

# Bacterial Communities of *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) Damaged in Strawberry in Turkey<sup>1</sup>

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**Abstract** *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) is an invasive species originating from Southeastern Asia and spreads in a fast manner. It is among major threats in soft-shell fruit cultivation in the whole world. It was detected in 2014 in Turkey. According to international criteria, it is considered that it has the potential of threatening the fruit cultivation in Turkey where garden plants are grown widely. In this study, a total of 39 bacterial strains were isolated from 100 mature *Drosophila suzukii* individuals. Gram staining characteristics, catalase, oxidase and nitrate reductase activities and chitinase enzyme activities and hypersensitivity reaction of these strains were determined by using microscopical and visual inspection. The bacterial strains were identified according to their fatty acid methyl esters (FAME) analysis by using Sherlock Microbial Identification System (MIS). The identification test results of the bacterial strains were also confirmed by phylogenetic analysis and their closely related species based on the 16S rRNA sequence. The most abundant bacterial species were *Paenibacillus alvei* (31.57%) and *Bacillus amyloliquefaciens* (47.36%) according to the MIS and 16S rRNA sequence analysis results, respectively. According to the MIS results, a total of 6 strains identified as *Paenibacillus alvei* were identified as *Bacillus amyloliquefaciens* according to the 16S rRNA sequence analysis results. A total of three *Paenibacillus macerans* strains identified in MIS system were also identified as *Bacillus amyloliquefaciens* according to the 16S rRNA sequence analysis. Morphological and biochemical characteristics results of all of *Bacillus amyloliquefaciens* strains showed the some results. According to the 16S rRNA sequence analysis results, the other bacterial strains consist of 1 *Bacillus atrophaeus* (5.2%), 1 *Bacillus safensis* (5.2%), 1 *Paenibacillus motobuensis* (5.2%) and 1 *Staphylococcus epidermidis* (5.2%) strains. To our knowledge, this is the first study characterizing the

bacterial communities of *Drosophila suzukii*.

**Keywords** Bacteria, Biological Control, *Drosophila suzukii*, Microbiota

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## 1. Introduction

*Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), an indigenous species to the continent of Asia, was first reported outside this continent in 1980 in Hawaiian Islands of the North America [1]. Within two years, it completely invaded the northern parts of Continent of America from west to the east, and consequently reached Canada at north and Mexico at south [2-5]. In the field study in which European Drosophilidae species were recorded, *D. suzukii* emerged as a predominant Drosophilidae species in the highlands where it habitates. From this date on, the first records in Italy [6], France [7], Switzerland [8], Slovenia [9], Croatia [10], Austria [11], United Kingdom, Portugal [12], Germany [13], Belgium [14], Hungary [15], Serbia [16], Bosnia and Herzegovina [17], Bulgaria [18], Greece (Crete Island) [19], Poland [20] and Japan [21] were reported. The first record in Turkey was in Erzurum in August-September 2014 [22].

Although the primary hosts of *D. suzukii* are cherry, sour cherry, strawberries, blackberries, raspberries and blueberries, a very wide spectrum of fruits can be affected. It can cause also serious damage to fruits such as fig, apricot, peach, plum, grape, medlar, greenhouse mandarin, kiwi, persimmon, and fallen or cracked apple and orange [2, 4, 23, 24]. It is estimated that these losses have affected 14% of all potential fruit production worldwide [25].

Due to the wide range of host fruit selection and rapid spreading, it is stated that this species is an important pest that is likely to cause major losses to the European and

American fruit industry in the near future [3-4].

While other *Drosophila* species feed on rotten fruits, *D. suzukii* prefers newly ripening fruits on the tree, and using their saw-shaped ovipositor, female individuals lay their eggs inside the fruits prior to their maturation for harvesting, which all make this organism a very important agricultural pest [2]. While the larvae feed on the rich protein content of the flesh of the fruit, the synthesis of inherent ethylene is increased in areas where tissue integrity is compromised. Ethylene synthesis locally accelerates maturity and causes collapse/softening (rotting) of the flesh of the fruit. As a result, these products lose their market value within a short period of time. In addition, the wounds that these flies open on the fruit for laying their eggs lead to additional losses caused by pathogens, including fungi and bacteria [26].

Few studies have been carried out to identify the microflora of this pest. The first study that was conducted to identify the bacterial flora of *D. suzukii* revealed species belonging to the genera *Gluconobacter* and *Acetobacter* [27]. Another study reported that *Wolbachia* spp.

(Rickettsiales: Rickettsiaceae) could be used in biological control of this pest [28-29]. To our knowledge, there is not any study characterizing the microbial communities of *D. suzukii*.

The present study aims to determine the microflora of *D. suzukii* which continues to spread rapidly and cause economic losses.

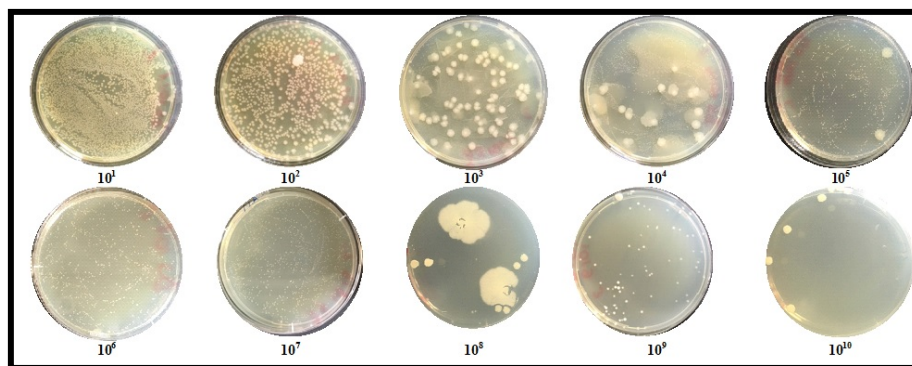
## 2. Materials and Methods

### 2.1. Pest Samples

The study was conducted on 100 healthy adult *D. suzukii* individuals collected on July 25th-26th, 2016 in Erzurum province of Turkey using traps prepared with cider vinegar (Figure 1) in strawberry trial fields where neither insecticide nor fungicide was used. Collected adult individuals were put into tubes and brought to Atatürk University Plant Protection Department, Plant Clinical Laboratory.



Figure 1. The traps used for *Drosophila suzukii*



**Figure 2.** Serial dilutions were obtained from *Drosophila suzukii* adults

## 2.2. Isolation of Bacterial Strains

Superficial sterilization was applied to *D. suzukii* adults in tens with 95% ethyl alcohol for 5 minutes. Adults were homogenized by pulverizing in a sterile mortar with sterile saline solution and serial dilutions were obtained from this homogenate [30] (Figure 2). The dilutions prepared from the adults were inoculated on Nutrient Agar (NA) for bacterial growth. Then, Bacterial cultures were incubated at 30°C, and at the end of 24-72 hours. The bacterial strains with the dominant character were selected and purified [31]. Each of single colonies was purified and streaked on agar plate. For each pure culture was given a separate code number, and information regarding the isolation conditions (location, altitude, insect form, date, etc.) was noted. The samples were kept at -86°C in stock growth media containing 30% glycerol and Loria Broth (LB) for routine use.

## 2.3. Determination of Morphological and Biochemical Properties of Bacterial Strains

The colony morphology and color of the bacterial strains were determined microscopical and visual inspection.

The Gram staining characteristics of the bacteria were determined according to the method described by Sands [32]. Presence of catalase and oxidase enzymes [33] and nitrate enzyme was assessed by the method of Harley and Prescott [34]. Chitinase enzyme activity was determined according to the method reported by Ortucu [35].

## 2.4. Polymerase Chain Reaction (PCR) of the Bacterial Strains

The strains were also identified in the molecular system. Total 19 bacterial strains selected from 39 bacterial strains obtained from *D. suzukii* adults according to high colony density were identified by sequencing a fragment of genome [36]. Bacterial DNA was amplified by a two-step PCR targeting the 16S rDNA gene with primers 27F and 907R, designed to include Illumina adaptor and barcode sequences. Sequencing was performed on an

IlluminaMiSeq at the UC Davis Genomics Core Facility generating 963 basepair paired-end reads. Samples were OTUs are identified by their closest hit in the SILVA SSU Reference Database Release 111. Number of sequences is after all quality-control steps.

## 2.5. Phylogenetic Relationship of the Bacterial Strains

The sequences obtained were used to perform BLAST searches using the NCBI GenBank database to confirm strain identification Altschul *et al.* [37]. Evolutionary relationships of the 19 bacterial strains 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 6.0 software [38] Reliability of dendograms was tested by bootstrap analysis with 1000 replicates using MEGA 6.0.

The evolutionary history was inferred using the Neighbor-Joining method [39]. The optimal tree with the sum of branch length = 0.56341095 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [40]. 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 [41] and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 823 positions in the final dataset. Evolutionary analyses were conducted [38].

## 3. Results

The identification test results of the isolated bacteria and their morphological and biochemical characteristics were given in Table 1.

**Table 1.** Identification test results of the isolated bacteria and their morphological and biochemical characteristics test results

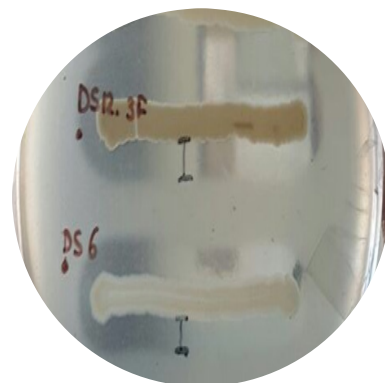
| Strains                                  | BLAST top hit                     | Identify (%) | Colony shape | Colony color | Gram staining | Catalase test | Oxidase test | Nitrate reduction | Chitinase activity |
|--|-----------------------------------|--------------|--------------|--------------|---------------|---------------|--------------|-------------------|--------------------|
| RK 1792                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1801                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1805                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1809                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1810                                  | <i>Bacillus amyloliquefaciens</i> | 98           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1812                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1813                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1814                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1815                                  | <i>Bacillus amyloliquefaciens</i> | 99           | rod          | cream        | +             | +             | +            | +                 | -                  |
| RK 1811                                  | <i>Bacillus atrophaeus</i>        | 99           | rod          | cream        | +             | +             | -            | +                 | +                  |
| RK 1807                                  | <i>Bacillus safensis</i>          | 93           | rod          | cream        | +             | +             | +            | -                 | -                  |
| RK 1770                                  | <i>Paenibacillus motobuensis</i>  | 99           | rod          | white        | -             | +             | +            | +                 | +                  |
| RK 1784                                  | <i>Proteus myxofaciens</i>        | 99           | rod          | cream        | -             | +             | -            | +                 | -                  |
| RK 1785                                  | <i>Proteus myxofaciens</i>        | 99           | rod          | Cream        | -             | +             | -            | +                 | -                  |
| RK 1787                                  | <i>Proteus myxofaciens</i>        | 99           | rod          | cream        | -             | +             | -            | +                 | -                  |
| RK 1768                                  | <i>Staphylococcus epidermidis</i> | 99           | cocci        | white        | +             | +             | -            | +                 | -                  |
| RK 1765                                  | nd                                | -            | rod          | cream        | -             | +             | +            | +                 | -                  |
| RK 1767                                  | nd                                | -            | rod          | cream        | -             | +             | -            | -                 | -                  |
| RK 1769                                  | nd                                | -            | rod          | cream        | -             | +             | -            | -                 | -                  |
| Other a total of 12 bacterial strains    |                                   |              | nt           | nt           | nt            | nt            | nt           | nt                | nt                 |
| Undefined a total of 8 bacterial strains |                                   |              | nt           | nt           | nt            | nt            | nt           | nt                | nt                 |

SIM: Similarity index, nd: Not determined; +: Positive reaction, -: Negative reaction, nt: Not tested

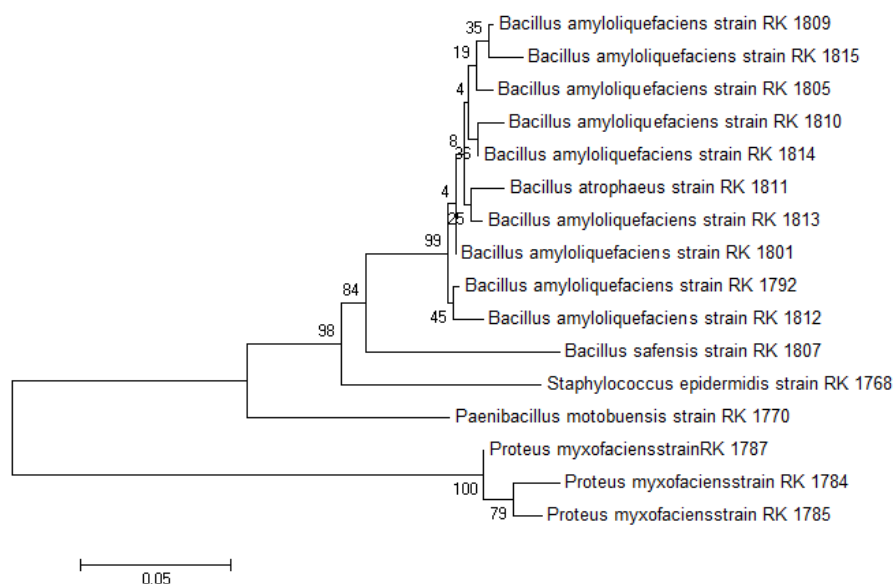
It was observed that the RK 1768 strain had a rod shaped colony, whereas other strains had a rounded colony shape, and that RK 1768 and RK 1770 strains had a white colony color while the other strains had cream colony color (Table 1). The strains RK 1770, RK 1784, RK 1785 and RK 1787 were gram-negative, while other strains were gram-positive. All strains had positive catalase and nitrate test results. Except RK 1770 and RK 1811 all other strains had negative chitinase test results. Chitinase activity of RK-1811 and RK-1770 strains was positive (Table 1) (Figure 3).

The bacterial strains with high colony density that were obtained from *D. suzukii* adults were identified molecular analysis. Molecular diagnostic results are given in Table 1. According to the 16S rRNA sequence analysis results, a total of 9 strains were identified as *Bacillus amyloliquefaciens*. The other bacterial strains consist of 3 *Proteus myxofaciens*, 1 *Bacillus atrophaeus*, 1 *Bacillus*

*safensis*, 1 *Paenibacillus motobuensis* and 1 *Staphylococcus epidermidis*. Percent identify of all the identified bacterial strains were 99%. But, a total of three strains were not identified (Table 1).

**Figure 3.** Chitinase positive bacterial strains





**Figure 4.** Evolutionary relationships of taxa

These identifications were also confirmed by phylogenetic analysis of the bacterial strains and their closely related species based on the 16S rRNA sequence (Figure 4).

## 4. Discussion

Chandler *et al.* [27], in their microbiota study on *D. suzukii*, isolated the genus *Tatumella* from 99% of both the adults and the larvae and Dunitz *et al.* [42] isolated *Tatumella* sp. from the larvae. Although this genus is not commonly found in *Drosophila* species, another study identified it also in *D. melanogaster*, which feeds on apples [43]. Again, Chandler *et al.* [27] reported that *Acetobacteraceae* and *Orbus* species were also associated with *Drosophila* population. However, Broderick and Lemaitre [43] noted that *Orbus* species were not associated with *Drosophila*. Brummel *et al.* [44] conducted molecular analyses on strains from the whole body of *D. melanogaster* adults, and they identified the genera *Lactobacillus*, *Gluconabacter*, *Enterobacter*, *Anaerococcus*, while Cox and Gilmore [45] identified *Wolbachia* sp., *Acetobacter aceti*, *A. cerevisiae*, *A. pasteurianus*, *A. pomorum*, *Gluconobacter cerinus*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Lactobacillus plantarum*, *Leuconostoc mersenteroide* and *Enterococcus faecalis* species.

In this study, the most abundant bacterial species in mature *D. suzukii* individuals were *B. amyloliquefaciens* (47.36%), *Proteus myxofaciens* (15.78%), *B. safensis*, *P. motubensis*, *S. epidermis* (%5.26) according to the 16S rRNA sequence analysis results. This identification results were supported with classical systems in this study. Morphological and biochemical characteristics tests results

of all of *B. amyloliquefaciens* strains showed the some results. According to the 16S rRNA sequence analysis results.

It suggests that differences in species distribution in microbial flora studies of the same insect species may be due to the differences in the body part where strains were obtained, the biological period of the pest, and dietary differences. Indeed, it has been stated that, in comparison to isolations made from the whole body, isolations from the gut yielded less microorganisms, that the sample preparation time may also influence variation due to the shorter passage time in the gut [46-47], and that microbiota could differ depending on the variations of consumed food, and whether the individual fed in its natural environment or in laboratory setting [47].

As it can be seen from all these studies, there is ongoing research on microbiota of *D. suzukii* species. It is thought that microbiota studies will guide biological pest control studies. The discovery of new pathogens and parasites of pest insects offers a chance to find organisms that may be useful for biological control [48]. For this purpose it has become increasingly important to identify microorganisms which are present in the microbiota of harmful organisms, and can be used in control of these pests, and to study them in biological control research. Identification of microorganisms that have the potential to be used in biological pest control against the invasive species *D. suzukii* will be possible detailed studies on this subject.

## 5. Conclusions

To our knowledge, this is the first study characterizing the bacterial communities of *Drosophila suzukii*. In conclusion, we think that especially *Bacillus*

*amyloliquefaciens* strains can be used as biological control agents against this economically important pest. Biological control studies will be planned with bacterial strains stored in the Atatürk University, Plant Protection Laboratory in future.

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