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The first study on bacterial flora and biological control agent of the little spruce sawfly, *Pristiphora abietina* (Christ.) (Hymenoptera: Tenthredinidae)

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Abstract

The aim of this study was to determine the bacterial flora of Pristiphora abietina and to find the performance of the members of this flora as a biocontrol agent for this pest. For this purpose, eleven bacteria were isolated from living, diseased and dead larvae. Morphological and biochemical properties, metabolic enzyme profiles by BIOLOG microtiter plate system and total cellular fatty acid profile by Microbial Identification Systems (MIS) of the bacterial isolates were determined. In addition, 16S rRNA gene sequence analysis was performed. The isolates were identified as Bacillus pumilus (Pa1), Lysinibacillus fusiformis (Pa2, Pa10), Stenotrophomonas maltophilia (Pa3), Acinetobacter johnsonii (Pa4, Pa9), Bacillus cereus (Pa5), Rhodococcus sp. (Pa6), Staphylococcus sciuri (Pa7), Ralstonia pickettii (Pa8), Neisseria perflava (Pa11). All these bacteria were tested against P. abietina larvae. The highest insecticidal activity was obtained from S. maltophilia and L. fusiformis (65.47%, 60.71%, respectively), ($p<0.05$), whereas the lowest insecticidal activity (17.26%) was obtained from N. perflava within seven days. Our result indicates that L. fusiformis (Pa2, Pa10) show potential to be used as biological control agents of P. abietina.

Keywords: *Pristiphora abietina*, bacterial flora, biological control, pest, entomopathogenic bacteria.

1. Introduction

The little spruce sawfly, *Pristiphora abietina* (Hymenoptera: Tenthredinidae), is one of the most important pest of spruce forests in Europe, including Turkey and also the other regions of the world [1,2,3,4]. It prefers 20-30 years old spruce stands and causes defoliation of the top whorls [5].

Up to now, an integrated pest management (IPM*)* strategy was not established for the pest [6]. Insecticides such as Dimilin 48 SC, Mimic 240 LV, Trebon 10 F have been utilized [7] but using insecticides may cause negativities on the useful fauna around. In addition, there are no biological methods that can be used to control its over-population [8]. Pathogenic fungi and bacteria will be potential agents in biological control of *P. abietina.* Some pathogenic species of the genera *Baeuvaria*, *Paecilomyces* and *Metarhizium* were found to be important natural enemies against *P. abietina* [9]. Furthermore, insecticidal effects of *B. bassiana* species isolated from *P. abietina* were determined [10]. A virus strain (CPV)

isolated from *Dasychira pudibunda* (Lep., Lymantriidae) showed pathogenicity against pest [11].

Surprisingly, although *P. abietina* is a very damaging pest species worlwide, bacterial pathogens of this pest have not been investigated so far. In this study, bacterial flora was investigated for the first time to be used in the biological control of *P. abietina* and their pathogenicity against third-fourth instar larvae of this pest was determined.

2. Materials and Methods

Collection of insects

Larvae of *P. abietina* were collected from spruce forests in Artvin, Turkey, in May 2011. The collected larvae in aseptic conditions were immediately transported to the laboratory.

Isolation of bacteria

After macroscopic examination of living, diseased and dead larvae were distinguished. The larvae were sterilized in 70% alcohol and then washed three times in sterile distilled water and homogenized in nutrient broth media by using a glass tissue grinder. Suspensions were diluted and 0.1ml suspension was plated on nutrient agar. Plates were incubated at 30ºC for 2-3 days. After the incubation period, the plates were examined and bacterial colonies were selected. Determined colonies were purified by a subculture on plates. Bacterial strains were maintained for long-term storage in nutrient broth with 15% glycerol at -80 ºC.

Identification of bacterial isolates

Bacterial isolates were examined in their some morphological (cell morphology, endospore formation and mobility) and biochemical properties (gram reaction, oxidase, catalase, gelatin hydrolysis and reduction of nitrate, urease test) [12].

FAME profiles

FAME profiles of bacterial isolates were determined using the Microbial Identification Systems. Preparation and analysis of FAME from whole cell fatty acids of bacterial isolates were performed according to the method described by the manufacturer's manual (Sherlock Microbial Identification System Version 4.0; Microbial ID, USA) [13,14]. FAMEs were separated by gas chromatography (HP-6890, Hewlett Packard, USA) FAME profiles of each bacterial isolates were identified by comparing the commercial databases (Tryptic soy broth agar 40) with the MlS software package. The identity of bacterial isolates was revealed by computer comparison of FAME profiles of the unknown test isolates with those in the library.

Metabolic enzyme profiles

Metabolic enzyme profiles of bacterial isolates were determined using Biolog GN and GP database microplate systems (Biolog, USA). One or two days before the inoculation of Biolog GN2 and GP2 plates (Biolog), bacterial isolates were streaked on TSA (Tripticase Soy Agar) or BUG (Biolog Universal Growth Agar) agar plates. Each well of Biolog GN2 or GP2 microtiter plates was inoculated with 125 µL of the Gram-negative or positive bacterial suspension and adjusted to an appropriate density (10^8cfu/ml) and incubated at 30 °C for 24 and 48 hours. The development of color was automatically recorded using a microplate reader with a 590-nm wavelength filter. Identification (Biolog Microlog 34.20 database) and ASCII file output of test results, applying the automatic threshold option, were performed using BI0L0G420/Databases/GN601 and GP601 KID software [15].

Genomic DNA extraction

Genomic DNA extraction was done with the Wizard Genomic DNA Purification Kit (Promega, Germany) according to manufacturer’s recommendations.

PCR amplification

PCR amplification of 16S rRNA genes of bacterial isolates was performed with the following universal primers: UNI16S-L: 5'-ATT CTA GAG TTT GAT CAT GGC TCA-3'as forward and UNI16S-R: 5'-ATGGTACCGTGTGACGGGCGGTGTGTA-3' as reverse. PCR conditions were adjusted according to Weisburg et al. [16]. The amplified 16S rRNA gene product was sent for sequencing to the RefGen Biotechnology Laboratory (Ankara, Turkey).

The obtained sequences were used to perform BLAST searches using the NCBIGenBank database to confirm isolate identification [17]. Evolutionary relationships of the eleven bacterial isolates were evaluated. Cluster analyses of the sequences were performed using Bio Edit (version7.09) with Clustal W followed by neighbor joining analysis on aligned sequences performed with MEGA 6.0 software [18]. Reliability of dendograms was tested by bootstrap analysis with 1000 replicates using MEGA 6.0.

The insecticidal effects of bacterial isolates

Healthy third-fourth instar larvae of *P. abietina* were used for the insecticidal assay of bacterial isolates. Bacterial isolates were incubated for 18 h (72 h for *Bacillus* to sporulation) at 30ºC in nutrient broth medium. After incubation, bacterial cells were centrifuged at 3000 rpm for 10 min [19]. The pellet was resuspended by adding sterile PBS. The optical density of the cells was adjusted to 1.89 at OD (optical density)$_{600}$ [20]. Fresh spruce exiles were inoculated by dipping into the bacterial suspensions and placed in a sterile plastic box (150ml). The control group was treated with sterile PBS. Ten healthy third-fourth instar larvae were placed into each box and fresh spruce exile were provided for seven days. At least thirty larvae were assayed for each isolate. The boxes were incubated at 26±2ºC and 60% RH under 12 h L: 12 h D photoperiod [21]. Insect mortality was recorded seven days later. All experiments were repeated three times.

Mortalities were corrected according to Abbott's formula [22]. The data were subjected to ANOVA and subsequently to LSD multiple comparison test to compare isolates against the control group and to determine differences among isolates using SPSS 15.0 statistical software.

3. Results and Discussion

Although *P. abietina* is one of the most important pest of spruce forests, no attemt was done to find its bacterial flora and their potentials as a biocontrol agent. In this study we determined the bacterial flora of *P.abietina* and also investigated their availability in the control of this pest for the first time.

We isolated eleven bacteria from *P. abietina*. Isolates Pa1, Pa2 and Pa3 were isolated from dead larvae, Pa4, Pa5, Pa6, Pa7, Pa11 from diseased larvae, Pa8, Pa9 and Pa10 from the healthy larvae. Pa7 and Pa11 were coccus, the other isolates were bacilli. Isolates Pa3, Pa4, Pa8, Pa9 and Pa11 were Gram negative, while the other isolates were Gram positive. Four spore-forming (isolates Pa1, Pa2, Pa5 and Pa10) and seven non-spore-forming (isolates Pa3, Pa4, Pa6, Pa7, Pa8, Pa9 and Pa11) isolates were obtained from the larvae. Some morphological and biochemical characteristics of bacterial isolates are summarized in Table 1. Besides, total cellular fatty acid profile by Microbial Identification Systems (MIS) and metabolic enzyme profile by BIOLOG microtiter plate system of the bacterial isolates were determined. Suggested identification of bacterial isolates according to MIS and BIOLOG was illustrated in Table 2.

In addition, molecular studies of isolates were performed by using 16S rRNA gene sequencing analysis. The 16S rRNA partial gene sequences generated in this study have been deposited with the GenBank database under the accession numbers KF111692, KF111693,

KF111694, KF111695, KF111696, KP128705, KP128706, KF111697, KF111698, KF111699 and KF111700, respectively. The results from these gene sequences were listed in Table 3 and phylogenetic tree constructed by use of the Neighbor Joining method (Figure 1). The isolates showed similarity between 98%- 99% compared to other species.

Table 1. Some morphological and biochemical characteristics of bacterial isolates*

	Isolates										
Tests	**Pa1**	**Pa2**	**Pa3**	**Pa4**	**Pa5**	**Pa6**	**Pa7**	**Pa8**	**Pa9**	**Pa10**	**Pa11**
Colony color	Cream	Cream	Cream	Cream	Cream	Pink	Cream	Yellow	Cream	Cream	Yellow
Shape	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Coccus	Bacillus	Bacillus	Bacillus	Coccus
Gram stain	+	+	-	-	+	+	+	-	-	+	-
Endospore	+	+	-	-	+	-	-	-	-	+	-
Motility	+	+	+	-	+	-	-	+	-	+	-
Nitrate reduction	-	-	+	-	+	+	+	+	-	-	+
Catalase test	+	+	+	+	+	+	+	-	+	+	+
Oxidase test	-	+	+	-	+	-	+	+	-	+	+
Gelatin Hydrolysis	+	+	+	-	+	ND	-	-	-	+	ND
Urea Hydrolysis	-	+	+	-	ND	+	-	+	-	+	-

* ND: not determined

Table 2. Suggested identification of bacterial isolates according to MIS and BIOLOG, their similarity index (SIM)

Isolates	**MIS results**	**SIM (%)**	**BIOLOG results**	**SIM (%)**
Pa1	*Bacillus pumilus*	33.5	*Bacillus psychrosaccharolyticus*	8.00
Pa2	*Bacillus sphaericus*	41.5	*Bacillus cereus/thuringiensis*	25.0
Pa3	*Stenotrophomonas maltophilia*	82.2	*Stenotrophomonas maltophilia*	47.0
Pa4	*Acinetobacter lwoffii*	26.7	*Acinetobacter johnsonii*	49.0
Pa5	*Bacillus cereus*	34.2	*Bacillus cereus/thuringiensis*	7.00
Pa6	*Rhodococcus erythropolis/ R. globerulus/N. globerula*	43.7	*Rhodococcus rhodocrous*	1.00
Pa7	*Staphylococcus xylosus*	40.1	*Staphylococcus sciuri*	1.00
Pa8	*Enterobacter cloacae*	58.4	*Ralstonia pickettii*	54.0
Pa9	*Acinetobacter calcaceticus*	29.4	*Acinetobacter johnsonii*	9.00
Pa10	*Bacillus sphaericus*	56.2	*Arcanobacterium bernardie*	19.0
Pa11	*Neisseria flavescens*	48.3	*Neisseria perflava*	61.0

The Pa1 isolate showed a low similarity with *Bacillus psychrosaccharolyticus* (8%) in the metabolic enzyme profile, but closely resembled *Bacillus pumilus* in 16S rRNA gene sequence analysis and the FAMES analyses (99% and 33.5% respectively). According to the FAME profile, major fatty acid components of this isolate were iso-C15:0, anteiso-C15:0, iso-C17:0 and anteiso-C17:0. These results were previously reported for *Bacillus pumilus* [23]. This bacterium can metabolize arbutin and salicin that is not metabolized by other isolates. *B. pumilus* has also been isolated from different insects in previous studies and has been determined insecticidal activity against pests [24,25,26]. Pa1 isolate caused 56.55% mortality in *P. abietina* larvae in our studies (Figure 2).

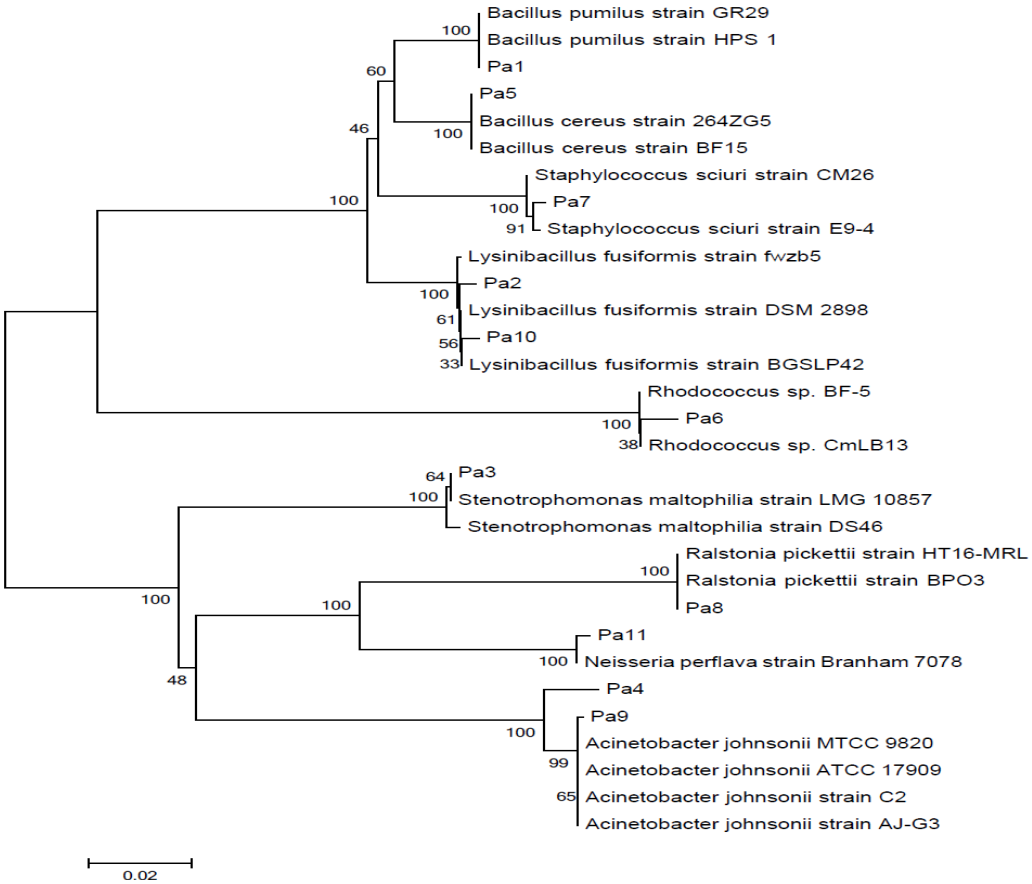

Figure 1. Neighbor-joining tree of bacterial isolates from *P. abietina* and their closely related 20 bacterial species. The dendrogram was constructed by MEGA 6.0 software based on the partial sequences of the 16S rRNA gene. Bootstrap values shown next to nodes are based on 1000 replicates. The scale on the bottom of the dendrogram shows the degree of dissimilarity.

Table 3. Conclusion identification and GenBank Accession numbers of bacterial isolates according to the partial 16S rRNA gene sequence

Isolates	GenBank	Conclusion	16S rRNA similarity(%)	Accesion numbers
Pa1	KF111692	*Bacillus pumilus*	99	KC771045
Pa2	KF111693	*Lysinibacillus fusiformis*	99	NR042072
Pa3	KF111694	*Stenotrophomonas maltophilia*	99	AJ131117
Pa4	KF111695	*Acinetobacter johnsonii*	98	NR117624
Pa5	KF111696	*Bacillus cereus*	99	KF831395
Pa6	KP128705	*Rhodococcus sp.*	99	KM282585
Pa7	KP128706	*Staphylococcus sciuri*	99	EU660348
Pa8	KF111697	*Ralstonia pickettii*	99	KP318066
Pa9	KF111698	*Acinetobacter johnsonii*	99	KJ995847
Pa10	KF111699	*Lysinibacillus fusiformis*	99	NR042072
Pa11	KF111700	*Neisseria perflava*	99	NR117694

Pa2 and Pa10 isolates were found to be similar to *Lysinibacillus fusiformis* (99%) using 16S rRNA sequencing. Dominate fatty acid components of *Lysinibacillus* sp. is iso-

C15:0, anteiso-C15:0, iso-C16:0, anteiso-C17:0 and 16:1w7c alcohol [27]. The fame profile is consistent with the results of our study. These bacteria can metabolize 12 different carbon sources from a total of 95 carbon sources tested. *Lysinibacillus* sp. has also been isolated from different insect in the earlier studies [28]. This is the first isolation of *L. fusiformis* from any insects. But, it hasn't been used for biocontrol of pests yet. We also observed high mortality against larvae of *P. abietina* from *L. fusiformis* (60.71%) (Figure 2).

Pa3 isolate was identified as *Stenotrophomonas maltophilia* according to both16S rRNA sequencing and the total cellular fatty acid and the metabolic enzyme profiles. Isolate Pa3 contained iso-C15:0, anteiso-C15:0, C16:0 and summed feature 3 (16:1 w7c/15:0 iso 2OH) as major fatty acids. This composition was similar to earlier reported for *S. maltophilia.* [29]. This isolate can perfectly metabolize 44 different carbon sources from a total of 95 carbon sources tested. *S. maltophilia* has been isolated from different insects in previous studies and its pathogenicity for other insects has also been determined [30,31,32]. Ryan et al. reported that *S. maltophilia* can be used for biological control of phytopathogenic fungi [33]. But, some *Stenotrophomonas* species are known as opportunistic human pathogens [34]. Therefore, we cannot recommend this bacterium to be used for biological control of *P. abietina.* In this study, we showed that *S. maltophilia* (Pa3) has an important mortality value against *P. abietina* larvae (65.47%) for the first time (Figure 2).

According to the metabolic enzyme profile and 16S rRNA gene sequencing, Pa4 and Pa9 isolates were determined as *Acinetobacter johnsonii*. C16:0, 18:1 w9c and 16:1 w7c/15:0 iso 2OH fatty acids dominate in these bacterium. These results were consistent with the related literature [29]. However, only summed feature 3 wasn't detected in isolate Pa4. Pa4 isolate can metabolize 18 different carbon sources from a total of 95 carbon sources tested. If Pa9 isolate can metabolize 22 different carbon sources. *A. johnsonii* has also been isolated from different insects in the earlier studies [29]. However, only summed feature 3 wasn't detected in isolate Pa4. Pa4 isolate can metabolize 18 different carbon sources from a total of 95 carbon sources tested. If Pa9 isolate can metabolize 22 different carbon sources. *A. johnsonii* has also been isolated from different insects in the earlier studies [29]. However, we did not observe notable activity against larvae of *P. abietina* from *A. johnsonii* (30.36%) (Figure 2).

Pa5 isolate was identified as *Bacillus cereus* according to 16S rRNA sequencing, the total cellular fatty acid and the metabolic enzyme profiles. The FAME profile of the isolate contained from iso-C15:0, iso-C13:0, summed feature. The profile was similar to Jung et. al. [35]. It can metabolize glycogen that is not metabolized by other Gram-positive isolates. *B. cereus* has been isolated from different insects in previous studies and has been showed pathogenity for insects [36,37,38]. We found that isolate *B. cereus*; Pa5 caused 43.45% mortality in *P. abietina* larvae (Figure 2).

Pa6 isolate was highly similar to *Rhodococcus sp.* (99%) using 16S rRNA sequencing. Gutierrez et al. [39] reported the presence of the following acids that dominate saturated fatty acid profile in *Rhodococcus* sp. 33. when grown in in the presence and absence of benzene: C14:0, C16:0, C18:0 10-Me. These results are similar to the findings of our studies. According to the metabolic enzyme profile, isolate Pa6 can metabolize a total of 48 different carbon sources, in which 11 of these cannot be metabolized by other gram positive isolates, Pa1, Pa2, Pa5, Pa7 and Pa10. *Rhodococcus* genus is reported to be isolated from different environments such as soil, marine habitats, groundwater, activated sludge, and the guts of insects [40,41]. We observed very low mortality against larvae of *P. abietina* from *Rhodococcus* sp. (22.02%) (Figure 2).

The Pa7 isolate resembled *S. xylosus*, according to FAME analyse (40.1%). However, 16S rRNA gene sequence analysis has shown that this isolate is *Staphylococcus sciuri* (99%). Pa7 isolate was identified as *Staphylococcus sciuri*. The major fatty acids of the Pa7 isolate contained from iso-C15:0, anteiso-C15:0, iso-C17:0 and anteiso-C170. It showed similar profiles with the related literature. [42]. This bacterium can perfectly metabolize 48 different carbon sources from a total of 95 carbon sources tested. *S. sciuri* has also been isolated from different insects in the earlier studies and has been determined insecticidal effect for insects [43,44,45]. In the present study, we found that it has 39.28% insecticidal effect on larvae of *P. abietina* (Figure 2).

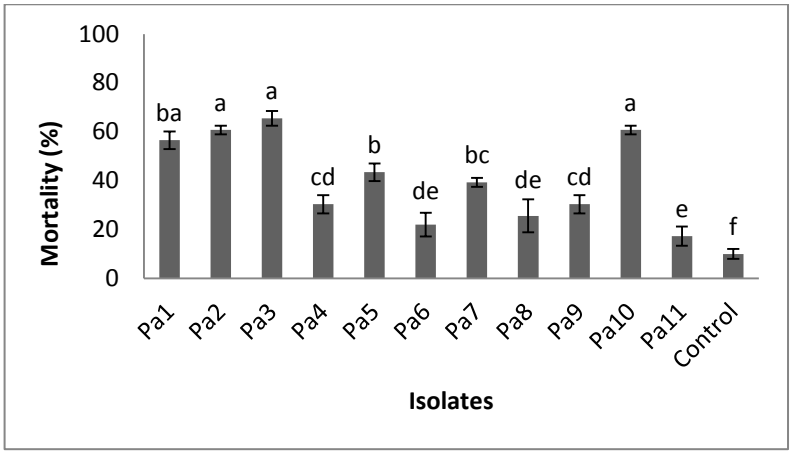

Figure 2. Mortality of bacterial isolates from *P. abietina* on larvaes of this pest within seven days. Pa1, *Bacillus pumilus*; Pa2 and Pa10, *Lysinibacillus fusiformis;* Pa3, *Stenotrophomonas maltophilia*; Pa4 and Pa9, *Acinetobacter johnsonii*; Pa5, *Bacillus cereus*; Pa6, *Rhodococcus sp.*; Pa7, *Staphylococcus sciuri*; Pa8, *Ralstonia pickettii*; Pa11, *Neisseria perflava*.

Pa8 isolate is similar to *Enterobacter cloacae* based on FAME analyses. Nevertheless, according to the metabolic enzyme profile and 16S rRNA gene sequence analysis (%54 and %99 similarity, respectively) results were identified as *Ralstonia pickettii*. The predominant fatty acids of the isolate are: 16:0, summed feature 2 (12: 0 ALDE), summed feature 3 and 18:1 w7c. These results were consistent with previous studies [46]. It can perfectly metabolize 56 different carbon sources from a total of 95 carbon sources tested. Accordingly, 17 of these cannot be metabolized by other gram negative isolates, Pa3, Pa4, Pa9 and Pa11. *R. pickettii* is a ubiquitous micro-organism found in water and soil [47]. This is the first isolation of *R. pickettii* from any insects. We determined that it has low mortality (25.59%) against the pest (Figure 2).

Pa11 isolate was identified as *Neisseria perflava* according to 16S rRNA sequencing, and the metabolic enzyme profiles (99% and 61%, respectively). The bacterium contained 12:0, 16:0, summed feature 3 and 18:1 w7c as major fatty acids. This composition was very similar to Vedros et al. [48]. This isolate can perfectly metabolize 18 different carbon sources from a total of 95 carbon sources tested. *Neisseria* sp. has also been isolated from different insects [49]. However, this is the first study showing that *N. perflava* has been isolated from insects up to now. In this study, the lowest insecticidal activity was found as 17.26% from *N. perflava* (Figure 2).

4. Conclusions

This is the first study conducted on the bacterial flora of *P. abietina* and pathogenicity of isolates on the larvae of *P. abietina*. Isolates were identified as *Bacillus pumilus* (Pa1), *Lysinibacillus fusiformis* (Pa2, Pa10), *Stenotrophomonas maltophilia* (Pa3), *Acinetobacter johnsonii* (Pa4, Pa9), *Bacillus cereus* (Pa5), *Rhodococcus sp.* (Pa6), *Staphylococcus sciuri* (Pa7), *Ralstonia pickettii* (Pa8), *Neisseria perflava* (Pa11). The highest insecticidal activity was obtained from *S. maltophilia and L. fusiformis* (65.47% and, 60.71%, respectively), ($p<0.05$), whereas the lowest insecticidal activity (17.26%) was obtained from *N. perflava* within seven days. Our result indicates that *L. fusiformis* (Pa2, Pa10) show potential to be used as biological control agents of *P. abietina*. Further studies will be directed to determine the effectiveness of the isolate in the field.

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Appendix

Table . The total cellular fatty acid profiles of bacterial isolates using Microbial Identification System (MIS)

	Percentage of cellular fatty acids (%)										
	Isolate number										
Fatty acids	Pa1	Pa2	Pa3	Pa4	Pa5	Pa6	Pa7	Pa8	Pa9	Pa10	Pa11
10:0	-	-	0.28	-	-	-	-	-	-	-	-
10:0 2OH	-	-	-	-	-	-	0.46	-	-	-	-
11:0 ISO	-	-	3.90	-	-	-	-	-	-	-	-
11:0 ANTEISO	-	-	0.15	-	-	-	-	-	-	-	-
11:0 ISO 3OH	-	-	1.79	-	-	-	-	-	-	-	-
12:0	4.48	3.29	2.05	12.84	5.66	0.26	-	4.27	12.29	3.54	11.21
12:0 2OH	0.36	-	-	1.62	-	-	-	0.20	1.91	-	-
12:0 ISO	-	-	-	-	1.44	-	-	-	-	-	-
12:0 3OH	-	-	-	-	-	-	-	-	-	-	3.41
12:0 ISO 3OH	-	-	0.32	-	-	-	-	-	-	-	-
13:0 ISO	0.43	-	0.26	-	11.26	-	0.72	-	-	-	-
13:0 ANTEISO	0.34	-	0.15	-	2.35	-	-	-	-	-	-
13:0 2OH	-	-	0.64	-	-	-	-	-	-	-	-
13:0 ISO 3OH	-	-	2.12	-	-	-	-	-	-	-	-
14:0	1.61	0.68	2.12	1.30	4.07	-	-	4.90	1.07	0.54	4.56
14:0 ISO	0.65	2.87	1.45	-	7.03	-	1.64	-	-	3.65	-
14:0 2OH	-	-	0.09	-	-	-	-	1.07	-	-	-
14:0 ISO 3OH	-	0.55	-	-	-	-	-	-	-	0.57	-
15:0 ISO	35.5	44.6	34.6	-	22.62	-	28.77	0.33	-	42.63	-
15:0 ANTEISO	38.67	18.00	13.94	0.39	6.00	-	43.59	-	-	13.56	0.35
15:0 2OH	-	-	-	-	0.66	-	-	-	-	-	-
15:1 ISOF	-	-	1.15	-	-	-	-	-	-	-	-
15:1 w6c	-	-	0.16	-	-	-	-	-	-	-	-
16:0	2.63	1.25	5.53	15.47	2.15	20.27	1.45	26.84	14.89	1.43	23.35
16:0 ISO	1.50	6.79	3.03	-	5.09	0.63	1.01	-	-	12.22	-
16:0 3OH	-	-	-	-	-	-	-	-	-	-	0.44
16:1 w7c alcohol	0.27	8.81	-	-	0.69	-	0.91	-	-	9.43	-
16:1 2OH	-	-	-	-	-	-	-	0.67	-	-	-
16:1 w11c	-	2.60	-	-	-	-	-	-	-	1.65	-
16:1 w5c	-	-	-	-	-	-	-	-	-	-	0.80
16:1 w9c	3.08	-	1.72	-	-	-	-	-	-	-	-
17:0	-	-	-	0.60	-	1.21	-	0.40	0.70	-	-
17:0 ISO	3.20	2.29	3.78	0.33	3.31	-	4.68	-	1.01	2.56	-
17:0 ANTEISO	5.48	3.25	0.72	-	0.83	-	5.86	1.27	-	3.62	0.67
17:0 CYCLO	-	-	-	-	-	-	-	1.14	-	-	-
17:0 10 methyl	-	-	-	-	-	0.87	-	-	-	-	-
17:0 ISO 3OH	-	-	0.21	-	-	-	-	-	-	-	-
17:1 w8c	-	-	0.70	0.44	-	4.21	-	-	0.67	-	-
17:1 ISO w10c	0.39	0.83	-	-	1.29	-	2.37	-	-	0.87	-
17:1 ISO w5c	-	-	-	-	6.27	-	-	-	-	-	-
17:1 ANTEISO A	-	-	-	-	1.74	-	-	-	-	-	-
17:1 ISO w10c	-	-	-	-	-	0.36	-	-	-	-	-
17:1 w6c	-	-	0.59	-	-	-	-	-	-	-	-
17:1 ISO w9c	-	-	4.92	-	-	-	-	-	-	-	-
18:0	0.67	0.71	0.45	3.65	1.56	0.94	-	1.10	3.17	0.84	1.91
18:0 10Me	-	-	-	-	-	9.53					
18:1 w7c	-	-	0.67	2.32	-	-	-	23.98	3.02	-	15.05
18:1 w9c	-	0.72	0.49	21.56	-	29.22	-	-	26.72	-	1.00
18:1 2OH	-	-	-	-	-	-	-	2.26	-	-	-
18:3 w6c(6,9,12)	-	-	-	-	-	-	-	-	0.46	-	-
19:0	-	-	-	-	-	4.32	-	-	-	-	-
19:0 ISO	-	-	-	-	-	-	0.69	-	-	-	-
19:0 ANTEISO	-	-	-	-	-	-	1.15	-	-	-	-
20:0	-	-	-	-	-	1.29	1.72	-	-	-	-
20:4 w6,9,12,15c	-	-	-	-	-	0.42	-	-	-	-	-
Unknown 11.799	-	-	0.99	-	-	-	-	-	-	-	-
Unknown 12.484	-	-	-	-	-	-	-	-	0.61	-	-
Unknown 15.669	-	-	-	-	-	-	2.18	-	-	-	-
Summed feature 2: 12:0 ALDE?	-	-	-	-	5.92	-	-	5.13	-	-	2.94
Summed feature 3: 16:1 w7c/15 iso 2OH	-	0.53	10.34	-	10.06	26.48	-	26.44	32.00	0.51	32.40
Summed feature 4: 17:1 ISO I/ANTEI B	0.74	2.17	0.65	-	-	-	2.81	-	1.47	2.36	1.89

Table . The metabolic enzyme profile of bacterial isolates using BIOLOG Microtiter Systems (GN and GP).[a]

Tests	Isolate number										
	Pa1	Pa2	Pa3	Pa4	Pa5	Pa6	Pa7	Pa8	Pa9	Pa10	Pa11
2- Aminoethanol	ND	ND	-	-	ND	ND	ND	-	-	ND	-
2'-Deoxy adenosine	+	+	ND	ND	-	-	-	ND	ND	+	ND
2,3-Butanediol	+	-	-	-	-	-	-	-	+	-	-
3-Methyl glucose	+	-	ND	ND	+	+	+	ND	ND	-	ND
Acetic Acid	-	-	+	+	-	+	+	+	+	-	-
Adenosine	+	+	ND	ND	+	-	-	ND	ND	+	ND
Adenosine-5'-monophosphate	-	-	ND	ND	-	-	-	ND	ND	+	ND
Adonitol	ND	ND	-	-	ND	ND	ND	-	-	ND	-
Amygdalin	-	-	ND	ND	-	+	+	ND	ND	-	ND
Arbutin	+	-	ND	ND	-	-	-	ND	ND	-	ND
α-Cyclodextrin	-	-	-	-	-	-	-	-	-	-	-
α-D-Glucose	+	-	+	-	+	+	+	+	+	-	+
α-D-Lactose	-	-	+	-	-	-	-	-	-	-	-
α-Hydroxy Butyric acid	-	-	+	+	-	-	-	+	+	-	+
α-Keto butyric acid	ND	ND	+	+	ND	ND	ND	+	+	ND	+
α-Keto glutaric acid	-	-	+	+	-	-	-	+	+	-	+
α-Keto valeric acid	-	+	+	-	+	-	-	+	+	-	-
α –Methyl-D-galactoside	-	-	ND	ND	-	-	-	ND	ND	-	ND
α –Methyl-D-glucoside	-	-	ND	ND	-	+	+	ND	ND	-	ND
α –Methyl-D-mannoside	-	-	ND	ND	-	-	-	ND	ND	-	ND
Bromo succinic acid	ND	ND	+	+	ND	ND	ND	+	+	ND	+
β- Cyclodextrin	-	-	ND	ND	-	-	-	ND	ND	-	ND
β-Hydroxy butyric acid	-	-	-	+	-	-	-	+	+	-	-
β-Methyl-D-galactoside	-	-	ND	ND	-	+	-	ND	ND	-	ND
β-Methyl-D-glucoside	+	-	+	-	-	+	-	-	-	-	-
cis-aconitic acid	ND	ND	+	-	ND	ND	ND	+	-	ND	-
Citric acid	ND	ND	+	-	ND	ND	ND	+	-	ND	-
Dextrin	+	-	+	-	+	+	-	+	-	-	-
D-Alanine	-	-	+	+	-	-	+	+	+	-	-
D-Arabitol	-	-	-	-	-	+	+	-	-	-	-
D-Cellobiose	+	-	+	-	-	+	+	-	-	-	+
D-Fructose	+	-	+	-	-	+	+	+	-	-	+
D-Galactose	+	-	-	-	-	+	-	+	-	-	-
D-Galactonic acid lactone	ND	ND	-	-	ND	ND	ND	-	-	ND	-
D-Galacturonic acid	-	-	-	-	-	+	+	+	-	-	-
D-Gluconic acid	-	-	-	-	-	+	-	+	-	-	-
D-Glucosaminic acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
D-Glucuronic acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
D-Lactic acid methyl ester	-	-	ND	ND	-	-	-	ND	ND	-	ND
D,L-Carnitine	ND	ND	-	-	ND	ND	ND	-	-	ND	-
D,L-Lactic acid	ND	ND	+	+	ND	ND	ND	+	+	ND	+
D-L-α-Glycerol phosphate	-	+	-	-	-	+	+	-	-	+	-
D-Malic acid	-	-	ND	ND	-	-	-	ND	ND	-	ND
D-Mannitol	+	-	-	-	-	+	+	-	-	-	-
D-Mannose	+	-	+	-	+	+	+	+	-	-	-
D-Melezitose	-	-	ND	ND	-	+	+	ND	ND	-	ND
D-Melibiose	-	-	+	-	-	-	+	-	-	-	-
D-Psicose	+	-	-	-	-	+	+	+	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-
D-Ribose	+	-	ND	ND	-	+	+	ND	ND	+	ND
D-Saccharic acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
D-Serine	ND	ND	-	-	ND	ND	ND	+	-	ND	-
D-Sorbitol	+	-	-	-	-	+	+	-	-	-	-
D-Tagatose	-	-	ND	ND	-	+	-	ND	ND	-	ND
D-Trehalose	+	-	+	-	+	+	+	-	-	-	-
D-Xylose	+	-	ND	ND	-	+	+	ND	ND	-	ND
Formic Acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
Fructose-6-phosphate	-	-	ND	ND	-	-	-	ND	ND	-	ND
Gentiobiose	-	-	+	-	+	+	+	-	-	-	-
Glucose–1 phosphate	-	-	-	-	-	+	-	-	-	-	-
Glucose–6 phosphate	-	-	+	-	-	+	-	-	-	-	-
Glucuronamide	ND	ND	-	-	ND	ND	ND	+	-	ND	-
Glycerol	+	+	-	-	-	+	-	-	-	+	-
Glycyl-L-aspartic acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
Glycyl-L-glutamic acid	-	-	+	-	-	-	+	+	-	-	+

Table (continued)

Tests	Isolate number										
	Pa1	Pa2	Pa3	Pa4	Pa5	Pa6	Pa7	Pa8	Pa9	Pa10	Pa11
Glycogen	-	-	+	-	+	-	-	+	+	-	-
γ-Amino butyric acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
γ-Hydroxy butyric acid	-	-	-	-	-	-	-	+	-	-	-
Hydroxy-L-proline	ND	ND	-	-	ND	ND	ND	-	-	ND	-
Inosine	+	+	+	-	-	-	-	-	-	+	-
Inulin	-	-	ND	ND	-	-	-	ND	ND	-	ND
Itonic acid	ND	ND	-	-	ND	ND	ND	-	-	ND	-
i-Erythritol	ND	ND	-	-	ND	ND	ND	-	-	ND	-
Lactamide	-	-	ND	ND	-	-	+	ND	ND	-	ND
Lactulose	-	-	+	-	-	+	-	-	-	-	-
L-Alanine	-	-	+	+	-	-	+	+	+	-	-
L-Alaninamide	-	-	+	+	-	-	-	+	+	-	-
L-Alanyl-glycine	+	-	+	-	-	-	-	+	-	-	-
L-Arabinose	-	-	-	-	-	+	+	+	+	-	+
L-Asparagine	-	-	+	+	-	-	-	+	-	-	-
L-Aspartic Acid	ND	ND	+	-	ND	ND	ND	+	-	ND	+
L-Fucose	-	-	-	-	-	+	+	-	-	-	-
L-Glutamic Acid	-	-	+	+	-	+	+	+	+	-	+
L-Histidine	ND	ND	-	-	ND	ND	ND	+	-	ND	-
L-Lactic acid	-	-	ND	ND	-	+	-	ND	ND	-	ND
L-Leucine	ND	ND	-	-	ND	ND	ND	+	-	ND	-
L-Malic Acid	-	-	ND	ND	-	-	-	ND	ND	-	ND
L-Ornithine	ND	ND	-	-	ND	ND	ND	-	-	ND	-
L-Phenylalanine	ND	ND	-	-	ND	ND	ND	+	-	ND	-
L-Proline	ND	ND	+	+	ND	ND	ND	+	+	ND	+
L-Pyroglutamic Acid	-	-	-	+	-	-	-	+	+	-	-
L-Rhamnose	-	-	-	-	-	+	+	-	-	-	-
L-Serine	-	-	+	-	-	-	+	+	-	-	-
L-Threonine	ND	ND	+	-	ND	ND	ND	+	-	ND	+
m-Inositol	-	-	-	-	-	+	+	-	-	-	-
Malonic acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
Maltose	+	-	+	-	-	+	+	-	-	-	+
Maltotriose	+	-	ND	ND	+	+	+	ND	ND	-	ND
Mannan	-	-	ND	ND	-	-	-	ND	ND	-	ND
Methyl pyruvate	+	-	+	+	-	-	-	+	+	-	+
mono-Methyl -succinate	-	-	+	-	-	+	-	+	-	-	+
N-Acetyl-D-galactosamine	ND	ND	+	-	ND	ND	ND	-	-	ND	-
N-Acetyl-D-glucosamine	+	-	+	-	-	+	-	-	-	-	-
N-Acetyl- β-D-mannosamine	+	-	ND	ND	-	+	+	ND	ND	-	ND
N-Acetyl-L-Glutamic Acid	-	-	ND	ND	-	-	-	ND	ND	-	ND
p-Hydroxy-Phenly Acetic Acid	-	-	-	-	-	-	-	+	-	-	-
Phenylethyl-amine	ND	ND	-	-	ND	ND	ND	-	-	ND	-
Palatinose	-	-	ND	ND	-	+	+	ND	ND	-	ND
Propionic acid	-	-	+	-	-	+	-	+	-	-	-
Pyruvic acid	+	-	ND	ND	+	-	-	ND	ND	-	ND
Putrescine	-	-	-	-	-	-	-	-	-	-	-
Quinic acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
Salicin	+	-	ND	ND	-	-	-	ND	ND	-	ND
Sebacic acid	ND	ND	-	-	ND	ND	ND	+	-	ND	-
Sedoheptulosan	-	-	ND	ND	-	-	-	ND	ND	-	ND
Stachyose	-	-	ND	ND	-	-	+	ND	ND	-	ND
Succinic acid	-	-	+	+	-	-	-	+	+	-	+
Succinamic acid	-	-	-	-	-	-	-	+	-	-	-
Sucrose	+	-	+	-	-	+	+	-	-	-	+
Thymidine	+	+	-	-	-	-	-	-	-	+	-
Thymidine–5'-monophosphate	+	+	ND	ND	+	-	-	ND	ND	+	ND
Turanose	-	-	+	-	+	+	+	-	-	-	-
Tween 40	-	+	-	+	+	+	-	+	+	-	-
Tween 80	-	+	-	+	+	+	+	+	+	+	-
Uridine	+	+	+	-	-	-	-	-	-	+	-
Uridine-5'-monophosphate	-	+	ND	ND	-	-	+	ND	ND	+	ND
Urocanic acid	ND	ND	-	-	ND	ND	ND	-	-	ND	-
Xylitol	-	-	-	-	-	+	-	-	-	-	-

[a] ND: No data