

# Research Article

# Screening of Essential Oils for the Inhibition of *Enterobacter ludwigii* Isolated from Tomato Fruits

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Tomato is perishable and requires preservation to extend its shelf life. In this study, we conducted selection processes to identify essential oils that can help to avoid spoilage and deterioration during tomato storage and extend the shelf life. Thereafter, we determined the phosphatase activity assay, potassium ion concentration, and electron microscopy to study the antibacterial mechanism of essential oil. We found that Enterobacter ludwigii W01 was the dominant spoilage bacterium in tomatoes with cracked and curled skin. We selected oregano essential oil from 12 essential oils (oregano essential oil, lemon essential oil, osmanthus essential oil, cypress wood essential oil, tea tree essential oil, licorice essential oil, Baili essential oil, white camphor essential oil, Shancang seed essential oil, rosemary essential oil, rose essential oil, and cinnamon essential oil) which could significantly inhibit the activity of E. ludwigii W01. However, the diameter of the inhibition zone for Wh, Te, Cy, Li, Rm, Le, and Os is 0 mm, the diameter of the inhibition zone for Ba, Sh, and Ro was less than 1.0 mm, whereas the diameter of the inhibition zone for Ci and Or was greater than 2.0 mm. The diameter of the suppression circle for Ci and Or was greater than 2.0 mm, while Ci was lesser than Or. Oregano essential oil can damage the cell wall of E. ludwigii W01, leading to the leakage of the alkaline phosphatase stored between the cell wall and the cell membrane which can increase the alkaline phosphatase activity in the bacterial solution. Meanwhile, the addition of oregano essential oil significantly altered the cellular morphology of E. ludwigii W01. Spraying the surface of fresh tomato fruits with 1 MIC (0.125%) of oregano essential oil prolonged the storage time to 15 days, without significant changes in its sensory attributes. Those results indicated that oregano essential oil was a potential preservative for tomatoes.

# 1. Introduction

Tomato (*Lycopersicon esculentum* L.) is one of the most cultivated fruit vegetables in the world and is available all year round. Tomato fruits are rich in vitamins, minerals, malic acid, citric acid, and sugars, and they are highly nutritious, with established anticancer and detoxifying effects [1]. With a thin skin and tender flesh, fresh tomatoes usually have a shelf life of 3–4 days at room temperature and can be stored for 7–10 days at 4°C in a foam box.

Both physiological softening and microbial infestation can shorten the shelf life of tomatoes, with fruit decay caused by spoilage bacteria being common [2–5]. The postharvest spoilage bacteria of tomatoes comprise *Botrytis* spp., Alternaria Nees, Rhizopus spp., Penicillium spp., Aspergillus spp., Fusarium spp., Bacillus anthracis, Pseudomonas spp., Xanthomonas spp., Bacillus spp., and Erwinia spp. [2, 6–8].

To extend the shelf life of tomatoes, biological breeding techniques, physical preservation techniques, and preservative addition are commonly being used [9]. In addition, hard fruit varieties have been selected by crossbreeding, and genetic engineering has been used to introduce antiripening genes or modify the genes encoding pectin lyase to produce new varieties of tomatoes with a prolonged shelf life. However, the resulting new varieties often have a poor taste and are less accepted by consumers. Physical methods for preservation include low-temperature air-conditioning storage, ozone preservation, high-voltage electrostatic preservation, irradiation preservation, and other methods, which are effective but technologically intensive and costly [10]. Preservatives are categorized into chemical and natural extract preservatives [11]. Although chemical preservatives are widely used for the preservation of tomatoes, their corresponding residues can pose a threat to human health and are disliked by consumers [12, 13]. Consequently, research on preservation methods has gradually shifted to the development of natural biological preservatives, including essential oils, chitosan, *Bacillus* spp., and *Lactobacillus* spp. [14].

Essential oils are volatile liquids that are secondary plant metabolites extracted from various parts (e.g., bark, seeds, flowers, peel, fruits, roots, leaves, wood, or whole plants) of aromatic plants, and their names depend on the names of these plants [15]. Volatile oily liquids can be obtained by methods such as pressing and distillation [16]. These extracts comprise several compounds with relatively small molecular weights, such as monoterpenes, sesquiterpenes, and aromatic derivatives, and usually have an aromatic odor. Essential oils are widely used in the food industry due to their natural antibacterial, antioxidant, and insect-repellent properties, which extend the shelf life of food. Fruits and vegetables are the most common types of foods where essential oils are used [17]. Researchers have explored the use of plant essential oils to make edible coatings to extend the shelf life of food. Significant progress has been made in the study of edible coatings in Satureja khuzistanica Jamzad essential oil [18] and Zataria multiflora Boiss essential oil [19].

This study is aimed at identifying essential oils with an inhibitory effect through screening. We evaluated the inhibitory ability and underlying mechanisms of oregano essential oil against *Enterobacter ludwigii*, which causes spoilage in tomatoes, and we assessed its effects on the shelf life and sensory attributes of tomatoes.

#### 2. Materials and Methods

2.1. Determination of Tomato Spoilage Bacteria. The spoilage bacterium of *E. ludwigii* W01 was confirmed to cause cracked and curled skin and moist fruit in tomatoes, identified by Koch's four postulates [20].

*E. ludwigii* W01 was inoculated in the LB liquid mediums and incubated at 37°C for 24 h with shaking. Sampling was conducted at a time interval of 2 h. The optical density (OD) of each sample was determined by the microplate reader (HBS-1096, BioTek Instruments, Inc., USA) at a wavelength of 600 nm. The OD<sub>600</sub> was then plotted against inoculation time to obtain the OD<sub>600</sub>-time relationship curve, which is the growth curve of *E. ludwigii* W01. In the late logarithmic phase, the total number of bacteria is approximately  $1 \times 10^8$  CFU/mL.

2.2. Molecular Biology Identification of Tomato Spoilage Bacteria. The genomic DNA of dominant spoilage bacteria was extracted, and PCR amplification was conducted to obtain conserved fragments. The gel-recovered PCR products were sent to GenScript Biotech Corporation for molecular biological characterization. The resulting sequencing results were compared using the database of the National Center for Biotechnology Information (NCBI). MEGA11 software was used to construct a phylogenetic tree from the sequence alignment results, using the neighbor-joining (NJ) method for preliminary identification.

2.3. Inhibition Test. A total of 12 essential oils—white camphor essential oil (Wh), Baili essential oil (Ba), Shancang seed essential oil (Sh), tea tree essential oil (Te), cypress wood essential oil (Cy), licorice essential oil (Li), rose essential oil (Ro), rosemary essential oil (Rm), oregano essential oil (Or), lemon essential oil (Le), cinnamon essential oil (Ci), and osmanthus essential oil (Os)—were provided by Jiangxi Hengcheng Natural Essential Oil Co., Ltd. The oils were diluted to 10% solutions using Tween-80 (Tw) and set aside.

The Oxford cup method that examines the inhibition zones was used for the inhibition tests on the spoilage bacteria, with Tw as the control. The spoilage bacterium strain W01 was evenly spread onto LB agar plates (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, agar 20 g/L, and pH 7.4). Holes with a diameter of 0.5 cm were made with a puncher on the medium, and 100  $\mu$ L of the 10% essential oil solutions were added [20]. The diameter of the inhibition zone was measured after 24 h incubation.

Determination of the Minimum Inhibitory 2.4. Concentration. A total of 1 mL of oregano essential oil was emulsified with 20% Tw and diluted with broth to make a 10% oregano essential oil master mix. Next, seven small test tubes were labeled; tube 1 was filled with the stock solution diluted to 0.5% with broth; tubes 2-6 were subsequently diluted to 0.25%, 0.125%, 0.0625%, 0.03125%, and 0.015625%, respectively, through the twofold serial dilution method; tube 7 was used as a blank control, containing only an equal amount of broth. Tubes 1-6 were inoculated with activated E. ludwigii bacterial solution and incubated at 30°C for 12 h. After the incubation was completed, the minimum concentration at which the bacterial solution remained clear was taken as the Minimum Inhibitory Concentration (MIC) of oregano essential oil against the spoilage bacterial strain W01, and the assay was repeated three times.

2.5. Determination of Cell Membrane Permeability. E. ludwigii W01 was inoculated in four flasks containing 100 mL of nutrient broth and incubated at 37°C and 150 r/min (until stationary phase). Then, oregano essential oil was added at final concentrations of 0 MIC, 1/2 MIC, 1 MIC, and 2 MIC, respectively. The incubation was continued, and samples were taken at 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h of incubation to determine the alkaline phosphatase activity and potassium ion concentration in each group using a kit.

After 12 hours of cultivation, collect the bacterial cells by centrifugation at 6 000 r/min and 4°C for 10 minutes. After gently rinsing with PBS, discard PBS and fix at room temperature for 2 hours with 2.5% glutaraldehyde solution. The sample is then rinsed three times with 0.1 M phosphate buffer (pH 7.4) for 15 minutes each time. Subsequently, the mixture was washed with 30%, 50%, 70%, 80%, 90%, 95%,

100%, and 100% alcohol for 15 minutes each time, followed by 15 minutes of isoamyl acetate. After drying the sample in the critical point dryer (K850, Quorum Technologies Co. Ltd, Laughton, UK), place it tightly onto the double-sided adhesive of the conductive carbon film and spray gold on the sample stage of the lon sputtering apparatus (MC1000, Hitachi High-Tech Co., Tokyo, Japan) for about 30 seconds and observe and collect images under a scanning electron microscope (SU8100, Hitachi High-Tech Co., Tokyo, Japan).

2.6. Effect of Oregano Essential Oil on E. ludwigii W01 Inoculated onto Tomato Wounds. Make a cut in the skin of fresh tomatoes, with the depth of the cut reaching the outer skin through the cube, but not the vascular bundles in the skin (Figure 1). 2 mL of E. ludwigii W01 bacterial solution (approximately 10<sup>8</sup> CFU/mL) was inoculated on the incisions and was subsequently sprayed with 0 MIC, 1/2 MIC, 1 MIC, and 2 MIC of oregano essential oil (200 µL per 100 g tomato). Each treatment group included 20 fresh tomatoes of similar ripeness (degree of redness and hardness) and weight  $(175 \pm 10 \text{ g})$  [20], and each group was replicated three times. The tomatoes of each group were placed in a foam box  $(42 \text{ cm} \times 20 \text{ cm} \times 20 \text{ cm})$  with an observation window and stored at 4°C in a refrigerator. The tomatoes were observed daily until the skin at the incised area was cracked, curled, and moist [21].

2.7. Effect of Oregano Essential Oil on Sensory Attributes of Tomatoes. Oregano essential oil at 1 MIC was sprayed on the surface of fresh tomatoes at a concentration of  $200 \,\mu\text{L}$  of oregano essential oil per 100 g of tomatoes, after which, the tomatoes were stored in a foam box at 4°C. Sensory evaluations were performed on days 1, 7, 10, 12, 15, 17, and 18. Twenty-five teachers and students experienced in food sensory evaluation were invited to score the appearance, odor, flavor, and texture of the tomatoes. Sensory profiles were evaluated based on a 9-point hedonic scale (1-9 denoted dislike extremely, dislike very much, dislike moderately, dislike slightly, neither like nor dislike, like slightly, like moderately, like very much, and like extremely, separately) [22].

2.8. Data and Statistical Analysis. All experiments were performed in triplicate. The data were analyzed for statistical significance using SPSS 19.0 software. The data were expressed as the mean  $\pm$  SD. One-way analysis of variance (ANOVA) was used to express the significance of differences ( $P \le 0.05$ ) between means. Figures were drawn using the SigmaPlot 14.0 software.

# 3. Results

3.1. Molecular Biology Identification of the Tomato Spoilage Bacteria. The genomic DNA of W01 was used as a template to amplify the 16S rDNA fragment using PCR, and one amplification product with a clear band of about 1500 bp was obtained. The fragment underwent recovery and 16S rDNA sequencing. Using 16S rDNA sequencing for molecular biology identification, the unknown strain showed 100% homology with *E. ludwigii* (accession number MN636674.1 in GenBank), consistent with its identification by colony

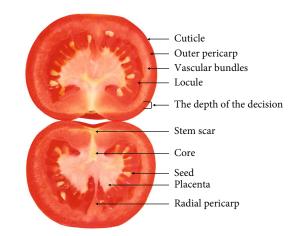


FIGURE 1: The structure of tomatoes.

and cellular morphology. This confirmed that the strain W01 was *Enterobacter ludwigii*. A phylogenetic tree was constructed using the neighbor-joining method (Figure 2).

Figure 3, among the 12 kinds of essential oils, the diameter of the inhibition zone for Wh, Te, Cy, Li, Rm, Le, and Os was 0 mm, the diameter of the inhibition zone for Ba, Sh, and Ro was less than 1.0 mm, whereas the diameter of the inhibition zone for Ci and Or was greater than 2.0 mm, while Ci was lesser than Or. So, oregano essential oil (Or) showed the best inhibitory effect of *E. ludwigii* W01.

3.2. Evaluation of the Minimum Inhibitory Concentration. To further investigate the antibacterial activity of oregano essential oil against *E. ludwigii*, the MIC was determined using the twofold serial dilution method. The results are shown in Figure 4. The  $OD_{600}$  of *E. ludwigii* bacterial solution remained below 0.1 when the concentration of oregano essential oil was greater than or equal to 0.125%, with no significant difference compared to that of the blank control group. Therefore, the MIC of oregano essential oil against *E. ludwigii* W01 was 0.125%.

3.3. Effect of Oregano Essential Oil on Alkaline Phosphatase Activity in E. ludwigii Bacterial Solution. The change in alkaline phosphatase activity in the E. ludwigii W01 bacterial solution after the addition of different concentrations of oregano essential oil was calculated by determining the absorbance value of the bacterial solution at 520 nm (Figure 5). The alkaline phosphatase activity in E. ludwigii bacterial solution after the addition of oregano essential oil was significantly higher than that of the control group, and the alkaline phosphatase activity of the solution increased with the increase in the concentration of oregano essential oil.

3.4. Effect of Oregano Essential Oil on Potassium Ion Leakage in E. ludwigii Bacterial Solution. After the addition of different concentrations of oregano essential oil to the E. ludwigii W01 bacterial solution, the change in potassium ion leakage was calculated by determining the absorbance value of the bacterial solution at 440 nm (Figure 6). However, no significant difference (P > 0.05) was observed in potassium ion

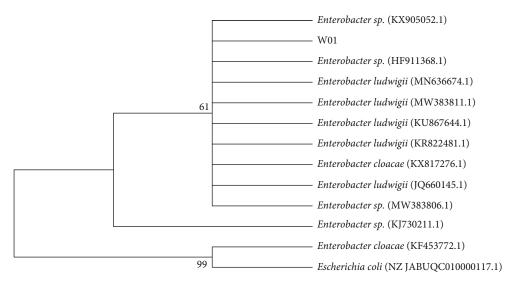


FIGURE 2: Phylogenetic tree constructed with the 16S rDNA showing the W01 strain.

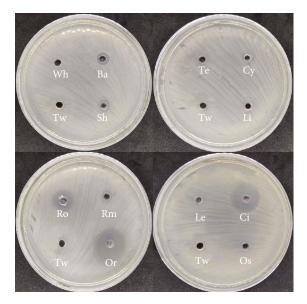


FIGURE 3: Assessment of 12 essential oils for their inhibitory effects against *E. ludwigii* W01 (note: the diameter of the puncher is 0.5 cm).

leakage in the bacterial solution with different concentrations of oregano essential oil, compared to the control group. This indicated that oregano essential oil did not induce a release of  $K^+$  from the cytoplasm of *E. ludwigii*.

3.5. Changes in the Microscopic Morphology of E. ludwigii Induced by Oregano Essential Oil. Subsequent to the addition of different concentrations of oregano essential oil to the E. ludwigii W01 bacterial solution and cultivation for 12 h, the cellular morphology of the bacteria was observed under SEM (Figure 7).

The microscopic morphology of *E. ludwigii* treated with 1 MIC oregano essential oil showed a significant reduction in size, along with prominent surface wrinkling and a severe disruption of the morphology. The depth of the surface wrinkles deepened with the addition of 2 MIC of oregano essential oil.

3.6. Oregano Essential Oil Prevents the Growth and Multiplication of E. ludwigii W01 in Tomato Wounds. E. ludwigii W01 was inoculated onto the wounds of tomatoes, which were then stored in the presence of oregano essential oil for 7 days in a refrigerator at 4°C. The appearance of the tomatoes is shown in Figure 8. Spraying 1 MIC and 2 MIC oregano essential oil maintained the intrinsic firmness and odor of the tomato even after 7 days of storage. However, tomatoes sprayed with 0 MIC and 1/2 MIC oregano essential oil showed cracking, curling, and moistness on the skin around the wound. These results indicate that oregano essential oil inhibits the growth and multiplication of *E. ludwigii* W01 on tomatoes with damaged skin, effectively preventing tomato spoilage.

3.7. Effect of Oregano Essential Oil on Sensory Attributes of Tomatoes. Sensory quality is an important evaluation index of fruit and vegetable products. As shown in Figure 9, after spraying 1 MIC oregano essential oil on the surface of fresh tomatoes and storing them in a single layer in a foam box, their sensory quality gradually decreases with time. The sensory acceptability of texture and flavor was above 6.5 at 15 days of storage. However, compared to fresh tomatoes (the first day), the smell and appearance significantly declined after 15 days, receiving a sensory rating of neither like nor dislike, revealing an impact on the sensory quality of the tomatoes. These results indicate that spraying 1 MIC oregano essential oil on the surface of fresh tomatoes and storing them in a single layer in a foam box extended their shelf life to 15 days.

# 4. Discussion

The aim of this study was to identify the bacteria that cause spoilage in tomatoes to subsequently identify the best natural preservative against this bacterial strain among common types of essential oils.

In the present study, the dominant spoilage bacterium, identified as *E. ludwigii* W01, was isolated from tomatoes

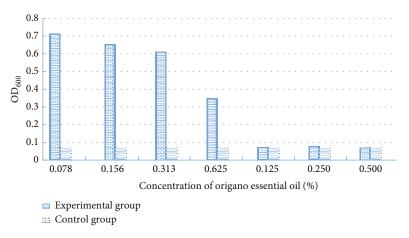


FIGURE 4: Absorbance values of E. ludwigii W01 bacterial solution after incubation with different concentrations of oregano essential oil for 12 h.

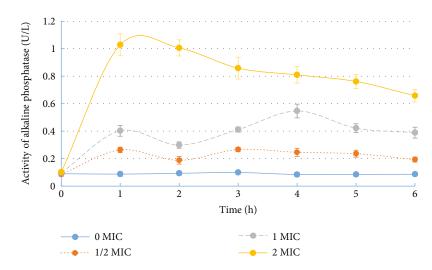


FIGURE 5: Changes in alkaline phosphatase activity of *E. ludwigii* W01 bacterial solution after the addition of different concentrations of oregano essential oil.

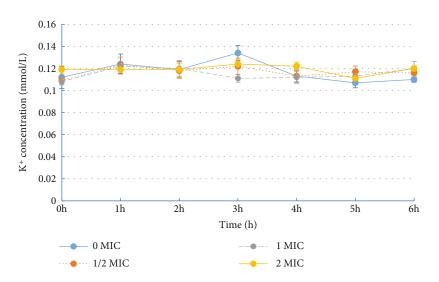


FIGURE 6: Changes in potassium ion leakage in *E. ludwigii* W01 bacterial solution after the addition of different concentrations of oregano essential oil.

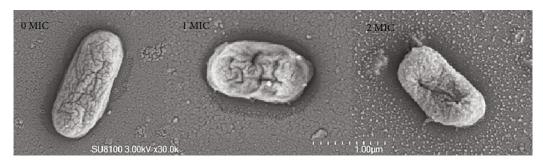


FIGURE 7: Microscopic morphology of *E. ludwigii* W01 after the addition of different concentrations of oregano essential oil to the bacterial solution.



FIGURE 8: Appearance of tomatoes after inoculating the wound with E. ludwigii W01 and storing it with oregano essential oil for 7 days.

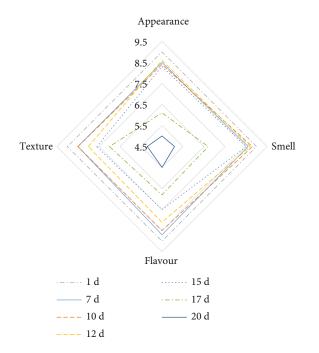


FIGURE 9: Sensory attributes of tomatoes with different preservation times after the application of oregano essential oil.

with cracked, curled, and moist skin. This bacterium has not been found in previous reports on food spoilage. It is commonly found in soil and sewage and is a commensal gut bacteria in the human gastrointestinal tract. It can cause a variety of infections, including bacteremia, endocarditis, infectious arthritis, osteomyelitis, skin, and soft tissue infections, as well as lower respiratory tract infections, urinary tract infections, and abdominal infections [23].

Oregano essential oil is widely used as a food preservative, containing different active phenolic compounds, such as thymol and carvacrol. In addition, its attractive palatability allows it to be successfully incorporated into food formulations [24].

4.1. Inhibition of E. ludwigii by Oregano Essential Oil. The addition of oregano essential oil to E. ludwigii bacterial solution resulted in elevated alkaline phosphatase activity without a significant change in potassium ion leakage. This indicated that oregano essential oil could disrupt the cell wall of E. ludwigii, causing leakage of the alkaline phosphatase stored between the cell wall and the cell membrane, indicated by the increasing alkaline phosphatase activity in the bacterial solution. However, this disruption did not damage the integrity of the cell membrane, preventing potassium ions from leaking from the cytoplasm into the bacterial solution. The size of E. ludwigii cells shrunk, and the surface appeared to be wrinkled with the addition of oregano essential oil, causing more significant damage as the amount of oregano essential oil increased. The microscopic morphological images also demonstrated the inability of oregano essential oil to completely disrupt the integrity of the cell wall and cell membrane of *E. ludwigii* W01.

However, previous research had yielded inconsistent results. Oregano essential oil might affect the membrane permeability and membrane integrity of the bacteria and cause the intracellular content to flow out, leading to the original morphological change of bacteria and eventually causing irreversible damage to the bacteria. This had been confirmed on *Staphylococcus aureus* [25], *Alicyclobacillus* spp. [26], *Shigella flexneri* [27], and *Vibrio vulnificus* [28]. Oregano essential oil against the gram-negative bacteria (*Vibrio vulnificus*) may enhance the level of reactive oxygen species which causes lipid peroxidation of cell membranes, thereby increasing the permeability and reducing the integrity of cell membranes and causing morphological changes to cells [28]. Therefore, oregano essential oil cannot alter the membrane permeability of *E. ludwigii* W01 cells because it could not promote the level of reactive oxygen species.

4.2. Preservation Effect of Oregano Essential Oil on Tomatoes. Oregano essential oil has been used with remarkable results in aiding disease treatment, animal healthcare, food pest control, and the preservation of meat products and fruits, such as grapes [29] and pears [30]. Among the 12 kinds of essential oils evaluated in this study, oregano essential oil had the highest inhibitory effect on E. ludwigii W01, with a MIC of 0.125%. In a previous study, Pirozzi et al. extended the freshness of tomatoes stored at 4°C to 15 days by spraying the tomatoes with oregano essential oil and using foam boxes for preservation. A coating of oregano essential oil nanoemulsion can reduce the growth of endogenous microbial flora of tomatoes within 14 days at room temperature, resulting in a reduction in the total amount of microbe [31]. Kwon et al. utilized a polyvinyl alcohol film containing oregano essential oil to impart antimicrobial properties to the packaging of cherry tomatoes, which showed strong antimicrobial effects against Salmonella enteritidis, molds, yeasts, and mesophilic aerobic bacteria [32]. Thus, the 15day shelf life obtained in the present study did not present a significant difference from previous studies; however, in our study, the tomatoes were stored at 4°C and were conventional varieties that had not undergone any cultivar improvements. The similar effects achieved using oregano essential oil when preserving tomatoes at different temperatures may be due to the differences in tomato varieties. In the present study, spraying 1 MIC of oregano essential oil on tomatoes also achieved significant results in inhibiting the growth and multiplication of E. ludwigii W01 in tomatoes with damaged skin and preventing the spoilage of tomatoes.

#### 5. Conclusion

*E. ludwigii* W01 was identified as the dominant spoilage bacterium in tomatoes, causing cracked, curled, and wet skin in them. After screening 12 common essential oils, oregano essential oil was found to inhibit *E. ludwigii* W01 the most, inhibiting its growth by disrupting the integrity of the bacterial cell wall. After spraying 200  $\mu$ L of 1 MIC (0.125%) oregano essential oil on the surface of 100 g fresh tomatoes and storing them in foam boxes at 4°C for 15 days, no significant differences in the sensory attributes of the tomatoes were observed.

#### **Data Availability**

All data generated or analyzed during this study are included in this published article.

# **Additional Points**

*Practical Applications*. Tomato with thin skin and tender flesh, fruit decay caused by spoilage bacteria is a common phenomenon. The dominant spoilage bacterium *E. ludwigii* W01 was isolated from the skin of tomatoes with cracked, curled, and moist. The most effective antibacterial agent was screened from 12 essential oils. The results demonstrated that spraying oregano essential oil was a natural and effective approach and could be kept fresh for 15 d in the 4°C foam box.

#### **Ethical Approval**

The study design was approved by the appropriate ethics review boards.

# Consent

All study participants provided informed consent.

# **Conflicts of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

# **Authors' Contributions**

WMC, WGL, LDH, XHL, and LEZ designed the study. WMC, JY, XLL, LDQ, and LEZ performed the experiments. WMC drafted the work. WMC, WGL, QZ, XHL, and LDH wrote and revised the manuscript. WMC, WGL, LEZ, and QZ revised the final version to be published. All authors approved the manuscript and agreed to its submission to your esteemed journal.

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