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Effect of Different Conditions of Citric Acid and Acetic Acid Decontamination against *Escherichia coli* in Lettuce

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ABSTRACT

This study was conducted to determine the effect of different conditions of citric acid and acetic acid decontamination against *Escherichia coli* in lettuce. The samples were inoculated with *E. coli* and kept for 24 h at 4°C. The samples were decontaminated by using different concentrations of citric acid and acetic acid (0, 0.5, 1.0 and 1.5%), different exposure times (0, 15 and 30 min) and different physical applications (agitation and without agitation). The number of *E. coli* was counted after incubation at 37°C for 24 h. The result shows that citric acid and acetic acid were effective in removing *E. coli* at concentration of 1.0% without agitation while application of physical forces significantly increases the efficiency of citric acid and acetic acid in eliminating *E. coli* with concentration at 0.5% after 15 min. There is no significant difference with regards to 30 mins decontamination duration compared to 15 mins. The citric acid and acetic acid with the application of physical force are more effective compared to citric acid and acetic acid without the agitation. In conclusion, citric acid and acetic acids could be used as a disinfecting agents for decontamination of fresh produce in home application and food service sectors.

Keywords: *Escherichia coli,* lettuce, decontamination, citric acid and acetic acid

ABSTRAK

Kajian ini dijalankan untuk menentukan kesan keadaan yang berbeza asid sitrik dan asid asetik dalam dekontaminasi *Escherichia coli* dalam sayur salad. Sampel telah diinokulasi dengan *E. coli* dan disimpan selama 24 jam pada suhu 4°C. Sampel itu telah dibasuh dengan menggunakan kepekatan asid sitrik dan asid asetik (0, 0.5, 1.0 dan 1.5%), masa yang berbeza (0, 15 dan 30 min) dan cara fizikal yang berbeza (agitasi dan tanpa agitasi). Bilangan *Escherichia coli* dikira selepas pengeraman pada suhu 37°C selama 24 jam. Hasilnya menunjukkan bahawa asid sitrik dan asid asetik berkesan dalam menghilangkan *E. coli* pada kepekatan 1.0% tanpa tenaga fizikal manakala penggunaan tenaga fizikal dapat meningkatkan kecekapan asid sitrik dan asid asetik dalam menghilangkan *E. coli* dengan kepekatan 0.5% selepas 15 min. Tiada perbezaan yang signifikan bagi dekontaminasi selama 30 min berbanding dengan 15 min. Asid sitrik dan asid asetik dengan penggunaan tenaga fizikal lebih berkesan berbanding dengan asid sitrik dan asid asetik tanpa tenaga fizikal. Kesimpulannya, asid sitrik dan asid asetik boleh digunakan sebagai agen pembasmian untuk dekontaminasi produk segar dalam aplikasi rumah dan perkhidmatan makanan.

Kata Kunci: *Escherichia coli,* sayur salad, dekontaminasi, asid sitrik dan asid asetik

INTRODUCTION

Salad vegetables are commonly consumed raw and considered as an important part of daily diet. Sair et al. (2017) reported that the vegetables provide numerous nutrients, phytochemicals, and vitamins. Epidemiological evidence has clearly shown that diets based on fruits and vegetables reduce mortality from cerebrovascular and also cardiovascular diseases (Alia et al., 2013). However, contamination of fresh produce is emerging as a major food safety challenge.

A report by Caroline Smith DeWaal et al. (2014) showed that fresh produce attributed the highest number of outbreaks in the USA during 2002–2011. According to Yarahmadi et al. (2012), the American Food and Drug Administration reported that there are serious concerns about the consumption of vegetables, especially leafy vegetables such as lettuce. Between 1995–2006, 22 produce outbreaks were documented in the United States, with nearly half traced to lettuce or spinach grown in California (Laupland et. al., 2008). Heaton and Jones (2007) found lettuce, spinach and tomatoes are most commonly linked to pathogenic bacteria such as Salmonella and *Escherichia coli* O157: H7. Fresh produce can cause food-borne illness if they are contaminated with the pathogenic bacteria. Fresh produces can be contaminated in various ways; for example at the time of harvest, transport, distribution, sale, and during the preparation process at home or restaurants.

Escherichia coli is one of the pathogenic bacteria that has been observed in vegetables. *E. coli* is widely distributed in the environment, foods, and intestines of human and animals. *E. coli* consist of a diverse type of strains. The six strains of *diarrheagenic E.coli* are categorized as *enterohemorrhagic (EHEC)* O157: H7*, enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), enteropathogenic (EPEC), and diffusely adherent (DAEC)* (CDC, 2014). The worst type of *E. coli* known as *E. coli* 0157: H7 that can cause bloody diarrhea and sometimes kidney failure and even death while the other strains of *E. coli* can cause urinary tract infections, respiratory illness, and pneumonia (CDC, 2014).

Washing the vegetables with clean water is a simple but not an effective way to disinfect pathogenic microorganisms (Yarahmadi et al., 2012). A variety of disinfection methods used for fresh produce include non-thermal disinfection methods such as the application of chlorine oxide, ozone, acidic compounds, alkaline compounds and quaternary compounds. Organic acids such as citric acid, acetic acid, and lactic acid act as sanitizing agents for disinfection of fresh produce (Nascimento et al., 2003). WHO reported that the organic acids have potential in reducing the level of microorganisms on fruits and vegetables (Beuchat et al., 1995). However, there is a lack of published studies on the effect of citric acid and acetic acid decontamination on the survival of *E. coli* on lettuce. Therefore, the objectives of this study were to determine the efficacy of different concentrations of citric acid and acetic acid , time durations and agitation in the reduction of *E. coli* microbial load on the lettuce.

MATERIAL AND METHODS

Preparation of lettuce

Lettuce (*Lactuca sativa*) was purchased from local wet market at Kg Gong Bayor, Terengganu. The outer layer, damaged leaves and the core of the coral salad were removed and discarded. The inner leaves were cut into squares (4 cm x 4 cm) using a sharp sterile knife and each pieces were decontaminated with 100 ml of 70% of ethanol and then rinsed with sterile distilled water. Then, the lettuce was placed in a sterile plastic Petri dish.

Preparation of *Escherichia coli* inoculums The strain of *E. coli* was grown into a 10 ml Nutrient Broth (Merck, Germany) for 24 h at 37 °C. The culture was poured into a microcentrifuge tube and centrifuged using the centrifuging machine (5000 rpm, 5 min, Eppendorf Centrifuge 5418). Cell pellets were resuspended in saline solution and the optical density was measured using a spectrophotometer with a wavelength of 600 nm. The final concentration of the *E. coli* inoculum was approximately 6.56 x 10^8 cfu/ cm^2.

Inoculation of *E. coli* on lettuce

A 0.1 ml of the washed bacterial culture of concentration 10^8 cfu/ ml was inoculated onto the upper surface of the lettuce. The inoculum of *E. coli* was spot-inoculated in 6-8 droplets and spread around the entire surface using the pipette tip. While spreading the inoculums, the cut edge of the lettuce was avoided. Then, the inoculum was allowed for contact with the lettuce for 24 h and kept at 4 °C. Control lettuce was not inoculated with *E. coli.*

Decontamination of *E. coli* inoculated on lettuce

The decontamination study was carried out as described in Nastou et al. (2012) with modification. The decontamination process were conducted in three replicates are as below:

1) The lettuce was dipped into the citric acid solution with different concentration and time.
2) The lettuce was dipped into the acetic acid solution with different concentration and time.
3) Decontamination of inoculated lettuce with agitation was done using magnetic stirrer at 250 rpm.

The citric acid solution and acetic acid solution of concentration 0.5%, 1.0% and 1.5% v/v were prepared using sterile distilled water. The pH of the solutions were measured using the pH meter (CRISON micro pH 2001). Then, the samples that were inoculated earlier are treated earlier were treated with the different acid solutions of different concentrations and exposure times. The inoculated lettuce squares were immersed in the solution with different parameters. Lastly, the lettuce squares were drained and placed in a sterile bag that contains the saline solution.

Enumeration of *E. coli* in decontaminated lettuce

The lettuce samples were transferred into a stomacher bag containing 50 ml saline solution and homogenized for 1 min. For the enumeration of *E. coli*, the samples from the serial dilution were spread on the Nutrient Agar. The plates were incubated at 37 °C for 24 h. The colony counts were transformed to log cfu/cm^2.

Data analysis

The microbial counts were transformed to the logarithm before calculating the means and standard deviations. The population densities were expressed as $\log_{10}$ cfu/cm^2. The population densities of *E. coli* were compared to determine the effectiveness of the decontamination methods using one-way ANOVA test at significance level p = 0.05.

RESULTS AND DISCUSSION

In this study, the use of acetic acid solution and citric acid solution as a disinfectant for lettuce were examined. *E. coli* was not detected in the uninoculated sample because it was decontaminated with 70% ethanol and rinsed with distilled water. The samples was then inoculated with *E. coli* inoculums of approximately 6.56 x 10^8 cfu/ cm^2. The colony counts of the *E. coli* were observed after 24 h of incubation at 37°C in the incubator. The result of the effect of the concentration of acetic acid solution without agitation and the time on the survival of the *E. coli* inoculated onto the surface of lettuce is shown in the Table 1.

Table 1 The effect of the different concentration of acetic acid (0, 0.5, 1.0 and 1.5%) and exposure time (0, 15 and 30 min) on the survival of *E. coli* in lettuce without agitation.

Acid concentration (%)	Time		
	0 min	15 min	30 min
0	6.11 ± 0.76 ^{aA}	5.36 ± 0.02 ^{aB}	5.34 ± 0.16 ^{aB}
0.5	6.11 ± 0.76 ^{aA}	4.22 ± 0.13 ^{bB}	4.10 ± 0.13 ^{bB}
1.0	6.11 ± 0.76 ^{aA}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}
1.5	6.11 ± 0.76 ^{aA}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}

Data represent mean ± standard deviation of three replications.
^{a,b,c} Data in the same column with different letter is different significantly ($p < 0.05$).
^{A,B,C} Data in the same row with different letter is different significantly ($p < 0.05$).

At 0 min, the number of *Esherichia coli* for all concentrations of acetic acid were 6.11 log cfu/cm^2. However, increasing decontamination time from 15 to 30 mins did not significantly ($p > 0.05$) reduce the number of *E. coli*. Regardless of exposure time, *E. coli* was not detected in lettuce dipped in acetic acid with concentration of 1.0% and 1.5%.

Table 2 shows the effect of the concentrations of acetic acid solution and the exposure time on the survival of the *Escherichia coli* inoculated onto the surface of lettuce with agitation. The number of *E. coli* population was reduced significantly ($p < 0.05$) after 15 min in 0.5, 1.0 and 1.5% acetic acids. Log reductions increased with longer treatment times for *E. coli* bacteria on lettuce, but no significant differences were observed between 15 min and 30 min exposure times.

From Table 1 and 2, it is clear that the decontamination of *E. coli* is effective at concentration of 0.5% acetic acid with agitation compared to acetic acid without agitation at 1.0%. Reduction of *E. coli* in lettuce is obtained when the population of *E. coli* was not detected at the concentration of 0.5% with agitation but a higher concentration at 1.0% is required without agitation. Based on the result obtained, the applications of physical force and increasing the concentrations affect the reducing number of the *E. coli*.

Table 2 The effect of the different concentration of acetic acid (0, 0.5, 1.0 and 1.5%) and exposure time (0, 15 and 30 min) on the survival of *E. coli* in lettuce with agitation.

Acid concentration (%)	Time		
	0 min	15 min	30 min
0	6.11 ± 0.76 ^{aA}	4.52 ± 0.03 ^{aB}	4.51 ± 0.01 ^{aB}
0.5	6.11 ± 0.76 ^{aA}	0.00 ± 0.00 ^{bB}	0.00 ± 0.00 ^{bB}
1.0	6.11 ± 0.76 ^{aA}	0.00 ± 0.00 ^{bB}	0.00 ± 0.00 ^{bB}
1.5	6.11 ± 0.76 ^{aA}	0.00 ± 0.00 ^{bB}	0.00 ± 0.00 ^{bB}

Data represent mean ± standard deviation of three replications.
^{a,b,c} Data in the same column with different letter is different significantly ($p < 0.05$).
^{A,B,C} Data in the same row with different letter is different significantly ($p < 0.05$).

Table 3 shows the effect of the concentration of citric acid solution without agitation and the time on the survival of the *Escherichia coli* inoculated onto the surface of lettuce. At 0 min, the initial population of *E. coli* on lettuce was about 6.27 log cfu/cm^2. Increase in citric acid concentration and exposure time significantly reduce the number of *E. coli*. When the concentration of citric acid was increased from 1.0 to 1.5%, no significant ($p > 0.05$) reduction in the number of *E. coli* was observed. Maximum reduction in *E. coli* was obtained when the lettuce was dipped in 1.0 % and 1.5% citric acid for a period of 15 and 30 min.

Table 3 The effect of the different concentration of citric acid (0, 0.5, 1.0 and 1.5%) and exposure time (0, 15 and 30 min) on the survival of *E. coli* in lettuce without agitation.

Acid concentration (%)	Time		
	0 min	15 min	30 min
0	6.27 ± 0.21^{aA}	4.92 ± 0.42 ^{aB}	4.81 ± 0.43 ^{aB}
0.5	6.27 ± 0.21^{aA}	3.93 ± 0.99 ^{bB}	3.82 ± 0.14 ^{bB}
1.0	6.27 ± 0.21^{aA}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}
1.5	6.27 ± 0.21^{aA}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}

Data represent mean ± standard deviation of three replications.
^{a,b,c} Data in the same column with different letter is different significantly ($p < 0.05$).
^{A,B,C} Data in the same row with different letter is different significantly ($p < 0.05$).

For agitation method as shown in Table 4, the number of *E. coli* was reduced significantly in water without citric acid after 15 min (4.45 log cfu/cm^2). Increasing the treatment time from 15 to 30 min did not result in any further significantly ($p < 0.05$) decrease. *E. coli* was effectively removed with acid concentration of 0.5% after exposure time of 15 min.

From Table 3 and 4 the result shows that 0.5% citric acid with agitation for 15 min was found to be more effective compared with 1.0% citric acid without agitation for 15 min. This is because the population of *E. coli* was not detected at the concentration of 0.5% and 1.0% respectively. Based on the result obtained, increasing the concentration and application of physical forces affect the reducing number of the *E. coli*.

Table 4 The effect of the different concentration of citric acid (0, 0.5, 1.0 and 1.5%) and exposure time (0, 15 and 30 min) on the survival of *E. coli* in lettuce without agitation.

Acid concentration (%)	Time		
	0 min	15 min	30 min
0	6.27 ± 0.21^{aA}	4.45 ± 0.04^{aB}	4.44 ± 0.04^{aB}
0.5	6.27 ± 0.21^{aA}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}
1.0	6.27 ± 0.21^{aA}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}
1.5	6.27 ± 0.21^{aA}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}

Data represent mean ± standard deviation of three replications.
[a,b,c] Data in the same column with different letter is different significantly ($p < 0.05$).
[A,B,C] Data in the same row with different letter is different significantly ($p < 0.05$).

This study found that both the citric acid and acetic acid treatment with agitation is more effective compared to those without agitation. The combination of organic acids and agitation showed a greater reduction of *E. coli*. The concept of combining two factors for reducing the *E. coli* showed greater effectiveness at inactivating the microorganisms than the use of a single factor. Nastou et al. (2012) demonstrated that many factors could potentially influence the efficacy of agitation in removing the bacteria from the vegetables such as flow rate and turbulence to which the vegetables are exposed and the extent of abrasive contact with other vegetables pieces. In this study the magnetic stirrer was used as a physical force. It was agitated at medium speed which is increase the flow rate of acid solutions thus make the lettuce leaves contact each other and the result shows significantly reduce the number of *E. coli* in lettuce.

This study found that acid concentration is a significant factor that affect the reduction the *E. coli* population. This is because increasing the concentration of the acid solution resulted in lowering the pH value. Park et al. (2013) reported that the antimicrobial activity of organic acids is attributed to a reduction of pH by the ionization of undissociated acid molecules. A low external pH can disrupt the substrate transport system by altering cell membrane permeability. In another study, Jongen (2005) reported that the dissociation of hydrogen ions causes reduction in the internal cellular pH of the organism. Disruption in the ability of the cell maintaining the pH homeostasis results in disruption of membrane permeability and substrate transport. In this study, the measured pH of the solutions of concentration 0.5 to 1.5% acetic acid was 2.69 to 2.55 while the pH for 0.5 to 1.5% citric acid was 2.50 to 1.67. It indicates that these organic acids were present mostly in undissociated form. Moreover, the weak organic acids cause acid stress in bacteria because they are less dissociated at any given pH than strong acids such as HCl, organic acids can cross the inner membrane more freely in the uncharged form (Lund et al., 2014).

Citric acid and acetic acid are organic acids which are known to have bactericidal activity (Akbaz and Olmes, 2007). Organic acids are naturally found in a variety of fruits and fermented foods. Anti-microbial activity of acetic acid was shown against *E. coli, L. monocytogenes, Salmonella* Typhimurium*, Y. enterocolitica* while citric acid in the form of lemon juice has been demonstrated to reduce *S.* Typhimurium populations on some fresh fruits (Akbas and Olmez, 2007). Park et al., (2013) suggested that washing with organic acids could be as effective as hydrogen peroxide and sodium hypochlorite.

Inactivation of microorganism also depend on the types of organic acid. The organic acids such as citric acid, tartaric acid, malic acid, sorbic acid, lactic acid and acetic acid are known as weak acids having different inhibitory effects compared to strong acids because they are lipophilic and penetrate plasma membrane and thus acidify the cell's interior (Booth and Kroll 1989).

Another reason affecting the efficacy of decontamination is the types of fresh produce tested. Each vegetables have different in the microenvironment (topography, presence of stomata, chemical composition) which certain surface may protect the bacteria from coming in contact with organic acid. This study used green leaf lettuce (*Lactuca sativa*) which has smooth surface structure without deep crevises. According to Nastou et al., (2012), the parsley is more resistant to removal or inactivation of *Listeria monocytogenes* than that on lettuce because of parsley has small leaf and stick to each other when wet. Such condition reduce the effectiveness of washing as

many parts of the leaves might left unwashed. From this study, lettuce structure also plays important reason in reducing the number of *E. coli* colonies.

This study found that mainly citric acid and acetic acid concentration are significant factors that affect the reduction of reducing the *E. coli* population. Park et al, (2013) reported that the antimicrobial activity of organic acids is attributed to a reduction of pH by the ionization of undissociated acid molecules. A low external pH can disrupt the substrate transport system by altering cell membrane permeability. Reduction in pH causes in inhibition of *E. coli* bacteria.

CONCLUSION

As a conclusion, the result shows that the time, concentration, and the physical force can affect the total *E. coli* reduction on the lettuce. In this study, the citric acid and acetic acid are effective in removing *E. coli* at concentration of 1.0% without agitation while application of physical forces significantly increases the efficiency of citric acid and acetic acid in eliminating *E. coli* with concentration at 0.5%. However there is no significant differences in the number of *E. coli* when time duration was increased from 15 min to 30 min. Increasing citric acid and acetic acid concentrations showed significantly different bactericidal effects as higher acid concentration the greater the log reduction was observed. The log reduction for the citric acid and acetic acid treatment combined with agitation significantly higher than that for the citric acid and acetic acid treatment without agitation. These finding demonstrated the effective way on *E. coli* decontamination using commonly available organic acid in household setting.

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