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A Double-Edged Sword of Surfactant Effect on Hydrophobic Surface Broccoli Leaf as a Model Plant: Promotion of Pathogenic Microbial Contamination and Improvement to Disinfection Efficiency of Ozonated Water

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Abstract: Pathogenic microbial contamination is significantly influenced by the crop surface properties and surfactant use, which are crucial factors for the postharvest washing process. However, there is little information on the interaction between surfactant and food pathogens on food crops. Thus, this study (1) investigated whether the attachment of *Salmonella* increases as pesticides denature epicuticular wax crystals and (2) tested if the antibacterial effect of ozonated water can be improved on waxy produce surfaces by adding surfactant to ozonated water. As a result, significantly lower levels of *Salmonella* Typhimurium attached to waxy leaf surfaces than they did to glossy and pesticide-treated waxy leaf surfaces (3.28 as opposed to 4.10 and 4.32 Log colony forming units (CFU)/cm^2, respectively), suggesting that the pesticide containing a surfactant application increased the attachment of *S.* Typhiumurium on waxy leaf surfaces. There was no significant washing effect on waxy leaf surfaces washed with ozonated water. On the other hand, *S.* Typhimurium were not detected on waxy leaf surfaces after washing with surfactant-added ozonated water.

Keywords: epicuticular wax; microbial attachment; food safety; food-borne disease; cross-contamination; eco-friendly disinfection method

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1. Introduction

Salmonella enterica along with *Escherichia coli* O157: H7 are major causes of the food-borne diseases associated with fresh produce [1–3]. In the United States, produce (fresh, canned, or processed) is estimated to cause 20 million cases of illness (24%), costing USD 38.6 billion every year [4]. In particular, produce is estimated to be associated with 27% and 37% of *Salmonella* outbreaks and cases, respectively [4]. Salad vegetables are a type of produce that are susceptible to *Salmonella* contamination [5–7]. Leafy salad greens are classified as the second most frequent source of food-borne infections within the European Union, while *Salmonella* is associated with 30% of outbreaks associated with fresh produce [8].

Pathogenic microbial contamination on fresh produce can occur during pre- or postharvest stages [9,10]. Preharvest sources that contaminate field-grown fresh produce include irrigation water, soil, raw or inadequately composted manure, and wild or domestic animals [11]. Out of these factors, pathogens from irrigation water and soil are the primary sources for contamination due to their ability to survive for a long time, even during postharvest storage [12]. Notably, the rate of contamination via irrigation water is closely related to plant surface properties such as wettability [13]. One important property of some plants, such as apple, blueberry, broccoli, cabbage, and collard, is the presence of

an epicuticular wax layer; their leaf surfaces are coated with wax crystals, making them hydrophobic. The structure of these wax crystals and the concentration of epicuticular wax directly affect wettability [13]. Furthermore, the wax crystals minimize contact with water, reducing the adsorption of *Salmonella* via this medium. Additionally, investigations into bacterial attachment on the surface of lettuces and a near-isogenic line of broccoli concluded that waxy surfaces significantly decreased *Salmonella* attachment at harvest and during postharvest storage [14,15].

Although epicuticular waxes tend to reduce *Salmonella* attachment, hydrophobic surfaces are not beneficial for pesticide administration or foliar application of nutrients. Thus, pesticides require added surfactant in order to increase their efficiency when applied to certain crops with hydrophobic surfaces. Such surfactants may increase bacterial adhesion rates, leading to food-borne illness, as pesticidal surfactants significantly change the wettability of plant surfaces. The attachment of *Salmonella* varies depending on the surface properties of the produce [14–16]. It is therefore critical to understand the processes involved in *Salmonella* attachment to waxy plant surfaces after pesticide application. However, changes in *Salmonella* contamination due to pesticide application have not been studied to date.

One of the microbial contamination sources that occurs postharvest is cross-contamination, which occurs during the washing process of contaminated and non-contaminated produce together or when reusing a washing solution with residual pathogens [17]. For this reason, it has been argued that disinfection strategy should focus on preventing cross-contamination in the washing tank [18]. Several researchers [19–21] have highlighted the importance of the safety of washing solution (i.e., without residual pathogens) to the prevention to cross-contamination rather than the application of disinfectants against pathogens attached to produce surfaces.

Ozonated water, a low-cost oxidizing sanitizer and efficient antibacterial agent, is useful for the disinfection of fresh produce. Ozone dissolved in water easily decomposes and does not deposit toxic residues. Ozonated water (1–3 mg/L) shows a one hundred times higher lettuce disinfection rate when compared with that of chlorine 100 mg/L [22]. In addition, the combination of ozonated water and organic acids enhances disinfection efficiency [23,24]. To date, most studies regarding disinfection of fresh produce using ozonated water have focused on its efficiency and parameters such as time, temperature, and pH [25–27]. Xu et al. [26] showed that washing efficiency of ozonated water differed for green onions, grape tomatoes, and green leaf lettuces, suggesting that different surface structures influence the disinfectant properties of ozonated water. Many commonly consumed vegetables including apple, blueberry, broccoli, cabbage, and collard have hydrophobic surfaces owing to their epicuticular waxes. For these produce types, although ozonated water is expected to have an insignificant effect on disinfection rates, no studies have been undertaken to confirm this.

We hypothesized that pesticidal surfactants applied to waxy produce during preharvest significantly increase *Salmonella* attachment by changing the epicuticular wax structure. If this hypothesis is correct, the antibacterial effect of ozonated water can be improved for such produce by adding surfactant to ozonated water in the postharvest washing process. Therefore, the aims of this study were to determine whether the attachment of *Salmonella* increases as pesticides (surfactant) denature epicuticular wax crystals and to ascertain if the antibacterial effect of ozonated water on waxy produce can be improved by adding surfactant. A near-isogenic line of broccoli was chosen to investigate the effects of surfactant on epicuticular wax surfaces.

2. Materials and Methods

2.1. Near-Isogenic Broccoli: Greenhouse Production and Phenotype Description

Seeds of near-isogenic line (NIL) broccoli, USVL188-GL (glossy) and USVL188-NG (non-glossy, waxy), were obtained from the U.S. Department of Agriculture's Agricultural Research Service (USDA-ARS, U.S. Vegetable Laboratory, Charleston, South Carolina) for

use in this study. These lines were chosen because with the exception of the presence or absence of epicuticular waxes they are highly phenotypically homogeneous [28]. The seeds were planted in 50-cell plant plug trays and germinated with Bio Soil (Heungnong Seed, Imsil, Republic of Korea). Four weeks after germination, the seedlings were transferred to 1 L plant pots; two weeks later, they were transferred to 12 L pots. The plants were grown in the greenhouse at Chonnam National University under a night (1 °C) and day (2 °C) time air temperature regime. Conventional fertilizer (Novatec Supreme N-P-K: 21-5-10, Münster, Germany) was used as needed to supply basic nutrients. To induce sufficient epicuticular wax concentration for study purposes, the plants were moved to a dry room (30–35% relative humidity (RH), 2 °C) and grown under LED lighting (6000 lux) for two weeks. At that stage, broccoli had 12–15 leaves.

2.2. Scanning Electron Microscope (SEM) Imaging of Leaf Surfaces

Waxy and glossy broccoli leaf samples were freeze-dried before using a Cressington 108 Auto Sputter Coater (Cressington, Watford, UK) to sputter-coat them with platinum ions for 180 s at a sputter set-point of 20 mA. Images of the leaves' surfaces were captured using a Gemini 500 Field Emission Scanning Electron Microscope (FE-SEM; Zeiss, Oberkochen, Germany) at 10 KV accelerating voltage. Resolutions of 1000 and 5000× resolutions were used in order to observe the difference in epicuticular waxes between the two leaf types. Images were taken at 1000 × resolution for observations of waxy surfaces before and after surfactant treatment.

2.3. Leaf Disk Preparation and Spraying of Pesticidal Surfactant

Leaf disks were prepared as per the published method [14] and uneven leaf surfaces were avoided to improve experiment reproducibility. Broccoli leaf was used as a model system to test the effect of epicuticle waxes on *Salmonella* attachment and for ozonated water treatment. Broccoli belonging to *Brassica oleracea* has more epicuticular wax content than that of other fruits and vegetables [29]. Although broccoli leaf is not a popular leafy vegetable and is usually considered as a non-edible part, other *Brassica oleracea* vegetables including kale, cabbage, and collard are frequently used for salads and belong to the same species. Medium sized-leaves were selected and cut with a cork-borer (diameter: 24 mm) to obtain six leaf disks from each waxy and glossy broccoli plant. One broccoli plant was considered as a biological replicate in this study. Pesticide for cabbage lopper and white cabbage caterpillar (0.1% v/v; Captain; Kyungnong, Seoul, Republic of Korea) and surfactant (0.1% *w*/*w*; Tween 20; Sigma-Aldrich, St Louis, MO, USA) were sprayed on each adaxial leaf surface using a sprayer. Spraying continued until the leaf surface was completely wet. Each leaf was then dried for 30 min in a biosafety cabinet (Figure 1).

2.4. Quantification and Composition of Epicuticular Wax

First, 5 mL of chloroform was added into a 20 mL glass vial (Fisher Scientific, Waltham, MA) and each leaf disk was placed in chloroform for 5 s to extract the epicuticular wax. For epicuticle wax quantification, 3 waxy leaves and 15 glossy leaves were used for one biological replication. Following this, the organic solvent was evaporated in a nitrogen stream heated to 40 °C. After drying, 1 mL of 50 mg/L n-tetracosane (internal standard) in hexane was added to reconstitute the wax extraction. The extract (0.3 mL per vial) was then transferred to Reacti™-vials (Thermo Fisher Scientific Inc., Waltham, MA) and subsequently evaporated under a gentle stream of nitrogen. The extract was then redissolved in a mixture of 150 µL bis-N, N- (trimethylsilyl) trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS; Sigma-Aldrich) for derivatization. The vials were incubated at 75 °C for 70 min before the extract was injected into a gas chromatograph (Nexis GC-2030, Shimadzu, Japan) coupled to a GC-MS (GCMS-QP 2020 NX, Shimadzu, Japan) for quantification. A capillary column (Rxi-5Sil MS, Restek, Bellefonte, PA, USA; 30 m, 0.25 mm, 0.25 m size with 10 m Integra-Guard Column) was used for separation. Oven temperature was initially held at 150 °C for 1 min, then increased by 12 °C/min to reach 300 °C, and

held for 7 min. Both injector and detector temperatures were set to 270 °C. The flow rate of the helium carrier gas was 1.2 mL/min. The following mass spectrophotometry parameters were employed: inlet temperature, 250 °C; ion source temperature, 300 °C; mass scan range was from 40 to 500.

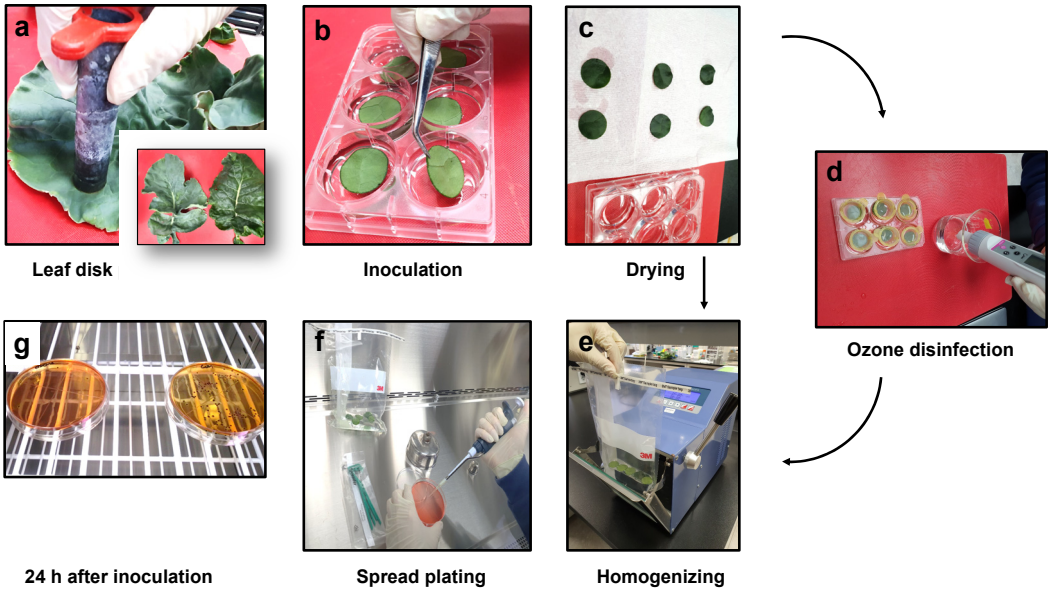

Figure 1. Leaf disk preparation and *S.* Typhimurium inoculation for quantification.

Compound identification was based on GC-MS analysis and the signature mass fragments of common *Brassica oleracea* wax components [30,31] in addition to the retention time of authentic standard compound injection in GC-MS. The C7-C40 saturated alkanes standard mixture (Supelco®, Bellefonte, PA, USA) was used for the standard compounds. Other compounds were identified using a signature mass fragment based on the previous literature and quantified with the above-listed authentic standards: C29 β-ketols and C29 ketones were quantified by C29 alkane.

2.5. Contact Angle Measurement

Six leaf disks (three waxy, three surfactant-treated waxy) were prepared to analyze contact angles between the leaf surfaces and water droplets. Each leaf disk was fixed flat to a table with tape. Distilled water (5 μL) was then dropped onto each leaf disk using a pipette. The contact angle was measured within 10 s of water application using the Angulus application for Android (Hanover, NH, USA).

2.6. S. Typhimurium Inoculation and Colony Number

The strain of *S.* Typhimurium used in this study was isolated from contaminated water in the greenhouse at Chonnam National University. The strain was identified by SolGent (Daejeon, Republic of Korea) using 16S rRNA, then selected from a single colony cultured on XLT-4 agar (35 °C, 24 ± 2 h; Sigma-Aldrich), and subsequently sub-cultured (0.1 mL, 35 °C, 24 ± 2 h) in buffered peptone water (BPW; Sigma-Aldrich). Bacterial cells were harvested by centrifugation at 5000 × *g* for 15 min. After supernatant removal, the cells were suspended in 10 mL of 0.1% BPW. This suspension was diluted in 100 mL of 0.1% BPW to adjust the concentration to approximately 7.5 Log colony forming units (CFU)/mL.

Broccoli leaf disks were placed on a sterile six-well cell culture plate containing 5 mL *S.* Typhimurium inoculum for 5 min. Although the 5 min inoculation time used in this study is relatively short compared to that used in other studies [32,33], it was considered necessary in order to ensure that *S.* Typhimurium adhesion resulted from the surface

wettability of the leaves and not from absorption. Leaves inoculated with *S.* Typhimurium were taken from the six-well plate and placed in a biosafety cabinet for 30 min to dry.

After inoculation and drying, each leaf disk was placed in an individual sterile filter bag (3 M, St Paul, MN USA) containing 200 mL of 0.1% BPW and then was mechanically homogenized at 10 times per second for 4 min using a stomacher (Biobase, Shangdong, China). Subsequently, the suspensions in the filter bags were serially diluted 10-fold in 0.1% BPW and spread plated on XLT-4 agar. The XLT-4 agar plates were incubated at 35 °C for 24 h following which the *S.* Typhimurium colonies were manually counted.

2.7. Ozonated Water Preparation

Ozone was generated using a microplasma ozone generator (MDP-1, CAST, Seoul, Korea). Oxygen prepared in an oxygen generator was introduced via a tube into the ozone generator and converted into ozone using the microplasma discharge method. The subsequent ozone gas was introduced via a tube into a tank of tap water and dissolved. Ozonated water has a short half-life; therefore, it is difficult to measure dissolved ozone concentration accurately. The indigo method [34] can accurately measure this parameter, but it is difficult to employ rapidly during experimentation. Thus, the dissolved ozone concentration in this study was estimated using data observed via the portable ozone meter (DOZ30, Clean Instruments, Shanghai, China). A regression model derived from both the portable ozone meter and the indigo method was developed and used to estimate the concentration level ($R^2 = 0.98$; $p < 0.01$).

2.8. Ozonated Water Treatment on Various Leaf Surfaces

Each leaf disk was subjected to the inoculation procedure described previously. Subsequently, ozonated water and ozonated water with 0.1% surfactant (Tween 20) were prepared using an ozone generator (MDP-1, CAST). Surfactant was also prepared as a washing solution. Using a cell strainer, inoculated and dried leaf disks were immersed on a sterile six-well plate containing 10 mL of each washing solution for 180 ± 5 s. Given the short half-life of ozonated water, the dissolved ozone concentration was measured at two different points in time. The initial concentration comprised averages of concentrations recorded before and after leaf disk immersion and the final concentration comprised concentration averages from before and after taking the leaf disks out. According to these calculations, 2.8 mg/L was used in the ozonated water treatment. The tap water in which the ozone was dissolved was maintained at 17 °C. After treatment, leaf disks were taken from the ozonated water, placed in separate sterile filter bags containing 200 mL of 0.1% BPW, and mechanically homogenized at 10 times per second for 4 min using a stomacher. The suspended leaf disks were then serially diluted 10-fold in 0.1% BPW and enumerated on XLT-4 agar after spread plating. *S.* Typhimurium is captured less frequently among waxy leaf disks than among glossy leaf disks [15]. Therefore, in the treatment with surfactant (Tween 20), spread plating was performed without dilution in order to make it easier to count colony numbers. The XLT-4 agar plates were incubated at 35 °C for 24 h and *S.* Typhimurium colonies were manually counted. To clarify the antibacterial performance, the surfactant used in the disinfection treatment was enumerated on the XLT-4 agar after spread plating. In the cases of ozonated water and ozonated water with surfactant, the washing solution was cultured (0.1 mL, 35 °C, 24 ± 2 h) in BPW and enumerated on XLT-4 agar after spread plating. The XLT-4 agar plates were incubated at 35 °C for 24 h and S. Typhimurium colonies were manually counted.

2.9. Statistical Analysis

All experiments were repeated three times. Statistical analyses were conducted to compare group means, with significant differences (p <0.05) between group means determined using the Student's *t*-test using Excel (Microsoft, Redmond, WA, USA).

3. Results and Discussion

3.1. Images of Scanning Electron Microscope and Wax Quantification from Waxy and Glossy Near Isogenic Broccoli Lines

As shown in Figure 2, between the two NIL broccoli lines (waxy and glossy), there was a significant difference in the three-dimensional epicuticular wax crystals observed on the leaf surfaces. While many epicuticular wax crystals were visible on the waxy leaves, there were few visible crystals on the glossy leaves. The concentration of epicuticular wax on waxy leaves (111.2 μg/cm^2) was significantly higher ($p < 0.05$) than on glossy leaves (10.4 μg/cm^2). These differences in the quantitative and visible levels of epicuticular wax are similar to those previously reported [15]. Therefore, NIL broccoli lines, which are highly phenotypically homogeneous with the exception of the presence or absence of epicuticular waxes[28], are suitable as a model plant for evaluating the effect of three-dimensional epicuticular wax.

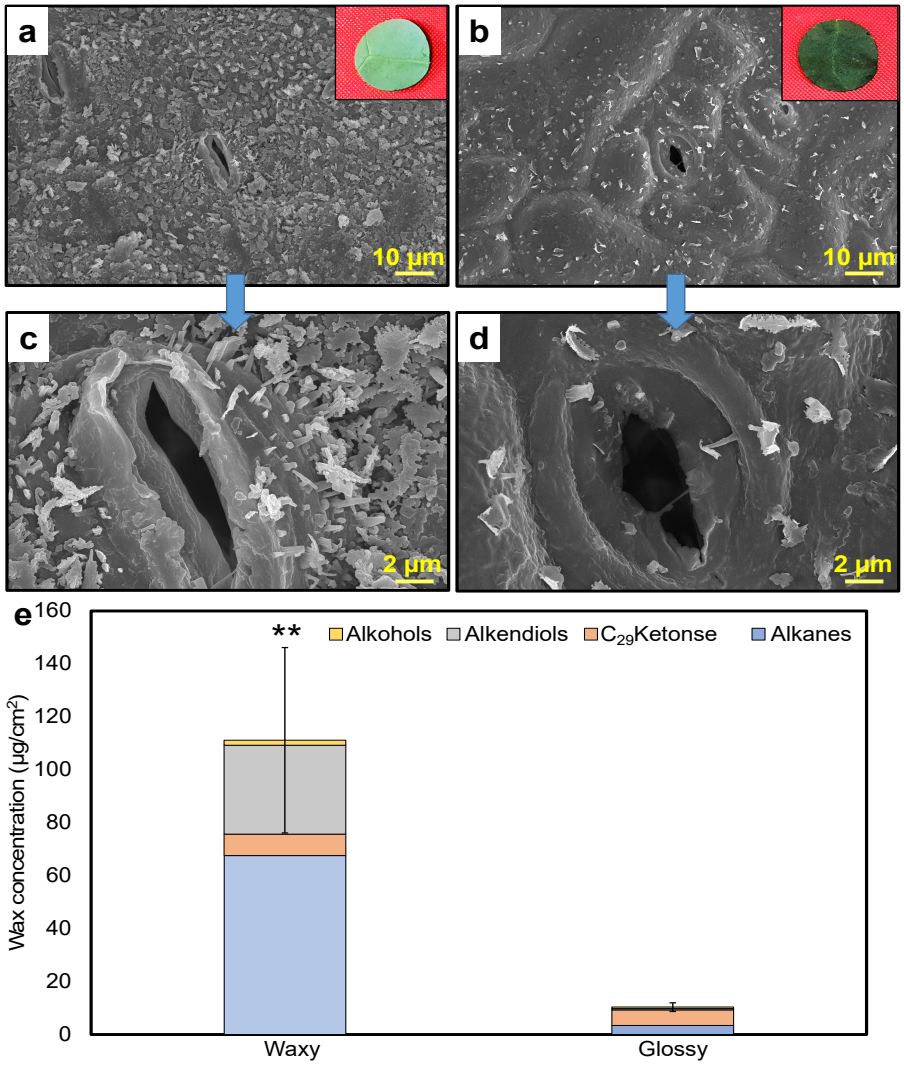

Figure 2. (**a**–**d**) Scanning electron microscope images of near isogenic broccoli lines; (**a**,**c**): USVL 188-NG (non-glossy; waxy), (**b**,**d**): USVL 188-GL (glossy). (**a**,**b**) were taken at 1000× resolution, (**c**,**d**) were taken at 5000× resolution using FE-SEM. Inner pictures of (**a**,**b**) are typical leaf phenotypes. The above images were chosen from three biological replications. (**e**) total epicuticular wax concentrations on leaves of near-isogenic broccoli USVL 188 lines, quantified using a GC-MS system. The asterisks above the bars indicate a significant difference between each group. ** indicates significant difference at $p < 0.01$ by Student's *t*-test. Error bars indicate the standard deviation of total epicuticular wax concentrations from three replicates (n = 3).

3.2. Pesticide Effects on S. Typhimurium Attachment to Waxy Leaves

As shown in Figure 3, the attachment of *S. Typhiumurium* on three different leaf surfaces (USVL-188 NG, USVL-188 GL, and the pesticide-treated USVL-188 NG) was 3.28, 4.10, and 4.32 Log CFU/cm^2, respectively. The attachment of *S. Typhiumurium* on glossy leaves was significantly higher ($p < 0.05$) than on waxy leaves. These results are similar to those reported in previous studies [15] and suggest that the presence of three-dimensional epicuticular wax crystals reduces the attachment of *Salmonella* on plant surfaces. Our pesticide-treated waxy leaf surfaces aimed to mimic the pesticide spraying of conventional farming and had a significantly higher ($p < 0.05$) *S.* Typhimurium attachment rate compared to that of untreated waxy leaves. These findings suggest that pesticidal surfactant, which reduces the hydrophobicity of waxy leaf surfaces to increase pesticide efficiency, may facilitate *S.* Typhimurium adhesion by increasing waxy leaf surface wettability. Preharvest contamination of produce often occurs from contact with contaminated irrigation water [11]. Our results therefore imply that pesticide use in conventional agriculture may be directly related to the adsorption of contaminated irrigation water onto the hydrophobic surfaces of produce such as broccoli. Consequently, although pesticide application on waxy leaves improves produce quality through pest control, it can also promote *Salmonella* attachment on waxy leaves by affecting epicuticular wax properties.

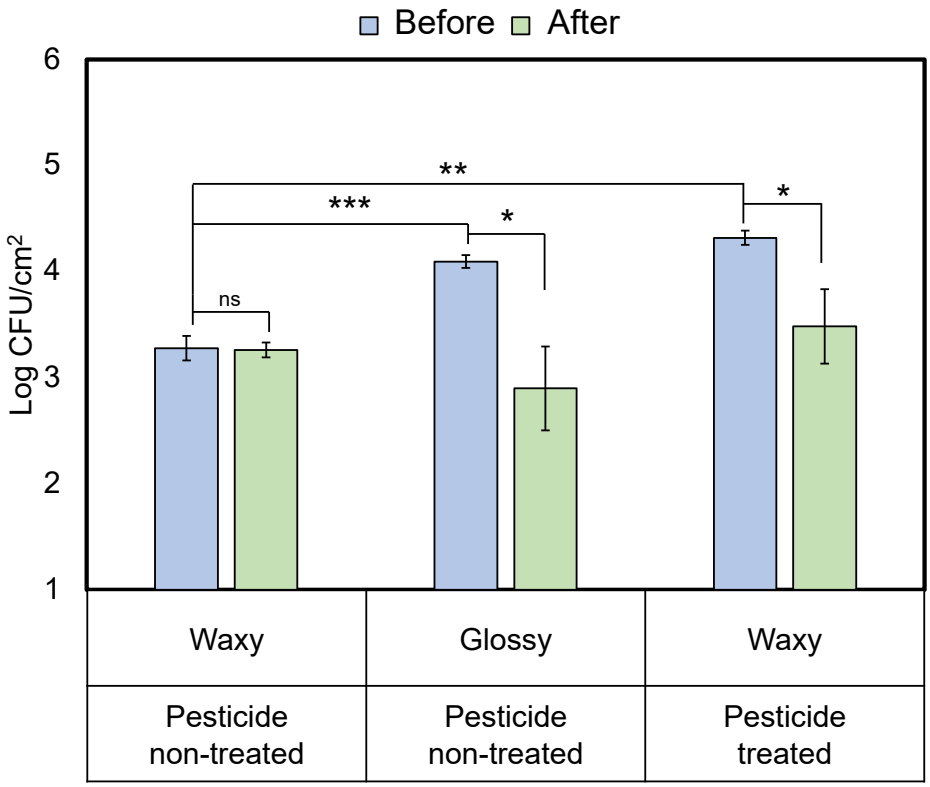

Figure 3. The number of *S.* Typhimurium before and after ozonated water (2.8 mg/L) treatment. Each leaf disk (diameter: 24 mm) was inoculated with 7.5 Log colony forming units (CFU)/mL *Salmonella* inoculum for 5 min and dried for 30 min. The *Salmonella*-inoculated each leaf disk was immersed with ozonated water for 180 ± 5 s. The asterisks above the bars indicate a significant difference between each group. ns, *, **, and *** correspond to non-significance, significant difference at $p < 0.05$, 0.01, and 0.001 by Student's T-test, respectively. Error bars indicate the standard deviation of *Salmonella* counts from three replicates. Six leaves were used for one biological replication.

3.3. Efficiency of Ozonated Water on Various Leaf Surfaces

A disinfection assay was conducted to compare the disinfection efficiency of ozonated water on leaf surfaces with different surface compositions and to examine the relation

between efficiency of ozonated water and epicuticular wax function. Figure 3 illustrates the number of *S.* Typhimurium present after washing NIL broccoli leaves (USVL-188 NG, USVL-188 GL, and pesticide-treated USVL-188 NG) with ozonated water (2.8 mg/L). The inactivation of *S.* Typhimurium by ozonated water varied with leaf surface conditions. Ozonated water treatment resulted in a significantly higher ($p < 0.05$) reduction in *S.* Typhimurium count on glossy leaves: from 4.10 to 2.90 Log CFU/cm^2. As observed in lettuce [35], bacterial inactivation by ozonated water was observed in glossy broccoli leaves but not on waxy leaves. There was no significant difference in *S.* Typhimurium counts before and after washing waxy leaves with ozonated water (a reduction from 3.28 to 3.26 Log CFU/cm^2). These results suggest that disinfection using ozonated water is significantly more effective for glossy leaves than for waxy leaves.

Ozonated water inactivates bacteria such as *Escherichia coli* O157:H7 and *Salmonella enterica* in both lettuce and tomato crops and inhibits the growth of bacteria on lettuce during storage at a lower concentration than is required with chlorinated water [35]. Despite its antibacterial efficacy, the effect of ozonated water on hydrophobic surfaces (waxy leaves) in this study was insignificant. Plants with three-dimensional epicuticular wax crystals, such as broccoli, are strongly hydrophobic [13], and the disinfection efficacy of ozonated water is known to be affected by the different surface structures of fresh produce [26]. In this context, it is possible that the limited antibacterial effect of ozonated water on waxy leaf surfaces is due to the presence of three-dimensional epicuticular wax crystals. Waxy leaves were less susceptible to *S.* Typhimurium contamination than were glossy leaves. However, after contamination, the disinfection efficiency of ozonated water was lower on waxy surfaces compared to that of glossy surfaces. As a result of the high viability of *S.* Typhimurium on waxy leaves, the application of ozonated water to a hydrophobic surface would not be an effective washing method when the contact angle between a water droplet and the surface is greater than 90° [13].

S. Typhimurium populations on pesticide-treated waxy leaf surfaces were significantly ($p < 0.05$) decreased from 4.32 to 3.48 Log CFU/cm^2 when treated with ozonated water, which is notable when compared with untreated waxy leaves. Although applying a pesticide to waxy leaves makes them vulnerable to *S.* Typhimurium adhesion, it also promotes a hydrophilic surface, improving the antibacterial activity of ozonated water. Our results indicate that the surface properties of waxy leaves are changed from hydrophobic to hydrophilic through pesticide application. This improves the washing efficiency of ozonated water and results in waxy leaves with properties similar to those of glossy leaves. Therefore, since pesticide application can increase the attachment of pathogens through irrigation, it would be desirable to irrigate crops with hydrophobic surfaces including broccoli, cabbage, kale, and collard in a manner that minimizes leaf contact, such as surface irrigation.

3.4. Surfactant Effects on Leaf Surface Properties

To confirm that the pesticide effects on *S.* Typhimurium attachment to waxy leaves is due to surfactant, the attachment of *S.* Typhimurium was also evaluated for waxy leaf surfaces treated with a surfactant (Tween 20). As shown in Figure 4a, surfactant-treated waxy leaf surfaces resulted in a significantly ($p < 0.05$) higher attachment (3.6 Log CFU/cm^2) when compared with waxy leaf surfaces (2.91 Log CFU/cm^2). This suggests that surfactant was a major factor affecting *S.* Typhimurium adhesion after pesticide application. We hypothesize that these results are due to the surfactant altering the morphology of waxy leaf surfaces, thereby reducing the contact angle.

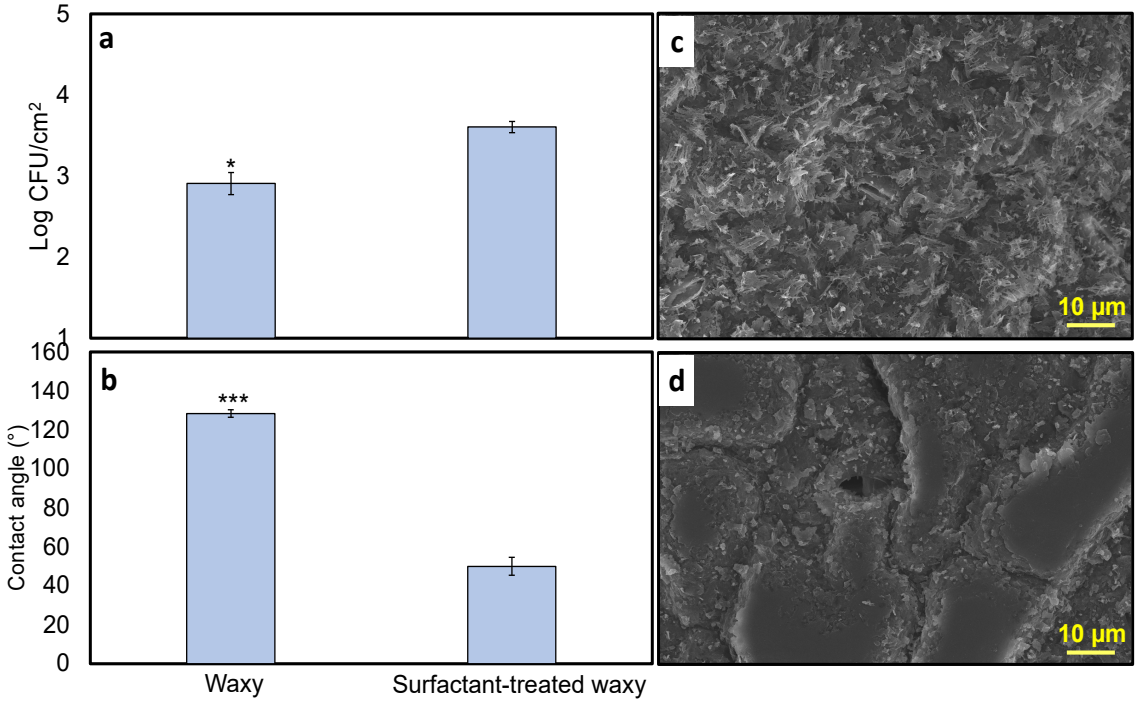

Figure 4. (**a**) The number of attached *S*. Typhimurium, (**b**) contact angle, and (**c**,**d**) scanning electron microscope images of waxy (non-treated) and surfactant-treated waxy leaves. In the experiment of (a), each leaf disk was inoculated with 7.5 Log CFU/mL *Salmonella* inoculum for 5 min and dried for 30 min. In the experiment of (**b**), the contact angle was measured by dropping distilled water (5 µL) onto each leaf surface and using the Angulus application. The asterisks above the bars indicate a significant difference between each group. * and *** correspond to a significant difference at $p < 0.05$ and 0.001 by Student's *t*-test, respectively. Error bars indicate the standard deviation of (**a**) contact angle and (**b**) *Salmonella* counts from three replicates. In the experiment of (**c**,**d**), (**c**): waxy, (**d**): surfactant-treated waxy. (**c**,**d**) were taken at 1000× resolution using FE-SEM. The above SEM images were selected as representative ones from three biological replications.

As shown in Figure 4b, the contact angle of waxy leaf surfaces was significantly ($p < 0.05$) reduced from 128.4° to 50° by surfactant treatment. Additionally, in surfactant-treated waxy leaves, epicuticular wax crystals were denatured (Figure 4c,d) while wax crystals on untreated waxy leaves were intact. This is possibly due to the surfactant causing re-coagulation of wax crystals and subsequent flattening of the waxy leaf surface. These results suggest that the attachment of *S*. Typhimurium is increased by the surfactant-induced change in surface morphology of waxy leaves.

As epicuticular wax crystals improve pathogen resistance by creating water repellent plant surfaces, it is important for them to function correctly [13]. Since pesticidal surfactants reduce the hydrophobicity of waxy leaves, excessive application of pesticides may increase the risk of food-borne pathogens being transferred through contaminated irrigation water, in cases where postharvest washing is insufficient. Therefore, understanding the interaction between surfactants and epicuticular wax crystals is important to predict and prevent produce contamination in the field.

3.5. Efficiency of Surfactant-Enhanced Ozonated Water on Waxy Leaves

In order to determine ways of improving disinfection strategies, we evaluated the antibacterial performance of ozonated water by adding surfactant on waxy leaves. As shown in Figure 5, the difference in the efficiency of ozonated water (2.8 mg/L) with and without surfactant was dramatic. *S*. Typhimurium populations were detected at similar levels on both unwashed and ozonated-water washed waxy leaves, with no significant reduction in population for washed leaves (2.91 to 2.86 Log CFU/cm^2). In contrast, *S*. Typhimurium were not detected on waxy leaves that had been washed with ozonated

water with added surfactant. Surfactant has been reported to influence the detachment of *Escherichia coli* O157:H7 and *Salmonella enterica* from lettuce and cilantro leaf surfaces, respectively [36,37]. In addition, previous studies have reported that surfactant removes *Escherichia coli* O157:H7 and *Listeria innocua* from lettuce leaf surfaces during the washing process [38,39]. Thus, it was necessary to clarify whether non-detection of *S*. Typhimurium was due to detachment or sterilization. After the disinfection experiment, the number of *S*. Typhimurium in washing solution used in each treatment was measured. *S*. Typhimurium were detected only in used surfactant (count), and not in used ozonated water with surfactant added. These results suggest that ozonated water with surfactant significantly inactivates *S*. Typhimurium on waxy leaves and its sterilization efficacy is not simply the ability to detach the bacteria cells from waxy leaves. In the washing process for fresh produce, surfactant may aid in the detachment of the bacterial cells from the crop surfaces [38,39], but the detached bacterial cells would remain viable in the washing solution, which could lead to cross-contamination if the bacteria attach to subsequent produce.

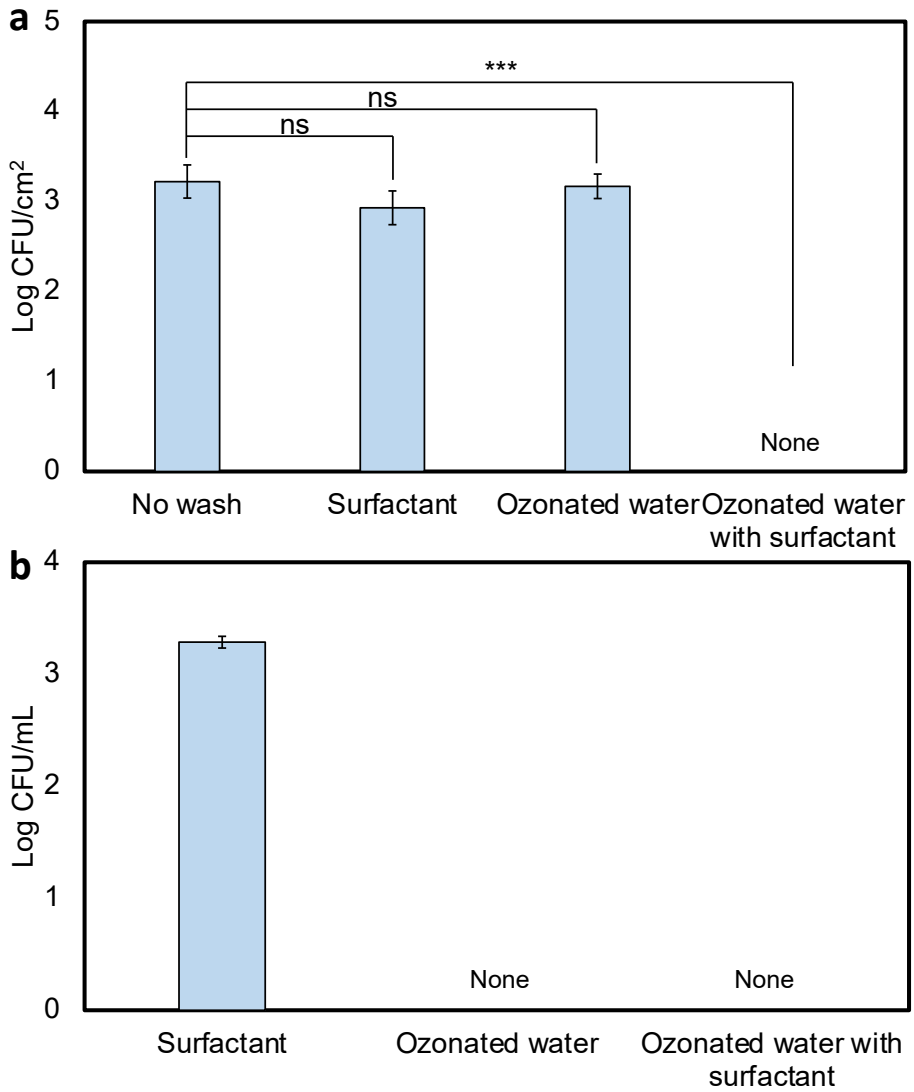

Figure 5. (**a**) Inactivation of *S*. Typhimurium on waxy leaves by ozonated water (2.8 mg/L) with added surfactant. (**b**) The number of *S*. Typhimurium detected in washing solution used in each treatment. *Salmonella*-inoculated waxy leaf disks were immersed with each washing solution for 180 ± 5 s. The asterisks above the bars represent significant differences between each group. ^{ns} and *** correspond to non-significance and significant difference at $p < 0.001$ by Student's *t*-test, respectively. Error bars indicate the standard deviation of *Salmonella* counts from three replicates.

Epicuticular wax crystals reduce *Salmonella* attachment to leaves, but there appears to be no difficulty in adhering an infective dose of *Salmonella* to waxy leaves [15]. Furthermore, early in plant growth, the formation of epicuticular wax may be such that its protective characteristics are comparatively less efficient. *Salmonella* may therefore readily attach to leaf surfaces at this stage, making it possible for food-borne diseases to occur via consumption of waxy produce. The concentration of epicuticular wax is affected not only by genetic factors but also by environmental conditions such as relative humidity and irrigation [40]. Especially, when a higher concentration of epicuticular wax is induced by environmental conditions, the common washing method may not be sufficient to prevent produce contamination. Unfortunately, ozonated water on its own appears ineffective on waxy leaves. Our findings suggest that washing efficiency could be enhanced by adding a surfactant to ozonated water in a such case.

3.6. The Potential and Disability of Surfactant for Decontamination on Waxy Leaves

The present study showed that there are contradictory aspects of surfactant on vegetable production: (1) As a pesticide component, surfactant promotes the attachment of *S*. Typhimurium on waxy surfaces during the preharvest stage and (2) the addition of surfactant to ozonated water remarkably improves its efficiency on waxy leaves during postharvest washing or disinfection. Excessive use of a pesticide may disrupt the role of three-dimensional epicuticular wax crystals against pathogenic microbial contamination by altering the crystal structure. This is an issue that must be carefully considered in agricultural production when using pesticides for food safety. In the cultivation of plants with abundant epicuticular wax, emulsion pesticides may serve the purpose of controlling pests, but they could provide food-borne pathogens with a better environment in which to live. Three-dimensional epicuticular wax crystals affect the efficiency of ozonated-water washing but washing using surfactant-added ozonated water eliminates this problem and may be a viable alternative for postharvest disinfection of waxy produce including broccoli, cabbage, blueberry, grapes, and others. In addition, ozonated water with surfactant may improve the storage of apple, pear, and other crops and fruits, which requires further study. For surfactant-based sanitizer, repeated use of sanitizer can lead to cross-contamination via the used sanitizer containing bacteria cells detached from the surface of the previous crop. As long as there is continuous ozone injection to surfactant-added water, ozonated water with surfactant will be continuously reusable and will be industrially useful as a sustainable washing method upon large-scale produce washing (Figure 6). Future studies should further investigate the safety and stability of surfactants as an additive to ozonated water and determine appropriate concentrations for their use that will not affect produce quality. For effective food-borne disease prevention, we have shown that it is necessary to use carefully selected washing methods that consider the surface characteristics of the produce.

In this study, the effects of surfactant on the hydrophobic surface of a crop were studied using a broccoli model (Figure 7). In the preharvest stage, pesticide application increased attachment of *Salmonella* by deformation of the epicuticular wax. This indicates that the function of epicuticular wax against microbial contamination can be hampered by emulsification of pesticides. Meanwhile, ozonated water showed a washing efficiency on waxy surfaces that was less than that on glossy surfaces. However, this problem could be improved by adding a surfactant to the ozonated water. Our results suggest that ozonated water with the addition of surfactant will be an effective disinfection method for crops with hydrophobic surfaces. Our results provide information on the attachment and disinfection of pathogens in crops with hydrophobic surfaces, which had not been previously studied, and will be applicable to fruits and vegetables with low washing efficiency due to hair (i.e., peach, okra, and mustards) or trichomes (cucumber, zucchini, perilla leaves, and cannabis).

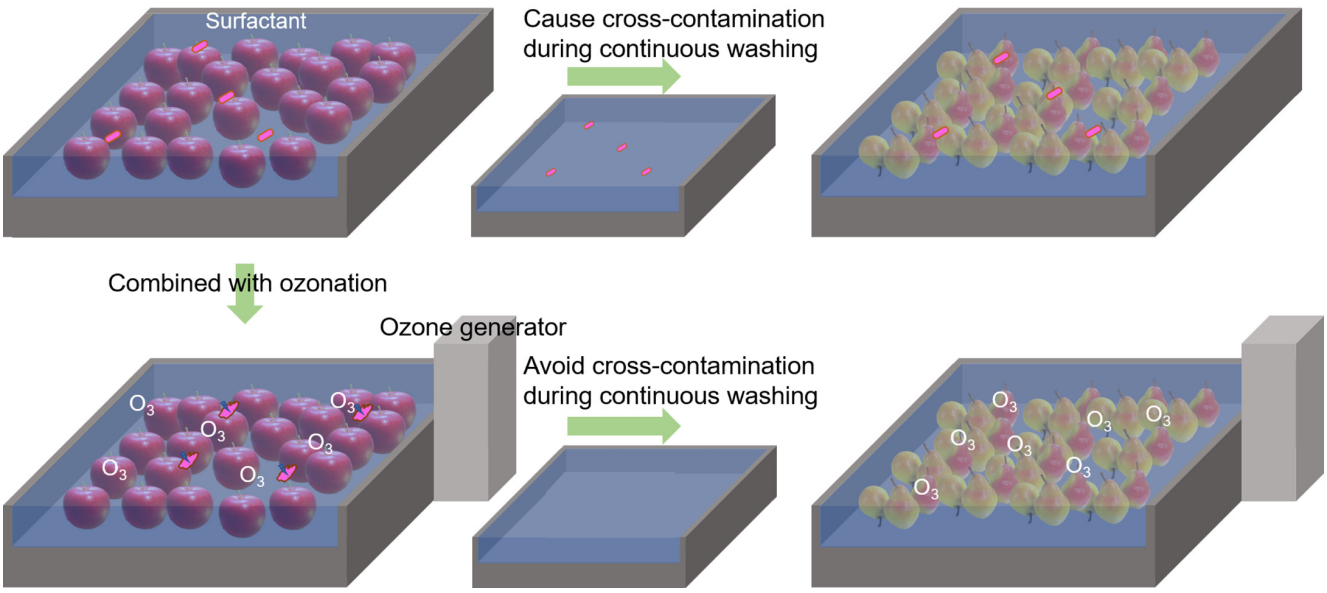

Figure 6. Industrial application model of improved washing process of fresh produce with a hydrophobic surface.

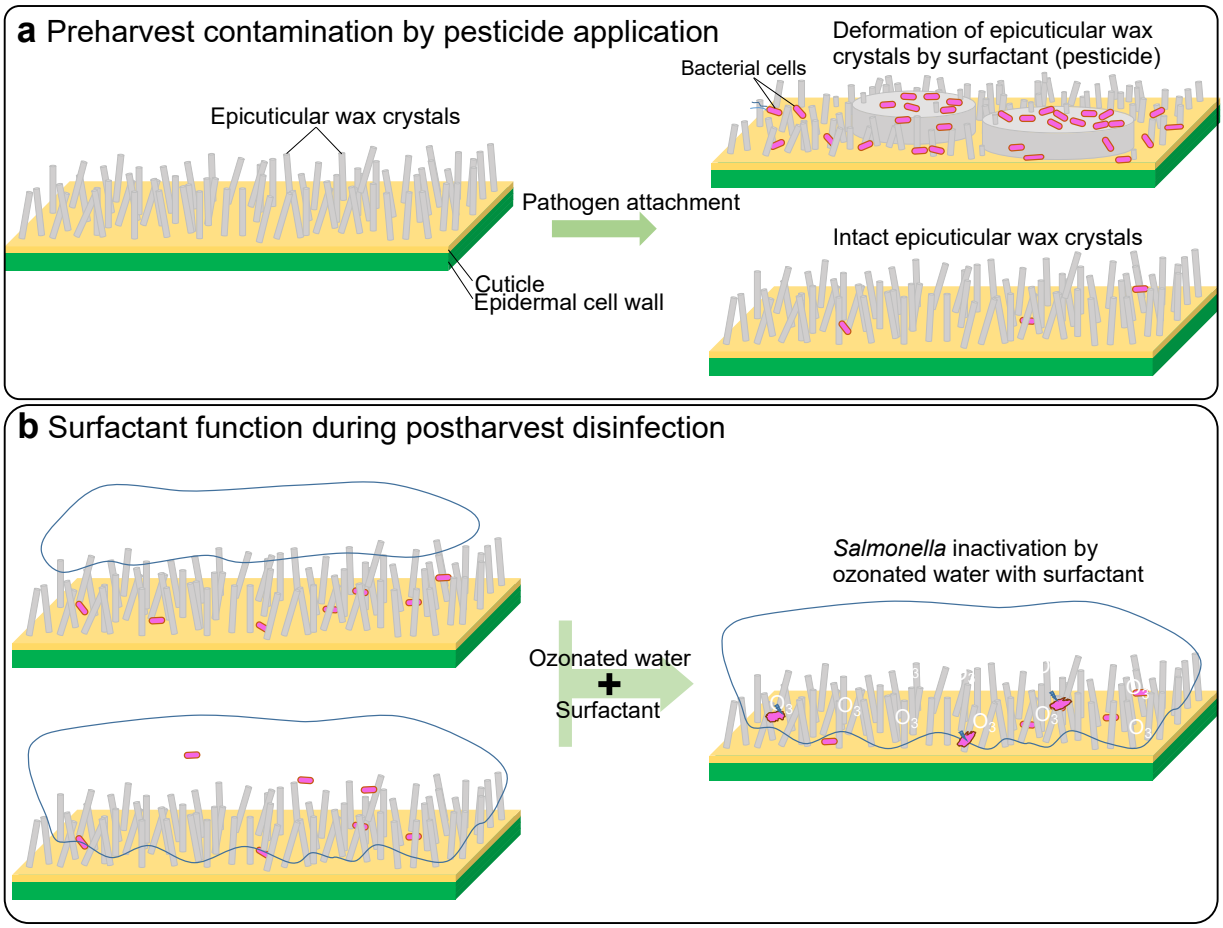

Figure 7. Surfactant effect on bacterial contamination during preharvest (**a**) and postharvest (**b**). Application of a pesticide containing surfactant increased the attachment of *Salmonella* by deformation of the epicuticular wax, whereas surfactant usage during postharvest washing increased disinfection efficiency of ozonated water by weakly changing the surface chemical structure, similarly to the preharvest surfactant reaction.

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