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# Investigating internal bacteria of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) larvae and some *Bacillus* strains as biocontrol agents

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**Abstract:** *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is one of the most destructive pests of several vegetables and fruits worldwide. In spite of various control methods, this pest has still continued to cause significant damage. In this study, the culturable bacterial flora of *S. littoralis* was determined. New isolates from *S. littoralis*, as well as 12 different *Bacillus* isolates belong to 5 species that were previously isolated from different pests, were tested on *S. littoralis* larvae. In total, 9 bacteria were characterized based on their morphological, biochemical, physiological, and molecular characteristics. The bacterial flora of *S. littoralis* was determined as *Flavobacterium* sp. (SL1), *Klebsiella pneumonia* (SL2), *Enterobacter* sp. (SL3), *Enterobacter* sp. (SL4), *Klebsiella* sp. (SL5), *Serratia marcescens* (SL6), *Pseudomonas aeruginosa* (SL7), *Acinetobacter baumannii* (SL8), and *Staphylococcus* sp. (SL9). The insecticidal activity tests were performed on the third-instar larvae of *S. littoralis*. SL1 and SL5 from *S. littoralis* caused the highest mortalities with 67% and 77%, respectively. Among previously isolated *Bacillus* isolates, *Bacillus thuringiensis* subsp. *kurstaki* (MnD) and *B. thuringiensis* subsp. *kurstaki* (BnBt) were found to be the most effective, causing 100% mortality within 10 days after treatment. A concentration-response test was conducted with these isolates and it was found that the isolate MnD was more effective than BnBt. Therefore, further bioassay experiments were conducted with the isolate MnD and results were discussed with respect to the biocontrol potential of the bacterial isolates.

**Key words:** Cotton leaf worm, bacterial flora, biocontrol, *Bacillus thuringiensis*

## 1. Introduction

The cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is considered one of the most important major pests of cotton plants (*Gossypium hirsutum* L.), as well as tobacco (*Nicotiana tabacum* L.) and corn (*Zea mays* L.), in Mediterranean and Asian countries. Larvae are polyphagous, causing important economic losses in both greenhouses and open fields on a broad range of ornamental, industrial, and vegetable crops (Martins et al., 2005).

Due to the severe damage to various vegetables, controlling this pest is an important part of integrated pest management systems where it exists. Up to now, management has mainly focused on chemical insecticides, particularly lufenuron, cyfluthrin, fenpropathrin, and mephospholan in Turkey (Ministry of Agriculture of Turkey, 2008). Numerous other organophosphorus synthetic pyrethroids and other insecticides have been used in many countries. However, these agents have potentially undesirable side effects on humans, plants, and other animal species, especially predators and parasitoids of important pests. Many populations of *S. littoralis* have also acquired resistance towards most insecticide groups (El-Guindy et al., 1983; Mosallanejad and Smagghe, 2009).

Microbial pesticides are becoming recognized as an important factor in crop and forest protection and in insect vector control. These pesticides are natural disease-causing microorganisms such as viruses, bacteria, nematodes, protozoa, and fungi that infect or intoxicate specific pest groups (Khetan, 2001). In addition to the chemical control of *S. littoralis*, numerous studies have been carried out on possible microbial control agents of the pest. Insect viruses, fungi, nematodes, and bacteria (mainly preparations based on *Bacillus thuringiensis*) have been investigated for the biological control of *S. littoralis* (Farag, 2008; Masetti et al., 2008). Direct use of these biocontrol agents to control *S. littoralis* has not apparently passed into practice extensively, except for *B. thuringiensis* (Kamel et al., 2010). The application of preparations of the bacteria *B. thuringiensis* ABG6104 and ABG6105, and their toxins, was highly effective against *S. littoralis*. A commercial formulation of *B. thuringiensis* (Agerin)

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has significant biochemical and physiological effect on the larvae of *S. littoralis* (Kamel et al., 2010). However, there is still a need to find new bacterial control agents against this pest since *S. littoralis* is developing resistance to many strains of *B. thuringiensis* (Salama et al., 1989).

Bacteria are the most common microorganisms associated with insects, most of them developing extracellularly except for the pathogenic *Rickettsia* (Wegensteiner, 2007). Symbiotic bacteria are ubiquitously located in insect guts with these symbioses ranging from pathogenic to mutualistic and from facultative to obligate. Determining the symbiotic bacteria in insect species can allow the development of new approaches to biological control since they can be genetically transformed to express insect-killing toxins or proteins (Li et al., 2005). Therefore, the bacterial flora of various harmful insects has been determined in both agriculture and forestry (Osborn et al., 2002; Sezen et al., 2004; Sezen et al., 2007; Yu et al., 2008; Consolo et al., 2010; Gökçe et al., 2010; Sevim et al., 2010; Danışmazoğlu et al., 2012; Demir et al., 2012; Seçil et al., 2012; Demirci et al., 2013). This is an important step towards understanding the role of symbiotic bacteria in the insect gut and in the process of using bacteria in the microbial control of pest species.

In the present study, we focused on the determination of the bacterial flora of *S. littoralis* in order to find more virulent microbial control agents against this insect pest. To this end, we isolated and characterized 9 bacteria from *S. littoralis* using morphological, biochemical, physiological, and molecular techniques. Additionally, we tested the insecticidal activity of these bacteria and 12 different *Bacillus* isolates belonging to 5 species against *S. littoralis*. This is the first study to determine the bacterial flora of *S. littoralis* and the species' virulence against it.

## 2. Materials and methods

### 2.1. Collection of larvae

*S. littoralis* larvae were collected from cotton fields in the vicinity of Adana, Turkey, on 3–4 September 2010. The collected larvae were placed in plastic boxes (25 cm in length and 18 cm in width) with ventilated lids, and small pieces of cotton leaves were provided as food until they were transported to the laboratory. After the transportation, larvae were fed lettuce at room temperature under a 12:12 photoperiod for 1 week. Healthy and diseased larvae were then separated and used for bacterial isolation.

### 2.2. Isolation of bacteria

Bacterial isolation was performed on dead and living larvae separately. Healthy and dead larvae were separated based on macroscopic examination, distinguishing between living larvae that showed general disease symptoms and dead larvae. Twenty living and 20 dead larvae were separately surface sterilized with 70% ethanol for 5 min and washed 3 times in sterile distilled water. The larval bodies were homogenized in nutrient broth using a glass tissue grinder and the homogenate was filtered through 2 layers of cheese muslin into sterile tubes to remove larval debris. From the larval extracts, 10, 25, and 50 µL were placed on nutrient agar and incubated at 30 °C for 2–3 days. The remaining mixtures were incubated at 30 °C for 3–4 h to increase the number of bacteria that had low concentrations. From these mixtures, 10, 25, and 50 µL were also placed on nutrient agar and incubated at 30 °C for 2–3 days. Isolates were distinguished based on colony color and morphology. Pure cultures of the bacterial colonies were prepared and stocked in 20% glycerol at the Laboratory of Microbiology of the Department of Biology, Faculty of Science, Karadeniz Technical University. Bacterial cultures were identified according to their morphology, nutritional features, and biochemical, physiological and molecular characteristics.

### 2.3. Identification of bacterial isolates

Bacterial isolates were identified by various tests, such as the utilization of organic compounds, spore formation, Gram staining, NaCl tolerance, optimum temperature, optimum pH, catalase and oxidase tests, and starch hydrolysis. The API 20E and API 50CH systems were also used for further characterization of the bacterial isolates. Test results were evaluated according to *Bergey's Manual of Systematic Bacteriology*, Vols. 1 and 2 (Krieg and Holt, 1986; Sneath et al., 1986). API test results were evaluated using IdBact v. 1.1 software by G. Kronvall, with Matrix for API20E from bioMerieux, France.

### 2.4. 16S rRNA gene sequencing

Partial sequencing of the 16S rRNA gene was used to confirm isolate identification. Total genomic DNA extraction was done according to the standard protocol of Sambrook et al. (1989). The isolated DNAs were stored at –20 °C until use.

PCR amplification of the 16S rRNA genes was performed using the universal primers UNI16S-L (5'-ATTCTAGAGTTTGATCATGGCTCA-3') as the forward primer and UNI16S-R (5'-ATGGTACCGTGTGA CGGGCGGTGTGTA-3') as the reverse primer (Weisburg et al., 1991). Amplification was carried out in a thermocycler (Eppendorf, Mastercycler Gradient, Hamburg, Germany) for 36 reaction cycles. Reactions were routinely performed in 50 µL including 1.5 µL of 10 mM dNTP mix, 1.5 µL of 10 pmol each of the opposing amplification primers, 1 µL of 5 U/µL Taq DNA polymerase (Fermentas), 3 µL of $MgCl_2$, 5 µL of Taq DNA polymerase reaction buffer, 1 µL of genomic DNA, and 35.5 µL of $dH_2O$. PCR conditions were 5 min at 95 °C for the initial denaturation of template DNA, 36 amplification cycles (1 min at 94 °C, 1 min at 56 °C, 2 min at 72 °C), and 10 min at 72 °C for the final extension. PCR products were separated on 1.0% agarose

gels, stained with ethidium bromide, and viewed under UV light. After checking the PCR products, they were sent to Macrogen (the Netherlands) for sequencing. The obtained sequences were used to perform BLAST searches (Altschul et al., 1990) using the NCBI GenBank database. Additionally, sequences were used for phylogenetic analysis for further characterization.

**2.5. Phylogeny**

Nucleotide sequences of the 16S rRNA genes were edited with BioEdit and aligned with ClustalW (Hall, 1999). A total of 36 16S rRNA gene sequences including 9 isolates from *S. littoralis* and their 27 closely related species were used in the phylogenetic analysis. The phylogenetic analysis was performed using the neighbor-joining (NJ) method, carried out using MEGA 5.0 software (Tamura et al., 2011). The NJ analysis was based on the Kimura 2-parameter test. Alignment gaps were treated as missing data. The reliability of the phylogram was tested by bootstrap analysis with 1000 replicates using MEGA 5.0.

**2.6. Preparation of bacterial isolates**

Bacterial isolates were streaked onto nutrient agar plates to obtain single colonies for each isolate. The obtained single colonies were inoculated into nutrient broth and incubated at 30 °C overnight. Some of the isolates were incubated at 30 °C for 2 days due to their slow growth. After incubation, the bacterial density was measured at optical density ($OD_{600}$) and adjusted to 1.89 (approximately $1.8 \times 10^9$ cfu/mL) (Moar et al., 1995). Five milliliters of this culture was centrifuged at 3000 rpm for 10 min. After that, the pellet was resuspended in 5 mL of sterile phosphate buffer solution (PBS) and used in bioassays.

**2.7. Bioassays with *Spodoptera littoralis* isolates and *Bacillus* species**

*S. littoralis* larvae were obtained from laboratory cultures at the Laboratory of Microbiology, Department of Biology, Faculty of Science, Karadeniz Technical University. The third-instar healthy larvae were selected at random and used in bioassays. A total of 9 isolates from *S. littoralis* and 12 *Bacillus* isolates (Table 1) were used in the initial bioassay experiments.

Fresh lettuce leaves (approximately 10 $cm^2$) were used as the diet in the initial infections. Experiments were performed with 10 larvae per replicate, and 3 replicates of each treatment group were used. A 1 mL bacterial suspension of each isolate prepared as described above was saturated on lettuce leaves and placed in individual plastic boxes (30 cm in length and 18 cm in width), each containing a single bacterial isolate. Ten third-instar larvae of *S. littoralis* for each replicate were then placed on the lettuce in the plastic boxes for 10 days.

**2.8. Concentration-response test**

Concentration application experiments were conducted with isolates MnD and BnBt (see Table 1 for details) based on their superior pathogenic effects on *S. littoralis* larvae according to the screening test. Three different bacterial concentrations ($0.9 \times 10^9$ cfu/mL, $1.8 \times 10^9$ cfu/mL and $3.6 \times 10^9$ cfu/mL, based on $OD_{600}$ values) were used in the concentration-response test. One milliliter of bacterial suspensions of different concentrations was saturated onto lettuce leaves (approximately 10 $cm^2$) and placed in individual plastic boxes (30 cm in length and 18 cm in width). After that, 10 third-instar *S. littoralis* larvae for

**Table 1.** *Bacillus* species used in this study and their hosts.

| Isolates | Species | Hosts | References |
|---|---|---|---|
| Lyd6 | *B. thuringiensis* | *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) | Demir et al., 2012 |
| Lyd7 | *B. thuringiensis* | *L. dispar* (L.) (Lepidoptera: Lymantriidae) | Demir et al., 2012 |
| Lyd8 | *B. thuringiensis* | *L. dispar* (L.) (Lepidoptera: Lymantriidae) | Demir et al., 2012 |
| Ar1 | *B. circulans* | *Anoplus roboris* (Suffr.) (Coleoptera: Curculionidae) | Demir et al., 2002 |
| Ar4 | *B. sphaericus* | *A. roboris* (Suffr.) (Coleoptera: Curculionidae) | Demir et al., 2002 |
| MnD | *B. thuringiensis* subsp. *kurstaki* | *Malacosoma neustria* (L.) (Lepidoptera: Lasiocampidae) | Sevim et al., 2012 |
| Xd3 | *B. thuringiensis* subsp. *tenebrionis* | *Xyleborus dispar* (F.) (Coleoptera: Scolytidae) | Sezen et al., 2008 |
| As3 | *B. cereus* | *Amphimallon solstitiale* (L.) (Coleoptera: Scarabaeidae) | Sezen et al., 2005 |
| Mm2 | *B. thuringiensis* | *Melolontha melolontha* (L.) (Coleoptera: Scarabaeidae) | Sezen et al., 2007 |
| Mm5 | *B. sphaericus* | *M. melolontha* (L.) (Coleoptera: Scarabaeidae) | Sezen et al., 2007 |
| Mm7 | *B. weihenstephanensis* | *M. melolontha* (L.) (Coleoptera: Scarabaeidae) | Sezen et al., 2007 |
| BnBt | *B. thuringiensis* subsp. *kurstaki* | *Balanius nucum* (L.) (Coleoptera: Curculionidae) | Sezen and Demirbağ, 1999 |

each replicate were placed on the lettuce in the plastic boxes for 10 days. Three replicates of each treatment group were used, each containing a single bacterial isolate and 10 third-instar larvae.

**2.9. Influence of larval stages and diets on the effect of bioassays**

More detailed bioassay experiments were conducted with the isolate MnD because of its high virulence compared to the isolate BnBt.

For the bioassays on different larval stages, the density of the bacterial cells was adjusted to 1.89 ($1.8 \times 10^9$ cfu/mL) at $OD_{600}$ and 1 mL of bacterial suspension of the isolate MnD was prepared as described above (Moar et al., 1995). After that, lettuce leaves (approximately 10 $cm^2$) were contaminated with the bacterial suspension and individually placed in plastic boxes (30 cm in length and 18 cm in width). Finally, 10 larvae belonging to the first-, second-, third-, and fourth-instar for each replicate were put into these plastic boxes. Three replicates of each treatment group were used, each containing a single concentration of isolate MnD and 10 larvae belonging to different development stages. The mortalities of larvae were checked on day 10.

For the effect of different diets on bioassays, the bacterial suspension of isolate MnD was prepared as described above, and freshly collected leaves (approximately 10 $cm^2$) of different diets [chard (*Beta vulgaris* var. *cicla* L.), parsley (*Petroselinum crispum* Mill.), corn (*Zea mays* L.), bean (*Phaseolus vulgaris* L.), cabbage (*Brassica oleracea* var. *capitata* L.), and lettuce (*Lactuca sativa* L.)] were contaminated with 1 mL of bacterial suspension. After that, 10 third-instar larvae of the pest for each replicate and the different diets were placed together in each plastic box (30 cm in length and 18 cm in width). Three replicates of each treatment group were used, each containing isolate MnD, different diets, and 10 third-instar larvae.

For the control group of all experiments, lettuce leaves were saturated with sterile PBS. All boxes used in all bioassay experiments were kept at 25 °C and 60% relative humidity with a 12:12 photoperiod. After the different diets were eaten completely, fresh untreated leaves were provided for the larvae for the remainder of the 10 days of bioassays. The mortalities of larvae were checked on day 10 after putting the larvae in plastic boxes including contaminated diets with bacterial suspensions.

**2.10. Data analysis of bioassays**

Mortality data were corrected by Abbott's formula (Abbott, 1925). The data were subjected to analysis of variance (ANOVA) and subsequently to least significant difference (LSD) multiple comparison tests to compare test isolates with each other and the control group with respect to mortality. *S. littoralis* and *Bacillus* isolates were separately evaluated. The effects of different developmental stages of *S. littoralis* and different diets were also analyzed by ANOVA, followed by LSD multiple comparison post hoc testing with respect to mortality of the pest. Concentration-response testing was analyzed by one-way ANOVA. All analyses were performed using SPSS 15.0.

**2.11. GenBank accession numbers of bacterial isolates**

The GenBank accession numbers for the partial sequence of the 16S rRNA gene sequences for the isolates SL1, SL2, SL3, SL4, SL5, SL6, SL7, SL8, and SL9 are JQ066774, JQ066775, JQ066776, JQ066777, JQ066778, JQ066779, JQ066780, JQ066781, and JQ066782, respectively.

## 3. Results

**3.1. Isolation and identification of bacteria from *Spodoptera littoralis***

A total of 9 bacteria were isolated from living and dead *S. littoralis* larvae. Colonies were observed in different colors on nutrient agar. Eight isolates were smooth-round, and only 1 isolate (SL7) was wavy-round. Eight gram-negative bacteria and 1 gram-positive bacterium were determined, and none of them formed spores. Only 1 bacterium (SL9) was isolated from the dead larvae. Other morphological properties of the isolates are given in Table 2. The biochemical characteristics of the bacterial isolates varied depending on the isolate. An API20E test was used for gram-negative bacteria and an API50CH test was used for gram-positive bacteria. Tables 3 and 4 summarize the biochemical properties of the bacterial isolates. Growth of the bacterial isolates at different pH levels, NaCl concentrations, and temperatures also varied depending on the isolate. Although all isolates grew at pH 10, only SL4 and SL8 grew at pH 3. As all isolates grew in 3% NaCl, the increment of the concentration influenced the bacterial growth. SL9 was the only isolate that grew in 15% NaCl concentration. Moreover, none of the isolates could grow at 10 or 50 °C. Other physiological characteristics of the bacterial isolates are given in Table 5.

We also sequenced approximately 1.350 bp of the 16S rRNA gene for each isolate to confirm the isolate's identification. Based on all the identification tests and sequencing analysis, isolates from *S. littoralis* were identified as *Flavobacterium* sp. (SL1), *Klebsiella pneumonia* (SL2), *Enterobacter* sp. (SL3), *Enterobacter* sp. (SL4), *Klebsiella* sp. (SL5), *Serratia marcescens* (SL6), *Pseudomonas aeruginosa* (SL7), *Acinetobacter baumannii* (SL8), and *Staphylococcus* sp. (SL9) (Table 6). Phylogenetic analysis of the 16S rRNA genes also supports this identification (Figure 1).

**3.2. Virulence of the bacterial isolates**

Bacterial isolates that were obtained from *S. littoralis* larvae produced different mortalities in comparison to each other and the control group ($F = 36.17$; $df = 9, 20$; $P < 0.05$). The highest mortalities were obtained from isolates SL1 and SL5 with 67% and 77%, respectively, within 10

**Table 2.** The morphological characteristics of the bacterial isolates from *S. littoralis* larvae.

| Isolates | Tests | | | | | | |
|---|---|---|---|---|---|---|---|
| | Colony color | Shape of colonies | Shape of bacteria | Gram stain | Spore stain | Source | Growth in NB* |
| SL1 | Yellow | Smooth-round | Bacillus | – | – | Healthy larvae | Turbid |
| SL2 | Cream | Smooth-round | Bacillus | – | – | Healthy larvae | Turbid |
| SL3 | Dark cream | Smooth-round | Bacillus | – | – | Healthy larvae | Turbid |
| SL4 | Cream | Smooth-round | Bacillus | – | – | Healthy larvae | Turbid |
| SL5 | Cream | Smooth-round | Bacillus | – | – | Healthy larvae | Turbid |
| SL6 | Red | Smooth-round | Bacillus | – | – | Healthy larvae | Turbid |
| SL7 | Green | Wavy- round | Bacillus | – | – | Healthy larvae | Turbid |
| SL8 | Dark cream | Smooth-round | Coccobacillus | – | – | Healthy larvae | Turbid |
| SL9 | Yellow | Smooth-round | Coccus | + | – | Dead larvae | Turbid |

*NB: Nutrient broth.

**Table 3.** The biochemical characteristics of the bacterial isolates from *S. littoralis* larvae based on conventional and API20E bacterial identification system.

| Tests | Isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | SL1 | SL2 | SL3 | SL4 | SL5 | SL6 | SL7 | SL8 | SL9 |
| Catalase | + | + | – | + | – | + | + | + | + |
| Oxidase | + | – | – | – | – | – | + | – | + |
| Starch hydrolysis | – | – | – | + | – | + | – | – | – |
| β-Galactosidase | + | + | + | + | + | – | – | + | + |
| Arginine dihydrolase | – | – | + | – | – | + | + | – | – |
| Lysine decarboxylase | – | + | – | + | + | – | – | – | + |
| Ornithine decarboxylase | – | – | + | + | + | – | – | – | – |
| Citrate utilization | – | + | + | + | + | + | + | – | + |
| $H_2S$ | – | – | – | + | + | – | – | – | – |
| Urease | – | + | + | – | – | – | – | – | + |
| Tryptophan deaminase | – | – | – | – | + | – | – | – | – |
| Indole | + | – | – | – | + | – | – | + | – |
| Acetoin | – | + | + | + | + | – | – | – | + |
| Gelatinase | + | – | – | – | + | + | – | + | – |
| Glucose | – | + | + | + | + | – | WP* | – | + |
| Mannitol | – | + | + | + | WP | – | – | – | + |
| Inositol | – | + | – | + | WP | – | – | – | + |
| Sorbitol | – | + | – | + | WP | – | – | – | + |
| Rhamnose | – | + | + | + | – | – | – | – | + |
| Saccharose | – | + | + | + | WP | – | – | – | + |
| Melibiose | – | + | – | + | WP | + | + | – | + |
| Amygdalin | – | + | + | + | WP | – | – | – | + |
| Arabinose | – | + | + | + | WP | + | + | – | + |

*WP: Weak positive.

**Table 4.** The biochemical characteristics of the gram-positive bacterial isolate (SL9) based on API50CH bacterial identification system.

| Source | Time | | Source | Time | |
|---|---|---|---|---|---|
| | 24 h | 48 h | | 24 h | 48 h |
| Glycerol | + | + | N-Acetylglucosamine | + | + |
| Erythritol | – | – | Amygdalin | + | + |
| D-Arabinose | - | - | Arbutin | + | + |
| L-Arabinose | + | + | Esculin-ferric citrate | + | + |
| D-Ribose | + | + | D-Cellobiose | + | + |
| D-Xylose | – | – | D-Maltose | + | + |
| L-Xylose | – | – | D-Lactose (bovine origin) | + | + |
| D-Adonitol | – | – | D-Melibiose | + | + |
| Methyl-βD xylopyranoside | – | – | D-Saccharose (sucrose) | + | + |
| D-Galactose | + | + | D-Trehalose | + | + |
| D-Glucose | + | + | Inulin | – | – |
| D-Fructose | + | + | D-Melezitose | – | – |
| D-Mannose | + | + | D-Raffinose | – | – |
| L-Sorbose | – | – | Amidon (starch) | – | – |
| L-Rhamnose | – | – | Glycogen | – | – |
| Dulcitol | – | – | D-Lyxose | – | – |
| Inosidol | + | + | D-Tagatose | – | – |
| D-Mannitol | + | + | D-Fucose | – | – |
| D-Sorbitol | + | + | L-Fucose | – | + |
| Methyl-αD-mannopyranoside | – | – | D-Arabitol | – | + |
| Methyl-αD-glucopyranoside | – | – | L-Arabitol | – | – |
| Potassium gluconate | + | – | Potassium 2-ketogluconate | – | – |

days after treatment. The other mortalities ranged from 3% to 57%. Only isolate SL3 caused statistically the same mortality as the control (Figure 2a).

The *Bacillus* species isolated from different pest species caused different mortalities in comparison to the control group ($F = 35.96$; $df = 12, 26$; $P < 0.05$). There was also a significant difference among treatments. Among *Bacillus* isolates, the highest mortalities were obtained from isolates MnD and BnBt with 100% mortality within 10 days after application. Other mortalities ranged from 30% to 77% (Figure 2b). Statistical differences were found between the MnD and the BnBt with respect to mortality after application of different concentrations ($F = 208.79$; $df = 2, 24$; $P < 0.05$) (Figure 3).

The MnD isolate was applied to different development stages of *S. littoralis* larvae at a concentration of $1.89 \times 10^9$ cfu/mL. There was a significant difference among the development stages of the pest with regard to mortality ($F = 62.38$; $df = 4, 10$; $P < 0.05$). It was found that the second-instar larvae were more resistant than those in the other development stages (Figure 4).

There was a significant difference among the chard, parsley, corn, bean, cabbage, and lettuce diets with regard to larval mortality after application of the isolate MnD ($F = 46.07$; $df = 6, 14$; $P < 0.05$). It was found that *S. littoralis* larvae were more susceptible to the isolate MnD when lettuce and beans were used in the bioassay (Figure 5).

## 4. Discussion

To date, all bacterial species determined in this study have been isolated from various insect species, except for *Acinetobacter baumannii* (Inglis et al., 2000; Dugas et

**Table 5.** The physiological characteristics of the bacterial isolates from *S. littoralis* larvae.

| Tests | Isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | SL1 | SL2 | SL3 | SL4 | SL5 | SL6 | SL7 | SL8 | SL9 |
| pH 3 | – | – | – | + | – | – | – | + | – |
| pH 4 | – | + | – | + | + | – | – | + | – |
| pH 5 | + | + | + | + | + | + | + | + | + |
| pH 6 | + | + | + | + | + | + | + | + | + |
| pH 7 | + | + | + | + | + | + | + | + | + |
| pH 8 | + | + | + | + | + | + | + | + | + |
| pH 9 | + | + | + | + | + | + | + | + | + |
| pH 10 | + | + | + | + | + | + | + | + | + |
| 3% NaCl | + | + | + | + | + | + | + | + | + |
| 5% NaCl | – | + | + | + | + | + | + | – | + |
| 7% NaCl | – | + | + | + | + | + | – | – | + |
| 10% NaCl | – | – | – | – | – | – | – | – | + |
| 12% NaCl | – | – | – | – | – | – | – | – | + |
| 15% NaCl | – | – | – | – | – | – | – | – | + |
| Growth at 10 °C | – | – | – | – | – | – | – | – | – |
| Growth at 15 °C | + | + | + | + | + | + | + | + | + |
| Growth at 30 °C | + | + | + | + | + | + | + | + | + |
| Growth at 37 °C | + | + | + | + | + | + | + | + | + |
| Growth at 45 °C | – | + | + | + | + | – | + | + | + |
| Growth at 50 °C | – | – | – | – | – | – | – | – | – |

al., 2001; İnce et al., 2008; Ademolu and Idowu, 2011). *Flavobacterium* (SL1) is a genus of gram-negative, nonmotile or motile, rod-shaped bacteria that consists of 10 recognized species. The members of this genus are widely found in soil and fresh water in a variety of environments. This genus is also closely associated with insects (Dugas et al., 2001). We also isolated a *Flavobacterium* sp. (SL1) from *S. littoralis* larvae and this strain caused a significant mortality (67%) in larvae of *S. littoralis* based on the preliminary pathogenicity test, indicating that this strain might be a potential insect pathogen and might be beneficial for the control of *S. littoralis*.

*Klebsiella* spp. and *Enterobacter* spp. are closely associated with many insect species and species belonging to these genera are not generally insect pathogens. They probably play roles in the digesting processes in the insect gut and in the physiological developments of *S. littoralis* larvae (Demir et al., 2002; Ademolu and Idowu, 2011). In this study, we also isolated 2 *Klebsiella* species (SL2 and SL5) and 2 *Enterobacter* (SL3 and SL4) strains from *S. littoralis*. The *Enterobacter* isolates did not show good activity against *S. littoralis*, while the *Klebsiella* species caused significant mortalities in *S. littoralis* larvae.

*S. marcescens* (SL6) is a well-known insect pathogen that can produce several hydrolytic enzymes, some of which have been shown to be toxins. This bacterium is a member of the family Enterobacteriaceae (facultative anaerobic, gram-negative rods that are cytochrome oxidase negative and catalase positive), which usually produces a characteristic red or pink pigment, although white to rose-red strains can occur. *S. marcescens* is not usually pathogenic to insects when present in the digestive tract in small numbers, but once it enters the hemocoel, it multiplies rapidly and causes death in 1–3 days (Sikorowski et al., 2001). Studies have shown the pathogenic properties of this bacterium against many insect pest species. Gökçe

**Table 6.** Identification of the bacterial isolates from *S. littoralis* larvae based on the BLAST searches using 16S rRNA gene sequences.

| Isolates | Suggested identification from GenBank | Query coverage (%) | 16S rRNA similarity (%) | GenBank accession numbers |
|---|---|---|---|---|
| SL1 | *Flavobacterium* sp. YD4 | 99 | 99 | GU458295 |
| | *Empedobacter brevis* LMG 4011 | 99 | 99 | NR042471 |
| | *Flavobacterium* sp. ANU301 | 99 | 99 | EF192137 |
| SL2 | *Klebsiella* sp. ICB477 | 96 | 99 | HM748075 |
| | *Klebsiella pneumoniae* | 96 | 99 | HE578781 |
| | *Klebsiella variicola* JDM-14 | 96 | 99 | JF690980 |
| SL3 | *Enterobacter* sp. B901-2 | 97 | 99 | AB114268 |
| | *Enterobacter hormaechei* subsp. *steigerwaltii* | 97 | 99 | HM058581 |
| | *Enterobacter* sp. HaNA17 | 97 | 99 | HM352360 |
| SL4 | *Enterobacter* sp. SCPB-2 | 96 | 99 | AB425051 |
| | *Enterobacter aerogenes* BPRIST043 | 96 | 99 | JF700493 |
| | *Kluyvera* sp. ES392 | 96 | 99 | GQ402165 |
| SL5 | *Klebsiella* sp. DB-3 | 97 | 99 | FJ711774 |
| | *Klebsiella* sp. SeLB3 | 97 | 99 | HM352414 |
| | *Klebsiella pneumoniae* SDM45 | 97 | 99 | GU997596 |
| SL6 | *Serratia marcescens* M14 | 99 | 99 | JN596118 |
| | *Serratia marcescens* Ki | 99 | 99 | JN201947 |
| | *Serratia* sp., endosymbiont of *Nilaparvata* | 99 | 99 | GU124496 |
| SL7 | *Pseudomonas aeruginosa* strain F1 | 98 | 99 | JN412064 |
| | *Pseudomonas aeruginosa* strain JL091016 | 98 | 99 | HM224410 |
| | *Pseudomonas aeruginosa* strain CGR | 98 | 99 | JN128893 |
| SL8 | *Acinetobacter baumannii* SRR-5 | 98 | 100 | DQ379505 |
| | *Acinetobacter baumannii* bpoe1351 | 98 | 100 | FN563422 |
| | *Acinetobacter* sp. TW | 98 | 100 | FJ753401 |
| SL9 | *Staphylococcus* sp. MIS10 | 95 | 99 | JN660071 |
| | *Staphylococcus* sp. ORG01 | 95 | 99 | AY940424 |
| | *Staphylococcus sciuri* RPa1 | 95 | 99 | JN559391 |

et al. (2010) showed that *S. marcescens* Rb2, which was isolated from *Rhynchites bacchus* (L.), had 73% mortality against *R. bacchus* larvae under laboratory conditions. Lauzon et al. (2003) showed that a nonpigmenting strain of *S. marcescens* was pathogenic to *Rhagoletis pomonella* (Walsh) flies. In the present study, we also showed that *S. marcescens* (SL6) was pathogenic to *S. littoralis* larvae (57%) under controlled laboratory conditions and that this strain may be considered as a possible microbial control agent against *S. littoralis*.

*Pseudomonas aeruginosa* is a member of the family Pseudomonadaceae, which is gram-negative and a well-known bacterial pathogen of many insects including adult grasshoppers, *Melanoplus bivittatus* (Say) and *Camnula pellucida* (Scudder). This species can also produce lethal septicemia in *Galleria mellonella* (L.) larvae, locusts, and cutworms. Banerjee and Dangar (1995) found that *P. aeruginosa* is a facultative pathogen of the red palm weevil, *Rhynchophorus ferrugineus* (Oliver). Jander et al. (2000) showed that *P. aeruginosa* was pathogenic to *G. mellonella* larvae. Inglis et al. (2000) demonstrated that *P. aeruginosa* was the most frequently isolated bacterium from larval and pupal cadavers of the southwestern corn borer (*Diatraea grandiosella* Dyar) and southern corn stalk borer (*Diatraea crambidoides* Grote). In this study, we also determined that *P. aeruginosa* (SL7) had a low pathogenic effect (30%) on *S. littoralis* larvae under laboratory conditions.

*Acinetobacter* (SL8) has been isolated from many insect species and is very common in insects, widely distributed in nature, and normally found in soil and water (Geiger et al., 2009). Although this genus is very common in insect populations, this study was the first to isolate *A. baumannii*

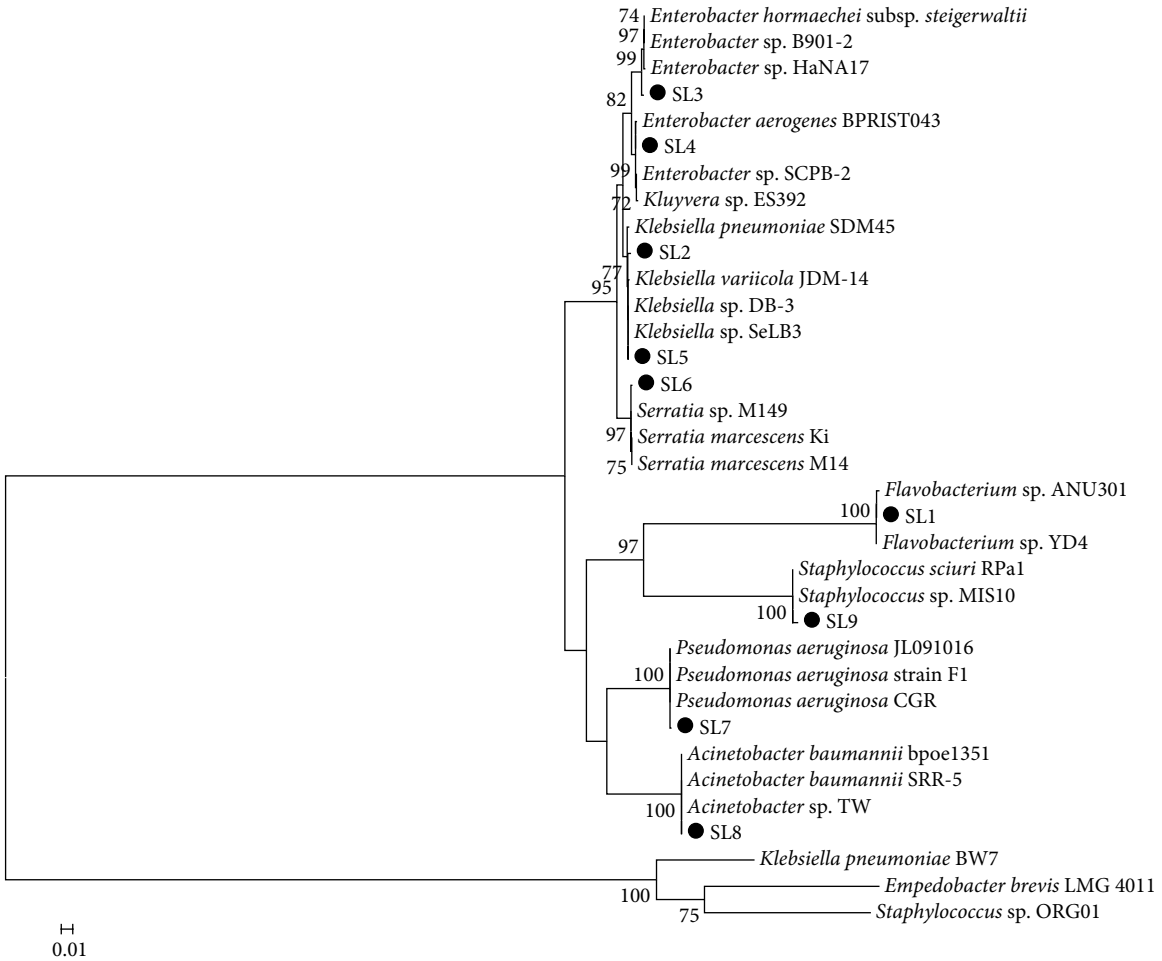


**Figure 1.** Neighbor-joining tree of 9 isolates from *S. littoralis* and their 27 closely related species based on partial sequencing of the 16S rRNA gene. Isolates from *S. littoralis* are indicated with black dots. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values C ≥ 70% are labeled.

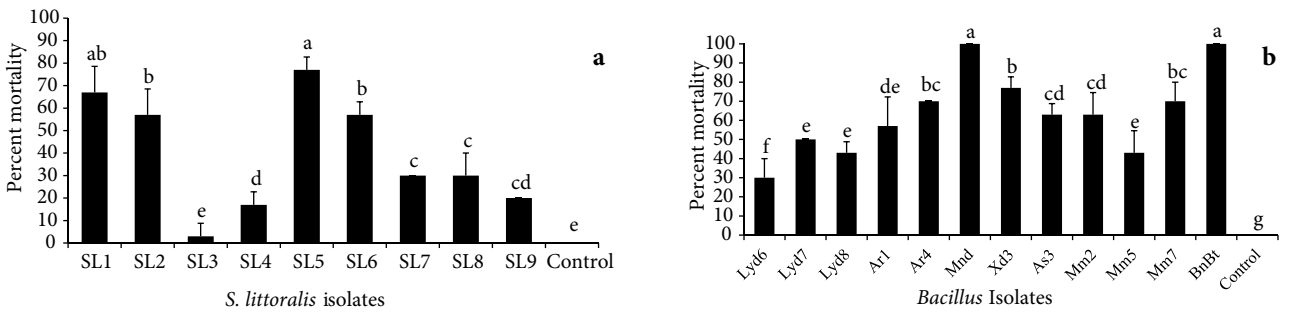


**Figure 2.** Pathogenicity of bacterial isolates against the third-instar larvae of *S. littoralis* after 10 days. **a)** Pathogenicity of *S. littoralis* isolates. SL1: *Flavobacterium* sp., SL2: *Klebsiella pneumoniae*, SL3: *Enterobacter* sp., SL4: *Enterobacter* sp., SL5: *Klebsiella* sp., SL6: *Serratia marcescens*, SL7: *Pseudomonas aeruginosa*, SL8: *Acinetobacter baumannii*, SL9: *Staphylococcus* sp. **b**) Pathogenicity of *Bacillus* isolates that were isolated from different pests. Lyd6, Lyd7, and Lyd8: *Bacillus thuringiensis*, Ar1: *B. circulans*, Ar4: *B. sphaericus*, MnD: *B. thuringiensis*, Xd3: *B. thuringiensis* subsp. *tenebrionis*, As3: *B. cereus*, Mm2: *B. thuringiensis*, Mm5: *B. sphaericus*, Mm7: *B. weihenstephanensis*, BnBt: *B. thuringiensis* subsp. *kurstaki*. Mortality data were corrected by Abbott's formula (Abbott, 1925). Different lowercase letters represent statistically significant differences among mortalities according to LSD multiple comparison test ($P < 0.05$). Bars show standard deviations.

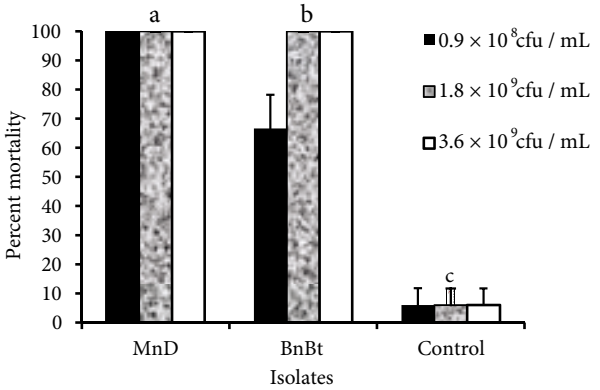


**Figure 3.** Mortality of the third-instar *S. littoralis* larvae 10 days after the application of *Bacillus thuringiensis* subsp. *kurstaki* strains MnD and BnBt. Different lowercase letters represent statistically significant differences among mortalities ($P < 0.05$). Bars show standard deviations.

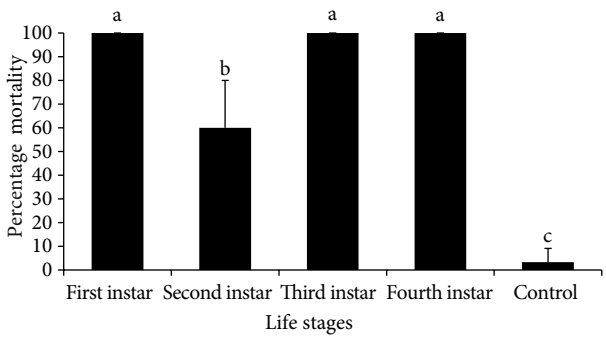


**Figure 4.** Mortality of different development stages of *S. littoralis* larvae 10 days after application of the isolate MnD ($1.89 \times 10^9$ cfu/mL). Different lowercase letters represent statistically significant differences among mortalities according to LSD multiple comparison test ($P < 0.05$). Bars show standard deviations.

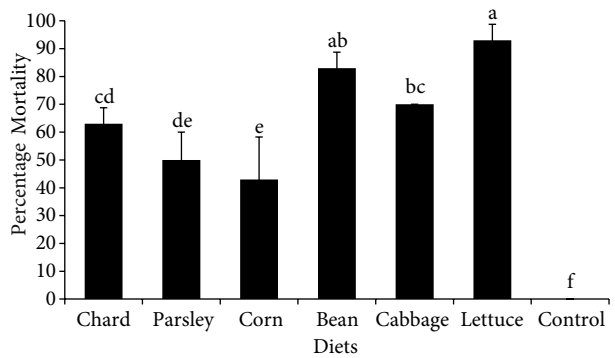


**Figure 5.** Mortality of the third-instar larvae of *S. littoralis* 10 days after application of the isolate MnD using different diets. Different lowercase letters represent statistically significant differences among mortalities according to LSD multiple comparison test ($P < 0.05$). Bars show standard deviations.

from any insect. This species also caused 30% mortality in *S. littoralis* larvae.

*Staphylococcus* species have been isolated from different insect species (İnce et al., 2008), although Bucher (1981) indicated that *Staphylococcus* species are rarely associated with insects. We also isolated *Staphylococcus* sp. SL9 from *S. littoralis* larvae, but this isolate did not show significant mortality in the pest.

Gram-positive entomopathogenic bacteria represent the most studied group of bacteria in insect pathology and include members of the genus *Bacillus* (Boemare and Tailliez, 2010). This genus is commonly recognized as a definitive insect pathogen, as are *B. lentimorbus*, *B. larvae*, *B. thuringiensis*, and certain strains of *B. sphaericus* (Stahly et al., 2006). Among *Bacillus* species, *B. thuringiensis* is the most commonly used entomopathogenic bacterium against many insect species belonging to a variety of different orders (Sevim et al., 2012; Al-Momani and Obeidat, 2013; Yılmaz et al., 2013). Although different *B. thuringiensis* isolates have been used against *S. littoralis*, we showed that 2 isolates of *B. thuringiensis* subsp. *kurstaki* (MnD and BnBt), which were isolated from *M. neustria* (L.) and *B. nucum* (L.), respectively, had potential in control of *S. littoralis*. The isolate MnD in particular seems to be the most effective and promising biocontrol agent against this pest based on a variety of bioassay experiments. Investigation of new *B. thuringiensis* isolates is always desirable because of insect resistance against *B. thuringiensis* strains (Navon et al., 1983). In this sense, these isolates could be useful in future biocontrol programs of *S. littoralis*.

We determined that all larval stages of *S. littoralis* were susceptible to the isolate MnD at the same rate, except for the second-instar larvae. The second-instar larvae were also infected with MnD, but they were found to be more resistant than the other development stages. In most cases, all larval stages of insect pests are susceptible to pathogens; therefore, often the larval stage is the preferred stage in field studies (Ravensberg, 2011). Indeed, maximum effectiveness of a biopesticide is sometimes limited because of the susceptibility of the particular developmental stage of the pest (Khetan, 2001). In this study, we showed that the isolate MnD can infect all larval development stages of *S. littoralis*. This could be a very promising advantage in the biocontrol of this pest because it may not be necessary to consider targeting the most susceptible stage of the pest during field application.

The insecticidal activity of bacterial pathogens under laboratory conditions is greatly influenced by several

factors such as diet, incubation time, and incubation temperature (Khetan, 2001). Diet can also influence either larval development or insect immunity. Recent studies on *S. littoralis* and its baculovirus (SlNPV) have shown that a high protein diet after virus challenge can significantly increase larval survival and there are even indications that larvae can alter their diets to promote this (Lee et al., 2006). In this study, we demonstrated that larval mortalities of *S. littoralis* when using a diet of beans and lettuce were significantly higher than the other diets used in bioassays. This may indicate that other diets might strengthen the immunity of *S. littoralis* larvae, and thus they make larvae less susceptible to the isolate MnD. In addition, interaction of bacteria used in bioassays with plants and their chemical compositions might influence larval mortality in positive ways. Therefore, the isolate MnD might be especially used in bean and lettuce fields to control *S. littoralis*.

In conclusion, we isolated and characterized different bacteria from *S. littoralis* larvae and tested their effectiveness against it. In addition, 12 different *Bacillus* species were tested against *S. littoralis* larvae. Some of the isolates appeared to be significant candidates for biological control programs of this pest. The isolate MnD (*B. thuringiensis* subsp. *kurstaki* from *Malacosoma neustria* L.) in particular was the most promising one. This study also showed that all larval stages of *S. littoralis* were susceptible to the isolate MnD at the same rate, except for the second-instar larvae. Finally, we determined that isolate MnD might be especially useful in bean and lettuce fields to control *S. littoralis*. However, further studies should include investigation of the predisposition of the isolate MnD in terms of mass production, formulation studies, toxicity assays against various organisms, and finally the field efficacy of isolate MnD.

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