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First Isolation and Characterization of *Serratia liquefaciens* Associated with Rot Disease of *Malus domestica* (Apple) Fruit and Its Inhibition by *Origanum vulgare* (Oregano) Oil

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Abstract: Microbial food spoilage has been a major source of concern because it widens the global food production-to-consumption gap. Thus, in the current study, we aimed to isolate, characterize and identify bacteria associated with spoiled apple fruit in Al-Ahsa city, Saudi Arabia. MDK33, a bacterial strain isolated from rotten apple fruit, was characterized at the phenotypic and genotypic levels. Furthermore, the antibacterial effect of oregano essential oil (OEO) against MDK33 was investigated. MDK33 formed circular colonies with entire margins, and the cells were Gram-negative rods with no endospores. Biochemical characterizations of MDK33, as revealed by the Biolog Gen III MicroPlate test system, indicated that the strain utilized 66 (~70%) of the 94 diverse metabolites and did not utilize 29 (~30%). MDK33 grew well on a variety of substrates, including pectin, gelatin, Tween 40 and dextrin, at pH 5 and 6; tolerated salts up to 4% NaCl; and was resistant to multiple antibiotics. Furthermore, based on the sequences of the 16S rRNA gene, MDK33 was identified as *Serratia liquefaciens* at a 99.73% identity level. Koch's postulates were affirmed, confirming that *S. liquefaciens* MDK33 is the causative agent of apple rot disease in Al-Ahsa, Saudi Arabia. The minimum inhibitory concentration (MIC) of 0.23 mg/mL for oregano oil against *S. liquefaciens* MDK33 demonstrated remarkable antibacterial and antibiofilm activities. This is the first isolation of pathogenic *S. liquefaciens* as the causative agent of rot disease of apple fruit and its inhibition by oregano oil. Furthermore, the findings pave the way for oregano oil to be evaluated as a natural preservative to reduce post-harvest losses while maintaining high quality for sustainable food security in future studies.

Keywords: *Malus domestica*; *Serratia liquefaciens*; oregano oil

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1. Introduction

Food spoilage has been a major source of concern because it accounts for approximately one-third of the food produced globally every year [1]. Many microbial groups cause food spoilage, rendering it unsafe to consume and widening the global food production-to-consumption gap. According to estimates of the global burden of foodborne diseases, nearly one in ten people become ill each year from eating contaminated food, and children under the age of five are extremely vulnerable, especially in Africa and southeast Asia. Food pathogens include various species within *Bacillus*, *Clostridium*, *Escherichia*, *Listeria*, *Salmonella*, *Serratia* and *Staphylococcus* [2], posing a potential risk to human health.

Malus domestica (apple) is a perennial tree in the Rosaceae family that is cultivated worldwide [3]. Apple fruits have a high nutritive value because they are rich in antioxidants, including vitamin C, fibers and carbohydrates [4]. Eating multiple apple fruits on a daily basis may aid in the prevention of many chronic diseases, such as cardiovascular and neurodegenerative disorders [5]. In addition, despite their high carbohydrate and sugar content, apple fruits have a relatively low glycemic index [6]. Apple fruits are easily

attacked by microbial pathogens, reducing shelf life and economic value. It is estimated that approximately 25% of harvested apple fruits decay due to microbial infection via secretion of hydrolytic enzymes, such as amylases and proteases, during post-harvest handling [7]. Members of the bacterial genera *Burkholderia*, *Hymenobacter*, *Methylobacterium*, *Pseudomonas*, *Pantoea*, *Ralstonia*, *Sphingomonas* and *Massilia* have been found on apples [8]. A vast majority of apple diseases are caused by fungal pathogens [9]. However few bacterial diseases have been reported on apples, including blister spot caused by *Pseudomonas syringae* pv. *papulans* [10], crown gall and hairy root caused by *Rhizobium rhizogenes* [11,12] and fire blight caused by *Erwinia amylovora* [13]. *Serratia* spp. are Gram-negative rods in the *Enterobacteriaceae* family that exist in various ecological niches, including water, soil, air, plants and animals [14,15]. They can also survive on hospital instruments and farm equipment and are involved in food spoilage, with potential socioeconomic consequences [16].

Origanum vulgare (oregano) is a perennial herbal plant that belongs to the Lamiaceae family and is recognized by the presence of aromatic compounds, primarily essential oils (EOs). EOs are secondary metabolic aromatic products derived from plant roots, stems, leaves and fruit and are volatile at room temperature. EOs inhibit the growth of some microorganisms [17] by disrupting cell membranes, causing cell death or inhibiting the sporulation and germination of food spoilage microbes [7]. Several lines of research support the efficacy of EOs as safe antimicrobial agents, and they can be used as an efficient strategy to control fruit pathogens instead of using chemicals with potential health and environmental consequences [18–20]. Oregano essential oil (OEO) is used as an additive in the production of Minas cheese to prevent microbial spoilage [21] and to control gray mold disease in table grapes caused by *Botrytis cinerea* [22].

Few studies have been focused on bacteria associated with deteriorated apple fruit, particularly in the Al-Ahsa region, Saudi Arabia. Therefore, the aim of the current study was to isolate, characterize and identify bacteria associated with spoiled apple fruit in Al-Ahsa region. Furthermore, the antibacterial effect of OEO against such bacteria was investigated.

2. Materials and Methods

2.1. Isolation of MDK33 from Spoiled Apple Fruit

Unwashed and unprocessed spoiled apple fruits (*M. domestica*, cv. Golden Delicious) were collected in sterilized plastic zip bags from a local market in Al-Ahsa city, Saudi Arabia. In our microbiology laboratory, exactly 1 g the spoiled apple fruit was crushed in a mortar and pestle with 9 mL of saline solution (0.85% NaCl). The suspension was serially diluted from 10^1 to 10^6 dilutions. Aliquots (100 µL) were spread onto nutrient agar plates and incubated at 30 °C for 48h. Nutrient agar medium contained the following ingredients (g/L): peptone, 5.0; sodium chloride, 5.0; peptone, 1.5; yeast extract, 1.5; and agar, 15.0). Purification was carried out by restreaking the obtained isolates onto fresh agar plates to obtain pure colonies, which were then grown overnight in nutrient broth (NB). An aliquot of active culture was mixed with glycerol (total concentration, 15%) and preserved at −80 °C. For routine use, the isolates were maintained in agar-slant test tubes at 4 °C and subcultured on fresh slants every 4 weeks. MDK33 was grown on nutrient agar plates and incubated at 30 °C for 48 h. Subsequently, the colony features, including color, margin, elevation, diameter and shape of the developed colonies, were determined. The reaction to Gram staining was also checked for MDK33 cells according to the method described in [23].

2.2. Determination of Biochemical Traits Using BIOLOG System

The BIOLOG Gen III MicroPlate test system (BIOLOG Inc., Hayward, CA, USA) was used to determine the biochemical features of MDK33 according to the recommendations of the manufacturer. The bacterial strain was grown on nutrient agar (YNA) (Gibco, Eggenstein, Germany) for 24 h at 30 °C. Actively growing MDK33 from an overnight culture was suspended in inoculating fluid to generate a bacterial cell suspension, the

transmittance of which was adjusted to between 90% and 98%. Then, 100 µL of the suspension (1.5 × 10^6 cells ml) was added to BIOLOG GN microplates. After 24 h of incubation at 30 °C, the results were visually recorded. The reproducibility of the technique was determined by testing in duplicate.

2.3. Amplification of 16S rRNA Gene Using PCR

MDK33 was identified using 16S rRNA sequencing. The extraction of genomic DNA, primers, PCR conditions, and sequencing were all carried out as described in [24]. Briefly, amplification of the 16S rRNA locus of the MDK33 strain was carried out using PCR in a total 20 µL reaction using the following primers: 27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′ [25]. Appropriate positive and negative controls were included as *Escherichia coli* genomic DNA and water, respectively. The obtained amplicons were then purified using a clean-up kit (Millipore, Fisher Scientific, Loughborough, UK) following the manufacturer's recommendations.

2.4. 16S rRNA Sequencing and Phylogenetic Analyses

The 16S rRNA locus was sequenced using a Big Dye terminator cycle sequencing kit (Applied BioSystems, Foster City, CA, USA). Products of sequencing were resolved on an Applied Biosystems 3730XL automated DNA sequencing system. The evolutionary history was inferred using the neighbor-joining method [26]. The optimal tree with a sum of branch length = 0.20093730 is displayed. The evolutionary distances were computed using the maximum composite likelihood method [27] and are presented in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Included codon positions were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were 1303 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [28]. The 16S rRNA gene sequence of MDK33 was deposited in the NCBI GenBank under accession number ON797448.

2.5. Pathogenicity Test

Healthy and unwounded mature apple fruits (*M. domestica*, cv. Golden Delicious) were purchased from a commercial market in Al Ahsa city, Saudi Arabia. Externally similar-sized fruits were selected, washed with sterilized distilled water, surface-sterilized for 3 min with sodium hypochlorite (1%) and washed five times with sterilized, distilled water to remove any traces of sodium hypochlorite. After drying in a laminar flow hood, apple fruits were placed on a sterilized plastic saucer in a sterilized plastic box with wet tissue. To inoculate the disinfected apple fruits, a bacterial suspension of MDK33 was used from an 18-h-old nutrient agar culture. Before infection, fruits (three fruits for each treatment) were wounded (depth: 10 mm) at four locations around their equator with a sterilized cork borer. The wounds were artificially infected with 100 µL of bacterial solution (10^6 CFU/mL), whereas control fruit was injected with 100 µL sterile distilled water. Fruits were incubated in a dark room with a constant high relative humidity of 95% and a temperature of 22 °C. The progression of symptoms was monitored by measuring two perpendicular diameters 2, 4, 6 and 8 days after infection. The experiment was repeated twice, separated by two weeks (repetitions). For both inoculation methods, three apples were tested, and the experiment was repeated twice.

2.6. In Vitro OEO Efficacy against S. liquefaciens Growth

OEO (100% pure) was purchased from NATURAL, a commercial herb store in Al-Ahsa, Saudi Arabia. OEO was prepared as a stock solution by mixing 1mg of OEO with 16 mL of dimethyl sulfoxide (DMSO). Various dilutions (0.09–2.82 mg/mL) were prepared from the stock solution to be used for antibacterial activity using the agar well diffusion method (Vasconcelos et al., 2019). DMSO alone was used as a negative control. Exactly 100 µL (1.5 × 10^6 CFU/mL) of the actively growing MDK33 strain was equally distributed onto Mueller-Hinton agar (Oxoid, Basingstoke, UK). Then, wells were made in the surface of

the agar plates using a sterilized cork borer (6 mm in diameter). Various concentrations of OEO were added to the wells. Inoculated plates were then incubated at 30 °C for 24 h. After the incubation period, the plates were visually checked for the appearance of zones of inhibition around the wells. The diameter of the inhibition zones around the wells were estimated, excluding well diameter. The experiments were conducted in triplicate, and the means of the values were reported. Wells with 10 µL of 0.1% DMSO (Centralchem, Bratislava, Slovakia) were used as a negative control.

2.7. Minimum Inhibitory Concentration (MIC)

The MIC of *S. liquefaciens* (MDK33) was determined using the agar microdilution method [29]. The inoculum was cultured for 24 h in Mueller-Hinton Broth (MHB, Oxoid, Basingstoke, UK) at 30 °C. The MDK33 suspension was adjusted with sterile saline solution until a concentration of 1.5×10^6 CFU/mL was reached. Then, 50 µL of MHB and 50 µL of inoculum were applied to a 96-well microtiter plate. Stock solution of OEO was prepared at 47 mg/mL in DMSO, and twofold dilutions (0.09–2.82 mg/mL) of the stock OEO in MHB were mixed thoroughly with MDK33 inoculum in the wells. The volume in each well was adjusted to 150 µL by adding MHB. The microplates were incubated in a rotary agitator (160 rpm) for 24 h at 30 °C. MHB with OEO was used as a negative control, and MHB with inoculum was used as a positive control for maximal growth. The inhibitory concentration was determined using p-iodonitrotetrazolium (MP Biomedicals, Salon) salt. After incubation, bacterial growth was tested by adding 10 µL of (0.1 mg/mL) p-iodonitrotetrazolium violet to each microtiter well and reincubated at 30 °C for 1 h. Change in color to orange–red indicated that the cells were still viable. The oxidation of metabolically active cells resembled the inhibitory concentration. The prepared 96-well microtiter plates were measured at 630 nm with a Biorad ELISA reader. The analysis was performed in triplicate. The concentration of the first well that showed no turbidity was taken as the MIC value [30].

2.8. Activity against Biofilms

MDK33 was incubated in trypticase soy broth (TSB) supplemented with glucose (0.1%) at 30 °C for 18 h. Cells were collected by centrifugation at 10,000 rpm for 10 min and washed twice with PBS. Bacterial suspensions with an optical density (OD600) of 0.1 in TSB were added to 96-well plates at 100 µL/well, followed by a 24 h incubation at 37 °C under static conditions. After incubating the biofilms with OEO at the indicated concentrations for 1 h, each well was filled with 0.1% crystal violet to allow for staining of the remaining microbial biofilms for 10 min. The crystal violet was removed from the biofilm by adding 30% (*v/v*) acetic acid to each well to solubilize the crystal violet before measuring the optical density of each sample at a wavelength of 540 nm using a microplate reader (Rayto, Germany) [31,32]. Each experiment was carried out in triplicate.

2.9. In Vivo Rot Suppression Using OEO

Healthy, unwounded and similar-sized fruits were sterilized, then wounded, as previously mentioned (pathogenicity test). Exactly 3 mL of bacterial suspension of MDK33 from an 18-h-old nutrient agar culture (10^6 CFU/mL) was mixed with the MIC value of OEO (0.23 mg/mL) and incubated at 30 °C for 30 min. The wounds were artificially infected with 100 µL of bacterial solution (10^6 CFU/mL), whereas control fruits were injected with 100 µL sterile distilled water. Incubation of the inoculated fruits was carried out as mentioned previously. The development of symptoms was recorded by measuring two perpendicular diameters 2, 4, 6 and 8 days after infection. The pathogen was then isolated and reidentified from the inoculated fruit. The resulting colony matched the colony obtained from the initially diseased fruit. The experiment was repeated twice, and three apples were tested.

2.10. Statistical Analysis

Data are represented by the mean of three replicates ± standard deviation (SD). Statistical significance was assessed with one-way analysis of variance (ANOVA), and mean comparisons were performed by LSD post hoc test using Microsoft Excel. Differences between means were considered statistically significant at $p < 0.05$.

3. Results

Three rotten apple fruits (*M. domestica*, cv. Golden Delicious) were purchased from a commercial market in Al Ahsa city, Saudi Arabia and used in the current study. The symptoms of rotting were brown lesions that ranged from 10 to 30 mm in diameter. Of 115 morphologically similar isolates, one most dominant bacterial isolate, designated MDK33, was obtained from deteriorated apple fruit and was further characterized based on phenotypic and genotypic traits (Figure S1). Some of the characteristics of MDK33 are presented in Table 1.

Table 1. Some characteristics of MDK33.

Feature	MDK33
Colony shape	Circular
Color	White
Colony elevation	Raised
Colony margin	Entire
Gram reaction	Gram-negative rods
MIC (OEO against MDK33)	0.23 mg/mL
Closest strain	*Serratia liquefaciens*
Identity (%) of the 16S rRNA locus	99.73
Accession number (Partial 16S rRNA locus)	ON797448

MDK33 formed circular smooth colonies with entire margins (Figure 1).

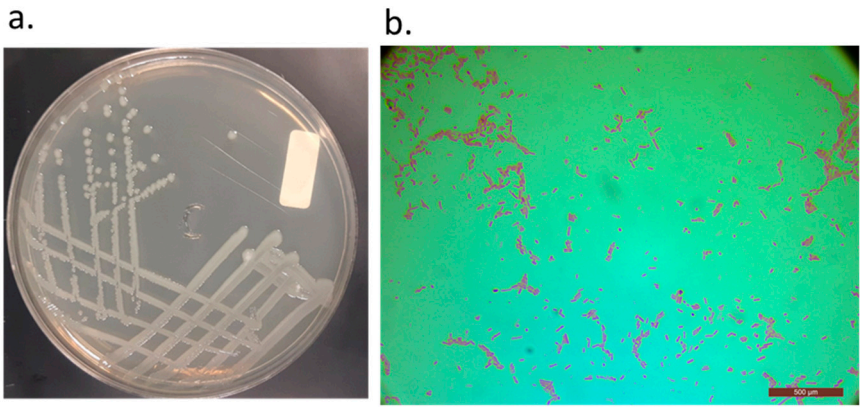

Figure 1. Pure colonies of the MDK33 strain from deteriorated apple fruit (**a**) and Gram staining of MDK33 cells (**b**). Note red rods indicating Gram-negative cells. Magnification was 100×; bar = 500 µm.

The cells were Gram-negative rods without endospores (Figure 1b and Table 1). These findings are identical to those reported for *S. liquefaciens* [14,15].

Table 2 shows the results of the biochemical characterization of *S. liquefaciens* revealed by the Biolog Gen III MicroPlate test system. MDK33 utilized 66 (~70%) of the 94 diverse metabolites (Table 2) and did not utilize 29 (~30%) traits. It grew well on several substrates, including gelatin, Tween 40 and dextrin, at pH 5 and 6 and tolerated salts up to 4% NaCl (Table 2). MDK33 displayed no growth on L-rhamnose, methyl pyruvate and at 8% NaCl. In 13 tests, borderline growth was obtained, such as that on pectin. Furthermore, MDK33 exhibited resistance to six antibiotics, namely troleandomycin, lincomycin, vancomycin,

aztreonam, fusidic acid and rifamycin SV, suggesting potential multiple-drug resistance. These observations provided additional evidence that the described strain was *S. liquefaciens*, as the traits of MDK33 were in general agreement with those described previously [14,15]. Furthermore, the Biolog GEN III system was applied as a powerful tool for phenotypic-based description and identification of bacterial strains.

Table 2. Biochemical characterization of strain MDK33 based on Biolog Gen III MicroPlate.

Positive Results with the Following Substrates			**Borderline Results**	**Negative Results with the Following Substrates**	
Gelatin	D-Glucuronic Acid	1% Sodium Lactate	D-Raffinose	Methyl Pyruvate	Propionic Acid
Tween 40	Citric Acid	Troleandomycin	α-D-Glucose	γ-Amino-Butyric Acid	L-Rhamnose
Dextrin	N-Acetyl-D-Glucosamine	Lincomycin	D-Sorbitol	D-Lactic Acid Methyl Ester	L-Pyroglutamic Acid
D-Mannitol	D-Glucose	Vancomycin	Pectin	α-Hydroxy-Butyric Acid	Quinic Acid
Glycyl-L-Proline	Glucuronamide	Aztreonam	p-Hydroxy-Phenylacetic Acid	D-Trehalose	Stachyose
D-Galacturonic Acid	α-Keto-Glutaric Acid	pH 6	α-D-Lactose	β-Methyl-D-Glucoside	N-Acetyl Neuraminic Acid
D-Maltose	Acetoacetic Acid	1% and 4% NaCl	D-Mannose	D-Galactose	Inosine
D-Melibiose	Sucrose	Fusidic Acid	L-Glutamic Acid	L-Arginine	D-Saccharic Acid
D-Fructose	N-Acetyl-b-D-Mannosamine	Rifamycin SV	D-Turanose	β-Hydroxy-D,L-Butyric Acid	Bromo-Succinic Acid
D-Arabitol	L-Fucose	Guanidine HCl	N-Acetyl-D-Galactosamine	D-Cellobiose	8% NaCl
L-Alanine	D-Fructose-6-PO4	Tetrazolium Violet	D-Aspartic Acid	D-Salicin	Potassium Tellurite
L-Galactonic Acid Lactone	D-Malic Acid	Lithium Chloride	Minocycline	α-Keto-Butyric Acid	Sodium Bromate
myo-Inositol	L-Malic Acid	Sodium Butyrate	D-Raffinose	Gentiobiose	
D-Gluconic Acid	Acetic Acid	pH 5		D-Fucose	
L-Lactic Acid	D-Serine	D-Serine		Mucic Acid	
3-Methyl Glucose	L-Serine	Niaproof 4		L-Aspartic Acid	
Glycerol	Formic Acid	Tetrazolium Blue		L-Histidine	

Comparative analyses based on the sequences of the 16S rRNA locus revealed that MDK33 (accession number: ON797448, (Table 1)) is identified as *S. liquefaciens* ATCC 27592 CP006252 at 99.73% identity (Table 1). Furthermore, based on the phylogenetic tree inferred after applying the neighbor-joining method to the 16S rRNA gene sequences of MDK33 and closely related sequences of reference strains, MDK33 clearly clustered within the *Serratia* clade, particularly with *S. liquefaciens* (Figure 2). *Streptomyces variabilis* (MN135855), which belongs to *Streptomycetaceae*, formed a distinct outgroup. MDK33 was assembled with members of the *Enterobacteriaceae* family, confirming its taxonomic status.

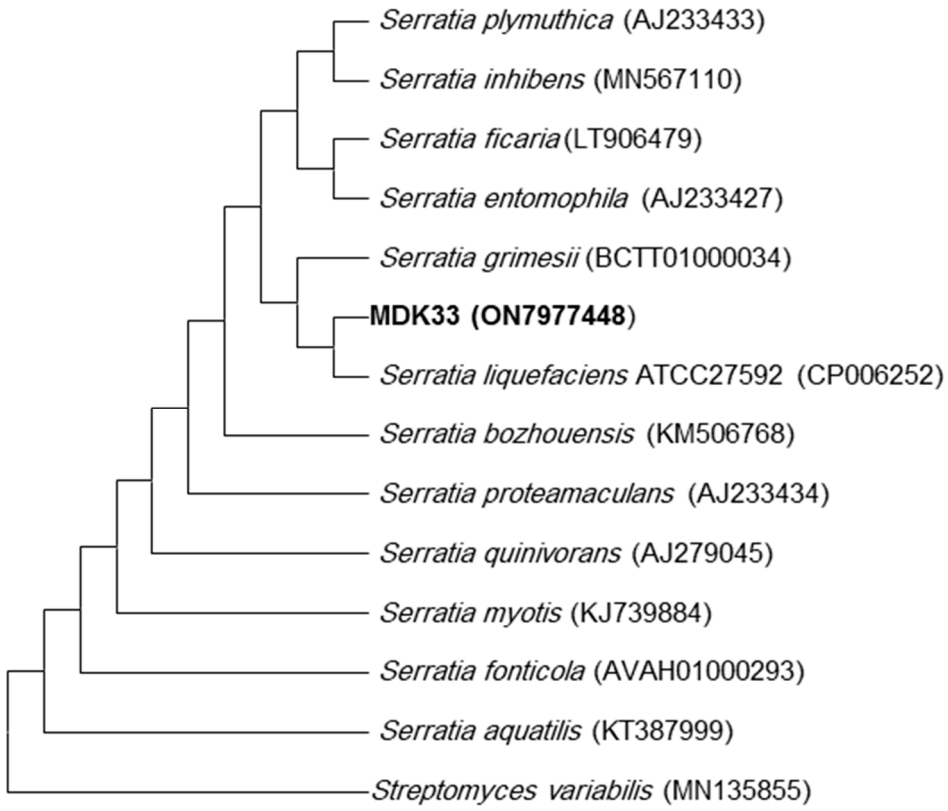

Figure 2. Evolutionary relationships of MDK33 (accession number: ON797448) and other related bacterial species as inferred using the neighbor-joining method. Accession numbers of the 16S rRNA gene sequences are given between parentheses adjacent to each bacterial species.

The results of pathogenicity tests are presented in Figure 3. After two days of inoculation with the bacterial strain, slight brown rot appeared (1mm diameter) around the wounds and extended outward after four days of incubation. An area of decay appeared in the infected tissues. These symptoms worsened as the incubation period extended, compared with the control uninoculated fruit. The control fruit developed discoloration around the hole, but no disease symptoms appeared (Figure 3 control). After, 8 days of inoculation, the pathogen was isolated again from the inoculated fruit and reidentified. The resulting colony matched the colony obtained from the initially diseased fruit, fulfilling Koch's postulates. The pathogen was identified as *S. liquefaciens* MDK3 based on the observed pathological symptoms, morphology and pathogenicity.

Figure 3. Inoculation of apple fruit with *S. liquefaciens*. Apple fruits were wounded (depth: 10 mm) on four locations around the equator of each fruit with a sterilized corker borer. The wounds were artificially infected with 100 µL of bacterial solution (10^6 CFU/mL), whereas control fruits were injected with 100 µL sterile distilled water. Fruit were incubated in a dark room with a constant high relative humidity of 95% and a temperature of 22 °C. The progression of symptoms was monitored by calculating the average of two perpendicular diameters measured in mm; the rotten areas were 0, 2 ± 0.3, 4 ± 0.5, 7 ± 0.4 and 9 ± 0.2 mm 2, 4, 6 and 8 days after infection, respectively.

The effects of different OEO concentrations on the growth of *S. liquefaciens* MDK3 are presented in Figure 4. As the OEO concentration increased, the survival percentage of the bacterial strain decreased. Good growth was observed under 0.06 and 0.12 mg/mL of OEO, whereas complete inhibition was reported at 1.88 mg/mL of OEO, compared with the untreated control. OEO displayed remarkable antibacterial activity against *S. liquefaciens* MDK33, as the lowest concentration that inhibited the growth (MIC) was 0.23 mg/mL.

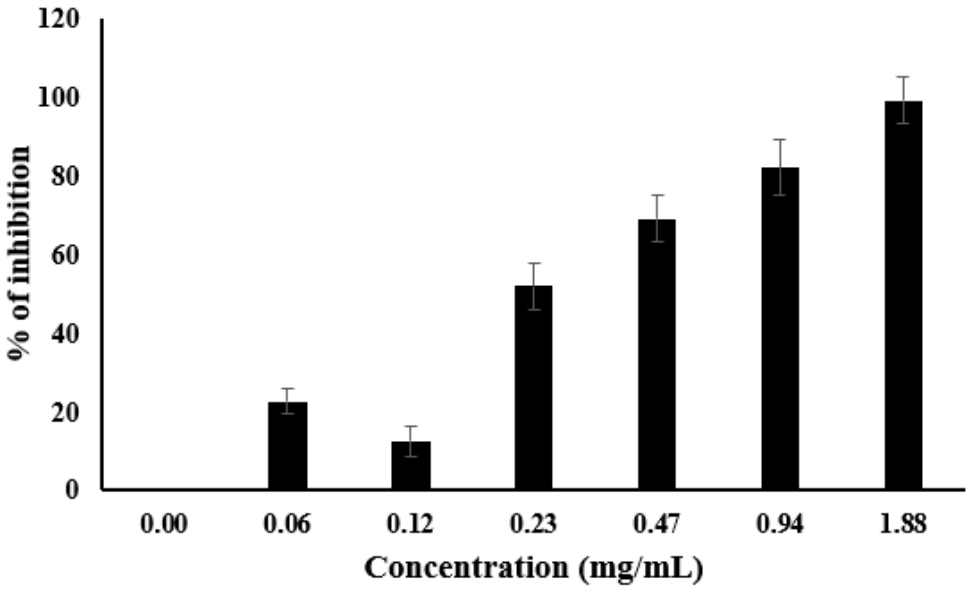

Figure 4. Effects of different OEO concentrations (mg/mL) on *S. liquefaciens* MDK3 growth on TSB medium.

The crystal violet test is utilized to measure biofilm formation [32]. The effects of different OEO concentrations, including MIC, MIC, 2 × MIC, 4 × MIC and control (no added OEO) were assessed, revealing that the treated cells exhibited a significant reduction in biofilm formation compared to the control, as shown in Figure 5. As the concentration of OEO increased, the percentage of biofilm formation decreased. After treatment with OEO at MIC, 2 × MIC and 4 × MIC, biofilm formation was substantially decreased by 50%, 66% and 88%, respectively, compared with the control. OEO demonstrated similar antibacterial activity against established biofilms (24-h-old) formed by *S. liquefaciens* MDK33, with a significant inactivation of biofilms at concentrations of 0.23 mg/mL, in general agreement with the MIC value (Figure 5).

The results of the in vivo inhibition test with *S. liquefaciens* MDK33 using OEO are shown in Figure 6. Inoculation was carried out with the bacterial strain treated with OEO at the MIC. There were no significant differences in the symptoms displayed on the inoculated apple fruits compared to the control fruits on days 2, 4 and 6. On day 8, minor brown rot appeared on the treated fruit. The control fruit became discolored around the hole, but no disease symptoms arose.

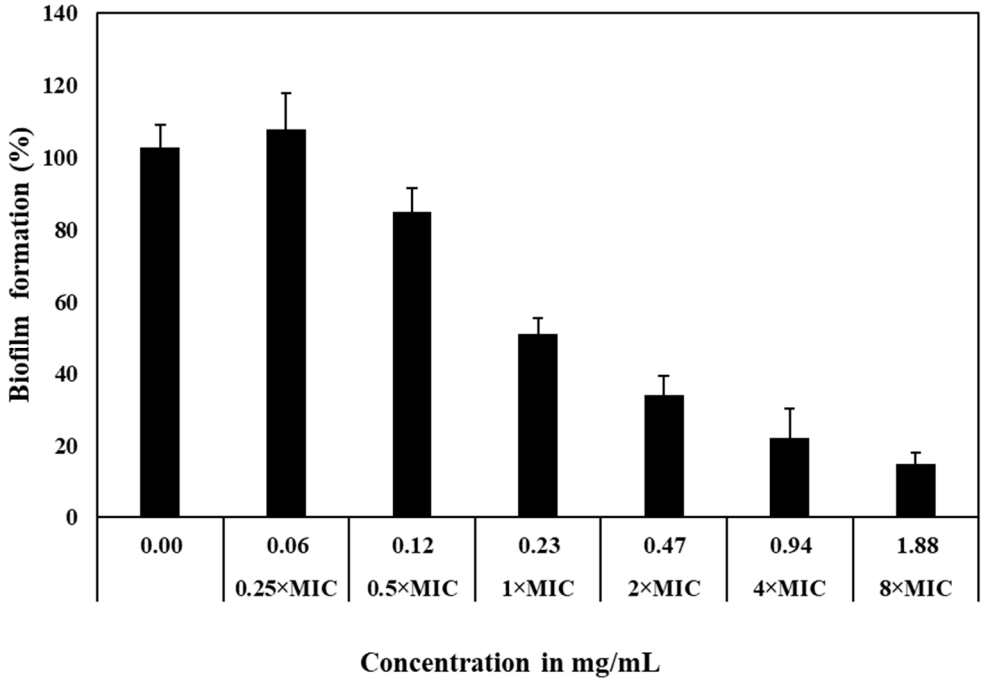

Figure 5. Effects of different OEO concentrations (mg/mL) on biofilm formation (%) of *S. liquefaciens* MDK33. Various concentrations of OEO were used: 0.25 × MIC (0.06 mg/mL), 0.5 × MIC (0.12 mg/mL), 1 × MIC (0.23 mg/mL), 2 × MIC (0.47 mg/mL), 4 × MIC (0.94 mg/mL) and 8 × MIC (1.88 mg/mL). Biofilm formation of 100% in the control corresponds to an OD_{530} of 0.48 ± 0.05 where no OEO was added (0 mg/mL) and the bacterial strain was able to form biofilm without inhibition.

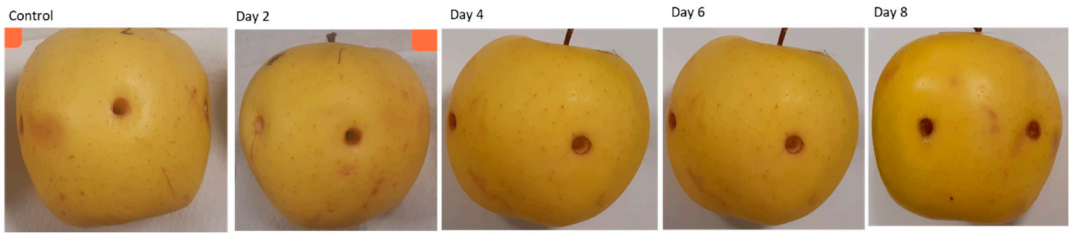

Figure 6. In vivo inhibition of *S. liquefaciens* on apple fruits using OEO. Exactly 3 mL of bacterial suspension of *S. liquefaciens* MDK33 from an 18-h-old nutrient agar culture (10^6 CFU/mL) was mixed with the MIC value of OEO (MIC 0.23 mg/mL) and incubated at 30 °C for 30 min. The wounds were artificially infected with 100 µL of bacterial solution (10^6 CFU/mL). Treatment with sterile distilled water represented a negative control. Infected apples were monitored by measuring two perpendicular diameters of the rotten areas (average in mm: 0, 1 ± 0.1, 1 ± 0.2, 1 ± 0.3 and 1 ± 0.3) 2, 4, 6 and 8 days after infection, respectively.

4. Discussion

Nearly one-tenth (300) of 3000 known essential oils are classified by the United States Food and Drug Administration as generally recognized as safe (GRAS) for human use [33]. Over the past century, various EOs have been used in a wide array of industrial, medicinal and pharmaceutical applications, such as food additives, preservatives and flavors [34]. EOs are considered promising efficient, cost-effective and biocompatible natural antimicrobial agents [35]. In the current study, we explored the antibacterial effectiveness of OEO against *S. liquefaciens* MDK33 isolated from spoiled apple fruit. Our results highlight that OEO effectively inhibited *S. liquefaciens* MDK33 and their biofilms, as evidenced by the MIC value 0.23 mg/mL.

The Biolog GEN III microtest is a rapid, low-cost and powerful tool for physiological and biochemical screening of bacterial species. This system comprises 23 tests for chemical

sensitivity and 71 tests for the consumption of carbon and nitrogen sources, in addition to control negative and positive microtubes. The biochemical pattern of *S. liquefaciens* MDK33 was in general accordance with that documented for *Serratia* spp. [14,15]. The variability in utilization of the substrates contained in the Biolog GEN III microtest by MDK33 highlights the versatility in enzymatic machineries and metabolic networks. Interestingly, *S. liquefaciens* MDK33 exhibited the ability to consume pectin, a complex carbohydrate in plant cell walls. This could be attributed to the activity of polygalacturonase (pectin depolymerase), which is a hydrolytic enzyme that breaks the alpha-1, 4 glycosidic bonds between galacturonic acid residues in pectin. Hence, polygalacturonase has a pivotal role in weakening the apple fruit cell wall, thereby facilitating infection with phytopathogens [36]. Similar results were reported for *Serratia marcescens*, a pectinase-producing bacterium from avocado peel waste [36].

Microbial enzymes such as amylases and pectinases have been recognized as fruit softeners [37]. MDK33 displayed a remarkable resistance to various antibiotics, such as troleandomycin and rifamycin SV (macrolide category), lincomycin (lincosamide category), vancomycin (glycopeptide category), aztreonam (beta-lactam category) and fusidic acid (fusidane class), highlighting the probability of multiple antibiotic resistance. Resistance to antibiotics is mediated by various mechanisms [38]. It was recently reported that various genes conferring resistance to macrolides, tetracycline Beta-lactams, fluoroquinolones and cephalosporins are present in the genome of *Serratia* sp. [38], supporting the multidrug resistance feature.

Comparative 16S rRNA gene sequence analysis has been applied as the gold standard for not only assigning bacterial genera and species but also inferring evolutionary history among bacteria and archaea [39]. Analyses of the 16S rRNA gene of MDK33 revealed its close relationship with the genus *Serratia*. This strain displayed 99.73% identity with *S. liquefaciens* ATCC 27592 (CP006252) (Figure 2). MDK33 grouped with members of the *Enterobacteriaceae* family. Similar findings were reported by Castro-Saines et al. [40], who characterized *Serratia* sp., reporting ixodicide activity against the cattle tick *Rhipicephalus microplus*, with 99.86% identity to recognized species. Although analyses of 16S rRNA sequences can be used to define species, this is not always the case because 16S rRNA genes may contain highly conserved regions that make discrimination between some species and subspecies within this group difficult [39], indicating the importance of including other housekeeping genes with powerful taxonomic indication. Previous reports have indicated that *Serratia* spp. are involved in plant diseases, such cotton (*Gossypium hirsutum*) boll infection [41], bell pepper (*Capsicum annuum*) soft rot ([42] and tomato (*Solanum lycopersicum*) fruit dark brown spots [43]. Koch's postulates were satisfied, confirming *S. liquefaciens* MDK33 as the causative agent of brown rot of apple fruit.

OEO displayed remarkable antibacterial activity against *S. liquefaciens* MDK33, as revealed by the MIC of 0.23 mg/mL. This MIC value is higher than that obtained with *Acinetobacter baumannii* (MIC of 0.16 mg/mL) but lower than that reported for methicillin-resistant *Staphylococcus aureus* (MRSA) (MIC of 0.32 mg/mL), *P. aeruginosa* (MIC of 0.64 mg/mL) [35] and *Listeria monocytogenes* (MIC of 2.56 mg/mL) [44]. Comparable results were obtained with *S. marcescens* [45]. The bacterial strain examined, cultural growth conditions and measurement parameters could all contribute to the variation in MIC values of OEO. It has been reported that Gram-positive bacteria are more susceptible to EOs than Gram-negative strains [35]. This could be attributed to the difference in the chemical composition of their cell walls. The presence of an outer membrane in Gram-negative bacteria, such as the investigated strain, *S. liquefaciens* MDK33, providing an additional barrier restricting the penetration of OEO due to its lipophilic nature. Additionally, periplasmic-associated enzymes could have the ability to degrade OEO once it enters cells. The antibacterial action of OEO is mediated primarily via damage of both cell wall and cell membrane, as revealed by TEM examination [35]. The authors of previous studies reported the chemical ingredients of oregano oil, revealing carvacrol, thymol and sesquiterpenes as the predominant components [46,47]. Such compounds exhibit potent antibacterial activities by interfering with

the plasma membrane, resulting in loss of membrane integrity and selective permeability; disturbing efflux pump, ATPase activity and RNA synthesis; and unbalancing osmotic pressure inside bacterial cells.

Bacterial biofilms are cell aggregates that adhere to an interface or a surface and become embedded in a self-produced matrix of extracellular substances, such as polysaccharides and proteins [48]. Biofilm is critical for bacterial survival, especially in harsh environments, such as those containing antimicrobial agents [48]. In our study, OEO demonstrated antibiofilm-forming activity comparable to that of antibacterial agents. Several lines of evidence suggest that antibiotic resistance in bacteria may be due to their ability to form biofilms rather than their existence as free bacteria. Biofilm has a strong potential to inactivate antimicrobial agents, preventing their access to bacterial cells [48]. Preventing biofilm formation by OEO would thus restrict the emergence of antibacterial resistance and pave the way for applications of OEO in food safety control. Furthermore, to avoid the spread of foodborne diseases to apple consumers, sanitary and hygienic protocols should be followed during pre- and post-harvest procedures.

To confirm its antimicrobial activity, OEO was tested in an in vivo study on apple fruit to inhibit *S. liquefaciens* MDK33. As expected, there were no significant differences in the symptoms displayed on the inoculated apple fruit (treated with OEO at the MIC), indicating its effectiveness to substantially decrease disease caused by strain MDK33. This might be explained by OEO inhibiting the bacterial strain by interfering with the plasma membrane, causing death. However, the detailed mechanism of pathogenicity of and the infection process of *S. liquefaciens* in apple fruit and its implications for human health could serve as an interesting basis for future investigations.

5. Conclusions

Collectively, this is the first isolation pathogenic *S. liquefaciens* as the causative agent of rot disease in apple fruit and its inhibition by oregano oil. Furthermore, the findings of the present study pave the way for oregano oil to be used as a natural preservative to reduce post-harvest losses while maintaining high quality for food security in future studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8080752/s1, Figure S1: Isolation source and initial isolation, deteriorated apple fruit (**a**); microorganisms obtained from rotten apple (**b**).

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