A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Application of vacuum impregnation to washing process for enhancement of antimicrobial effect

미생물 저감화 효과 증진을 위한 세척 과정으로의

진공함침 기술 적용

February, 2016

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석사학위논문

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이 논문을 석사학위 논문으로 제출함

2016 년 2 월

서울대학교 농생명공학부

강준원

강준원의 석사 학위논문을 인준함

2016 년 2 월

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ABSTRACT

To enhance antimicrobial effect of sanitizer washing, vacuum impregnation was applied to sanitizer washing to increase the penetration activity of sanitizer into protected site where microbes could escape from contacting with sanitizer. This study was undertaken to evaluate the effect of VI applied to the washing process for removal of pathogens from fresh produce surfaces. At first, its antimicrobial effect was verified by applying to inoculated broccoli. Broccoli was inoculated with *Salmonella* Typhimuruim and *Listeria monocytogenes* and treated with simple dipping washing or with VI in 2 % malic acid for 5, 10, 20, or 30 min. There were two methods of VI: continuous and intermittent. When 2 % malic acid alone was applied to inoculated broccoli, mean log reductions of 1.5 and 1.3 $\log_{10}$ CFU/g were observed for *S.* Typhimurium and *L. monocytogenes*, respectively, after 5 min. However, there were no further reductions even though treatment times extended from 5 min to 30 min. When VI was applied, there were significant increases ($P < 0.05$) of reduction of pathogens with increasing vacuum for both continuous and intermittent treatment. In continuous VI treatment, there were no significant ($P \geq 0.05$) additional antimicrobial effect with increasing treatment time. In intermittent VI treatment,

however, there were significant ($P < 0.05$) additional antimicrobial effect with increasing treatment time. Scanning electron photomicrographs showed that bacteria tend to attach to or become entrapped in protective sites after simple wash processing (dipping). However, most bacteria were washed out of protective sites after intermittent treatment. Direct treatment of cell suspensions with VI showed that it had no inactivation capacity in itself since there were no significant differences ($P \geq 0.05$) between the reduction rates of non- and VI treatment. These results demonstrate that the increased antimicrobial effect of VI can be attributed to increased accessibility of sanitizer and an enhanced washing effect in protected sites on produce. Color, texture and titratable acidity values of broccoli treated with intermittent VI in 2 % malic acid for 30 min were not significantly ($P \geq 0.05$) different from those of untreated samples even though a storage interval was needed for titratable acidity values to be reduced to levels comparable to those of untreated controls. On the basis of this result that VI has possibility to be applied to washing process to improve antimicrobial effect, this technique was extended to various samples. VI was applied to organic acid washing against *Salmonella* Typhimurium, *Escherichia coli* O157:H7 and *Listeria monocytogenes* on paprika fruit, carrots, king

oyster mushrooms and muskmelons. The samples were treated with intermittent VI with 21.3 kPa and compared with dipping washing in 2 % malic acid for 3, 5, 10, 15 or 20 min. For simple dipping, the reduction rate of paprika was highest among the samples tested and followed by carrots, king oyster mushrooms and muskmelons. For VI treatment, a significant ($P < 0.05$) enhanced antimicrobial effect occurred with paprika and carrots. However, there were no significant ($P \geq 0.05$) differences in pathogen reductions between dipping and VI treatment for both king oyster mushrooms and muskmelons. This might be due to surface roughness. King oyster mushrooms ($R_a = 6.02 \pm 1.65$) and muskmelons ($R_a = 11.43 \pm 1.68$) had relatively large roughness values compared to those of paprika ($R_a = 0.60 \pm 0.10$) and carrots ($R_a = 2.51 \pm 0.50$). Adequate space for microbes to escape contact with sanitizer could be possible during VI treatment due to the relatively coarse surface roughness present in king oyster mushrooms and muskmelons. This view was based on scanning electron photomicrographs that showed many deep protected sites in king oyster mushrooms and muskmelons with many microbes located deep in these sites following VI treatment. Color, texture and titratable acidity values of paprika and carrots subjected to VI washing treatment with 2 % malic acid for 5 and

20 min were not significantly ($P \geq 0.05$) different from those of untreated control samples during 7 day storage.

Keywords: Fresh produce, sanitizer, Foodborne pathogens, organic acid, vacuum impregnation.

Student Number : 2014-20697

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I. INTRODUCTION

Fresh produce is popular worldwide because it is recognized as an important source of nutrients, vitamins and fiber which are potentially beneficial for our health (EUFIC, 2012; Olaimat and Holley, 2012) and consumers are increasingly concerned about staying healthy through proper diet. Also, a large variety of domestic and imported produce has become available year round. For these reasons, consumption of fresh produce has increased over the past two decades (Warriner et al., 2009).

However, foodborne illness outbreaks linked to fresh produce have been on the rise due to increased consumption of fresh produce resulting in significant numbers of illnesses, hospitalizations, and deaths. (Bennett et al., 2015; Callejón et al., 2015; Painter et al., 2013; Warriner et al., 2009). Pathogen contamination can occur during any of many steps along the farm-to-consumer continuum such as untreated manure used for fertilization, contaminated irrigation water, infected workers, the presence of domestic or wild animals and birds, and unclean containers and tools used in harvesting, packing, transporting, or processing (FDA, 2014). Although there are preharvest strategies which may help decrease the risk of contamination such as GAPs during growing and harvesting,

there is still much reliance on produce decontamination strategies applied by the processing industry (Goodburn and Wallace, 2013).

Generally, the food industry applies a washing process such as su bmersion or spray with chlorinated water containing 50-200 ppm to fresh produce to control pathogens (Wu and Kim, 2007). However, chlorine has serious drawbacks such as rapid depletion under conditions of high organic loading and formation of carcinogenic halogenated by-products generated by reaction of chlorine with organic matter (Wang et al., 2006). Therefore, alternative sanitizers are needed to overcome these limitations of using chlorine.

Organic acids, found in a variety of fruits and fermented foods, are one type of alternative sanitizer. They are generally recognized as safe (GRAS) and are known to have bactericidal activity (Dickson, 2006). Thus, they can be applied to inactivate foodborne pathogens on organic fresh produce. Organic acids act rapidly and kill a broad spectrum of bacteria. Moreover, they are effective within a wide temperature range and are not affected by water hardness (Marriott and Gravani, 2006).

Moreover, decontamination of produce by conventional washing and sanitizing is only marginally effective and often can only reduce numbers of pathogenic organisms by less than 2-3 log units (Gil et al.,

2009; Niemira, 2012). Although many past studies have demonstrated that this washing process cannot eliminate pathogens on fresh produce, however, it is still critically important in fresh produce processing as it provides a crucial chance to focus on pathogen inactivation and remove soil, dust and insects from fresh produce in the absence of practical strategies which could help to reduce the risk of fresh produce without causing significant deterioration of produce quality (Luo et al., 2012; Huang et al., 2012; Gil et al., 2009; Niemira, 2012). The efficacy of washing is mainly influenced by surface properties of produce (Fransisca and Feng, 2012; Wang et al., 2009). In part, the inefficacy of aqueous sanitizers is thought to be due to lack of ability to access protected sites (such as cut surfaces, stomata, bacterial aggregates and crevices) on the surface of fresh produce (Bernett and Beuchat, 2001; Olaimat and Holley, 2012). Because of this, it is crucial to develop effective sanitization strategies to control pathogens on produce surfaces and thus reduce foodborne illness outbreaks related to consumption of fresh produce.

Vacuum impregnation is a technique which exchanges the internal gas or liquid of a porous product occluded in open pores for an external liquid phase. It is the action of hydrodynamic mechanisms (HDM)

promoted by pressure changes (Fito et al., 1994). Two steps are needed to perform this operation after product immersion in a tank containing the liquid phase. In the first step, vacuum is applied to the system to promote the expansion and outflow of the product's internal gas. In the second step, atmospheric pressure is restored and compression leads to a great volume reduction of the remaining gas in the pores. As a result, external liquid flows subsequently into the porous structure (Fito et al., 2001). Thus, it can be a useful tool to introduce sanitizers into inaccessible sites. Therefore, the efficacy of washing with sanitizers is expected to increase by using this technique.

II. MATERIALS AND METHODS

2.1. Bacterial cultures and cell suspension

Three strains each of *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104), *E. coli* (ATCC 35150, ATCC 43889 and ATCC 43890) and *L. monocytogenes* (ATCC 19111, ATCC 19115, and ATCC 15313) were provided by the bacterial culture collection of the School of Food Science, Seoul National University (Seoul, South Korea), for this study. Stock cultures were prepared by growing strains in 5 ml of tryptic soy broth (TSB; Difco, BD) at 37 ℃ for 24 h, combining 0.7 ml with 0.3 ml of sterile 50 % glycerol and then storing at -80 ℃. Working cultures were streaked onto Tryptic Soy Agar (TSA; Difco, BD), incubated at 37 ℃ for 24 h and stored at 4 ℃ for less than 1 mo.

Each strain of *S.* Typhimurium, *E. coli* and *L. monocytogenes* was cultured in 10 ml TSB at 37 ℃ for 24 h, harvested by centrifugation at 4000 g for 20 min at 4 ℃ and washed three times with sterile 0.2 % peptone water (PW, Bacto, Sparks, MD). The final pellets were resuspended in 10 ml 0.2 % PW, corresponding to approximately 10^7 to

10^8 CFU/ml. Suspended pellets of all strains of the three pathogens were combined into a mixed culture cocktail for use in this study.

2.2. Sample inoculation

Broccoli (*Brassica oleracea L. var Italica*), whole fresh paprika (*Capsicum annuum* L.), carrots (*Daucus carota* subsp. *sativus*), king oyster mushrooms (*Pleurotus eryngii*) and muskmelons (*Cucumis melo* L.) used in this evaluation were purchased from a local market (Seoul, South Korea) and stored at refrigerator temperature (4 ± 2 °C) until experiments were conducted. Broccoli florets including 1 cm of stem were cut into segments with a diameter of 4 cm. Each of the final broccoli florets were of uniform size and color without any visible decay and weight of between 8 and 9 g. For inoculation of broccolil, the dipping method was choose to inoculate samples and needed a culture cocktail of high cell concentration because inoculation was conducted with a comparative high volume (1.5 L of peptone water (PW)). Therefore, 10 ml of culture cocktail (approximately 10^8-10^9 CFU/ml) was made as described previously and mixed with 1.5 L of 0.2 % sterile PW for a final concentration of approximately 10^6-10^7 CFU/ml. Samples

were completely submerged into this inoculum solution and shaken by hand for 10 min to ensure even distribution of the bacteria. After dipping, samples were separated from the cell suspension by draining the mixture on a sterilized rack and drying for 2 h in a laminar flow biosafety hood at 22 ± 2 ℃ to allow the attachment of bacteria, and used in each experimental trial. Three pieces of broccoli (approximately 25 g each) comprised a single set in each treatment. For inoculation of the others, Intact portions of produce surfaces were cut into 2 by 5 cm (=10 cm^2) pieces. Samples were placed on sterile aluminum foil in a laminar flow biosafety hood and 0.1 ml of previously described culture cocktail was evenly inoculated onto the surface of samples by depositing small droplets at 15-20 locations with a micropipettor. The inoculated samples were dried for 2 h in the laminar flow biosafety hood at room temperature (22 ± 2 ℃) to allow attachment of bacteria, and used in each experimental trial.

2.3. Procedure of treatment

2.3.1 Broccoli treatment

For organic acid treatment alone, inoculated samples were immersed in 1 L glass beakers containing 500 ml of 2 % malic acid (99.0 %; Samchun Chemical Co. Ltd., Pyeongtaek, Korea, pH 2.16) for 5, 10, 20, or 30 min at room temperature (22 ± 2 °C).

For vacuum impregnation, inoculated samples were immersed in 1 L glass beaker containing 500 ml of 2 % malic acid and treated with vacuum impregnation in a vacuum oven (OV-11, JEIO TECH Co., Ltd., Daejeon, Korea) for 5, 10, 20, or 30 min at room temperature (22 ± 2 °C). A vacuum of 61.3 kPa (=8.9 psi) or 21.3 kPa (=3.1 psi) was applied to the system (Atmospheric pressure is 101.3 kPa = 14.7 psi). In the experiment, two vacuum treatment methods were conducted: continuous treatment or intermittent treatment. Continuous treatment comprised of a single vacuum time interval and a single atmospheric pressure time interval. Continuous treatments consisted of 5 min treatment (comprised of 2.5 min vacuum followed by 2.5 min of atmospheric pressure), 10 min treatment (comprised of 5 min vacuum followed by 5 min atmospheric pressure), 20 min treatment (comprised of 10 min vacuum followed by 10 min atmospheric pressure), and 30 min (comprised of 15 min vacuum followed by 15 min atmospheric pressure). Intermittent treatment was made up of a collection of 5 min

treatment cycles which consisted of 2.5 min vacuum treatment followed by 2.5 min atmospheric pressure treatment. Therefore, 5, 10, 20 or 30 min of intermittent treatment had 1, 2, 4, or 6 cycles, respectively. All experiments were performed using a reticulated stainless steel instrument to keep the samples submerged to prevent their being on top of the washing solution. Also,

2.3.2 Whole paprika, carrot, king oyster mushroom and muskmelon treatment

For simple dipping treatment, inoculated samples were immersed in 1 L glass beakers containing 300 ml of 2 % malic acid (99.0 %; Samchun Chemical Co. Ltd., Pyeongtaek, Korea, pH 2.16) for 3, 5, 10, or 20 min at room temperature (22 ± 2 °C).

For VI treatment, inoculated samples were immersed in 1 L glass beakers containing 300 ml of 2 % malic acid and immediately treated with VI in a vacuum oven (OV-11, JEIO TECH Co., Ltd., Daejeon, Korea) for 3, 5, 10, 15, or 20 min at room temperature (22 ± 2 °C). In this treatment, intermittent VI of 21.3 kPa (=3.1 psi) was applied because i found it is more effective than continuous vacuum treatment

based on previous study. Intermittent treatment was comprised of a collection of 5 min treatment cycles, each of which consisted of 2.5 min vacuum treatment followed by 2.5 min atmospheric pressure (=101.3 kPa or 14.7 psi) treatment, except for 3 min treatment (which consisted of 1.5 min vacuum treatment followed by 1.5 min atmospheric pressure treatment) . Therefore, 5, 10, and 20 min of intermittent treatment had 1, 2, and 4 cycles, respectively. All experiments were performed using a reticulated stainless steel instrument to keep the samples submerged to prevent their rising to the top of the washing solution.

2.3.3 Cell suspension treatment

1 ml of cell suspension was directly placed into 100 ml of treatment solution (with no broccoli sample) and treated with non-vacuum impregnation (101.3 kPa) and intermittent vacuum impregnation (21.3 kPa) as above for 30 min to ascertain if vacuum impregnation in itself has any inactivation effect on bacterial cells (1 ml of cell suspension placed into 100ml of deionized water (DW) constituted the control). In this case, 0.7% malic acid was used instead of 2% malic acid because the antimicrobial effect of 2% malic acid with no broccoli sample was too

strong to observe differences of inactivation tendency between each treatment.

2.4. Bacterial enumeration

After treatments were performed, the treated samples (25 g of broccoli and 1 piece of 10 cm^2 of the others) and 1 ml of treatment solution containing cell suspension were immediately transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie,Quebec, Canada) containing 225 ml (for broccoli) and 100 ml (for the others) of Dey-Engley (DE) neutralizing broth (Difco) and test tubes containing 9 ml of DE neutralizing broth, respectively. Stomacher bags containing treated samples were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min and test tubes containing treatment solution including cell suspension were mixed using a vortex mixer for 10 s. After homogenization, 1 ml aliquots of stomached samples and mixed treatment solution containing cell suspension were tenfold serially diluted in 9 ml of sterile 0.2 % buffered peptone water and 0.1 ml aliquots of the samples or diluents were spread plated onto selective media. Xylose lysine desoxycholate agar (XLD; Difco),

Sorbitol MacConkey Agar (SMAC; Difco) and Oxford agar base with Bacto Oxford antimicrobial supplement (MOX; Difco) were used as selective media for the enumeration of *S.* Typhimurium, *E. coli* and *L. monocytogenes*, respectively. Where low bacterial numbers were anticipated, 250 µl of undiluted sample were plated onto 4 plates of each respective medium. All plates were incubated at 37 °C for 24 h before counting. Colonies were counted and calculated as $\log_{10}$ CFU/(g or ml or cm^2) and the detection limit was 1 $\log_{10}$ CFU/(g or ml or cm2).

2.5. Scanning Electron Microscopy (SEM)

In order to visually ascertain the effect of vacuum impregnation on attachment and removal of bacteria, surfaces were photographed using scanning electron microscopy (SEM). Broccoli samples were treated using two methods. One was vacuum impregnation of inoculated broccoli (intermittent treatment) for 30 min in 2 % malic acid. The other was simple dipping of broccoli in 2 % malic acid for 30 min. After treatment, cut and intact stem surfaces were shaved into thin slices (0.5 cm x 0.5 cm). Carrots, king oyster mushrooms, and muskmelons were each treated for 20 min. However, paprika was treated for 5 min because

microbial populations on samples treated with both simple dipping and VI were below the detection limit (1 log CFU/cm^2) after 5 min, and thus visual differences between the two treatments could not compared after 5 min. Following treatments, surfaces were shaved into thin slices (0.5 cm x 0.5 cm). The sample slices were immersed in 2 % Karnovsky's fixative for 2 h and washed three times with 0.05 M sodium cacodylate buffer for 10 min each. For post-fixation, sample slices were immersed in a solution of 2 % osmium tetroxide mixed with 0.1 M cacodylate buffer (1:1 v/v) for 2 h and briefly washed twice with distilled water. The fixed sample slices were dehydrated with a graded ethanol series (once in 30, 50, 70, 80, 90 %, and three times in 100 %) for 10 min each. The sample slices were then completely dried in a Balzers CPD 030 critical point drying apparatus (BAL-TEC, Balzers, Lichtenstein). Dried sample slices were mounted on aluminum stubs and then sputter-coated with gold using a vacuum coater (EM ACE200, Leica, Germany). Finally, photomicrographs were obtained using a Field-Emission Scanning Electron Microscope (SIGMA, Carl Zeiss, Germany).

2.6. Surface roughness analysis

Scanning interferometry was performed for quantitative analysis of sample surfaces. The samples were mounted on a noncontact 3D surface profiler (NanoView-E1000, Nanosystem, Daejeon, Korea) which was used to measure the surface roughness of the scan area (125 μm × 95 μm) using a 50 × objective lens. Average roughness as topography parameters were acquired using a software package (NanoMap version 2.5.17.0, Nanosystem, Daejeon, Korea) from five randomly selected scan areas

2.7. Quality measurement (Color, texture, and titratable acidity)

Broccolis were treated with intermittent vacuum impregnation in 2 % malic acid for 30 min. Pieces (10 cm^2) of paprika and carrots were treated with intermittent VI in 2 % malic acid for 5 and 20 min, respectively. Untreated samples were used as a control. All treated samples were rinsed with distilled water and stored at 4 °C for 7 days. All quality measurements were taken of these samples.

Color changes of surfaces were measured using a Minolta colorimeter (model CR400; Minolta Co., Osaka, Japan) at 3 random locations on each floret and expressed as L^*,a^*,and b^* values. L^*,a^*,and b^* values

indicate color lightness, redness, and yellowness of the sample, respectively.

Texture of the samples was measured by texture profile analysis (TPA).). Broccoli stem, paprika and carrots were cut into 2 × 2 × 1 cm pieces and a TA-XT2i texture analyzer (Stable Microsystems Ltd., Surrey, England) was used for performing texture tests. The operating parameters were that the pre-test speed, test speed, post-test speed and compression strain were 2.00 mm/s, 1.00 mm/s, 2.00 mm/s, and 50 %, respectively. An aluminum cylindrical probe with a diameter of 20 mm was used. The time interval and trigger force were 5 s and 0.05 N, respectively. Hardness was used as indicator of texture change and was measured by reading the maximum peak value of deformation curve. Five samples were measured and then average hardness values of the five samples were expressed.

Titratable acidity was measured by titration of samples with 0.1N NaOH to a pH of 8.1. Twenty g broccoli samples and 2 × 5 × 1 cm pieces of paprika and carrots were homogenized in a blender with 100 ml DW and this mixture was filtered through filter paper. Twenty ml of filtrate was titrated to an endpoint at pH 8.1 using 0.1N NaOH and a pH meter (Mettler Toledo, Greifensee, Switzerland). The results were

expressed as percentage of malic acid (grams of malic acid per 100 g or cm^2 of sample).

2.8. Statistical analysis

All experiments were repeated 3 times. Triplicate data were analyzed by the ANOVA procedure of SAS (Version 9.4. SAS Institute Inc., Cary, NC, USA) and LSD t-test was used to determine significant differences at a probability level of $p < 0.05$.

III. RESULTS AND DISCUSSION

In my study, 2 % malic acid was used as a sanitizer for treatment because it is an organic acid known to have powerful antimicrobial activity due to its low pH (Park et al., 2011). Most other research studies investigating antimicrobial effects of organic acids set a maximum concentration of organic acid to around 2 % (Huang and Cheni, 2011; Park et al., 2011; Raybaudi-Massilia et al., 2009; Sagong et al., 2011; Singla et al., 2011), so i chose to use this percentage in my experiments.

The results of the effectiveness of organic acid alone (evaluated at atmospheric pressure) compared with vacuum impregnation (61.3, 21.3 kPa) for reduction of *S.* Typhimurium and *L. monocytogenes* on broccoli are shown in Fig. 1, 2, 3, and 4. The initial levels of *S.* Typhimurium on inoculated broccoli were 5.7 ± 0.15 and 6.1±0.14 $\log_{10}$ CFU/g for continuous and intermittent tests, respectively (Fig. 1 and 3). And the initial levels of *L. monocytogenes* on inoculated broccoli were 4.9±0.04 and 5.4±0.29 $\log_{10}$ CFU/g for continuous and intermittent tests, respectively (Fig. 2 and 4). When 2 % malic acid alone was applied to inoculated broccoli, mean log reductions of 1.5 and 1.3 $\log_{10}$ CFU/g were observed for *S.* Typhimurium (Fig. 1 and 3) and *L. monocytogenes*

(Fig. 2 and 4), respectively, for samples treated with atmospheric pressure for 5 min. Similar results were reported by other researchers. Hung et al. (2010) demonstrated that 1.4 and 1.3 $\log_{10}$ CFU/g reduction of *E. coli* O157:H7 were achieved with broccoli using electrolyzed oxidizing water 20 A (97.8 mg/L of residual chlorine) and chlorine water (99.3 mg/L of residual chlorine), respectively. However, in my study, even though treatment times extended from 5 min to 30 min, there were no further reductions of *S.* Typhimurium and *L. monocytogenes*. Seo and Frank (1999) suggested that bacteria tend to be located in indentations, pores, natural irregularities, cracks, and cut surfaces when they attach to surface of fruits and vegetables. Sapers et al. (2008) demonstrated that there was more attachment of *E. coli* in the calyx and stem areas of inoculated apple than elsewhere, and more bacteria in these areas could survive after washing than elsewhere on the apple surface. Therefore, if bacteria attach to inaccessible sites, they can escape contact with washing or sanitizing agents. Also, Wang et al. (2006) reported that the bacterial reduction tendency resulting from washing is represented by 2 stages due to surface morphology of the sample. The first stage consists of rapid reduction of bacteria attributed to removal and inactivation of loosely attached bacteria cells located in shallow areas where sanitizers

or water can easily reach, and the second stage involves a decrease of bacterial inactivation rates after most bacteria distributed in the shallow surface sites are inactivated or removed. In my study, Fig. 5 (A, B, and C) and Fig. 6 (A, B, and C) show that most bacteria were located and survived in irregularities and rough sites of broccoli after simple dipping treatment for 30 min. Therefore, there were no further reductions of bacteria after 5 min due to insufficient penetration of organic acid into protected sites harboring bacteria.

To overcome inaccessibility of sanitizers into protective sites, vacuum impregnation can be incorporated with the washing process. I postulated that sanitizers could access protected sites such as irregularities, indentations, pores, cracks, puncture sites, and cut surfaces by using vacuum impregnation, since it can facilitate external liquid flow into the porous structures containing protected sites through exchanging internal gas or liquid within food products for externally applied liquids. With this in mind, i tried including vacuum impregnation with the washing process and then investigated efficacy of treatment. Furthermore, i applied two vacuum (intermittent or continuous) treatment methods explained above to ascertain which is the more important factor involved in increasing accessibility of sanitizers.

For continuous treatment (Fig. 1 and 2), there were significant increases ($P < 0.05$) of reduction of *S.* Typhimurium and *L. monocytogenes* with increasing vacuum at every treatment time except between atmospheric pressure and 61.3 kPa for *L. monocytogenes* at 30 min. On the whole, reductions of bacterial populations increased with increasing vacuum for continuous treatment.

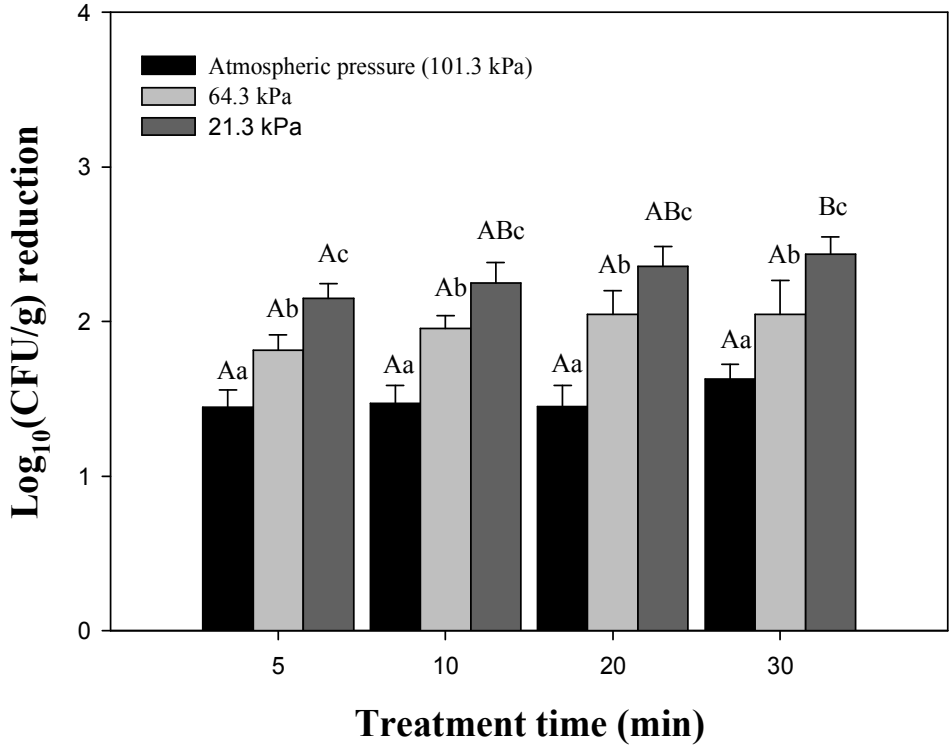

Fig. 1. Log_{10}(CFU/g) reduction[log(N0/N)] levels of *Salmonella* Typhimurium on broccoli treated with simple dipping (Atmospheric pressure) and continuous vacuum impregnation (61.3, 21.3 kPa) in 2% malic acid solution. Error bars indicate standard deviations calculated from triplicates.

* Different uppercase letters within the same pressure indicate significant differences ($P < 0.05$)

* Different lowercase letters within the same treatment time indicate significant differences ($P < 0.05$).

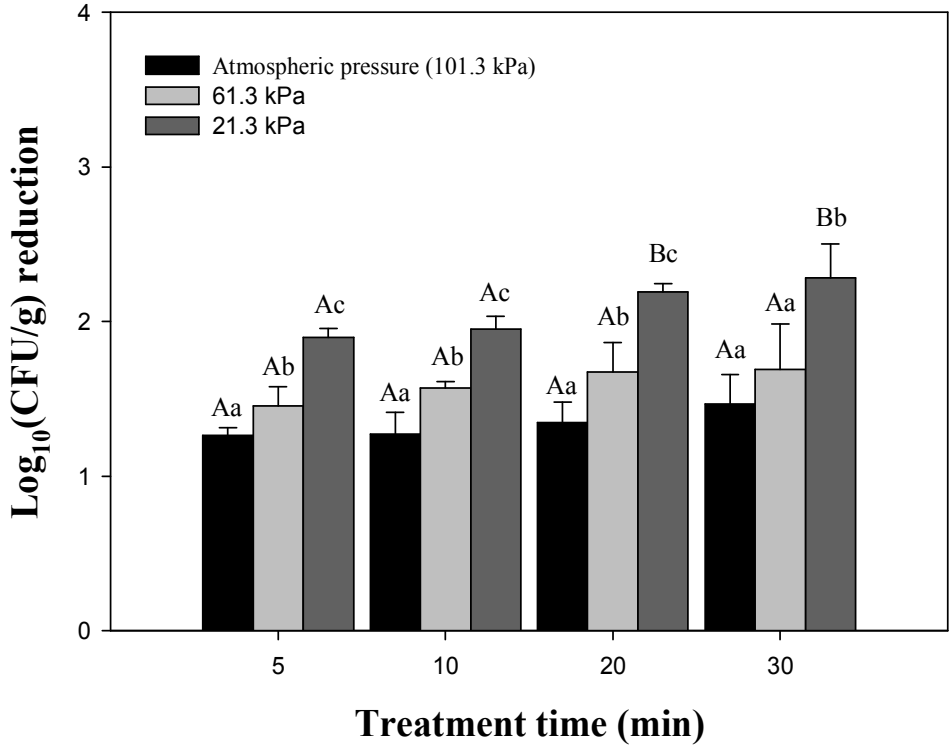

Fig. 2. Log_{10}(CFU/g) reduction[log(N0/N)] levels of *Listeria monocytogenes* on broccoli treated with simple dipping (Atmospheric pressure) and continuous vacuum impregnation (61.3, 21.3 kPa) in 2% malic acid solution. Error bars indicate standard deviations calculated from triplicates.

* Different uppercase letters within the same pressure indicate significant differences ($P < 0.05$)

* Different lowercase letters within the same treatment time indicate significant differences ($P < 0.05$).

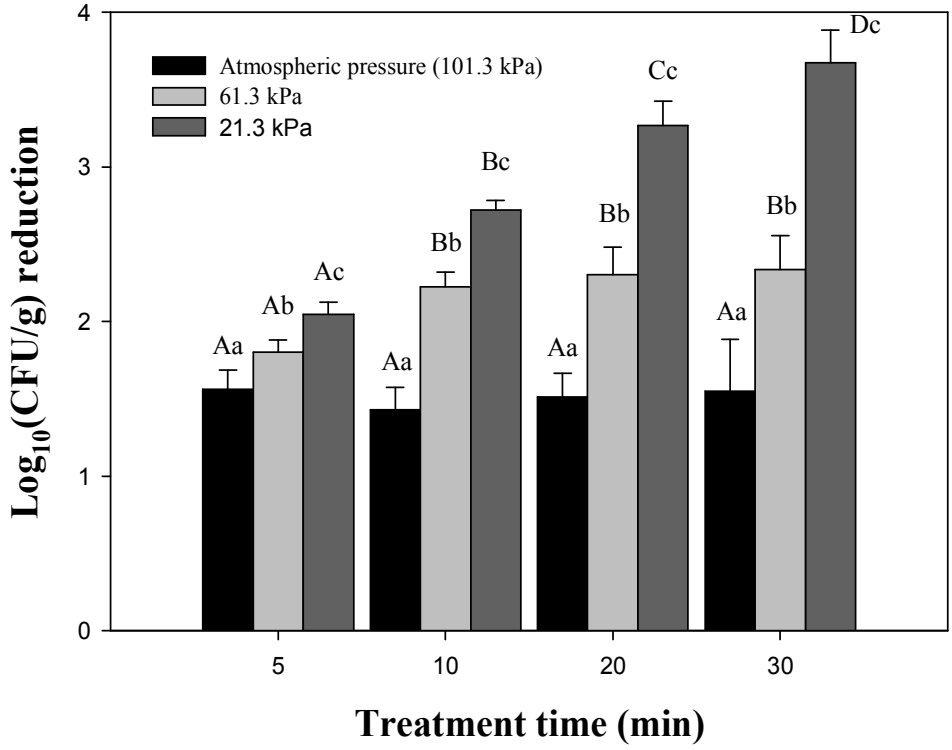

Fig. 3. Log_{10}(CFU/g) reduction[log(N0/N)] levels of *Salmonella* Typhimurium on broccoli treated with simple dipping (Atmospheric pressure) and intermittent vacuum impregnation (61.3, 21.3 kPa) in 2% malic acid solution. Error bars indicate standard deviations calculated from triplicates.

* Different uppercase letters within the same pressure indicate significant differences ($P < 0.05$)

* Different lowercase letters within the same treatment time indicate significant differences ($P < 0.05$).

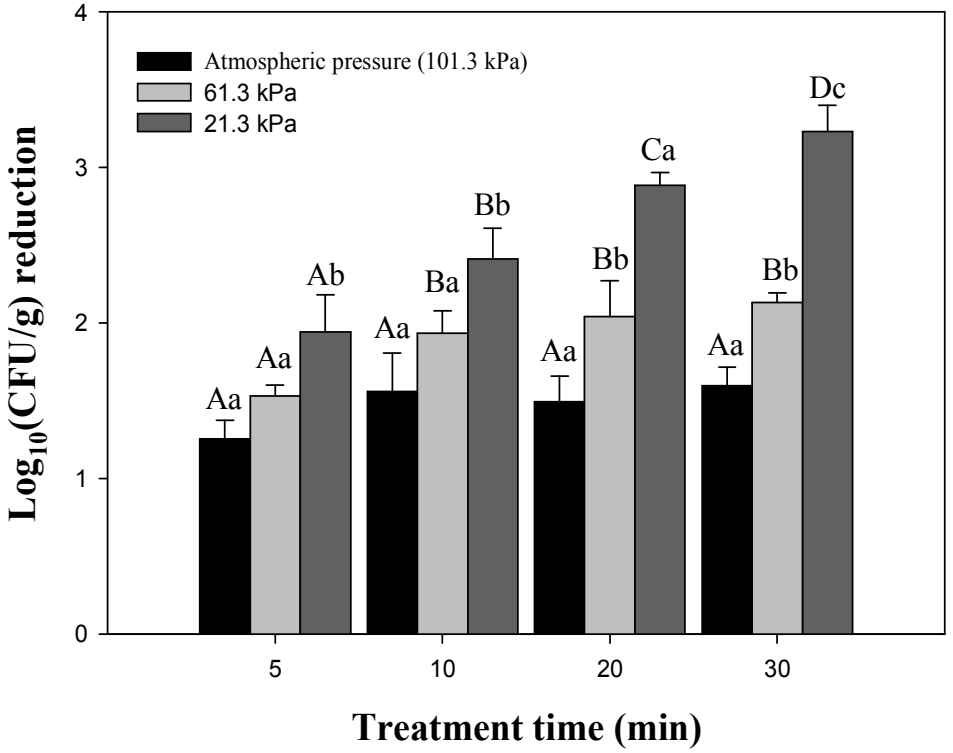

Fig. 4. Log_{10}(CFU/g) reduction[log(N0/N)] levels of *Listeria monocytogenes* on broccoli treated with simple dipping (Atmospheric pressure) and intermittent vacuum impregnation (61.3, 21.3 kPa) in 2% malic acid solution. Error bars indicate standard deviations calculated from triplicates.

* Different uppercase letters within the same pressure indicate significant differences ($P < 0.05$)

* Different lowercase letters within the same treatment time indicate significant differences ($P < 0.05$).

Therefore, it can be expected that increasing vacuum level increases sanitizer access into protected sites. And, at 61.3 kPa, reductions of *S.* Typhimurium (1.8, 1.0, 2.1, and 2.0 $\log_{10}$ CFU/g) and *L. monocytogenes* (1.5, 1.6, 1.6, and 1.7 $\log_{10}$ CFU/g) after 5, 10, 20, and 30 min, respectively, were achieved with increasing treatment time. Also, At 21.3 kPa, reductions of *S.* Typhimurium (2.1, 2.4, 2.4, and 2.4 $\log_{10}$ CFU/g) and *L. monocytogenes* (1.9, 2.0, 2.2, and 2.3 $\log_{10}$ CFU/g) after 5, 10, 20, and 30 min, respectively, were achieved with increasing treatment time and significant differences ($P < 0.05$) between 5 and 30 min and between 5 and 20 min, respectively. Even though slight reductions continued to occur with increasing treatment time, these reductions did not significantly differ ($P \geq 0.05$) at 61.3 kPa and the significant differences ($P < 0.05$) of 21.3 kPa treatment were not cost-effective from an economic standpoint because these reductions were very small relative to treatment time. Therefore, if vacuum and atmospheric pressure treatment time exceeds the optimum time interval, further treatment time increases cannot achieve effective reductions of bacteria using a continuous treatment system. So, setting an optimum vacuum and atmospheric pressure treatment time is important for effective vacuum impregnation.

Regarding intermittent treatment (Fig. 3 and 4), there were also significant ($P < 0.05$) increased reductions of both *S.* Typhimurium and *L. monocytogenes* with increasing vacuum levels at every treatment time, similar to continuous treatment. Therefore, it is also concluded that the more vacuum levels are intensified, the more sanitizers can access protected sites via intermittent treatment. Also, reductions of *S.* Typhimurium (1.80, 2.2, 2.3, and 2.3 $\log_{10}$ CFU/g) and *L. monocytogenes* (1.5, 1.9, 2.0, and 2.1 $\log_{10}$ CFU/g) after 5, 10, 20, and 30 min, respectively, displayed significant differences ($P < 0.05$) between 5 and 10 min, but no additional significant reductions ($P \geq 0.05$) occurred from 10 to 30 min in the case of 61.3 kPa. Thus, the most significant reductions ($P < 0.05$) of both *S.* Typhimurium and *L. monocytogenes* using intermittent treatment occurred within 10 min. In the case of 21.3 kPa, reductions of *S.* Typhimurium (2.1, 2.7, 3.7, and 3.7 $\log_{10}$CFU/g) and *L. monocytogenes* (1.9, 2.4, 2.9, and 3.2 $\log_{10}$ CFU/g) after 5, 10, 20, and 30 min showed significant differences ($P < 0.05$) between treatment time intervals. These results can be interpreted into two parts:

The first part is that unlike continuous treatment, which could not achieve further reductions despite vacuum and atmospheric pressure

treatment time, intermittent treatment achieved further reductions as the number of treatment cycles (vacuum pressure → atmospheric pressure) increased. Therefore, it appears that the number of treatment cycles is important for promoting efficacy of vacuum impregnation which facilitates sanitizer access into protected sites. On the basis of deformation-relaxation phenomena during intermittent treatment, Fito and Pastor (1993) and Fito et al. (1994) suggested a physical explanation for this behavior. In theory, the exchange of sanitizer for trapped air at each intermittent cycle step can facilitate the vacuum impregnation treatment. Therefore, this phenomenon could promote better sanitizer penetration into protected sites by removing air from these locations, such as cracks, pores, indentations, and cut surfaces during the vacuum cycle, and then allowing sanitizer to infiltrate as atmospheric pressure is restored. Hofmeister et al. (2004) reported similar results that when cheese was treated with continuous and intermittent vacuum impregnation in dyed solution; the intermittent treatment promoted greater degasification of the sample than continuous treatment and thus a more liquid penetration occurred.

The second part is that although increasing the number of treatment cycles significantly ($P < 0.05$) enhanced the reduction of bacteria, this

result was limited in accordance with the vacuum level. Consequently, in 61.3 kPa treatment (Fig 3 and 4), significant ($P < 0.05$) reductions of bacteria occurred only by the second cycle. However, with 21.3 kPa treatment, significant ($P < 0.05$) reductions of bacteria occurred by the fourth cycle. Therefore, it can be concluded that the number of cycles associated with significant ($P < 0.05$) reduction is predicated by vacuum level. In my study, i could not determine if 21.3 kPa treatment for more than four cycles would produce additional significant ($P < 0.05$) reductions, since only that number of cycles was utilized. However, it is clear that with the number of cycles applied, significant ($P < 0.05$) reductions increased with increasing pressure.

I investigated the effect of vacuum impregnation on bacteria attached to broccoli surfaces through scanning electron photomicrographs (Fig. 5 and 6). Broccoli surfaces were divided into intact and cut surfaces and then photographed since each surface had different morphology. In the case of intact broccoli surfaces, most bacteria aggregated in groove sites (Fig. 5A, as indicated by arrows) since surfaces were uneven. In the case of cut broccoli surfaces, most bacteria were aggregated in pits and gaps (Fig. 6A, as indicated by arrows). However, efficacy of the washing process could be increased by using vacuum impregnation.

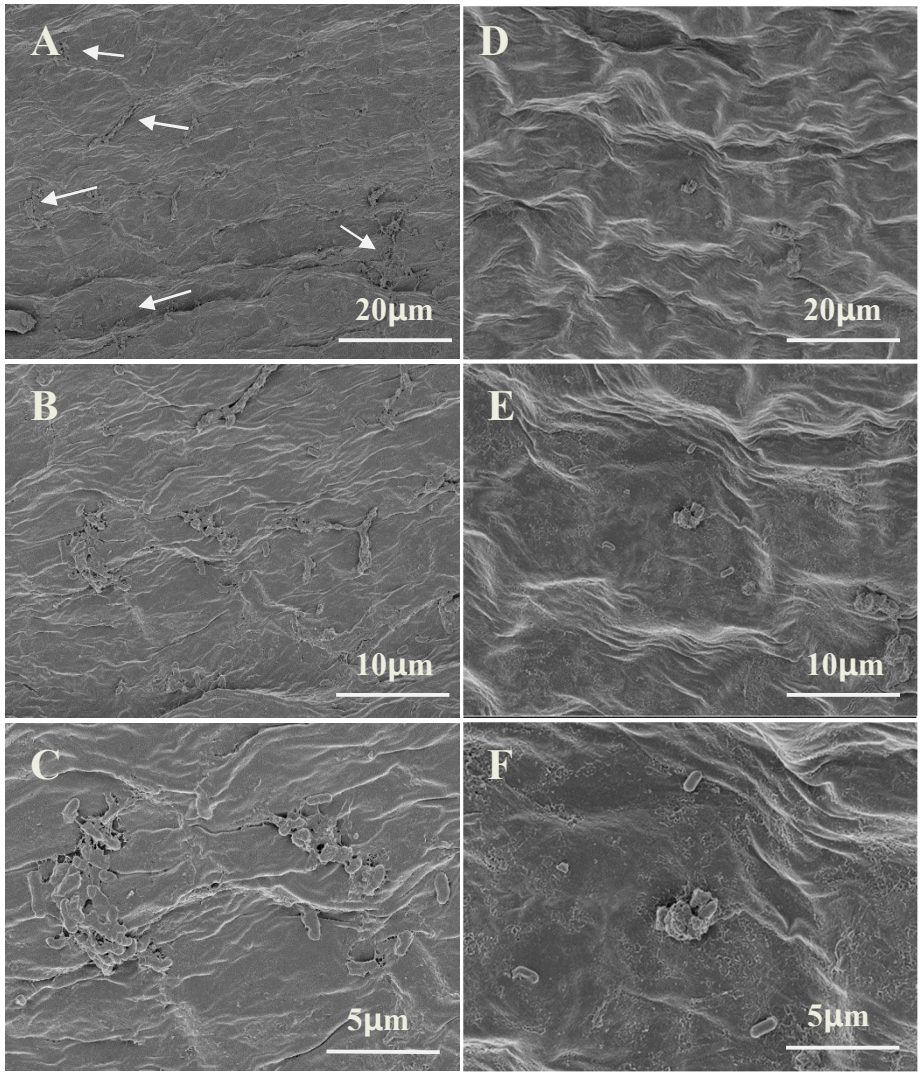

Fig. 5. SEM photomicrographs of intact surfaces of inoculated broccoli. (A), (B), (C) show the same sample field (treated with simple dipping in 2 % malic acid for 30 min) visualized at different magnifications : (A) 1000X, (B) 2000X, (C) 4000X. (D), (E), (F) show the same sample field (treated with intermittent vacuum impregnation of 21.3 kPa in 2 % malic acid for 30 min) visualized at different magnifications : (D) 1000X, (E) 2000X, (F) 4000X.

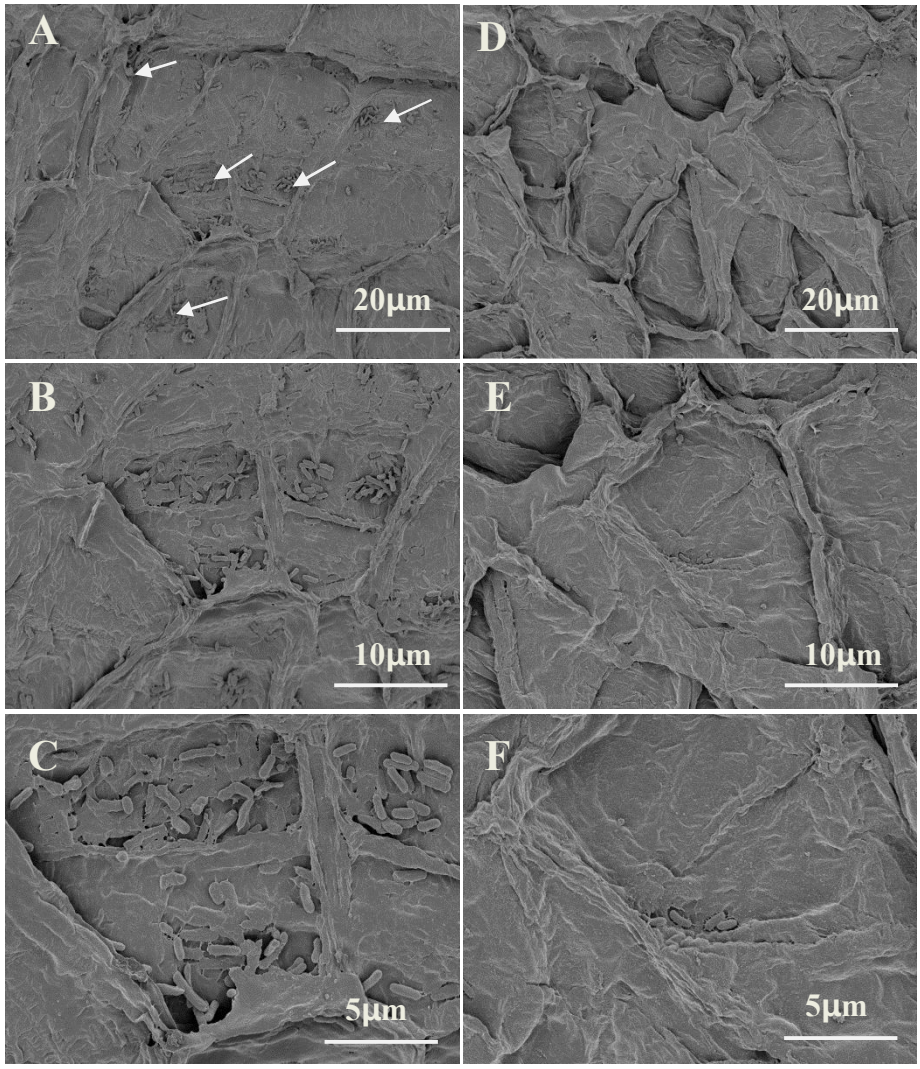

Fig. 6. SEM photomicrographs of cut surfaces of inoculated broccoli. (A), (B), (C) show the same sample field (treated with simple dipping in 2 % malic acid for 30 min) visualized at different magnifications : (A) 1000X, (B) 2000X, (C) 4000X. (D), (E), (F) show the same sample field (treated with intermittent vacuum impregnation of 21.3 kPa in 2 % malic acid for 30 min) visualized at different magnifications : (D) 1000X, (E) 2000X, (F) 4000X.

Fig. 5 and 6 (D, E, and F) showed that most bacteria were removed from broccoli surfaces by washing. The difference of bacterial reduction between vacuum impregnation and simple dipping may be attributed to limited sanitation accessibility due to surface characteristics and conditions of produce since most produce washing methods are surface treatments (Wang et al., 2009). Other researchers observed similar surface protection phenomena through examining SEM images. Wang et al. (2009) reported that an increase of surface roughness would provide protection to bacteria entrapped on sample surfaces. There were more bacteria on stub surfaces of increased surface roughness after washing with water. Their SEM images indicated that many cells remained and were concentrated in crevices and along grooves. Han et al. (2000) investigated the efficacy of gaseous ClO_2 on green peppers having artificially increased roughness due to surface injury with a sterile blade. They found that inoculated bacteria on uninjured surfaces showed significantly more inactivation than those on injured surfaces. Their confocal laser scanning microscopy (CLSM) photomicrographs also showed that more bacteria were found on injured surfaces. Therefore, their study also showed that rough surfaces can harbor and limit the effectiveness of washing due to the protective effect of surface

roughness. However, this could be overcome by vacuum impregnation, which can exchange internal gas within protected sites with an externally applied liquid (organic acid in the case of my study). I thus postulate that the antimicrobial effect of vacuum impregnation was attributed to enhanced contact with pathogens due to increased accessibility of liquid sanitizer into protected sites of sample surfaces and removal of pathogens by washing as demonstrated by SEM images. This idea was supported by tests in which cell suspensions were treated directly in acidic solutions ; the results of these tests had no significant differences ($P \geq 0.05$) between reductions of non- and vacuum impregnation treatments (data not shown). This means that vacuum impregnation treatment had no inactivation capacity in itself against pathogens. Based on SEM images of these two tests on broccoli plus results of direct placement of cell suspension into acidic solution, it is apparent that the antimicrobial effect of vacuum impregnation applied to sanitizer was entirely due to increased accessibility of sanitizer into protected sites and consequent enhanced washing ability.

In this study, the antimicrobial effect of vacuum impregnation applied to organic acid treatment varied according to the target pathogen. *Salmonella* Typhimurium was relatively more sensitive than *Listeria*

monocytogenes to the treatment. Even though statistically significant differences of reduction between *Salmonella* Typhimurium and *Listeria monocytogens* occurred only for some treatment times, the reduction rate of *Salmonella* Typhimurium was numerically larger than that of *Listeria monocytogens* throughout the whole treatment. Because enhanced washing capacity was a factor contributing to the antimicrobial effect of vacuum impregnation applied to organic acid treatment, microbial reduction might vary according to pathogen strains relative to differences in their adhesion to sample surfaces in this study. As a pathogen's adhesion force increased, resistance of the pathogen to vacuum impregnation treatment increased. Adhesion force of a pathogen onto a sample is associated with multiple factors and mediated by these complex relationships, although the exact mechanisms are not completely understood. Specific factors such as lipopolysaccharides (Walker et al., 2004), flagella (Erdem et al., 2007; Gabriel Piette and Idziak, 1991), and non-specific factors such as hydrophbocity and surface charge (Boyer et al., 2011; Dickson and Koohmaraie, 1989; Harimawan et al., 2011; Van Loosdrecht et al., 1987) are considered to be involved. Therefore, it is postulated that these factors affected differences of reduction between *Salmonella* Typhimurium and *Listeria*

monocytogenes after vacuum impregnation treatment, and further research into differences of the antimicrobial effect of vacuum impregnation according to bacterial strain should be performed to avoid underestimating the scale of treatment needed for commercial application of this novel intervention.

Quality changes should be investigated throughout the storage interval prior to commercial application of this enhanced sanitation process. Color changes of broccoli surfaces were measured since produce washing is a surface treatment, and texture changes were measured since variations in applied pressure and organic acid may influence texture. Also, if malic acid remains on broccoli surfaces after treatment, it could increase sourness and thus adversely affect the sensory quality of broccoli. Therefore, the presence of sour taste resulting from residual malic acid should be confirmed. Even though sour taste intensity due to presence of acid cannot be entirely explained by various factors, titratable acidity (%) is considered to be one key factor indicating sour taste intensity (Da Conceicao Neta et al., 2007; Lugaz et al., 2005); thus titratable acidity of broccoli was measured. The two quality measurements of color and texture were conducted on different parts of the broccoli plant; florets were subjected to color

analysis and stems to texture measurement. In the case of color measurement, florets, having a deeper color, were considered to be more suitable than stems for testing since they are more sensitive to color changes resulting from changes in the environment. Conversely, stems are a more appropriate subject for texture measurements due to their major contribution to the crunchiness of broccoli and for their greater firmness compared to florets. Accordingly, i tested different parts of broccoli for evaluating each quality parameter, and many other authors also conducted color measurements on florets and texture measurements on stems for their quality tests much as i did (Ansorena et al., 2011; Fernández-León et al., 2013; Gomes et al., 2008; Jacobsson et al., 2004; Serrano et al., 2006). As Table 1 shows, after treatment with intermittent impregnation (21.3 kPa) for 30 min in 2% malic acid, color values (L*, a* and b*) and maximum load value (N) of broccoli samples were not significantly different ($P \geq 0.05$) from those of untreated controls during 7 day storage periods. In the case of titratable acidity, there was a significant difference ($P < 0.05$) between untreated controls and treated broccoli at 0 day. However, titratable acidity of treated broccoli gradually decreased over time and was not significantly different from that of untreated controls.

Table 1. Comparison of quality values of broccoli between untreated (control) and treated samples stored at 4°C for 7 days.

Quality value	Sample	Storage day		
		0	3	7
L*	Control[a]	41.99±0.60Aa	42.03±0.74Aa	43.48±0.75Ba
	Treated[b]	41.25±0.26Aa	43.07±0.29Ba	42.50±0.84Ba
a*	Control	-7.56±0.43Aa	-7.29±0.47Aa	-7.22±0.05Aa
	Treated	-7.95±0.39Aa	-6.83±0.24Ba	-7.43±0.15Aa
b*	Control	8.18±0.61Aa	7.83±0.51Aa	8.41±0.31Aa
	Treated	8.36±0.67Aa	7.46±0.25Ba	8.41±0.30Aa
Maximum load (N)	Control	120.2±15.1Aa	118.4±9.4Aa	120.8±5.2Aa
	Treated	119.3±12.6Aa	120.7±9.1Aa	122.3±6.4Aa
Titratable Acidity(%)	Control	0.09±0.01Aa	0.09±0.01Aa	0.09±0.01Aa
	Treated	0.15±0.03Ab	0.12±0.02Aa	0.09±0.01Aa

[a]Untreated broccoli.

[b]Broccoli treated with intermittent vacuum impregnation (21.3 kPa) in 2 % malic acid for 30 min.

* Different uppercase letters within the same row indicate significant differences ($P < 0.05$) between storage intervals (days).

* Different lowercase letters within the same column indicate significant differences ($P < 0.05$) at each value (L*, a*, b*, N, %).

It can be surmised that malic acid penetrating into protective sites such as cut surfaces, cracks, indentations, pores, and so on remained without being removed by washing with DW, but then dissipated from protective sites over time. So, malic acid could easily dissipate from produce surfaces due to physical factors such as transfer to storage bag surfaces, tumbling or flowing by gravity, shaking and bumping against each other in a processing line. The reduction of residual malic acid over time could be related to resulting color changes. Residual malic acid could affect color by reacting with the sample, but no color changes associated with decreasing residual malic acid over time were observed. Also, texture quality of product subjected to vacuum impregnation is mainly related to the type of treatment solution (Xie and Zhao, 2003; Zhao and Xie, 2004). I conclude that 2% malic acid used in my study can be considered a proper sanitizer to use for vacuum impregnation washing because no significant quality changes occurred in my study. According to these results, my study demonstrates the feasibility of utilizing organic acid produce washing in concert with vacuum impregnation as a possible commercial intervention for controlling pathogens on broccoli without affecting product quality.

On the basis of these results, this technique was applied to various types of produce as a next study since bacterial inactivation may vary according to different surface properties.

The levels of surviving cells of the three pathogens on samples after dipping and VI washing are shown in Fig. 7, 8 and 9, respectively. The initial populations of *S.* Typhimurium, *E. coli* and *L. monocytogenes* on samples were approximately 10^5-10^6 CFU/cm^2 and the limit of detection was 1.0 log CFU/cm^2. In the case of VI treatment of paprika, counts of S. Typhimurium, *E. coli* and *L. monocytogenes* were reduced to below the detection limit after 5, 5, and 3 min, respectively. At each time point when pathogen populations were reduced to below the detection limit by VI treatment, corresponding populations following dipping treatment for the same time intervals were 1.2 ± 0.35 log CFU/cm^2 for *S.* Typhimurium, 1.2 ± 0.35 CFU/cm^2 for *E. coli* and 1.9 ± 0.30 log CFU/cm^2 for *L. monocytogenes*. In the case of carrots, counts of *S.* Typhimurium, *E.coli* and *L. monocytogenes* were reduced to below the detection limit after 15, 20, and 20 min by VI treatment, respectively. For each time point where pathogen populations were reduced to below the detection limit by VI treatment, populations of 2.8 ± 0.66 log CFU/cm^2 of *S.* Typhimurium, 2.8 ± 0.15 log CFU/cm^2 of *E. coli* and

2.45 ± 0.15 log CFU/cm^2 of *L. monocytogenes* survived on samples after dipping treatment. In case of king oyster mushrooms and muskmelons, populations of *S.* Typhimurium, *E. coli* and *L. monocytogenes* were not reduced below the detection limit after20 min of either dipping or VI treatment. Populations of 3.5 ± 0.14 log CFU/cm^2 of *S.* Typhimurium, 4.1 ± 0.31 log CFU/cm^2 of *E. coli* and 3.5 ± 0.24 log CFU/cm^2 of *L. monocytogenes* survived on king oyster mushrooms after 20 min of dipping treatment, and populations of 3.0 ± 0.39 log CFU/cm^2 of *S.* Typhimurium, 3.6 ± 0.40 log CFU/cm^2 of *E. coli* and 3.4 ± 0.15 log CFU/cm^2 of *L. monocytogenes* survived on king oyster mushrooms after 20 min of VI treatment. There were no significant ($P \geq 0.05$) differences in pathogen reductions between dipping and VI treatment for king oyster mushrooms. Cell levels of 4.1 ± 0.26 log CFU/cm^2 of *S.* Typhimurium, 4.4 ± 0.51 log CFU/cm^2 of *E. coli* and 3.3 ± 0.43 log CFU/cm^2 of *L. monocytogenes* survived on muskmelons after 20 min of dipping treatment, and populations of 3.9 ± 0.03 log CFU/cm^2 of *S.* Typhimurium, 4.1 ± 0.52 log CFU/cm^2 of *E. coli* and 3.1 ± 0.39 log CFU/cm^2 of *L. monocytogenes* survived on muskmelons after 20 min of VI treatment. Moreover, there were no significant ($P \geq 0.05$) differences in reduction between dipping and VI treatments for inoculated

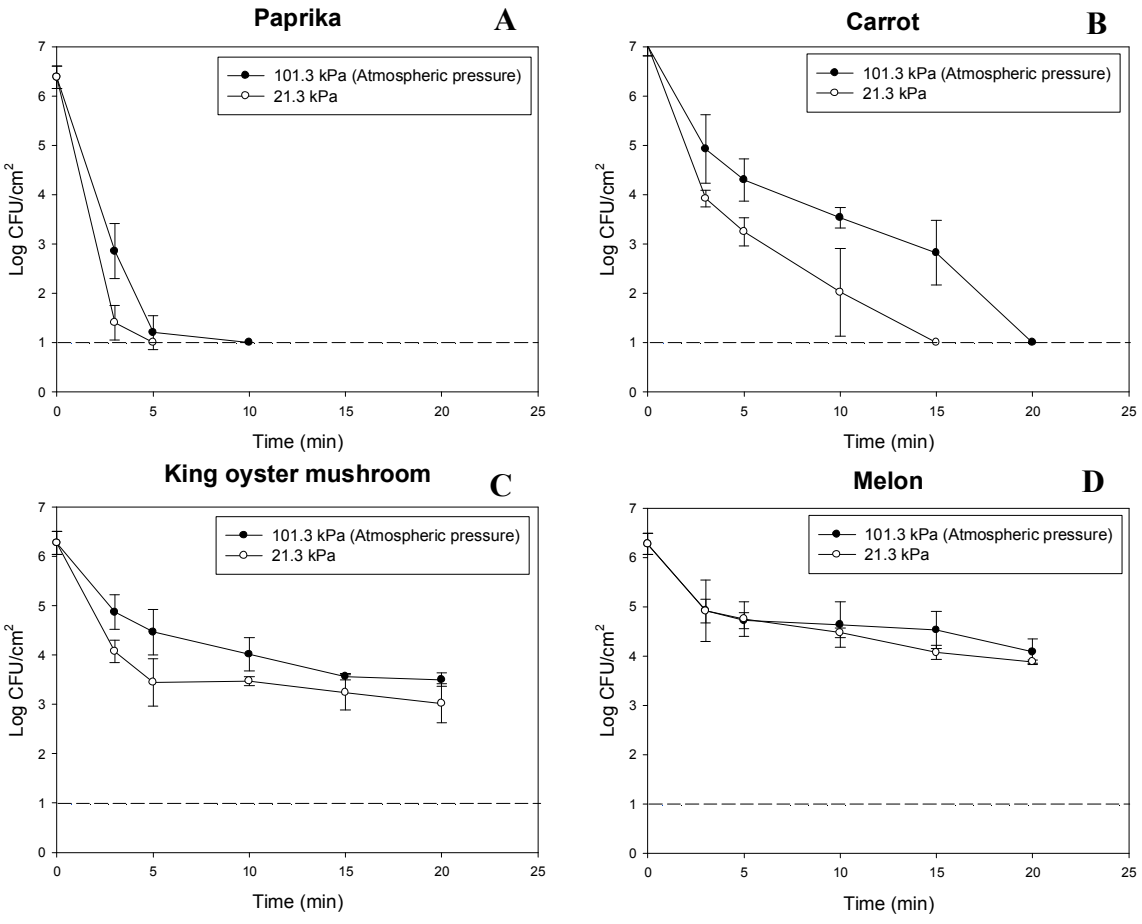
Paprika
A
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
Carrot
B
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
King oyster mushroom
C
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
Melon
D
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)

Fig. 7. Log CFU/cm^2 population of *Salmonella* Typhimurium on paprikia [A], carrot [B], king oyster mushroom [C] and melon [D] treated with simple dipping (101.3 kPa) and intermittent vacuum impregnation (21.3 kPa) treatment in 2% malic acid solution. Error bars indicate standard deviations calculated from triplicates.

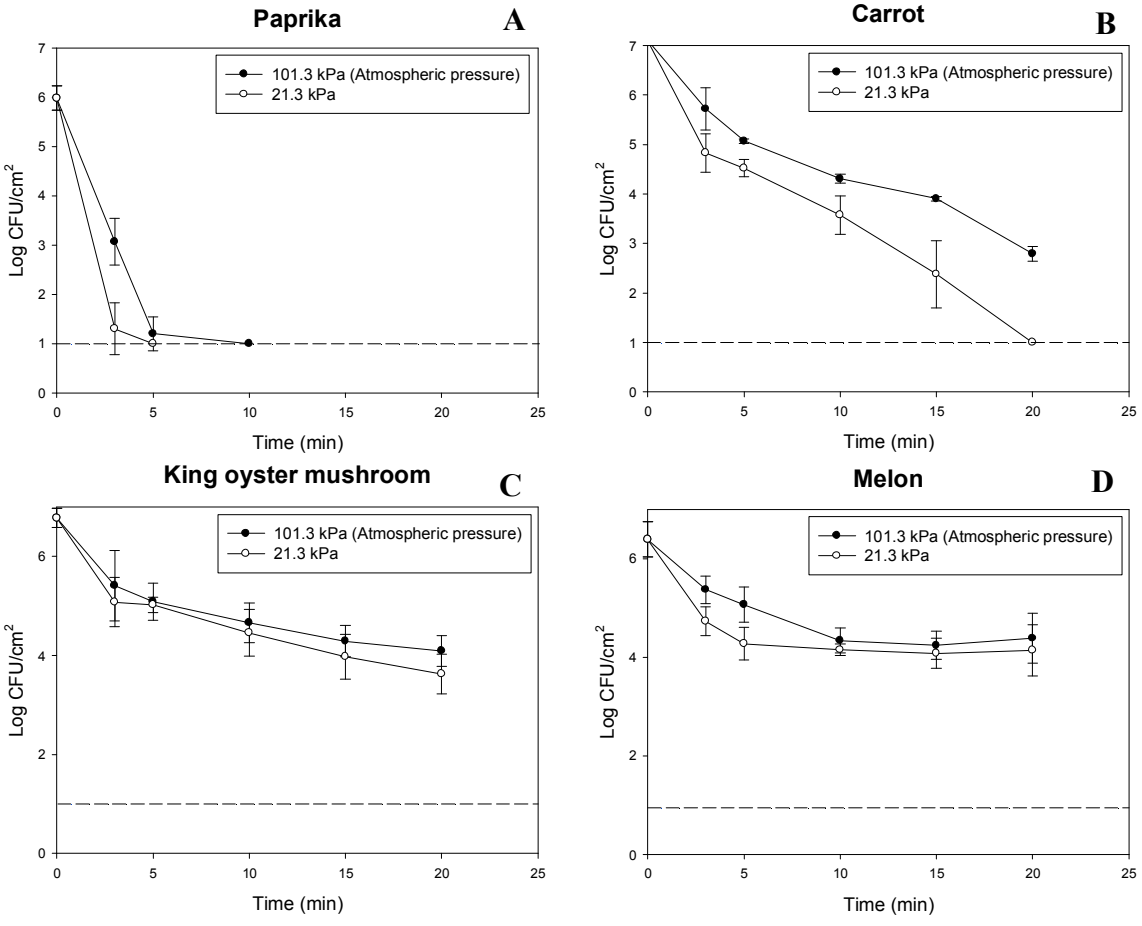
Paprika
A
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
Carrot
B
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
King oyster mushroom
C
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
Melon
D
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)

Fig. 8. Log CFU/cm^2 population of *Escherichia coli* on paprikia [A], carrot [B], king oyster mushroom [C] and melon [D] treated with simple dipping (101.3 kPa) and intermittent vacuum impregnation (21.3 kPa) treatment in 2% malic acid solution. Error bars indicate standard deviations calculated from triplicates.

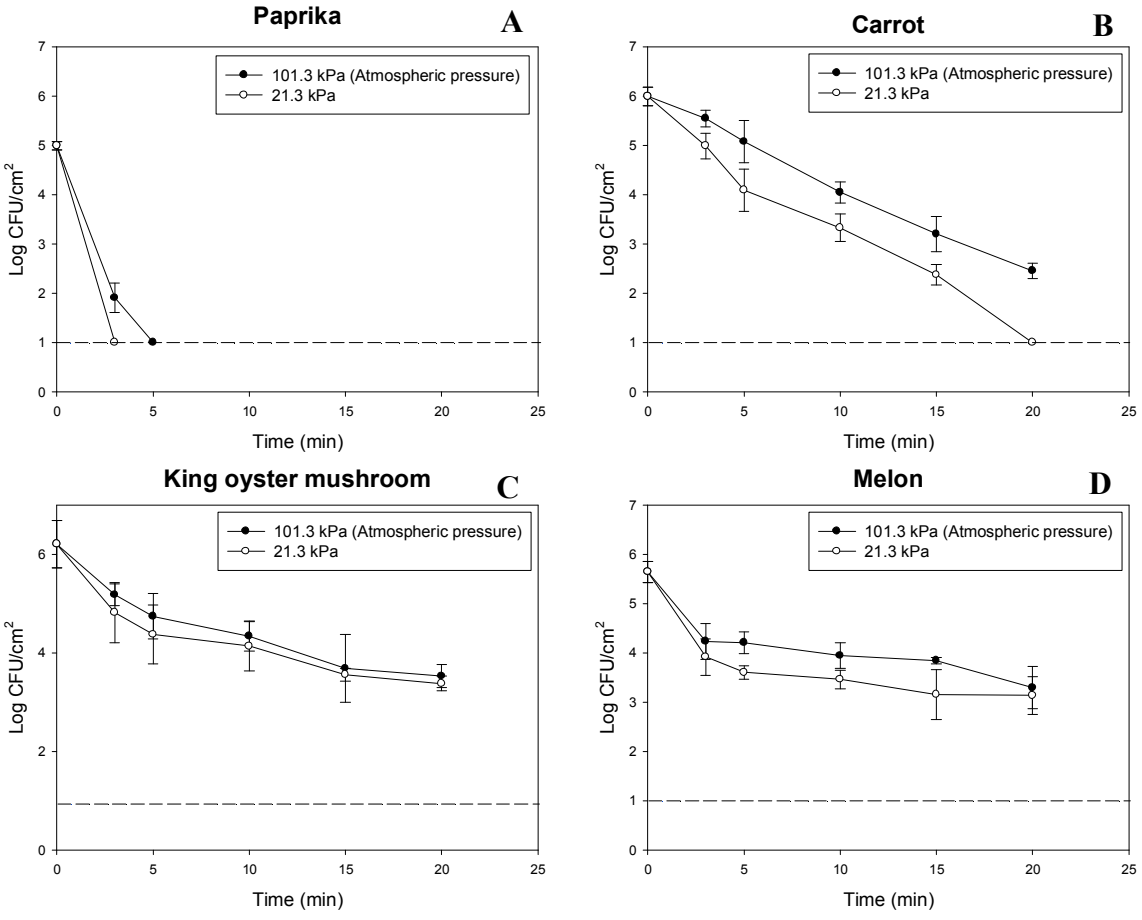
Paprika
A
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
Carrot
B
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
King oyster mushroom
C
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)
Melon
D
101.3 kPa (Atmospheric pressure)
21.3 kPa
Log CFU/cm2
Time (min)

Fig. 9. Log CFU/cm^2 population of *Listeria monocytogenes* on paprikia [A], carrot [B], king oyster mushroom [C] and melon [D] treated with simple dipping (101.3 kPa) and intermittent vacuum impregnation (21.3 kPa) treatment in 2% malic acid solution. Error bars indicate standard deviations calculated from triplicates.

muskmelons. From these data of sample populations remaining after dipping and VI treatment, two prominent trends emerged.

First, the pathogen reduction rate varied according to the type of produce. Many studies have shown that the same treatment with the same sanitizer had different antimicrobial efficacy against pathogens according to the type of sample (Park et al., 2011; Singh et al., 2002; Alvarado-Casillas et al., 2007; Rodgers et al., 2004; Sengun and Karapinar, 2005; Wang et al, 2009). Since most washing of fresh produce involves surface treatment, it would seem that differences of antimicrobial efficacy mainly stem from differences in surface properties of produce. Several studies have investigated differences of pathogen reduction according to differences of sample topography (Felkey et al., 2006; Yuk et al., 2006; Yuk et al., 2005). These investigators inoculated pathogens onto several different sites of the samples and then treated with sanitizers. A comparison of these studies revealed that sanitizer treatments were more effective in reducing levels of pathogens on smooth surfaces such as an intact surface rather than on rough surfaces such as punctures, stem scars, or scrapes. That is, rough surfaces that provided protected sites for pathogens in an aqueous sanitizer environment enabled pathogens to have high resistance to sanitizer

washing treatments. Wang et al. (2009) quantified the surface topography of samples to roughness parameters such as average roughness (R_a) and compared sanitizer efficacy according to differences in roughness measurements. They found that increased surface roughness provided more protected sites where microbes could escape contact with sanitizer resulting in reducing washing efficacy. I also quantified surface topography using average roughness (R_a) and investigated the correlation between the reduction rate of pathogens and surface roughness of the samples. R_a is the average absolute deviation of the roughness irregularities from the mean line over one sampling length giving a general description of height variation (Gadelmawla et al, 2002) and it was calculated using the roughness tester using phase shifting interferometry which is a common optical technique for non-contact surface profilometry. The R_a values of paprika, carrots, king oyster mushrooms and muskmelons are shown in Table 1; the R_a of muskmelons (11.4 ± 1.7) was highest among the samples tested and followed by king oyster mushrooms (6.0 ± 1.7), carrots (2.5 ± 0.50) and paprika (0.60 ± 0.16). For the dipping treatment, the reduction rate of pathogens was inversely proportional to the R_a. These results were convincingly explained by past studies conducting comparisons of

Table 2. Surface roughness (R_a, μm) of paprika, carrot, king oyster mushroom and melon.

Sample	Surface roughness (R_a, μm)
Paprika	0.60 ± 0.16A
Carrot	2.51 ± 0.50B
King oyster mushroom	6.02 ± 1.65C
Muskmelon	11.43 ± 1.68D

* Different uppercase letters within the same column indicate significant differences ($P < 0.05$).

sanitizer efficacy according to surface properties as mentioned above. That is, surface features could affect the antimicrobial effect of sanitizer washing and this effect could be predicted by quantifying surface roughness parameters such as R_a. It became more evident that surface features are a critical factor affecting the antimicrobial effectiveness of sanitizer washing after examining the scanning electron photomicrographs (Fig. 10 and 11). Fig 10 A and C and Fig 11 A and C show micro surface images of paprika, carrots, king oyster mushrooms and muskmelons, respectively, after dipping treatment. It was evident to the unaided eye that samples with higher Ra values had visibly rougher surfaces. After dipping treatment, samples with high Ra values had more bacteria located on surfaces, especially those with prominent valleys and hills, and thus many bacteria could escape contact with sanitizer. Similar results were reported by other studies (Fransisca and Feng, 2012; Wang et al., 2009) which conducted comparisons using SEM images to correlate R_a values with bacterial location on surfaces.

Second, an enhanced antimicrobial effect of organic acid washing incorporating application of VI only occurred with certain types of samples. In the case of paprika and carrots, there was a significant ($P < 0.05$) additional antimicrobial effect related to the inclusion of VI

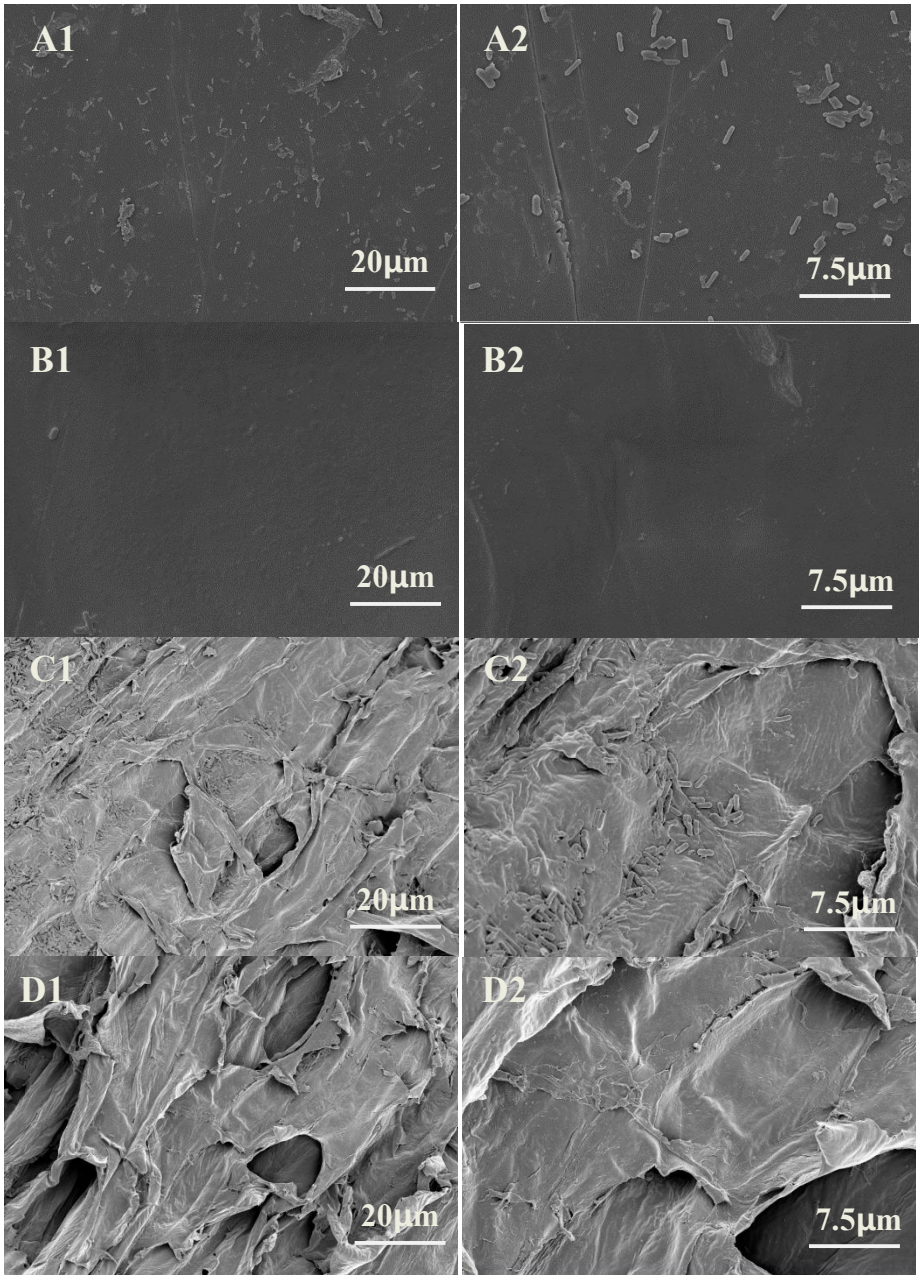

Fig. 10. SEM photomicrographs of surfaces of inoculated paprika (A and B) and carrot (C and D). (A) and (C) show the sample field treated with simple dipping in 2 % malic acid for 5 min (A) and 20 min (C) visualized at different magnifications : (A1 and C1) 1000X, (A2 and C2) 3000X. (B) and (D) show the sample field treated with intermittent vacuum impregnation in 2 % malic acid for 5 min (B) and 20 min (D)

visualized at different magnifications : (B1 and D1) 1000X, (B2 and D2) 3000X.

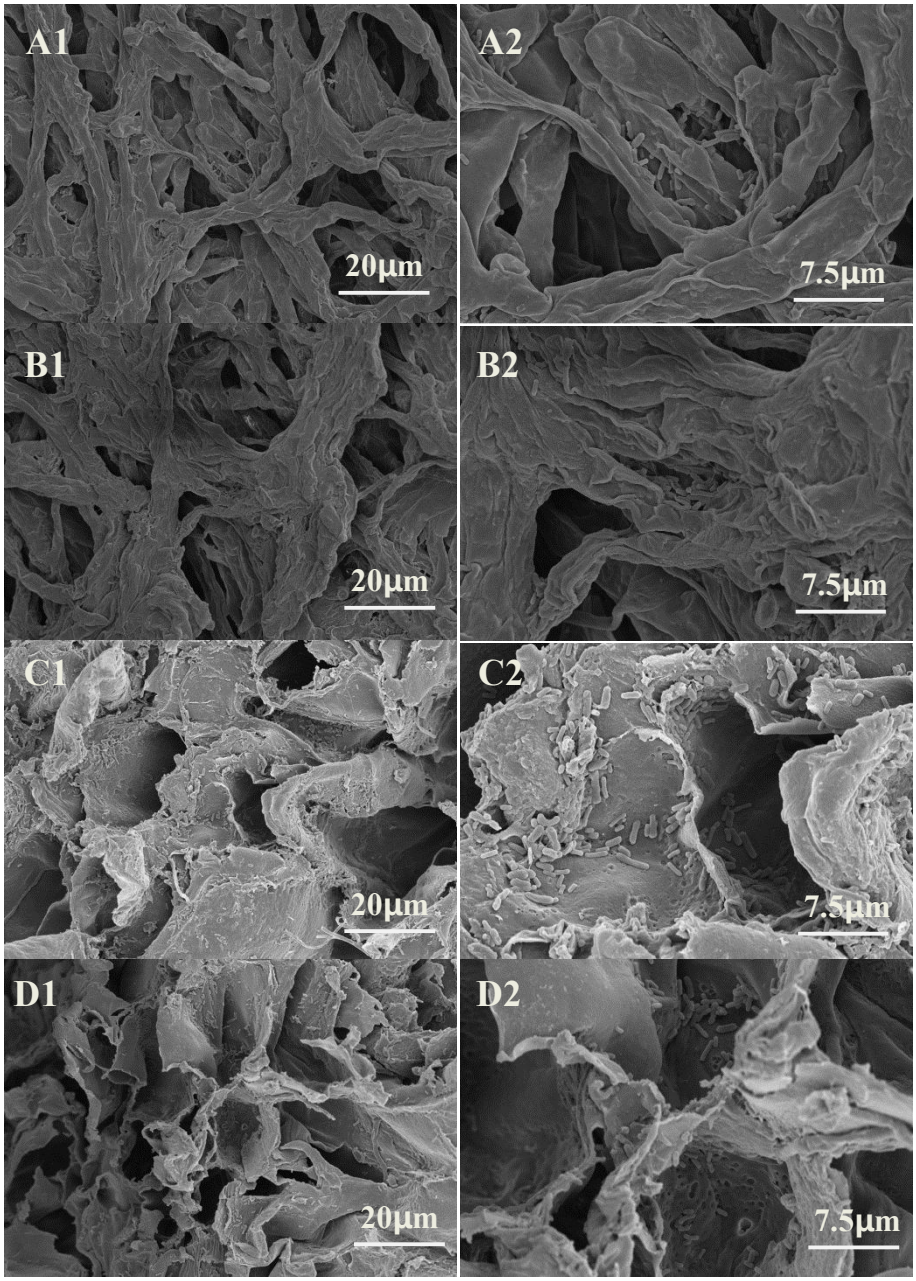

Fig. 11. SEM photomicrographs of surfaces of inoculated king oyster mushroom (A and B) and muskmelon (C and D). (A) and (C) show the sample field treated with simple dipping in 2 % malic acid for 20 min visualized at different magnifications : (A1 and C1) 1000X, (A2 and C2) 3000X. (B) and (D) show the sample field treated with intermittent vacuum impregnation in 2 % malic acid for 20 min visualized at

different magnifications : (B1 and D1) 1000X, (B2 and D2) 3000X.

treatment. However, there were no significant additional antimicrobial effects due to addition of VI in the case of king oyster mushrooms and muskmelons. This also may be attributed to surface properties of samples, especially roughness. During the vacuum step internal gas in protected sites such as cracks, pores and valleys expand and partially flow out. This flow continues until the pressure of internal gas and external solution reach equilibrium, and its volume is proportional to the decompression level. During the return to atmospheric pressure following vacuum exposure, the residual gas is compressed and external solution flows into the protected site as a ratio of flowing out gas volume. Actually, it is impossible for internal gas to flow out completely and thus protected sites cannot be completely filled with external solution. Therefore, it is inevitable that empty spaces within protected sites are not completely filled with external solution following VI treatment. This empty space volume might be related to surface roughness. Samples with greater rough surface properties have bigger empty spaces even after VI treatment. Consequently, enough spaces where microbes could escape contact with sanitizer would be created after VI treatment in produce of relatively large surface roughness such as king oyster mushrooms and muskmelons (Table 2). Conversely, there were not

enough spaces where microbes could escape contact with sanitizer after VI treatment in samples of relatively small surface roughness such as paprika and carrots (Table 2). Through SEM images (Fig. 11) it was observed that there were many deep protected sites in king oyster mushrooms and muskmelons and many microbes were located deep in these sites after VI treatment (Fig. 11 B and C). Relatively speaking, paprika and carrots had shallow protected sites and most microbes were washed out during VI treatment (Fig. 10 B and C).

Quality changes need to be investigated to evaluate the suitability of VI washing for commercial application. I only conducted quality analysis on paprika and carrots because VI washing did not increase the antimicrobial effect of malic acid washing in the case of king oyster mushrooms and muskmelons versus paprika and carrots. All quality measurements were conducted after treatment with intermittent VI on paprika and carrots for 5 min and 20 min, respectively, in 2% malic acid and compared with untreated control samples. In general, previous researchers have analyzed color changes after sanitizer washing treatments of fresh produce since sanitizer washing is a surface treatment (Yang et al., 2003; Koseki et al., 2004; Wang et al., 2004; Park et al., 2011; Kim et al., 2011). Accordingly, i evaluated color changes due to

VI washing compared to untreated controls. As table 3 shows, color values (L*, a* and b*) of both paprika and carrots were not significant different ($P \geq 0.05$) from those of untreated controls during 7 days of storage. Texture was also evaluated because sample structure might change due to pressure fluctuations during VI treatment. Maximum load value (N), an indicator of textural integrity, was measured and no significant differences ($P \geq 0.05$) were observed between VI treated paprika and carrots and their untreated controls during 7 days storage (Table 3). Sour taste resulting from residual malic acid was also evaluated for its adverse effect on sensory quality. In this evaluation, titratable acidity (%) measurement was conducted because it is considered to be a key factor indicating sour taste intensity (Da Conceicao Neta et al., 2007; Lugaz et al., 2005). Titratable acidity (%) of samples treated with VI was not significant different from those of untreated controls during 7 days storage (Table 3). Based on these results, it was concluded that VI could be effectively incorporated into a washing process for control of pathogens while simultaneously maintaining color, texture and not inducing sourness. Water rinsing after treatment was used in this study which might minimize quality changes.

Therefore, proper water rinsing following this treatment is necessary to maintain original produce quality.

Table 3. Comparison of quality values of paprika and carrot between untreated (control) and treated samples stored at 4°C for 7 days.

Sample	Storage day	Condition	Quality value				
			L*	a*	b*	Maximum load(N)	Titratable Acidity(%)
Paprika	0	Control[a]	37.84 ± 0.18Aa	18.47 ± 2.63Aa	16.84 ± 1.13Aa	117.7 ± 6.0Aa	0.19 ± 0.03Aa
		Treated[b]	37.27 ± 0.38Aa	16.77 ± 1.52Aa	16.22 ± 0.87Aa	116.8 ± 6.4Aa	0.19 ± 0.03Aa
	3	Control	38.01 ± 0.14Aa	18.09 ± 1.62Aa	17.24 ± 1.16Aa	114.4 ± 8.7Aa	0.18 ± 0.02Aa
		Treated	37.23 ± 1.03Aa	17.11 ± 1.00Aa	16.46 ± 0.70Aa	121.7 ± 6.2Aa	0.18 ± 0.01Aa
	7	Control	37.42 ± 1.33Aa	17.90 ± 0.59Aa	17.11 ± 0.82Aa	120.0 ± 9.8Aa	0.19 ± 0.01Aa
		Treated	36.69 ± 1.55Aa	17.59 ± 0.97Aa	16.78 ± 1.88Aa	111.3 ± 9.9Aa	0.20 ± 0.02Aa
Carrot	0	Control[c]	55.78 ± 0.55Aa	19.16 ± 1.67Aa	33.45 ± 2.96Aa	205.5 ± 10.3Aa	0.15 ± 0.02Aa
		Treated[d]	55.31 ± 0.86Aa	18.31 ± 1.31Aa	32.26 ± 1.12Aa	216.3 ± 18.0Aa	0.15 ± 0.04Aa
	3	Control	55.60 ± 1.22Aa	17.43 ± 1.68Aa	31.50 ± 1.13Aa	229.4 ± 34.6Aa	0.15 ± 0.05Aa
		Treated	55.27 ± 0.63Aa	18.76 ± 0.67Aa	33.35 ± 1.65Aa	240.3 ± 30.1Aa	0.14 ± 0.03Aa
	7	Control	56.44 ± 2.04Aa	18.25 ± 1.63Aa	32.23 ± 1.12Aa	228.9 ± 11.4Aa	0.14 ± 0.02Aa
		Treated	55.79 ± 0.80Aa	18.27 ± 0.45Aa	32.31 ± 2.13Aa	217.1 ± 21.9Aa	0.14 ± 0.03Aa

[a]Untreated paprika.
[b]Paprika treated with intermittent vacuum impregnation (21.3 kPa) in 2 % malic acid for 5 min
[c]Untreated carrot
[d]Carrot treated with intermittent vacuum impregnation (21.3 kPa) in 2 % malic acid for 20 min.
* Different uppercase letters between control and treated sample within the same storage day indicate significant differences at each value (L*, a*, b*, N, %).
* Different lowercase letters within the same condition indicate significant differences ($P < 0.05$) between storage intervals (days) at each value (L*, a*, b*, N, %)

IV. CONCLUSION

Most consumers and processers have thought that washing and sanitizing will achieve a remarkable reduction in microbial load. However, published data about washing efficacy indicate that these conventional methods are not sufficiently adequate to reduce microbial loads on produce by more than 2 to 3 log (Gil et al., 2009; Niemira, 2012). This limited efficacy of produce washing is due to conditions causing contamination, an excessive interval between contamination and washing, bacterial attachment in inaccessible sites, biofilm formation, and internalization of bacteria within produce (Sapers et al., 2008). I suggest that limited efficacy caused by attachment in inaccessible sites can be overcome by applying vacuum impregnation. However, there has been little research into the efficacy of vacuum impregnation incorporated with the washing process. Furthermore, to date, there have been no studies on the efficacy of intermittent vacuum impregnation on bacterial reduction compared with continuous treatment. Gurtler et al. (2012) combined continuous vacuum perfusion (the term perfusion can be used interchangeably with impregnation) of 15" Hg (= 50.8 kPa or 7.4 psi) with the washing process (200 ppm chlorine) for 2 min, resulting

in increased inactivation of *Salmonella* in tomato stem scars by 0.7 log CFU over simple treatment at atmospheric pressure. However, Gurtler et al. (2014) found that incorporating vacuum perfusion in the washing (200 ppm chlorine or 90 ppm peracetic acid) of strawberries did not significantly reduce *Salmonella* populations more the same treatment without vacuum perfusion. This finding is in contrast to the previously mentioned result of log 0.7 CFU bacterial reduction in tomato stem scars. Gurtler et al. (2014) suggested that the difference of reduction between tomato stem scars and strawberries could be attributed to differences in their porosity. In other words, because tomato stem scars have more protected sites than strawberries, more bacteria could lodge in these protected sites and escape contact with sanitizer agents. Therefore, surface properties can influence the efficacy of vacuum impregnation on the inactivation of bacteria. As a result, based on my study, i postulate that Gurtler et al. could have observed greater bacterial reduction had they utilized intermittent treatment, since i achieved 2.5 $\log_{10}$ CFU/g further reduction compared to simple dipping by applying intermittent treatment, and this reduction was greater than that of continuous treatment (0.8 $\log_{10}$ CFU/g). Therefore, my study validates the improved application of vacuum impregnation into commercial produce washing

by overcoming the limitations of continuous treatment through the incorporation of intermittent treatment.

In addition, VI washing with malic acid had greater antimicrobial activity than dipping washing with malic acid on paprika and carrots. This enhanced effect could be attributed to increased accessibility of sanitizer into protected sites. On the other hand, there were no significant differences ($P \geq 0.05$) of antimicrobial activity between VI and dipping washing for king oyster mushrooms and muskmelons. King oyster mushrooms and muskmelons, which have relatively greater surface roughness than paprika and carrots, could provide enough space for microbes to escape contact with sanitizer after penetration of sanitizer into protected sites resulting from VI treatment. That is, VI washing is ineffective when applied to produce having large average roughness values (R_a), similar to those of king oyster mushrooms or muskmelons. However, we investigated surface roughness values of 12 additional types of fresh produce (data not shown) and fortunately, most had R_a similar to those of paprika and carrots. Therefore, this study supports the possibility of VI washing with organic acid by industry to help improve fresh produce safety through effective control of pathogens on various produce.

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VI. 국문초록

세척의 효과를 증진시키기 위해 진공함침 (Vaccum impregnation) 기술이 세척과정에 적용되어 미생물이 세척액의 접촉을 피할 수 있는 표면 틈 사이로의 접근성을 높이고자 하였다. 이 연구는 세척과정에 적용된 진공함침의 신선 농산물 표면의 병원성 미생물에 대한 저감화 효과 측정을 수행하였다. 우선, 병원성 미생물이 접종된 브로콜리에 적용하여 이 기술의 저감화 효과를 검증하였다. 브로콜리에 *Salmonella* Typhimurium 과 *Listeria monocytogenes* 를 접종하고 침지 세척과 진공함침 세척을 2 % malic acid 용액에서 5, 10, 20, 30 분간 처리하였다. 이 때, 진공함침은 연속적 처리와 간헐적 처리 두가지 방법이 이용되었다. 접종된 브로콜리에 5 분간 침지 세척을 하였을 때 1.5 와 1.3 log CFU/g 의 저감화가 *S.* Typhimurium 과 *L.* monocytogenes 에서 각각 발생했다. 그러나 5 분에서 30 분간의 처리에서는 추가적인 저감화가 발생하지 않았다. 진공함침 세척의 경우, 연속적 처리와 간헐적 처리 모두에서 진공압력이 커질수록 병원균의 저감화가 유의적으로 ($P < 0.05$) 증가했다. 연속적 처리의 경우, 시간의 증가했지만

유의적인 추가적 저감화는 발생하지 않았다 ($P \geq 0.05$). 그러나 간헐적 처리의 경우, 시간의 증가에 따른 추가적인 유의적 ($P < 0.05$) 저감화가 발생하였다. 표면을 관찰한 주사전자현미경 사진은 침지 세척 후에 표면 틈 사이에 균이 많이 부착되어 있는 것을 나타내었다. 그러나 간헐적 처리의 진공함침 세척의 경우, 대부분의 미생물이 씻겨 내려졌다. 미생물 현탁액에 직접 진공함침을 처리하였을 때, 진공함침 처리와 비 진공함침 처리 사이에 저감화 경향의 차이가 없었기 때문에 진공함침 자체가 미생물을 저감화 시키는 역할을 하지 않는 것을 나타냈다. 이 결과는 진공함침 세척의 증가된 미생물 저감화 효과는 세척용액의 표면 틈 사이로의 접근성의 증가에 의한 것임을 나타낸다. 간헐적 진공함침을 2 % malic acid 용액에서 30 분간 처리한 후 색, 물성 및 적정산도를 측정한 결과 비록 적정산도 값의 경우 처리되지 않은 대조군과 같아지기 위해서는 저장 기간이 필요하지만 모두 처리하지 않은 대조군과 유의적인 차이는 나타나지 않았다 ($P \geq 0.05$). 진공함침이 세척과정에 적용되어 저감화 효과를 개선할 가능성을 가진다는 이 결과를 토대로, 이 기술을 다양한 식품에 확대 적용해 보았다.

S.Typhimurium, *L. monocytogenes*, *Escherichia coli* O157:H7 을 접종한 파프리카, 당근, 새송이 버섯, 메론에 대한 유기산 세척에 진공함침을 적용했다. 이 샘플들은 21.3 kPa 의 간헐적 진공함침 세척을 3, 5, 10, 15 및 20 분간 처리하여 침지세척과 비교하였다. 침지세척의 경우, 파프리카의 저감화 경향이 가장 컸으며 당근, 새송이 버섯, 메론이 그 뒤를 따랐다. 진공함침 세척의 경우, 파프리카와 당근에서 유의적인 ($P < 0.05$) 향상된 저감화 효과가 나타났다. 하지만 새송이 버섯과 메론에서는 침지와 진공함침 세척 사이에 유의적인 저감화 차이가 나타나지 않았다 ($P \geq 0.05$). 이것은 표면 거칠기 때문일 것이다. 새송이 버섯 ($R_a = 6.02 \pm 1.65$) 과 메론 ($Ra = 11.43 \pm 1.68$)은 파프리카 ($Ra = 0.60 \pm 0.10$) 와 당근 ($Ra = 2.51 \pm 0.50$) 보다 상대적으로 큰 표면 거칠기 값을 가졌다. 새송이 버섯과 메론에서 상대적으로 거친 표면이 존재하기 때문에 미생물이 세척액과 접촉을 피할 수 있는 충분한 공간이 진공함침 처리 동안에도 생길 가능성이 있다. 이것은 새송이 버섯과 메론에서 진공함침 세척 후에 세척액으로부터 보호를 받을 수 있는 공간에서 많은 미생물이 깊게 위치해 있는 것을 보여주는

주사전자현미경의 사진에 근거한다. 각각 5 분, 20 분간 2 % malic acid 에서 진공함침 처리를 받은 파프리카와 당근에서 7 일의 저장기간동안 색, 물성 및 적정산도의 유의적 변화는 없었다 ($P \geq 0.05$)

주요어 : 신성 농산물, 세척액, 식품매개 병원균, 유기산, 진공함침

학번 : 2014-20697