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Abstract: Different cutting types not only affect the quality and bioactive substances of broccoli but may produce wounding stress that influences the quantity and diversity of microorganisms and finally leads to safety concerns. In this study, three different fresh-cutting types (floret (F), quarterly cut floret (QF) and shredded floret (FS)) and four storage stages (0, 1, 2 and 3 day(s)) were designed to detect the bacterial diversity in broccoli. Results showed that the quantity and growth of microorganisms in fresh-cut broccoli were positively correlated with the intensity of fresh-cut damage, and the 97 strains isolated using 16S rRNA gene sequences were mainly classified into four phyla: Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes. The predominant phylum was Proteobacteria, dominated by *Pseudomonas* spp. and *Acinetobacter* spp. Four representative strains of different species were selected to analyze their spoilage potential on fresh-cut broccoli by metabolizing to produce nitrite and consuming nutrition. Moreover, *Comamonas* spp. and *Acinetobacter* spp. also affected the quality of broccoli, but received little attention previously. This study provides a reference for precise bacteriostasis and preservation of fresh-cut vegetables such as broccoli.

Keywords: broccoli; bacterial diversity; cutting type; microbiology; food safety

1. Introduction

Broccoli is a health-promoting vegetable known for its rich content of bioactive components, such as vitamins, antioxidant substances, and anticarcinogenic precursors. It is cultivated worldwide due to high consumer popularity [1]. Studies show that glucosinolates have recently attracted great research interest because of their potential health benefits in cardiovascular diseases such as hypertension, atherosclerosis and cholesterol reduction [2,3]. Fresh-cut vegetables refer to those processed by washing, sorting, peeling, and slicing, and the most popular ones among them are cruciferous vegetables such as broccoli, cauliflower and cabbage [4]. Fresh-cut fruits and vegetables, which originated in the United States in the 1950s, have received much attention due to their nutrition, convenience and flavor [5]. While fresh-cut vegetables are rich in essential nutrients, they are susceptible to external biological, chemical, and physical processes, which may create a variety of safety issues. During the process of fresh-cut produce, cutting is an essential procedure that unavoidably produces wounding stress, which may cause fresh-cut broccoli to be infected by microorganisms [6]. Moreover, the mechanical damage caused by primary processing, such as peeling, cutting, and slicing, leads to the release of intracellular products in broken cells and tissues, enhancing the risk of bacterial contamination compared with vegetables without processing. Although fresh-cut fruits and vegetables will be contaminated by a variety of microorganisms during the process of picking and transportation, it is not



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). easy to determine the quality of vegetables solely on the total bacterial count at the end of storage, because not all bacteria are capable of proliferating on vegetables behaving as spoilage bacteria.

In addition to enzymolysis of nutrients, spoilage of fruits and vegetables can be caused by the activity of a wide variety of microorganisms. Thus, a great number of studies have focused on identifying the bacteria responsible for vegetable spoilage. Most of the microorganisms causing fruits and vegetables to spoil are aerobic bacteria or molds, while a few are anaerobic microorganisms [7]. Multiple bacteria have been identified as spoilage microorganisms in fruits and vegetables, including *Pseudomonas* [8], *Bacillus* [9], and *Erwinia* [10]. Because vegetables are easily infected by bacteria in soil, the number of microbial colonies is high even in the primary stage and the common ones belonged to *Pseudomonas* and *Bacillus*, as reported in [11]. The total number of colonies in some vegetables can even reach up to a high level, which may be caused by mechanical damage and environmental pollution in the process of picking and processing [12].

To investigate the microorganisms in the process of broccoli becoming putrid, the culture-dependent method, relying on separation and identification methods, is considered to be a wide and conventional isolation technique, which lays a solid foundation for the study of microorganisms in foods. In addition, culture-independent molecular techniques can also be used to fully estimate microbial communities [13–16].

The objective of this study was to evaluate the effect of cutting types and storage on bacterial diversity of fresh-cut broccoli with culture-dependent methods and to conduct the spoilage potential analyses of dominant spoilage microorganisms. Many studies have investigated the contamination processes of specific bacteria, but the overall microbial diversity of rotten vegetables is still unclear. In order to keep vegetables fresh and prolong their shelf life, it is important to determine which kinds of microorganisms will actually deteriorate vegetables. The findings could provide information to food scientists and food industries in controlling microbial contamination and extending the shelf life of foods.

2. Materials and Methods

2.1. Sample Collection and Preparation

The raw material broccoli was harvested in April at Beijing National Vegetable Research Center (Beijing, China). Healthy broccoli with the same maturity and size were selected and transported to the lab with ice and stored at 20 °C for further analysis. The experiments were performed with three randomly selected biological replicates.

2.2. Pretreatment of Sample

The selected samples were washed with distilled water and then placed in a ventilator to dry naturally. The broccoli was subjected to three different cutting types using a sterile scalpel: (1) the broccoli was cut into single floret (floret, marked as F); (2) the broccoli floret was cut into four parts quarterly (quarterly cut floret, marked as QF); (3) the broccoli was shredded into pieces (shredded floret, marked as FS). All broccoli samples were packaged in a polyethylene plastic bag and then stored at 20 °C to study the change of microorganisms on it. During storage, broccoli was subsequentially sampled for further analyses at 0, 1, 2, 3 day(s). Three biological replicates were used for each sample. The characteristics of samples are shown in Figure 1.

2.3. Isolation of Bacteria

During 3 days of storage at 20 °C, the isolation and purification of bacteria were performed as Li et al. [17]. The total number of bacteria were determined by the standard plate count method using Plate Count Agar (PCA) medium. Briefly, 25 g broccoli samples were mixed with 225 mL sterilized 0.85% NaCl solution, placed in a sterilized homogenous bag, and homogenized at the speed of 10,000 r/min for 2 min to prepare a 1:10 sample homogenate. The sample suspension was serially diluted with sterilized 0.85% NaCl solution, 1 mL of which was spread, in triplicates, on PCA medium to enumerate bacteria.

The PCA plates were incubated at 37 $^{\circ}$ C for 2 days and the bacterial counts were expressed as lg CFU/g with 3 replicates. In addition, different single colonies were picked out according to the morphology, color and size, and grown continuously on the plate until pure single colonies were obtained.



Figure 1. Plant materials and sampling design. Broccoli was harvested, prepared and cut with three different types, floret, quarterly cut floret and shredded floret. Then they were stored at 20 °C and subsequentially collected at 0, 1, 2, 3 days for further analyses.

2.4. DNA Extraction

Strains were harvested for DNA extraction at 37 °C. Genomic DNA was extracted from cell suspension cultures using TIANamp Bacteria Genomic DNA kits (TIANGEN BIOTECH) according to the manufacturer's instructions. DNA concentration and quality were determined spectrophotometrically at 260 and 280 nm.

2.5. PCR Amplification

After the extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were performed according to Li et al. [18]. Amplification of the 16S rRNA gene was conducted using a pair of universal primers, forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1525R (5'-AGAAAGGAGGTGATC CAGCC-3'). The PCR amplification were carried out under the following conditions: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. The products above were purified and sequenced by BGI (The Beijing Genomics

Institute). The 16S rRNA gene sequences of the isolates were sequenced with the Sanger sequencing method.

2.6. 16S rRNA Gene Phylogeny

After sequencing, the 16S rRNA gene sequences of different strains in broccoli were obtained and similarity searches were performed using the EzBioCloud server (http://www.ezbiocloud.net) [19]. The 16S rRNA sequencing data of all the isolates were aligned using Molecular Evolutionary Genetics Analysis 11 (MEGA 11) software and analyzed by Basic Local Alignment Search Tool (BLAST) with those of the closest strains retrieved from the National Center for Biotechnology Information (NCBI) database. The phylogenetic tree was constructed using the neighbor-joining method [20] in MEGA 11 software with representative type strains to determine the evolutionary relationship among the strains. Kimura's two-parameter model was used to calculate the evolutionary distances [21]. Bootstrap values were determined based on 1000 replications [22].

Based on the results of identification, four representative strains of different species were selected as dominant spoilage bacteria to study their impacts on the spoilage of broccoli.

2.7. Spoilage Characteristics of Representative Strains in Spoilage of Broccoli

The broccoli samples were washed with sterile water three times, soaked in 75% alcohol for 10 s, then picked up and dried naturally. After sterilization, they were washed with sterile water and dried. The total number of bacterial colonies of sterile broccoli should be less than $2 \log_{10} (\text{CFU/g})$.

The dominant spoilage bacteria were inoculated on broccoli again, and the effects of different dominant spoilage bacteria on the physicochemical properties and nutritional quality of fresh-cut broccoli were measured.

2.7.1. Chlorophyll Content Determination

The content of total chlorophyll was determined by spectrophotometry according to Lichtenthaler [23], and the content of chlorophyll a, chlorophyll b was measured as described by Fang et al. [24] with slight modifications. An amount of 0.1 g plant tissues was homogenized in 80% acetone and extracted until the plant tissue completely became white and then centrifuged at 8000 rpm for 15 min. The optical density of the supernatant was recorded at 645 nm and 663 nm.

2.7.2. Nitrite Content Determination

The total content of nitrite was measured according to the naphthyl ethylenediamine hydrochloric acid spectrophotometric method by Nerdy et al. [25] with minor modifications. Approximately 5.0 g broccoli was crushed, deproteinated and defatted by precipitation followed by filtration. 40 mL of the filtrate was transferred into a 50 mL colorimetric tube with stopper, added 2 mL of p-Aminobenzenesulfonic Acid solution and 1 mL of N-(1-Naphthyl) Ethylenediamine Dihydrochloride solution, shaken until homogeneously mixed, diluted with distilled water to the marked line, shaken until well mixed. The mixtures were then kept at room temperature for 15 min. OD of the colored mixtures was read at 538 nm against the reagent blank as described by An et al. [26]. The experiments were carried out in triplicates and the results were calculated as the average obtained from them.

2.7.3. MDA Content Assay

Malondialdehyde (MDA) content was measured by the thiobarbituric acid (TBA) chromogenic method as described by Liu et al. [27]. Extraction was carried out by homogenizing 1.0 g of broccoli samples with 5 mL of 10% trichloroacetic acid (TCA), and then the homogenate was centrifuged at 8000 rpm at 4 °C for 20 min. The supernatant was collected and 2 mL of extract was mixed with 2 mL of 0.67% TBA solution to react in a boiling water bath for 20 min. The solution was cooled and centrifuged again. The extraction of supernatant was determined in absorbance at 450 nm, 532 nm, and 600 nm,

respectively. The amount of MDA was expressed as nmol per gram (nmol/g fresh weight) of broccoli.

2.7.4. Soluble Sugar Content Analysis

The total soluble sugar content in each broccoli sample was measured by the anthrone colorimetric method [28] with slight modifications. Broccoli samples weighing 0.2 g were taken in test tubes. Then, to each test tube was added 1 mL of distilled water to disperse it and the samples were grinded into homogenate. After placing the test tubes in boiling water for 10 min, the extracts were collected and the volumes of the extracts were adjusted to 10 mL using distilled water. The absorbances of the extracts were determined at 620 nm wavelength after adding sulfuric acid and anthrone. The results were presented as milligram per gram (mg/g fresh weight) of broccoli, and the experiments were repeated three times.

2.7.5. Statistical Analysis for Spoilage Characteristic of Microorganisms in Broccoli

All data were analyzed statistically using a one-way analysis of variance (ANOVA) with SPSS software 23.0. Statistical differences between samples (* p < 0.05 was considered to be significant; ** p < 0.01 was considered to be extremely significant) were determined by Duncan's multiple range test. All data were recorded as the mean \pm standard errors (SE) of the three independent biological replicates.

3. Results

3.1. Changes in Bacterial Counts and Isolates Separated from Broccoli

Fresh-cut broccoli was easily infected by bacteria during storage. At the harvest period, the bacterial counts of broccoli were up to 5.12 lg (CFU/g), which might be contaminated with a large number of microorganisms during sample collection and transportation (Figure 2). After 3 days, the bacterial counts in broccoli which was cut into a single floret reached up to 8.54 lg (CFU/g), and different kinds of bacteria were observed. In addition, the number of microorganisms in QF (quarterly cut floret) and FS (shredded floret) reached up to 8.66 lg (CFU/g) and 9.60 lg (CFU/g), respectively. It can be seen from Figure 2 that mechanical damage had a great impact on the amounts of microbial colonies. The total number and growth rate of microorganisms on severely damaged broccoli, referring to the broccoli floret cut quarterly into four parts. Therefore, in the postharvest treatment, mechanical damage to broccoli should be lessened as much as possible, or supplementary strategies (such as cold storage) should be applied to prolong its shelf life.



Figure 2. Effects of different cutting types on the growth of total bacterial counts in fresh-cut broccoli during storage.

The microorganisms could be separated from the broccoli during storage (Table 1). The analysis of the growth features and corruption characteristics of the spoilage microorganisms were, therefore, of great significance in assisting the control of quality and safety of fresh-cut broccoli.

Sample	Strain Number	Cutting Type	Color of Broccoli	Peculiar Smell	Storage Time (d)
А	BRO-A1~BRO-A20	Floret	Green	No	0
В	BRO-B1~BRO-B21	Floret	Green	No	1
С	BRO-C1~BRO-C12	Floret	Light green	Minor	2
D	BRO-D1~BRO-D13	Floret	Light yellow	Minor	3
E	BRO-E1~BRO-E9	Quarterly cut floret	Light yellow	Minor	3
F	BRO-F1~BRO-F6	Shredded floret	Yellow	Intense	3
G	BRO-G1~BRO-G16	Shredded floret	Yellow	Intense	3

Table 1. The descriptions of the sampled broccoli and the isolates.

3.2. Identification of Bacterial Isolation

To identify key microbes that accounted for the observed microbiota separation, community composition was analyzed. According to the different colonial morphologies, a total of ninety-seven bacterial colonies that appeared on culture medium were selected for obtaining pure cultures to be used in characterization for further research. The screened strains were sequenced, and the phylogenetic tree of the spoilage strains is shown in Figure 3. According to the 16S rRNA gene sequencing, the bacterial isolates were grouped into 21 different bacterial genera (Table 2). And the culturable bacterial composition of freshcut broccoli at the phylum and genus levels is shown in Figure 4. A similarities research based on near-complete 16S rRNA sequences of the isolates showed that there was a high similarity (>97%) between the test isolates and the representative strains of *Pseudomonas*, Stenotrophomonas, Acinetobacter, Alcaligenes, Enterobacter, Delftia, Comamonas, Agrobacterium, Sphingobacterium, Brevundimonas, Lysinibacillus, Brevibacterium, Glutamicibacter, Lelliottia, Myroides, Pectobacterium, Proteus, Microbacterium, Leclercia, Escherichia and Rothia. The majority of bacteria belonged to three phyla: Proteobacteria, Actinobacteria and Bacteroidota, accounting for more than 90% of the total. Furthermore, Proteobacteria was the predominant phylum, and the majority were *Pseudomonas* spp. and *Acinetobacter* spp. A few genera with the least number of isolates were also obtained, such as Lysinibacillus, a genus of phylum Firmicutes. Isolates belonging to the phyla Actinobacteria and Bacteroidetes were found in a low number. A diversity of microorganism distribution was observed in bacterial isolates.

 Table 2. Taxonomic identification and detailed information of culturable bacteria isolated from fresh-cut broccoli based on 16S rRNA gene sequences.

Phylum	Species	Strain No.	Similarity Range (%)	Reference Strain	Percentage (%)
Proteobacteria	Pseudomonas capeferrum	BRO-B1 BRO-B7 BRO-B3 BRO-D9 BRO-C1 BRO-B18 BRO-C9 BRO-C3 BRO-C11	99.29–99.60	Pseudomonas capeferrum WCS358 ^T	9.28
	Pseudomonas alloputida	BRO-A11 BRO-E2 BRO-E3 BRO-G13 BRO-F2 BRO-E1 BRO-G12	99.86–100.00	Pseudomonas alloputida Kh7 ^T	7.22

Phylum	Species	Strain No.	Similarity Range (%)	Reference Strain	Percentage (%)
	Pseudomonas rhizoryzae	BRO-A19	99.47	Pseudomonas rhizoryzae RY24 ^T	1.03
	Pseudomonas qingdaonensis	BRO-D11	100.00	Pseudomonas qingdaonensis JJ3 ^T	1.03
	Pseudomonas baetica	BRO-C4	99.58	Pseudomonas baetica a390 ^T	1.03
	Pseudomonas rhodesiae	BRO-C6	99.72	Pseudomonas rhodesiae CIP 104664 ^T	1.03
	Pseudomonas mosselii	BRO-D8	100.00	Pseudomonas mosselii CIP 105259 ^T	1.03
	Pseudomonas juntendi	BRO-B16	99.64	Pseudomonas juntendi BML3 ^T	1.03
	Pseudomonas entomophila	BRO-B19	99.83	Pseudomonas entomophila L48 ^T	1.03
	Pseudomonas atacamensis	BRO-E4 BRO-A1 BRO-A7 BRO-B2	99.84-100.00	Pseudomonas atacamensis M7D1 ^T	4.12
Proteobacteria	Pseudomonas sichuanensis	BRO-B8	100.00	Pseudomonas sichuanensis WCHPs060039 ^T	1.03
	Pseudomonas oleovorans subsp. oleovorans	BRO-B14 BRO-G9 BRO-G10	98.87–99.71	Pseudomonas oleovorans subsp. oleovorans DSM 1045 ^T	3.09
	Acinetobacter johnsonii	BRO-B5 BRO-B10 BRO-D10 BRO-F3 BRO-B21	98.67–99.30	Acinetobacter johnsonii CIP 64.6 ^T	5.15
	Acinetobacter pittii	BRO-B11 BRO-D3 BRO-B4 BRO-G7 BRO-A5 BRO-G6 BRO-G1	99.86–100.00	Acinetobacter pittii CIP 70.29 ^T	7.22
	Acinetobacter oryzae	BRO-B12 BRO-B15 BRO-D5 BRO-F1 BRO-B20 BRO-C12 BRO-E6	98.98–99.36	Acinetobacter oryzae B23 ^T	7.22
	Acinetobacter guillouiae	BRO-D13 BRO-G16	98.75–98.86	Acinetobacter guillouiae CIP 63.46 ^T	2.06
	Acinetobacter junii	BRO-A17 BRO-D6	99.33	Acinetobacter junii CIP 64.5 ^T	2.06

Table 2. Cont.

Phylum	Species	Strain No.	Similarity Range (%)	Reference Strain	Percentage (%)
	Acinetobacter proteolyticus	BRO-A13	100.00	Acinetobacter proteolyticus NIPH 809 ^T	1.03
	Acinetobacter bereziniae	BRO-C8 BRO-D7	99.85–100.00	Acinetobacter bereziniae LMG 1003 ^T	2.06
	Acinetobacter calcoaceticus	BRO-G4	100.00	Acinetobacter calcoaceticus DSM 30006 ^T	1.03
	Comamonas aquatica	BRO-B6 BRO-D12 BRO-G3	99.85–100.00	Comamonas aquatica NBRC 14918 ^T	3.09
	Comamonas koreensis	BRO-A12	98.86	Comamonas koreensis KCTC 12005 ^T	1.03
	Comamonas testosteroni	BRO-A14 BRO-A16 BRO-G8 BRO-C5	99.85–100.00	<i>Comamonas</i> testosteroni ATCC 11996 ^T	4.12
	Stenotrophomonas maltophilia	BRO-A2 BRO-F5	99.21–99.34	Stenotrophomonas maltophilia MTCC 434 ^T	2.06
	Stenotrophomonas pavanii	BRO-F4	99.86	Stenotrophomonas pavanii DSM 25135 ^T	1.03
	Stenotrophomonas rhizophila	BRO-A9	99.86	Stenotrophomonas rhizophila DSM 14405 ^T	1.03
Proteobacteria	Stenotrophomonas terrae	BRO-A10	99.59	Stenotrophomonas terrae DSM 18941 ^T	1.03
	Stenotrophomonas lactitubi	BRO-C10	99.37	Stenotrophomonas lactitubi M15 ^T	1.03
	Stenotrophomonas indicatrix	BRO-F6	99.72	Stenotrophomonas indicatrix WS40 ^T	1.03
	Brevundimonas vesicularis	BRO-A8	100.00	Brevundimonas vesicularis NBRC 12165 ^T	1.03
	Brevundimonas diminuta	BRO-A18	99.35	Brevundimonas diminuta ATCC 11568 ^T	1.03
	Agrobacterium arsenijevicii	BRO-A4	98.13	Agrobacterium arsenijevicii KFB 330 ^T	1.03
	Alcaligenes faecalis subsp. phenolicus	BRO-G2 BRO-G5	99.72–99.73	Alcaligenes faecalis subsp. phenolicus DSM 16503 ^T	2.06
	Escherichia hermannii	BRO-A3	99.87	Escherichia hermannii CIP 103176 ^T	1.03
	Leclercia adecarboxylata	BRO-B13	100.00	Leclercia adecarboxylata NBRC 102595 ^T	1.03

Table 2. Cont.

Phylum	Species	Strain No.	Similarity Range (%)	Reference Strain	Percentage (%)
	Lelliottia jeotgali	BRO-C7	99.28	Lelliottia Jeotgali PFL01 ^T	1.03
	Enterobacter chengduensis	BRO-G14	99.85	Enterobacter chengduensis WCHECI-C4 ^T	1.03
Proteobacteria	Delftia tsuruhatensis	BRO-G11	100.00	Delftia tsuruhatensis NBRC 16741 ^T	1.03
	Proteus mirabilis	BRO-E8	99.86	Proteus mirabilis ATCC 29906 ^T	1.03
	Pectobacterium carotovorum	BRO-D2	99.72	Pectobacterium carotovorum NCPPB 312 ^T	1.03
	Glutamicibacter arilaitensis	BRO-D4	99.13	Glutamicibacter arilaitensis Re117 ^T	1.03
	Glutamicibacter nicotianae	BRO-C2	99.55	Glutamicibacter nicotianae NBRC 14234 ^T	1.03
Actinobacteria	Microbacterium algeriense	BRO-B9	99.47	Microbacterium algeriense G1 ^T	1.03
	Rothia marina	BRO-D1	99.75	Rothia marina JSM 078151 ^T	1.03
	Brevibacterium iodinum	BRO-A6	99.13	Brevibacterium iodinum NCDO 613 ^T	1.03
Bacteroidetes	Sphingobacterium faecium	BRO-E5 BRO-A15	99.26–99.43	Sphingobacterium faecium DSM 11690 ^T	2.06
	Myroides odoratus	BRO-E9 BRO-E7 BRO-B17 BRO-G15	99.17–100.00	Myroides odoratus DSM 2801 ^T	4.12
Firmicutes	Lysinibacillus fusiformis	BRO-A20	99.59	Lysinibacillus fusiformis NBRC 15717 ^T	1.03

Table 2. Cont.





Bacteroidetes Firmicutes

Actinobacteria Proteobacteria



Figure 4. The culturable bacterial composition of fresh-cut broccoli at the phylum (**A**) and genus (**B**) levels.

3.3. Effects of Spoilage Microorganisms on the Quality of Broccoli

Regardless of the cutting types, the diversity of spoilage bacteria isolated from broccoli was similar. In the present study, we collected more than ninety colonies of bacteria from broccoli and selected four major isolates based on colony phenotype grown on PCA medium. The phylogenetic trees of the four strains are shown in Figure 5 and their impacts are shown in Table 3. The results showed that each of the strains had different effects on the quality of the broccoli, *Pseudomonas* spp. particularly.

Table 3. The influence of spoilage microorganisms on the chlorophyll, nitrite, MDA and soluble sugar content of fresh-cut broccoli during 72 h of storage at 20 °C. All data were recorded as the mean \pm standard errors (SE). Values with different letters were significantly different at p < 0.05. Lowercase letters represent significant differences among treatment factors; capital letters represent significant differences.

	Storage Time (d)	Chlorophyll (mg/g(F _W))	Nitrite (mg/kg)	MDA (nmol/g(F _W))	Soluble Sugar (mg/g(F _W))
	0	$0.39\pm0.01~\text{A}$	$0.31\pm0.03~BCD$	$1.62\pm0.07\text{CD}$	$2.66\pm0.03~BCD$
	1	$0.33\pm0.02~\mathrm{aB}$	$0.35\pm0.02~\mathrm{cA}$	$4.65\pm0.04~\mathrm{aB}$	$2.70\pm0.04~bB$
control	2	$0.29\pm0.00~\mathrm{aC}$	$0.11\pm0.02~\mathrm{cD}$	$7.39\pm0.22~\mathrm{cA}$	$2.83\pm0.01~\mathrm{dA}$
	3	$0.27\pm0.01~\text{aC}$	$0.16\pm0.01~\text{eC}$	$4.79\pm0.07~aB$	$2.86\pm0.06bA$
D1	1	$0.19\pm0.01~\text{dB}$	$1.61\pm0.09~\mathrm{aA}$	$3.85\pm0.15bB$	$3.17\pm0.02~aB$
Pseudomonas sp.	2	$0.18\pm0.01~\text{bB}$	$0.77\pm0.02~\mathrm{aB}$	$11.67\pm0.04~\mathrm{aA}$	$3.54\pm0.05~\mathrm{aA}$
BRO-CII	3	$0.13\pm0.01~\text{dC}$	$0.66\pm0.00~\text{aC}$	$3.20\pm0.15bC$	$2.94\pm0.05bC$
D1	1	$0.23\pm0.01~\mathrm{cB}$	$0.71\pm0.04\mathrm{bA}$	$3.25\pm0.03~\mathrm{cB}$	$3.16\pm0.03~\mathrm{aB}$
Pseudomonas sp.	2	$0.17\pm0.01~{ m bC}$	$0.23\pm0.04~\mathrm{bC}$	$8.72\pm0.03bA$	$3.17\pm0.02~\mathrm{bB}$
BRO-G12	3	$0.16\pm0.01~\text{cC}$	$0.34\pm0.01~\text{cB}$	$3.03\pm0.11bC$	$3.25\pm0.03~aA$
A size of all a stars are	1	$0.32\pm0.01~\text{aB}$	$0.36\pm0.02~\text{cA}$	$3.59\pm0.06~bB$	$2.69\pm0.03~bB$
Acinetobucter sp.	2	$0.27\pm0.02~\mathrm{aC}$	$0.12\pm0.01~\mathrm{cD}$	$7.23\pm0.02~\mathrm{cA}$	$2.85\pm0.04~\mathrm{dA}$
BKO-FI	3	$0.24\pm0.00b\text{D}$	$0.24\pm0.02~dC$	$3.24\pm0.15bC$	$2.90\pm0.05bA$
Comamonas or	1	$0.27\pm0.01~\mathrm{bB}$	$0.35\pm0.02~\text{cB}$	$2.77\pm0.11~\mathrm{cB}$	$2.73\pm0.06~\text{bB}$
Comumonus sp.	2	$0.17\pm0.02~\mathrm{bC}$	$0.23\pm0.04~\mathrm{bC}$	$6.59\pm0.04\mathrm{dA}$	$2.96\pm0.03~\mathrm{cA}$
вко-с5	3	$0.14\pm0.01~dD$	$0.38\pm0.01bA$	$2.47\pm0.19~\text{cC}$	$2.66\pm0.02~\text{cB}$





(b)



(c)

Figure 5. Cont.



Figure 5. Phylogenetic trees of the four selected spoilage microorganisms BRO-C11 (**a**), BRO-G12 (**b**), BRO-F1 (**c**) and BRO-C5 (**d**).

Chlorophyll is an important indicator of plant vital signs in the photosynthesis of plant cells, which can absorb light energy to synthesize carbohydrates, carbon dioxide, and water. Thus, the change of chlorophyll content was considered to be an important factor affecting the quality of broccoli during the preservation. As shown in Table 3, the content of total chlorophyll in broccoli decreased during the storage. After 72 h, the total chlorophyll content in fresh-cut broccoli inoculated with *Pseudomonas* sp. BRO-C11 and *Comamonas* sp. BRO-C5 decreased by 51.9% and 48.1%, respectively, compared with the control. At the end of storage, the total chlorophyll content was decreased by 30.8–66.7% compared with the initial value, but *Acinetobacter* sp. BRO-F1 showed little effect on the change of chlorophyll content compared with the control.

Furthermore, the accumulation of nitrite is another common problem in the process of broccoli becoming putrid. Nitrite can react with amines or amides to form carcinogenic, mutagenic, and teratogenic N-nitroso compounds. High nitrate dietary intake was found to be associated with gastric cancer [29]. In addition, nitrosamine products, once accumulated to a certain amount, likely cause teratogenicity, carcinogenesis and mutagenicity. Therefore, it is of great importance to detect the content of nitrite in vegetables. It can be seen from Table 3 that with the time of storage, the content of nitrite had the law that first it increased, next decreased, then increased again to reach a relatively steady state in most samples. The content of nitrite in newly harvested broccoli was relatively low. During storage, the physiological activities of bacteria might be an important factor leading to the change of nitrite content. Among them, the nitrite in the group inoculated with *Pseudomonas* sp. BRO-C11 reached up to a maximum, 1.61 mg/kg fresh weight, indicating that the *Pseudomonas* spp. could promote the accumulation of nitrite. After 24 h, the nitrite content in broccoli inoculated with Pseudomonas sp. BRO-C11, Pseudomonas sp. BRO-G12 and Acinetobacter sp. BRO-F1 showed downward trends, which may be caused by the activity of microorganisms. Under the metabolism of microorganisms in broccoli, nitrate was greatly reduced to nitrite, and the content of nitrite increased. With the consumption of oxygen, the growth of aerobic bacteria was inhibited, resulting in the activity of nitrate reductase being inhibited, the effect of nitrate reduction to be reduced, and large amounts of nitrite to be decomposed. The nitrite content in the group inoculated with *Pseudomonas* sp. BRO-C11 increased at the end of storage, with significant differences among the other groups (p < 0.05). In addition, Acinetobacter sp. BRO-F1 and Comamonas sp. BRO-C5 had little effects on the content of nitrite.

The effects of spoilage bacteria on the content of MDA in fresh-cut broccoli is shown in Table 3. In all samples, the content of MDA increased first then decreased, and with storage, the content of MDA showed a downward trend, and the final contents were 2.47–4.79 nmol/g fresh weight. The MDA content in all samples reached a maximum at 48 h. In general, *Pseudomonas* spp. accelerated the accumulation of MDA in fresh-cut broccoli, which might be due to that the bacteria gradually infected and destroyed the membrane structure, resulting in the formation of malondialdehyde (MDA).

The effects of bacterial infection on the content of soluble sugar in fresh-cut broccoli are shown in Table 3. The soluble sugar content in fresh-cut broccoli was 2.66 mg/g fresh weight in the initial value. At 48 h of storage, the soluble sugar in broccoli inoculated with *Pseudomonas* sp. BRO-C11 and *Pseudomonas* sp. BRO-G12 increased by 33.08% and 19.17% compared with the initial value. However, there was no significant difference in the content of soluble sugar between the group inoculated with *Acinetobacter* sp. BRO-F1 and the control group. The soluble sugar content in broccoli inoculated with *Pseudomonas* sp. BRO-C11 was significantly increased under the bacterial infection for 48 h, and it displayed an increase of 25.09% compared with control.

4. Discussion

The cutting types had great impacts on the amounts of microorganisms in broccoli but had little impact on the species of microorganisms. The amounts of microorganisms gradually increased and conformed to the S-shaped curve of microbial growth. During storage, the total number of colonies of broccoli treated with different cutting types had the same trend, but the average count of bacteria in broccoli subjected to different cutting types showed a significant difference. The processed fruits and vegetables were more perishable than the raw products from which they were prepared. Cutting of fruits and vegetables might cause mechanical damage, outflow of nutrients, and provide favorable living conditions for microorganisms to grow and multiply. In addition, it increased the chance of contamination of fruits and vegetables and resulted in the microbial spoilage of fruits through the transfer of microflora on the outer surfaces to the interior tissue, where microorganisms had access to nutrient-laden juice. On the other hand, fresh-cut fruits and vegetables lost the barrier and protection of the epidermis, and the surface exposed to the air increased, resulting in the disorder of the original physiological function and the destruction of the self-protection system, so that the resistance of the body decreased, resulting in more microorganisms invading the internal tissues of fruits and vegetables from the wound. The larger the specific surface area formed by cutting treatment, the more serious the microbial infection was, and the worse the storage of fresh-cut products. The resistance of freshcut fruits and vegetables to microbial infection in the environment gradually decreased. However, even in the early storage, the amounts of microorganisms can also be large. There were three main ways fresh-cut fruits and vegetables were shown to be infected by microorganisms before and during storage. Firstly, fresh-cut fruits and vegetables were easily infected by microorganisms living in manure, fertilizer, soil and irrigation water sources during planting or harvest. The microorganisms contained were usually bacteria, molds, yeasts, and actinomycetes [30]. Secondly, in the process of cutting, the main pollution sources of fresh-cut fruits and vegetables were usually from cutting equipment or the environment [31]. Moreover, the cross contamination of microbial communities between fruits and vegetables could accelerate their decay and deterioration. Thirdly, fresh-cut fruits and vegetables were easily infected by microorganisms during storage. The resistance of harvested fruits and vegetables to microbial infection in the environment gradually decreased, and the number of microorganisms on the surface of fruits and vegetables increased.

From this study, the phylogenetic relationships of the 97 bacterial isolates were determined in comparison to type strains with the neighbor-joining method. Among them, *Pseudomonas* spp. were the most predominant and might have the greatest impact on food quality. The physiological properties of *Pseudomonas* spp. were adapted to the environment of vegetables. At the end of storage, the relative abundance of other flora decreased gradually. The main reason was that the growth of dominant bacteria was too fast, which inhibited the growth of other microorganisms. *Pseudomonas* spp. are aerobic, Gram-negative bacteria, and are widely distributed in nature. Different from enzymatic browning of vegetables, *Pseudomonas* spp. mainly cause the tissue of fresh-cut vegetables to become rotten and proved to result in a rapid browning reaction on the surface of fresh-cut vegetables [32]. *Pseudomonas* spp. can utilize organic compounds, oxidize glucose and maltose to produce acid, resulting in the decline of food quality. In addition, *Pseudomonas* spp. can also produce catalase and oxidase, and hydrolyze or lipolyze frozen food, so as to make it corrupt. Moreover, they produced pectolytic enzymes to hydrolyze the cell walls of fruits and vegetables, resulting in rot and deterioration of fruits and vegetables, such as pectolytic enzymes produced in *Pseudomonas viridiflava* and *Pseudomonas chlororaphis*, which caused organoleptic properties to decline as well as spoilage in fresh-cut vegetables [33].

The color of vegetables is considered to be an extremely crucial factor for consumer acceptance, especially the unique green color in vegetables, and the dominant reason for de-greening of broccoli was chlorophyll deterioration. Compared with the control group, the chlorophyll of broccoli infected by bacteria decreased in varying degrees, especially the group inoculated with *Pseudomonas* sp. BRO-C11, *Pseudomonas* sp. BRO-G12 and *Comamonas* sp. BRO-C5, which also indicated that the infected group was more prone to yellowing in the experiment, explaining that bacterial infection promoted the yellowing of broccoli and destroyed the appearance of broccoli. This result may explain the yellowing of fresh-cut broccoli after longer storage periods. In order to maintain the pleasant appearance of broccoli, measures to avoid broccoli being polluted by bacteria, *Pseudomonas* sp. particularly, should be taken.

Agricultural standards clearly stipulate the limit standards of nitrate and nitrite in vegetables. Our research investigated potential correlations between bacterial infection and the formation or depletion of nitrite during storage, and microorganisms in fresh-cut fruits and vegetables were the main cause of nitrite accumulation. With storage, the concentration of nitrite reached a maximum, the nitrite peak, and then began to decrease. Among the microorganisms, *Pseudomonas* sp. BRO-C11 had the most important effects on the content of nitrite in broccoli. In the harvest period, *Pseudomonas* sp. BRO-C11 with nitrate reductase proliferated in large numbers, resulting in the nitrate in fresh-cut broccoli to be reduced to nitrite. However, the growth of aerobic *Pseudomonas* sp. BRO-C11 was inhibited when the oxygen was consumed, resulting in the activity of nitrate reductase to be inhibited, promoting large amounts of nitrite to be decomposed. In general, controlling the content of nitrite and nitrate in vegetables was the key to scientific production and edible vegetables. In the follow-up study, the spoilage bacteria that had important impacts on nitrite content should be strictly controlled.

Malondialdehyde (MDA), as the main product of membrane lipid peroxidation, indicates the integrity of cell membranes. The content of malondialdehyde in plant is positively correlated with the degree of cell membrane damage. In our study, the content of MDA in all samples exhibited approximately the same first increased then decreased trend, and the group inoculated with *Pseudomonas* sp. BRO-C11 and *Pseudomonas* sp. BRO-G12 increased at a higher rate than the control group at 48 h of storage. The results showed that after being infected by bacteria, the permeability of the cell membrane increased, which might be due to the damage by bacteria to plant cells by secreting hydrolases and toxins, resulting in the loss of contained logistics in plant cells. The substance in cells, which was conducive for the growth and reproduction of microorganisms, flowed out due to the rupture of the cell membrane, and further aggravated the damage to the cell membrane, leading to the increase of MDA content. Overall, the content of MDA increased, and the senescence process of broccoli was enhanced after being infected.

Soluble sugar has the function to maintain the turgidity of cells, and it is able to protect the membranes of cells from damage caused by the abiotic stress. In this study, under infection, the soluble sugar content of broccoli inoculated with *Pseudomonas* sp. BRO-C11, *Pseudomonas* sp. BRO-G12 and *Comamonas* sp. BRO-C5 increased at 48 h, which meant that the broccoli could adapt to bacterial infection in the short term. Moreover, the soluble sugar had the function of acting as an osmotic agent, which was expressed directly by the content

in plants. In a word, plants could adapt to the bacterial infection by regulating the content of soluble sugar.

The chlorophyll of the experimental group inoculated with bacteria was decreased, which affected the sensory quality of fresh-cut broccoli. In addition, the content of MDA in broccoli infected by *Pseudomonas* sp. BRO-C11 and *Pseudomonas* sp. BRO-G12 increased rapidly, and broccoli inoculated with *Pseudomonas* sp. BRO-C11 produced more nitrite, indicating that the *Pseudomonas* had great impacts on the quality and safety of vegetables and led to the production of harmful substances in fresh-cut broccoli, which should be paid more attention for food safety.

5. Conclusions

The results showed that cutting types had significant effects on the quantity of microorganisms in fresh-cut broccoli, and the number and growth rate of colonies of broccoli with severe mechanical damage were much higher than those with mild mechanical damage. The ninety-seven strains of spoilage microorganisms in broccoli samples were obtained by isolation. Through 16S rRNA gene sequencing, the main microorganisms in the samples were classified into four phyla: Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes. Furthermore, Proteobacteria was the predominant phylum, and the majority were the Pseudomonas spp. and Acinetobacter spp. Pseudomonas spp. was not only dominant in quantity, but also had great impacts on the quality of broccoli. *Comamonas* spp. and Acinetobacter spp. also affected the quality of broccoli to varying degrees, but received little attention in previous studies. The corruption phenomena included the production of harmful substances, such as nitrite, the consumption of nutrients and active components, and inhibition of key enzyme activities, causing spoilage characteristics such as malodor, softness, and browning. This study provided a foundation for targeted bacteriostasis, delaying the corruption and deterioration of fresh-cut vegetables and prolonging the edible period of broccoli. Therefore, necessary measures, such as ensuring a clean processing environment and storage environment, the addition of antibacterial preservative, and less mechanical damage need to be taken into account to reduce contamination from corrupt microorganisms and ensure the safety and flavor of broccoli.

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References

- Fernández-León, M.F.; Fernández-León, A.M.; Lozano, M.; Ayuso, M.C.; González-Gómez, D. Different postharvest strategies to preserve broccoli quality during storage and shelf life: Controlled atmosphere and 1-MCP. *Food Chem.* 2013, 138, 564–573. [CrossRef] [PubMed]
- Li, Y.; Zhang, T.; Korkaya, H.; Liu, S.; Lee, H.F.; Newman, B.; Yu, Y.; Clouthier, S.G.; Schwartz, S.J.; Wicha, M.S.; et al. Sulforaphane, a dietary component of broccoli/broccoli sprouts, inhibits breast cancer stem cells. *Clin. Cancer Res.* 2010, 16, 2580–2590. [CrossRef] [PubMed]

- 3. Wang, H.; Wang, L.; Cao, L.; Zhang, Q.; Song, Q.; Meng, Z.; Wu, X.; Xu, K. Inhibition of autophagy potentiates the anti-metastasis effect of phenethyl isothiocyanate through JAK2/STAT3 pathway in lung cancer cells. *Mol. Carcinog.* **2018**, *57*, 522–535. [CrossRef]
- Alvarez, M.V.; Moreira, M.; Roura, S.I.; Ayala-Zavala, J.F.; González Aguilar, G.A. Using natural antimicrobials to enhance the safety and quality of fresh and processed fruits and vegetables: Types of antimicrobials—ScienceDirect. In *Handbook of Natural Antimicrobials for Food Safety and Quality*; Woodhead Publishing: Cambridge, UK, 2015; pp. 287–313.
- Barrett, D.M.; Beaulieu, J.C.; Shewfelt, R. Color, flavor, texture, and nutritional quality of fresh-cut fruits and vegetables: Desirable levels, instrumental and sensory measurement, and the effects of processing. *Crit. Rev. Food Sci. Nutr.* 2010, 50, 369–389. [CrossRef]
- 6. Oliveira, M.; Abadias, M.; Usall, J.; Torres, R.; Teixid, N. Application of Modified Atmosphere Packaging as a safety approach to fresh-cut fruits and vegetables—A review. *Trends Food Sci. Tech.* **2015**, *46*, 13–26. [CrossRef]
- 7. Wang, C.W.; Wang, Y. First report of postharvest fruit rot caused by Fusarium equiseti on stored cerasus Pseudocerasus in China. *Plant. Dis.* **2017**, *101*, 1451. [CrossRef]
- 8. Dakwa, V.; Powell, S.; Eyles, A.; Gracie, A.; Tamplin, M.; Ross, S. Effect of peroxyacetic acid treatment and bruising on the bacterial community and shelf-life of baby spinach. *Int. J. Food Microbiol.* **2021**, *343*, 109086. [CrossRef]
- Bah, A.; Ferjani, R.; Fhoula, I.; Gharbi, Y.; Najjari, A.; Boudabous, A.; Ouzari, H.I. Microbial community dynamic in tomato fruit during spontaneous fermentation and biotechnological characterization of indigenous lactic acid bacteria. *Ann. Microbiol.* 2019, 69, 41–49. [CrossRef]
- Voidarou, C.; Alexopoulos, A.; Tsinas, A.; Rozos, G.; Tzora, A.; Skoufos, I.; Varzakas, T.; Bezirtzoglou, E. Effectiveness of bacteriocin-producing lactic acid bacteria and bifidobacterium isolated from honeycombs against spoilage microorganisms and pathogens isolated from fruits and vegetables. *Appl. Sci.* 2020, *10*, 7309. [CrossRef]
- 11. Maffei, D.F.; Batalha, E.Y.; Landgraf, M.; Schaffner, D.W.; Franco, B.D. Microbiology of organic and conventionally grown fresh produce. *Braz. J. Microbiol.* **2016**, *47*, 99–105. [CrossRef]
- 12. Barbosa, A.A.T.; Mantovani, H.C.; Jain, S. Bacteriocins from lactic acid bacteria and their potential in the preservation of fruit products. *Crit. Rev. Biotechnol.* **2017**, *37*, 852–864. [CrossRef] [PubMed]
- 13. Stoll, D.A.; Müller, A.; Meinhardt, A.K.; Dötsch, A.; Greiner, R.; Kulling, S.E.; Huch, M. Influence of salt concentration and iodized table salt on the microbiota of fermented cucumbers. *Food Microbiol.* **2020**, *92*, 103552. [CrossRef] [PubMed]
- 14. Nahidul-Islam, S.M.; Takashi, K.; Hajime, T.; Bon, K. Bacterial and fungal microbiota in traditional Bangladeshi fermented milk products analysed by culture-dependent and culture-independent methods. *Food Res. Int.* **2018**, *111*, 431–437. [CrossRef]
- Rocha, K.E.M.; Milani, C.; Ventura, M.; Valencia, F.E.; Madiedo, R.; Rusa-Madiedo, P.; Delgado, S. Bacterial diversity of the Colombian fermented milk "Suero Costeño" assessed by culturing and high-throughput sequencing and DGGE analysis of 16S rRNA gene amplicons. *Food Microbiol.* 2017, 68, 129–136.
- Liang, H.; Yin, L.; Zhang, Y.; Chang, C.; Zhang, W. Dynamics and diversity of a microbial community during the fermentation of industrialized Qingcai paocai, a traditional Chinese fermented vegetable food, as assessed by Illumina MiSeq sequencing, DGGE and qPCR assay. *Ann. Microbiol.* 2018, 68, 111–122. [CrossRef]
- 17. Li, R.; Cai, L.; Gao, T.; Li, C.; Ye, K. Comparing the quality characteristics and bacterial communities in meatballs with or without blown pack spoilage. *LWT Food Sci. Technol.* **2020**, *130*, 109529. [CrossRef]
- Li, W.J.; Xu, P.; Schumann, P.; Zhang, Y.Q.; Pukall, R.; Xu, L.H.; Stackebrandt, E.; Jiang, C.L. *Georgenia ruanii* sp. nov. a novel actinobacterium isolated from forest soil in Yunnan (China), and emended description of the genus *Georgenia*. *Int. J. Syst. Evol. Microbiol.* 2007, 57, 1424–1428. [CrossRef]
- 19. Yoon, S.H.; Ha, S.M.; Kwon, S.; Lim, J.; Chun, J. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1613–1617. [CrossRef]
- 20. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425.
- 21. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef]
- 22. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985, 38, 14–26.
- 23. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods Enzymol.* **1987**, 148, 350–382.
- 24. Fang, H.; Luo, F.; Li, P.; Zhou, Q.; Zhou, X.; Wei, B.; Cheng, S.; Zhou, H.; Ji, S. Potential of jasmonic acid (JA) in accelerating postharvest yellowing of broccoli by promoting its chlorophyll degradation. *Food Chem.* **2020**, *309*, 125737. [CrossRef] [PubMed]
- 25. Nerdy, N.; Putra, E. Spectrophotometric method for determination of nitrite and nitrate levels in broccoli and cauliflower with different fertilization treatment. *Orient J Chem.* **2018**, *34*, 2983–2991. [CrossRef]
- 26. An, F.; Sun, H.; Wu, J.; Zhao, C.; Li, T.; Huang, H.; Fang, Q.; Mu, E.; Wu, R. Investigating the core microbiota and its influencing factors in traditional Chinese pickles. *Food Res. Int.* **2021**, *147*, 110543. [CrossRef]
- 27. Liu, H.; Jiang, W.; Cao, J.; Ma, L. A combination of 1-methylcyclopropene treatment and intermittent warming alleviates chilling injury and affects phenolics and antioxidant activity of peach fruit during storage. *Sci. Hortic.* **2018**, 229, 175–181. [CrossRef]
- 28. Fairbairn, N.J. A modified anthrone reagent. Chem. and Ind. 1953, 4, 86.
- 29. Rezaei, M.; Fani, A.; Moini, A.L.; Mirzajani, P.; Malekirad, A.A.; Rafiei, M. Determining Nitrate and Nitrite content in beverages, fruits, vegetables, and stews marketed in Arak, Iran. *Int. Sch. Res. Not.* **2014**, 2014, 1–5. [CrossRef]

- Devleesschauwer, B.; Marvasi, M.; Giurcanu, M.C.; Hochmuth, G.J.; Speybroeck, N.; Havelaar, A.H.; Teplitski, M. High relative humidity pre-harvest reduces post-harvest proliferation of Salmonella in tomatoes. *Food Microbiol.* 2017, 66, 55–63. [CrossRef]
- 31. Shen, C.; Rao, J.; Wu, Q.; Wu, D.; Chen, K. The effect of indirect plasma-processed air pretreatment on the microbial loads, decay, and metabolites of Chinese bayberries. *LWT Food Sci. Technol.* **2021**, *150*, 111998. [CrossRef]
- Pinto, L.; Ippolito, A.; Baruzzi, F. Control of spoiler *Pseudomonas* spp. on fresh cut vegetables by neutral electrolyzed water. *Food Microbiol.* 2015, 50, 102–108. [CrossRef] [PubMed]
- 33. Dong, H.L.; Kim, J.B.; Kim, M.; Roh, E.; Jung, K.; Choi, M.; Changsik, O.H.; Choi, J.; Yun, J.; Heu, S. Microbiota on spoiled vegetables and their characterization. *J. Food Prot.* **2013**, *76*, 1350–1358.