Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 6 Special issue [2] 2017: 441-451

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804
NAAS Rating 4.95

FULL LENGTH ARTICLE **OPEN ACCESS**

Diversity of culturable and unculturable gut bacteria associated with field population of *Spodoptera litura* (Fab.)

Sanjay Yalashetti, Mahesh Shankarappa Yandigeri*, Onkarappa Rudrappa[2], Mohan Muthugounder[1], Sivakumar Gopalasamy[1]
[1]ICAR-National Bureau of Agricultural Insect Resources, Hebbal, Bengaluru, India-560024
[2]Sahyadri Science College, Vidya Nagar, Shivamogga, India-577 203
Corresponding author: E-mail: micromahesh@gmail.com, Telephone: +91 9480046850

ABSTRACT
Polyphagus crop pest Spodoptera litura (Fab.) harbor various symbiotic microorganisms providing several benefits to the host with various types of interactions. The field collected and laboratory reared populations of S. litura were subjected to isolation of culturable endosymbiotic gut microflora and 16S rDNA sequence based identification. Enterobacter cloacae, Lysinibacillus macroides, Pseudomonas stutzeri, Staphylococcus saprophyticus and S. sciuri were identified as culturable gut bacterial flora. These bacteria possessed enzymatic activity aiding in digestion. Apart from this, E. cloacae, P. stutzeri and S. saprophyticus were found common in both rhizospheric soil and insect gut possibly indicating their movement from soil to plants and further to the insects through feeding. Metagenomic studies by traditional cloning of 16S rDNA resulted in identification of uncultured Enterococcus sp., Parvimonas sp., Erysipelatoclostridium sp., Dysgonomonas sp. and Gilliamella sp. from the field population. An attempt was made to study the endosymbiont Wolbachia using wolbachia surface protein gene revealed absence of Wolbachia infection. Ability of S. litura gut bacteria for degradation of insecticides leading to the insecticide resistance of the host was studied by in vitro microbial degradation of insecticide imidacloprid. Luxurious growth of gut symbiont Lysinibacillus macroides SPL-1 in insecticides amended M9 medium implied the utilization of insecticides as carbon sources for growth and its possible involvement in degradation of insecticides.
Keywords: diversity, gut bacteria, insecticide degradation, Spodoptera litura

Received 02.07.2017 Revised 19.08.2017 Accepted 29.08.2017

INTRODUCTION

Insects are the most diverse group among all invertebrates on the earth. They are having interactive relationships with all living organisms like plants, animals and microbes. They are having their own array of functions such as pest, parasites, parasitoids, predators, pollinators, and scavengers. Insects and microbes established symbiotic relationship (Kikuchi et al. 2009), and transmission of microbes to insects is through diet or vertical transmission (Yun et al. 2014). The role of symbiotic microbes includes cellular digestion of host food *viz*., nitrogen fixation, cellulose digestion, nutritional support and protection of the host (Wenzel et al. 2003; Behar et al. 2005; Kikuchi et al. 2005).

Spodoptera litura belongs to the insect order Lepidoptera within the family Noctuidae and distributed worldwide. It is a polyphagus pest, feeds on more than 120 species of field crops, vegetables, ornamentals, wild plants, weeds and shade trees leading to great economic loss. Larvae feed gregariously during early instar by scraping the leaves. Later instar may completely devour the leaves and fruits resulting in reduced crop growth.

Insect gut possesses a large source of microbial diversity. The endosymbionts are having different classes include fungi, bacteria and yeast with different functions *viz.,* sterol utilization and nitrogen recycling in hosts (Suh et al. 2001). These microflora utilize a wide range of organic polymers and can be involved in methanogenesis and nitrogen fixation (Nardi et al. 2002). The gut bacteria play an important role in pheromone production, pesticide degradation, vitamin synthesis, and pathogen prevention (Dillon and Charnley 1995; Tokuda et al. 1997; Dillon et al. 2002; Reeson et al. 2003). Therefore, analysis of the composition and diversity of the intestinal bacteria of insects have various applications. Bacterial

communities in insect guts have been studied mainly by the cultivation dependent technique, which does not reflect the entire microbial communities (Gilliam 1997). 16S rDNA genes, which exist in all nuclear biological cells, have been widely used to estimate the bacterial diversity of the insect gut bacterial microbiota (Reeson et al. 2003; Broderick et al. 2004). Apart from culturable microbes many obligate symbionts are known to harbor insects. *Wolbachia* a common and widespread group of symbiotic unculturable bacteria transmitted maternally and is known for reproductive exploitations in many arthropods (Dobson et al. 2002). However, little information is available on the gut bacterial communities of many insects. In this study, a detailed analysis of bacterial diversity and community structure in the gut of the *S. litura* larvae is reported with possible movement of microflora from soil to insects through host plants.

MATERIALS AND METHODS

Insect collection

The laboratory experiment was conducted during 2015 at ICAR-National Bureau of Agricultural Insect Resources, Bengaluru, India to explore the culturable and unculturable microbial diversity associated with the *Spodoptera litura* larval gut of field collected and laboratory reared (third generation) populations along with soil microflora. The larvae were collected from tomato fields sprayed with insecticide imidacloprid from Battlahalli (13°31' N, 78°60' E), and field with no insecticide spray from Yelahanka (13°09'N, 77°56'E), Karnataka, India.

Rearing of insects

Field collected population of *S. litura* larva was mass reared on artificial diet from neonate to adult stage as described by Gupta et al. (2005) with following modifications: chickpea flour 100g, yeast powder 10g, L-ascorbic acid 3.2g, two multivitamin multimineral capsules, vitamin E liquid capsule 2 ml, methyl-p-hydroxybenzoate 2g, Sorbic acid 1g, streptomycin sulphate 0.25g, formaldehyde solution 5 mL, agar agar 12.75g and 780 ml distilled water. Kidney bean flour, wheat germ, casein, castor oil and cholesterol were not used in the diet. Mass rearing was done in BOD incubator at 26±2 °C, 70±5% RH and 16:8 h dark/light phase regime in the plastic trays amended with artificial diet.

Isolation of culturable gut microflora

Culturable microflora was isolated from gut of *S. litura* larvae. A total of ten fifth instar larvae samples were collected, starved for 3-4 h, and later killed by lethal freezing at 4 °C. The dead larvae were washed with 70% ethanol (×4) and then with sterile water (×4) for surface sterilization. The larvae were dissected aseptically in 1ml of saline phosphate buffer (pH 8.0) by removing anterior and posterior part of larvae. The gut was carefully transferred using sterile forceps to 1ml phosphate buffer solution and homogenised using micro pestle and mortar. The homogenate was serially diluted up to 10^{-6} and then 100µl of 10^{-6} dilution was spread plated on different laboratory media (nutrient agar, yeast peptone dextrose agar, potato dextrose agar) plates in triplicates. The faecal matter of *S. litura* was also subjected to isolation of culturable microflora after serial dilutions. A control plate was also maintained for confirmation of efficiency of surface sterilization using 100 µl final wash elute from surface sterilization step. The plates were incubated in BOD incubator at 28±2 °C and the colony forming units (CFU) count after incubation was enumerated after 24 h. The representative bacterial colonies on plates were purified using quadrant streak method on respective media. Microbial cultures were preserved in nutrient agar slants at 4°C and in 20% glycerol stocks at -80°C for further investigation.

Isolation of rhizosphere soil microflora

Soil sampling was carried out from rhizosphere of two tomato plants from where the *S. litura* larvae were collected at 15 cm depth and were transported to laboratory in sterile container. One gram of soil was serially diluted in sterile water up to 10^{-6} dilution. Aseptically 100µL of diluted sample was spread plated to different isolation media similar to isolation of culturable microflora from larval gut. The representative bacterial colonies growing on plates were purified using quadrant streak method on respective media.

Biochemical characterization

The purified bacteria from *S. litura* larval gut and rhizosphere soil were subjected to assess protease production (Rodarte et al. 2011; El-latif 2014), starch hydrolysis, gelatin liquefaction, casein hydrolysis, and H_2S production (Vashist et al. 2013). The bacterial colonies were subjected to differentiation using Grams' staining technique.

Insecticide degradation assay

Bacteria isolated from larval gut were examined *in vitro* for degradation of insecticide imidacloprid amended in M9 minimal media. The M9 minimal medium constituted 5X salts of 800ml with 64g $Na_2HPO_4.7H_2O$, 15g KH_2PO_4, 2.5g NaCl, 5.0g NH_4Cl which were sterilised by autoclaving. M9 salts solution (1X) (200 ml per liter) was added with 15g of agar and autoclaved. Sterile 20 ml of 1M $MgSO_4$ solution

and 0.1ml of 1M $CaCl_2$ solution were added when media cooled to 45±3°C. Medium was amended with 100ppm, 50 ppm, 25 ppm and 12.5 ppm of imidacloprid (Pestinal®, Sigma Aldrich Laborchemicalen, GmbH) insecticides and to the plates. Minimal medium agar plates were inoculated with larval gut and rhizosphere microflora, incubated at 28±2 °C for 24 h in BOD incubator. Microbial cultures exhibiting luxuriant growth on M9 medium embedded with imidacloprid were recorded. The microbes exhibiting luxuriant growth were subcultured in respective broth without insecticide for 24 h at 150 rpm in orbital shaker (Orbitek, Scigenics Biotech Pvt. Ltd., India). 0.5 ml overnight grown culture (0.05 optical density at 600 nm) was inoculated in 50 ml of M9 broth amended with 150ppm of insecticides and incubated 28±2°C at 150 rpm in orbital shaker for monitoring microbial growth. The microbial growth measurements at absorbance of 600 nm were recorded using UV-Vis spectrophotometer (BioMate-5, Thermospectronic, UK) at 24h intervals till 120 h. A control was maintained with M9 media with insecticides devoid of microbes.

Molecular identification of gut microflora from *S. litura* larva

The purified bacterial isolates were inoculated in Luria broth medium and incubated overnight and DNA was extracted using HiPura DNA extraction kit (HiMedia India Pvt. Ltd., India) as per manufacturers' instructions. 16S rRNA gene was amplified using universal 16S rRNA primers (forward primer pA5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer pH5' AAGGAGGTGATCCAGCCGCA 3'). The final volume of reaction mixture of 50 µl contained 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 1 mM of each primer, 1.0U of *Taq* polymerase and 100 ng of template DNA. The amplification was performed on thermal cycler (Qantarus, UK) (initial denaturation step at 95°C for 3 min, after which *Taq* polymerase was added, followed by 35 amplification cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and 72°C for 10 min for final elongation). DNA template replaced with sterile water served as negative control. The amplified products were purified with PCR purification kit (Qiagen GmbH, Hilden, Germany) and sequenced directly with the *Taq*-mediated dideoxy chain terminator cycle sequencing in ABI 3130*xl* automated genetic analyzer (Applied Biosystems, UK) according to manufacturers' instructions.

Isolation of metagenomic DNA:

DNeasy blood and tissue DNA kit (Magspin 35, APS Lifetech, India) was used for isolation of DNA from insect gut tissue as per manufacturer instructions. Isolated DNA was checked using agarose gel (1% w/v) electrophoresis using 2 µl of each DNA and gel was documented (GelDoc-XR, Bio-Rad Laboratories, Inc.). DNA concentration was estimated using 260/280 nm absorbance using UV-Vis spectrophotometer (BioMate-5, Thermospectronic, UK).

PCR amplification of 16S rDNA and gene cloning

PCR amplification of the bacterial 16S rDNA was performed using universal bacterial 16S rDNA primers (27F 5'AGAGTTTGATCMTGGCTCAG3' and 806R 5'GGACTACHVGGGTWTCTAAT3') generating amplicon of ~790 bp. PCR reaction mixture contained 5.0 µl of genomic DNA; 10 mM of each primer; 200 mM each dNTP, and 1.0U of *Taq* DNA polymerase in the PCR buffer. The molecular sizes of PCR amplicons ~798 bp was purified using PCR purification kit (Qiagen GmbH, Hilden). Agarose gel electrophoresis was performed for confirmation of PCR products. Cloning of 16S rDNA amplicons was performed using TA cloning kit (Promega) consisting of pGEMT vector and *E. coli* XL1 blue chemical competent cells followed by transformation and screening as per manufacturers' instructions. The white coloured colonies were picked up and gridded on a fresh LB agar medium plates amended with Ampicillin (100 µg ml^{-1}). The plates were incubated for 37°C overnight. Each colony was further subjected to boiling lysis followed by colony PCR using T7 and SP6 Primers. (initial denaturation step at 94°C for 5 min, after which *Taq* polymerase was added, followed by 36 amplification cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 90s and 72°C for 7 min for final elongation). Transformants were identified based on size of amplicons in agarose gel electrophoresis (1%) and the PCR products generated in clones showing presence of insert were further subjected to DNA sequencing directly with the *Taq*-mediated dideoxy chain terminator cycle sequencing in ABI 3130*xl* automated genetic analyzer (Applied Biosystems, UK).

Amplification of *Wolbachia* gene

Wolbachia infection in *S. litura* larva was screened by amplifying fragment of the ***Wolbachia*** surface protein (*wsp*) gene using specific primers (forward primer Wsp81 5'TGGTCCAATAAGTGATGAAGAAC3' and reverse primer Wsp 691 5'AAAAATTAAACGCTACTCCA 3') (Zhou ***et al.*** 1998). PCR reaction mixture contained 5.0 µl of genomic DNA; 10mM of each primer; 200mM each dNTP, and 1.0U of *Taq* DNA polymerase in the PCR buffer. The amplification of ***wsp*** gene was performed as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, followed by a final extension at 72°C for 7 min for 32 cycles (Chen et al. 2013).

Phylogenetic analysis:

All sequences were compared with 16S rRNA gene sequences (closest representatives) available in public databases (Genbank, NCBI) by BLASTn search. Sequences were aligned by ClustalW, ver 1.8 and the phylogenetic tree was constructed from evolutionary distances using neighbor joining method implemented through the MEGA software version 4.0 (Saitou and Nei 1987). Gaps were cured by pairwise deletions and bootstrap analysis was done by using 900 pseudo replications. The sequences of microflora obtained under the study were assigned to different taxonomic classes based on data available in public databases.

RESULTS

In larval *S. litura,* five culturable and ten unculturable bacteria were isolated and identified. A total of eight culturable microflora were isolated from tomato rhizosphere soil samples. All the culturable bacteria were characterised through morphological and biochemical methods.

Culturable gut microflora

A total of 78 culturable bacteria were isolated from the guts of dissected larvae, among which 26 isolates showing wide variation in the colony morphology were chosen for further studies. Among culturable bacteria five bacteria were chosen as representatives based on colony morphology, biochemical tests and identified using molecular tools, the isolates as *Enterobacter cloacae, Lysinibacillus macroides, seudomonas stutzeri, Staphylococcus sciuri* and *Staphylococcus saprophyticus* (Table 1;). Qualitative biochemical tests for insect gut isolated bacteria inferred that 100% isolates as protease positive, 40% starch hydrolysis positive, 20% casein hydrolysis positive and none of bacteria were positive for gelatin hydrolysis and hydrogen sulphide tests (Table 1). Culturable bacteria isolated from faecal matter of *S. litura* were repetitive with gut isolated microflora and hence were eliminated from the study.

Culturable rhizosphere soil microflora

Microflora isolated from two tomato plant rhizosphere soil consisted of 82 colonies out of which 22 isolates showing variation in the colony morphology were chosen for further studies. Among the 22 isolates, 8 isolates were identified using molecular tools (Table 1) as belonging to *Bacillus cereus*, *B. firmus*, *Enterobacter cloacae*, *Pseudomonas stutzeri*, *Stenotrophomonas maltophilia*, and *Staphylococcus saprophyticus*. 37.5% *Bacillus* sp., 25% *Stenotrophomonas* sp., *Staphylococcus*, *Enterobacter* and *Pseudomonas* sp. 12.5% each of representative of total isolated bacteria. Host plant rhizosphere soil sample isolated microbes revealed that 100% isolates were protease positive, 62% for starch hydrolysis, 50% for casein hydrolysis and none of organisms were positive for gelatin hydrolysis and hydrogen sulphide tests (Table 1).

Unculturable microflora

Metagenomic study of insect DNA along with consecutive amplification and cloning resulted in 10 clones comprised unculturable *Enterococcus* sp. 40%, *Gillimella* sp. 30%, 10% each to *Parvimonas* sp., *Erysipelatoclostridium* sp. and *Dysgonomonas* sp. To confirm the presence of *Wolbachia* infection, PCR detection of *Wolbachia* from metagene of *S. litura* was conducted using the *wsp* gene primers, with the *Wolbachia* infected *Bemisia tabaci* strain as a positive control. Positive amplification of ~600bp fragment was observed in the DNA samples of *Bemisia tabaci*, whereas metagene of *S.litura* did not yield any amplification. The results indicated absence of *Wolbachia* in the metagene of *S. litura*.

Insecticide degradation assay

Culturable gut bacteria *Lysinibacillus macroides* SPL-1, *Pseudomonas stutzeri* SPL-2, *Staphylococcus sciuri* SPLN-1, *S. saprophyticus* SPLN-2 and *Enterobacter cloacae* SPLN-3 from *S. litura* were subjected *in vitro* for degradation of insecticides both on M9 agar media and broth. Among all culturable microflora, *Lysinibacillus macroides* SPL-1 revealed profuse growth on M9 media and was further confirmed by optical measurement using spectrophotometer in M9 broth containing insecticide imidacloprid as alternative source of carbon (Fig. 4).

Phylogenetic analysis

Phylogenetic analysis for culturable microflora was carried out for bacterial sequences similarity to known bacteria aligned together with the sequences (closest representatives), available in public databases (GenBank, NCBI) (Fig 1). Two genetic groups were formed from 13 bacterial strains from *S. litura* gut and host plant rhizosphere soil samples. Group I represented 6 bacterial isolates belonging to gram negative bacteria with >99% similarity (*Pseudomonas stutzeri* BTS-1, *P. stutzeri* SPL-2, *Enterobacter cloacae* SPLN-3, *E. cloacae* NTS-2, *Stenotrophomonas maltophilia* NTS-4 and *S. maltophilia* BTS-3). Group II consisted of 7 isolates belonging to gram positive bacteria with >97% similarity *Bacillus cereus* BTS-2, *Staphylococcus saprophyticus* NTS-1, *B. firmus* NTS-3, *B. cereus* NTS-5, *Lysinibacillus macroides* SPL-1, *S. sciuri* SPLN-1 and *S. saprophyticus* SPLN-2 (Table 1, 2; Fig.1).

The nucleotide sequences of the unculturable bacterial strains were subjected to homology searches in DNA databases revealed that the sequences of five isolates belonging to uncultured *Enterococcus* sp. SL-2,

uncultured *Enterococcus* sp. SL-4, uncultured *Parvimonas* sp. SL-6, uncultured *Gilliamella* sp. SL-9 and uncultured *Enterococcus* sp. SL-10 showed 99% and uncultured *Gilliamella* sp. SL-1 with 97% similarity with their closest representatives. Besides, four isolates had <97% similarity namely uncultured *Enterococcus* sp. SL-5 (95%), uncultured *Erysipelatoclostridium* sp. SL-7 (94%), uncultured *Gilliamella* sp. SL-3 and uncultured *Dysgonomonas* sp. SL-8 (93%) to their closest representatives available in public database (Table 2, Fig. 2). Genus affiliation of the 16S rDNA sequences of culturable and unculturable microbes from the *S. litura* gut and host plant rhizosphere soil exhibited 5 different taxonomic classes of microflora. Class Bacilli and Gammaproteobacteria represented 43.48% each of the total number of isolates whereas, 4.35% each was represented by classes Erysipelatotrichia, Tissierellia and Bacteroidia (Fig. 3).

Nucleotide accession numbers

A total of 23 sequences of 16S rRNA gene were deposited in public databases (GenBank, NCBI) under the accession numbers from KT818800 to KT818802, KT818804 to KT818805, KU960901 to KU960910 and KX290304 to KX290311 (Table 2).

DISCUSSION

Insect gut can adapt suitable gut-microbiome according to their habitual diet. Insect gut supports different enzymes like cellulases, hemicellulases, amylases and proteases produced either by symbiotic microbes or insect itself, aiding in digestion of ingested food containing complex carbohydrates and proteins (Gosalbes et al. 2010). Cigarette beetle endosymbiont *Symbiotophrina kochii* produces detoxifying enzymes to utilize diet toxins as carbon sources (Shen and Dowd 1991). Termites gut is evidenced for different types of enzymes *viz.* cellulases, hemicellulases, α-amylases and proteases to degrade ingested lignocellulosic materials (Lima et al. 2014). Insect endosymbionts play role in digestion of ingested carbohydrates, proteins and cellulose, which is necessary to utilize nutrients present in the diet. Biochemical characterization of bacteria for protease, amylase and caseinase inferred that majority of endosymbionts produced these enzymes aiding in digestion of ingested food. Bacterial enzymes also aided in insecticide degradation by utilizing insecticides as carbon source.

Endosymbiont microflora may be culturable and more than 90% environmental unculturable microflora is unrevealed. From metagenomic studies pertaining to unculturable microflora, we report bacterial isolates *Gilliamella apicola* SL-1 and SL-3 belonging to Gammaproteobacteria from larval gut of *S. litura.* This isolate has been reported from gut of honey bee *Aphis mellifera* and bumble bees *Bombus bimaculatus* (Kwong and Moran 2013), having role of detoxification of toxic carbohydrates present in honeybee diet (Zheng et al. 2016). *Enterococcus* sp. are dominant bacterial group in insects that are reported to be associated with guts of Gypsy moth (Allen et al. 2009), *Manduca sexta* (Brinkmann et al. 2008) and *S. litura* (Tang et al. 2012). We identified *Enterococcus termitis* SL-2, *Enterococcus rotai* SL-4, *Enterococcus moraviensis* SL-5 and *Enterococcus* sp. SL-10 from larval gut of *S. litura*. *Dysgonomonas* sp. SL-8 reported from current metagenomic study has also been reported from gut of termites (Pramono et al. 2015).

Wolbachia are commonly transmitted intracellular bacteria of arthropods, crustaceans, and arachnids. It reportedly enhances susceptibility to NPV in *Spodoptera exempta* (Graham et al. 2012). *Wolbachia* infection in insects *Drosophila melanogaster* and *Culex quinquefasciatus* showed enhanced resistance to West Nile virus (Glaser and Meola 2010). *Wolbachia* are involved in sexual manipulation like reducing host viability, parthenogenesis, and Feminization etc. (Sumithra et al. 2013). *Wolbachia* surface protein (*wsp*) gene sequences are most widely used to identify infection of *Wolbachia*. *wsp* is reported from common odonate species *Agroicnemis femina* (Thipaksorn et al. 2003). Our attempt to amplify *wsp* gene and identify *Wolbachia* infection from laboratory and field larval populations in *S. litura* did not yield positive results for *wsp* gene amplification.

Culturable bacteria *Lysinibacillus macroides* SPL-1 in this study isolated from gut of *S. litura* larva, is also represented in gut of *Drosophila melanogaster* (Maji et al. 2012) and found associated with rice plant (Shreshta et al. 2016). *Bacillus cereus* has been reported pervasive from soil and insects (Vilain et al. 2006; Arnesen et al. 2008). In the current study, common culturable bacteria *Bacillus cereus* was isolated from rhizosphere soil samples from two different locations and was designated as *B.cereus* BTS-2 and NTS-5 after elimination of repetitive microflora from study. *B. cereus* is reported to be present in soil and adequately known for biological control of phytopathogenic fungi (Silo-Suh et al. 1994), and involved in two types of relationship *viz.,* infective in animals and symbiotic with insects. They are implied as rhizosphere bacteria for mediating commensalism (Peterson et al. 2006) which may decide suitable host for their growth and functions (Jensen et al. 2003). *Pseudomonas stutzeri* is prominent organism isolated from rhizosphere soil having role of nitrogen fixing and enhance root colonization in paddy fields (Rediers et al. 2009). In our study we isolated culturable organism *P. stutzeri* BTS-1 from soil sample and

P. stutzeri SPL-2 from insect gut. *P. stutzeri* antagonistic against pathogenic bacteria and functioning as probiotics in *Artemia* larvae (Abdelkarim et al. 2010), although possesses feasibility to soil cyanide remediation (Nwokoro and Dibua 2014). *Staphylococcus saprophyticus* SPLN-2 is reported in current study has also been recorded from other insects like mealy bug *Rhizoecus amorphophalli* (Sreerag et al. 2014) and from rhizospheric soil as *S. saprophyticus* NTS-1 from current study. *Enterobacter cloacae* was recorded in soil as NTS-2 and laboratory reared insect gut SPLN-2. This organism has been reported as endosymbiont in silkworm larvae (Watanabe and Sato 1998) and *Bemesia tabaci* (Davidson et al. 2000). *E. cloacae* isolated from *S. litura* acts as bacterial pathogen against host insect (Thakur et al. 2015).

The presence of *P. strutzeri*, *S. saprophyticus* and *E. cloacae* in both rhizospheric soil and insects in the present study may be due to possible movement of these microorganisms from soil to plants and further to the insects through feeding. These microbes return to soil possibly through excreta or upon the death of insects. Symbionts are transmitted vertically in host insects, although they are acquired from environments during different nymph stages of Stink bug (Kikuchi et al. 2007; 2011). Similar study on movement of microflora from soil to plants and plants to insects have been studied and established with *Burkholderia cepacia* in soyabean plant bean bug *Riptortus pedestris* (Kikuchi et al. 2012). *R. pedestris* endosymbiont *B. cepacia* is capable of degradation of fenitrothion in sugarcane fields and confer fenitrothion resistance to the host insects. Gut microbes are capable of establishing a specific and beneficial symbiosis with the hosts (Kikuchi et al. 2012). *Bacillus* sp., *Brevibacterium* sp., *Pseudomonas putida*, *Bacillus subtilis* and *Rhizobium* sp. bacteria are reported to be involved in degradation of insecticide imidacloprid (Sabourmoghaddam et al. 2015).

Phylogenetic analysis for 13 culturable microflora categorised under two distinct phyloclades based on grouping and isolates represented were obviously grouped themselves based on Gram's reaction as Gram positive and Gram negative. Also, the isolates had >97% similarity to the closest representatives from the public databases. In case of unculturable microflora, distinct phylogenetic grouping was not observed due to variation in sequence lengths and alignment with the closest sequences available in public databases (GenBank, NCBI). Despite majority (60%) of unculturable bacterial sequences had >97% similarity and only 4 isolates had <97% similarity with their closest representatives. Genus affiliation exhibited 5 different taxonomic classes based on 16S rDNA sequences of culturable and unculturable microbes from the *S. litura* larval gut and tomato rhizosphere soil. Gammaproteobacteria represented 43.48% of the total number of isolates which is due to presence of secondary endosymbionts belonging to genus *Enterobacteria, Stenotrophomonas, Pseudomonas* and *Gilliamella.* Bacilli class (43.48%) represented genus belonging to *Bacillus, Enterococcus Lysinibacillus* and *Staphylococcus*. *Parvimonas* sp. SL-6 represented Tissierellia class, whereas uncultured *Erysipelatoclostridium* sp. SL-7 and uncultured *Dysgonomonas* sp. SL-8 represented Erysipelatotrichia and Bacteroidia classes respectively, representing 4.35% each of the total population.

The *S. litura* gut endosymbiont *L. macroides* SPL-1 revealed luxurious growth in imidacloprid insecticide amended M9 medium, implying the utilization of insecticides as carbon sources for growth and possible role of endosymbiont involvement in degradation of these insecticides. The present study revealed the occurrence of several culturable and unculturable microflora in the larval gut of *S. litura* and tomato rhizosphere. Further studies are on the way to find out role played by these bacteria for the fitness attributes including insecticide resistance which will be beneficial for taking suitable pest management strategies against *S. litura.*

ACKNOWLEDGMENTS

This study was funded by the Indian Council of Agriculture Research (ICAR) by a network project 'Application of Microorganisms in Agriculture and Allied Sectors (AMAAS)' under the award number NBAIM/AMAAS/2014-15/61. Authors are thankful to Director, ICAR-National Bureau of Agricultural Insect Resources, Bengaluru for providing necessary infrastructure and facilities to undertake research wok.

REFERENCES

1. Abdelkarim M, Kamel C, Fathi K, and Amina B (2010) Use of *Pseudomonas stutzeri* and *Candida utilis* in the improvement of the conditions of *Artemia* culture and protection against pathogens. Braz J Microbiol 41(1):107–115.
2. Allen HK, Cloud-Hansen KA, Wolinski JM, Guan C, Greene S, Lu S, Boeyink M, Broderick NA, Raffa KF, Handelsman J (2009) Resident microbiota of the gypsy moth midgut harbors antibiotic resistance determinants. DNA Cell Biol 28(3):109-117.
3. Arnesen SLP, Fagerlund A, Granum PE (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Review. 32(4):579-606.

4. Behar A, Yuval B, Jurkevitch E (2005) Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. Mol Ecol 14(9):2637-2643.
5. Brinkmann N, Martens R, Tebbe CC (2008) Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of *Manduca sexta* (Sphingidae, Lepdidoptera, Insecta). Appl Environ Microbiol 74(23):7189-7196.
6. Broderick NA, Raffa KF, Goodman RM, Handelsman J (2004) Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl Environ Microbiol 70(1):293-300.
7. Chen L, Zhu C, Zhang D (2013) Naturally occurring incompatibilities between different *Culex pipiens pallens* populations as the basis of potential mosquito control measures. PLoS Negl Trop Dis 7(1):e2030.
8. Davidson EW, Rosell RC, Hendrix DL (2000) Culturable bacteria associated with the whitefly, *Bemisia argentifolii* (Homoptera: Aleyrodidae). Fla Entomol 83(2):159-171.
9. Dillon RJ, Charnley AK (1995) Chemical barriers to gut infection in the desert locust: *In vivo* production of antimicrobial phenols associated with the bacterium *Pantoea agglomerans.* J Invertebr Pathol 66:72-75
10. Dillon RJ, Vennard CT, Charnley AK (2002) A Note: Gut bacteria produce components of a locust cohesion pheromone. J Appl Microbiol 92:759–763.
11. Dobson SL, Marsland EJ, Veneti Z, Bourtzis K, O'Neill SL (2002) Characterization of *Wolbachia* host cell range *via* the *in vitro* establishment of infections. Appl Environ Microbiol 68(2):656-660.
12. El-latif AOA (2014) Biochemical characterization of the midgut serine proteases of the Egyptian cottonworm, *Spodoptera littoralis* and their interactions with standard protease inhibitors. Int J Chem Environ Biol Sci 2(3):157-162.
13. Gilliam M (1997) Identification and roles of non-pathogenic microflora associated with honey bees. FEMS Microbiol Lett 155(1):1-10.
14. Glaser RL, Meola MA (2010) The native *Wolbachia* symbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile infection. PLoS One 5(8):e11977.
15. Gosalbes MJ, Latorre A, Lamelas A, Moya A (2009) Genomics of intracellular symbionts in insects. Int J Med Microbiol 300(5):271-278.
16. Graham RI, Grzywacz D, Mushobozi WL, Wilson K (2012) *Wolbachia* in major African crop pest increases susceptibility to viral disease rather than protects. Ecol Lett 15(9):993-1000
17. Gupta GP, Rani S, Birah A, Raghuraman M (2005) Improved artificial diet for mass rearing of the tobacco caterpillar, *Spodoptera litura* (Lepidoptera: Noctuidae). Int J Trop Insect Sci 25(1):55-58.
18. Jensen GB, Hansen BM, Eilenberg J, Mahillon J (2003) The hidden lifestyles of *Bacillus cereus* and relatives. Environ Microbiol 5(8) 631-640.
19. Kikuchi Y, Hosokawa T, Fukatsu T (2007) Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment in every generation. Appl Environ Microbiol 73(13):4308-4316.
20. Kikuchi Y, Hosokawa T, Fukatsu T (2011) Specific developmental window for establishment of an insect-microbe gut endosymbiosis. Appl Environ Microbiol 77(12):4075-4081.
21. Kikuchi Y, Hosokawa T, Fukatsu T (2012) An ancient but promiscous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. ISME J 5(3):446-460.
22. Kikuchi Y, Hosokawa T, Nikoh N, Meng XY, Kamagata Y, Fukatsu T (2009) Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. BMC Biol 7:2.
23. Kikuchi Y, Meng XY, Fukatsu T (2005) Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). Appl Environ Microbiol 71(7): 4035-4043.
24. Kwong WK, Moran NA (2013) Cultivation and characterization of the gut symbionts of honey bees and bumble bees: description of *Snodgrassella alvi* gen. nov., sp. nov., a member of the family *Neisseriaceae* of the *Betaproteobacteria* and *Gilliamella apicola* gen. nov., sp. nov., a member of *Orbaceae* fam. nov.,*Orbales* ord. nov., a sister taxon to the order '*Enterobacteriales*' of the *Gammaproteobacteria*. Int J Syst Evol Microbiol 63:2008-2018.
25. Lima Tde A, Pontual EV, Dornelles LP, Amorim PK, Sá RA, Coelho LC, Napoleao TH, Paiva PM (2014) Digestive enzymes from workers and soldiers of termite *Nasutitermes corniger.* Comp Biochem Physiol B Biochem Mol Biol 176:1-8.
26. Maji P, Chakrabarti C, Chatterjee S (2012) Phenotyping and molecular characterization of *Lysinibacillus* sp.P-011 (GU288531) and their role in the development of *Drosphila melanogaster*. Afr J Biotechnol 11(93):15967-15974.
27. Nardi JB, Mackie RI, Dawson JO (2002) Could microbial symbionts of arthropod guts contribute significantly to nitrogen fixation in terrestrial ecosystems? J Insect Physiol 48(8):751–763.
28. Nwokoro O, Dibua ME (2014) Degradation of soil cyanide by single and mixed cultures of *Pseudomonas stutzeri* and *Bacillus subtilis*. Arh Hig Rada Toksikol 65(1):113-119.
29. Peterson SB, Dunn AK, Klimowicz AK, Handelsman J (2006) Peptidoglycan from *Bacillus cereus* mediates commensalism with rhizosphere bacteria from the *Cytophaga-flavobacterium* group. Appl Environ Microbiol 72(8): 5421–5427.
30. Pramono AK, Sakamoto M, Iino T, Hongoh Y, Ohkuma M (2015) *Dysgonomonas termitidis* sp. nov., isolated from the gut of the subterranean termite *Reticulitermes speratus*. Int J Syst Evol Microbiol 65:681-685.
31. Rediers H, Vanderleyden J, De Mot R (2009) http://www.sciencedirect.com/science/article/pii/S0944501307000493 - aff1Nitrate respiration in *Pseudomonas stutzeri* A15 and its involvement in rice and wheat root colonization. Microbiol Res 164 (4):461-468.

32. Reeson AF, Jankovic T, Kasper ML, Rogers S, Austin AD (2003) Application of 16S rDNA-DGGE to examine the microbial ecology associated with a social wasp *Vespula germanica*. Insect Mol Biol 12(1):85-91.
33. Rodarte MP, Dias DR, Vilela DM, Schwan RF (2011) Proteolytic activities of bacteria, yeasts and filamentous fungi isolated from coffee fruit (*Coffea arabica* L.). Acta Sci Agron 33(3):457-464.
34. Sabourmoghaddam N, Zakaria MP, Omar DO (2015) Evidence for the microbial degradation of imidacloprid in soils of Cameron Highlands. J Saudi Soc Agri Sci 14(2):182-188.
35. Saitou N, Nie M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.
36. Shen SK, Dowd PF (1991) Detoxification spectrum of the cigarette beetle symbiont *Symbiotophrina kochii* in culture. *Entomol Exp Appl* 60:51-59.
37. Shreshta BK, Karki HS, Groth DE, Jungkhun N, Ham JH (2016) Biological control activities of rice-associated *Bacillus* sp. strains against sheath blight and bacterial panicle blight of rice. PLoS One 11(1): e0146764.
38. Sreerag RS, Jayaprakas CA, Ragesh L, Kumar SN (2014) Endosymbiotic bacteria associated with the mealy bug, *Rhizoecus amorphophalli* (Hemiptera: Pseudococcidae). Int Sch Res Notices 2014:268491.
39. Silo-Suh LA, Lethbridge BJ, Raffel SJ, He H, Clardy J, Handelsman J (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl Environ Microbiol 60(6):2023-2030.
40. Suh SO, Noda H, Blackwell M (2001) Insect symbiosis: derivation of yeast-like endosymbionts within an entomopathogenic filamentous lineage. Mol Biol Evol 18(6):995-1000.
41. Sumithra NM, Guruprasad, Puttaraju HP (2013) Molecular detection of *Wolbachia* and phage WO infection in *Spodoptera litura*. (Lepidoptera: Noctuidae). J Entomol Zool Stud 1(4):54-57.
42. Tang X, Freitak D, Vogel H, Ping L, Shao Y, Cordero EA, Andersen G, Westermann M, Heckel DG, Boland W (2012) Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. PLoS One 7(7):e36978.
43. Thakur A, Dhammi P, Saini HS, Kaur S (2015) Pathogenicity of bacteria isolated from gut of *Spodoptera litura* (Lepidoptera: Noctuidae) and fitness costs of insect associated with consumption of bacteria. J Invertebr Pathol 127:38-46.
44. Thipaksorn A, Jamnongluk W, Kittayapong P (2003) Molecular evidence of *Wolbachia* infection in natural populations of tropical odonates. Curr Microbiol 47(4):314-8.
45. Tokuda G, Watanabe H, Matsumoto T, Noda H (1997) Cellulose digestion in the wood-eating higher termite, *Nasutitermes takasagoensis* (Shiraki): distribution of cellulases and properties of endo-beta-1,4-glucanase. Zoolog Sci 14(1):83-93.
46. Vashist H, Sharma D, Gupta A (2013) A review on commonly used biochemical test for bacteria. Innovare J Life Sci 1(1):1-7.
47. Vilain S, Luo Y, Hildreth MB, Brozel VS (2006) Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. Appl Environ Microbiol 72(7):4970-4977.
48. Watanabe K, Sato M (1998) Plasmid-mediated gene transfer between insect-resident bacteria, *Enterobacter cloacae*, and plant-epiphytic bacteria, *Erwinia herbicola*, in guts of silkworm larvae. Curr Microbiol 37(5):352-355.
49. Wenzel WW, Bunkowski M, Puschenreiter M, Horak O (2003) Rhizosphere characteristics of indigenously growing nickel hyper accumulator and excluder plants on serpentine soil. Environ Pollut 123(1):131-138.
50. Yun JH, Roh SW, Whon TW, Jung MJ, Kim MS, Park DS, Yoon C, Nam YD, Kim YJ, Choi JH, Kim JY, Shin NR, Kim SH, Lee WJ, Bae JW (2014) Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. Appl Environ Microbiol 80(17):5254-5264.
51. Zheng H, Nishida A, Kwong WK, Koch H, Engel P, Steele MI, Moran NA (2016) Metabolism of toxic sugars by strains of the bee gut symbiont *Gilliamella apicola*. MBio 7(6):e01326-16.
52. Zhou W, Rousset F, O'Neil S (1998) Phylogeny and PCR-based classification of ***Wolbachia*** strains using wsp gene sequences. Proc Biol Sci 265(1395):509-515.

Table 1 Morphological and biochemical characterization of culturable gut bacteria from *S. litura* larva and tomato rhizosphere soil

Bacterial strain	Colony morphology	Color	Gram's reaction	Protease production	Starch hydrolysis	Gelatin liquefaction	Casein hydrolysis	H_2S production
SPL-1	Filamentous	Pale yellow	+	+	-	-	-	-
SPL-2	Radiate, smooth	Reddish brown	-	+	-	-	-	-
SPLN-1	Irregular, raised	White	+	+	-	-	+	-
SPLN-2	Glossy, smooth	Pale yellow	+	+	+	-	-	-
SPLN-3	Smooth, flattened	White	-	+	+	-	-	-
BTS-1	Radiate, smooth	Reddish brown	-	+	+	-	+	-
BTS-2	Radiate, small	Pale brown	+	+	-	-	-	-
BTS-3	Radiate,	Cream	-	+	-	-	-	-

	smooth	color						
NTS-1	Glossy, smooth	Pale yellow	+	+	+	-	+	-
NTS-2	Smooth, flattened	White	-	+	-	-	-	-
NTS-3	Irregular, smooth	Pale brown	+	+	+	-	+	-
NTS-4	Radiate, smooth	Pale brown	-	+	+	-	-	-
NTS-5	Radiate, small	Pale brown	+	+	+	-	+	-

+ indicates positive and – indicates negative for the respective test

Table 2 Closest BLASTn matches for the 16S rDNA sequences and their percentage similarity with the closest bacterial strains in GenBank

Host	Culture type	Strain	Most similar species	Accession No.	Similarity %
Tomato rhizosphere soil	Culturable	BTS-1	*Pseudomonas stutzeri*	KX290304	99
	Culturable	BTS-2	*Bacillus cereus*	KX290305	99
	Culturable	BTS-3	*Stenotrophomonas maltophilia*	KX290306	99
	Culturable	NTS-1	*Staphylococcus saprophyticus*	KX290307	99
	Culturable	NTS-2	*Enterobacter cloacae*	KX290308	99
	Culturable	NTS-3	*Bacillus firmus*	KX290309	97
	Culturable	NTS-4	*Stenotrophomonas maltophilia*	KX290310	99
	Culturable	NTS-5	*Bacillus cereus*	KX290311	99
Spodoptera litura (Fab.) larval gut	Culturable	SPL-1	*Lysinibacillus macroides*	KT818804	100
	Culturable	SPL-2	*Pseudomonas stutzeri*	KT818805	100
	Culturable	SPLN-1	*Staphylococcus sciuri*	KT818800	99
	Culturable	SPLN-2	*Staphylococcus saprophyticus*	KT818801	100
	Culturable	SPLN-3	*Enterobacter cloacae*	KT818802	100
	Unculturable	SL-1	Uncultured *Gilliamella* sp.	KU960901	97
	Unculturable	SL-2	Uncultured *Enterococcus* sp.	KU960902	99
	Unculturable	SL-3	Uncultured *Gilliamella* sp.	KU960903	93
	Unculturable	SL-4	Uncultured *Enterococcus* sp.	KU960904	99
	Unculturable	SL-5	Uncultured *Enterococcus* sp.	KU960905	95
	Unculturable	SL-6	Uncultured *Parvimonas* sp.	KU960906	99
	Unculturable	SL-7	Uncultured *Erysipelatoclostridium* sp.	KU960907	94
	Unculturable	SL-8	Uncultured *Dysgonomonas* sp.	KU960908	93
	Unculturable	SL-9	Uncultured *Gilliamella* sp.	KU960909	99
	Unculturable	SL-10	Uncultured *Enterococcus* sp.	KU960910	99

Fig. 1 Molecular phylogenetic analysis of culturable microflora from tomato rhizospheric soil samples and *S. litura* larval gut by maximum likelihood method using 16S rRNA gene sequences

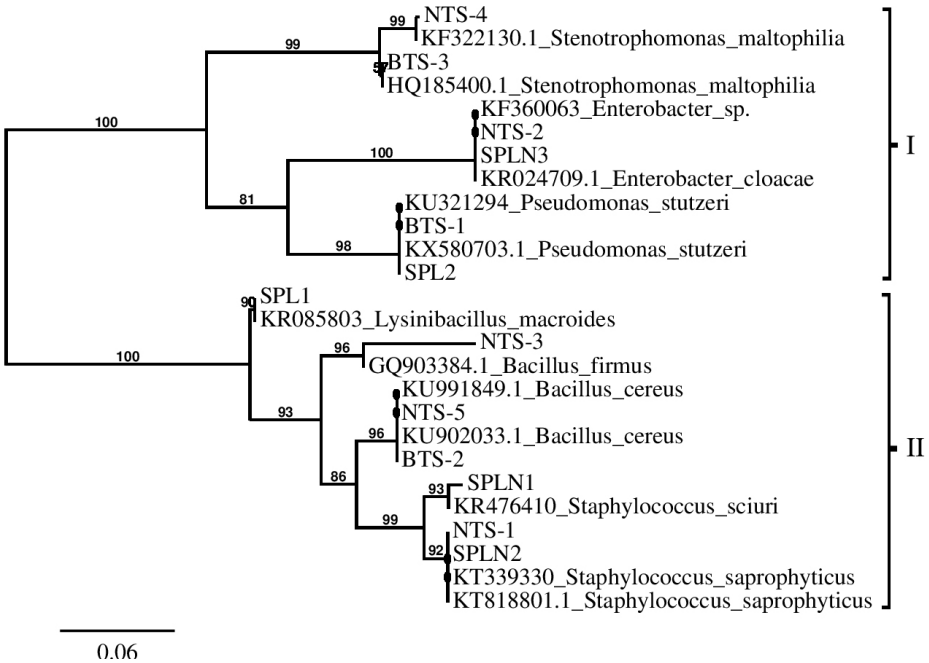

NTS-4
KF322130.1_Stenotrophomonas_maltophilia
BTS-3
HQ185400.1_Stenotrophomonas_maltophilia
KF360063_Enterobacter_sp.
NTS-2
SPLN3
KR024709.1_Enterobacter_cloacae
KU321294_Pseudomonas_stutzeri
BTS-1
KX580703.1_Pseudomonas_stutzeri
SPL2
SPL1
KR085803_Lysinibacillus_macroides
NTS-3
GQ903384.1_Bacillus_firmus
KU991849.1_Bacillus_cereus
NTS-5
KU902033.1_Bacillus_cereus
BTS-2
SPLN1
KR476410_Staphylococcus_sciuri
NTS-1
SPLN2
KT339330_Staphylococcus_saprophyticus
KT818801.1_Staphylococcus_saprophyticus
I
II
0.06

Fig. 2 Molecular phylogenetic analysis of unculturable microflora from *S. litura* larval gut by maximum likelihood method using 16S rRNA gene sequences of NCBI GenBank. The numbers at branch points of the tree designate boot strap values.

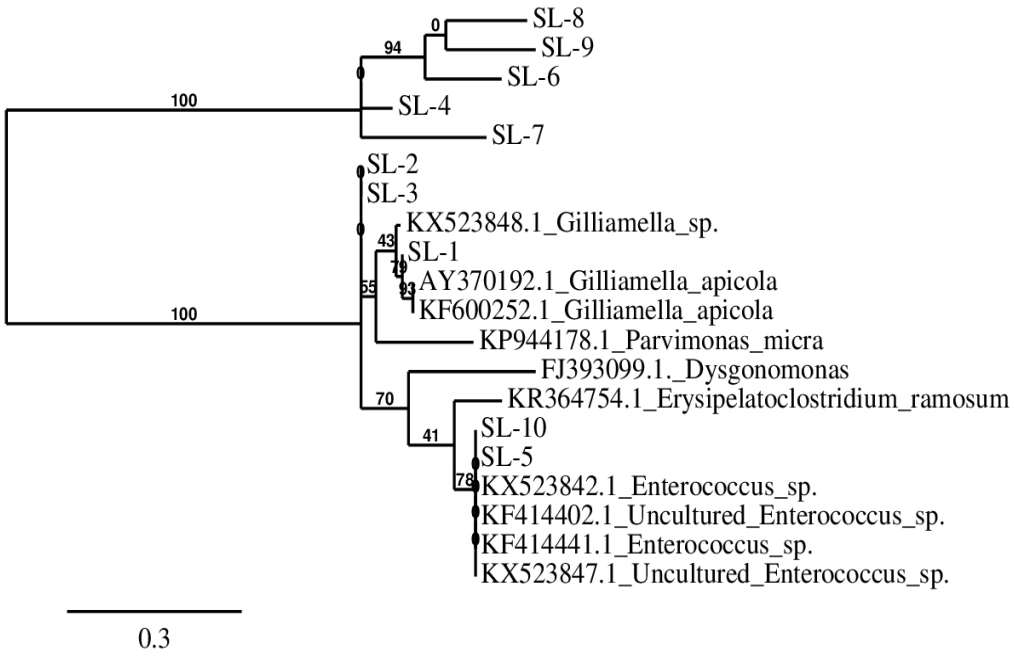
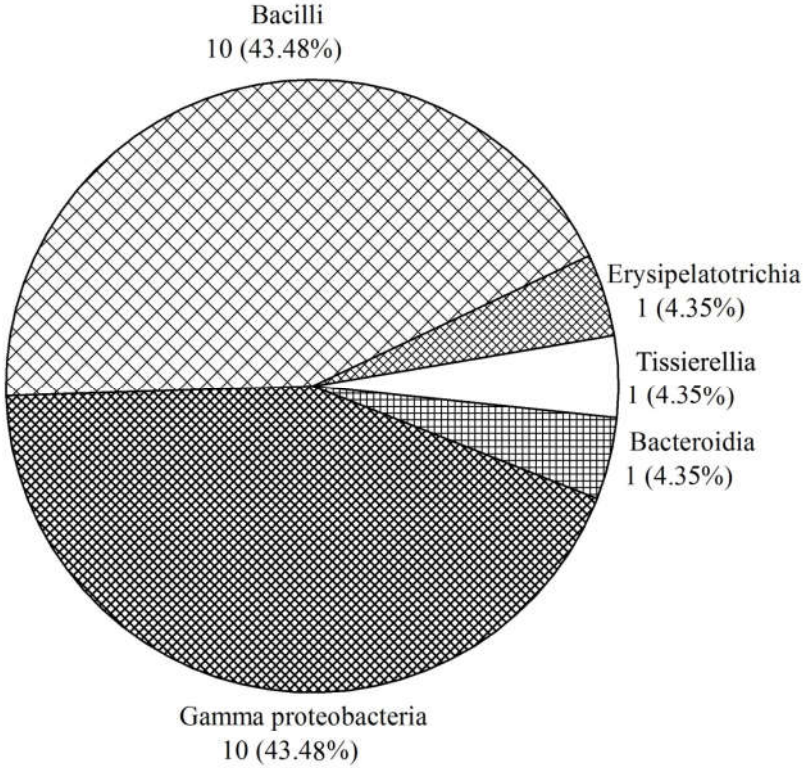


Fig. 3 Genus affiliation of 16S rDNAs sequences obtained from *S. litura* larval gut and tomato rhizosphere soil samples to different taxonomic classes. Classes followed by number indicate the total number of bacteria and figures in parenthesis indicate per cent of isolates from total population. Sequences were classified using BLAST search results and phylogenetic analysis.



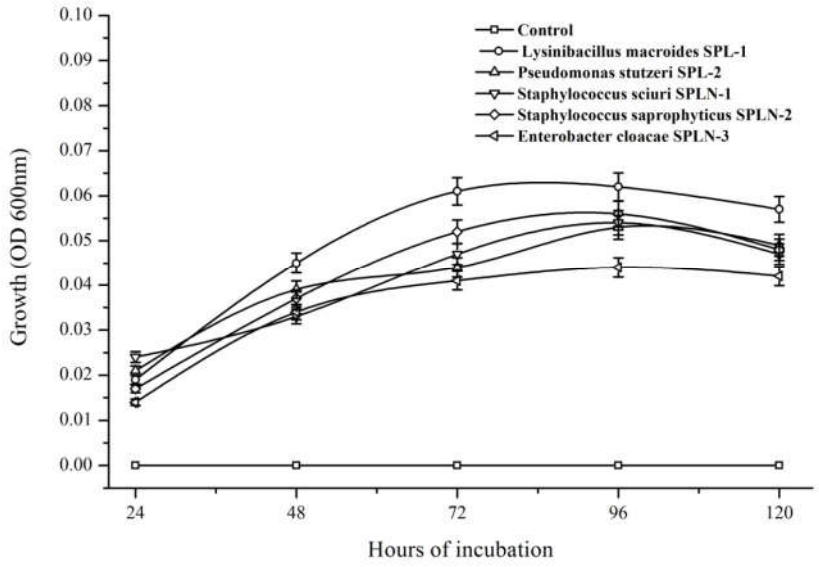


Fig. 4 Microbial growth curve in imidacloprid amended M9 medium

CITATION OF THIS ARTICLE

Sanjay Y, M Shankarappa Yandigeri, O Rudrappa, M Muthugounder, S Gopalasamy. Diversity of culturable and unculturable gut bacteria associated with field population of *Spodoptera litura* (Fab.). Bull. Env. Pharmacol. Life Sci., Vol 6 Special issue 2, 2017: 441-451