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Bacterial Flora of *Lobesia botrana* ([Denis & Schiffermüller]) (Lepidoptera: Tortricidae) as a Possible Microbial Control Agent

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Abstract: The European grapevine moth, *Lobesia botrana* ([Denis & Schiffermüller, 1775]) (Lepidoptera: Tortricidae) is one of the most harmful insect pests of grape species in many countries, including Turkey and Iran. The purpose of this study was to determine the bacterial flora and to find a safe and an effective microbial control agent against *L. botrana*. Bacterial isolates, obtained from larvae of the target insect, were identified using morphological, physiological, biochemical and molecular methods. The insecticidal effects of the bacterial isolates on 3rd and 4th instar larvae of *L. botrana* were assessed and the results were statistically compared with a control group. The bacterial flora of *L. botrana* was determined as *Enterococcus faecalis* (Lb4), *Klebsiella pneumonia* (Lb6), *Enterobacter ludwigii* (Lb12, Lb17), *Rhodococcus erythropolis* (Lb13), *Enterobacter aerogenes* (Lb15) and *Serratia marcescens* (Lb21). Bioassay tests showed that the Lb21 had the highest mortality (93%, $P < 0.0001$) on the larvae of *L. botrana* within ten days of inoculation. Therefore, *S. marcescens* Lb21 is a suitable candidate for the development of an effective microbial control agent against *L. botrana*.

Key words: *Lobesia botrana,* bacterial flora, biological control, *Serratia marcescens, Vitis vinifera*

Introduction

Vitis vinifera L., the most widely cultivated species of grape, has been grown in Asia Minor between the Caspian Sea and the Black Sea since the beginning of civilisation (Winkler et al. 1974) and continued to flourish through the 14th century, especially in Central Europe. Vineyards and wine production have always played a significant role in finances and livelihoods of people in different parts of the world. With respect to the amount of land used for grapevine planting, Turkey, with around 590,000 ha of vineyard, is holding the 4th position in the world after Spain, Italy and France. With respect to grape production, Turkey falls behind Italy, Spain, France, the USA and China with 3,650,000 t of grape production (Castellucci 2004). On the other hand, with respect to the amount of land used for grapevine planting, Iran comes behind Turkey, getting the 5th place with 306,000 ha of vineyard and the 7th place with 3,000,000 t of grape production (Nazli 2007).

Grapevine moth, *Lobesia botrana* ([Denis & Schiffermüller, 1775]) (Lepidoptera: Tortricidae), is a major pest of berries and berry–like fruits in Europe, the Mediterranean countries, Southern Russia, Japan, the Middle East, the Near East and Northern and Western Africa (Avidov & Harpaz, 1969, Venette et al. 2003). The pest has three or four generations per year depending on environmental conditions in late summer (Thiery et al. 2013).

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The first generation of larvae causes damage to the flowers, while the second and third generations cause damage to unripe and ripening berries, respectively (Cozzi et al. 2013). Thus the species is considered among the main pests of vineyards.

Recently, the importance of healthy food and identification of environmental hazards inclined the research field toward alternative control disease strategies by focusing on biological control agents. Biological control using entomopathogenic bacteria of harmful insects has been considered as an alternative method (Demirbag et al. 2008) for keeping these insects under control. In the last decade, many bacterial pathogens have been isolated from insects and their insecticidal effects have been determined on agricultural pests and studied as potential agents for biocontrol of harmful pests (Demir et al. 2012, Danismazoglu et al. 2012, Demirci et al. 2013). In order to find a safer and more effective biological control agent against *L. botrana,* the present study focused on isolation and identification of the bacterial flora of this serious pest.

Materials and Methods

Collection of larvae

During this study different instars of larvae of *Lobesia botrana* were collected from 20 stations (vineyards). Larvae, which are usually found on flowers or fruit clusters covered with webs produced by the insect, were collected and placed in plastic boxes with perforated covers to permit airflow, together with some flowers or grapes. The boxes were then taken to the laboratory. Larvae were fed with grapes at room condition until the bacterial isolation was done. Since the detection and determination of the dead larvae on grapes is difficult, we used artificial diets for larvae during our further experiments as suggested by Rauscher et al. (1984).

Isolation and purification of bacterial samples

Two hundred larvae, which seemed diseased and moving slowly, were used for the isolation of bacteria. Larvae were surface sterilised using 70 % ethanol for 1.5–2 minutes and then rinsed twice with sterile distilled water (Lipa & Wiland, 1972). They were homogenised in nutrient broth by using a glass tissue grinder. Bacterial purification was done in two steps: *i*) a portion of larval homogenate was heated at 80 °C for 10 min in a water bath to eliminate non–spore forming organisms (Ohba & Aizawa, 1986). One hundred µl of this suspension was then cultured on each nutrient agar (NA) (Difco) plate and incubated aerobically at 30 °C for 2–3 days; *ii*) the other portion was incubated at 30 °C for 5–6 hrs for the purpose of bacterial enrichment and a series of dilutions (from 10^{-1} to 10^{-5}) were prepared. Serially diluted suspensions were spread on NA plates and incubated under the same conditions. Towards the end of the incubation period, isolates were selected according to the colour and morphology of the colonies. Individual colonies were isolated, sub–cultured twice to guarantee purity and stored in 20 % sterilised glycerol at -80 °C for further studies.

Identification of the bacterial isolates according to their conventional tests

Pure cultures of bacterial colonies were identified according to their morphology, spore formation, nutritional features and physiological, biochemical and molecular characteristics following Bergey's Manual of Systematic Bacteriology (Holt et al. 1994). The API 20E and API 50CH panel test and VITEK2 identification systems were also used for determining the biochemical features of bacterial isolates.

The API 20E and API 50CH panel tests were performed according to the procedure suggested by Alsina & Blanch (1994), with some modifications. Bacterial colonies of each isolate on tryptic soy agar medium were diluted using API special solution ampoules. The quantity of bacteria was adjusted to 1 McFarland standard. Sufficient amounts of this solution were poured into each well of API 20E and API 50CHB panels. To make them air–tight, some wells were filled up with mineral oil. The panels were read after 18–24 hrs of incubation at 30 °C. Reading of panels was repeated after 48 hrs. The results of the tests were evaluated by "IdBact v. 1.1, G. Kronvall" programme, with Matrix for API 20E and API 50CHB from bio–Merieux, France.

The VITEK2 identification system was used for the isolates which were not identified by the API tests. The VITEK2 is an automated identification system for bacteria that have been commercialised. Analysis of the results was based on the computerised software of VITEK2, according to the percent identification accuracy (ID %). Gram–negative and Gram–positive identification cards were used according to manufacturer's instructions (bio–Merieux). The inoculum was prepared in 3 ml of sterile saline and the turbidity was adjusted to match that of 0.5–0.63 McFarland Standard. The cards were inoculated by using the evacuator–sealer module then placed into the 30 °C reader–incubator module and automatically read.

16S rRNA gene sequence analysis

Total genomic DNA of all isolates was extracted using the Wizard Genomic DNA purification kit

(Promega, Germany) according to the manufacturer's instructions. Universal oligonucleotide primers were used to amplify the 16S rRNA gene from genomic DNA extracted from the bacterial isolates. UNI16S–L; 5'–
ATTCTAGAGTTTGATCATGGCTTCA–3'
and UNI16S–R; 5'–
ATGGTACCGTGTGACGGGCGGTGTTGTA–3'
were used as forward and reverse primers, respectively (Weisberg et al., 1991). Polymerase chain reaction (PCR) was performed using BioRad Thermal Cycler (USA) in a total volume of 50 µl consisting of 1.5 µl (0.5 µg) genomic DNA, 1 µl 10 mM dNTP mix, 1 µl 10 pM of each of the opposing amplification primers, 3 µl of 25 mM $MgCl_2$, 10 µl *Taq* DNA polymerase reaction buffer (× 5), 0.5 µl 5 U/µl of GoTaq DNA polymerase (Promega) and 32 µl dH_2O. PCR conditions were used for 2 min at 95 °C for initial denaturation of the template DNA, with 35 amplification cycles (45 sec at 95 °C, 45 sec at 56 °C and 1 min 30 sec at 72 °C) and for 5 min at 72 °C for final primer extension. All PCR products were analysed on agarose gel electrophoresis and were sent to Macrogen Inc. (Korea) for sequencing. The obtained sequences were used to perform BLAST searches (Altschul et al. 1990) using the NCBI GenBank database. Comparison of approximately 1,400 bp fragments of 16S rRNA gene sequences of each isolates with known 16S rRNA sequences in the NCBI GenBank database were performed.

The taxonomic identification of bacterial isolates was finalised by comparing the results obtained from all tests according to the Bergey's Manual of Systematic Bacteriology, API 20E, API 50CHB, VITEK2 and 16S rRNA gene sequence analyses.

The insecticidal effects of bacterial isolates

Bioassays were carried out for determination of insecticidal effects of bacterial isolates from *L. botrana.* Third and 4th instars of *L. botrana* larvae were used in the assay. Overnight broth culture of each bacterial isolate was calculated to be 1.8×10^9 CFU/ml per OD_{600} (1.89). The larvae used in the insecticidal activity tests were nurtured using an artificial diet. The surface of artificial food (20 g) in the plastic boxes (10 × 10 cm) was contaminated with bacterial suspension approximately at 1.8×10^9 CFU/ml concentration. Each group had ten 3rd and 4th instars of larvae in the boxes containing artificial food and was maintained in a growth chamber at 24 ± 1 ºC, 60 ± 10% RH with a 16:8 h (L:D) photoperiod. Each test was repeated three times and a total of 30 larvae were used in each group. The mortality of larvae was recorded daily for ten days. Data were statistically analysed using SPSS, version18 for Windows OS. Mortality rate of each isolate was statistically compared between control and exposed groups using correlation and Chi–square tests. We used Pearson correlation test for the comparison of two groups. Also survival statistical analysis was used for detecting the significant differences of mortality rate between the days of studies.

Results

Two hundred larvae of *L. botrana* were used for the isolation of bacteria. In order to purify spore forming bacteria, homogenates were incubated at 80°C for 10 minutes before usual growth condition (30 °C for insect originating bacteria). Bacterial growth on plates was not observed in the heat-treated samples. However, several bacterial colonies were observed on the nutrient agar plates after 24 h at 30 °C for the homogenates which were not heat-treated. Based on colony morphology and colour, seven distinct bacterial isolates were selected for the identification.

In order to identify bacterial isolates, morphological, physiological, biochemical and molecular tests were used. The results are shown in Table 1 and Table 2. NaCl tolerance test in a range of 2–9 % and growth in different pH (3–10) were used for physiological tests. NO_2 reduction, oxidase and catalase tests and MacConkey agar culture were also done. Biochemical tests were performed using API 20E, API 50CHB and VITEK2 systems. 16S rRNA gene sequences were also compared with the existing database in GenBank.

Lb4 isolate was observed as Gram–positive coccus and identified as *Enterococcus faecalis* according to its properties (Tables 1–3). In addition to morphological results, the VITEK2 analysis showed that Lb4 had 95 % similarity to *Enterococcus faecalis*. Also, the 16S rRNA gene sequencing showed that it had 100 % similarity to many strains of *Enterococcus faecalis* in GenBank database (Table 3).

Lb6, Lb12, Lb15 and Lb17 were determined as Gram-negative rod and non–spore forming bacteria. VITEK2 analysis showed that isolate Lb6 belonged to the genus of *Klebsiella*. Also, API 20E tests confirmed that this isolate was *Klebsiella pneumonia* with 97.7 % homology. In addition, 16S rRNA gene sequence results showed 99 % similarity to *K. pneumonia* and *K. variicola.* Furthermore, Lb6 showed a negative result for indol and a positive result for adonitol fermentation (ADO) tests. Adonitol fermentation (ADO), the only test proposed by Alves et al. (2006) to differentiate *K. pneumoniae* (positive) from *K. variicola* (negative), revealed that Lb6 was a *K. pneumonia* isolate (Table 3).

Table 1. Morphological and physiological characteristics of bacterial isolates of *Lobesia botrana*.

Isolates	Gram staining	Motility	Bacterial morphology	Growth in different pH	Growth in NaCl (%)	Growth in McC. agar
Lb4	+	-	Coccus	5≤ pH≥7	2≤NaCl≥7	-
Lb6	-	-	Small rod	4≤ pH≥9	2≤NaCl≥7	+
Lb12	-	+	Small rod	4≤ pH≥10	2≤NaCl≥8	+
Lb13	+	-	Rod	5≤ pH≥9	2≤NaCl≥7	-
Lb14	-	w+	Small rod	4≤ pH≥10	2≤NaCl≥8	+
Lb15	-	w+	Small rod	4≤ pH≥10	2≤NaCl≥8	+
Lb17	-	+	Small rod	4≤ pH≥10	2≤NaCl≥8	+
Lb21	-	+	Small rod	4≤ pH≥10	2≤NaCl≥8	+

w+, weak motile

Table 2, Biochemical characteristics of bacterial isolates of *Lobesia botrana*.

Tests / Isolates	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	Oxidase	Catalase
Lb4	-	+	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-	-	+	-	-
Lb6	w+	-	+	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+
Lb12	+	+	-	+	+	-	-	-	-	+	-	+	+	-	+	-	+	+	+	+	+	-	-	+
Lb13	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
Lb14	+	+	-	+	+	-	-	-	-	+	-	+	+	-	+	-	+	+	+	+	+	-	-	+
Lb15	+	w+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+
Lb17	+	+	-	+	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+
Lb21	+	-	+	+	+	-	-	-	-	+	+	+	+	-	+	-	+	+	+	-	+	-	-	+

w+, weak positive

For Lb12 and Lb17, the API test showed 99.4 % and 95 % identity to *Enterobacter sp.*, respectively. VITEK2 system (Gram–negative) showed 96 % and 99 % identity, respectively. *Enterobacter sp*p. comprised six species: *Enterobacter cloacae, E. asburiae, E. hormaechei, E. kobei, E. ludwigii* and *E. nimipressuralis.* Since these bacteria were motile (except *E. asburiae)* with negative urease and a positive sorbitol test, despite a single difference in RHA (Rhamnose, Lb12 was negative and Lb17 was positive), they have been identified as *Enterobacter ludwigii.* These results were also confirmed by 16s rRNA sequencing with 97 % similarity, indicating that they were *Enterobacter ludwigii*.

Lb15 isolate had positive lysine decarboxylase (LDC) and positive inositol fermentation (INO) tests. This isolate was identified as *Enterobacter aerogenes* based on VITEK2 (99 %) and API 20E (99.7 %) results. These results were also confirmed by 16S rRNA sequence analysis.

Lb13 isolate is a Gram–positive rod, non–motile and non–spore forming bacterium. Results of 16S rRNA sequencing showed 100 % similarity to *Rhodococcus erythropolis* and *Rhodococcus baikonurensis* species. In addition to morphological and specific biochemical characteristics (Tables 1–3), inositol negative result and sucrose fermentation, Lb13 was identified as *Rhodococcus erythropolis* (inositol and sucrose fermentation of *R. baikonurensis* are positive). Since *R. erythropolis* is a nonclinical isolate, it was not identified by the API test.

Lb21 isolate is a Gram–negative rod and non–spore forming bacterium. The other properties of this bacterium are shown in Tables 1–3. Based on the analysis, with 99 % probability by VITEK2 (GN) system, 96 % probability by API 20E and 99 % similarity by the 16S rRNA GenBank database, Lb21 was identified as *Serratia marcescens.*

The phylogenetic tree supported the identification results of morphological, physiological, biochemical and molecular studies by locating the isolates near the proposed species (Fig. 1).

After identification, bacterial isolates were assayed for their insecticidal activity. There were 30 healthy larvae in each bio test, repeated three times. Mortality tests of isolates on larvae within ten days

Table 3. Identification of bacterial isolates from *Lobesia botrana* based on molecular, API and VITEK tests.

Bacteria	Result of sequencing	Accession	Max ident	Result of API & VITEK2	Identification
Lb4	*Enterococcus faecalis* *Enterococcus rivorum* *Enterococcus moraviensis* *Enterococcus silesiacus* *Enterococcus termitis* *Enterococcus caccae*	AJ420803 FN822765.1 AF286831 AM039966 AM039968 AY943820	100% 98% 97% 97% 97% 97%	*Enterococcus faecalis,* Bacillus licheniformis	Enterococcus faecalis
Lb6	*Klebsiella variicola* *Klebsiella pneumoniae* *Enterobacter cloacae*	AJ783916 AJ233420 HE978272.1	99% 99% 98%	*Klebsiella pneumoniae ssp pneumoniae*	Klebsiella pneumoniae
Lb12	*Enterobacter ludwigii* *Enterobacter cloacae* *Enterobacter cancerogenus* *Pantoea agglomerans*	JN644609.1 GU186117.1 FJ009374.1 AM184214	97% 96% 96% 96%	*Enterobacter cloacae complex (E. cloacae, E. asburiae, E. hormaechei, E. kobei, E. ludwigii and E. nimipressuralis)*	Enterobacter ludwigii
Lb13	*Rhodococcus erythropolis* *Rhodococcus baikonurensis* *Rhodococcus globerulus* *Rhodococcus erythreus* *Rhodococcus coprophilus* *Nocardia calcarea*	EU729738KC315768 JF820113 EU647695 X80626 AB037105	100% 100% 100% 99% 99% 98%	*Brevibacillus laterosporus* *Bacillus lentus*	*Rhodococcus erythropolis*
Lb15	*Enterobacter aerogenes* *Klebsiella pneumonia* *Enterobacter cancerogenus* *Enterobacter hormaechei* *Enterobacter cloacae* *Enterobacter asburiae*	FJ976592 JQ680798 JQ682639 AJ853890 EU073021 HM854374	99% 99% 99% 99% 98% 98%	*Enterobacter aerogenes*	Enterobacter aerogenes
Lb17	*Enterobacter ludwigii* *Enterobacter asburiae* *Enterobacter hormaechei* *Enterobacter cancerogenus* *Enterobacter cloacae* *Enterobacter kobei*	JX500182 JQ682630 JF690889 JX262394 EU047702 JQ680938	99% 99% 99% 99% 99% 98%	*Enterobacter cloacae complex (E. cloacae, E.asburiae, E. hormaechei, E. kobei, E. ludwigii and E.nimipressuralis)*	Enterobacter ludwigii
Lb21	*Serratia marcescens* *Serratia ureilytica* *Serratia nematodiphila*	JN596118 JQ680894 JQ281540	99% 99% 99%	*Serratia marcescens*	Serratia marcescens

are shown in Table 4. Mortality of Lb4, Lb6, Lb13 and Lb15 achieved 70 %, 77 %, 63 % and 73 %, respectively. These mortality rates were not significantly different ($P > 0.05$) from the mortality of the control group. On the other hand, the insecticidal effects of Lb12, Lb17 and Lb21 were 87 %, 87 % and 93 %, respectively, and they were significantly different from the control group (Wilcoxon = 21.637, $P < 0.003$) . The highest efficacy was observed in the first six days of the study period (Table 5). Kaplan-Meier model was used for paired comparison of bacteria. Lb21 was the most efficient , however, the difference with Lb12 was not significant while with Lb17 it was significant (Table 6).

Discussion

This study focused on the isolation and identification of bacteria from *Lobesia botrana,* and determination of their insecticidal effect on the respective host. Reviews of previous studies showed that, the current study is the first to determine the bacterial flora of *L. botrana* and the insecticidal effect of the bacterial isolates against the host insect.

We found that the bacterial flora of *L. botrana* included *Enterococcus faecalis* (Lb4), *Klebsiella pneumonia* (Lb6), *Enterobacter ludwigii* (Lb12), *Rhodococcus erythropolis* (Lb13), *Enterobacter aerogenes* (Lb15), *Enterobacter ludwigii* (Lb17) and

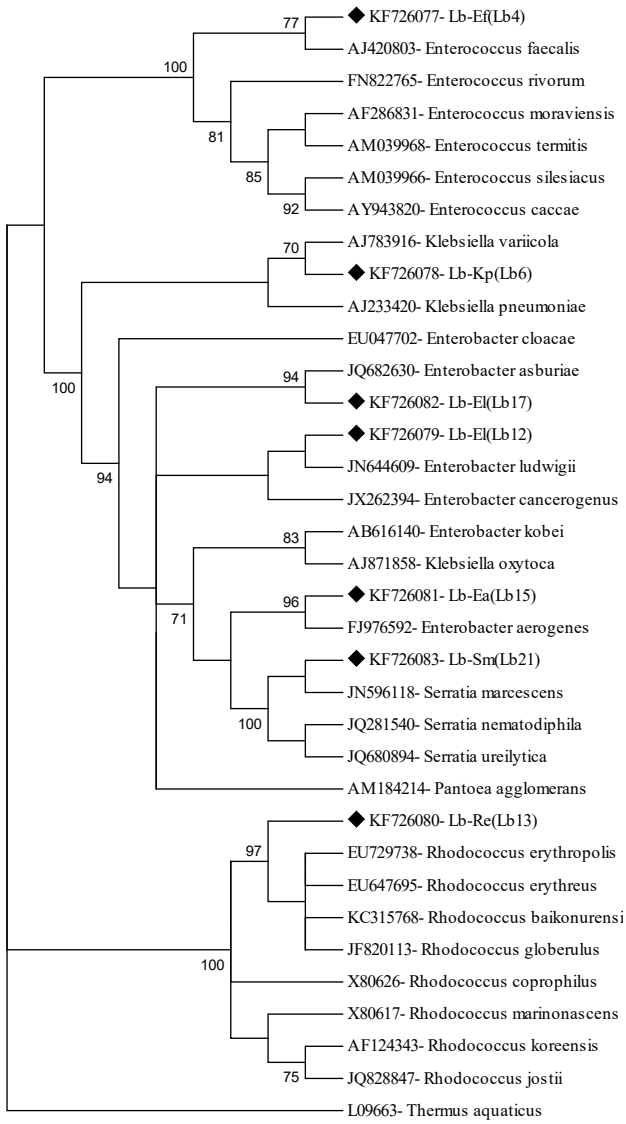

Fig. 1. Maximum-likelihood tree of the bacterial isolates of *Lobesia botrana* and their closely related bacterial species. The approximately1400-bp sequence of the 16S rRNA gene was used to construct the dendrogram. Bootstrap values based on 1000 replicates were indicated above nodes. *Lobesia botrana* isolates (4, 6, 12, 13, 15, 17, 21) were indicated ♦ Accession number - Lb species name for isolates

Serratia marcescens (Lb21) (Table 3).

Enterococcus faecalis is a common member of the normal gut microbiota in diverse species. Previous studies indicated that *E. faecalis* is common in vertebrates and insects, such as *Manduca sexta* (Mason et al. 2011), *Acrosternum hilare* and *Nezara viridula* (Hirose et al. 2006).

We also isolated *Klebsiella pneumonia* from the larvae of *L. botrana.* This bacterium has been isolated from several insects, such as *Bactrocera tryoni* (Thaochan et al. 2010) and green stink bug, *Nezara viridula* (Hirose et al. 2006).

Table 4. Mortality percentage of *Lobesia botrana* and statistical values

Bacteria	Mortality %	Significance (P)	Chi-square value
Lb4	70	0.211	1.56
Lb6	77	0.099	2.7
Lb12	87	0.013*	6.1
Lb13	63	0.614	0.25
Lb15	73	0.240	1.37
Lb17	87	0.019*	5.5
Lb21	93	0.000*	16.18
control	57		

*significant

It has been shown that many insects have Enterobacteriaceae bacteria in their gut system (De Vries et al. 2008). Gayatri Priya et al. (2012) isolated *Enterobacter* sp from midgut of larvae of *Helicoverpa armigera.* We detected two species of the family of Enterobacteriaceae, *Enterobacter ludwigii* and *E. aerogenes*, from *L. botrana.* Lighthart (1988) isolated *E. aerogenes* from gut of *Peridroma saucia*. Dong et al. (2009) have identified *E. aerogenes* from midgut microbiota of mosquito *(Anopheles gambiae)*. Also, Sevim et al. (2010) isolated this bacterium from *Agrotis segetum.*

Pittman et al. (2008) isolated *E. ludwigii* from the hindgut wall of *Dermolepida albohirtum larvae.* Hosokawa et al. (2012) showed that the gut symbionts of *Adrisa magna* and *Macroscytus japonensis* were allied with *E. ludwigii.*

Rhodococcus species have been isolated from a large variety of sources, including soils, rocks, groundwater, seawater, plants, animals and guts of insects (Gürtler et al. 2004). We also isolated *Rhodococcus* from the larvae of *L. botrana* and identified it *as R. erythropolis.*

Serratia marcescens, another isolate of *L. botrana,* is an important insect pathogen that has been isolated from many insects. Kleespies et al. (2008) have reported the isolation of *Serratia marcescens* from *Blatta orientalis, B. germanica (Phyllodromia germanica), Rhyparobia maderae (Leucophae maderae), Epilachuna varivestis, Nympalis antiopa, Operophthera brumata, Ostrinia nubilalis, Chrysoperla carnea and Achaeta domesticus, Zonocerus variegates.* Thaochan et al. (2010) isolated it from *Bactrocera tryoni,* Gökce et al. (2010) isolated it from *Rhynchites bacchus* and *Secil* et al. (2012) isolated it from *Ostrinia nubilalis.*

After identification, bacterial isolates were assayed for insecticidal activities against Lobesia

Table 5. Survival of the study groups in a period of ten days.

Group	Day	Number Entering Interval	Number of Terminal Events	Cumulative Proportion Surviving at End of Interval	Group	Day	Number Entering Interval	Number of Terminal Events	Cumulative Proportion Surviving at End of Interval
Control	1	30	0	1.00	Lb13	1	30	1	.97
	2	30	2	.93		2	29	1	.93
	3	28	7	.70		3	28	3	.83
	4	21	4	.57		4	25	6	.63
	5	17	0	.57		5	19	1	.60
	6	17	2	.50		6	18	0	.60
	7	15	2	.43		7	18	7	.37
	8	13	0	.43		8	11	0	.37
	9	13	0	.43		9	11	0	.37
	10	13	0	.43		10	11	0	.37
Lb4	1	30	1	.97	Lb15	1	30	2	.93
	2	29	3	.87		2	28	0	.93
	3	26	2	.80		3	28	4	.80
	4	24	6	.60		4	24	3	.70
	5	18	4	.47		5	21	5	.53
	6	14	2	.40		6	16	3	.43
	7	12	3	.30		7	13	4	.30
	8	8	0	.30		8	9	1	.27
	9	4	0	.30		9	8	0	.27
	10	4	0	.30		10	8	0	.27
Lb6	1	30	1	.97	Lb17	1	30	0	1.00
	2	29	0	.97		2	30	1	.97
	3	29	4	.83		3	29	7	.73
	4	25	8	.57		4	22	6	.53
	5	17	4	.43		5	16	4	.40
	6	13	3	.33		6	12	6	.20
	7	10	3	.23		7	6	2	.13
	8	7	0	.23		8	4	0	.13
	9	6	0	.23		9	4	0	.13
	10	6	0	.23		10	4	0	.13
Lb12	1	30	3	.90	**Lb21**	1	30	7	.77
	2	27	2	.83		2	23	4	.63
	3	25	0	.83		3	19	6	.43
	4	25	3	.73		4	13	4	.30
	5	22	7	.50		5	9	3	.20
	6	15	6	.30		6	6	1	.17
	7	9	0	.30		7	5	1	.13
	8	9	0	.30		8	4	2	.07
	9	9	0	.30		9	2	0	.07
	10	9	0	.30		10	2	0	.07

botrana. Lb21 (*Serratia marcescens*) isolate had the highest mortality (93 %) on larvae within ten days of inoculation; it was significantly higher than the one in the control group. The insecticidal effects of Lb12 and Lb17 were also significantly different from the control group, while the effect of the other isolates were not (Table 4).

Campos et al. (2007) have studied the effect of *Serratia marcescens* and *Enterobacter cloacae* against *Phyllocnistis citrella* (Gracillariidae). The mortality of *P. citrella* larvae exposed to *S. marcescens* and *E. cloacae* was about 48 % at both the assayed concentrations (10^{10} and 10^{7} UCF/mL). However, the mortality of *L. botrana*'s larvae exposed to *S. marcescens* and *E. cloacae* were determined as 93 % and 87 %, respectively, in this study. These were significantly different from control group, despite the partly high mortality that was observed in the control group.

Inglis & Lawrence (2001) studied the effects of S. marcescens on the F1 generation of laboratory–reared *Heliothis virescens* (Noctuidae). They

Table 6. The Kaplan-Meier survival analysis between study groups.

Group Chi-Square		Control		Lb4		Lb6		Lb12		Lb13		Lb15		Lb17		Lb21	
		Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	
Log Rank (Mantel-Cox)	control			.644	.422	1.271	.260	4.406	.036	.002	.963	.697	.404	4.085	.043	12.773	.000
	Lb4	.644	.422			.155	.694	1.725	.189	.712	.399	.008	.929	1.614	.204	8.427	.004
	Lb6	1.271	.260	.155	.694			.925	.336	1.715	.190	.288	.591	.893	.345	7.415	.006
	Lb12	4.406	.036	1.725	.189	.925	.336			5.290	.021	2.218	.136	.000	.991	3.289	.070
	Lb13	.002	.963	.712	.399	1.715	.190	5.290	.021			.726	.394	5.509	.019	13.792	.000
	Lb15	.697	.404	.008	.929	.288	.591	2.218	.136	.726	.394			2.314	.128	9.386	.002
	Lb17	4.085	.043	1.614	.204	.893	.345	.000	.991	5.509	.019	2.314	.128			3.919	.048
	Lb21	12.773	.000	8.427	.004	7.415	.006	3.289	.070	13.792	.000	9.386	.002	3.919	.048		

observed a higher prevalence of mortality of F1 (n = 2,888) larvae for the *Serratia* (3.5–4.6 %) than for the control (1.1–1.5 %) group. Moreover, up to now, only the effect of *B. thuringiensis* on *Lobesia botrana*'s larva was tested as entomopathogen. In the current study seven culturable bacteria from larva of *L. botrana* were isolated and the insecticidal activity of each isolate was assessed. As a result two bacteria, *Serratia marcescens* and *Enterobacter ludwigii* had statistically significant effect as compared to the control group. The mortality rate of *S. marcescens* was considerable. Therefore, *Serratia marcescens* and *Enterobacter ludwigii* can be considered as potential biological control agents for this pest. Thus we suggest that, in manufacturing biological products, the effect of this bacterium should be taken into consideration.

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