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Susceptibility Response of *Spodoptera littoralis* to Insecticides of Assorted Classes Via Insecticide-Degrading Bacteria in Its Gut

Wael M. Khamis[1] and Said I. Behiry[2]

1-Plant Protection Research Institute, Agricultural Research Center, Al-Sabhia, Alexandria, Egypt. https://orchid.org/0000-0002-9736-362X

2-Agricultural Botany Department, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria 21531, Egypt. https://orchid.org/0000-0001-5701-2254

*E-mail: waelmkhamis2019@yahoo.com

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ABSTRACT

Changes in susceptibility of *Spodoptera littoralis* (Lepidoptera: Noctuidae) were investigated against some insecticides. Newly hatched eggs collected from "Al-Qnawiyah," in El-Behira governorate were reared on cotton leaves of the same location up to the 4th instar in the laboratory to gain the 1st generation of field strain (1GFS) owned habitual gut-bacteria (HGB) versus free gut-bacteria (FGB) reared on leaves treated with antibiotics. HGB revealed less susceptibility than FGB realized by LC_{50} ratios (HGB/ FGB) of lufenuron, emamectin benzoate, chlorpyrifos and alpha-cypermethrin with 15.38, 5.08, 3.39 and 2.17 folds in, respectively. Isolated strains from the 4th instar's gut were molecularly sequenced for 16S rRNA gene as *Enterobacter cloacae* Ag10, *Pseudomonas aeruginosa* Fs40 and *Pseudomonas aeruginosa* Fs30. These isolates were submitted for growth activity confirmation test for 3 days of cultivation on insecticide-based minimal medium 9 (from 20 to 500 mg L^{-1}) versus glucose-based medium (control). *E. cloaca* Ag10 grew on all concentrations of alpha-cypermethrin likewise *P. aeruginosa* Fs40 on lufenuron and chlorpyrifos. *P. aeruginosa* Fs30 did not grow on lufenuron but was limited on the others. Semi-field trials in two successive seasons of cotton crop in the foregoing site against the 4th instar of 1GFS showed the long-term toxicity of these insecticides against FGB more than HGB. Therefore, the identified gut-bacterial strains are the hidden cause behind susceptibility shifts in *S. littoralis* to these insecticides.

INTRODUCTION

The cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is a prevalent polyphagous pest in the tropical and subtropical regions of the world. The harmful larvae stages have the ability to cause severe significant damage to the foliage of many economic crops including 112 plants belonging to 44 families (Voirol *et al.*, 2018; Mohamed *et al.*, 2019; Mazumdar *et al.*, 2021).

The lepidopteran's Larva is a haven for diverse bacteria that habit the cavity of the gut throughout the insect's life (Mazumdar *et al.*, 2021). The diversity of gut-bacteria depends on the abundance and variety of nutrition sources of plant leaves exhibited in their

habitat area (Xia *et al.,* 2020). Reared *S. littoralis* on plants collected from different regions showed a significant diversity of gut-bacteria. Further studies declared that the gut bacteria of *Heliothus armigera* owned similar symbiotic bacteria communities, which had been influenced by the source of the leaves surface where they fed (Priya *et al*., 2012; Bapatla *et al.,* 2018; Xia *et al.,* 2020). However, the gut of the larval stage of *S. littoralis* (Boisd.) features alkalinity media, and symbiotic bacteria of the phylum *Firmicutes, Enterococci* and *Clostridium* sp. dominate the cavity of the gut. *Enterococcus mundtii* has a high tolerance to alkaline stress (Tang *et al.,* 2012; Voirol *et al.,* 2018). Moreover, the persistence of *Enterococci* mostly overrides the larva's gut of many other Lepidopterans (Mazumdar *et al.,* 2021). Proteobacteria in Kenya and Firmicutes in Nigeria dominated the gut-bacterial communities of most *Spodoptera frugiperda* samples (Gichuhi *et al.,* 2020). The biodiversity of bacteria in the third and fifth instar larvae of *Spodoptera exigua* included members of the phyla Firmicutes and Proteobacteria. The mid-gut of *S. exigua* possessed the highest diversity and multitude of *Halomonas, Pseudomonas* and *Methylobacterium* communities. In contrast, intestinal contents owned the lowest diversity and highest abundance of *Enterococcus* (Gao *et al*., 2020). Dominated bacterial families in larvae and adults of *Pieris canidia* comprise Burkholderiaceae (Betaproteobacteria), Enterobacteriaceae and Moraxellaceae (Gammaproteobacteria), Pseudomonadaceae and Xanthomonadaceae (Gammaproteobacteria), and Brucellaceae (Alphaproteobacteria). Levels of bacterial community vary sometimes between individuals at the same life stage. In addition, families of Pseudomonadaceae, Moraxellaceae and Xanthomonadaceae significantly shifted in relative multitude across the developmental stages and sexes of *P. canidia* (Wang *et al.,* 2020a).

Insecticides belong to different common groups that are widely used in agricultural fields and are the sole carbon sources for gut-bacteria in the target insect pest. Carbon and nitrogen sources provide sufficient nutrients for gut-bacteria in different insect pests (Indiragandhi *et al.,* 2007; Shankar *et al.,* 2011; Latifi *et al.,* 2012; Chu *et al.,* 2013; Almeida *et al.,* 2017). Living microorganisms have been used in the bioremediation process to degrade pesticides into lesser toxic moieties depending on the type of agro-pesticide, environmental matrix and the organisms present in the ecosystem (Abatenh *et al.,* 2017; Khalil *et al.,* 2021). Degradation of a wide spectrum of pyrethroids into non-toxic compounds by many degrading bacteria and fungi isolated from fauna where the insect pest live as a mean to gain their needs for carbon and nitrogen sources. Pyrethroid-degrading bacteria owned the enzyme esterase/carboxyl esterase which contributes to the initial step of ester bond hydrolysis in pyrethroid biodegradation. In addition, these microorganisms had the ability to use the degrading moieties resulting from parent compounds of pyrethroids (Cycon and Piotrowska-Seget, 2016; Bhatt *et al.,* 2019). On the other hand, organic phosphorus such as fenitrothion insecticide had the ability to be degraded by *Burkholderia* genera strains that developed in the soil and gut of resistant insects (Tago *et al.,* 2015). Enormous members of bacteria, especially from *Bacillus* and *Pseudomonas* genera were able to degrade OPs to gratify their needs for a xenobiotic single carbon source for growth activity (Kumar *et al*., 2017; Patyka *et al.,* 2016). Insect growth inhibitor like lufenuron was used as a carbon source was resultant of the metabolization process via many enterobacteria (*Enterococcus, Klebsiella* and *Leclercia*) that habit the larvae's gut of *S. frugiperda* (Gomes *et al*., 2020; Polenogova, 2021). Information regarding the biodegradation of avermectin compounds via microflora community in agricultural environments has been reviewed in some recent research, but still no information about their degradations via gut-bacteria in insects (Rahman *et al.,* 2018; Cenkseven *et al*., 2019).

Investigations of our research were carried out on two groups of 1st generation of field strain (1GFS) of *S. littoralis* (Boisd.) larvae collected from certain locations of a cotton

field. One of them owned habitual degrading gut bacteria versus another that owned mostly free gut bacteria. This study revolves around the following pivots: a) comparisons between these two groups were made to stand on the role of degrading bacteria that affected the toxicity of chlorpyrifos, emamectin benzoate, lufenuron and alpha-cypermethrin. b) Isolation, molecular identification and sequencing of symbiotic bacteria habit the larvae's gut was accomplished for the 16S rRNA gene. c) Confirmative steps were conducted on isolated gut bacteria to study their capabilities to consume and degrade different concentrations of these insecticides as a glucose substitute in a nutritive medium supplement. d) Semi-field evaluations were achieved on residual toxicities of the selected insecticides against the two groups of rearing larvae.

MATERIALS AND METHODS

Tested Insecticides:

All the selected insecticides were sprayed in the field with applied dosage rates following the recommendations of the Agriculture Pesticides Committee of the Egyptian Agriculture ministry. Chlorpyrifos (Dorsban 48 % EC belongs to organophosphates, Dow AgroSciences) was sprayed with a dosage rate of 1 L acre^{-1}. Emamectin benzoate (Vertimic 1.8 % EC belongs to avermectins, Synginta Agro Egypt) was sprayed with a dosage rate of 250 cm^3 acre^{-1}. Lufenuron (Match 5 % EC belongs to benzoylureas, Synginta Agro Egypt) sprayed with the field dosage rate of 160 cm^3 acre^{-1}. Alpha-cypermethrin (Super Alpha 10 % EC belongs to pyrethroids, El-Helb Pesticides and chemical co) was sprayed with a dosage rate of 250 cm^3 acre^{-1}.

Chemicals of Isolation and Molecular Identification of Gut Bacteria:

Chemicals, media and solvents of laboratory quality were obtained from Sigma Aldrich in the United States. All the glassware, media and other materials were autoclaved at 121 °C for 15 min in accordance with the laboratory protocol, and reagents were prepared according to the protocol's recommendations.

Insect Collecting and Rearing:

Egg masses of cotton leafworm (CLW), *S. littoralis*, were obtained from Al-Qnawiyah village, El-Behira governorate, Egypt (31º05'16.9''N: 30º17'26.6''E) coinciding to cotton plant in seasons of 2020 and 2021. The reared larvae stage of the 1st generation of field strain (1GFS) was reared under laboratory conditions (27 ± 2 °C, RH 65 %) according to the method of Eldefrawi *et al.,* (1964). Feeding the 1GFS during the larval stage till the 4th instar (about 17 days) was accomplished on samples of untreated cotton leaves collected from the control area in the same foregoing origin. The 1GFS comprised two rearing groups reared in a plastic box from egg mass to the 4th instar. The first group contained larvae fed on cotton leaves treated with antibiotics to obtain mostly free bacteria content (FGB) in their gut. The second group contained larvae fed on cotton leaves without antibiotics (control) to maintain habitual gut bacteria (HGB). The two groups were completely isolated aside from each other during rearing time in the laboratory. Homogeneous larvae at the 4th instar of each group were obtained to submit the laboratory and semi-field bioassay tests.

Antibiotic Treatment:

The group of FGB was obtained through consecutive feeding for every 50 homogeneous larvae from the 2nd up to 4th instar (sufficient time to obtain mostly FGB at the 4th instar) on contaminated cotton leaves with antibiotics solution (1 mg ml^{-1} ciprofloxacin +1 mg ml^{-1} levofloxacin + 2 mg ml^{-1} metronidazole in sterile distilled water) containing 1 % surfactant of Tween-20 to enhance antibiotics intake (Xia *et al.,* 2020). Adequate quantities of cotton leaves for the daily feeding of 50 larvae were soaked for 20 min in the solution of antibiotics. The treated leaves resided to dry before being introduced to the larvae. The

treated leaves were changed once a day. In the same foregoing conditions, the group of HGB was obtained by consecutive feeding for the larvae on cotton leaves soaked in sterile water only without antibiotics (control).

Toxicity Studies:

Laboratory bioassay was conducted on the two groups of the 4th instar of *S. littoralis* (Boisd.) (1GFS) obtained from egg masses of 31º05'16.9''N: 30º17'26.6''E in season 2020. The study aimed at the influences of gut bacteria in the larvae on the susceptibility of selective insecticides. Toxicity tests of the selected insecticides on each rearing group were performed by the leaf dipping method of El-defrawi *et al*., (1964) using cotton leaves. Each insecticide had gradual sub-lethal concentrations. Dipping time was about 20 seconds for leaf discs in each diluted concentration. The treated leaf discs were left to dry at room temperature. Each concentration contained four replicates. Ten larvae were used for each replicate. Mortality percentages after 48 hrs of treatment were corrected by the formula of Abbott (1925). Mortality percentages of each tested insecticide were submitted to probit analysis (Finney 1971) to estimate their LC_{50} and the LC_{50} ratio (RR) of HGB/FGB.

Semi-Field Trials on Long-Term Toxicity:

Two field experiments were achieved on the cotton plant after 30 days of plantation in seasons of 2021 and 2022 at 31º05'16.9''N: 30º17'26.6''E. Cotton crop plantations (variety, Giza 86) disciplined the guidelines of crop management practices (Gibbs *et al*., 2005; Directorate Plant Production 2016). The semi-field trial followed the design of a randomized complete block. The selected insecticides were applied alone at their recommended rates. All treatments were applied in four replicated plots (45 m^2). Sprayer equipment Knapsack (CP3) was assigned for the foliar spray trial. The total spray volume was 10 liters per four replicated plots. The control treatment was sprayed with water only in four replicated plots. Adequate samples of mid-aged leaves were collected from the treated and untreated (control) plots in perforated bags. The samples were transferred to the laboratory to resume the susceptibility tests of each treatment along the 48 hrs separately on each group of FGB and HGB of the 4th instar of *S. littoralis* (Boisd.) (1GFS) under certain conditions of 27 ± 2 °C, RH 60 ± 5 %. Each treatment had 4 replicates of ten larvae in glass cubs (200 cm^3) exposed to a fixed portion of the treated leaves (20 gm daily) collected at 0, 2, 4, 6, 8, 10 and 12 days after treatments (DAT). Mortality percentages and long-term toxicity were corrected according to Abbott (1925). The lethal time required to kill 50 % (LT_{50}) of treated larvae was estimated by probit analysis (Abd El Rheem, 2005; Patil, 2015). LT_{50} was calculated by at least five values of mortality percentages amidst the range of ≤ 95 and ≥ 10 %.

Schedule Time For Implementation in Laboratory and Field Experiments:

Toxicity tests in the laboratory were achieved in a short time lag prior to a semi-field trial on cotton crops in the first season of 2021 against the two tested groups of 1GFS of the 4th instar of *S. littoralis*. In the second season of 2022, the semi-field trial was repeated in the same site on another 1GFS collected in this season at the 4th instar. Meanwhile, isolates strains of gut-bacteria obtained from 1GFS of the 4th instar in both seasons were submitted to molecular identification and sequencing based on the 16S rRNA gene. Thereafter, the dose-dependent test of the growth activity (GA) of the isolated bacteria was conducted on sub-lethal gradual concentrations of the tested insecticides.

Statistical Analysis:

All the obtained data were subjected to analysis of variance (ANOVA). Means were determined for significance at 0.05 using LSD test using SAS software (2002).

Isolation of Gut-Bacteria:

The larvae's guts of *S. littoralis* (Boisd.) (1GFS at 4th instar) in the groups of HGB and FGB in both seasons were submitted to bacterial isolation. In both groups, the larvae

were surface-sterilized for 5 min in a solution of 5 % sodium hypochlorite dissolved in 70 % ethanol, followed by three 1-min rinses in sterile distilled water. Under sterile circumstances, larvae were dissected in minimal medium 9 (MM9), and then taken out guts were rinsed in clean, sterile MM9 (Sambrook and Russell 2001). Six guts from each raised group were taken and macerated in sterile plastic homogenizer tubes with pestles in 6 mL of MM9. Samples were vortexed, and one gut's worth of material was transferred to 6 mL of MM9.

Samples were plated on MM9 agar after being cultured at 28 °C with continual shaking (7 g x 10 days). The plated samples underwent bacterial growth and colony isolation incubation at 28 °C. Experiments were replicated three times. Thereafter, Bacterial colonies were isolated according to morphological and growth characteristics. Three or more bacterial colonies were selected for each morphotype and then submitted to re-isolation prior to molecular characterization. Each selected colony was grown in a liquid medium of MM9. The selected colonies were incubated at 28 °C under continual shaking (7 g x 5 days). The samples were centrifuged and bacterial cell pellets were re-suspended in MM9. Sufficient portions of the obtained bacterial cells were centrifuged (2000 g x 5 min) to be ready-made for genomic DNA extraction. The rest of the excess cells were preserved in 20 % sterile glycerol at -80 °C.

Molecular Identification of Bacterial Isolates:

Sunnucks and Hales' genomic DNA (gDNA) extraction method was applied to bacterial cells (1996). Gel-electrophoresis in a slab of 0.8% (w/v) agarose gel containing 0.5 µg mL^{-1} ethidium bromide adjusted at 70 voltages for 1 hr and 7.2 pH by using 40 mM Tris-acetate buffer and 1 mM ethylene diamine tetra acetic acid was used to verify the gDNA's purity. Additionally, gDNA samples were detected using ultraviolet (UV) measurements at an optical density ratio of 260: 280 nm. The isolates were molecularly identified using an almost full sequencing of the 16S rRNA gene. Each isolate's 10 - 20 ng gDNA was subjected to polymerase chain reaction amplification using the universal primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACTT-3') (Weisburg et al 1991). The reaction mixture included 1 x enzyme buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 320 pM of each primer, 95 °C for 4 min (1x), 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min (35x) and with latest prolong at 72 °C for 10 min (1x). As previously mentioned, amplicons were separated by gel-electrophoresis in a 1 % agarose slab. Amplicons were detected by the use of a UV transilluminator. The Macrogen Company in Seoul, Korea performed amplicon sequencing. To get the sequence, amplicons were submitted to sequencing utilizing the original amplification primer set and the universal primer 27F.

The sequences were examined using FinchTV v1.4.0 (Geospiza Inc.), and the blast tool was used to combine several readings into a single sequence. Putatively identification for the isolates against reference sequences required selected trimmed sequences for indicative blast searches versus the National Center for Biotechnology Information (NCBI).

After alignment using ClustalW, which is a feature of Mega 6.0, phylogenetic analyses of the collected sequences for the various isolates were attained (Tamura et al, 2013). To create a phylogenetic tree, Neighbor-Joining was used (Saitou and Nei, 1987) to infer evolutionary relationships. From the NCBI databases, 16S rDNA sequences of bacteria that are closely akin to those isolates of the tested *S. littoralis* larvae's gut were retrieved and used for alignment.

Active Growth Response of Bacteria on Insecticide-Based Media:

To determine which bacteria were most suited for each insecticide in order to conduct additional research on insecticide degradation, the growth activity of the isolates in insecticide-based media compared to control (20 µg of glucose mL^{-1}) and free nutrition-

based media (FNM) were examined. By inoculating 120 µL 10^5 Colony forming units (CFU) mL^{-1} in 3 mL MM9 supplemented with 10 µg mL^{-1} of the tested insecticide, under constant shacking as before, the rate of bacterial growth was measured. CFU was measured daily by plating 10 µL of serial 100 µL aliquot dilutions, followed by 48 hrs of incubation at 28 °C. Up to 5 days were spent cultivating isolates. Each treatment underwent 3 replications, and each sampling period had 3 technical duplicates.

Bacterial Growth Response to Different Concentrations of Insecticides:

For the best GA of isolated bacteria acquired from the prior experiment, dose-dependent investigations of insecticide effects on the growth activity of the isolates were done. Selected isolates were cultured in MM9 with insecticide concentrations (20, 50, 75, 100, 125, 150, 200, 300, 400, and 500 µg mL^{-1}), as well as a control without insecticides (20 µg of glucose mL^{-1}) and FNM. As previously mentioned, samples were produced and bacterial GA was monitored for 3 days of incubation or until no viable cells were seen.

RESULTS

Toxicity of the Selected Insecticides Against Larvae of *Spodoptera littoralis* (Boisd.):

Laboratory studies were carried out to determine the acute LC_{50} values of the selected insecticides on the two groups of HGB and FGB of the 4th instar of *S. littoralis* (Boisd.) (1GFS) at 48 hrs of exposure (Table 1). The LC_{50} values of the selected insecticides in HGB showed their highest toxicity in lufenuron 5 % EC (0.020 mg L^{-1}) followed by alpha-cypermethrin 10 % EC (0.527 mg L^{-1}), emamectin benzoate 1.8 % EC (0.655 mg L^{-1}) and chlorpyrifos 48 % EC (1.085 mg L^{-1}). Meanwhile, all the LC_{50} values in FGB were shifted below those in the HGB and realized the highest toxicity in lufenuron 5 % EC (0.0013 mg L^{-1}) followed by emamectin benzoate 1.8 % EC (0.129 mg L^{-1}), chlorpyrifos 48 % EC (0.320 mg L^{-1}) and alpha-cypermethrin 10 % EC (0.243 mg L^{-1}). In addition, the LC_{50} LC_{50} ratios of HGB / FGB were carried out to evaluate the influences of the susceptibility of gut bacteria in the larvae, to the tested insecticides. The results of LC_{50} ratios revealed its highest levels in lufenuron 5 % EC (15.38 fold) followed by emamectin benzoate 1.8 % EC (5.08 fold), chlorpyrifos 48 % EC (3.39 fold) and alpha-cypermethrin 10 % EC (2.17 fold).

Table 1: LC_{50} values of the selected insecticides on free and habitual gut bacteria of *S. littoralis* (Boisd.) (4th instar, 1st field strain) at 48 hrs of exposure.

Tested insecticides	Rearing group	LC_{50} (mg L^{-1})	Confidence limits (mg L^{-1})	Slope	x^2	*df*	LC50 ratio (HGB / FGB)
Alpha-cypermethrin 10 % EC	HGB	0.527	(0.387-0.717)	1.094	3.18	6	2.17
	FGB	0.243	(0.160-0.369)	0.702	4.51	6	
Chlorpyrifos 48 % EC	HGB	1.085	(0.703-1.675)	1.018	2.058	4	3.39
	FGB	0.320	(0.210-0.487)	0.981	12.34	4	
Emamectin benzoate 1.8 % EC	HGB	0.655	(0.232-1.851)	0.511	0.85	4	5.08
	FGB	0.129	(0.072-0.233)	0.718	6.11	4	
Lufenuron 5 % EC	HGB	0.020	(0.009-0.044)	0.530	3.11	4	15.38
	FGB	0.0013	(0.0001-0.013)	0.301	2.62	4	

Long-term Toxicity of The Selected Insecticides Against Larvae of *S. littoralis*:

The obtained results of semi-field trials in seasons 2021 and 2022 were carried out on the toxic effect of field rates of the selected insecticides against HGB and FGB of *S. littoralis* (Boisd.) (4th instar, 1GFS) at 48 hrs of exposure. The LT_{50} values expressed the certain times at which the long-term toxicity of each treatment could achieve 50 % of mortality against *S. littorals* (Tables 2 and 3).

The results of in season 2021 showed that the toxic effects of chlorpyrifos 48 % EC and alpha-cypermethrin 10 % EC on FGB were significantly higher than those on HGB from 4 to 12 DAT. In the same trend, the toxic effects of emamectin benzoate 1.8 % EC and lufenuron 5 % EC on FGB significantly prevailed over HGB at 6 and 0 up to 10 DAT, respectively. The total mean percentages of long-term toxicity of chlorpyrifos 48 % EC, emamectin benzoate 1.8 % EC, lufenuron 5 % EC and alpha-cypermethrin 10 % EC on FGB (83.57, 46.07, 50.00 and 73.21 %, respectively) were significantly higher than those effects on HGB (70.00, 35.71, 31.79 and 56.43 %, respectively). Moreover, The LT_{50} values of chlorpyrifos 48 % EC, emamectin benzoate 1.8 % EC, lufenuron 5 % EC and alpha-cypermethrin 10 % EC on FGB (12.85, 5.19, 6.16 and 9.63 days, respectively) apparently surpassed those on HGB (9.36, 4.27, 2.79 and 6.51 days, respectively) (Table 2).

Table 2: Long-term toxicity of the tested insecticides on free and habitual gut bacteria of *Spodoptera littoralis* (Boisd.) (4^{th} instar, 1^{st} field strain) at 48 hrs of exposure, season 2021.

Tested insecticides	Rearing group	Mortality % ± SE[1] along intervals of DATs							Total mean of long-term toxicity % ± SE	LT_{50} (days)
		0	2	4	6	8	10	12		
Chlorpyrifos 48 % EC	HGB	97.50^{a}	92.50^{ba}	82.50^{b}	80.00^{a}	72.50^{b}	32.50^{b}	32.50^{b}	70.00^{b}	9.36 (7.03-12.46)
		± 5.00	± 5.00	± 5.00	± 0.00	± 5.00	± 5.00	± 5.00	± 1.17	
	FGB	100.00^{a}	100.00^{a}	97.50^{a}	90.00^{a}	87.50^{a}	57.50^{a}	52.50^{a}	83.57^{a}	12.85 (8.43-19.58)
		± 0.00	± 0.00	± 5.00	± 8.16	± 5.00	± 9.57	± 5.00	± 2.97	
Emamectin benzoate 1.8 % EC	HGB	72.50^{cb}	67.50^{c}	60.00^{c}	22.50^{d}	10.00^{e}	10.00^{e}	7.50^{c}	35.71^{e}	4.27 (2.91-6.27)
		± 9.57	± 5.00	± 0.00	± 5.00	± 8.16	± 0.00	± 5.00	± 1.65	
	FGB	80.00^{b}	72.50^{c}	62.50^{c}	40.00^{c}	35.00^{d}	20.00^{dc}	12.50^{c}	46.07^{d}	5.19 (3.53-7.64)
		± 8.16	± 5.00	± 5.00	± 0.00	± 5.77	± 0.00	± 5.00	± 1.37	
Lufenuron 5 % EC	HGB	70.00^{c}	50.00^{d}	32.50^{d}	30.00^{dc}	22.50^{e}	10.00^{e}	7.50^{c}	31.79^{e}	2.79 (0.88-8.83)
		± 8.16	± 8.16	± 5.00	± 8.16	± 9.57	± 8.16	± 5.00	± 2.95	
	FGB	80.00^{b}	70.00^{c}	67.50^{c}	60.00^{b}	40.00^{d}	22.50^{c}	10.00^{c}	50.00^{d}	6.16 (4.71-8.06)
		± 8.16	± 8.16	± 9.57	± 8.16	± 8.16	± 5.00	± 8.16	± 2.02	
Alfa-Cypermethrin 10 % EC	HGB	100.00^{a}	90.00^{b}	80.00^{b}	62.50^{b}	40.00^{d}	12.50^{de}	10.00^{c}	56.43^{c}	6.51 (5.34-7.94)
		± 0.00	± 8.16	± 8.16	± 9.57	± 8.16	± 5.00	± 0.00	± 2.74	
	FGB	100.00^{a}	95.00^{ba}	92.50^{a}	80.00^{a}	60.00^{c}	52.50^{a}	32.50^{b}	73.21^{b}	9.63 (7.48-12.39)
		± 0.00	± 5.77	± 5.00	± 8.16	± 8.16	± 5.00	± 5.00	± 4.86	

- SE means standard error.
- Each column of mortality percentages and overall mortality with the same letter are not significantly different according to the $LSD_{0.05}$.

In season 2022, chlorpyrifos 48 % EC, emamectin benzoate 1.8 % EC and alpha-cypermethrin 10 % EC showed significantly higher toxic effects on FGB compared to HGB from 4 to 12 DAT as well as lufenuron 5 % EC realized along 0 to 12 DAT. At 0 DAT, both emamectin benzoate 1.8 % EC and alpha-cypermethrin 10 % EC showed surpasses for their toxicity on FGB more than HGB. In the same trend of the first season, the total mean percentages of long-term toxicity of chlorpyrifos 48 % EC, emamectin benzoate 1.8 % EC, lufenuron 5 % EC and alpha-cypermethrin 10 % EC on FGB (75.36, 63.22, 64.29 and 71.79 %, respectively) overpassed HGB (61.43, 47.50, 47.14 and 55.71 %, respectively). Otherwise, values of LT_{50} of chlorpyrifos 48 % EC, emamectin benzoate 1.8 % EC, lufenuron 5 % EC and alpha-cypermethrin 10 % EC on FGB (18.41, 12.75, 10.19 and 14.11 days, respectively) almost exceeded those on HGB (11.09, 2.34, 3.85 and 6.28 days, respectively) (Table 3).

Table 3: Long-term toxicity of the tested insecticides on free and habitual gut bacteria of *Spodoptera littoralis* (Boisd.) (4th instar, 1st field strain) at 48 hrs of exposure, season 2022.

Tested insecticides	Group	Mortality % ± SE[1] along intervals of DATs							Total mean of residual toxicity %[a] ± SE	LT_{50} (days)[b]
		0	2	4	6	8	10	12		
Chlorpyrifos 48 % EC	HGB	87.50^{bdc}	82.50^{ba}	62.50^{dc}	60.00^{bc}	52.50^{c}	47.50^{bc}	37.50^{c}	61.43^{b}	11.09 (4.13-29.77)
		± 5.00	± 5.00	± 5.00	± 8.16	± 5.00	± 5.00	± 5.00	± 1.65	
	FGB	90.00^{bac}	87.50^{a}	82.50^{a}	77.50^{a}	70.00^{a}	62.50^{a}	57.50^{a}	75.36^{a}	18.41 (5.12-66.12)
		± 0.00	± 5.00	± 9.57	± 5.00	± 8.16	± 9.57	± 9.57	± 4.10	
Emamectin benzoate 1.8 % EC	HGB	70.00^{e}	65.00^{de}	50.00^{e}	45.00^{d}	40.00^{d}	35.00^{d}	27.50^{d}	47.50^{d}	2.34 (0.50-10.98)
		± 8.16	± 5.77	± 8.16	± 5.77	± 8.16	± 5.77	± 8.16	± 5.52	
	FGB	90.00^{bac}	67.50^{dc}	67.50^{c}	62.50^{bc}	57.50^{bc}	52.50^{ba}	45.00^{cb}	63.22^{b}	12.75 (2.63-61.72)
		± 8.16	± 9.57	± 9.57	± 9.57	± 5.00	± 5.00	± 5.77	± 4.10	
Lufenuron 5 % EC	HGB	80.00^{d}	57.50^{e}	55.00^{de}	42.50^{d}	40.00^{d}	32.50^{d}	22.50^{d}	47.14^{d}	3.85 (1.71-8.64)
		± 8.16	± 5.00	± 5.77	± 5.00	± 8.16	± 12.58	± 9,57	± 3.09	
	FGB	95.00^{ba}	75.00^{bc}	70.00^{bc}	60.00^{bc}	55.00^{c}	50.00^{b}	45.00^{cb}	64.29^{b}	10.19 (4.03-25.76)
		± 5.77	± 5.77	± 8.16	± 8.16	± 10.00	± 8.16	± 5.77	± 5.08	
Alfa-Cypermethrin 10 % EC	HGB	82.50^{dc}	77.50^{b}	62.50^{dc}	52.50^{dc}	52.50^{c}	37.50^{dc}	25.00^{d}	55.71^{c}	6.28 (3.78-10.43)
		± 5.00	± 5.00	± 5.00	± 5.00	± 5.00	± 9.57	± 9.57	± 3.09	
	FGB	97.50^{a}	82.50^{ba}	80.00^{ba}	70.00^{ba}	67.50^{ba}	57.50^{ba}	47.50^{b}	71.79^{a}	14.11 (4.89-44.73)
		± 5.00	± 5.00	± 0.00	± 8.16	± 5.00	± 5.00	± 5.00	± 1.37	

- SE means standard error.
- Each column of mortality percentages and overall mortality with the same letter are not significantly different according to the $LSD_{0.05}$.

Selective Isolation of Insecticide-Degrading Bacteria:

Our data show that no bacterial isolates were found in the FGB lines in the FGB larvae's gut in the 4th instar of *S. littoralis* (Boisd.). Meanwhile, the HGB lines in HGB larvae's gut have identical phylotypes. Isolates of three bacterial strains were discovered in the culture of selective medium MM9 with each tested insecticide as a substitution for the nutritional source of glucose.

Isolation and Molecular Characterization of Gut Bacteria:

The isolation trails of gut-bacterial strains from the 4th instar of *S. littoralis* (Boisd.) in lines of HGB revealed three Egyptian isolated strains of *Enterobacter cloacae* Ag10, *Pseudomonas aeruginosa* Fs40 and *Pseudomonas aeruginosa* Fs30 that were molecularly characterized and accessioned in the Genbank as OP218481, OP218483 and OP218482, respectively. Furthermore, these isolates were aligned in the same cluster with a similarity of up to 99 % according to the NCBI databases. These isolates were illustrated by a phylogenetic tree using ClustalW, which is implemented in Mega 6.0. (Fig. 1).

Bacterial Growth Response to Sub-Lethal Concentrations of Insecticides:

The previous isolated bacterial strains were cultured in insecticide-based media of MM9 at different concentrations of 20, 50, 75, 100, 125, 150, 200, 300, 400, and 500 mg L^{-1}. After three days of cultivation, the data revealed that the *E.cloacae* Ag10 strain could grow on lufenuron 5 % EC up to 200 ppm compared to the control. At the same time, the GA of the isolated Ag10 strain was ended by 100 ppm of chlorpyrifos 48 % EC. The *Enterobacter* isolates have a full GA on all concentrations of alpha-cypermethrin 10 % EC but no GA has appeared on emamectin benzoate 1.8 % EC at 400 and 500 ppm. Meanwhile, the *P. aeruginosa* Fs40 strain energetically grew on all concentrations of lufenuron 5 % EC and chlorpyrifos 48 % EC. On the other hand, the Fs40 bacterial strain has limited activity up to 200 and 300 ppm on emamectin benzoate 1.8 % EC and alpha-cypermethrin 10 % EC, respectively. On the other hand, the strain of Fs30 has no GA but only a viable appearance at lufenuron 5 % EC, while it was viable at 20 ppm in chlorpyrifos 48 % EC. In addition, the rest two insecticides were very effective on GA of Fs30 at low doses. Meanwhile, all the isolated bacteria could not grow on FNM (Table 4).

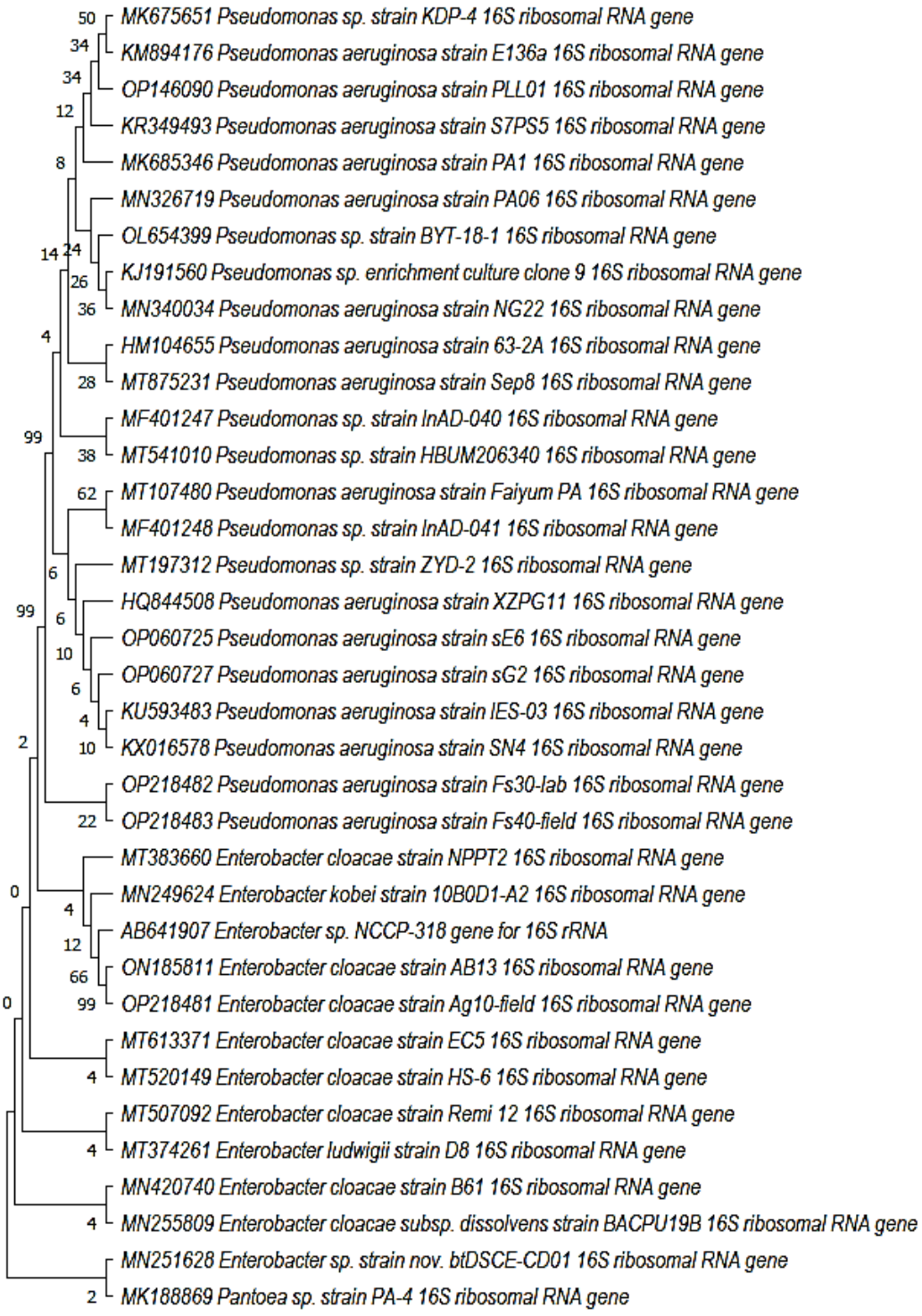

Fig. 1. Phylogenetic tree of all the Egyptian isolates from the 4th instar larvae's gut of *S. littorals* was calculated upon the neighbor-joining method. The Egyptian isolates were *Enterobacter cloacae* strain Ag10 (OP218481), *Pseudomonas aeruginosa* strain Fs40 (OP218483), and *P. aeruginosa* strain Fs30 (OP218482).

Table 4: Bacterial growth response of the isolated gut-bacterial strains to sub-lethal concentrations of insecticides of the tested insecticides.

Treatments	Concentrations (mg L^{-1})	Growth activity of isolated strains		
		Enterobacter cloacae Ag10	*Pseudomonas aeruginosa* Fs40	*Pseudomonas aeruginosa* Fs30
Lufenuron 5 % EC	20	+	+	-
	50	+	+	-
	75	+	+	-
	100	+	+	-
	125	+	+	-
	150	+	+	-
	200	+	+	-
	300	-	+	-
	400	-	+	-
	500	-	+	-
Chlorpyrifos 48 % EC	20	+	+	+
	50	+	+	-
	75	+	+	-
	100	+	+	-
	125	-	+	-
	150	-	+	-
	200	-	+	-
	300	-	+	-
	400	-	+	-
	500	-	+	-
Emamectin benzoate 1.8 % EC	20	+	+	+
	50	+	+	+
	75	+	+	+
	100	+	+	-
	125	+	+	-
	150	+	+	-
	200	+	+	-
	300	+	-	-
	400	-	-	-
	500	-	-	-
Alpha-cypermethrin 10 % EC	20	+	+	+
	50	+	+	+
	75	+	+	+
	100	+	+	+
	125	+	+	-
	150	+	+	-
	200	+	+	-
	300	+	+	-
	400	+	-	-
	500	+	-	-
Control (glucose)	0.02	+	+	+
FNM	0	-	-	-

- (+) means viable bacterial growth activity, (-) means no growth change.

DISCUSSION

As far as we know, various groups of conventional agro-insecticides are the sole carbon and nitrogen sources for gut bacteria in the lepidopteran larvae (Almeida *et al.,* 2017; Mazumdar *et al*., 2021). The gut bacteria degrade various pesticides into less toxic moieties depending on the pesticide structure and the abundance of fauna matrix in the area where the insect pest lives (Abatenh *et al*., 2017; Xia *et al*., 2020; Khalil *et al*., 2021). Consequently, these symbiotic bacteria habit the larvae's gut could lessen its susceptibility to many insecticides (Mazumdar *et al*., 2021). Thus, our study tried to perceive and determine how far the abundance of the symbiotic gut-bacterial strains could affect the chemical control of *S. littoralis* (Boisd.) in one of the certain sites in agro-environmental regions in Egypt.

Our obtained data of the LC_{50} of HGB / FGB for each tested insecticide at LC_{50} in the range of 2.17 up to 15.38 folds. These obvious folds could point out the influences of the gut-bacteria on the susceptibility of *S. littoralis* (Boisd.) larvae to these insecticides. Thus, we resorted to isolating and identifying the gut-bacterial strains from the 4th instar (1GFS). These isolated strains were molecularly characterized and accessioned in the Genbank as *E. cloacae* Ag10 (OP218481), *P. aeruginosa* Fs40 (OP218483) and *P. aeruginosa* Fs30 (OP218482). Moreover, residual toxicity and LT_{50} values of all tested insecticides in both seasons on cotton crops showed significant surpassed and lasting longer in FGB than those in HGB. Gut-associated bacteria are an important ecological resource that involves the digestion and multi-trophic interactions of the host habitat (Schmidt and Engel, 2021). The mid-gut of fall armyworm *S. frugiperda* and *Helicoverpa zea* had different bacterial communities at the same host plant species and sites of collection (Jones *et al*., 2019). Recently, several studies demonstrated several genera of gut symbionts (*Acetobacter*, *Actinobacteria*, *Aeromonas*, *Arsenphonus*, *Burkholderia*, *Citrobacter*, *Clostridium*, *Arthrobacter*, *Enterococcus*, *Lactobacillus*, *Lysinibacillus*, Exiguobacterium, *Lachnospiracease*, *Microbacterium*, *Pseudomonas*, *Symbiotaphrina*, *Staphylococcus* and *Wolbachia*) that habit different orders of insects (Lepidoptera, Hemiptera, Diptera and Coleoptera) could aid in the detoxification of many insecticides classes such as Benzoylurea, Pyrethroids, Avermectin and Organophosphate depending on their biochemical systems to satiate their need of carbon source (Lourthuraj *et al*., 2022; Lin *et al*., 2022; Siddiqui *et al*., 2022).

For more confirmation step, we stand out for the GA of these isolated strains from the gut of the 4th instar for 3 days of cultivation on different concentrations (20 to 500 µg mL^{-1}) of the tested insecticides in MM9 media. The strain of *P. aeruginosa* strain Fs40 had a distinguished GA on all concentrations of chlorpyrifos 48 % EC while strain Fs30 and *E. cloaca* Ag10 had a limited GA on chlorpyrifos 48 % EC. These data came in accordance with the microbial degradation of chlorpyrifos 48 % EC in the gut of the 4th instar of *S.frugiperda* that has been accessed by *Enterobacter asburiae* (Singh *et al.*, 2004; Almeida *et al*., 2017). Moreover, enormous members of *Pseudomonadaceae* bacteria are able to degrade pesticides to gratify their needs for xenobiotic single carbon, nitrogen and phosphorous sources for growth and development (Patyka *et al*., 2016). *Pseudomonas stutzeri* (Sasikala *et al*., 2011), *Pseudomonas kilonensis* (Khalid *et al.,* 2016) and *P. aeruginosa* (Gilani *et al*., 2016) are the few microbial reported to degrade chlorpyrifos 48 % EC. Our data of bacterial GA on alpha-cypermethrin 10 % EC showed potent GA of *E.cloacae* Ag10 on all concentrations while strains of *P. aeruginosa* Fs40 and Fs30 showed restricted growth. Previous findings have been registered on many pyrethroid-degrading bacteria and fungi in the gut of lepidopterans such as *Bacillus* spp., *Raoultella ornithinolytica*, *Psudomonas flourescens*, *P. stutzeri*, *Enterococcus casseliflavus*, *E. mundtii*, *Arthrobacter nicotinovorans*, *Brevibacterium* sp., *Acinetobactor* sp., *Aspergillus* sp., *Trichoderma* sp., and *Candia* spp., which have the capability to realize a direct degradation for a wide spectrum of pyrethroids into non-toxic moieties and use it as a sole carbon and nitrogen source. Pyrethroid-degrading bacteria owned the enzyme esterase/carboxyl esterase that contributes to the initial step of ester bond hydrolysis in pyrethroids biodegradation (Cycon and Seget 2016; Almeida *et al*., 2017; Bhatt *et al*., 2019). Our data showed active bacterial growth on lufenuron 5 % EC at all concentrations for *P. aeruginosa* Fs40 whereas no viable appearance has occurred for the Fs30 strain. Meantime, *E. cloacae* Ag10 revealed a restricted GA on lufenuron 5 % EC. Further studies found that degradation of benzoylurea class such as lufenuron 5 % EC in the gut of *S. frugiperda* based on microbial activity of *E. mundtii*, *Microbacterium arborescens* and *Staphylococcus sciuri subsp. Sciuri* (Almeida *et al*., 2017; Siddiqui *et al*., 2022). The data of our present research

found a limited GA on emamectin benzoate 1.8 % EC for strains of *E. cloacae* Ag10, *P. aeruginosa* Fs40 and Fs30. Wang *et al*., (2020b) found out that Ivermectin, one of the widely used anti-parasitic agents and acaricides, against arthropods and nematodes could undergo a biodegradation process for about 15.7 % in 30 days at 30 ^{o}C in the presence of *Pseudomonas* sp. strain. Polenogova *et al*., (2021) elucidated a counter-unexpected role for gut-bacteria *Enterobacteriaceae*, *Enterobacteria* in increasing and accelerating the susceptibility of Colorado potato beetle *Leptinotarsa decemlineata* (Say) to avermectins class due to destructive changes in the activity of digestive enzymes in its gut tissues.

Conclusion:

Referring to the obtained data, we can deduce that the toxicity of the tested insecticides in the laboratory and their residual toxicities in field trials showed significant transcend in the larvae of *S. littoralis* (Boisd.) owned free gut-bacteria than those in the habitual gut-bacteria group. Three gut-bacterial strains of *Enterobacter cloacae* Ag10, *Pseudomonas aeruginosa* Fs40 and *Pseudomonas aeruginosa* Fs30 were isolated, molecularly identified and sequenced for 16S rRNA gene. *E. cloaca* strain Ag10 had a well-growth on all concentrations of alpha-cypermethrin 10 % EC as well as *P. aeruginosa* strain Fs40 on lufenuron 5 % EC and chlorpyrifos 48 % EC. On the other hand, *P. aeruginosa* Fs30 had no GA on lufenuron 5 % EC but a limited GA on the other tested insecticides. Therefore, the GA study of these isolated strains could elucidate their role in consuming the tested insecticides as an alternative carbon source and consequently influence the susceptibility of *S. littoralis* (Boisd.) larvae to these insecticides (Mazumdar *et al*., 2021) in Al-Qnawiyah village, El-Behira governorate, Egypt.

Declaration:

Competing interests:

The authors confess that they do have not any competing interests.

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ARABIC SUMMARY

إستجابة قابلية التأثر فى دودة ورق القطن لمبيدات الحشرات من مجموعات مختلفة من خلال البكتيريا المحطمة لمبيدات الحشرات فى معدتها

وائل محمود خميس*؛ سعيد إبراهيم عبد الله البحيرى**

* قسم إختبار مبيدات آفات القطن، معهد بحوث وقاية النباتات، مركز البحوث الزراعية، الصبحية، الأسكندرية، جمهورية مصر العربية

**قسم النبات الزراعى، كلية الزراعة (سبا باشا)، الأسكندرية، جامعة الأسكندرية ص. ب 21531، جمهورية مصر العربية

فى هذا البحث تم التحقيق فى التغيرات الطارئة فى قابلية التأثر فى دودة ورق القطن عند تعرضها لبعض المبيدات. حيث تم تربية الفقس الحديث للطع البيض التى تم تجميعها من قرية القناوية بمحافظة البحيرة على أوراق القطن من نفس المنطقة فى المعمل وصولا حتى العمر اليرقى الرابع للحصول على الجيل الأول من السلالة الحقلية والتي تحتوي على البكتيريا المعدية وذلك مقارنة مع نظيرتها التى تغذت على نفس الأوراق بعد معاملتها بالمضادات الحيوية بهدف الحصول على مجموعة من اليرقات خالية إلى حد كبير من البكتيريا المعدية. يظهر انخفاض فى قابلية التأثر فى مجموعة اليرقات التى تحتوي على البكتيريا المعدية بفارق ملاحظ عن نظيرتها ذات المعدة الخالية من البكتيريا من خلال المقاومة النسبية بين التركيز النصف قاتل لليرقات المحتوية على البكتيريا منسوبة إلى نظيرتها الخالية من البكتيريا فى اللوفينيورون، الإيماميكن بنزوات، الكلوربيريفوس والألفاسيبرميثرين بقيم 15.38، 5.08، 3.39 و2.17 ضعف، على التوالى. وقد تم التعريف الجزيئى بناء على الحمض النووى الريبوزى للسلالات المعزولة من معدة يرقات العمر الرابع للجيل الحقلى الأول والتى تحددت فى عزلات هى الإنتيروباكتر كلواكاِ Ag10، السيدوموناس أريجنوزا Fs40والسيدوموناس أريجنوزا FS30. هذه العزلات خضعت لمدة 3 أيام للتنمية على بيئة متخصصة من المركبات الحشرية كمصدر غذائى (من 20 إلى 500 جزء فى المليون) مقارنة ببيئة متخصصة غذائية من الجلوكوز (الكنترول). حيث تبين نمو بكتيريا الإنتيروباكتر كلواكا Ag10 على جميع تركيزات الألفاسيبرمثرين كما هو الحال السيديموناس أريجنوزا FS40 على اللوفينيورون والكلوربيريفوس. وتبين عدم نمو السيديموناس أريجنوزا FS30 على اللوفنينيورون ولكن نمت بشكل محدود على باقى المبيدات الأخرى. ومن خلال التجارب النصف حقلية على مدار موسمين زراعيين تبين أن مدة الأثر الإبادى والزمن اللازم للقتل النصفى لهذه المبيدات المختبرة ضد يرقات العمر الرابع الخالية من البكتيريا المعدية فد تفوقت على نظيرتها التى تحتوي تلك البكتيريا. لذي، فإنه من خلال نشاط النمو للعزلات البكتيريا المعدية السابق تعريفها فإنها تعد السبب الخفى وراء إنخفاض قابلية التأثر فى دودة ورق القطن لتلك المبيدات محل الدراسة فى بيئتها الزراعية.