into the ovipositional cage (35x35x35 cm). The walls of the cage were provided with white paper as a supporting platform for egg laying by the moths and castor leaves also placed inside the cage as substrate for oviposition. A piece of cotton soaked with 10 percent honey solution was provided as a source of food for the adults. The next-generation larvae were used for studies.

Experimental design

From the laboratory-reared population, 10 individuals from each developmental stage (5^{th} instar larvae, pupae, and adults) of *Spodoptera litura* were selected. Each treatment group consisted of three biological replicates. Before the experiment, the 5th instar larvae and adults were starved for 2 hours and immobilized by freezing at -20 °C for 5 minutes. The surfaces of the 5th instar larvae, pupae, and adults were then sterilized by washing with 0.5% NaOCl for 2 minutes, followed by 75% ethanol for 1 minute, and finally rinsed three times with sterilized deionized water (Chen *et al*., 2016) [7].

Collection of hemolymph samples and isolation of bacteria

Haemolymph was collected from the dorsal prothoracic region of 5th instar larvae, pupae, and adults using a sterile, ice-chilled Hamilton needle (Plate 1a, 1b, 1c) and drained into sterile 1.5 mL tubes. A 0.1 mL sample of haemolymph was mixed with 0.9 mL of sterile water in 1.5 mL tubes. Serial dilutions were prepared up to 10^{-7}, and 0.1 mL from each dilution was plated onto nutrient agar (NA) for bacterial isolation and potato dextrose agar (PDA) for fungal isolation. The spread plate technique was used to evenly distribute the samples across the media. The plates were incubated at 28 °C for 24 hours and then examined for bacterial growth. Bacterial colonies displaying different morphological characteristics (Table 1) were picked and pure cultured on NA plates using the quadrant streak method. Pure bacterial colonies were isolated and transferred to NA slants. The slants were incubated at 28 °C until bacterial growth was observed and then stored at -20 °C for future use.

DNA extraction by CTAB method

Total nucleic acid was extracted using the CTAB method. Sterile PBS without insect tissue was used as a negative control during both DNA extraction and PCR amplification to check for reagent and environmental contamination. The integrity and quality of the extracted DNA were assessed using 1% agarose gel electrophoresis and a NanoDrop® ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The 16S rRNA fragment was amplified using a thermo cycler (Eppendorf - vapo. Protect, Germany) with the following primers: forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-ACGGCTACCTTGTTACGACTT-3'). All samples were amplified in triplicate. The PCR products were then subjected to 1% agarose gel electrophoresis (Lee *et al*., 2012) [25] and documented using a gel documentation system (Fig. 1). PCR amplified products were sent to outsourcing for sanger sequencing.

Sequencing of 16S rRNA Gene

Nucleotide sequencing was carried out 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). The raw reads with maximum coverage were deposited in the NCBI GenBank, and accession numbers were obtained for all bacterial strains. A Neighbor-Joining phylogenetic tree of the 16S rRNA sequences was constructed using MEGA 11 software (Version 11.0.13) with 1,000 bootstrap replications.

Trans-stage transmission of bacteria

The transmission of bacteria between developmental stages in insects is a complex and dynamic process, influenced by the specific functions of the bacteria involved. Understanding these dynamics is crucial for studying insect-bacteria interactions. Bacterial transmission may be more prevalent or significant at certain developmental stages. For example, some bacteria may be more abundant in the larval stage due to their role in nutrient acquisition and digestion, while others may be more prominent in the adult stage, aiding in reproduction and dispersal. However, certain bacteria are consistently present across all developmental stages due to their consistent role in all the developmental stages and transmission from one stage to the next. The percentage of bacterial transmission (From larva to pupa and pupa to adult) was calculated using a specific formula.

$$\text{Bacterial transmission (\%)} = \frac{\text{Number of bacterial species transmitted to next stage}}{\text{Total number of bacterial species in current stage}} X\ 100$$

Results and Discussion

16S rDNA sequencing data

Negative controls are essential for identifying potential contamination. In this study, no bacteria were detected in the negative controls, effectively ruling out contamination from environmental or reagent sources. Data sequencing and analysis of 45 samples from all three developmental stages were conducted to study bacterial diversity. Phylogenetic trees were constructed for each stage based on the bacterial sequences obtained.

Hemolymph Microbiota Composition of *S. litura* across Different Developmental Stages

Twenty haemolymph Bacteria isolated from lab-reared *Spodoptera litura*, Phylum *Bacillota* was the dominant with 55 percent followed by *Pseudomonadota* with 45% (Fig. 1a). Orders Enterobacterales (40%) was dominant followed by *Bacillales* (35%), *Lactobacillales* (20%) and Moraxellales (5%) (Fig.1b). family Enterobacteriacae (40%) was dominant followed by Staphylococcaceae (25%), Enterococcaceae (20%), Bacillaceae (10%) and Moraxellaceae (5%) (Fig. 1c). genera Klebsiella was abundant (25%) followed by Enterococcus (20%), *Mammaliicoccus* (15%), Enterobacter and Staphylococcus contributing 10% each and *Bacillus*, Atlantibacter, *Acinetobacter* and Lysini *Bacillus* contributed 5% each (Fig.1d).

Larvae of *Spodoptera litura* found to inhabit Twelve bacterial species belongs to eight genera *Klebsiella* (16.6%), *Staphylococcus* (16.6%), *Enterococcus* (16.6%), *Enterobacter* (16.6%) and *Bacillus*, *Atlantibacter*, *Acinetobacter*, *Mammaliicoccus* contributes 8.33 percent each. Pupa of *Spodoptera litura* found to inhabit four bacterial species belongs to four genera *Klebsiella*, *Mammaliicoccus*, *Enterococcus* and *LysiniBacillus* contribute 25 percent each. Adult of *Spodoptera litura* found to inhabit four bacterial species belongs to three genera *Klebsiella* (50%) *Mammaliicoccus and Enterococcus* with 25 percent each (Fig. 2)

Trans-stage transmission of bacterial species between developmental stages of *S. frugiperda*

S. litura Klebsiella variicola and *Mammaliicoccus sciuri* were found common in all three developmental stages, showing 20 percent transmission from larva to pupa and 50 percent from pupa to adult (Fig.3). This implies that bacterial community members are consistently passed on from one developmental stage to another, possibly leading to the development of a more mutually beneficial relationship between these bacteria and their hosts.

Discussion

Analyzing the diversity of microbial communities presents significant challenges due to the complexity of sampling volume, method, and developmental stage (Li *et al*., 2022). For instance, because pupal and adult stages of *S. litura* contain less hemolymph, a larger number of samples is required for sequencing. Our research has assessed the microbial abundance and diversity in the hemolymph across all developmental stages of laboratory-reared *S. litura***.**

We observed that, *Bacillota* as the dominant phylum followed by *Pseudomonadota*. This finding is consistent with the results of Devi *et al.* (2022) [9], Xiang *et al*. (2006) [46], Xia *et al*. (2020) [45], Chen *et al*. (2016) [7], Snyman *et al*. (2016) [40] who found that Firmicutes (*Bacillota*) was the dominant phylum followed by Proteobacteria (*Pseudomonadota*) and Actinobacteria in the gut of *S. litura*. In the current investigation, Enterobacteriaceae was the predominant family in all the developmental stages (larvae, pupae, and adults) of S.litura. Composed of *Enterobacter cloacae*, *Enterococcus mundtii* and *Enterococcus casseliflavus* encodes the enzymes that are involved in the synthesis of amino acids and the metabolism of carbohydrates this finding is consistent with the results of (Xia *et al.*, 2020; Morales *et al*., 2012; Lilburn *et al*., 2001, Xu *et al*., 2003) [45, 32, 28, 47] 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) [5, 44]. 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) [1]. 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*., 2022) [4, 48, 17, 19, 29]. 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) [28, 32]. The higher abundances of Enterococcus and Enterobacter at the larval and adult stages implies that they may contribute to *S. litura* nutrient absorption.

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, 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) [18].

Table 1: Colony characteristics bacteria isolated from haemolymph of *S. litura*

Sl. No	Isolates	Shape	Colour	Margin	Elevation	Opacity	Branching	Cell shape	Gram staining
5th Instar larva									
1	R1 5f	Circular	Creamy	Smooth	No	Opaque	No	Rod	Negative
2	R2 4c	Circular	Yellow	Smooth	Yes	Opaque	No	Ovoid	Positive
3	R1 4d	Circular	Yellow	Smooth	Yes	Opaque	No	Circular	Positive
4	R1 4c	Circular	Creamy	Smooth	Yes	Opaque	No	Ovoid	Positive
5	R25i	Circular	Cream	Smooth	Yes	Opaque	No	Rod	Negative
6	R1 7g	Circular	Yellow	Smooth	Yes	Opaque	No	Circular	Positive
7	R1 2c	Circular	White	Irregular	Yes	Opaque	No	Circular	Positive
8	R36f	Circular	White	Smooth	Yes	Transparent	No	Rod	Negative
9	R3 4e	Circular	Yellow	Smooth	Yes	Transparent	No	Rod	Negative
10	R3 5i	Circular	White	Smooth	Yes	Transparent	No	Rod	Negative
11	R2 7j	Circular	Yellow	Smooth	Yes	Transparent	No	Globular	Positive
12	R3 2c	Circular	Cream	Smooth	Yes	Opaque	No	Rod	Negative
Pupa									
1	R12a	Circular	Creamy	Smooth	Flat	Opaque	No	Rod	Negative
2	R25L	Circular	White	Smooth	Yes	Transparent	No	Circular	Positive
3	R1 2b	Circular	Yellow	Smooth	Yes	Opaque	No	Circular	Positive
4	R37P	Circular	Brown	Irregular	Yes	Opaque	No	Rod	Positive
Adult									
1	R4 3c	Circular	Creamy	Irregular	Yes	Opaque	No	Rod	Negative
2	R13a	Circular	Creamy	Smooth	Yes	Opaque	No	Ovoid	Positive
3	R2 5d	Circular	Brown	Smooth	Yes	Opaque	No	Rod	Negative
4	R2 4b	Circular	Creamy	Smooth	Yes	Opaque	No	Rod	Negative

Table 2: Culturable bacteria in the haemolymph of different developmental stages of *Spodoptera litura*

Sl. No.	Isolates	Bacterial species	Similarity (%)	Phylum	Class	Order	Family	Accession ID
5th Instar Larva								
1	R1 5f	*Klebsiella variicola*	96.94%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR088578
2	R2 4c	*Enterococcus casseliflavus*	90.90%	*Bacillota*	Bacilli	Lactobacillales	Enterococcaceae	OR074488
3	R1 4d	*Mammaliicoccus sciuri*	98.89%	*Bacillota*	Bacilli	Bacillales	Staphylococcaceae	OR073651
4	R1 4c	*Enterococcus mundtii*	96.41%	*Bacillota*	Bacilli	Lactobacillales	Enterococcaceae	OR073759
5	R25i	*Enterobacter cloacae*	100%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR074442
6	R1 7g	*Staphylococcus gallinarum*	100%	*Bacillota*	Bacilli	Bacillales	Staphylococcaceae	OR073816
7	R1 2c	*Bacillus paramycoides*	100%	*Bacillota*	Bacilli	Bacillales	Bacillaceae	OR074135
8	R36f	*Atlantibacter subterranea*	94.07%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR098641
9	R3 4e	*Acinetobacter rhizosphaerae*	88.09%	*Pseudomonadota*	Gammaproteobacteria	Moraxellales	Moraxellaceae	OR074179
10	R3 5i	*Klebsiella pneumoniae*	98.99%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR074744
11	R2 7j	*Staphylococcus saprophyticus*	95.55%	*Bacillota*	Bacilli	Bacillales	Staphylococcaceae	OR074916
12	R3 2c	*Enterobacter bugandensis*	87.94%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR098503
Pupa								
1	R12a	*Klebsiella variicola*	96.46%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR095020
2	R25L	*Enterococcus faecium*	92.72%	*Bacillota*	Bacilli	Lactobacillales	Enterococcaceae	OR091338
3	R12b	*Mammaliicoccus sciuri*	100%	*Bacillota*	Bacilli	Bacillales	Staphylococcaceae	OR083324
4	R37P	*LysiniBacillus mangiferihumi*	99.49%	*Bacillota*	Bacilli	Bacillales	Bacillaceae	OR088569
Adult								
1	R4 3c	*Klebsiella variicola*	99.41%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR095018
2	R1 3c	*Enterococcus mundtii*	100%	*Bacillota*	Bacilli	Lactobacillales	Enterococcaceae	OR073759
3	R2 5d	*Mammaliicoccus sciuri*	100%	*Bacillota*	Bacilli	Bacillales	Staphylococcaceae	OR079439
4	R2 4b	*Klebsiella pneumoniae*	94.95%	*Pseudomonadota*	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	OR074510

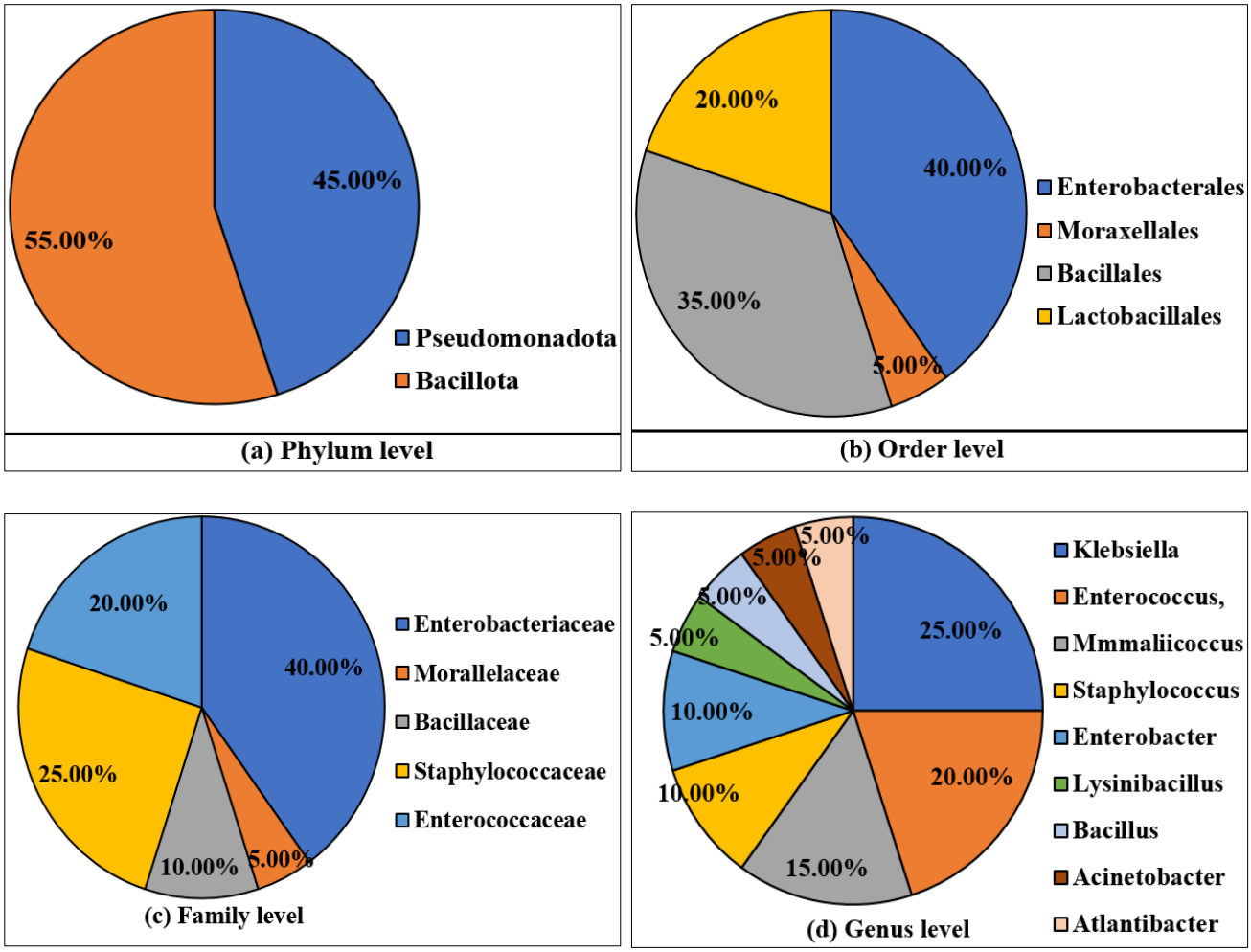

Fig 1: Diversity of bacterial endosymbionts isolated from the haemolymph of developmental stages of *S. litura*

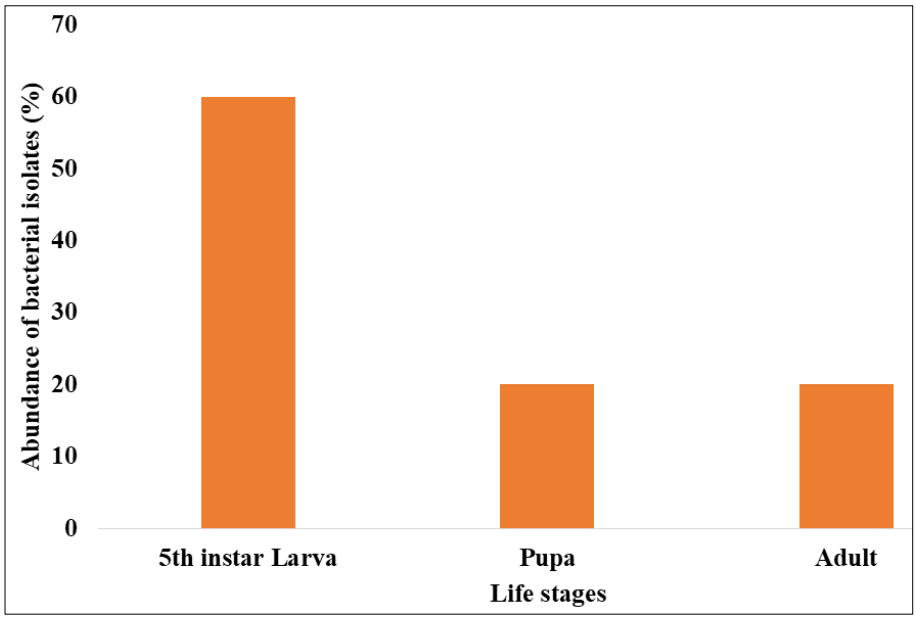

Fig 2: Abundance of bacterial isolates developmental stages of *S. litura*

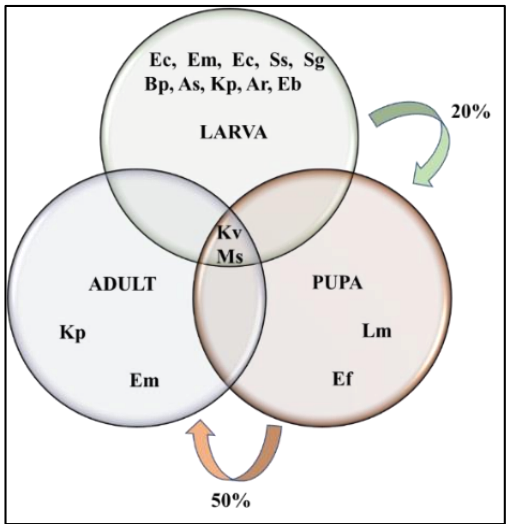

Fig 3: Trans-stage transmission of bacterial species between developmental stages of *Spodoptera litura*

Larva	Pupa
Ec-*Enterococcus casseliflavus*	Lm-*LysiniBacillus mangiferihumi*
As-*Atlantibacter subterranea*	Ef- *Enterococcus faecium*
Em- *Enterococcus mundtii*	Adult
Ar-*Acinetobacter rhizosphaerae*	Em-*Enterococcus mundtii*
Ec- *Enterobacter cloacae*	Kp-*Klebsiella pneumoniae*
Kp- *Klebsiella pneumoniae*	Common isolates
Sg- *Staphylococcus gallinarum*	Kv- *Klebsiella variicola*
Bp- *Bacillus paramycoide*	Ms- *Mammaliicoccus sciuri*
Ss-*Staphylococcus saprophyticus*	
Eb-*Enterobacter bugandensis*	

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Author Contribution Statement
All authors contributed equally to the conception and design of the study. All authors read and approved the final manuscript.

Conflict of interest
No conflict of interest.

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