RESEARCH PAPER

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16S rRNA gene based identification of gut bacteria from field collected larvae of *Helicoverpa armigera* (Lepidoptera: Noctuidae) from tomato ecosystem

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Tomato fruit borer *Helicoverpa armigera* (Hübner) is a polyphagous pest of different host plants and has developed resistance to most of the insecticide groups. In order to know the gut microbial flora of the insecticide resistant field collected larvae of *H. armigera*, whole gut was dissected from the fourth to fifth instar larvae of the *H. armigera* and the culturable bacterial species were identified by sequence analysis of 16S rRNA gene. Altogether eleven bacterial species of different genera were identified were *Stenotrophomonas* sp., *Enterococcus casseliflavus, Enterococcus* sp., *Enterococcus gallinarum, Enterococcus feacium, Bravundimonas diminuta, Staphylococcus* sp., *Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Bacillus subtilis and Rhodococcus* sp., of which genera *Enterococcus* were found to be predominant. The nucleotide sequences of 11 isolates were submitted to NCBI-Gen Bank and accession numbers (HM446252, HM446253, HM446254, HM446256, HM446258, HM446260, HM446261, HM446263, HM446264, HM446265, and HM446266) were obtained.

Key words : *Helicoverpa armigera,* Gut bacteria, 16S rRNA, Nucleotide, Sequencing

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INTRODUCTION

Microorganisms play a key role in both host physiology and nutrition (Dillon and Charnley 1995; Nardi *et al.*, 2002). Bacteria and insects have evolved a diverse array of symbiotic interactions, which play a role in insect nutrition (Bernays and Klien, 2002; Bracho *et al.,* 1995; Douglas, 1988; Douglas and Prosser, 1992; Lal, *et al.,* 1994; Wicker, 1983), defence (Ferrari *et al.,* 2004; Kellner and Dettner, 1996; Oliver *et al.,* 2003; Piel, 2002), reproduction and development (Caspari and Watson 1959; Gherna *et al.,* 1991; Hurst *et al.,* 1999). Bacteria are associated with a number of different insect species across all major orders of the insects (Buchner, 1965; Dillon and Dillon 2004). The insect gut provides a suitable habitat for bacteria (Bignell *et al.,* 1984). In many insect species the gut possess different types of bacteria, which are transient and do not remain in the gut during all life stages. However, in some cases, a variety of permanent microorganisms are present that supply essential nutrients to their host and some posses obligate microbial endosymbionts that benefit the insects (Bridges, 1981).

Although cultivation based biochemical techniques have been used for analysis of the specific groups of bacteria, several limitations are associated with such approaches, particularly for surveying intestinal bacterial ecosystem. The introduction of high resolution molecular techniques has improved the analysis of diverse microbial populations (Muyzer, 1999). The important advance has been the use of 16S rRNA as a molecular finger print to

identify and classify organisms (Ohkuma and Kudo, 1996). Until recently little was known about the bacteria associated with Lepidoptera, those studies on Lepidopteron gut microbiota suggested the possibility that microorganisms provided essential nutrients or assisted in important biochemical function related to host food ingestion (Broderick *et al.,* 2004)

The cotton bollworm or tomato fruit borer *Helicoverpa armigera* (Hübner) is a polyphagos lepidopteron pest that infests important crops like cotton, tomato, sunflower and corn all over the world. The fifth and sixth instar larva of *H. armigera* feeds voraciously and damages agricultural crops and hence reduces the yield (Sarode, 1999). Tomato (*Lycopesicon esculentum* Mill.) is one of the most important vegetables grown in the world, which is good source of vitamins. A wide range of insects attack tomato and forms major limiting factor in its successful cultivation and improvement in yield. Among them fruit borer, *H. armigera* is the most destructive insect pest causing the loss in tomato yield to the tune of 50 to 80 per cent (Tewari and Krishnamoorthy, 1984). Control measures are difficult because the larvae feed inside the host plant and are difficult to kill with insecticides and also have gained resistance to variety of insecticides (Kranthi *et al.,*2001). Knowledge of the gut microbiota of the tomato fruit borer and the roles, it might play in the larval biology may lead to new target for the management of the pest.

In the present study, we isolated gut microbes from insecticide resistant fourth to fifth instar field collected larvae of *H. armigera*. The DNA extracted from the microbes was amplified in PCR for 16S rRNA gene. The gene was partially sequenced and the gut microbial communities were identified using NCBI databases.

RESEARCH METHODOLOGY

Collection of field larvae:

The larvae of *H. armigera* were collected during June 2009 in the tomato fields of Mallur, (Karnataka-Lat-13º43'60N; Lon-75º19'60 E), India, the crops were frequently sprayed with insecticides by the farmers. Fourth to fifth instar larvae which were collected in the plastic container brought to the laboratory and was immediately used for isolation of gut microbial flora.

Isolation of gut bacteria:

Prior to dissection, ten larvae of *H. armigera* were immobilized by chloroform (100%) and sterilized in 0.1% sodium hypochlorite and 70% aqueous ethanol for five seconds to remove the adhering contaminants (Gebbardi *et al.,* 2001). Such larvae were dissected and the entire gut was removed under aseptic conditions in laminar air flow hood. The gut was placed in a micro tube containing 500 µl of sterile peptone water, crushed mechanically and vortexed thoroughly. Hundred µl of the gut homogenate was plated on sterile nutrient agar and nutrient glucose agar plates in replicates and incubated at 30^0C for 48 h. The colonies obtained after 48 h incubation in the plates were further screened for colony morphology.

Identification of colony morphology:

Eleven colonies isolated from 48 h nutrient agar and nutrient glucose agar plates were further sub cultured on fresh sterile nutrient agar plates by streak plate method and again incubated at 30^0C for 48 h. All eleven pure isolated colonies in nutrient agar plates were Gram stained and colony characteristics were analysed by visual investigation and light microscope (Labmed, Binocular) and the characteristics were tabulated (Table 1).

Bacterial DNA isolation:

Bacterial genomic DNA was isolated as per the standard protocol (Hoffman and Winston, 1987). Single colony from each of the fifteen bacterial cultures was inoculated in nutrient broth and grown for 48 h at 30ºC. Cells were harvested from 5 ml of the culture and to this 100 µl of lysozyme was added and incubated at RT for 30 min, followed by the addition of 700 µl of cell lysis buffer (Guanidium isothiocyanate, SDS, Tris-EDTA). The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked transparent. Seven hundred µl of isopropanol was added on top of the solution. The two layers were mixed gently till white strands of DNA were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 100 µl of 1X TE buffer. The quality of the DNA was checked by running on 0.8% agarose gel stained with ethidium bromide (0.5 µg/µl). A single intense band with slight smearing was noted. The extracted genomic DNA of the 11 bacterial isolates was used as template DNA for amplification of the 16S rRNA gene.

Oligonucleotide primers:

16S rRNA gene primers were procured from Aristogene Biosciences (P) Ltd, Bangalore. The oligonucleotides were reconstituted to 100 ng/ µl stocks in sterile TE buffer. The primers were used at working concentration of 100 ng/ µl in sterile filtered distilled water. The sequence of the primers were as follows:

Forward primer – 5'-ACTCCTACGGGAGGCAGCAG-3'
Reverse primer – 5'-ATTACCGCGGCTGCTGG-3'

Amplification of 16S rRNA gene by PCR:

Optimum annealing temperature was determined by employing gradient PCR. Amplification reaction was performed in 0.5 ml tubes. Individual reaction (50 µl) contained 100 ng of the extracted DNA, 1X PCR assay buffer (250 mM Tris-HCl, 10 mM KCl, 1.5 mM $MgCl_2$), 100 mM dNTP's, 100 ng/ µl each of forward and reverse primers, 1 unit of *Taq* DNA polymerase (Sigma, USA). PCR was performed with forward and reverse primers with an initial denaturation for 2 min at 94 ºC, followed by 30 cycles of 94ºC denaturation for 1 min, 58ºC for annealing for 30 s and extension at 72ºC for 1 min 30 s. Finally the reactions were healed at 72ºC for 5 min. Specific and optimum amplification of the gene was seen at 58ºC of annealing temperature. Subsequently the gene was amplified at 58ºC and the amplified PCR product (1.5 kb) was purified from low melting agarose gel, stained with ethidium bromide (0.5 µg/µl) as per the standard protocols (Sambrook *et al.,* 2001) for further sequencing.

Sequence and Sequence Analysis:

Sequencing of the 16S rRNA gene of all the 11 bacterial isolates was done at sequencing facility of Aristogene Biosciences (P) Ltd, Bangalore, India from both the directions. The sequence obtained was subjected to BLAST search and the bacterial species were determined. The percentages of sequence matching were also analysed and the sequences were submitted to NCBI-Gen Bank and obtained accession numbers.

Phylogenetic tree construction:

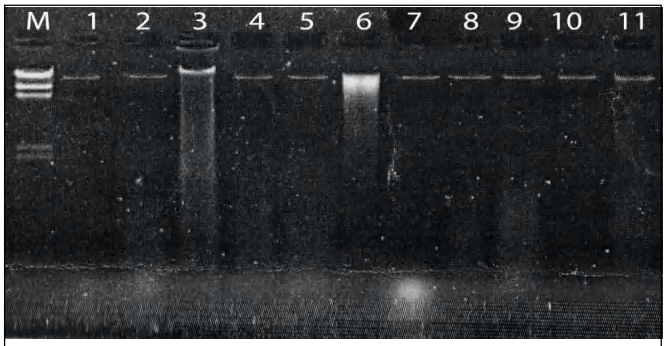
The phylogenetic tree was constructed using CLUSTAL-W bioinformatics tool and the similarity relatedness between the isolates were analysed.

RESULTS AND ANALYSIS

The results obtained from the present investigation are summarized below:

Bacterial isolation and characterisation:

Eleven randomly isolated colonies on nutrient agar plates were characterized and sub cultured to obtain pure cultures for Gram staining. Gram staining of the 11 isolates using light microscopy showed that 6/11 isolates were Gram positive cocci, 4/11 isolates were Gram negative rods and 1/11 isolates were Gram positive rods.



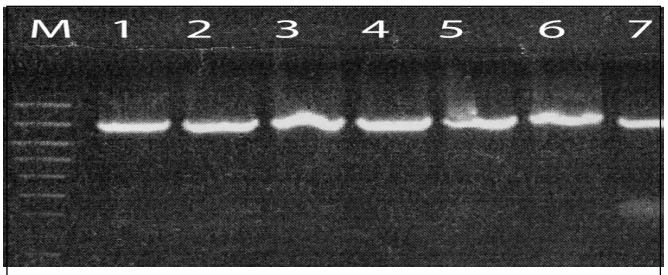
Lane M –Marker - Lambda/HindIII digest (sizes-23130, 9416, 6557, 4361, 2322, 2027, 564). Lane 1 – 15 – Genomic DNA from 11 bacterial isolates.

Fig. 1: Agarose gel electrophoresis of genomic DNA from eleven bacterial isolates

Table 1: Colony characteristics and gram nature of 11 pure gut microbial isolates from *H. armigera* on nutrient agar plates

Sr. No.	Isolate\ code	Shape	OD	Margin	Colour	Size	Elevation	Surface	Gram Nature
1.	HT1	Irregular	Opaque	Lobate	White	1mm	Flat	Mucoidal	Gram -ve cocci
2.	HT2	Irregular	Opaque	Lobate	Off white	2mm	Flat	Mucoidal	Gram +ve cocci
3.	HT3	Round	Opaque	Entire	White.	1mm	Convex	Smooth	Gram +ve cocci
4.	HT5	Rond	Opaque	Entire	Creamish	2mm	Flat	Mucoidal	Gram +ve cocci
5.	HT7	Irregular	Translusent.	Lobate	Brown	1mm	Raised	Smooth	Gram -ve rods
6.	HT9	Irregular	Opaque	Lobate	White	1mm	Raised	Smooth	Gram +ve cocci
7.	HT10	Round	Opaque	Lobate	Yelllowish	2mm	Raised	Smooth	Gram +ve cocci
8.	HT12	Pinpoint	Transluscent	Lobate	Creamish	1mm	Convex	Smooth	Gram –ve rods
9.	HT13	Irregular	Transluscent	Entire	White	1mm	Flat	Smooth	Gram --ve rods
10.	HT14	Irregular	Opaque	Lobate	Off white	3mm	Flat	Rough	Gram +ve rods
11.	HT15	Pinpoint	Opaque	Lobate	Brick red	1mm	Convex	Smooth	Gram +ve cocci

HT1-3, 5-7, 9, 10, 12-15 –Gut bacterial isolate from *Helicoverpa armigera* from tomato ecosystem.



Lane M - Marker - 0.1-2 k blow range marker, (sizes-100bp, 200bp, 300bp, 600bp. 1kb,1.5kb, 2 kb). Lane 1 – 11 – 1.5 kb 16S rRNA PCR amplicon from eleven bacterial isolates. 1=HT1, 2=HT2, 3=HT3, 4=HT5, 5=HT7, 6=HT9, 7=HT10, 8=HT12, 9=HT13, 10=HT14, 11=HT15.

Fig. 2: Agarose gel electrophoresis of 16S rRNA PCR amplicon from eleven bacterial isolates

Amplification of 1.5 kb 16S rRNA gene by PCR:

The presence of genomic DNA from all the 11 isolates was confirmed on 0.8% agarose gel stained with etihidium bromide (Fig. 1). An intense single band was seen in all the 11 wells along with the DNA marker. The extracted DNA was used as template for amplification of 16S rRNA gene.. The primers selected were specific. Initial standardisation by many gradient PCR has facilitated the specific amplification as observed by high intense band. The optimum annealing temperature was found to be 58^0C. An intense single band of size approximately 1.5 kb was visible on 1% agarose gel stained with ethidium bromide (Fig. 2) in all the 11 wells.

Sequence analysis, bacterial identification and phylogeny analysis:

The PCR amplified 16S rRNA gene from all the 11 isolates was gel eluted and was partial sequenced using forward and reverse primers, at sequencing facility of Aristogene Biosciences (P) Ltd., Bangalore, India. The partial sequence obtained from all the 11 isolates ranged from 852, 621, 616, 810, 681, 676, 840, 658, 706, 625, 834 bp, respectively in length and were analysed in BLASTn (www.ncbi.nlm.nih.gov) and the bacterial genera and species were determined. The partial 16S rRNAs sequence and the determined bacterial spp. along with the accession number have been shown in Table 2. The max identity of the sequence was 99-100%. The nucleotide sequences of 11 isolates were submitted to NCBI-Gen Bank and the accession numbers were obtained (Table 3). The determined bacterial communities were found to be *Stenotrophomonas* sp., *Enterococcus casseliflavus, Enterococcus* sp., *Enterococcus gallinarum, Bravundimonas diminuta, Enterococcus feacium, Staphylococcus* sp., *Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Bacillus subtilis and Rhodococcus* sp., of which genera *Enterococcus* were found to be predominant. The sequences of the 11 bacterial isolates were used for the construction of the phylogenetic dendrogram to know the genetic relatedness between the bacterial isolates. The dendrogram showing the relation between the bacterial species is shown in Fig. 3. The dendrogram showed genus *Enterococcus* and *Staphylococcus* were closely related and genus *Stenotrophomonas*, *Pseudomonas* and *Bravundomonas* were closely related, similarly genus *Acinetobacter*, *Bacillus* and *Rhodococcus* were closely related to each other.

In the present study the identified bacterial isolates were found to be the inhabitants of many insect species. Thakur *et al.* (2005) have isolated *Bacillus* sp., *Pseudomonas* sp. from the gut of the *Discladispa armigera* (Olivier). Mishra and Tandon (2003) have reported presence of *Staphylococcus* sp., *Pseudomonas stutzeri, Enterobacter aerogens,* and *Bacillus subtilis*, from the gut of third instar larvae of *H. armigera*. Bacterial isolates from the gut of *H. armigera Enterococcus casseliflavus, Enterococcus gallinarum, Pseudomonas* sp. *Acinetobacter* have been reported from China (Hui Xiang *et al.,* 2006). In the present study out of the 11 bacterial isolates determined genera *Enterococcus* was predominant, this was in accordance with the studies on microbial communities present in the mid gut of *H. armigera* where in *Enterococcus* was predominant (84%) (Hui Xiang *et al.,* 2006). Presence of genera *Stenotrophomonas* sp. have been reported as midgut bacteria from the field *Anopheles* mosquitoes

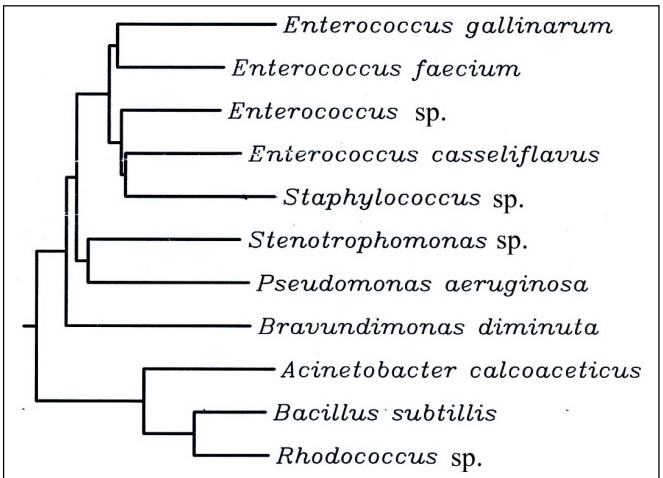


Fig. 3: Phylogenetic dendrogram of the bacterial isolates from the gut of *H. armigea*

Table 2: Bacterial species of 11 isolates determined with partial 16S rRNA gene sequence from BLAST search

Isolate	Partial 16S rRNA gene sequence	Identified bacteria By BLASTn	Max identity
HT1	AGTCGAACGGCAGCACAGTAAGAGCTTGCTCTTATGGGTGGCGAGT GGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGA TAACGTAGGGAAACTTACGCTAATACCGCATACGACCTTCGGGTGA AAGCAGGGGACCTTCGGGCCTTGCGCGGATAGATGAGCCGATGTCG GATTAGCTAGTTGGCGGG…	*Stenotrophomonas* sp. (HM13683)	100%
HT2	AACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA CACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGG AAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCG GTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATG CATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG……...	*Enterococcus casseliflavus* (GU904691)	100%
HT3	TGCAAGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAG AAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAT CAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAC TATTTTCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGAT GGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAAC……	*Enterococcus* sp. (GU827515)	100%
HT5	CCCTTAGAGTTTGATTCCTGGCTGAGGACGAACGCTGGCGGCGTGCC TAATACATGCAAGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCA CCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAAC CTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCG TATAACACTATTTTCCGCA……..	*Enterococcus gallinarum* (FN821377)	100%
HT7	TCGAACGGACCCTTCGGGGTTAGTGGCGGACGGGTGAGTAACACGT GGGAACGTGCCTTTAGGTTCGGAATAGCTCCTGGAAACGGGTGGTA ATGCCGAATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGCGG CCCGCGTCTGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG ACGATCAGTAGCTGGTCTGAGAGGATGACCAGCCACACT……	*Bravundimonas diminuta* (GU397389)	100%
HT9	TGCAAGTCGACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGA AAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTACCTGCCCATCA GAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACTA TTTTCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGG ATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACG……..	*Enterococcus faecium* (GU460391)	100%
HT10	AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATA CATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTA GCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTG GGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATATTGAAC CGCATGGTTCAATAGTGAAA……	*Staphylococcus* sp. (GU797289)	100%
HT12	TGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCG GACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAAC GTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGT GGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATT AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATC……..	*Pseudomonas aeruginosa* (HM036358)	100%
HT13	GTCCTCCTTGCGGTTAGACTACCTACTTCTGGTGCAACAAACTCCCA TGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC GGCATTCTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCGA GTTGCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGCAT	*Acinetobacter calcoaceticus* (FJ867364)	100%
HT14	GGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACT CTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCA CCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAG TCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATT GGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGCCCATT……	*Bacillu subtilis* (AB501113)	100%
HT15	GGGGTTAGGCCACCGGCTTCGGGTGTTACCGACTTTCATGACGTGAC GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCT GATCTGCGATTACTAGCGACTCCGACTTCACGGGGTCGAGTTGCAGA CCCCGATCCGAACTGAGACCGGCTTTAAGGGATTCGCTCCACCTCAC GGTATCGCAGCCCTCTGTACCGACCATTGTAGCATGTGT……	*Rhodococcus* sp. (HMOO4214)	100%

Table 3: Accession number obtained from Gen Bank for 11 isolates

Sr. No.	Isolate	Bacteria determined from BLASTn	Gen Bank Accession Number Obtained
1.	HT1	*Stenotrophomonas* spp.	HM446252
2.	HT2	*Enterococcus casseliflavus*	HM446253
3.	HT3	*Enterococcus* spp.	HM446254
4.	HT5	*Enterococcus gallinarum*	HM446256
5.	HT7	*Bravundimonas diminuta*	HM446258
6.	HT9	*Enterococcus faecium*	HM446260
7.	HT10	*Staphylococcus* spp.	HM446261
8.	HT12	*Pseudomonas aeruginosa*	HM446263
9.	HT13	*Acinetobacter calcoaceticus*	HM446264
10.	HT14	*Bacillu subtillis*	HM446265
11.	HT15	*Rhodococcus* spp.	HM446266

(Jenney *et al.,* 2005). *Stenotrophomonas maltophilia* were also found in one of the caterpillar mid gut microflora (Hui Xiang *et al.,* 2006). Genera *Bravundimonas* and *Rhodococcus* have not been reported in any of the insect groups.

There have been studies on the gut microbial flora of Lepidoptera (Broderick *et al.,* 2004). In current study we studied the gut flora of *H. armigera* using culture dependent methods. Seven genera of bacteria were identified in the field collected insecticide resistant larvae and the gut microbial communities were quite complex, consisting mostly *Enterococcus*. *Enterococcus* sp. is common members of the gut microbial communities in insect and other animals (Reeson *et al.,* 2003; Broderick *et al.,* 2004). Mead *et al.,* (1988) also found that *Enterococcus* were common in the gut of the grass hopper *Melanoplus sanguinipe*. Despite the possible influence of food or environment on gut microbial diversity, present study revealed that *Enterococcus* was the dominant member of the gut microbial flora. *Acinetobacter* are commonly found in soil, plant, animal and water systems and there are reports on their capacity to degrade large molecules such as polycyclic aromatic hydrocarbons (Lei *et al.,* 2004) or pesticides such as polychlorinated compounds (Hao *et al.,* 2002). In field conditions *H. armigera* are polyphagos in nature and are exposed to several complex factors like pesticides and *H. armigera* may be acquiring wide range of microbes from various host plants. They might consume diverse variety of phytochemicals and are shown to have most diet-related plasticity by means of complicated mechanisms of altering their gut composition (Patankar *et al.,* 2001). Both conditions may be challenging with their associated microorganisms. Ingestion of toxic compounds might result in their detection by bacteria that metabolise such compounds and therefore these bacteria help the insects in degrading the ingested compounds (Liebhold *et al*., 1995). However, interaction between gut microbe and insect host should not be simply regarded as helping nutritional balance or overcoming the insect pathogens. A more complicated polytrophic interaction between the insect or plant or animal host were taken into consideration by Dillon and Dillon (2004), who analysed that diverse group of microorganism inhabit in gut of *H. armigera* in the field environment, but their role in the host interaction is unclear. However, if they have functional significance with regards to the detoxifying any toxic compounds, physiology and nutrition of the cotton bollworm or tomato fruit borer *H. armigera* remains to be further studied.

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