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Sanitizing action of triple-strength vinegar against *Escherichia coli* on lettuce

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ABSTRACT

Vegetable sanitization protocols recommend the use of chlorine, which has adverse effects on the environment and carcinogenic effects on humans. Acetic acid is an interesting alternative to chlorine because it possesses no risk to human health and is widely available in the form of vinegar. This study aimed to evaluate the sanitizing action of vinegar, 130 g L^{-1} total titratable acidity expressed as acetic acid, on lettuce. Vinegar was chosen because it is a low-cost product widely available in the Brazilian market. The minimum inhibitory concentration and minimum bactericidal concentration of vinegar against *Escherichia coli* were 2.5 and 15 g L^{-1} total acidity, respectively. Lettuce leaves artificially contaminated with *E. coli* or naturally contaminated with total coliforms were washed with water and immersed in vinegar solution (15 g L^{-1} total acidity) for 15 min. This period was sufficient to reduce *E. coli* counts in artificially contaminated samples and total coliforms in naturally contaminated samples. There were no visual changes in lettuce leaves, which indicates that vinegar at 15 g L^{-1} total acidity can be used to sanitize vegetables without affecting their appearance.

Keywords: *Lactuca sativa*, acetic acid, sanitization.

RESUMO

Ação sanitizante de vinagre triplo sobre *Escherichia coli* em alface

Protocolos de sanitização de hortaliças utilizam cloro, que apresenta efeitos adversos sobre o meio ambiente e na produção de compostos cancerígenos. Uma alternativa para substituir o cloro é o uso do ácido acético, devido à disponibilidade na forma de vinagre e por não apresentar riscos à saúde humana. O trabalho teve como objetivo avaliar a ação do fermentado acético de álcool com 130 g L^{-1} de acidez total, expressa em ácido acético, para uso na sanitização de alfaces. A escolha deste fermentado é justificada por ser um produto de baixo preço e disponível no mercado brasileiro. A concentração inibitória mínima e a concentração bactericida mínima (concentração que eliminou a bactéria) para *Escherichia coli* foi de 2,5 e 15 g L^{-1} de acidez total, respectivamente. Amostras de alface artificialmente contaminadas com suspensões de *E. coli* e amostras com contaminação de origem foram lavadas com água e imersas em solução com 15 g L^{-1} de ácido acético, por 15 minutos. Esse tempo foi adequado para a redução da contagem de *E. coli* nas amostras artificialmente contaminadas e na contagem de coliformes totais nas amostras com contaminação de origem. Nenhuma alteração visual das folhas de alface foi observada, indicando que a concentração de 15 g L^{-1} de acidez total pode ser utilizada na sanitização da hortaliça sem prejuízo à sua aparência.

Palavras-chave: *Lactuca sativa*, ácido acético, sanitização.

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Foodborne diseases are still a public health problem, and the supply of safe food products has been a constant concern for government health agencies, epidemiological surveillance agencies, and the food industry.

Lettuce (*Lactuca sativa*) is one of the most consumed vegetables in salads in Brazil. Chlorinated compounds are widely used to sanitize lettuce due to low cost and recommendations of the National Agency of Sanitary Surveillance (ANVISA). The formation of trihalomethanes from chlorinated sanitizers, however, is a cause for concern, as these compounds are carcinogenic. Sanitization alternatives that provide economic and safety benefits by replacing chlorine are of interest.

Acetic acid, produced by fermentation of ethanol by acetic acid bacteria, may be an alternative to chlorine in food sanitization. Acetic acid is the main component of vinegar and can be produced by *Acetobacter* spp. fermentation of fruit must, honey, cereals, hydroalcoholic mixtures, and vegetables (Spinosa *et al*., 2015). An important characteristic of vinegar is its low toxicity. Moreover, its use as sanitizer does not alter the taste or smell of foods (Nascimento *et al*., 2003).

According to Brazilian legislation, the product of acetic fermentation of a hydroalcoholic mixture containing potable ethanol of agricultural origin is denominated alcohol vinegar. Vinegar with volatile acidity over 12 g of acetic acid per 100 mL is classified as triple and called triple-strength vinegar. The product of acetic fermentation of ethanol produced from one or more cereals is denominated cereal vinegar (BRASIL, 2012).

Brazilian industries have sufficient capacity to meet the demand for diversified products based on vinegar. Triple-strength vinegar is commercialized in the Brazilian market for $ 0.15/L (FOB, Free On Board). Noteworthy, vinegar production facilities have a low impact on the environment (ANAV, 2015).

The aim of this study was to evaluate the efficiency of triple-strength vinegar for sanitization of artificially contaminated lettuce with *Escherichia coli* and naturally contaminated lettuce with total coliforms and *E. Coli* determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) using the macrodilution method.

MATERIAL AND METHODS

Twenty-seven crisphead lettuce heads were randomly obtained and purchased at different commercial establishments in Londrina, in different weeks from October to December 2014. The vegetables were marketed as products grown by conventional agricultural methods.

Triple-strength alcohol vinegar was provided by Tecnologia em Saúde, Indústria e Comércio de Alimentos Ltda., Assis, São Paulo, Brazil. The vinegar was produced by biological fermentation of alcohol. Its total titratable acidity expressed as acetic acid was 130 g L^{-1}, and its pH was 2.50.

Alcohol vinegar and cereal vinegar were purchased from commercial establishments in Londrina. Alcohol vinegar and cereal vinegar presented pH 2.3 and 2.6, respectively, and total titratable acidity expressed as acetic acid was 40 g L^{-1}.

Production of E. coli suspension

E. coli ATCC 25922 cephae was inoculated into brain heart infusion (BHI) and incubated for 24 h at 35±2°C. Serial dilutions were performed in 1 g L^{-1} peptone water (Himedia). and put on MacConkey agar (Himedia) to determine bacterial counting (log CFU/mL).

Determination of the minimum concentration to inhibit (MIC) *E. coli* growth

The MIC of triple-strength alcohol vinegar, and cereal vinegar against *E. coli* was determined by microdilution in tubes. Aliquots of 100 µL of *E. coli* suspension containing approximately 6 log CFU/mL were added to tubes containing BHI and vinegar at final concentrations of 0, 2.5, 5.0, 10.0, 15.0, 20.0, or 40.0 g L^{-1} total acidity in a final volume of 5 mL. After 15 min, 5 mL of BHI was added to all tubes. Tubes were incubated at 35°C for 24 h. MIC was determined in the tube with no turbidity and with the lowest concentration of acetic acid, that is, the minimum concentration that did not allow bacterial growth.

Determination of the minimum bactericidal concentration (MBC) of vinegar

The determination of MBC of vinegar against *E. coli* was performed by plate counting using MacConkey solid medium. Subsequent to the determination of MIC, 10 µL aliquots from the test tubes containing *E. coli* and vinegar at different concentrations were inoculated onto MacConkey agar plates. After 24 h of incubation, MBC was determined as the lowest concentration of acetic acid that completely inhibited *E. coli* growth.

Efficiency evaluation of the sanitization procedure using triple-strength vinegar on artificially contaminated lettuce leaves with *E. coli*

Six lettuce heads were analyzed individually, representing six replicates of the sanitization procedure. From each lettuce head, the inner and outer leaves were discarded and two 25 g portions of leaves were weighed. The two portions, identified as A and B, were contaminated with 4 mL each of *E. coli* suspension containing approximately 6 log CFU/mL. The suspension was randomly distributed over the leaves using a pipette. After the leaves had dried at room temperature for 3 to 4 h, the units of *E. coli* on portion A were counted. Portion B leaves were immersed for 15 min in a triple-strength vinegar solution containing 15 g L^{-1} acetic acid, considered the MBC. Portion B leaves were washed with sterile distilled water and centrifuged in a centrifuge suitable for handling leafy vegetables. *E. coli* counts were then determined.

Portions A (not sanitized) and B (sanitized) were homogenized separately in 50 mL of 1 g L^{-1} buffered peptone water, and serial dilutions from 10^{-2} to 10^{-4} were carried out in 1 g L^{-1} buffered peptone water. Subsequently, 100 µL of each dilution was plated on MacConkey agar medium in duplicate. Colonies were counted after 24 h of incubation at 35±2°C.

Evaluation of the efficiency of sanitization procedure using triple-strength vinegar on naturally contaminated lettuce leaves

For this analysis, 31 lettuce heads were purchased commercially in Londrina, and analyzed individually. Three 25 g portions of leaves were collected from each lettuce head and labelled as A, B, and C. Portions B and C were washed under chlorinated running water. Portion C was immersed in a triple-strength vinegar solution (15 g L^{-1} acetic acid) for 15 min; the leaves were then washed in sterile distilled water and centrifuged in a centrifuge suitable for handling leafy vegetables. The most probable numbers (MPN) of total coliforms and *E. coli* on portions A, B, and C were determined by the multiple-tube fermentation technique (Silva *et al*., 2007).

Determination of total titratable acidity of vinegar

To determine total titratable acidity, 1 mL of triple-strength vinegar and two drops of a 10 g L^{-1} phenolphthalein solution were added to a 100 mL Erlenmeyer flask. Titration was performed with 0.1 M sodium hydroxide until the solution turned red. Each mL of sodium hydroxide solution corresponded to 6 g L^{-1} total titratable acidity expressed as acetic acid (H_3CCOOH) (Spinosa *et al*., 2015).

Statistical analysis

E. coli counts on the six artificially contaminated lettuce samples were converted into decimal logarithms (log CFU/mL). Data were submitted to statistical analysis in Microsoft Excel 2013. Student's *t*-test was used to determine whether *E. coli* counts before and after sanitization with triple-strength vinegar differed at 5% significance level.

RESULTS AND DISCUSSION

The concentrations of triple-strength vinegar, alcohol vinegar, and cereal vinegar used for determining MIC and MBC were 0, 2.5, 5.0, 10.0, 15.0, 20.0, and 40.0 g L^{-1} total titratable acidity expressed as acetic acid. The addition of 5 mL of BHI after 15 min of exposure of the *E. coli* inoculum to vinegar was essential for the determination of MIC and MBC values. Without this step, it was not possible to recover viable *E. coli* cells, independently of the concentration of acetic acid used in the experiment. This procedure allowed the recovery of *E. coli* cells damaged by the action of vinegar after 15 min sanitization probably because of dilution of the acetic acid present in the medium at the end of the experiment. The MIC and MBC values of the vinegars against *E. coli* were respectively 2.5 and 15.0 g L^{-1} total titratable acidity expressed as acetic acid.

A significant reduction was observed in the mean *E. coli* count of artificially contaminated lettuce leaves after sanitization using 15 g L^{-1} acetic acid solution prepared with triple-strength vinegar (Table 1).

Nascimento *et al*. (2003) reported similar results to those of the present study. The authors compared the efficiency of acetic acid, chlorine, and peracetic acid for sanitizing lettuce and recommended the use of 20 g L^{-1} acetic acid for 15 min by immersion. Other studies have also evaluated the bactericidal action of acetic acid on *E. coli* using artificially contaminated lettuce, but assays were performed with different concentrations from those used in the present study. Park *et al*. (2011) tested the antimicrobial efficiency of different organic acids and obtained a 1.57-log CFU/g reduction in *E. coli* count using 20 g L^{-1} acetic acid for 5 min, a result similar to that reported by Akbas & Olmez (2007), a 1.5-log CFU/g reduction after 2 min of exposure of lettuce leaves to 10 g L^{-1} acetic acid solution. Vijayakumar & Wolf-Hall (2002) evaluated the effect of apple vinegar, white vinegar, chlorine, and lemon juice against *E. coli* on lettuce. The most effective result was achieved with white vinegar at 19 g L^{-1} total titratable acidity expressed as acetic acid. There was a 5-log reduction in *E. coli* counts when samples were immersed in white vinegar solution for 5 min under shaking or for 10 min without shaking.

Bjornsdottir *et al.* (2006) stated that many factors affect the antimicrobial activity of organic acids, such as pH, acid concentration, bacterial species, and the environment of bacterial cultures. Several studies have been carried out on the bactericidal action of acetic acid against other pathogenic bacteria, using varied vegetables and acetic acid concentrations. Reductions of up to 1.4 log CFU/g of *Listeria monocytogenes* were observed in artificially contaminated lettuce and leafy salads sanitized with acetic acid solutions at concentrations ranging from 5 to 10 g L^{-1} (Porto & Eiroa, 2006; Samara & Koutsoumanis, 2009; Nastou *et al*., 2012; Ramos *et al*., 2014). A reduction of 1.74 log CFU/g in *Salmonella* spp. count was obtained by sanitizing alfalfa and lettuce with 50 and 20 g L^{-1} acetic acid solution for 10 min, respectively (Weissinger & Beuchat, 2000; Park *et al*., 2011). Karapinar & Gonul (1992) achieved a 7-log CFU/mL reduction in *Yersinia enterocolitica* counts using 20 g L^{-1} acetic acid.

Other important factors to be considered in studies that evaluate food sanitization using vinegar are the food's inherent characteristics. Differences in bacterial microenvironments formed on the surfaces of different vegetables should be taken into consideration when extrapolating the findings of this study to other foods. Washing processes may not be as efficient when there are cracks, crevices, or interstices on food surfaces (Ramos *et al.*, 2014).

Studies that tested the performance of 50 g L^{-1} acetic acid in the sanitization of vegetables reported negative changes to the sensorial characteristics of the product (Chang & Fang, 2007; Wu *et al.*, 2000). The results obtained in the present study using triple-strength vinegar containing 15 g L^{-1} acetic acid are satisfactory from an application point of view, as the higher the concentration of acetic acid in the sanitizing solution, the greater the probability of sensorial changes in vegetables.

Most of the studies on the efficacy of sanitizing agents on fruits and vegetables were carried out with artificially contaminated products. In the present study, in addition to the analysis of lettuce artificially contaminated with *E. coli*, naturally contaminated lettuce leaves were analyzed. Table 2 shows the results of log MPN/g of total coliforms on lettuce samples. Contamination ranged from 1.63 to 3.38 log MPN/g. Brazilian legislation does not establish limits for total coliforms, and their presence is natural in fresh vegetables because of the type of cultivation. Berbari *et al.* (2001) stated, however, that total coliform counts can be used

Table 1. *E. coli* counts in artificially contaminated lettuce leaves before and after sanitization with vinegar (15 g L^{-1} total titratable acidity expressed as acetic acid). Londrina, UEL, 2014.

Sample	*E. coli* count before sanitization (log CFU/g)	*E. coli* count after sanitization (log CFU/g)
1	2.99a	2.11b
2	3.00a	0.60b
3	3.04a	0.78b
4	3.36a	0.00b
5	3.36a	0.78b
6	3.88a	0.30b
Mean	3.27 ± 0.31^{a}	0.76 ± 0.66^{b}

Counts and mean counts followed by different letters differ significantly ($p \leq 0.05$).

Table 2. Total coliform counts in naturally contaminated lettuce leaves[2] and count reductions after washing samples under running chlorinated water[3] and after washing samples under running chlorinated water and sanitizing with vinegar solution (15 g L^{-1})[4] (total titratable acidity expressed as acetic acid). Londrina, UEL, 2014.

Sample[1]	Total coliform count (log MPN/g)[2]	Total coliform count after washing (log MPN/g)[3]	Total coliform count after washing and sanitizing (log MPN/g)[4]
1	2.38	0.00	2.38
2	2.38	1.75	2.38
3	2.38	1.41	2.02
4	2.38	1.93	2.02
5	1.66	1.49	1.66
6	2.38	1.41	2.38
7	2.38	1.41	1.75
8	2.38	0.34	2.02
9	2.38	0.72	1.20
10	2.38	2.20	2.38
11	2.38	1.20	1.41
12	1.66	1.03	2.06
13	2.04	1.07	2.04
14	2.38	1.41	1.75
15	2.38	1.06	2.38
16	1.63	0.79	1.03
17	1.97	1.37	1.97
18	2.66	1.34	2.66
19	3.38	2.54	2.78
20	3.04	2.44	3.04
21	2.32	0.69	1.72
22	3.04	2.09	3.04
23	3.04	0.87	2.44
24	3.38	1.41	3.38
25	2.32	1.00	1.72
26	3.04	0.00	2.09
27	3.38	0.73	2.02
28	3.04	0.39	1.41
29	3.38	0.73	3.38
30	3.04	1.68	3.04
31	3.38	1.00	2.78

[1]Samples purchased at different commercial establishments in Londrina, Paraná, Brazil.

as an indicator of hygiene and are considered high when above 5.0 log MPN/g in foods. All samples analyzed in this study were contaminated with total coliforms but none showed counts greater than 5.0 log MPN/g, as opposed to other studies, in which high counts of total coliforms were identified in the samples (Cabrini *et al.*, 2002; Oliveira & Figueiredo, 2006).

The results of the sanitization of naturally contaminated lettuce using the triple-strength vinegar solution containing 15 g L^{-1} acetic acid are in Table 2. A reduction of 1.03 to 3.38 log MPN/g in total coliform bacteria was observed. This reduction was greater than that obtained by washing samples in running chlorinated water (0-2.5 log MPN/g).

Statistical analysis was performed only on the results of *E. coli* counts of the six artificially contaminated lettuce samples before and after vinegar sanitization (Table 1). The results of total coliform counts in naturally contaminated lettuce before and after sanitization (Table 2) were not submitted to statistical analysis because of the great variation between initial and final bacterial counts. The purpose was to present the reduction in bacterial count promoted by the treatments as raw data.

Other studies also evaluated the performance of acetic acid solutions in the sanitization of naturally contaminated vegetables. Oliveira *et al.* (2012) reported a reduction of 2.09 log CFU/g in total coliforms on lettuce using 8 g L^{-1} acetic acid. Nascimento *et al.* (2003) used 20 g L^{-1} acetic acid and obtained a 3.57-log CFU/g reduction in mesophilic aerobes on lettuce samples. Fantuzzi *et al.* (2004) obtained a reduction of 1.8 log CFU/g in mesophilic aerobes after sanitizing cabbage samples for 10 min with 10 g L^{-1} acetic acid at room temperature.

A total of 23 (74.2%) of the 31 naturally contaminated lettuce samples analyzed in this study were contaminated with *E. coli* before washing with water. All counts were below the limit established by Brazilian legislation and ranged from 0.4 to 46 log MPN/g. *E. coli* was isolated from nine of the 23 contaminated samples after sanitization with running chlorinated water and from one sample after sanitization using triple-strength vinegar solution containing 15 g L^{-1} acetic acid.

Washing lettuce leaves under running chlorinated water and immersing them in 15 g L^{-1} acetic acid solution for 15 min promoted a reduction in *E. coli* counts on artificially contaminated samples and a reduction in total coliform counts in naturally contaminated samples. There were no visual changes in treated lettuce leaves, which indicates that vinegar at 15 g L^{-1} total titratable acidity can be used to sanitize vegetables without affecting their appearance.

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