Surface decontamination of *Salmonella Typhimurium* and *Escherichia coli* on sweet basil by ozone microbubbles

Asama Phaephiphat\(^1,2\) and Warapa Mahakarnchanakul\(^2\)*

**Abstract:** The objective of this study was to investigate the effectiveness of ozone microbubbles water (OMB) compared to microbubbles water (MB), and sodium hypochlorite (NaOCl) on surface decontaminating inoculated *Salmonella Typhimurium* and *Escherichia coli* on sweet basil. The washing of a heavy initial load of *S. Typhimurium* on sweet basil was done by washing at 30\(^\circ\)C or 10\(^\circ\)C with OMB (at 0.5, 1.0, or 2.0 mg/L), MB and tap water (TW). Exposure times of 2, 5, or 7 min under shaking at 60 rpm. The results indicated that washing for 7 min showed the highest reduction of *S. Typhimurium*, 2.6 log CFU/g at 30\(^\circ\)C with 1.0 mg/L of OMB and 2.2 log CFU/g at 10\(^\circ\)C with 2.0 mg/L of OMB. Washing treatments with MB or TW at 10\(^\circ\)C did not result in greater bacterial reductions compared to 30\(^\circ\)C, similar results ranged from 1.3 to 1.8 log reductions. The optimal condition was selected and applied to washing a low initial load (4 log CFU/g) of *S. Typhimurium* and *E. coli*. Using OMB compared to MB and the common sanitizer, NaOCl (at 50 or 100 mg/L) reduced the populations of *S. Typhimurium* by 2.8, 1.9, 4.2, and 4.2 log CFU/g, while the populations of *E. coli* were reduced 2.1, 1.0, 1.4, and 1.6 log CFU/g. Micrographs from Scanning Electron Microscope (SEM) revealed that microbubbles has no bactericidal effect, but microbubbles could help to remove contaminants on leaf surfaces. Bacterial cells lysis was achieved when using OMB and NaOCl and caused cell death.

**Subjects:** Agriculture and Food; Food Microbiology; Food Safety Management

**Keywords:** ozone microbubbles; washing process; decontamination; leafy vegetables; food safety

**ABOUT THE AUTHOR**

Asama Phaephiphat Our group conduct a research about food safety especially washing process to reduce pathogens contaminated in fresh produces.

**PUBLIC INTEREST STATEMENT**

Recently, the consumption of sweet basil as a side dish herb has been increasing. When it is eaten fresh, consumer illness and outbreaks are frequently reported which related to fresh-produce consumption. Therefore, washing process is a necessary step to apply for reducing microorganism contamination on vegetables.

This article reveals the efficiency of using microbubbles technology combined with sanitizers in the washing process. This alternative method will help to achieve higher efficacy on microbial decontamination of leafy vegetables surfaces which can be used at the household level.
1. Introduction

World Trade Organization (2017) has reported that world exports of agricultural products have increased by 70% since 2006. Thailand, an agricultural country, has increased vegetable production for domestic consumption and export markets. Fresh produce export is one of the major economic drivers. It has contributed more than 30 million US dollars per year (The Centre for Agricultural Information, Office of Agricultural Economics, 2017). One of the valuable export vegetables is sweet basil (*Ocimum basilicum* L.), a leafy green herb which has a unique taste and odor, and is good source of vitamin A, vitamin C, minerals and phenolic compounds associated with antioxidant capacity (Nguyen, Kwee, & Niemeyer, 2010). Typically, sweet basil can be eaten as raw vegetables as side dishes or it may be added to cooked food as an ingredient. According to the style of use, especially where it is eaten fresh, consumer illness and outbreaks are reported frequently that are related to fresh-produce consumption. Some pathogenic bacteria, *Escherichia coli* and *Salmonella* spp. have been detected and associated with food-borne outbreaks from (imported) fresh leafy herbs particularly sweet basil (Delbeke, Ceuppens, Jacxsens, & Uyttendaele, 2015). There are report supported that *E. coli* has the ability to attach well to leafy structures, then they are difficult to be removed from leaf surfaces (Bermúdez-Aguirre & Barbosa-Cánovas, 2013; López-Gálvez, Allende, Selma, & Gil, 2009). This occurrence leads to detention of these vegetable products and may cause illnesses. To address this problem, an effective decontamination protocol is needed to apply in vegetable production.

The washing of fresh produce is not only an important step to remove dirt, soil, and debris but also helps to reduce microbial contamination on vegetables surfaces. In addition, adding sanitizers into the wash water could help to achieve higher efficacy with microbial decontamination of leafy vegetables surfaces. However, not all washing procedures and washing treatments are effective (Olaimat & Holley, 2012), the success of washing depends on many factors such as type of washing, exposure time, the concentration of sanitizing agent, pH, temperature as well as the target microorganisms, the characteristics of produce surface, the attachment of cell to produce surface, the formation of resistant biofilms and the internalization of microorganisms (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008). Thus, the selection of sanitizers applied in the washing step is a critical step for achieving the quality and safety of fresh produces. Ideal sanitizer should perform sufficient level of antimicrobial activity, and be a negligible effect on the sensory quality of the product (Allende et al., 2008). Most of the common type of sanitizers are oxidizing agents, working by increasing the oxidation potential in the water system, and are considered to be the Generally Recognized As Safe (GRAS). Chlorinated water is by far the most-used sanitizing method for washing produce; however, its effectiveness in sanitizing vegetables is minimal, with less than 99–99.9% reduction (Chang & Schneider, 2012; Park, Alexander, Taylor, Costa, & Kang, 2008). Moreover, the use of chlorine has been associated with the formation of carcinogenic compounds in the last few years, and some pathogens have been shown to be more resistant.

Ozone has been suggested as an alternative in the washing process. Ozone has been approved in the United States to be used in its gaseous or liquid phase as a sanitizer of food because it has better antimicrobial properties than chlorine Kim, Yousef, & Khadre, 2003). Ozone is commonly used to sanitize drinking water; however, one of the main challenges with ozone as a sanitizer is its poor stability when organic matter is present (Selma, Allende, López-Gálvez, Conesa, & Gil, 2008). There are reports on the efficiency of ozone in vegetable products that present with some injuries and wounds, where the bacteria can be protected from the action of ozone and not be inactivated (Kim et al., 2003). This occurrence may intercept ozone to reach its full potential in eliminating bacteria. Thus, sanitation of fresh produce sometimes requires a combination of several treatments, such as washing with mechanical treatment, to achieve higher inactivation.

Microbubble technology has been extensively used for improving ozone-based, advanced oxidation processes for water and wastewater treatment (Chu et al., 2008; Khuntia, Majumder, & Ghosh, 2012; Li, Tsuge, & Itoh, 2009). Nowadays, microbubbles technology has been applied in the washing process in a few studies with food materials, particularly to reduce the microbial
contamination on leafy vegetables. Klintham, Tongchitpakdee, Chinsirikul, and Mahakarnchanakul (2017) studied a combination of microbubbles with oxidizing sanitizers to eliminate E. coli and S. Typhimurium on Thai leafy vegetables. Following its success of using microbubbles for sanitation, the use of ozone microbubbles water may be a good alternative method for the decontamination of pathogenic bacteria on fresh produce. Microbubbles combined with ozone known as OMB is a new technology that converts ozone gas to small sized bubbles (<50 µm) in water while the diameter of bubbles generated by this machine was in the range of 1.23–3.41 µm. This new sterilization method has been applied in many fields except in food processing. There have been only a few studies on the effect of microbubbles combined with sanitizer in washing fresh produce (Klintham et al., 2017; Kobayashi, Ikeura, Ohsato, Goto, & Tamaki, 2011; Lee, Lee, Yoo, Kim, & Jang, 2011; Lee, Youn, Kwon, Kim, & Cha, 2009). Therefore, the objective of this study was to investigate the effectiveness of microbubbles water (MB) and ozone microbubbles water (OMB) compared with traditional sanitizer (sodium hypochlorite solution) in decontaminating artificially inoculated S. Typhimurium and E. coli on sweet basil.

2. Materials and methods

2.1. Sample preparation

The sweet basil used in this study was purchased from the fresh market, Bangkok during 2015–2017. The plant samples were transported to the Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand within an hour. Flowers, bruised leaves and yellowed decay tissue were removed. Then, the sweet basil was cut into lengths approximately 16–18 cm before use. Pre-washing with tap water removed dirt on samples, followed by draining on a clean, stainless steel mesh in a bio-safety cabinet (Model: Microflow Advance Biosafety cabinet, Astec Microflow Ltd, UK) for 30 min. Then, all vegetable samples were placed in sealed polyethylene (PE) plastic bags (40 × 50 cm). Before inoculating samples with tested bacterial strains, vegetable samples were randomly taken to test the natural presence of E. coli and Salmonella spp. by selective plating agar. The spread plate technique was used to culture onto MacConkey agar (MAC, Merck Chemical, Germany) for E. coli and onto Xylose lysine deoxycholate agar (XLD, Merck Chemical, Germany) for Salmonella spp. In addition, the background flora was enumerated by standard plate count agar (PCA, Merck Chemical, Germany).

2.2. Bacterial strains

A culture of Salmonella Typhimurium DMST 562 and the Escherichia coli DMST 4212, used as a surrogate for E. coli O157: H7, were obtained from the Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand (DMST). The bacteria were maintained on nutrient agar (NA) slant (Merck Chemical, Germany) held at 4°C. A loopful of bacteria was transferred into 10 mL of tryptic soy broth (TSB, Merck Chemical, Germany) and incubated for 18–24 h at 35–37°C consecutively for two days before inoculum preparation. Inoculum was prepared as 1 L with an incubation period of 18 h. The population of inoculum was between 8 and 9 log CFU/mL before inoculating on sweet basil.

2.3. Inoculation of sweet basil

The preparation of sweet basil was done by pre-washing, soaking in tap water for 5 min. After that, 100 g of sweet basil was submerged in 200 mL of bacterial inoculum in a polyethylene bag and then manually shaken for 2 min and left for 20 min after which the samples were placed in a laminar flow cabinet and allowed to dry for 20 min. Inoculation was done to achieve final number of cells of 4 log CFU/g for a low initial load and 6–7 log CFU/g for a heavy initial load. At these high amounts of the initial load, the tested bacterial strains that present on inoculated sweet basil will be able to grow over the natural flora. Then, the contaminated samples were divided into two sets. One set was analyzed for the initial load of S. Typhimurium and E. coli cells before washing. The other set was washed and then analyzed for survivors after the different treatments.
2.4. Preparation of sanitizers
Forty liters of tap water (10°C and 30°C) were used to generate microbubbles water (MB) using a microbubbles generator model Microstar FS101-1 (Fuki Manufacturing Co, Ltd., Japan) in an acrylic tank. The machine was kept running for 5 min. The pH and temperature of the wash water at before and after washing were determined using an oxidation-reduction potential (ORP) and pH meter (Model: Cyberscan pH 510, EUTECH Instruments, Singapore). Ozone microbubbles (OMB) were generated in tap water (10°C and 30°C) using a microbubbles generator which mentioned above together with the ozone generator model ED-0GR6 (Ecodesign.inc., Japan) at a flow rate of 0.5 L/min in an acrylic tank (Figure 1). Ozonated water solutions were produced containing 0.5, 1.0, or 2.0 mg/L dissolved ozone. The dissolved ozone concentration was measured using an N,N-diethyl-p-phenylenediamine (DPD) measuring photometer with a DPD tablet No.4 (ProMinent® HD-MMP 01, Germany) immediately before use.

Sodium hypochlorite solutions (50 and 100 mg/L) were freshly prepared immediately before use by diluting 6% (w/v) sodium hypochlorite (Kao Industrial, Thailand) in tap water to achieve concentrations of 50 or 100 mg/L free chlorine. The best compromise of activity and stability was achieved by maintaining a water pH between 6.5 and 7.5 adjusted using 1.0% v/v acetic acid solution (Merck Chemical, Germany). The standard method was followed for analysis of residual chlorine (IS: 3025 (Part 26)—Reaffirmed, 2003).

2.5. Washing process
For all experiments, 100 g of sweet basil was submerged in 4 L of each sanitizer (ratio 1:40 of sample: water) and agitated on an orbital shaker (SK 300, JeioTech, UK) at 60 rpm for 2, 5 or 7 min. The washed sweet basil was drained and dried in a laminar flow cabinet at 26 ± 2°C (65–70% relative humidity) for 30 min before microbial analysis. The control sample was made using inoculated vegetables before washing.

2.6. Bacterial analysis
After washing, vegetables were kept in the laminar flow cabinet for 30 min; then random samples from each treatment were taken for microbiological analysis. Twenty-five grams of vegetable sample were mixed with 225 mL of 0.1% w/v peptone water as a diluent in a sterile stomacher bag, and the solids were broken down using a laboratory stomacher (Model 400, Stewart, UK). Serial dilutions (1:10) with 0.1% peptone water were performed and then spread-plated onto XLD for S. Typhimurium and onto MAC for E. coli. All plates were incubated at 37 ± 2°C for 24 h. The populations of microorganisms in 10 mL of wash water were determined. The colony counts were expressed as log CFU/g and log reductions were determined by subtracting the log CFU/g for the corresponding treatment from the log CFU/g of the control sample. The control sample was made using inoculated vegetables before washing.

Figure 1. Experimental setup for microbubbles and ozone microbubbles generation. Laboratory setup (a); and schematic diagram (b).
2.7. Microscopic study of sweet basil
The treated sweet basil samples using different sanitizers were cut into small pieces (0.5 × 0.5 cm) using a sterile knife. After that, samples underwent primary fixation with a fixative buffer (2.5% glutaraldehyde in 0.2 M sodium phosphate buffer, pH 7.2) at 12°C and left overnight at 4°C. Then, they were washed in 0.1 M sodium phosphate buffer (pH 7.2) and post-fixed with 1% osmium tetroxide for 1 h, before rinsing with sterile distilled water. Then, samples were dehydrated in an ethyl alcohol series (20%, 40%, 60%, 80% and 100%). The dehydrated samples were dried immediately using a critical point dryer (Emitech; K850, England) for 1 h. Then, samples were coated with platinum using an ion coater (Q150RES, Quorum, England) and the specimens were examined under a scanning electron microscope (SEM, HITACHI, SU 8020, Japan) operating at 5.0 kV.

2.8. Statistical analysis
The experiments were carried out in duplicate. Data were converted to log CFU/g and recorded as mean ± standard deviation and analyzed using the SPSS software, version 12 (SPSS Inc., Chicago, IL. USA). Differences between groups were analyzed using one-way analysis of variation and mean comparisons were conducted using Duncan’s multiple range test. Differences between means at 5% (p< 0.05) level were considered significant.

3. Results and discussion

3.1. Effects of microbubbles and ozone microbubbles on reducing S. Typhimurium on artificially inoculated sweet basil
The results from studying the efficacy of tap water, MB, and OMB for the decontamination of S. Typhimurium are shown in Figure 2. Different temperatures of wash water at 30°C, and 10°C were chosen to compare the stability of ozone when it was used to generate microbubbles. The highest bacterial reduction was 2.6 log CFU/g or 99.8% after washing with OMB at 1.0 mg/L for 7 min. While using 0.5 and 2.0 mg/L showed less reduction at the same temperature, 30°C. The exposure time at 2, 5 and 7 min did not result in any significant differences in log reduction of bacterial populations (p> 0.05) (Figure 2(a)). OMB at 10°C was chosen to compare the stability of ozone when it was used to generate microbubbles. However, the results on using this low temperature did not show better efficacy. The highest bacterial reduction was 99.4% after washing with OMB at 2.0 mg/L for 7 min (Figure 2(b)). Washing with MB performed similar results on both temperatures, reduction in the S. Typhimurium population varied from 93.4 to 97.9% which not significantly different when compare to using tap water, the results showed 92.1–98.4%. According to the results, the optimal condition for washing sweet basil at ambient temperature (30°C) was selected at 1 mg/L OMB for 7 min and microbubbles has no bactericidal effect but helped to reduce bacteria by detaching contaminated bacteria from sweet basil.

Our results from using OMB were in accordance with Inatsu et al. (2013) studied the effectiveness of stable ozone microbubbles water on reducing bacteria on the surface of lettuce, Chinese-cabbage, spinach and cabbage. Their results indicated that E. coli viable cells were reduced 0.8–1.2 log CFU/g (84.1–93.7%) after washing with ozone microbubbles water for all tested leafy vegetables. However, Inatsu et al. (2013) found no significant differences were observed in the effectiveness of ozone microbubbles water, ozonated water and distilled water which contradicts to our study which showed the highest of bacterial reductions when using ozone microbubbles. It may explain about the difference of bubbles’ stability and ozone concentration.

Fukumoto, Hashizume, and Nishimura (2010) reported that OMB water was effective in inactivate the growth of bacterial withering disease of tomato, brassica and strawberry with no harm to postharvest quality. Not only OMB effects on bacterial reduction, there was a report that OMB can inactivate fungal particular plant pathogen such as Fusarium spp. Kobayashi et al. (2011) reported that OMB had sanitizing activity against Fusarium oxysporum f. sp. melonis and Pectobacterium carotovorum subsp. carotovorum in infected plant roots. OMB had a higher solubility and remained in the water for a longer period than ozone millibubbles, the diameter less than 3 mm, resulting in...
extremely high sanitizing activity against both phytopathogens. Kwack, Kim, Hwang, and Chun (2014) investigated the efficacy of ozone microbubbles water in reducing microbial populations on alfalfa seeds. These successful results from decontaminating bacteria and inactivating plant pathogens might result from the specific properties of microbubbles such as a slow rising velocity that could help the ozone to remain in the water for a long time.

For more understanding, some researchers considered that microbubbles are small bubbles made up in part of fine bubbles. The MB diameter range is in the range 10–50 μm (Takahashi, 2005). As the MB are very tiny, they can remain stably suspended in the water for a longer period (over several months) gradually decreasing in size and finally disappearing in the water (Tsuge, 2014). However, the efficacy of washing depends on many factors, the selection of the optimum washing process is important. The appearance of the vegetables is another factor of concern. Preliminary study found that increasing the OMB concentration up to 3.0 mg/L and the washing
time to 10 min affected the appearance of sweet basil compared with lower concentrations. At this high dose of ozone and long washing time caused stem and leaves wilt, but no color changes.

In the fresh produce washing process, some food industries use a thermal sanitation method and/or radiation (Birmpa, Sfika, & Vantarakis, 2013). Tirawat, Phongpaichit, and Benjakul (2016) studied the efficacy of lactic acid (LA) and acidic electrolyzed water (AEW) against mesophilic bacteria on sweet basil. A combination treatment using mild heat with the selected sanitizer to disinfect sweet basil was also investigated. The results indicated that LA can be more effective than AEW in disinfection and provides greater efficiency when combined with mild 50°C heat. Even thermal sanitation can be effective in microbial elimination, but the cost is high and the process could affect the appearance of fresh produce and consequently, chemical sanitizing agents are more often used. For example, chlorine is often used in sanitation due to its advantages; easy to apply, inexpensive, effective against all microbial forms, easy to monitor and FDA approved (Sapers, 2014). Ozone is also used as a sanitizing agent due to its properties such as more potent antimicrobial than chlorine, no chlorinated reaction products formed, economical to operate, self-affirmed GRAS (Sapers, 2014). Therefore, finding new technologies to enhance ability and reduce the use of chemical sanitizing agents could be possible.

Based on the current results, the main effect was caused by the ozone acting on the microbial cells (Hampson & Fiori, 1997). Ozone is one of the most efficient sanitizing agents; it has high sanitizing activity and is widely used. However, ozone dissolves in water, decreasing its oxidation properties. Some researchers have studied the efficacy of ozone in the fresh produce washing process. Ölmez and Temer (2010) studied the effects of ozone (2 mg/L) treatment at 10°C for 2 min on the removal of E. coli and L. monocytogenes cells embedded inside biofilms on the surface of lettuce leaves. The populations of E. coli and L. monocytogenes were reduced 1.2–3.75 log CFU/g. Barak, Jahn, Gibson, and Charkowski (2007) reported that ozone at 5 mg/L was effective to inactivate bacteria in tomatoes (2.2 log) after only 3 min. The inactivation efficacy of ozone is an oxidation-reduction potential (ORP), considered as a primary factor affecting microbial inactivation, as the oxidizing chemical pulls an electron away from the cell membrane causing the cell to destabilize and leak, which then destroys the integrity of the cell membrane leading to rapid death. The water used in the washing system should be maintained with an ORP value higher than 650 mV for an adequate bactericidal effect (Suslow, 2004). Klintam et al. (2017) observed that ORP was rapidly decreased when washing vegetables with oxidizing agents such as acidic electrolyte water (AEW, 20 and 40 mg/L, ORP 910–1010 mV, pH 2.7–3.1), chlorine dioxide (ClO2, 3 and 5 mg/L, ORP 550–680 mV, pH 7.1–7.5), and sodium hypochlorite (NaOCl, 40 and 80 mg/L, ORP 900–990 mV, pH 6.5–6.7). In our study, ORP of wash water was measured. Before washing at the concentrations of OMB 0.5, 1.0 and 2.0 mg/L for 7 min, the ORP showed 695, 927 and 1070 mV, respectively. Another parameter that related the efficiency of ozone is pH; high or low pH can alter ORP readings involving dissolved ozone due to the rapid decomposition of ozone at elevated pH. Optimal accuracy requires pH levels within 6.58 (Tapp & Rice, 2012). Washing solutions in this experiment were in the pH range of 6.97–7.78.

According to the results, using 1.0 mg/L OMB at 30°C for 7 min showed the highest on bacterial reduction. Then, it was selected to apply in the next experiment.

3.2. Effects of MB, OMB and NaOCl on reducing S. Typhimurium and E. coli on inoculated sweet basil
In the second experiment, OMB at 1.0 mg/L and 30°C was selected from the previous experiment to decontaminate a low initial load of S. Typhimurium and E. coli on sweet basil, as it showed the best treatment condition on decontamination, less smell of ozone on sweet basil after washing and safe for workers on the generation site. In this experiment, comparison of washing efficacy between OMB and sodium hypochlorite solution (NaOCl). Two different concentrations of NaOCl, 50
and 100 mg/L, were selected by the recommendation of using chlorine dosage in fresh produce sanitization, 50–200 mg/L (Itoh et al., 1998; Martínez-Hernández et al., 2015).

Sodium hypochlorite with high concentration, 100 mg/L, was expected to perform good result on decontamination. In our study revealed that 50 and 100 mg/L of NaOCl and an exposure time of 7 min could result in 96.2% and 97.5% reductions, respectively, in E. coli (from the initial population 3.94 log CFU/g) as shown in Figure 3(a). When washing with OMB at 1.0 mg/L and 30°C, the higher reduction was observed of 99.1% (from the initial population 3.84 log CFU/g). In contrast, S. Typhimurium had a 99.6% reduction for tested vegetables (from the initial population 4.09 log CFU/g) after being treated under the same conditions, 1.0 mg/L of OMB (Figure 3(b)). The highest decontamination efficacy was shown after washing with either concentration of 50 or 100 mg/L of NaOCl, with a reduction of more than 4 log CFU/g of S. Typhimurium population (99.9% reduction). The results revealed that S. Typhimurium was more sensitive to sanitizers than E. coli when exposing to sodium hypochlorite.

Figure 3. Effects of microbubbles water, ozone microbubbles water at 1.0 mg/L and 30°C and NaOCl at 50 or 100 mg/L on decontamination of E. coli (a) and S. Typhimurium (b). Error bars indicate ± standard deviation of the mean of replicated experiments (n = 4). Different letters represent significant differences at p < 0.05.
This occurrence may be explained by the difference of the attachment ability of each bacterial strain to the leafy surfaces. São José, Medeiros, Bernardes, and Andrade (2014) reported that the total energy of adhesion for *S*. Enteritidis was less than *E*. coli when measured the hydrophobicity of the microorganisms and the vegetable surfaces, in our study *S*. Typhimurium cells were easily detached and expected to expose to the sanitizers. In case of low load contamination, MB detached both bacteria on tested sweet basil. The results indicated that *E*. coli and *S*. Typhimurium were reduced 90.2% and 98.8%, respectively. Moreover, survival of bacteria in wash water was determined. The result shows in Table 1 indicated that adding sanitizing agents to the wash water can significantly eliminate the population bacteria cells which help to reduce the risk of cross-contamination, corresponding to the observation has been mentioned by Sapers (2014).

Increasing the concentration of sanitizer could result in greater microbial log reductions (Neo et al., 2013), but not always when using chlorine. Similar results were reported by Klintham et al. (2017) that no significant difference in log reductions between 40 and 80 mg/L of NaOCl used to wash sweet basil or Thai mint. Furthermore, they also discovered that removal contaminated bacteria on Thai mint was more difficult than sweet basil. This result could be elucidated by different type of vegetable influences in washing. It is possible that roughness of leaves surface could effect on bacterial attachment. Using scanning electron microscope will help to clarify in the next experiment.

### 3.3. Micrographs of untreated and treated sweet basil

The microstructures of bacterial cells on tested sweet basil were observed using a scanning electron microscope (SEM) to understand the difference mechanism of OMB and chlorine on elimination of contaminated bacteria. Figure 4 shows the microstructures of *S*. Typhimurium cells attached to the sweet basil surface. In the untreated control sample (Figure 4(a)), *S*. Typhimurium cells remained on leaf surface. Figure 4(b) shows the microstructures of *S*. Typhimurium cells on sweet basil leaf surface after treated with MB for 7 min. According to the observation, *S*. Typhimurium cells were less dense when compared to the control sample but cells still intact. It may expect that microbubbles could help to remove contaminated bacteria from the sweet basil surfaces, albeit absence of bactericidal activity.

When adding sanitizers (NaOCl at 50 mg/L) into the wash water to observe the changes of cells under SEM (Figure 4(c)). The micrograph indicated that almost all bacterial cells were damaged. Chlorine compounds are broad spectrum germicides which act on microbial membranes, inhibit cellular enzymes involved in glucose metabolism, have a lethal effect on DNA and oxidize cellular protein (Schmidt, 1997). However, major disadvantages are also associated with chlorine byproducts, such as chlorine cause the metal surfaces corrosion, skin irritation, and mucous membrane damage. Moreover, under certain conditions, chlorine can form chlorinated organic derivatives, some of which are the potentially carcinogenic such as: trihalomethanes (THMs), chloroform, and

### Table 1. Survival of *E*. coli and *S*. Typhimurium in wash water at 30°C after washing sweet basil with MB, OMB and NaOCl

| Treatments     | pH     | *E*. coli (log CFU/mL) | pH     | *S*. Typhimurium (log CFU/mL) |
|----------------|--------|------------------------|--------|------------------------------|
| MB             | 7.67 ± 0.14 | 2.27 ± 0.03          | 7.75 ± 0.11 | 2.77 ± 0.21                |
| OMB 1.0 mg/L   | 7.56 ± 0.08 | ND                    | 7.58 ± 0.15 | ND                          |
| NaOCl 50 mg/L  | 6.80 ± 0.12 | ND                    | 6.75 ± 0.10 | ND                          |
| NaOCl 100 mg/L | 6.67 ± 0.10 | ND                    | 6.77 ± 0.09 | ND                          |

Note: Values are mean ± standard deviation of the mean of replicated experiments (n = 2), ND = Not detected (counts below 1 CFU/mL)
Chlorophenols carry a health risk because they are potentially mutagenic (Owusu-Yaw, Toth, Wheeler, & Wei, 1990). Even NaOCl gave good result but due to concerns about its safety, ozone was combined with MB to achieve great efficacy without using harsh chemicals.

Previous experiments have shown that OMB at 1.0 mg/L produced the highest efficacy to eliminate S. Typhimurium on tested sweet basil. Subsequently, it was selected to treat sweet basil and observed under an SEM (Figure 4(d)). The micrograph indicated that it gave a good result on bacterial decontamination treated with OMB at 1.0 mg/L. The white arrow in Figure 4(d) pointed bacterial cells lysis which caused by ozone. Thanomsub et al. (2002) mentioned that ozone has bactericidal activity; destroying the bacterial cell membrane, subsequently producing intracellular leakage and finally causing cell lysis.

Similar treatment conditions were applied to observe the microstructure of E. coli cells on the sweet basil surface. The untreated control sample (Figure 5(a)) shows the cells of E. coli have spread out into the leaf surface near stomata. Many researchers have been reported that E. coli can attach at inaccessible sites like the stomata on the leaves of leafy vegetables (Itoh et al., 1998; Seo & Frank, 1999). Figure 5(b) shows the microstructure of E. coli cells on the sweet basil leaf surface that was removed after treatment with MB for 7 min, similar to the scenario of Salmonella (Figure 4(b)). The micrograph indicates that the visible cell populations were less dense compared to the control sample. The E. coli cells showed signs of damage (Figure 5(c)) after being subjected to the same treatment conditions (50 mg/L of NaOCl) as S. Typhimurium. In Figure 5(d) which showed bacterial cell lysis on sweet basil surfaces after exposed by OMB 1.0 mg/L for 7 min. Ozone microbubbles (OMB) lysed bacterial membrane and caused cell death. According to Kim et al. (2003) ozone causes cells death due to the oxidation of lipids on the cells. Ozone acts on the unsaturated lipids of the cell membrane, and in the lipopoly-saccharides coat of Gram-negative bacteria, enzymes and genetic material and promoting the death of the microorganism.
The ability of Salmonella to attach to our tested vegetable, sweet basil, may differ from the other types of vegetable such as cabbage, lettuce, spinach and tomato (Patel & Sharma, 2010; Patel, Singh, Macarisin, Sharma, & Shelton, 2013; Weissinger, Chantarapanont, & Beuchat, 2000). Microorganisms preferred to attach around the oil glands rather than on the stomata walls (Aharoni et al., 2010). In this study, micrographs showed many oil glands on sweet basil provided spaces for bacterial cells to attach (Figure 4(c,d)). Bacteria with greater hydrophobic characteristics may attach to the cuticle, a hydrophobic layer on plant surfaces composed of fatty acids, polysaccharides and waxes (Solomon & Sharma, 2009). Besides the topography of vegetables, the attachment depends on the bacterial strains. Researchers have shown that S. Enteritidis strains lacking genes involved in producing cellulose (bcsA) and capsule assembly genes (yihO) attached to alfalfa sprouts in lower numbers than wild-type strains (Barak et al., 2007). These same workers showed that strains lacking the bcsA could not attach to surfaces in high numbers in vitro but could still attach to produce surfaces in numbers similar to the wild-type strains and they reported that the attachment strength of Salmonella serovars was significantly higher on Romaine lettuce over intact cabbage.

Apart from the ability of bacteria to attach to the vegetables surface, the surface of intact produce covered by a hydrophobic waxy cuticle may allow hydrophobic Salmonella cells to attach to the waxy cuticle. However, breaks in the cuticle may expose hydrophilic structures from within allowing intimate contact between bacterial cells and the leaf, and may release previously unavailable nutrients to enteric bacteria, making them good sites for colonization (Patel & Sharma, 2010). Bacterial cells can survive on fresh produce and can be present on the surface and in subsurface structures including stomata and damaged tissues (Seo & Frank, 1999; Takeuchi & Frank, 2001; Takeuchi, Matute, Hassan, & Frank, 2000). In our study, micrographs indicated the distribution of E. coli cells occurred on surfaces, stomata and oil glands while Salmonella cells frequently attached to oil glands.
The type of vegetable is an importance factor that influences microbial adhesion as well as the effectiveness of washing. Leaf topography presents the surface roughness, crack in the cuticle and other damages are often sites at which bacteria colonize. Leaf stomata provide protective niches for the bacteria. Seo and Frank (1999) reported that E. coli O157:H7 cells have been found that cells could adhere better to cut lettuce leaf surfaces than intact or normal lettuce leaf surfaces. The reduction of E. coli and S. Typhimurium on sweet basil appeared to be higher than on Thai mint. Differences in surface roughness of these two leafy vegetables may effect on the washing efficacy (Klintham et al., 2017).

Removal of food-borne pathogen and spoilage bacteria from fresh produce surfaces has proved difficult. Surface adherence of bacteria may serve to enhance survival of bacteria during washing or sanitizing process. In our observation, the micrographs showed the difference between adhesions of each bacterial strain on the sweet basil leaf surface. It seems that the attachment of E. coli cells was greater than S. Typhimurium cells, corresponding to the observation has been mentioned by Klintham et al. (2017). In vegetables with smooth surfaces are easy for bacterial removal, but when the roughness is higher with some deep valleys, bacteria is not totally exposed to the mechanical forces/wash water of washing. In vegetables with high roughness with valleys and big cavities, bacteria are well protected themselves from mechanical forces and sanitizing agents (Wang, Feng, Liang, Luo, & Malyarchuk, 2009).

4. Conclusion
Applying microbubbles technology combined with ozone represents a novel method for new prospective applications in the food industry particularly fresh produce production (washing process). Ozone microbubbles (OMB), an alternative sanitizer produced good results regarding microbial decontamination on leaf surfaces as well as prevent cross contamination. The highest reduction of S. Typhimurium (2.6 log CFU/g or 99.7%) was obtained when using 1.0 mg/L of OMB at 30°C for 7 min. Using microbubbles alone performed similar results on bacterial reduction compared to using tap water at any washing conditions. However, after observation by using SEM revealed that microbubbles could help to remove contaminants on leaf surface but none of the bactericidal effect was observed. At low initial load of bacterial populations, although NaOCl showed higher bacterial reduction (99.9%) than OMB (99.6%) in case of S. Typhimurium. But for E. coli, using 1.0 mg/L of OMB at 30°C gave better results on bacterial reduction than using 50 mg/L of NaOCl. According to the results, this novel washing solution (OMB) could be potentially implemented in the washing step to enhance the safety of fresh vegetables production in both ways; achieve the general requirement for microbiological safety of fresh produce and reduce the use of chemical sanitizing agents.

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