Full Title

Enhanced removal of *Escherichia coli* O157:H7 and *Listeria innocua* from fresh lettuce leaves using surfactants during simulated washing

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Abstract:

Removal of foodborne bacterial pathogens from fresh produce during washing and sanitation process can significantly improve inactivation of the bacteria. Currently, produce wash systems mainly rely on mechanical forces to aid in removal of bacteria attached to the produce surface during washing and sanitation. This study evaluates the potential of surfactants to enhance removal of pathogens from the surface of fresh produce. Influence of three types of commercial food-grade surfactants, including Tween-20, sodium dodecyl sulfate (SDS), and lauric arginate (LAE), on the mechanical removal of pathogenic bacteria and viruses from fresh lettuce leaves in the presence of soil was evaluated. The addition of surfactants did not increase the removal of T7 phages from lettuce leaf surface ($P > 0.05$). The improvement of bacterial removal by addition of surfactants to wash water is corresponding to the decrease of the contact angle between wash water and leaf surface. The most effective *Escherichia coli* O157:H7-*lux* removal was obtained by washing with 0.1% LAE, followed by 0.1% Tween-20, then 0.1% SDS. The most enhanced detachment of *Listeria innocua* was achieved by 0.1% LAE, followed by 0.1% SDS, then 0.1% Tween-20. The presence of soil resulted in an increased resistance of bacterial cells to the washing process. There was no significant difference in the cell persistence on the lettuce surface within the extended incubation period ($P > 0.05$). The evaluation of lettuce quality indicated that the introduction of surfactants during the washing procedure may affect the firmness of leaves, but the color and electrolyte leakage rate were not affected by the exposure to wash water with surfactants ($P > 0.05$). Overall, these results suggest the potential of food grade surfactants to enhance the removal of bacteria particularly foodborne pathogens from the surface of fresh produce.

Keywords:

Decontamination, *Escherichia coli* O157:H7, *Listeria innocua*, Virus, Surfactant, Fresh produce

1. Introduction

Consumption of fresh produce has significantly increased over the last two decades in the United States (Olaimat and Holley, 2012). This increase in consumption is attributed to growing consumer awareness of the health benefits of fresh produce (Wiseman, 2008). Considering most of the fresh produce is consumed raw or after minimal processing, it is important to reduce the risk of its contamination with pathogenic bacteria and viruses throughout the production chain (Predmore and Li, 2011; Scallan et al., 2011). The risk of contamination of fresh produce is highlighted by the fact that about 46% of the foodborne illnesses in the US from 1998 to 2008 can be attributed to the contamination of fresh produce with pathogens (Braden and Tauxe, 2013; Painter et al., 2013). Among those, norovirus accounted for more than 40% of outbreaks in fresh produce in the United States (Doyle and Erickson, 2008). The contamination of fresh produce can occur via soil, water, processing equipment, and human handling (Beuchat and Ryu, 1997).

Washing and sanitation of fresh produce after harvest is a critical step to reduce microbial load on fresh produce and reduce cross-contamination (Artés et al., 2009; Banach et al., 2015). To address the issues of microbial contamination and cross-contamination, a number of decontamination methodologies for fresh produce have been reported (Allende et al., 2006; Ölmez and Kretzschmar, 2009). The limitations of those methods include: a) Many of chemical-based decontamination methodologies (such as chlorine, hydrogen peroxide, organic acids, and quaternary ammonium) are effective in reducing microbial load in wash water environment but have limited efficacy in reducing microbial load on the surface of fresh

produce (Allende et al., 2007; Allwood et al., 2004; Chaidez et al., 2007; De Giusti et al., 2010). This limited activity is attributed to non-specific reactivity of sanitizers with plant organic content (Banach et al., 2015; López-Gálvez et al., 2009), restricted diffusion of sanitizers through the self-produced matrix of extracellular polymeric substances by bacteria on the plant surface (Ölmez and Temur, 2010; Shen et al., 2012). Also, the structural features of the plant surface, such as crevices, cracks, and small fissures, along with the hydrophobic nature of waxy cuticle, prevent sanitizers from inactivating microorganisms on fresh produce (Gil et al., 2009; Yaron and Römling, 2014). b) The presence of soil on the surface of produce may harbor pathogenic microorganisms and may resist effective sanitation of the produce (Beuchat and Ryu, 1997; Warning and Datta, 2013). c) As one of major causes of foodborne illnesses, the prevalence and behavior of viruses in post-harvest environments and on vegetable commodities are seldom studied. In summary, the current decontamination methodologies for fresh produce is limited by the physical and chemical barriers on the surface of fresh produce and effective removal of microbes from the fresh produce during washing may improve sanitation efficacy by reducing the physical and chemical barriers for inactivation of bacteria.

The detachment of bacteria from the plant surface may depend on mechanical forces during washing as well as the surface energetics of the microbe-plant interaction. The surface energetics can be influenced by several factors including bacterial cell hydrophobicity, produce-water contact, bacterial distribution of plant surface and surface roughness (Faille et al., 2013). Few studies have been conducted to illustrate the effect of surface hydrophobicity on detachment of microorganisms from the plant surface. Ukuku and Fett (2002) reported a linear correlation between the surface hydrophobicity of bacterial cells and the strength of

bacterial attachment to cantaloupe surfaces (Ukuku and Fett, 2002). In this study, the cell surface hydrophobicity was measured based on partitioning of cells to octan-water interface. Use of surfactants is one of the potential approaches to influence the surface energetics of bacteria-produce interactions. Surfactants are surface-active compounds that can lower the interfacial tension between a liquid and a solid (Banat et al., 2000; Mukherjee and Das, 2010). Based on this property, surfactants can enable release of tightly bound contaminations such as foodborne pathogens from the surface, which led us to hypothesize that surfactants may enhance the removal of foodborne pathogens from fresh produce. Out of numerous ionic and nonionic surfactants, we chose sodium dodecyl sulfate (SDS), Tween-20, and lauric arginate (LAE) for this study. Tween-20 is a nonionic surfactant, and LAE is a cationic surfactant, both of which are the GRAS (generally recognized as safe) substances recognized by the FDA. SDS is an anionic surfactant and an FDA-approved food additive.

To address the hypothesis, the goal of this study was to evaluate the effect of three types of commercial food-grade surfactants (including Tween-20, SDS, and LAE) on the removal of pathogens and viruses from fresh lettuce surfaces. A shiga toxin negative strain of *Escherichia coli* O157:H7-*lux* was selected as a model for a gram-negative foodborne pathogen, *Listeria innocua* was selected as a surrogate for a gram-positive human pathogenic *Listeria monocytogenes*, and bacteriophage T7 was selected as a model for a foodborne virus. Cell surface hydrophobicity, cell surface charge, surface tension of wash water with and without surfactants, and contact angle between leaf and wash water were measured. Efficacy of microbial removal from lettuce surface was quantified by enumeration of microbial population, and further validated by bioluminescence imaging. The influence of soil particles and extended incubation period on removal of bacteria from fresh produce was also evaluated.

Finally, changes in total color, electrolyte leakage rate, and plant tissue compressibility were quantified to examine the effect of surfactants on the quality of fresh lettuce during the simulated washing process. The results of this study have a potential to improve postharvest processing of fresh produce to reduce microbial load including any foodborne pathogen on fresh produce.

2. Materials and methods

2.1 Bacterial strain, media and growth condition

Gram-negative bacterial strain *Escherichia coli* O157:H7-*lux* (ATCC, 700728, Manassas, VA, USA) and gram-positive bacterial strain *Listeria innocua* (ATCC, 33090, Manassas, VA, USA) were used in this study. The stocks of *E. coli* O157:H7-*lux* and *L. innocua* were maintained in liquid nitrogen in Luria-Bertani and tryptic soy broth supplemented with 15% (vol/vol) of glycerol, respectively. The bacteria were streaked on to agar plates and grown overnight. Before each experiment, one colony was picked from the agar plate, cultured in the liquid medium, and incubated to achieve the stationary phase cultures. *E. coli* strain was grown in a Luria Bertani (LB) broth (Fisher Scientific, Pittsburg, CA, USA) supplemented with 25 µg/ml tetracycline with constant shaking (250 rpm) at 37 °C. *L. innocua* strain was grown in a tryptic soy broth (TSB) (Sigma-Aldrich, St. Louis, MO, USA) with constant shaking (250 rpm) at 30 °C. For plate cultures, LB agar and tryptic soy agar plates were used for *E. coli* O157:H7-*lux* and *L. innocua*, respectively.

2.2 Bacteriophage culture and propagation

E. coli BL21 (ATCC, BAA-1025, Manassas, VA, USA) was cultured in LB broth and LB agar. 10 ml of broth was inoculated with a colony of *E. coli* BL21 and grown at 37 °C at 150

rpm. The following day, 100 µl of the overnight culture was inoculated in 10 ml of pre-warmed LB broth and incubated until an absorbance of 1.5 was reached at 600 nm (approximately 2×10^9 CFU/ml). Bacteriophage T7 (ATCC, BAA-1025-B2, Manassas, VA, USA) was cultured in stationary-phase *E. coli* BL21 cells in LB medium, and allowed to incubate in a shaking incubator at 37 °C and 250 rpm for 4 h to allow several phage lifecycles to complete. Phage propagation was blocked with a chloroform treatment, and the recovery of lysate was performed as previously described (Han et al., 2014).

2.3 Lettuce leaf preparation

Pre-bagged romaine lettuce leaves were purchased from a local supermarket one day before the experiments. Exterior leaves of the lettuce were removed and discarded. The leaves that had no evidence of tissue damage were selected and washed with deionized water. Then the chosen leaves were cut into squares (5 by 5 cm) with a sterile scalpel for the bacterial attachment experiments.

2.4 Inoculation of bacteria/bacteriophages on lettuce leaf surface

To address the limitation of spot inoculation methods and to foster interactions between leaf and bacteria, this study illustrates a novel inoculation approach. In this approach bacteria are uniformly inoculated on the surface of a leaf to form a uniform thin coating across the leaf surface. This approach was critical for measuring changes in contact angle and to reduce bias by the surface inoculation approaches in some prior studies. The inoculation of lettuce leaf surface was performed using a commercial desktop spin coater (Model VTC-100-50, MTI Corporation, Richmond, CA, USA). Before inoculation, the bacteria were centrifuged at 3,100 ×g for 10 min at room temperature, washed twice with phosphate buffered saline (PBS) (Fisher Scientific, Pittsburg, CA, USA), and then resuspended in the same buffer to obtain a

final bacterial cell concentrations of 10^4 , 10^6 , and 10^8 CFU/ml, respectively. The T7 phages were centrifuged at $11,000 \times g$ for 15 min at room temperature, washed twice with PBS, and then resuspended in the same buffer to obtain a final viral concentration of 10^6 PFU/ml. Leaf slices ($5 \times 5 \text{ cm}^2$) were placed in a petri dish, and 0.5 ml of microorganism suspension was dropped by pipette onto each leaf surface (Chen and Zhu, 2011). Then the petri dish was sealed by a para film (Fisher Scientific, Pittsburg, CA, USA) and fixed on the disk of a spin coater. The coatings of microorganisms on the leaf surface were produced by spinning the petri dish at 500 rpm for about 30 s. The inoculated leaf slices were stored for an additional 1 h to allow for attachment of microorganisms to lettuce leaf surface.

To evaluate the influence of soil on removal of bacteria from lettuce leaves, the leaves were inoculated with the mixture of bacteria and soil using a spin-coating method. A mixture of 1 ml of bacterial suspension (10^7 CFU/ml), 9 ml of PBS, and 2 g of sterile clay soil (Fisher Scientific, Pittsburg, CA, USA) was prepared to obtain a suspension with an initial load of bacteria at 10^6 CFU/ml and the initial load of soil at 0.2 g/ml. After the leaf sample was placed in a petri dish, 0.5 ml of bacterial suspension with soil was dropped by pipette onto each leaf surface. Then the petri dish was sealed by parafilm and fixed on the disk of a spin coater. The bacterial suspension with soil was coated on a leaf surface using a spin coater with a spinning speed of 500 rpm for about 30 s. After inoculation, the leaf samples were stored at 4°C for 1, 24 and 48 h, respectively. All the leaf samples after incubation were cut into 3 cm diameter disks with a sterile cork borer before bacterial detachment assay.

In order to demonstrate the uniformity of bacterial deposition on the lettuce surface, bioluminescence imaging was performed using a widefield bioluminescence imaging system. In this assay, the leaf slice was inoculated with *E. coli* O157:H7-*lux* cells, in which the

plasmid pSB401 (Winson et al., 1998) contains a complete lux operon, *luxCDABE* (bioluminescence genes). After inoculation, the leaf samples were imaged to characterize the spatial distribution of the bacteria on a lettuce leaf surface. The images illustrated uniformity in deposition of bacteria on a lettuce leaf surface using a spin coating process (Fig. S1).

2.5 Preparation of wash water with different surfactants

The surfactants used in this study were: (1) Tween-20, purchased from Sigma-Aldrich (St. Louis, MO, USA); (2) SDS, purchased from Sigma-Aldrich (St. Louis, MO, USA); (3) LAE solution (CYTOGUARD™ LA20, 10 v/v%), kindly provided by A&B Ingredients (Fairfield, NJ, USA). The wash water with different surfactants were prepared by adding Tween-20, SDS, and LAE respectively to the sterilized distilled water to obtain a solution of wash water with a final surfactant concentration of 0.1% w/w.

2.6 Washing of fresh produce

To quantify the effect of different surfactants on removal of microorganisms from lettuce leaves, the leaves inoculated with different initial loads of bacteria/viruses were exposed to simulated washing process in the presence of individual surfactants. In our recent study, we have reported the design and fabrication of a benchtop scale device for evaluating the efficacy of bacterial detachment from fresh produce by shear stress (Huang et al., 2016). Briefly, the leaf inoculated with microorganisms was fixed to the rotating disk and the rotating disk was inverted into the wash tank. During the washing process, the leaf surface was exposed to the wash water. In this study, the disk was rotated at a constant speed of 200 rpm. The samples were exposed to wash water for 20 min. All samples were treated in triplicate for each experimental condition. After each treatment, the leaf was collected and rinsed twice by sterile DI water to remove any loosely adhered microorganisms. The leaf samples were then used for

enumeration of the attached microorganisms on the leaf surface using plate counting.

2.7 Recovery of bacteria/viruses from lettuce leaves for enumeration

For enumeration of bacterial population on lettuce leaves, the leaf samples after treatment were transferred to a sterile test tube containing 10 ml of maximum recovery diluent (MRD, Sigma, St. Louis, MO, USA), and allowed it to stand for 2min. After vortexing at full speed for 1 min in the tube by a vortex mixer (Fisher Scientific, Waltham, MA), quantification of bacterial number was performed by serial dilution and plate counting. Bacterial counts were determined after overnight incubation at 37 °C. The results were reported in colony forming units (CFU/ml).

For enumeration of phage population on lettuce surface, the leaf samples after washing were transferred to a sterile test tube containing 10 ml of MRD, then exposed to homogenization for 1 min. Quantification of phage number in the homogenized solution was performed by serial dilution and the standard soft agar overlay plating assay (Han et al., 2014). Briefly, top agar (0.75% agar) was melted, and 3 ml aliquots were kept at 45 °C in water bath until use. Phage samples were serially diluted in PBS, and 100 µl of each sample was combined with 250 µl of fresh log phase *E. coli* BL21 culture. The samples were incubated for 10 min and then combined with an aliquot of molten agar. The molten agar mixture was poured onto a prewarmed LB agar plate and allowed to solidify. Once the plates solidified, they were inverted and incubated at room temperature overnight, and then the plaques were counted. The results were reported in plaque forming units (PFU/ml).

2.8 Determination of cell surface hydrophobicity

In order to assess cell surface hydrophobicity, the cell partition to octan-water interface was measured as described by (Rosenberg et al., 1983). Bacteria were harvested by

centrifugation at 3,100 $\times g$ for 10 min, washed twice with PBS and re-suspended in the same buffer. The bacterial suspension with the concentration of approximately 10^8 CFU/ml was used for the cell surface hydrophobicity assay. The optical density of suspension at 640 nm (A_1) was measured using Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Rochester, NY, USA). The 0.5 ml of Octan (Alfa Aesar, Haverhill, MA, USA) was added into 3.5 ml of bacterial suspension, then mixed using a vortex mixer for 2 min and allowed to stand for 10 – 15 min until the phase separated. The upper aqueous phase was discarded, then the lower aqueous phase was selected and optical density at 640 nm was determined (A_2). The hydrophobicity index (HPBI) was calculated as:

$$\text{HPBI} = \frac{(A_1 - A_2)}{A_1} \times 100 \quad (1)$$

2.9 Surface charge of bacterial cells

The surface charge of *E. coli* O157:H7-*lux* and *L. innocua* cells was measured using a Malvern Zetasizer nano-ZS (Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom). Briefly, a 1: 10 dilution of the stationary-phase culture was made in sterile DI water and gently vortexed to ensure thorough mixing. Then 1 ml of bacterial suspension was aliquoted into a cuvette and inserted into the measurement chamber of the zetasizer. Measurements were taken in triplicate for each bacterial strain.

2.10 Surface tension of washing solution

The surface tension of washing solution, including DI water, Tween-20 (0.1%), SDS (0.1%), and LAE (0.1%), was measured by the Wilhelmy method on a Krüss Model K10T digital tensiometer (Krüss, Hamburg, Germany), with a roughened platinum plate. All the measurements were performed in a circular temperature controlled dish with an area of 63.6

cm². A minimum of 3 replicates measurements were carried out for each sample at a temperature at 25 ± 0.5 °C.

2.11 Contact angle measurement

The advancing contact angles of four liquids, including DI water, Tween-20 (0.1%), SDS (0.1%), and LAE (0.1%) were measured at room temperature using the Theta Optical Tensiometer (Attension, Biolin Scientific, Stockholm, Sweden). Contact angles were measured on clean leaves and leaves coated with suspension of *E. coli* and *L. innocua* (with the initial load of 10^8 CFU/ml), respectively. Leaf samples of about 1 cm diameter disk were cut with a cork borer from the middle part of the leaf discarding the veins. These were mounted on a microscope slide with a double-sided tape. Droplet volumes of 15 µl of wash solution were dropped on the leaf surfaces. The advancing contact angles were recorded and calculated using the sessile drop method (OneAttension version1.8, Biolin Scientific, Stockholm, Sweden). A total of 3 scans were performed for each evaluation.

2.12 Widefield bioluminescence imaging

To characterize spatial distribution of *E. coli* O157:H7-*lux* on lettuce samples, bioluminescence imaging was performed using a widefield bioluminescence imaging system equipped with an ICCD camera and a band pass emission filter (558-583 nm) (IVIS 100 Series, Caliper Life Sciences Inc., Hopkinton, MA). The integration time for each image was 1 s. Bioluminescence signals were represented using a pseudocolor signal intensity scale.

2.13 Evaluation of quality of fresh lettuce leaves

For color measurement, lettuce leaves were selected and analyzed using the ColorFlex EZ Spectrophotometer (Hunter Lab, Reston, VA, USA). Hunter's color values (*L*, *a*, *b*) were analyzed by the EasymatchQC software (Hunter Lab, Reston, VA, USA). Triplicate

measurements were performed for each of the four slices of lettuce leaves, and the average and standard deviation based on 12 readings for each treatment were reported. Total color difference (ΔE) was determined using the following equation:

$$\Delta E_{ab}^* = \sqrt{(L_1^* - L_0^*)^2 + (a_1^* - a_0^*)^2 + (b_1^* - b_0^*)^2} \quad (2)$$

where L_0^* , a_0^* , and b_0^* are Hunter's color values from a reference and, L_1^* , a_1^* , and b_1^* are Hunter's color values from the treated samples. L^* , a^* , and b^* indicate luminosity, chromaticity on a green (-) to red (+) axis, and chromaticity on a blue (-) to yellow (+) axis, respectively (Chen et al., 2010).

Electrolyte leakage from fresh lettuce was measured immediately after washing to determine the rate of tissue deterioration. One slice of lettuce leaf were submerged in 100 ml of deionized water in a beaker and incubated at 23 °C. During incubation the samples were agitated at a speed of 100 rpm. Electrical conductivity ($\mu\text{S}/\text{cm}$) of the bathing solution was measured at 1 min (C_1) and 60 min (C_{60}) using a conductivity meter (Accumet, Fisher Scientific, Pittsburg, CA, USA). The samples were then autoclaved (121 °C) for 25 min, and total conductivity (TC) was measured after cooling. Electrolyte leakage rate (ELR) was calculated based on the following equation (Salgado et al., 2014):

$$\text{ELR} = \left[\frac{C_{60} - C_1}{TC} \right] \times 100 \quad (3)$$

The compressibility of fresh lettuce leaves was measured using a TA-TXPlus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA). A slice of leaf sample was positioned in the press holder and the plunger was moved down at a velocity of 1 mm/s to 2 mm below the bottom of the holder. The maximum compression force (MCF) was recorded using the Texture Expert Software (version 2.64, Texture Technology Corp., Scarsdale, NY, USA). A total of six slices of lettuce leaves were measured for each treatment set.

2.14 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software V.5.04 (Graphpad Software, Inc., La Jolla, CA). Data sets were analyzed using one-way analysis of variance ($p < 0.05$) to determine statistical significance of the selected measurements. The measurement sets evaluated using the statistical analysis were cell surface hydrophobicity, contact angle, surface tension, microbial enumeration, and determination of lettuce quality.

3. Results and Discussion

3.1 Cell surface hydrophobicity, surface tension, and contact angle measurement

Several studies have suggested that the cell surface hydrophobicity may be an important factor influencing adherence of bacteria to solid-liquid or air-liquid interfaces (Gibbons and Etherden, 1983; Hermansson et al., 1982). However, only few studies have focused on the influence of these surface properties on attachment/removal of bacteria from fresh produce (Hassan and Frank, 2003; Ukuku and Fett, 2002). To further develop this understanding, this study compared the relative hydrophobicity of a gram positive and a gram-negative bacteria, i.e. *E. coli* O157:H7-*lux* and *L. innocua*, respectively. Based on the relative partitioning of bacterial cells on Octan-water interface, the cell surface hydrophobicity (HPBI) was evaluated as described in the materials and methods section (Fig. 1A). The values of HPBI for *E. coli* O157:H7-*lux* and *L. innocua* cells were 15.1% and 61.7%, respectively. This result indicates that the cell surface of *L. innocua* was significantly ($p < 0.05$) more hydrophobic than that of *E. coli* O157:H7-*lux*. This result also concurs with those of other prior studies (Hsu et al., 2013; Ukuku and Fett, 2002). The net electrical charge on the cell surface was quantified by measuring the electrophoretic mobility of bacterial cells. Both *E. coli* O157:H7-*lux* and *L.*

innocua cells had a negative surface charge, as the electrophoretic mobility of *E. coli* O157:H7-*lux* and *L. innocua* cells in stationary phase were -2.61 ± 1.41 mV and -38.00 ± 5.43 mV, respectively (Fig. 1B). This observation is in agreement with the previous publications (Dickson and Koohmaraie, 1989; Ukuku and Fett, 2002), reporting the net surface charge of *E. coli* O157:H7 and *L. monocytogenes* ranged $-1.5 \sim -3.0$ mV and $-37 \sim -39$ mV, respectively. Overall, these results indicate that both cell surface hydrophobicity and surface charges of the selected bacterial strains are different and these differences may influence binding or removal of the bacteria from fresh produce.

The surface tension of wash water-air interface with different surfactants was determined by the Wilhelmy plate method. The surface tension of the water-air interface (without any organic content) was 71.17 mN/m. The surface tension of water-air interface decreased significantly to (46.67 mN/m) upon addition of SDS (0.1 %). Similarly, with the addition of Tween-20 (0.1 %) or LAE (0.1 %) to water, the surface tension of water-air interface decreased to approximately 36 mN/m. Thus, addition of surfactants to the wash water lowered the interfacial tension and is expected to improve removal of bacteria from the fresh produce surface.

Figure 1

Mean contact angle between wash water and leaf surface were measured for the wash water without surfactant and wash water with the selected surfactants, i.e., Tween-20 (0.1%), SDS (0.1%), and LAE (0.1%), respectively. The results in Fig. 2 represent the mean contact angle $\pm$ standard deviation based on the three independent replicate measurements. The contact angle of DI water on the leaf surfaces was $78.95^\circ \pm 2.63^\circ$, which decreased to $66.25^\circ \pm 3.08^\circ$ and $75.98^\circ \pm 2.01^\circ$ when the leaf surfaces were coated with *E. coli* O157:H7-*lux* and *L.*

innocua cells, respectively (Fig. 2A). This observation suggests that the presence of *E. coli* on the surface of lettuce leaves decreased the leaf surface hydrophobicity and reduced the water contact angles. The results also indicate that the reduction of water contact angle caused by *L. innocua* was significantly smaller than that observed with *E. coli* O157:H7-*lux* on fresh produce.

As expected, introduction of surfactants into the wash solution definitely enhanced the solutions' ability to wet the leaf surface in the absence of bacteria. The average contact angles of wash water on the lettuce surface decreased to 63.28°, 66.18°, and 59.42°, upon addition of Tween-20 (0.1%), SDS (0.1%) and LAE (0.1%) in the wash solution, respectively. Similar to the trend observed for the control leaf surface, the leaf surface coated with *E. coli* O157:H7-*lux* cells also showed similar reduction in contact angles upon addition of surfactants. In this case, similar to the clean leaf surface, the contact angle was highest for the DI water, followed by DI water with 0.1% SDS, with 0.1% Tween-20, and with 0.1% LAE respectively. For the leaf surface coated with *L. innocua*, however, the introduction of SDS in wash water led to the most significant decrease in contact angle between the wash water and the leaf surface coated with *L. innocua*. This observation can be attributed to the differences in cell surface characteristics, such as surface hydrophobicity, net surface charge, cell membrane structure of the selected bacterial cells, and topographical distribution of bacterial cells on the leaf surface, and resulting differences in interactions among bacteria, produce and surfactants. To the best of our knowledge, these measurements of contact angle have not been reported in any of the previous studies. These measurements characterize surface activity of the surfactants on the fresh produce surface. The results in this study illustrate that the decrease in surface contact angles improves the removal of bacteria. This knowledge can aid

in rational selection of surfactant to aid in washing of fresh produce.

Figure 2

3.2 Effect of different surfactants on microbial removal

To study the influence of surfactants on the removal of bacteria from lettuce surface, the number of bacterial cells, i.e. colony forming unit/leaf surface area, adhered on the leaf surface before and after washing procedure were quantified by the plate counting method. The efficacy of bacterial detachment by wash water with different surfactants was evaluated on the lettuce leaf surface inoculated with bacterial loads of 4-, 6-, and 8-log CFU/ml, respectively (Figs. S2, 3A, and S3). The increase in removal efficiency of bacteria with surfactants was independent of the initial load of bacteria on lettuce surface. Washing by sterile DI water at the rotating speed of 200 rpm for 20 min gave a 1.47-log and 0.84-log reduction of *E. coli* O157:H7-*lux* and *L. innocua*, respectively. A solution with 0.1% of the nonionic surfactant Tween-20 brought about a slight increase in the detachment of both *E. coli* O157:H7-*lux* and *L. innocua* (by approximately 0.5-log CFU/cm²) in comparison with DI water alone. Interestingly, the removal of *L. innocua* was significantly improved upon washing with an SDS solution, resulting in a reduction of 1.79 ± 0.12 log CFU/cm². However, there was no significant improvement in the reduction of *E. coli* O157:H7-*lux* adhered on the leaf surface when a solution with 0.1% SDS was used, indicating that 0.1% of SDS is significantly more efficient in the removal of *L. innocua* than *E. coli* O157:H7-*lux*. The addition of LAE enhanced the efficiency of both *E. coli* O157:H7-*lux* and *L. innocua* removal at a concentration of 0.1%, resulting in reduction of more than 2 log CFU/cm². LAE is a

surfactant derived from lauric acid and arginine. As a result of the net positive charge, prior studies have reported the antimicrobial properties of this surfactant (Becerril et al., 2013; Woodcock et al., 2009). Therefore, the enhanced bacterial reduction by 0.1% of LAE can be attributed to the synergistic effect of surfactant and antimicrobial properties of LAE.

The efficacy of viral removal by wash water with different surfactants was evaluated on the lettuce leaf surface inoculated with T7 phages at an initial load of 6-log PFU/ml (Fig. 3B). Washing by sterile DI water at the rotating speed of 200 rpm for 20 min resulted in a viral reduction of 1.20 ± 0.11 PFU/cm². In contrast with bacterial detachment, all three types of surfactants did not significantly increase the efficiency of removal of T7 phages from lettuce leaves ($P > 0.05$). This result was in agreement with the previous study on the removal of human norovirus from lettuce surface (Predmore and Li, 2011). This result indicates that due to their size (~100 nm), viral particles can persist on leaf surface and are not influenced by the presence of surfactants in wash water. This result illustrates the need to further understand distribution of viral particles on the leaf surface and its impact on accessibility of viral particles to wash water and surfactant. This results also indicate potential challenges for inactivation of viral particles on the surface of fresh produce.

Notably, the results suggest that enhancement in removal of bacteria with the addition of surfactants is corresponding to the decrease in the contact angle between wash water and leaf surface. These results are significant as introduction of surfactants to washing process can reduce the hydrophobic barrier that limits the mechanical contact of the wash solution with the leaf surface (Hassan and Frank, 2003; Singh et al., 1984). This conclusion was also demonstrated by the removal of viruses from lettuce surface, in which surfactants were not able to affect the decontamination efficacy due to the small size of viruses.

Figure 3

The washing efficacy of surfactants was further validated by widefield bioluminescence imaging to characterize the spatial distribution of residual *E. coli* O157:H7-*lux* cells on lettuce leaves after each treatment (Fig. 4A). The widefield imaging results showed a decrease in bioluminescence signal intensity indicating that the amount of bacterial cells adhered on the leaf surfaces decreased with the use of surfactants. The highest reduction of *E. coli* from lettuce leaves was achieved by washing with LAE, followed by Tween-20 and SDS, which resulted in the least reduction of bacteria. In addition, it is observed that the distribution of bioluminescence signal on the leaf surface treated by surfactants was relatively uniform when compared to those treated by water, indicating surfactants are helpful to obtain a relatively uniform removal of bacteria from leaf surface.

Figure 4

3.3 Bacterial detachment in the presence of soil

The presence of soil particles can be an important factor influencing both binding and removal of bacteria from fresh produce. Often the bacteria on the surface of fresh produce can be embedded into the soil. This layer of soil can act as a barrier in washing and sanitation of produce. To mimic this, this study measures the influence of surfactants to aid in removal of bacteria from fresh produce in the presence of soil. It is interesting to note that the presence of soil resulted in an increased resistance in removal of bacterial cells from fresh produce surface (Fig. 5). For example, the removal of *E. coli* adhered on the leaf surface decreased significantly by ~ 0.4 log CFU/cm² in the presence of soil as compared to the leaf samples inoculated with *E. coli* without soil (Fig. 5A). Similarly, the removal of *L. innocua* also

decreased by approximately $0.47 \log \text{ CFU/cm}^2$ as compared to the number of bacteria removed from lettuce leaf surfaces without soil (Fig. 5E). In addition, the presence of soil on leaf surface also reduced the decontamination efficacy of wash water with surfactants, which was also demonstrated by widefield imaging (Fig. 4B). But the decrease in bacterial reduction caused by LAE was lower than those of Tween-20 and SDS, which may be due to the antimicrobial activity of LAE against a wide range of food pathogens and spoilage organisms (Asker et al., 2009; Bonnaud et al., 2010). The results highlight that despite some decrease in the efficiency of removal of bacteria in the presence of soil, one of the selected surfactants, LAE was quite effective for the removal of both selected *Listeria* and *E. coli* strains from the produce surface.

Overall, the decrease in the efficacy of bacterial detachment may be a result of the increased hydrophobicity of leaf surface due to the presence of soil particles. This increased hydrophobicity may limit the mechanical contact of wash water with leaf surface (Hassan and Frank, 2003; Singh et al., 1984). It is also likely that the sticking efficiency of bacterial cells increased in the presence of soil (Farrokhian et al., 2015; Foppen and Schijven, 2005). Comparing the detachment efficacies of two tested strains, it was found that the efficacy of removal of *E. coli* O157:H7-*lux* was higher than *L. innocua*. This result could be explained by the differences in cell surface properties such as cell surface hydrophobicity between *E. coli* O157:H7-*lux* and *L. innocua*. Increased cell hydrophobicity may also enhance cell-to-cell adhesion and aggregation. Together these changes may reduce the efficiency of removing bacteria from fresh produce surface with mechanical agitation of wash water (Kristian Stevik et al., 2004; Liu et al., 2009).

The results of bacterial detachment from lettuce leaf surfaces after extended incubation

periods of 24 h and 48 h are presented in Fig. 5. The bacterial counts revealed that there was no significant difference in the cell persistence on the lettuce surface after 24 h of incubation. After 48 h of incubation period of bacteria on lettuce surface, the numbers of detached bacterial cells decreased slightly. In the published reports (Luo et al., 2004; Ölmez, 2010), the retention of bacteria on fresh produce surface in the absence of soil increased significantly with extended incubation period of 18 hours, resulting from increased adhesion of bacterial cells as well as formation of bacterial aggregates on leaf surface (Singh et al., 2002). In this study, however, the presence of soil may limit the contact of bacteria and leaf surface, which can reduce the formation of bacterial aggregates but also lead to more bonding of bacteria with soil particles. Since the extended incubation period may not increase the adhesion of soil particles to leaf surface, the removal of bacteria from lettuce surface in the presence of soil did not decrease significantly with an increase in incubation time to 24 hours.

Figure 5

3.5 Effect of surfactants on the quality of fresh lettuce

Influence of surfactants on the quality of fresh lettuce after washing was evaluated by measuring changes in color, electrolyte leakage rate and compressibility of fresh lettuce leaves. Color is one of the most important attributes in determining freshness of the produce. In lettuce, it can be affected by chlorophyll degradation, edge browning, and russet spotting (Rico et al., 2007). The representative photographs suggested no significant visible differences between the lettuce leaf surfaces before and after washing process (Fig. 6A). The total color changes of lettuce leaves subjected to different wash water are shown in Fig. 6B. Only a marginal change in the color of the lettuce leaves was observed upon washing with

and without surfactants as compared to the control leaves. According to the classification proposed by a published report (Chen and Mujumdar, 2009; Klug et al., 2016), the appreciable color differences in fresh cut lettuce ranged from 3.1 to 6.0. All the values of total color difference fell in the acceptable range of 0 – 3 (Chen and Mujumdar, 2009). These results agree with those of the published data (Izumi, 1999; Park et al., 2001; Salgado et al., 2014). The results of these prior studies suggest that the addition of surfactants (including Tween-20, SDS, and LAE) did not influence the color and general appearance of lettuce leaves.

Changes in electrolyte leakage rate (ELR) in Romaine lettuce before and immediately after washing are presented in Fig. 6C. Without any treatment, the leakage rate in lettuce was 1.31 ± 0.27 , which was similar to the published report (Sagong et al., 2013). Overall, there was no significant differences between the leaves before and after washing in the presence of surfactants. Lettuce leaves treated with SDS had the highest leakage rate, but it was still not significantly different from the ELR of the untreated leaves ($P > 0.05$).

The effect of washing process on lettuce texture are shown in Fig. 6D. The maximum compression force for the treated samples were smaller than the untreated ones, indicating a loss of turgor in the treated samples. The washing procedure resulted in a significant difference between the leaves before and after washing procedure for 20 min, except for the leaves washed by water. A similar trend was reported by (Manolopoulou et al., 2010; Sagong et al., 2013), indicating that the introduction of surfactants caused a decrease in firmness of lettuce leaves when compared to those washed by water. Also relatively large standard deviation in the maximum compression force was observed. This relatively large standard deviation can be attributed to leaf-leaf variations as well as differences in the locations of

sampling during mechanical compression of leaf samples.

Figure 6

4. Conclusions

In this work, we evaluate the effect of three types of commercial food-grade surfactants (including Tween-20, SDS, and LAE) on the mechanical removal of pathogenic bacteria and viruses from fresh lettuce leaves in the presence of soil. The addition of surfactants did not increase the removal of viruses from lettuce leaf surface ($P > 0.05$). The improvement of bacterial removal by addition of surfactants to wash water is corresponding to the decrease of the contact angle between wash water and leaf surface. Further studies are required to validate the influence of contact angle between wash water and fresh produce and removal of bacteria from fresh produce surface. The most efficacious removal of *E. coli* O157:H7-*lux* was obtained by washing with 0.1% LAE, followed by 0.1% Tween-20, then 0.1% SDS. The most enhanced detachment of *L. innocua* was achieved by 0.1% LAE, followed by 0.1% SDS, then 0.1% Tween-20. The presence of soil resulted in an increased resistance of bacterial cells to the washing process. There was no significant difference in the cell persistence on the lettuce surface within the extended incubation period ($P > 0.05$). The evaluation of lettuce quality indicated that the introduction of surfactants during the washing procedure may affect the firmness of leaves, but the color and electrolyte leakage rate were not affected by the exposure to wash water with surfactants ($P > 0.05$). Further studies are needed to understand the mechanisms of bacterial adhesion on plant surface and the role of surfactants. These studies may be conducted using the real-time imaging of changes in the distribution of bacteria on leaf surface.

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Figure captions

Fig. 1 (A) Cell surface hydrophobicity of *E. coli* O157:H7-*lux* and *L. innocua*, cell surface charge of *E. coli* O157:H7-*lux* and *L. innocua*, and (C) surface tension of wash water with the selected surfactants. Treatments with different letters are significantly different ($p < 0.05$).

Fig. 2 Contact angles between wash water and leaf surface: (A) DI water, (B) Tween-20, (C) SDS, and (D) LAE. Treatments with different letters are significantly different ($p < 0.05$).

Fig. 3 Effect of surfactants on the removal of microorganisms from lettuce surface: (A) *E. coli* O157:H7-*lux* and *L. innocua*; (B) T7 phage. Treatments with different letters for the same strain are significantly different ($p < 0.05$). Asterisk (*) represents significant differences in removal of *E. coli* O157:H7-*lux* and *L. innocua* from the leaf surface ($p < 0.05$).

Fig. 4 Representative widefield bioluminescence images of lettuce leaves after exposure to different wash water: (A) leaf surface without soil; (B) leaf surface with soil.

Fig. 5 The influence of incubation time on bacterial removal in the presence of soil: (A-D) *E. coli* O157:H7-*lux* and (E-F) *L. innocua*. Treatments with different letters are significantly different ($p < 0.05$).

Fig. 6 Effect of surfactants on the quality of lettuce surface during washing: (A) representative photograph of leaf samples before and after washing process, (B) total color difference, (C) electrolyte leakage, and (D) texture analysis. Treatments with different letters are significantly different ($p < 0.05$). Asterisk (*) represents significantly different values from control samples (paired *t*-test, $p < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6