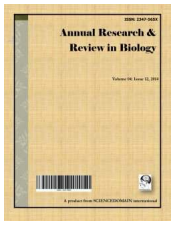

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Diversity of Gut Bacteria in *Spodoptera litura* (F) and *Helicoverpa armigera* (Hubn) Larvae

Hanamant Gadad[1*], A. S. Vastrad[2] and P. U. Krishnaraj[3]

[1]Department of Agricultural Entomology, University of Agricultural Sciences, Dharwad, Karnataka-580005, India.
[2]Agriculture Entomology (DDSW), Agriculture College, Vijaypur, University of Agricultural Sciences, Dharwad, Karnataka-580005, India.
[3]Department of Agriculture Microbiology, Agriculture College, Vijaypur, University of Agricultural Sciences, Dharwad, Karnataka-580005, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author HG conducted an experiment and involved in taking observations, tabulation and writing of the article. Authors ASV and PUK involved in planning, constant monitoring of experiment, tabulation, data analysis and interpretation. All authors read and approved the final manuscript.

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ABSTRACT

Gut bacterial diversity in field and laboratory strain of *S. litura* and *H. armigera* was studied USING Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Results showed that field collected larvae found to be had more diverse of gut bacterial community with greater Shannon diversity index and Operational Taxonomic Units (OUT's) as compared to laboratory strain. Shannon diversity index of field collected *S. litura* and *H. armigera* larvae recorded were 1.89 and 2.60 for the primer pair PRBA338-PRUN518 respectively. While another primer pair E1052-E1193 recorded Shannon diversity index of 2.65 and 2.03 respectively. On the contrary laboratory reared larvae of *S. litura* recorded Shannon diversity index of 1.60 for PRBA338-PRUN518 primer pair and 2.20 for E1052-E1193 primer pair. Shannon diversity index for *H. armigera* was 2.02 and 1.09 against

**Corresponding author: E-mail: hsgadad@gmail.com;*

PRBA338-PRUN518 and E1052-E1193 primer pairs respectively. With respect to Operational Taxonomic Units (OUT's) again field collected test insect larvae shown higher OUT's for both primer pairs. Field collected *S. litura* larvae shown 17.20 OUT's for PRBA338-PRUN518 primer and 13.60 OUT's for E1052-E1193 primer pair. Similarly Field collected *H. armigera* larvae shown 21.00 OUT's for PRBA338-PRUN518 primer and 15.00 OUT's for E1052-E1193 primer pair. Whereas laboratory reared larvae of *S. litura* recorded 9.00 OUT's for PRBA338-PRUN518 primer and 11.40 OUT's for E1052-E1193 primer pair and another test insect *H. armigera* recorded 11.00 OUT's for PRBA338-PRUN518 primer and 7.40 OUT's for E1052-E1193 primer pair. DGGE analysis indicated that field strains of both test insects showed more diversity in gut bacteria as compared to laboratory reared strains.

Keywords: Gut bacteria; Spodoptera litura; Helicoverpa armigera; denaturing gradient gel electrophoresis; diversity.

1. INTRODUCTION

In nature insects harbor bacteria, which occupy right niches in host bodies. Evidences support that interactive relationship between microbiota and their host exists for their survival. As early as 1929, the interaction between the microorganisms and insects inhabiting the gut was recognized [1]. Co-evolution of microorganisms and their insect hosts has led to a stable mutualistic relationship [2,3].

Insect gut presents a distinctive environment for microbial colonization, and bacteria in the gut potentially provide many beneficial services to their hosts. Insects display a wide range in degree of dependence on gut bacteria for its basic functions. Natural gut microflora of insects reflects the state of health of their host. Studies suggest that microorganisms provide essential nutrients or assist in important biochemical functions [4]. Loss of microorganisms often results in abnormal development and reduced survival of the insect host [5]. Most insect guts contain relatively few microbial species compared to mammalian gut, though some insects harbor large gut communities of specialized bacteria. Others are colonized only opportunistically and sparsely by bacteria common in environments. Insect digestive tracts vary extensively in morphology and physicochemical properties, these factors greatly influence microbial community structure [6].

Recently symbiotic relationships and diversity of gut microflora have attracted extensive Meta genomic approach. It is a culture-independent strategy involving direct extraction and cloning of DNA from an assemblage of microorganisms, thereby capturing their genetic diversity in a surrogate host. High-throughput functional screens and sequence-based analysis of metagenomic libraries have led to the identification of novel microbial genes and their products in environmental samples, such as insect gut [7]. Studying these functional genes with two important polypagaous herbivors *Spodoptera litura* (F.) and *Helicoverpa armigera* (Hubn) would be very useful since these herbiovors are experiencing wider adaptation to various hosts and are known to cause considerable yield loss in many commercial crops [8].

2. MATERIALS AND METHODS

Gut bacterial diversity of *Spodoptera* and *Helicoverpa* was studied by selecting pre-established lab population as well as field collected larvae in order to know the difference in the bacterial community of lab reared and field collected test insect larvae following the Denaturing Gradient Gel Electrophoresis (DGGE).

2.1 Gut Dissection and DNA Isolation

Third-instar larvae of the tests insect were surface sterilized for 5 sec in 95% ethanol prior to dissection. Dissecting scissors were used to cut laterally behind the head capsule, and the gut was removed from the cuticle with larval forceps. The whole gut including crop were collected and placed in a 1.5-ml microcentrifuge tube for processing. Samples were placed in a -80℃ freezer. Guts were analyzed individually in all experiments. The total microbial DNA was extracted from individual test insect by followinga protocol modified from the method described by [9] and the whole gut from individual larvae was used. Briefly, guts were homogenized in 500 µl TE buffer (Tris EDTA, 10 mM, pH 8.0) and sonicated (at 30 Amplitude, 1 s pulse), and total

volume was raised to 5.37 ml with TE buffer. The suspension was mixed thoroughly with 600 µl of 10% SDS and 5 µl of 20 mg/ml Proteinase K and incubated for 1 hour at 37℃. One ml of 5 M NaCl was added to each tube, followed by CTAB (Cetyl trimethylammonium bromide) and incubated for 30 min at 65℃. The genomic DNA from a single insect was purified by extraction with phenol: chloroform: isoamylalcohol (25:24:1) and then chloroform: isoamylalcohol (24:1). The DNA was precipitated with isopropanol and recovered by centrifugation and the pellets were resuspended in 100 µl of TE buffer. The quality of the DNA was checked by running on 0.8% agarose gel (Fig. 1) stained with ethidium bromide (0.5 µg/µL) and the DNA suspension was stored at -20℃ until it was used for PCR and further analysis.

2.2 PCR Amplification of 16S rRNA Using Universal Primers

DNA samples were used as template for carrying out PCR. Primer pair used for diversity study is detailed in Table 1 which is known to target different partial variable domains of 16S rRNA gene fragment. The reaction mixture was prepared for final volume of 10 µl which contained 0.25 pmol each of forward and reverse primers, 0.1 mM each of dNTP's, 1X Taq buffer A containing 1.5 mM $MgCl_2$ and 1 unit of *Taq DNA polymerase* (GeNei, India). The PCR was performed in automated thermal cycler (Eppendorf master cycler, Germany) with following PCR programme, initial denaturation of seven minutes at 95℃ followed by denaturation at 94℃ for 45 seconds, annealing at 55℃ for 45 seconds and 45 seconds primer extension for 32 cycles, followed by 10 minutes final extension at 72℃. After completion of PCR, amplified products were analyzed using 1 per cent agarose gel (Figs. 2 and 3).

2.3 Diversity Analysis

2.3.1 Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analysis was carried out to assess the difference in the microbial diversity in the gut of test insect larvae collected from field and lab reared population. Five samples of field and five samples of lab population were used in the study. Partial length 16S rDNA primer with GC clamp, PRBA 338f -PRUN 518r targeting variable V_3 domain and E 1052f-E 1193r targeting V_6 of 16S rRNA gene fragment were used for DGGE analysis [10,11]. The PCR product was subjected to DGGE analysis by following protocol [12]. Acrylamide gel gradient of 8-14% was used for proper migration of bands in the gel. The denaturing gradient maintained was 30-80% using gradient maker. The polyacrylamide gels were prepared with denaturing gradient from 30-80%, where 100% denaturant contained 7 M urea and 40% formamide. DGGE was performed with Ingeny Phor U-2 system (Leiden, The Netherlands). After the denaturant gel was set completely (3 hr), a 5% staking gel was prepared and slowly poured over the denaturant gel avoiding the formation of air bubbles or gaps in between the two gels. Once the staking gel was set, the comb was removed and the whole cassette was placed into a preheated tank buffer containing 1X Tris-Acetate EDTA buffer (TAE buffer, pH-8). The PCR products were mixed with 1 µl of loading dye and loaded onto the staking gel of the DGGE in aliquots of 20 µl per lane. Once the loading dye migrated to a length of staking gel, the tank buffer was kept in a circulating motion. The electrophoresis was performed at a constant voltage of 150 V for 18 h at 60℃ in 1X TAE buffer. After complete run, gels were carefully removed from the unit and transferred to the OHP sheets. Gels were stained by using silver staining protocol as given by [13] using the following four solutions.

I. Fixer solution
II. Impregnation solution
III. Developer solution
IV. Stop solution (Appendix I)

After staining, the gel was dried sufficiently and was analyzed using the SynGene Gene Tools. Bands were scored in the Gene Tools, by giving lowest score to the least intense band in the gel. On the basis of this scored data, Sorenson's similarity coefficient, Shannon's diversity index and Pareto Lorenz evenness curve were calculated to analyze the similarity, diversity and functional organization of species in the gut microbial population.

2.3.2 Shannon-weaver index (H)

The diversity of taxa present in each sample was determined using this index [14], which was calculated using the formula.

$$H = \sum_{i=1}^{S} (\mathbf{pi})[\mathbf{loge}(\mathbf{pi})]$$

Where,

S is the number of OTUs in one sample.
pi is the proportion of that OTU in the sample.

2.3.3 Sorenson's similarity index (Cs)

Similarity in species composition between two samples was determined using this parameter. The overall similarity was calculated as the average of the pair-wise similarities [15].

$$Cs = \frac{2S(A \times B)}{N_A + N_B}$$

Where,

S (A x B) is the number of similar bands in sample A and sample B,

N_A and N_B are the total number of bands in sample A and sample B

Table 1. Shannon diversity index of gut bacteria of *Spodoptera litura* and *Helicoverpa armigera*

Test insects	Primer pair	Field population (F_1)	Lab population (F_5)
Spodoptera litura	PRBA338-PRUN518	1.89	1.60
	E1052-E1193	2.65	2.20
Helicoverpa armigera	PRBA338-PRUN518	2.60	2.02
	E1052-E1193	2.03	1.09

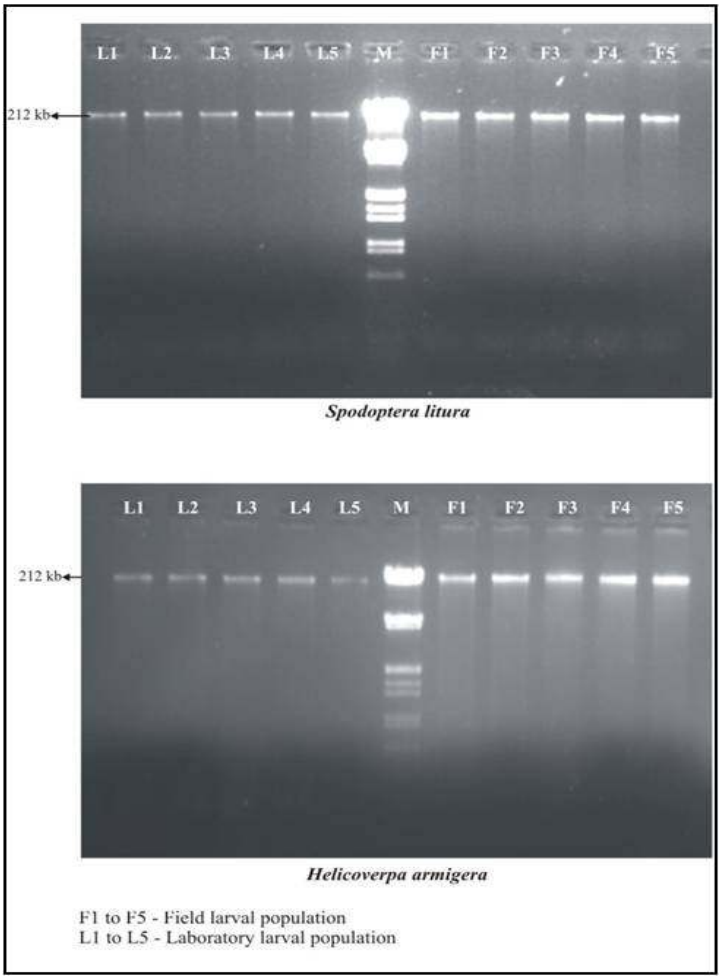

Fig. 1. An ethidium bromide stained agarose gel indicating DNA isolated from the gut of *Spodoptera litura* and *Helicoverpa armigera*

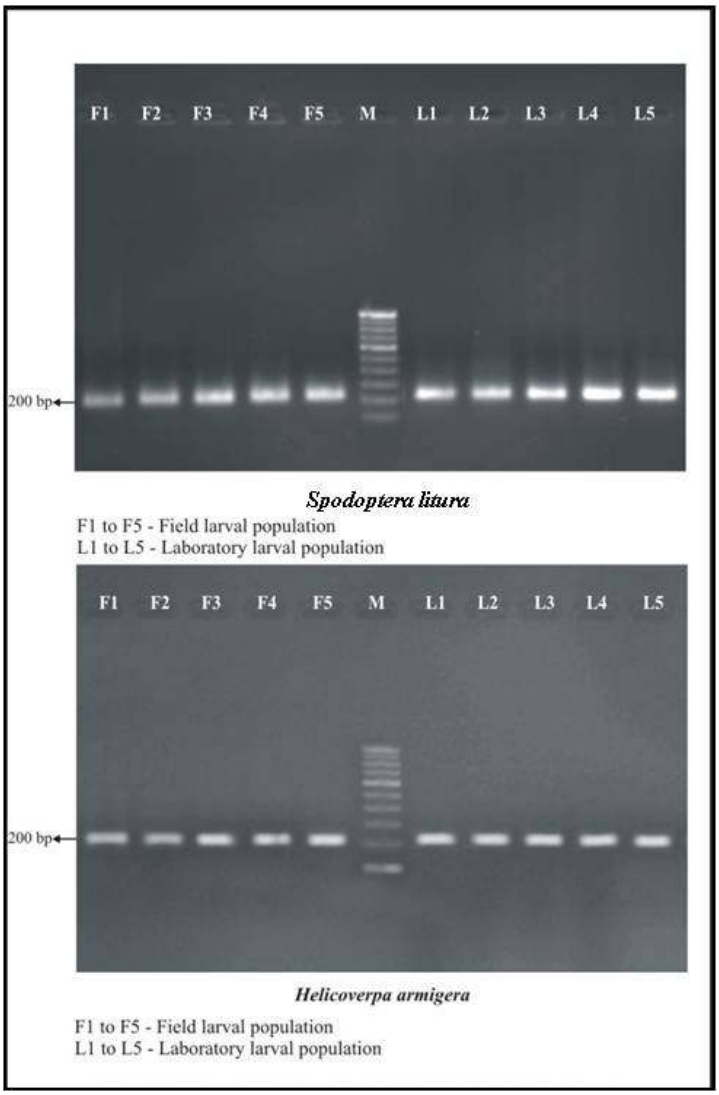

Fig. 2. PCR amplification of v_3 region of 16s rRNA gene of gut bacteria using PRBA-338 and PRUN-518 primer pair

3. RESULTS AND DISCUSSION

Gut microbial DNA obtained was pure enough to get amplification without dilution. The DNA samples subjected to PCR amplification targeting variable region of partial 16S rRNA gene.

Primer pairs PRBA338-PRUN518, and E1052-E1193 showed amplification at their expected size of 180 bp, and 141 bp respectively, indicating that primers targeted the exact region of 16S rRNA gene of gut bacteria isolated from *S. litura* and *H. armigera*.

3.1 Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

PCR amplified product obtained using different primer pairs were separated on the basis of different migration profile on denaturing gradient gel (Figs. 4 and 5). The banding patterns present in the DGGE profile of all the samples were scored using SynGene Tools. Further, the numerical data were analyzed using different statistical tools for its diversity and similarity using Shannon diversity index and Sorenson's similarity index.

3.2 Shannon Diversity Index (H) of Gut Bacteria of *Spodoptera litura* and *Helicoverpa armigera*

Microbial diversity in the field populations of both the test insects *viz*. *S. litura* and *H. armigera* was higher (1.89, 2.65 and 2.60, 2.03 respectively) compared to laboratory population (1.60, 2.20 and 2.02, 1.09 respectively) for PRBA338-PRUN518 and E1052-E1193. However, between the test insects, *H. armigera* recorded higher microbial diversity of 2.60 and 2.02 in field and laboratory population respectively for the PRBA338-PRUN518 primer pair, whereas *S. litura* recorded higher microbial diversity in field and laboratory population for the E1052-E1193 primer pair (Table 1).

3.3 Sorensons Similarity Index between Field and Laboratory Population of *Spodoptera litura* and *Helicoverpa armigera*

Field and laboratory population of *S. litura* recorded Sorensons similarity index of 22.96 per cent for PRBA338-PRUN518 primer pair and for E1052-E1193 the similarity was 14.22 per cent. Similarly, calculated per cent similarity for field and laboratory population of *H. armigera* was 21.22 and 20.09 for PRBA338-PRUN518 and E1052-E1193 primer pairs respectively (Table 2).

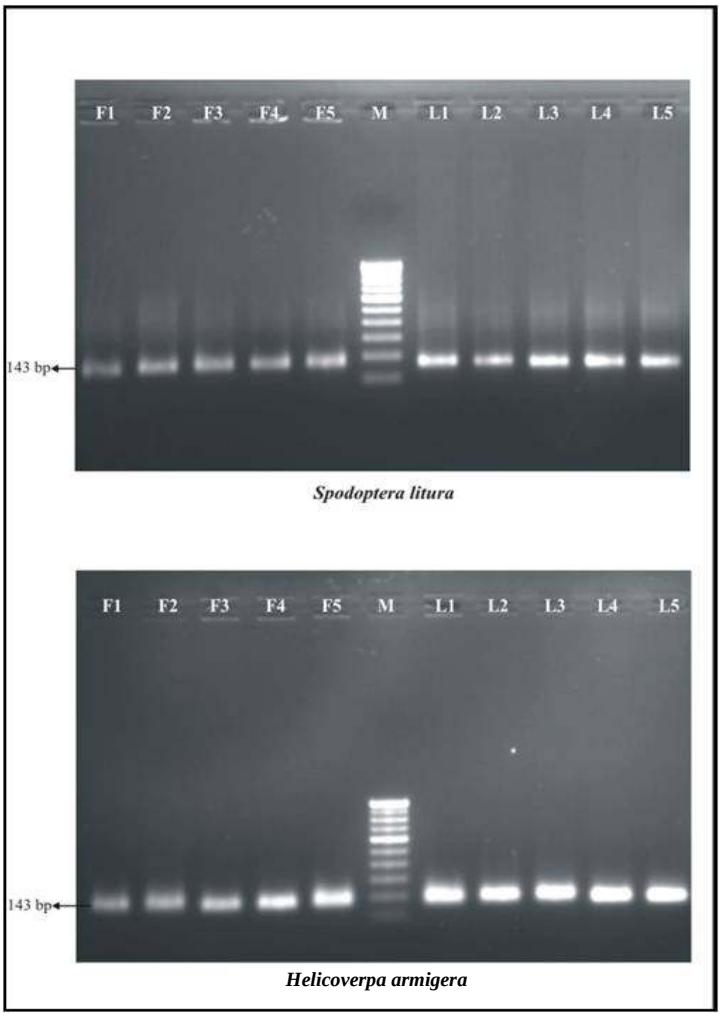

Fig. 3. PCR amplification of v_7 region of 16s rRNA gene of gut bacteria using E-1052 and E-1193 primer pair

3.4 Gut Bacteria OTUs in the Field and Laboratory Population of *Spodoptera litura* and *Helicoverpa armigera*

DGGE analysis indicated that field population of *S. litura* recorded higher number of gut bacterial OTUs of 17.20 and 13.60 for both primer pairs PRBA338-PRUN518 and E1052-E1193 respectively. The laboratory population however, recorded comparatively lower number of OTUs in the both PRBA338-PRUN518 and E1052-E1193 primer pairs with 9.00 and 11.40 OTUs respectively. Similarly *H. armigera* recorded more number of OTUs in field population (21 and 15.40) than laboratory population (11 and 7.40) respectively for both primers pair PRBA338-PRUN518 and E1052-E1193. Comparative DGGE analysis between *S. litura* and *H. armigera* indicated that irrespective of primers pair used *H. armigera* recorded more number of OTUs in both field and laboratory population (Table 3).

Table 2. Sorensons similarity index between field and lab population of *Spodoptera litura* and *Helicoverpa armigera*

Test insects	Primer pair	Sorensons similarity index (%)
Spodoptera litura	PRBA338-PRUN518	22.96
	E1052-E1193	14.22
Helicoverpa armigera	PRBA338-PRUN518	21.22
	E1052-E1193	20.09

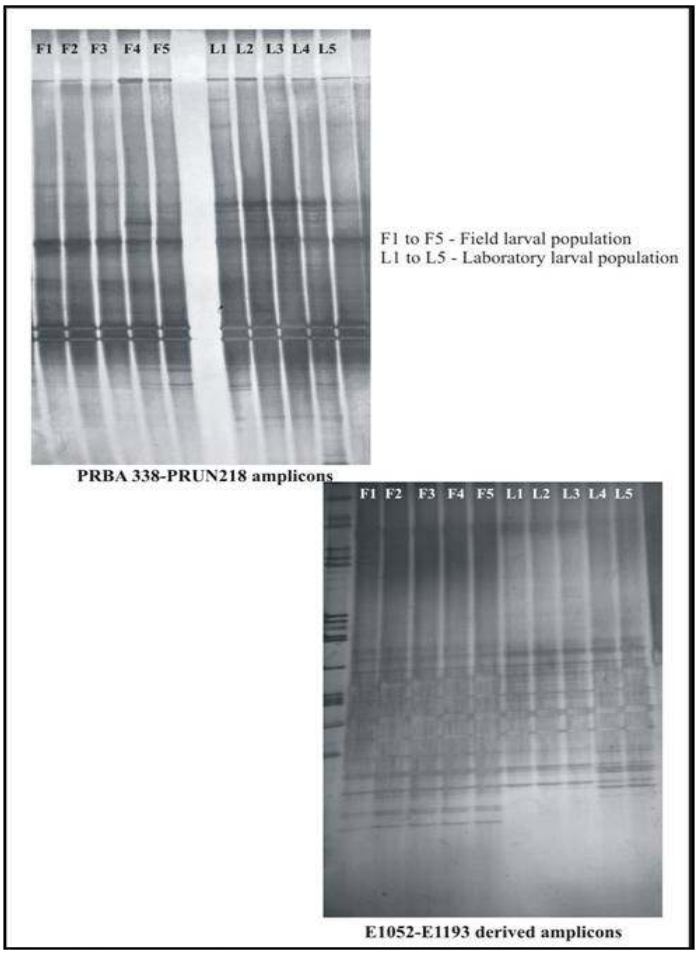

Fig. 4. Denaturing Gradient Gel Electrophoresis (DGGE) profiles of gut bacteria isolated from *Spodoptera litura*

Table 3. Gut bacteria OTUs in the field and lab population of *Spodoptera litura* and *Helicoverpa armigera*

Test insects	Primer pair	OTUs	
		Field population	Lab population (F_5)
Spodoptera litura	PRBA338-PRUN518	17.20	9.00
	E1052-E1193	13.60	11.40
Helicoverpa armigera	PRBA338-PRUN518	21.00	11.00
	E1052-E1193	15.40	7.40

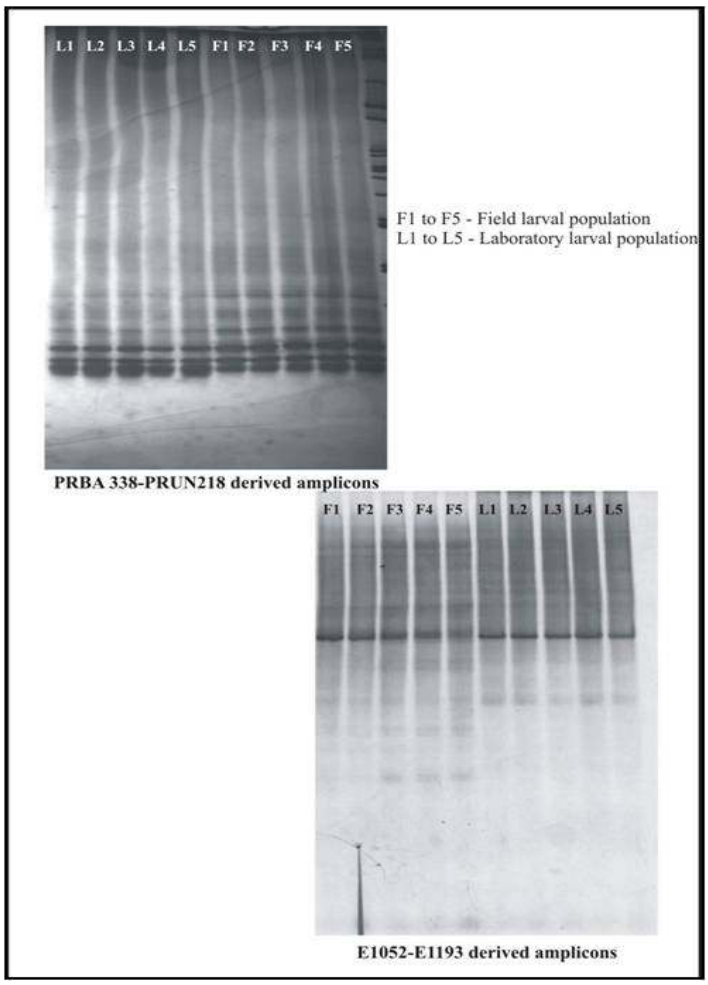

Fig. 5. Denaturing Gradient Gel Electrophoresis (DGGE) profiles of gut bacteria isolated from *Helicoverpa armigera*

DGGE analysis indicated that field population of both the test insects recorded higher gut bacterial diversity by recording the higher Shannon's diversity index and higher operational taxonomic units (OTUs) compared to laboratory reared larval population. Sorenson's similarity index revealed that the gut bacterial communities were not similar between field and laboratory population of *S. litura* and *H. armigera* larvae. These results indicate that field larval population was more diverse in gut bacterial community than the laboratory larval population of test insects.

Low diversity of the gut bacteria community in laboratory population was mainly due to single host plant in which laboratory population was reared. Field environments are more complex

than the laboratory. Because of the polyphagous nature of *S. litura* and *H. armigera*, they are likely to be exposed to a wider range of microbes in their natural environment. Since these two test insects are polyphagous in nature known to feed on variety of host plants, it may influence the composition of gut bacterial community. Results of the present findings are supported by [16] who reported that gut bacteria diversity of Lepidopteran larvae is influenced by the hosts they feed upon. Similarly field collected *S. litura* larvae recorded higher diversity of bacterial community than the laboratory reared larval population [17] and differential activity in their midgut originating biochemical enzymes and polymorphism in its gene [18].

Field collected *H. armigera* larvae harbored diverse group of gut bacteria compared to laboratory reared larvae also reported that field collected *H. armigera* larvae varied significantly compare to laboratory reared larvae. Similarly, laboratory and field collected larvae of *Heliothis virescens* (Fabricius) shared no OTUs in common. Laboratory larval population was dominated by two OTUs classified as *Enterococcus* sp. (Lactobacillales) and *Asaia* sp. (Rhodospirillales), whereas larvae of the field population did not contain any OTUs affiliated with these two genera. In fact, field collected larvae did not contain any dominant OTUs, but harbored higher percentage of Enterobacteriales, Burkholderiales and Rhizobiales [19-21].

4. CONCLUSION

Results of the present study give new insight to understand the variability of the gut bacteria between the field and laboratory strains of *S. litura* and *H. armigera.* Further this information can be used for the better understanding of influence of diet on the diversity of gut bacteria and what are the roles these gut bacteria plays in the host metabolism.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX I

Denaturant gradient gel electrophoresis

Sl. no	Preparation of denaturant gradient gel		
	Components	**Solution A 80 % denaturant – 8 % gel**	**Solution B 30 % denaturant – 8 % gel**
1	45 % polyacrylamide	7.8 ml	17.8 ml
2	50X TAE	2 ml	2 ml
3	Formamide	32 ml	12 ml
4	Urea	42 g	12.6 g

Note: The final volume made up to 100 ml of solution A and solution B using nanopure water

Sl. no	Preparation of staking gel	
	Components	**Volume (in µl)**
1	45% polyacrylamide	1111
2	50X TAE	200
3	20% APS	50
4	TEMED	5
5	Nanopure water	8620

Sl. no	Preparation of silver staining solutions	
	Solutions used	**Reagents**
1	Fixer	100 ml ethanol and 5 ml glacial acetic acid
2	Wash solution	Deionised water
3	Impregnation solution	1.5 gm $AgNO_3$ and 1 ml Formaldehyde
4	Rinse	Deionised water
5	Developer	15 gm NaOH and 2 ml Formaldehyde
6	Stop solution	100 ml ethanol and 5 ml glacial acetic acid

Note: Make up the volume of each solution to 1000 ml by using nanopure water

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