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RESEARCH

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Isolation and determination of bacterial microbiota of *Varroa destructor* and isolation of *Lysinibacillus* sp. from it

Mehtap Usta*

Abstract

Background: The importance of bees for environmental health is known. Within the scope of this importance, it is of great importance to protect the health of bees and to prevent colony extinction. In this context, it is very important to develop effective methods in combating microorganisms, parasitoids, mites and organisms that cause disease or harm in bees. Both use different methods in terms of bee health.

Result: In this study, the possibility of the bacteria isolated from *Varroa destructor* mite being bioinsecticide was investigated. Accordingly, six bacteria were isolated from the mite. Isolated bacteria were analyzed according to biochemical tests, molecular analysis, optimum growth pH and phylogenetic tree drawn as *Pantoea dispersa* (GV1), *Lysinibacillus macroides* (GV3), *Bacillus mycoides* (GV4), *Lysinibacillus fusiformis* (GV5), *Pseodomonas lutea* (GV5), *Lysinibacillus varians* (GV7). *Lysinibacillus* sp. The entomopathogenic feature of *Lysinibacillus* sp. ranked it as the most important species. When the insecticidal properties of bacteria were examined, they were determined as 53, 90, 62, 95, 74 and 83% for GV1, GV3, GV4, GV5, GV6 and GV7, respectively.

Conclusion: Based on these results and literature review, *Lysinibacillus* sp. species had a high potential to be used as bioinsecticide against *V. destructor* mite.

Keywords: *Varroa destructor*, Honey bee, Bacteria, Microbiology, Biological control

Background

Beekeeping makes significant contributions to the agricultural economy in Turkey and in the world. It is evaluated that vegetative production can be reduced by 47% in an environment where there are no bees. Colony extinction events, which have recently been observed in different countries and whose cause cannot be explained, are considered as a problem that may affect the biological balance in the future. It is noted that similar problems can be seen in Turkey. Viral, bacterial, parasitic and fungal diseases seen in honey bees are very important for the honey production sector. It is known that the rate of co-infection of honey bee colonies with colony extinction with viral and parasitic pathogens is higher than the rate of coexistence of other pathogens (Berényi et al. 2006). For this reason, many studies have been conducted to determine these diseases that cause economic efficiency losses. However, as a result of these studies, no medicine or therapeutic product for diseases could be obtained. Bacteria, fungi, viruses, parasitoids and protozoans in honey bees cause diseases in adults and juvenile bees. These diseases cause slow development of bees and limitation of their effective production and even extinction of hives (Williams 2000). Bee colonies are exposed to various agro-ecosystems throughout the year and to many environmental variables that can affect the microbial balance of the hive. Bee microflora is depended on different factors such as seasonal and geographical changes (Mattila et al. 2012), developmental stages from fertilized egg to adulthood (Martinson et al. 2012), honey

*Correspondence: myakupoglu@gmail.com
Tonya Vocational School, Trabzon University, Trabzon, Turkey

bee's age, nutrition and social life style (Linjordet 2016). Some studies show that as worker bees age and forage, their microbiota compositions shift slightly to different species (Saccà and Lodesani 2020). Varroosis, a parasitic disease, constitutes the biggest problem among these diseases. *V. destructor* is an external parasite that survives and reproduces in the colonies of bees and is highly harmful to beekeeping. Today, the disease that harms the world beekeeping the most is Varroosis (Williams 2000). Many studies have been conducted on the biology, epidemiology, parasitoid guest relations, population dynamics and hormonal, chemical, physical, genetic and biological struggles of *Varroa destructor* (Acari: Varroidae), and protection and control methods against *V. destructor* have been tried to be developed. One of the integrated control methods in many parts of the world in beekeeping; biotechnical methods, various organic acids, essential oils and the use of chemical substances together have started to be applied as combat methods (Škerl et al. 2011). Many researchers emphasized and advocated methods of combating *V. destructor* from colonies without using chemicals. Due to the intensive use of chemicals, residue problem in honey is one of the most important problems. Pesticides have many disadvantages such as the problem of residue as a result of polluting the environment and the deterioration of biological balance due to resistance to these chemicals. Most of these pesticides are used in *V. destructor* control. Today, organic acids, vegetable oils or mechanical methods are used for *V. destructor* control. In addition to these, the use of biological control methods should be preferred because they will be less harmful. Since *V. destructor* damages the colony by carrying viruses, its struggle is of great importance.

The aim of this study was to determine an effective screening method for mite detection in natural infested honeybee colonies with *V. destructor*, whose residue and resistance risk is low and will not adversely affect human health. For this purpose, bacteria were isolated from *V. destructor* and defined.

Methods

Sample collection

Samples of *V. destructor* mites from *Apis mellifera* were collected from hives located in Gümüşhane province, distinct of Kürtün and village of Günyüzü, Turkey (latitude 40.748302 and longitude 38.984703). The hives used didn't receive chemical applications. The samples were obtained using around 250 pieces *V. destructor* obtained from honey bees collected from approximately 20 hives. The collected *V. destructor* mites were placed in sterile tubes and transferred to the laboratory. The samples were collected twice a year, in the spring (May 2020) and the autumn (October 2020).

Isolation of bacteria

Before bacterial isolation, *V. destructor* mites were surface sterilized by 70% alcohol to remove possible contamination and then washed in sterile distilled water. The mite bodies were homogenized in 0.5 ml of sterilized phosphate buffer solution (0.01 M PBS, pH 7.4) using a glass tissue grinder and filtered twice through 2 layers of cheesecloth to remove debris. After preparing the homogenate for bacterial isolation, suspensions were diluted to 1×10^{-5} and 0.1 ml were spread on nutrient agar (Thiery and Frachon 1997). Plates were incubated at 30 °C for 2–3 days. Isolates were determined based on color and morphology of the colonies. Individual colonies were isolated, sub-cultured twice to ensure purity and then stored in 15% sterilized glycerol at − 80 °C for further studies. Pure cultures of bacterial colonies were identified by their morphology, spore formation, physiological, biochemical and molecular characteristics (16S rRNA).

Morphological, physiological, biochemical characterizations and scanning electron microscope of ısolates

Bacterial strains were selected based on their morphological biochemical, physiological features according to Bergey's Manual of Systematic Bacteriology (Sneath 1986). Phenotypic characteristics of the strains included cell and colony shape on NA. Optimum pH was determined, after 16 h incubation at 30 °C by measuring the densities using a spectrophotometer (Spectramax M2) at OD600 (Ben-Dov et al. 1995). Biochemical panel test system API 20E (bioMerieux, France) was handled according to the manufacturer's instructions. Then the panels were incubated for 18–24 h at 30 °C. The results of these tests were performed by referring to the API 20E reading table.

The bacterial samples (*Lysinibacillus sp.*) were grown on NA for 24 h at 30 °C on 20 ml nutrient agar. The bacteria were collected by sterile inoculating loops solid culture plate. The collected samples were added into 1 ml deionized water, vortexed and centrifuged for 5 min at 7500 rpm. The supernatant was discarded. This procedure was repeated 3 times. Finally, the washed bacteria were diluted 20 times with sterile water. Then, 5 µl of the bacteria solution was spotted and dried on a scanning electron microscope (SEM) specimen stub (Carl Zeiss, Germany). The accelerating voltage was in the range of 5–10 kV.

16S rRNA gene sequence analysis

Genomic DNA from all samples was extracted using the standard phenol/chloroform procedures (Sambrook et al. 1989). PCR amplification of 16S rRNA genes of bacterial isolates was performed with the following universal primers (William et al. 1991); UNI 16S-L: 5_- ATT CTAGAGTTTGATCATGGCTCA-3_ as forward and UNI 16S-R: 5_ ATGGTACCGTGTGTGACGGGCGGT G TGTA-3_ as reverse. PCR conditions were adjusted according to William et al. (1991). Reactions were totally in 50 µl; 1 µl of template DNA was mixed with 5 µl reaction buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.5 µM (each) with primer and 0.5 U with Taq DNA polymerase. Amplification was performed with 30 cycle program (each cycle consisting of denaturation at 94 °C for 3 min, annealing at 55 °C for 60 s and extension at 72 °C for 3 min), followed by a final extension step at 72 °C for 5° min, by using thermal cycler (BioRad). Each experiment was associated with negative (without DNA template) controls. PCR products were analyzed on a 1.2% agarose gel. The samples displayed on the gel were sent to the sequence after cleaning from the gel. Sequence analysis of the samples of 16S rRNA products was performed using 16S universal primers by SenteBiolab (Ankara/TURKEY). Obtained sequences were used to perform BLAST searches using the NCBI GenBank database. Comparison of approximately 1.400 bp fragments of 16S rRNA gene sequences of each isolate with other 16S rRNA sequences in the NCBI GenBank database (Altschul et al. 1990) was performed.

Phylogenetic relationship of the bacterial ısolates

The sequences obtained were used to perform BLAST searches using the NCBI GenBank database to confirm isolate identification (Altschul et al. 1990). Evolutionary relationships of the six bacterial isolates were evaluated. Cluster analyses of the sequences were performed using BioEdit (version 7.09) with Clustal W, followed by neighbor joining analysis on aligned sequences performed with MEGA 7.0 software (Tamura et al. 2013). Reliability of dendograms was tested by bootstrap analysis with 1000 replicates using MEGA 7.0.

Determination of toxin genes

Toxin genes were investigated according to the methodology described by Nishiwaki et al. (2007). PCR amplification was performed of the toxin genes using binary toxin genes, mtx1 and mtx2. Toxin gene primers are given in Table 1. PCR was constructed according to the following conditions: pre-amplification 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and elongation at 72 °C for 3 min. Reactions were totally in 50 µl; 1 µl of template DNA was mixed with 5 µl reaction buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.5 µM (each) with primer and 0.5 U with Taq DNA polymerase. PCR products were analyzed on a 1.2% agarose gel.

Table 1 Primers used for toxin gene sequencing

Toxin gene primers	Primer sequence (5′ → 3′)
Mtx1F	CAAGCTGCTTCACTTACATG
Mtx1R	GTCCAGTTACATCTTGAGCC
Mtx2F	GGAGACTAATTGAATTTTCGGTTTCC
Mtx2R	GCGATGCTGGGCTATGTTCGTTGTTA

Miticidal effects of bacteria

All of the bacterial isolates were tested as overnight cultures after removing the growth medium (NB). Bacterial isolates were incubated for 18 h (72 h for sporulation) at 30 °C in the nutrient broth medium (NB). After incubation, bacterial cells were centrifuged at 3000 rpm for 10 min (Ben-Dov et al. 1995). The pellet was resuspended by adding sterile PBS. The optical density of the cells was adjusted to 1.89 at OD (optical density) 600 (Moar et al. 1995).

Concentration–response assays of bacterial cultures against *V. destructor* and their impact on honey bee larvae. Adult females of *V. destructor* were collected from recently capped brood cells and placed in Petri dishes (35 mm diameter × 10 mm high) which had previously been filled with sterile and residue free wax. According to the trial, mites were sprayed or immersed by spent bacterial culture of each strain (GV1, GV3, GV4, GV5, GV6 and GV7). Each trial was carried out with 3 replicates. The application was done with a small spray dispenser flask or the mites were immersed in a test tube. Then, mites were left for 2 h before being fed with spinning larvae. Larvae of *A. mellifera* subspecies (one every 2 mites) were used in substituted every 48 or 24 h in case of need. The trial dishes were incubated at 26 °C and 70% RH, in the dark (Lodesani et al. 2017). Observations were carried out every 24 h in order to count and collect the dead mites, until at least 90% of the mites were dead. Each dish contained 4 mites and 2 larvae.

Results

In this study, 6 bacterial isolates; *Pantoea dispersa* (GV1), *Lysinibacillus* sp. (GV3), *Bacillus mycoides* (GV4), *Lysinibacillus fusiformis* (GV5), *Pseudomonas* sp. (GV6) and *Lysinibacillus* sp. (GV7) from were isolated and characterized from *V. destructor*. The colony colors of 5 isolates were creamy, and the other isolate was yellow. The

Table 2 Optimum pH range of bacteria

pH	GV1	GV3	GV4	GV5	GV6	GV7
pH3	–	–	–	–	–	–
pH4	–	–	–	–	–	–
pH5	–	–	–	–	–	–
pH6	+	+	+	+	+	+
pH7	+	+	+	+	+	+
pH8	+	+	+	+	+	+
pH9	+	+	+	+	+	+
pH10	+	+	+	+	–	+

Table 3. 16S results of bacteria

Isolate name	Bacterium name	Accession number	Similarity (%)
GV1	*Pantoea dispersa*	MW940887	99
GV3	*Lysinibacillus macroides*	MW940888	99
GV4	*Bacillus mycoides*	MW940889	99
GV5	*Lysinibacillus fusiformis*	MW940890	99
GV6	*Pseudomonas lutea*	MW940891	99
GV7	*Lysinibacillus varians*	MW940892	99

optimum pH range for the bacterial isolates was determined (Table 2).

16S rRNA gene sequence analysis results of the isolates are given in Table 3. The 16S rRNA partial gene sequences generated in this study have accession numbers MW940887, MW940888, MW940889, MW940890, MW940891 and MW940892, respectively.

GV3, GV5 and GV7 are identified as *Lysinibacillus* sp. and these are the first record from *V. destructor.* Scanning electron microscope images of these bacteria are shown in Fig. 1. Also, GV1 was identified as *Pantoea dispersa* and this is first record from *V. destructor.*

Phylogenetic tree was constructed by using neighbor joining method (Fig. 2). The similarities between isolates ranged between 93 and 100% compared to other species.

Based on the studies, isolates were identified as *Pantoea dispersa* (GV1), *Lysinibacillus* sp. (GV3), *Bacillus mycoides* (GV4), *Lysinibacillus fusiformis* (GV5), *Pseudomonas* sp. (GV6) and *Lysinibacillus* sp. (GV7) (Fig. 1). These identifications were also confirmed by phylogenetic analysis of the bacterial isolates and their closely related species based on the 16S rRNA sequence (Fig. 2).

In this study, PCR amplification of mtx toxin genes (mtx1 and mtx2) of all bacteria was scanned. According to amplification results, only *B. mycoides* (GV4) (Lane 3) had *mtx 1* gene (Fig. 3). The other five bacteria did not have both *mtx 1* and *mtx 2* genes.

As a result of the bioassay study, the highest rate (90, 95 and 83%) was observed in bacteria of the genus *Lysinibacillus* spp., which were the bacteria named: GV3, GV5 and GV7, respectively, within 10 days. Other bacteria also showed mortality rates of 53, 62 and 74% respectively (GV1, GV4 and GV6), within 10 days (Fig. 4). Mites sizes were 1.5 mm long and 1.7 mm wide. Observations were carried out every 24 h in order to count and collect the dead mites, until at least 90% of the mites were dead. In all the trials, the comparison took place between the uninoculated culture broth (control) and the bacteria culture (treatment). Each dish contained 4 mites and 2 larvae.

Discussion

In the present study, a culturable bacterial microbiota of *V. destructor* was determined to identify new candidate organisms as a possible biocontrol agents against the pest. Four different genera of bacteria were isolated. These are *Pantoea*, *Lysinibacillus, Bacillus* and *Pseudomonas*. And also 2 species (*Lysinibacillus* sp. and *Pantoea dispersa*) were determined for the first time from *V. destructor*. *Lysinibacillus* sp. is a naturally occurring, mesophilic, soil bacterium, toxic to insects. It was isolated from the dead larvae of *Culex pipiens* and *Palomena prasina* in previous studies and investigated on the pathogenicity of the isolated hosts. (Ozsahin et al. 2014) Insecticidal property of this organism is due to 2 proteins produced during sporulation. These proteins are the binary toxins, which accumulate as crystal inclusions and mosquitocidal toxins (Mtx proteins) are produced during vegetative growth of the bacteria. The bacteria are not harmful to humans and other animals, *Lysinibacillus* sp. is an ideal insecticide.

The insecticidal bacterium that has been in trade and has grown in importance in the last decade is *B. sphaericus*, with many isolates proven to be active against mosquito larvae. *B. sphaericus* strains first described as

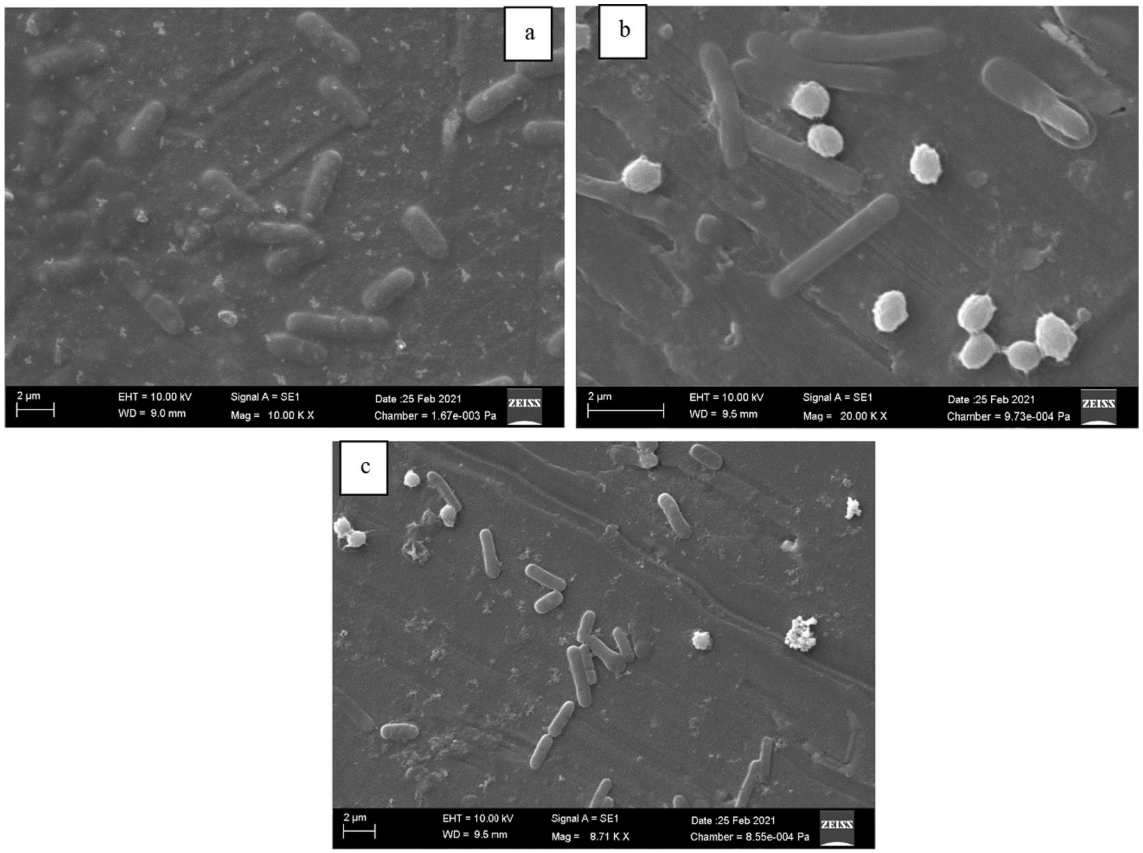

Fig. 1 Scanning electron microscope images of the bacteria GV3 (**a**), GV5 (**b**) and GV7 (**c**)

insect pathogens in 1965. Although this discovery led to further investigation of the bacterium, until 1973 it was not that species with potential use in insect control programs were discovered. (Singer 1973).

The genus *Pantoea* is a diverse group of yellow pigment, rod-shaped Gram-negative bacteria in Enterobacteriaceae. Some *Pantoea* isolates produce antimicrobials and have been developed as commercial biocontrol products The ubiquity, versatility and genetic traceability of *Pantoea* isolates make it an ideal group not only for exploring niche-specific adaptation and opportunism, but also for the development of commercially relevant medicinal, agricultural and environmental products. A wide variety of insect species appear to be associated with different *Pantoea* isolates (Jiang et al. 2019). Many of these relationships have been described mutually, where bacteria are found in specialized cells of the symbiont host in the insect intercellularly and in some cases intracellularly (Vorwerk et al. 2007). In such mutualisms, it has been suggested that the insect host can provide food and habitat to bacteria and possibly provide a direct propagation pathway (Sood and Nath 2002), while the insect may benefit from bacterial-mediated hydrolysis of proteins (Sood and Nath 2002), antagonism of pathogens (Vorwerk et al. 2007), breakdown of toxic substances (Sood and Nath 2002), nitrogen fixation, nutrition and digestion (Maccollom et al.2009). *Bacillus mycoides* has lower GC and a gram positive bacteria than its old phylogeny. It is a spore-forming rod that produces a characteristic spreading filament morphology when cultured on agar. *B. mycoides* is found in soils all over the world. (Ko et al. 2004). The closest relatives of *B. mycoides*—the *Bacillus cereus* subgenus—are human pathogens (*B. cereus, B. anthracis*) and other animals, including insects (*B. anthracis*) (*B. thurigiensis*) (Ko et al. 2004). *B. mycoides* was known since 1886 when the spreading rhizoidal colony structure was first observed by Flu¨gge in soil cultures (Flugge 1886). *B. mycoides* is considered a saprophytic organism that makes it ecologically distinct from its more pathogenic close relatives (Nakamura and Jackson 1995).

Microflora associated with honey bees was first investigated as techniques developed (Gilliam and Valentine 1976; Gilliam and Morton 1978). In various studies, Gram-variable pleomorphic bacteria, *Bacillus* species and some Enterobacteriaceae family members were find

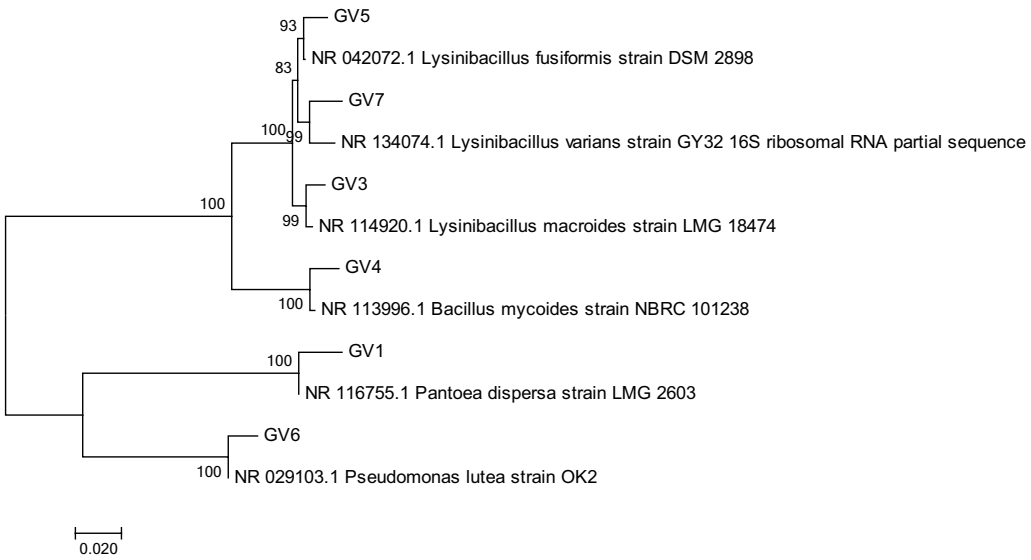

Fig. 2 Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbor joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.45843344 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1298 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

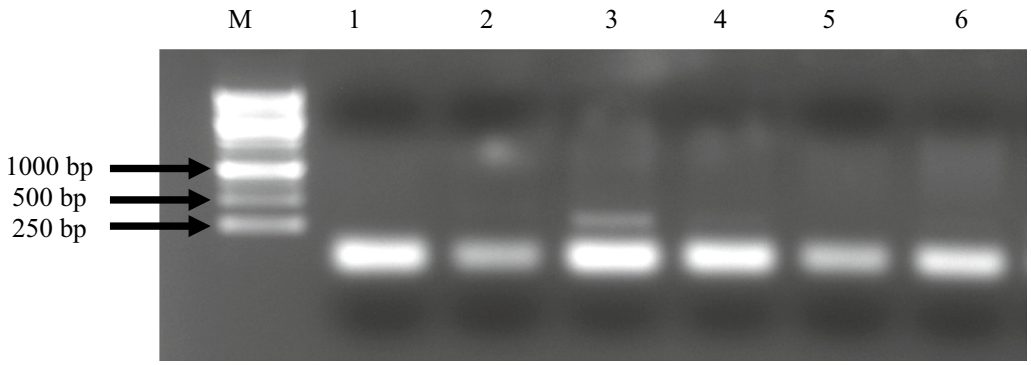

Fig. 3 PCR amplicon of *mtx 1* toxin genes of all bacteria. 1: GV1, 2: GV3, 3: GV4, 4: GV5, 5: GV6, 6: GV7. *Bacillus mycoides* (Lane 3: the size of band is approximately 300 bp)

on pollen, nectar, bees, honey, beeswax and royal jelly (Suyabatmaz et al. 2020).

Also *V. destructor* mites control is very important for honey bee viruses diseases. *V.* destructor is a vector for various honey bee viruses. Additionally, *Varroa* mites may induce immunosuppression in parasitized pupae and, thus, activate these covert virus infections (Noël et al. 2020). By feeding on bee tissues, *Varroa* acts as an efficient vector of pathogens. Vector-based disease transmission involves three main phases: Acquisition: varroa feed on bee tissues, ingesting the pathogens that reside in those tissues, Mobility: varroa moves freely between different individual hosts, Transmission: during feeding, varroa introduces the pathogen into the new host (Traynor et al. 2020; Light et al. 2020).

Conclusions

According to the results of the study, the bacteria belonging to the genus *Lysinibacillus* are potentially entomopathogenic as well as an effective bioinsecticide on many pests, especially *V. destructor* mite. It should be tested on different pests, especially bee pests. This study

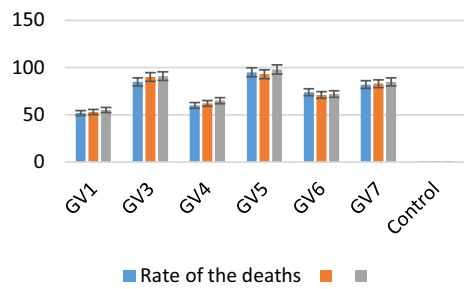

Fig. 4 Miticidal effects of bacteria against *V. destructor* mites

showed that the bacterium belonging to the genus *Lysinibacillus* was isolated for the first time from *V. destructor*. It is thought that a more advanced commercial preparation can be prepared by carrying out bioinsecticide studies with this bacterium. Thus, in addition to the currently used *B. thrugiensis* isolates, different preparations can also be applied.

Abbreviations
PCR: Polymerase chain reaction; OD: Optical density; SEM: Scanning electron microscope.

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Authors' contributions
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Ethics approval and consent to participate
Ethics certificate is not required in the study.

Consent for publication
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Competing interests
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