Entomology

Microbiota of the fall armyworm, *Spodoptera frugiperda* larvae (Lepidoptera: Noctuidae) infesting vegetables

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Abstract

The fall armyworm, *Spodoptera frugiperda* is a harbour of various microbial communities that varied from symbionts to pathogenic. So, identification of these communities is an important step towards exploring new native entomopathogens or attempting to cause a dysfunction in the symbiotic ones to disrupt the main biological functions of the insect in order to control it. The main objective of this study is isolation and identification of the microbial organisms naturally associated with *S. frugiperda* larvae. The entomopathogenic bacterium, *Bacillus velezensis* and three others symbionts, *Enterococcus durans*, *Klebsiella variicola* and *K. michiganensis* were identified using 16S rRNA gene sequencing. Moreover, two symbiotic yeasts, *Candida corydalis* and *Kodamaea ohmeri* were identified according ITS sequencing analysis. Also, the pathogenicity of *B. velezensis* against the third instar larvae was tested. It showed significant virulence making it eligible to be introduced as green sustainable insecticide.

Keywords: *Spodoptera frugiperda*, Bacteria, Yeasts, Identification and Pathogenicity

1. Introduction

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is an extremely dangerous invader causing substantial economic losses globally, owing to its high reproductive rate, long distance of adults migration (Prasanna *et al*., 2018) [1] and the gluttony of the larval stage which threatens about 353 host plants belongs to 76 different plant families (Agravante *et al*., 2022) [2]. The conventional insecticides was the quickest approach to restrain the pest outbreak, nevertheless, the excessive and indiscriminate use of them negatively impacted the human and animal health, environment, beneficial organisms and natural enemies (Meena *et al*. 2020; Martins and Oliveira 2022) [3, 4], in addition to evolving of insect resistance to most chemical insecticides (Yu, 1991, Carvalho *et al*., 2013, Nascimento *et al*., 2016 and Dos Santos *et al*. 2021) [5-8]. Moreover, it has also developed field-evolved resistance to transgenic crops that produce insecticidal proteins, a prominent example of this is developing resistance to *Bacillus thuringiensis* (Bt) maize (Storer *et al*., 2010; Farias *et al*., 2014; Huang *et al*., 2014; Omoto *et al*., 2016 and Chandrasena *et al*., 2018) [9-13]. *S. frugiperda* like other insects live in close association with many microorganisms. It is a harbor of various microbial communities such as bacteria, viruses, fungi, protozoa and archaea that influence the insect host itself due to their action and interaction between these communities (Gurung *et al*., 2019) [14] and manage the interaction of the insect host and other trophic levels and their environment (Acevedo *et al*., 2017) [15].

The main objective of the current study was isolation, identification of the microbiota especially, bacteria and yeasts of *S. frugiperda* larvae infesting vegetables. The diversity and potential functional roles of them were further discussed and evaluating of potentially pathogenic isolates.

2. Materials and Methods

2.1. Insect collection

Alive larvae of *S. frugiperda* with and/or without any disease symptoms were weekly collected from different vegetables fields: cabbage, *Brassica oleracea* var. capitate; lettuce, *Lactuca sativa* and Nalta jute, *Corchorus olitoroius*,in Dakahlia governorate, Egypt from March 2022 to February 2023. They were collected individually in sterilized eppendorf tubes then it had been rushed to the lab to subject to surface sterilization according to (Claire *et al*., 1997) [16].

2.2. Isolation and purification of the microbes

S. frugiperda cadavers were individually suspended in 1ml of sterilized water, then a drop of this suspension was cultured in Autoclaved SADYA media [40 g/l dextrose, 10g/l yeast extract, 10g/l peptone, and 20 g/l agar] at 27±2 C^0 and 65 ±5% RH until complete growth. Purification of the microbial cultures was performed by using streak plate method.

2.3. Microbial molecular identification

2.3.1. DNA extraction and Polymerase Chain Reaction (PCR)

QIAamp DNA Micro Kit (QIAGEN, Germany) was used for extracting the Genomic DNA from the tested microbial isolates in compliance with the instructions of manufacturer. Amplification of the PCR was conducted in 50 µl consisting of

25 µl of Master Mix (sigma), 3 µl of every primer (10pcmol/ µl) (Table 1: for bacteria & Table 2: for Yeasts), 3 µl of template DNA (10ng/ µl) in addition to 16 µl dH_2O. Perkin-Elmer/Gene Amp® PCR System 9700 (PE Applied Biosystems) was configured for PCR amplification through performing preliminary denaturation cycle for five min at 94ºC, then complete 40 denaturation cycles, each of them takes 30 sec. at 94ºC, an annealing step for 30 sec. at 45ºC and an elongation step for 1 min. at 72ºC. In the final cycle, segment extension of the primer was extended to 7 min at 72ºC.

Electrophoresis of amplification products was performed in a 1.5% agarose gel containing 0.5ug/ml ethidium bromide in 1X TBE buffer at 95 volts. The standard molecular size of DNA ladder is 100bp. The products of PCR were detected by UV light and photographed by using a Gel Documentation System (BIO-RAD 2000). PCR products were purified by using EZ-10 spin column PCR products purification. The mixture of PCR reaction was transferred to 1.5 ml microfuge tube containing three volumes of binding buffer 1, then, the tube was placed in the EZ-10 column and allow it standing for 2 min. at room temperature before centrifuge. The washing process was occurred twice by adding 750 ul of wash solution to the column and centrifuge for two minutes and repeated for an additional minute at 10.000 rpm to shed any residue of wash solution. In a clean 1.5 ml microfuge tube, the column was transferred in addition to 50 ul of elution buffer and incubated for 2 minutes at room temperature. The storage of purified DNA occurred at -20 °C.

2.3.2. Sequencing analysis

Automatic sequencer ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle. Sequencing Kits was used for sequencing the product PCR following the protocols of the manufacturer. Single-pass sequencing was performed on each template using Rbcl Forward primer for bacteria (16s sequencing) (Table 1) and using (ITS-1 and tef1) Forward primer for yeasts (ITS sequencing) (Table 2). Getting rid of unincorporated terminators that still remains with the fluorescent-labeled fragments was conducted by ethanol precipitation protocol. The samples were homogenated in distilled water and analyzed by electrophoresis in an ABI 3730xl sequencer (Microgen Company). Sequences analysis and sequences alignment were performed by using BLAST program and Align Sequences Nucleotide BLAST [17].

Table 1: Primers employed for bacteria PCR

Primer code	Sequence	Product size
27F	5′- AGAGTTTGATCCTGGCTAG -3′	1500bp
1492R	5′- GGTTACCTTGTTACGACTT -3′	

Table 2: Primers employed for yeasts PCR

Primer code	Sequence	Product size
(ITS-1) F	5′- TCCGTAGGTGAACCTGCGG -3′	600bp
(ITS-4) R	5′- TCCTCCGCTTATTGATATGC-3′	

2.4. Preparation of the tested microbial suspensions

The tested isolates were re-cultured and incubated on a fresh SADY broth at 27°C and 65% ± 5% relative humidity (RH) for complete growth. The microbial cells from ten days old culture were suspended in sterilized 0.05% aquoes Tween 80 and counted by using a haemocytometer Neeubauer in order to prepare series of concentrations of the tested isolates.

2.5. Rearing of *S. frugiperda*

Eggs of *S. frugiperda* were collected from a laboratorial culture. The eggs were kept in a ventilated plastic jars. The hatched larvae were individually reared and fed on insecticides-free lettuce leaves till pupation. Pupae awaiting moth emergence were maintained in ventilated cages. Moths were fed on 10% sugar solution. All the culture stages were kept at 27°C ± 2°C, 65% ± 5% RH and with a photoperiod of 12: 12 (light: dark).

2.6. Pathogenicity test

Pathogenicity test of the microbial isolates thought to be potent against *S. frugiperda* 3rd instar larvae was carried out. Fresh lettuce leaves were sterilized according to (Clair *et al*., 1997) [16] by 1% sodium hypochlorite and quickly washed by sterile water. Lettuce leaves were sprayed with the tested microbial suspensions and provided to *S. frugiperda* larvae in ventilated cups (one larva/cup) then incubated at 27± 2 ºC, 65±5 % RH, and photoperiod 12:12 hrs L:D. Each concentration test was performed in triplicate and each replicate contained ten larvae. Another three replicates were sprayed only with water and 0.05% aqueous Tween 80 to act as control. Mortality percentages were daily recorded along five days. Average mortality percentages of *S. frugiperda* larvae were assessed by Abbott's formula (1925) [18]. Estimation of LC_{50}, LC_{90} and slope values were conducted according to Finney (1971) [19].

3. Results and Discussions

3.1. Microbial molecular identification

3.1.1. Bacterial isolates identification

16Sr DNA BLASTx analyses detected four bacterial isolates naturally associated with *S. frugiperda* larvae. They were grouped into each taxonomic category from phyla level to genus level in Table 3. They were *Bacillus* velezensis, *Enterococcus durans, Klebsiella variicola* and *K. michiganesis*. The phylogenetic trees of them were constructed as shown in Fig. 1-4.

B. velezensis is a widespread Gram-positive aerobic, endospore-forming bacterium. It has plant growth promoting properties and support plant health by producing secondary metabolites such as lipopeptide antibiotics and β-1,3-1,4-glucanase (Kim *et al*., 2017 and Fan *et al*., 2018) [20, 21] which suppress many pathogenic bacteria, nematodes and fungi like *F. oxysporum* (Cao *et al*., 2018) [22] and *A. flavus* (Chen *et al*., 2019) [23]. Previous studies reported the high pathogenic activity of *B. velezensis* strains against *Diatraea saccharalis* caterpillars (de Castro *et al*., 2025) [24], immature stages of the Lepidopteran insects (Harun-Or-Rashid *et al*., 2018) [25] and nymphs of *Bemisia tabaci* (Ibrahim *et al*., 2024)

[26]. Moreover, the extracellular metabolites obtained from *B. velezensis* fermentation supernatant showed high aphicidal activities against *Aphis gossypii*, *A. craccivora*, *Myzus percicae* and *Uroleucon formosanum* (Liang *et al*., 2022) [27].

Enterococcus durans is a gram positive facultative anaerobic bacterium (Fisher and Phillips, 2009) [28]. Enterococcus is the most dominant symbiotic bacterium in the gut of order Lepidoptera. Likewise, *Enterococcus* is the prevalent flora in the gut of *S. frugiperda* (Almeida *et al*., 2017; Gichuhi *et al*., 2020, Liu *et al*., 2020 and Ugwu *et al*., 2020) [29-32]. High relative abundance of *E. durans* was recorded in *S. frugiperda* gut (Sahani *et al*., 2023) [33]. *Enterococcus* play a crucial role in carbohydrate transport, metabolic processes and energy production in *S. frugiperda* (Chen *et al*., 2021) [34]. Also, previous studies revealed that Enterococcus related to alkaloids and latex degradation, as well as the detoxification of plant secondary metabolites (Gomes, 2020 and Liu, 2020) [35, 36].

Klebsiella michiganensis and *K. variicola* are symbiotic bacteria isolated from Lepidoptera gut (Sahani *et al*., 2023 and Chu *et al*., 2025) [33, 37]. They are belonged to Enterobacteriaceae which support digestion and detoxification and provide the host insect with nutritional supplementation (Wang *et al*., 2020) [38]. They also play a role in carbohydrates metabolite, protection, courtship and reproduction (Adair *et al*., 2017, Liu *et al*., 2020 and Lv *et al*., 2021) [39,31,40]. *K. michiganensis* was significantly related to host fecundity enhancement, increasing egg production by modulating JH III levels (Chu *et al*., 2025) [37].

It was investigated that synergistic inhibition of *Klebsiella* and *Enterococcus* resulted in significant reduction of the food consumption and insect weight gain and cause prolongation of harmful larval stage (Lü *et al*., 2023) [41].

Table 3: Taxonomic categories of bacteria naturally associated with *S. frugiperda* larvae

S. No.	Phylum	Class	Order	Family	Genus	Species
1	Firmicutes	Bacilli	Bacilli	Bacillaceae	*Bacillus*	B. velezensis
2			Lactobacillales	Enterococcaceae	*Enterococcus*	*E. durans*
3	Proteobacteria	Gammaproteo-bacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella*	*K. variicola*
4						*K.michiganensis*

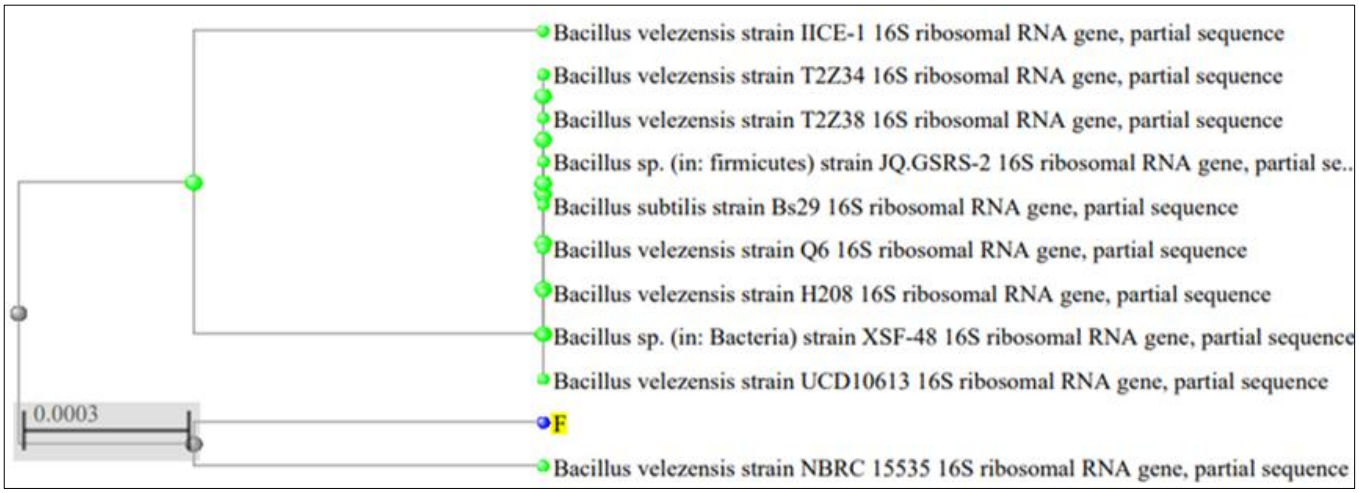

Fig 1: Phylogenetic tree of *B. velezensis*

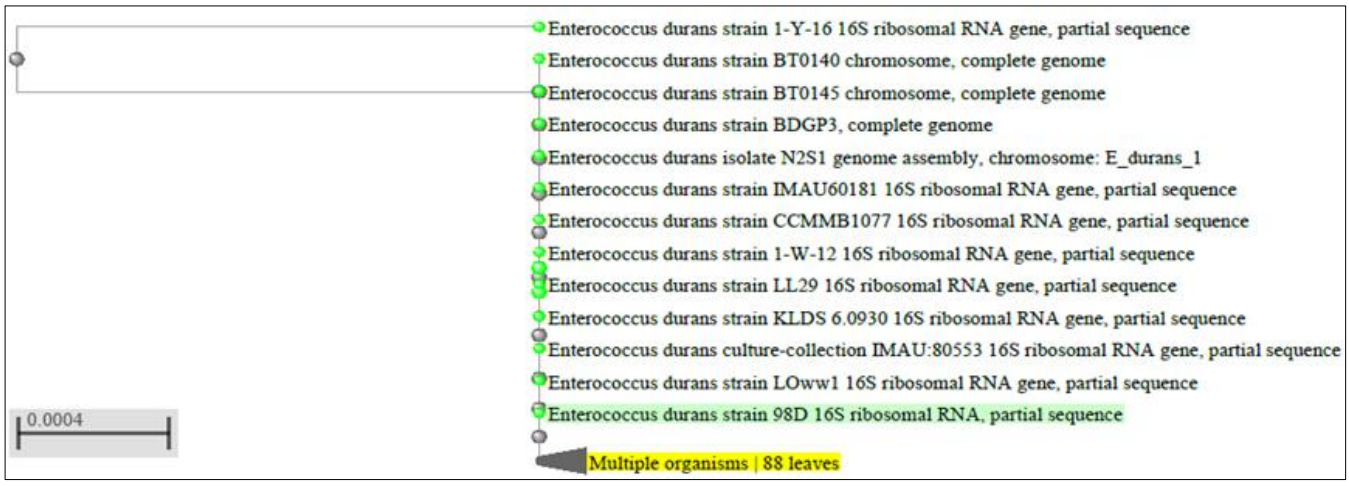

Fig 2: Phylogenetic tree of *E. durans*

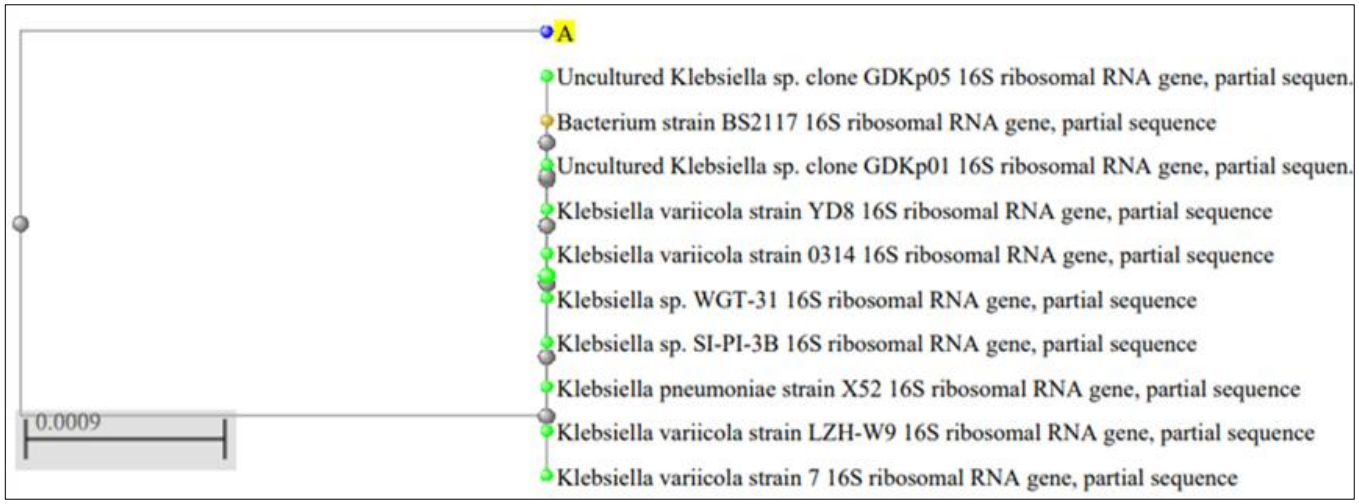

Fig 3: Phylogenetic tree of *K. variicola*

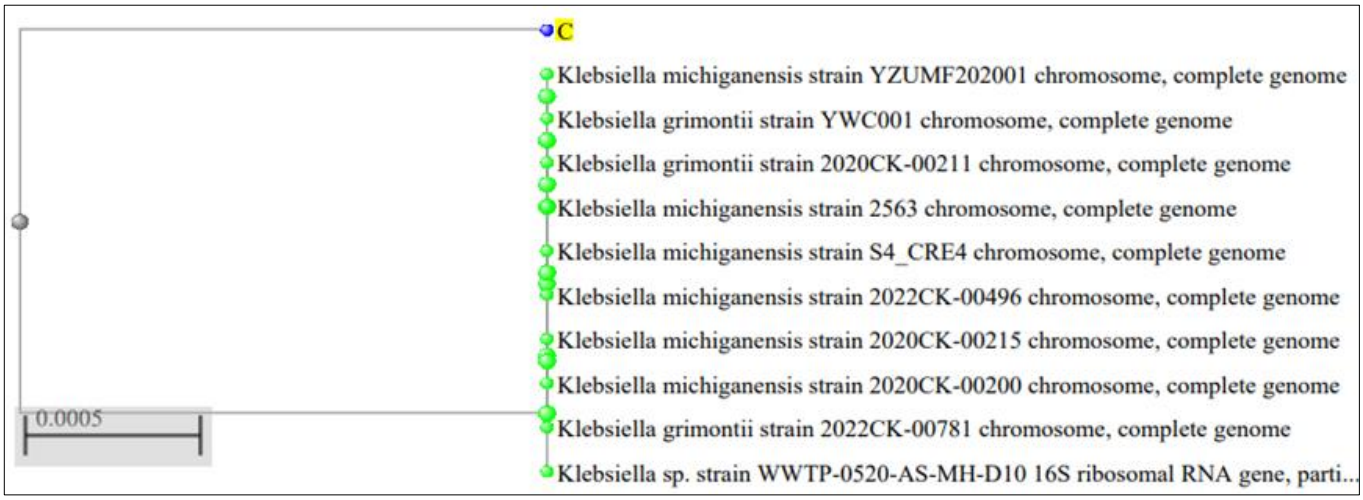

Fig 4: Phylogenetic tree of *K. michiganensis*

3.1.2. Identification of the yeasts

Identification of the yeast isolates led to the detection of two species recorded for the first time naturally associated with *S. frugiperda* larvae. They were identified as *Candida corydali* and *Kodamaea ohmeri*. They were grouped into their taxonomic categories in Table 4 and their phylogenetic trees were constructed as shown in Fig. 5 & 6.

In previous studies, *Candida species* but not the same in the current study was isolated from the intestinal tract of *S. frugiperda* (Wu *et al*., 2019) [42]. Also, few *Candida species* like *C. carpophila*, *C. quercitrusa* (Molnár *et al*., 2008) [43], *C. sake* and *C. zeylanoides* (Mankowski and Morrell, 2004) [44] were found in Lepidoptera intestines and in guts of beetles (Shu *et al*., 2005) [45].

Regarding to *K. ohmeri,* it was previously detected associated with small hive beetle, *Aethina tumida*, the pest of social bee colonies (Amos *et al*., 2018) [46].

It was proven that yeasts associated with insects are vital symbionts facilitating their insect host feeding on recalcitrant food (Ali, *et al*., 2017; Toki, 2021 and Soto-Robles *et al*., 2019) [47-49], promote digestion and supply their host insect with essential amino acids, nutrients and metabolic compounds (Vogeland Moran, 2013; Vogel,*et al*., 2017 and Yun *et al*., 2015) [50-52]. Also, they provide immunity to their insect host and protect them from various pathogens and parasites (Pozo *et al*., 2018 and Cappelli *et al*., 2019) [53, 54].

Table 4: Taxonomy of yeasts comunities naturally associated with *S. frugiperda* larvae

S. No.	Phylum	Class	Order	Family	Genus	Species
1	Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Candida*	*C. corydali*
2				Saccharomycetaceae	*Kodamaea*	*K. ohmeri*

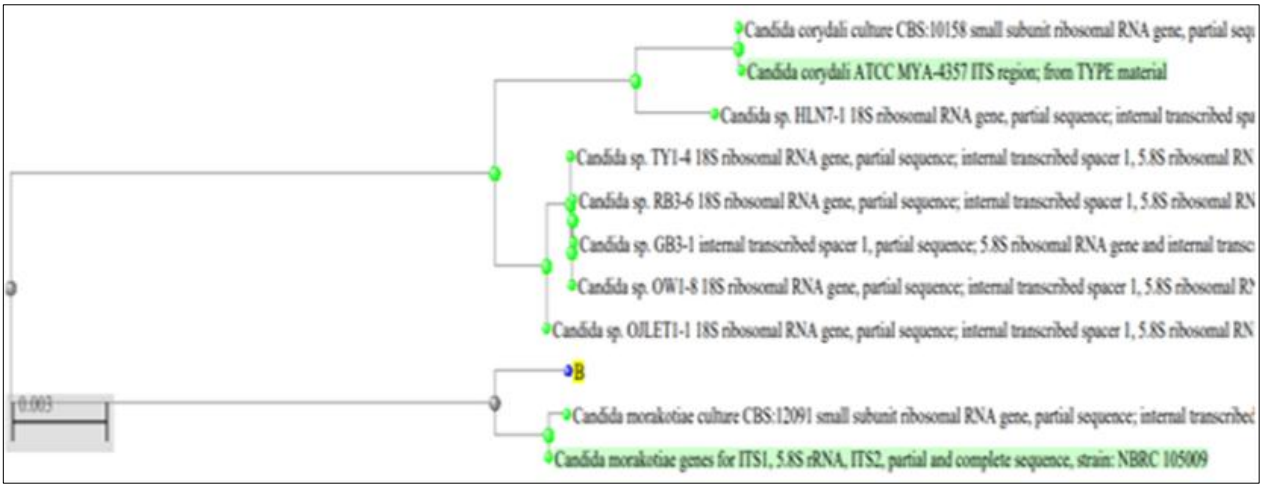

Fig 5: Phylogenetic tree of *C. corydalis*

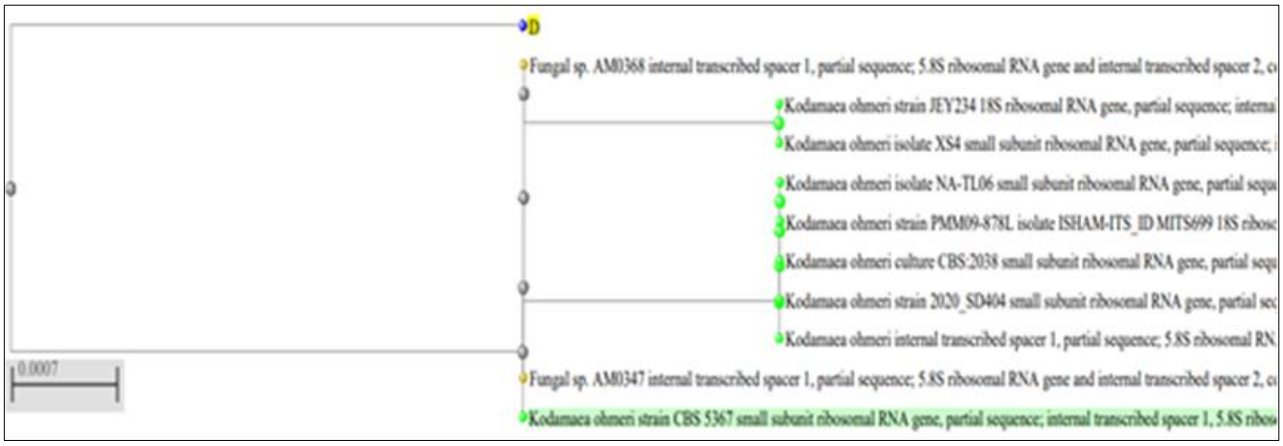

Fig 6: Phylogenetic tree of *K. ohmeri*

3.2. Pathogenicity test

Potentiality of *B. velezensis*, the only identified isolate possess history of pathogenicity unlike the other isolates whose symbiotic relationship has been proven, was conducted against the 3rd instar larvae of *S. frugiperda*. Data in Table 5 cleared that mortality percent of *S. frugiperda* is directly proportional to both of *B. velezensis* concentration and the elapsed time post treatment. *B. velezensis* showed peak of activity at the third day and continued till the fifth day post treatment. It showed LC_{50} of 1.1x 10^7 and LC_{90} of 6x 10^{10} cell/ml. It was noticed that the treated larvae showed significant reductions in growth and movement.

Previous study reported that the commercial product of *B. thuringiensis* subsp. *Kurstaki*, Dipel, was not doing well for controlling fall armyworm larvae (48.86% mortality and an LC_{50} of 116.239) (Priyanka *et al*. 2021) [55]. This means our native isolate of *B. velezensis* was much more efficient than the previously mentioned commercial product. So, exploration of native isolates is very important to introduce new promising candidates can be used in insect- microbial control strategy

Table 5: Efficiency of *B. velezensis* against *S. frugiperda* 3rd instar larvae under laboratory conditions of 27± 2 C°, 65±5 RH and 12:12 L: D

Treatment	Conc. (cell/ml)	Mortality % at indicated day after treatment			LC50 (cell/ml) and confidence limits at 95%		LC90 (cell/ml) and confidence limits at 95%		Slope ± SE	X^2
		1 day	3 day	5day						
B. velezensis	1x10^6	16.67	30.00	33.33	1.1x 10^7		6x 10^{10}		4.677 ± 0.1121	0.1761
	1x10^7	23.33	43.33	46.67						
	1x10^8	36.67	60.00	66.67	2.1x 10^6	3.5x 10^7	8.1x 10^8	9.7x 10^{11}		
	1x10^9	53.33	76.67	83.33						

4. Conclusion

The majority of traditional chemical insecticides now in use were relatively unsuccessful to control *S. frugiperda* due to appearance of insect resistance in addition to their negative environmental impacts. So, Insect microbial control strategy became a key pillar for controlling this invasive pest. The fundamental tenet of this strategy is understanding and characterizing the microbial symbionts and/or pathogens of this pest to adopt entomopathogen-based control strategy or attempting to disrupt the microbiome of the insect pest which serves as the backbone of its strength and adaptability. The current study investigated an entomopathogenic bacterium and three others symbionts in addition to two symbiotic yeasts associated with *S. frugiperda larvae.*

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