https://doi.org/10.17221/98/2022-CJFS

Effects of washing procedures in reducing *Listeria monocytogenes* on raw leafy vegetables

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Citation: Giang N.T.H., Nor-Khaizura M.A.R., Mahyudin N.A., Vu T.L.A. (2022): Effects of washing procedures in reducing *Listeria monocytogenes* on raw leafy vegetables. Czech J. Food Sci., 40: 422–426.

Abstract: The efficacy of potable water and antimicrobial agents, including turmeric extract, black pepper extract, sodium chloride and sodium bicarbonate, in reducing *Listeria monocytogenes* on the leaf mustard (*Brassica integrifolia*) and iceberg lettuce (*Lactuca sativa L.*) was compared. The uncontaminated samples of two raw leafy vegetables were inoculated with *L. monocytogenes* at a concentration of 5 log and 7 log colony-forming units (CFU) g^{-1} for 1 h. At the high *L. monocytogenes* contamination level, the treatment with black pepper extract 0.6 mg mL^{-1} for 5 min was found to produce the most considerable decrease in *L. monocytogenes* counts, resulting in 1.44 log and 1.68 log reduction on leaf mustard and iceberg lettuce, respectively. Similarly, the black pepper extract also showed the highest *L. monocytogenes* reduction, approximately 0.79 log CFU g^{-1} on two leafy vegetables at the low contamination level. However, the odour of black pepper extract was left on the vegetables after being washed.

Keywords: foodborne pathogen; leaf mustard; iceberg lettuce; black pepper extract; turmeric extract

A high intake of fruits and vegetables is widely recommended in the daily diet for human beings because of their high concentrations of dietary fibre, vitamins, and minerals. Moreover, fruits and vegetables increase appetite and perform as anti-ageing agents. However, due to a large amount of moisture content as well as the high surface area, raw vegetables are ideal habitats for microorganisms speeding up decomposition and causing spoilage on vegetables (Ragaert et al. 2007). Raw vegetables may be contaminated with human pathogens, including bacteria such as *Escherichia coli*, *Salmonella* spp., *Listeria* spp. and viruses or parasites through contact with soil, animal manure used as fertiliser or sewage and irrigation water (Cliver et al. 1992). The contamination can also occur throughout the postharvest handling, including the preparation by street vendors, in the food-service establishment and the home (Beuchat 1996).

Currently, foodborne illness caused by the ingestion of food containing live bacteria is one of the biggest

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public health issues. Among those, food poisoning associated with listeriosis is a major concern due to the great number of cases and the severity of the related harm (EFSA and ECDC 2021). The evidence of fresh produce-associated *Listeria monocytogenes* has been growing year by year. The outbreaks have been reported commonly in the US and Canada, and the risk originating from *Listeria*-contaminated foodstuffs is more serious in pregnant women, children and elders (Schlech et al. 1983; CDC 2011, 2016).

However, it should be noticed that most people have awareness of the importance of using safe animal meat but do not take into account the risk of food poisoning from vegetables. Although many farms and plantations have a strict system to grow and harvest vegetables as well as make efforts to protect them from primary sources of contamination, the hazards have never been completely eliminated. Therefore, in order to reduce the microorganism population on fresh produce to a safe level for consumption, not only should contamination be minimised, but also initial counts need to be decreased by decontamination treatments. Washing is considered critical decontamination before carrying out the next processing steps.

Nowadays, the application of disinfectant agents to decontaminate the surface of fruits and vegetables is more and more popular (Pezzuto et al. 2016). In response to the current public health concerns regarding the microbiological safety of fresh produce, many researchers have investigated the efficiency of numerous methods in reducing the microbiological loadings of produce and determined their optimum conditions for use, as well as the presence of adverse toxicological effects. Besides conventional sanitisers such as sodium chloride solution and chlorine, the current studies have made some efforts to find out substitutes having a better effect in order to replace chemical agents (Meireles et al. 2016). Specifically, there are many concerns about the application of plant and plant by-product extracts such as pomegranate pomace and peel extract (Kang and Song 2017), cinnamon, lemongrass essential oil (Agourram et al. 2013), thyme oil, oregano oil, basil methyl chavicol (Burt 2004) and so forth. Due to awareness of the importance of microbiological food safety and quality, consumers now demand clean and fresh produce. Therefore, this study aimed to determine the efficacy of five washing methods (including both chemical and natural agents) in reducing *L. monocytogenes* on two raw leafy vegetables and evaluate the effect of the identified washing methods on the quality (pH, colour, texture) of these vegetables.

MATERIAL AND METHODS

Materials. Unpacked leaf mustard (*Brassica integrifolia*) and iceberg lettuce (*Lactuca sativa* L.) were purchased from retail in Seri Kembangan, Selangor, Malaysia. *L. monocytogenes* Pirie (ATCC® 19112™) strain was obtained from Food Microbiology Research Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM). Turmeric and black pepper extract was based on supercritical fluid extraction and was obtained from the Supercritical Fluid Centre, UPM.

Washing methods. Washing methods were displayed to compare their effectiveness in the reduction of previously inoculated *L. monocytogenes*. Each washing method was carried out in duplicate. The five selected washing methods were listed as follows:
- Washing method 1 (WM1): a single 5 min immersion in potable water;
- Washing method 2 (WM2): a single 5 min immersion in turmeric extract (0.25 mg mL^{-1});
- Washing method 3 (WM3): a single 5 min immersion in 1 M sodium chloride;
- Washing method 4 (WM4): a single 5 min immersion in 100 mM sodium bicarbonate;
- Washing method 5 (WM5): a single 5 min immersion in black pepper extract (0.6 mg mL^{-1}).

Sample preparation. The leaf mustard and iceberg samples with a weight of 25 ± 0.5 g (B-220C; Fisher Scientific, Switzerland) were stored at fridge (GR-R48MD; Toshiba, Japan) temperature 4 °C before conducting the experiments. The samples were placed on a sterilised aluminium foil and treated with ultraviolet irradiation of biosafety cabinet (Esco Global; Changi South Lane, Singapore) for 15 min on each side to reduce the existing microorganisms. For each experimental washing method, inoculated vegetable samples were immersed in a washing solution (1 : 10 w/v) and then dried on a clean bench for 30 min.

L. monocytogenes suspensions were prepared in order to obtain a final microbial loading of 5 log and 7 log colony-forming units (CFU) g^{-1}. Each 25 g of leaf mustard and 25 g of iceberg lettuce were inoculated with 1 mL of a suspension containing *L. monocytogenes* for 1 h to allow the attachment of *L. monocytogenes*. The samples were subsequently washed using one of the five washing methods identified above.

Microbiological analyses. The concentration of microbial suspension was measured according to the 0.5 McFarland turbidity value (0.5 McFarland is approximately 1.5×10^8 CFU mL^{-1}) (McFarland 1907), and a dilution process was carried out that was confirmed using the plate count method. The dilution prepared for *L. monocytogenes* counts was inoculated onto Polymyxin-Acriflavin-Lithium chloride-Ceftazidime-Aesculin-Mannitol (PALCAM) agar, (Oxoid, United Kingdom) using the spread plate technique and incubated at 37 °C (loading model 100-800; Memmert, Germany) for 24 ± 2 h (Hitchins et al. 2017). Microbiological analyses were performed in duplicates.

Statistical analysis. All results were recorded as the mean ± standard deviation (SD) (duplicates). The analysis of significant differences between the means was determined by one-way analysis of variance (ANOVA) of MINITAB statistic 16. The difference between data was counted as significant when $P < 0.05$.

RESULTS AND DISCUSSION

The efficacy of the five washing methods in reducing *L. monocytogenes* on the leaf mustard (*B. integrifolia*) and iceberg lettuce (*L. sativa* L.). The efficacy of the five washing methods in reducing *L. monocytogenes* on leaf mustard and iceberg lettuce is shown in Tables 1, 2. The results were depicted in the presence of both high- and lower-level contamination at 5 log and 7 log CFU g^{-1}, respectively. Almost all washing methods in this study reduced *L. monocytogenes* populations significantly compared to the control ($P < 0.05$). In terms of the high-level contamination (7 log CFU g^{-1}), 0.6 mg mL^{-1} of black pepper extract (WM5) resulted in 1.44 log and 1.68 log reduction of *L. monocytogenes* on leaf mustard and iceberg lettuce, respectively, compared to the control ($P < 0.05$), and it was greater than in all the other washing methods. However, the efficacy of all washing methods in lower microbial loading was slightly lower than in higher microbial loading. In the low-level contamination (5 log CFU g^{-1}), even though WM5 also displayed the best performance, the highest reduction was just approximately 0.79 log CFU g^{-1} on both leaf mustard and iceberg lettuce. As regards the pairwise comparison between the five washing methods, WM5 and WM3 generally resulted in a significant reduction compared to WM1, WM2, and WM4.

The overall efficacy of the five different washing methods in reducing *L. monocytogenes* on leaf mustard and iceberg lettuce at high contamination was as follows: the most efficient was WM5, followed by WM3, WM2, WM4 and WM1. All washing treatments showed a significant reduction of *L. monocytogenes* ($P < 0.05$) in comparison with the control, except for WM1. A similar pattern of *L. monocytogenes* reduction by the washing methods was observed at low *contamination for both leafy vegetables*. However, only WM5 and WM3 significantly reduced ($P < 0.05$) the microbial contamination compared to the control.

Table 1. The efficacy of the five washing methods in reducing *Listeria monocytogenes* on leaf mustard at high and low contamination, 7 log and 5 log CFU g^{-1}, respectively (mean ± SD; $n = 2$)

Level of contamination	Concentration of *L. monocytogenes* (log CFU g^{-1})					
	control	WM1	WM2	WM3	WM4	WM5
7 log	7.0845 ± 0.0126^{a}	6.9315 ± 0.0717^{a}	5.9898 ± 0.0502^{c}	5.7168 ± 0.0764^{d}	6.3798 ± 0.0256^{b}	5.6450 ± 0.0763^{d}
5 log	5.1038 ± 0.0048^{a}	4.9215 ± 0.0184^{ab}	4.7362 ± 0.0169^{bc}	4.5863 ± 0.1343^{c}	4.8381 ± 0.0356^{b}	4.3116 ± 0.0150^{d}

$^{a–d}$Mean values with different superscripts in the same row are significantly different at $P < 0.05$; CFU – colony-forming units; WM1 – potable water; WM2 – turmeric extract; WM3 – sodium chloride; WM4 – sodium bicarbonate; WM5 – black pepper extract

Table 2. The efficacy of the five washing methods in reducing *Listeria monocytogenes* on iceberg lettuce at high and low contamination, 7 log and 5 log CFU g^{-1}, respectively (mean ± SD; $n = 2$)

Level of contamination	Concentration of *L. monocytogenes* (log CFU g^{-1})					
	control	WM1	WM2	WM3	WM4	WM5
7 log	7.1160 ± 0.0470^{a}	6.9088 ± 0.0643^{b}	5.8538 ± 0.0301^{d}	5.5434 ± 0.0351^{e}	6.3768 ± 0.0772^{c}	5.4311 ± 0.0228^{e}
5 log	5.1122 ± 0.0071^{a}	4.9966 ± 0.0267^{ab}	4.7348 ± 0.1291^{bc}	4.4969 ± 0.0488^{cd}	4.8862 ± 0.0239^{ab}	4.3266 ± 0.1009^{d}

$^{a–e}$Mean values with different superscripts in the same row are significantly different at $P < 0.05$; CFU – colony-forming units; WM1 – potable water; WM2 – turmeric extract; WM3 – sodium chloride; WM4 – sodium bicarbonate; WM5 – black pepper extract

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Table 3. The pH value of leaf mustard and iceberg lettuce after being washed by five washing methods (25 °C) (mean ± SD; $n = 2$)

Leafy vegetable	Control	WM1	WM2	WM3	WM4	WM5
Mustard leaf	6.2575 ± 0.1276^{b}	6.3400 ± 0.1458^{b}	6.1325 ± 0.2290^{b}	6.2150 ± 0.1561^{b}	7.9300 ± 0.1270^{a}	6.1700 ± 0.2085^{b}
Iceberg lettuce	5.9000 ± 0.1158^{b}	6.0100 ± 0.0990^{b}	5.7950 ± 0.0640^{b}	5.8325 ± 0.0675^{b}	7.4900 ± 0.2853^{a}	5.8025 ± 0.0793^{b}

[a, b]Mean values with different superscripts in the same row are significantly different at $P < 0.05$; WM1 – potable water; WM2 – turmeric extract; WM3 – sodium chloride; WM4 – sodium bicarbonate; WM5 – black pepper extract

As mentioned previously, among the five washing methods investigated, WM1 was the least effective in reducing *L. monocytogenes* populations. This proves that washing with only water should be considered insufficient to effectively reduce *L. monocytogenes* populations on leafy vegetables. Although *L. monocytogenes* has the ability to tolerate a concentrated salt solution of up to 25% (Khan et al. 2016), it does not mean that the salt solution does not perform its antibacterial activity on *L. monocytogenes*. In this study, 1 M of sodium chloride solution (WM3) produced a great *L. monocytogenes* reduction, approximately 1.37 log and 1.57 log on leaf mustard and iceberg lettuce in terms of the high-level contamination (7 log CFU g^{-1}), respectively. However, due to the extremely high concentration of the salt solution, the appearance of vegetables was quite unacceptable. Generally, the use of plant extracts in reducing the Gram-positive bacteria such as *L. monocytogenes* on a variety of vegetables showed a satisfactory result.

In fact, the structure of Gram-positive bacteria such as *L. monocytogenes* contains 90–95% peptidoglycan, which allows hydrophobic molecules to penetrate the cells and act within the cytoplasm (Nazzaro et al. 2013). Black pepper extract contains alkaloids such as piperine, terpenes, flavones and volatile oils such as piperyline (Zou et al. 2015). Among these compounds, piperine has performed as a main antimicrobial component (Cowan 1999). Black pepper extract has been reported to reduce the intracellular adenosine triphosphate (ATP) concentration by inhibiting the Krebs cycle in bacterial cells; it also leads to metabolic dysfunction, inhibits energy synthesis, and causes bacterial cell death by destroying the permeability of the cell membrane (Zou et al. 2015).

This study mainly imitates the common surface contamination of leafy vegetables with *L. monocytogenes*. The approach of different washing methods was revealed to reduce the *L. monocytogenes* growth. However, as *L. monocytogenes* could also be present in the tissues of leafy vegetables, the washing treatment alone may not be sufficient. Therefore, a combination of washing treatment with thermal or non-thermal treatment is required to inhibit both surface and internal *L. monocytogenes* contamination.

The effect of washing methods on the pH of leaf mustard and iceberg lettuce. The pH value of leaf mustard and iceberg lettuce before and after being washed by the identified five washing methods is shown in Table 3. The pH values changed insignificantly after being washed with all solutions except the baking soda solution. The immersion of vegetables into the baking soda solution for 5 min (WM4) increased the pH value up to 7.93 on leaf mustard and 7.49 on iceberg lettuce. Taormina and Beuchat (2001) reported that *L. monocytogenes* has the ability to survive in alkaline media at refrigeration and ambient temperature. The possibly alkaline compound residues in food-processing environments may prolong the survival of *L. monocytogenes* and enhance the cross-protection against other conditions. Therefore, the treatments of vegetables with alkaline compounds for the purpose of reducing *L. monocytogenes* are required for further studies.

The effect of washing methods on the appearance of leaf mustard and iceberg lettuce. The washing methods tested could affect the appearance of vegetables. The addition of black pepper extract, turmeric extract, and sodium chloride resulted in the most effective reductions of *L. monocytogenes* populations. Although negligible colour changes were observed in mustard leaves and iceberg lettuce after being washed with two plant extracts, the treated leaf appearance looked slightly oily and had the odour of black pepper and turmeric. The texture for both leafy vegetables after washing with the plant extracts was still firmly intact, which contributed to a good texture. For the sodium chloride washing method, due to the extremely high salt concentration, the water amount present in treated vegetables was drawn out of cells via the process of osmosis. The loss of water made vegetables wilted and shrunken.

https://doi.org/10.17221/98/2022-CJFS

CONCLUSION

The results from this study showed that the addition of black pepper extract (WM5), turmeric extract (WM2), and concentrated sodium chloride solution (WM3) reduced *L. monocytogenes* populations on both leaf mustard and iceberg lettuce. Among them, black pepper extract resulted in the most effective reduction. This indicates the potential of the plant extracts to be applied as the antibacterial ingredient in producing natural detergents for washing fresh produce.

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Received: June 1, 2022
Accepted: October 31, 2022
Published online: November 24, 2022