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Article

Evaluating cinnamaldehyde as an antibacterial agent in a produce wash for leafy greens

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SUMMARY

Escherichia coli **is a common foodborne pathogen in produce, especially leafy greens, which are often consumed raw. Post-harvest sanitizing is an essential step in mitigating the risk of foodborne illness associated with uncooked produce. We explore the potential antibacterial effects of cinnamaldehyde against *E. coli* when used as a produce wash for leafy greens. We hypothesized that treating leafy greens with cinnamaldehyde, a promising antibacterial agent, would yield an observable decrease in *E. coli* growth contamination, measured in colony-forming units (CFUs). In this study, we treated lettuce samples with various concentrations of cinnamaldehyde solution and compared the *E. coli* growth to water and bleach treatment controls. By analyzing either the leaf surface or the wash solution, we were able to detect the presence of *E. coli*. Lettuce treated with cinnamaldehyde in any concentration displayed significantly lower CFUs when compared to lettuce treated with chlorine bleach ($p<0.00001$). 0.5% and 1.0% cinnamaldehyde solutions were also more effective at inhibiting *E. coli* growth than 0.2% cinnamaldehyde ($p=0.00387$). Cinnamaldehyde had an effect on the survival rate of *E. coli* on lettuce that was equal to or greater than that of bleach. The concentration of cinnamaldehyde in the solution had a significant effect on the bacterial counts after washing; thus, we anticipate that cinnamaldehyde treatment may lead to a possible leafy green post-harvest wash solution.**

INTRODUCTION

Doctors widely regard a balanced diet with multiple servings of fresh fruit or vegetables each day to be essential to a healthy life (1). Fresh produce contains necessary vitamins and other nutrients that the human body cannot produce on its own. Despite the innumerable health benefits of eating fresh fruits and vegetables, a growing concern over produce safety prevents people from getting the nutrients they need (2). According to the Center for Disease Control (CDC), approximately 76 million cases of illness are caused by foodborne pathogens in the United States annually, resulting in 325,000 hospitalizations and 5,000 deaths (3). Contaminated produce is a leading carrier of foodborne illnesses, contributing to approximately 46.9% of total cases (4). Produce can become contaminated in numerous ways, including pathogens living in the soil, contaminated irrigation water, improperly composted manure used as fertilizer, and poor worker hygiene during processing or packaging (5). Leafy greens, especially romaine lettuce, are responsible for between 10% and 40% of produce-linked cases, likely because consumers often eat leafy greens raw, eliminating the cooking step that contributes to microbial safety (5, 6). Additionally, romaine lettuce grows in an open leaf formation, which causes dirt and manure to contaminate a greater area of the plant than closed-head iceberg lettuce.

According to the CDC, *Escherichia coli* is a species of bacteria that commonly lives in the intestines of humans and warm-blooded animals. Most strains are harmless and aid in digestion, but some strains are pathogenic (7). In the United States, *E. coli* causes over 265,000 cases of illness yearly, though a large number of cases go unreported due to patients not seeking medical treatment for mild cases (8). Leafy greens are a common culprit for *E. coli* outbreaks because greens are often eaten raw and can be exposed to this pathogen at multiple points in the growing and processing system.

The food processing industry has resorted to using chemical cleaners at numerous stages of the food supply chain to reduce the risk of food contamination. Sanitization of food-contact surfaces, produce wash water, and the produce itself minimizes the risk of contamination (10). Chlorine, often used in the forms of sodium hypochlorite or calcium hypochlorite, is most commonly used for its low price and its broad spectrum of effectiveness against numerous microorganisms (11). Sodium hypochlorite, commonly marketed as liquid bleach, is used in concentrations ranging from 200 parts per million (ppm) for sanitization to over 2400 ppm for complete disinfection. Despite the advantages of using chlorine-based cleaners, its effectiveness as a disinfectant rapidly decreases with increasing organic load and is highly pH-dependent (12). In addition to only being effective in limited situations, chlorine reacts with the natural organic material in the wash water, creating chlorination byproducts that are both carcinogenic and mutagenic(11, 13).

Recently, a growing consumer preference towards organic produce has emerged (14). The United States Department of Agriculture (USDA) requires that for a food to be marketed as organic, both the ingredients and the methods used for growing and processing must conform to strict regulations (15). As part of these regulations, the National List of Allowed and Prohibited Substances states that the “residual chlorine levels in the water in direct crop contact or as water from cleaning irrigation systems applied to soil must not exceed the maximum residual disinfectant limit under the Safe Drinking Water Act," which is currently 4 mg/L expressed as Cl_2 (16). Chlorine, when used as a sanitizer for fresh-cut fruit and vegetables, is commonly applied in concentrations ranging from 50-200 ppm (10). One ppm equals approximately 1 mg/L (17), so the lowest concentration of chlorine used as a produce disinfectant cannot be used for organic produce because it does not comply with the Safe Drinking Water Act. Organic produce must meet the same standards of microbial safety as produce farmed with other methods but without chemicals such as chlorine that do not comply with USDA standards.

Researchers are looking for plant-based solutions to meet the growing consumer demand for safe produce that meets organic standards. Spices, essential oils, and plant extracts are currently being evaluated for their antimicrobial properties (18, 19). These plant-based substances conform to USDA standards and could be used as sanitizers or antimicrobial additives in organic foods (16).

Cinnamaldehyde, also known as trans-cinnamaldehyde or 3-phenylpropenal, is an organic compound produced by the plant genus *Cinnamonum* (20). The primary antibiotic pathway of cinnamaldehyde is through disruption of the bacterial cell membrane (21). Cinnamaldehyde contains a six-carbon phenol group that allows it to pass through the phospholipid bilayer of bacterial cells and bind to proteins inside the cell, disrupting necessary functions (22). This disruption of cell proteins causes irreversible membrane damage through acidification and protein denaturation of the cell membrane, leading to cell death (21). Cinnamaldehyde is a commonly used food additive because it is the chemical compound that gives cinnamon its characteristic flavor and smell (22). In addition to its use in the food industry, cinnamaldehyde gives a spicy aroma to perfumes, soaps, and toothpaste in the fragrance and personal care industries (20). Because of its current use as a flavoring and aromatic additive, the industrial infrastructure already exists to transition the use of this chemical towards becoming a mass-produced sanitizer. It is generally recognized as safe by the Food and Drug Administration (FDA) for human consumption. (23). Cinnamaldehyde is a promising antibacterial agent that is both naturally based and approved by the FDA as a food additive (23,24).

Because of this, cinnamaldehyde has the potential to be used as an antibacterial agent to help improve the microbial safety of raw produce. This research seeks to assess the effectiveness of cinnamaldehyde as a postharvest sanitizing treatment of romaine lettuce. We will test cinnamaldehyde solutions against chlorine-based sanitizers, which are the current industry standard, in addition to water as a control. An observed decrease in bacteria growth after treatment by an optimal concentration of cinnamaldehyde will lead to a possible leafy green postharvest wash solution. If the optimal concentration of cinnamaldehyde decreases *E. coli* in the treated leafy greens, this will decrease contamination measured by colony-forming units (CFUs). Using the spread plate technique, samples from the surface of lettuce inoculated with *E. coli* and treated with various solutions will be incubated on agar plates. Cinnamaldehyde has been shown in this research to have an effect on the survival rate of *E. coli* on lettuce equal to or greater than that of bleach.

RESULTS

We evaluated cinnamaldehyde in various concentrations to assess its ability to reduce *E. coli* growth on leafy greens. We treated romaine lettuce samples with either a cinnamaldehyde or control solution (water or 100 ppm bleach), then we analyzed both the leaf surface and washing solutions for the presence of remaining *E. coli*. We created bar graphs to represent the average results from both the wash water and swab tests. The first graph represents the average CFU/mL (y-axis) of each test group (x-axis). Colony counts from the samples treated with water were too many to count reliably, which is represented as "TMTC". The second graph represents the number of colonies counted from each swab sample. Note that this number is labeled in colonies because we could not determine the CFU/mL based on the nature of the test. The untreated samples (none group) had colony counts that were too high to be counted reliably and are labeled as "TMTC".

After statistically analyzing the results of both the swab and wash water samples, several points became clear. In the wash water testing, we observed a statistically significant difference between the samples of test groups treated with 100 ppm of chlorine bleach and samples of test groups treated with varying concentrations of cinnamaldehyde (**Table 1,** F = 718238.35, p < 0.00001). Samples treated with 0.2% cinnamaldehyde had an approximately 2.25 x 10^4 greater reduction of E. coli when compared to the bleach test group **(Figure 1, Table 2)**. Samples treated with either 0.5% or 1.0% cinnamaldehyde experienced the most substantial reduction of bacteria, with no visible colonies observed when plated. The concentration of *E. coli* was statistically significantly lower in all cinnamaldehyde concentrations when compared to the bleach group, suggesting that cinnamaldehyde in any concentration is more effective than bleach at destroying

Source	*Sum of Squares (SS)*	*Degrees of Freedom (df)*	*Mean Square (MS)*	
Between Groups	1939433676	3	646477892.1	*F = 718238.4*
Within Groups	15301.5	17	900.0882	*P < .00001*
Total	1939448978	20		

Table 1: Wash water samples one-way ANOVA. Samples from the leftover treatment solution were evaluated to determine the presence of E. coli. There was a significant difference found between the results of the test groups studied. Significant values are indicated in blue text.

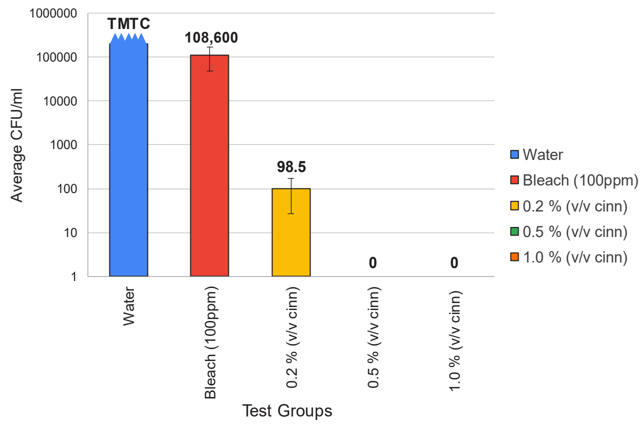

Figure 1: Average *E. coli* CFU isolated from wash water of inoculated lettuce treated with cinnamaldehyde or controls. The concentration of *E. coli* was statistically significantly lower in all cinnamaldehyde concentrations when compared to the bleach group (p < 0.00001). Columns sharing letters are not statistically different (column 4: c, and column 5: c are not statistically different). The broken line on the first column denotes a CFU that was too high to reliably count based on the dilution series performed.

any remaining bacteria left in the wash water (p < 0.00001, **Table 2**). Additionally, the 0.5% and 1.0% treatment groups significantly reduced *E. coli* more than the 0.2% treatment group (p = 0.00029, **Table 2**). Thus, increasing concentrations of cinnamaldehyde show greater effectiveness against *E. coli*.

However, the results of the swab test were less clear. The average number of colonies counted from each swab test yielded different results than the wash water tests. The 0.2% cinnamaldehyde solution was the least effective of the five tests, followed by water and bleach **(Figure 2)**.

Trials	Mean Values	Honest Significant Difference (HSD)	Q Values
T1:T2	T1= 22600.00 T2= 98.67	22501.33	Q = 1718.77 (p < .00001)
T1:T3	T1= 22600.00 T3= 0.00	22600	Q = 1726.31 (p < .00001)
T1:T4	T1= 22600.00 T4= 0.00	22600	Q = 1726.31 (p < .00001)
T2:T3	T2= 98.67 T3= 0.00	98.67	Q = 7.54 (p = .00029)
T2:T4	T2= 98.67 T4= 0.00	98.67	Q = 7.54 (p = .00029)
T3:T4	T3= 0.00 T4= 0.00	0	Q = 0.00 (p < .00001)

Table 2: Wash water samples Tukey post-hoc. Samples from the leftover treatment solution were evaluated to determine the presence of *E. coli*. The concentration of *E. coli* was statistically significantly lower in all cinnamaldehyde concentrations when compared to the bleach group (p<0.00001). Significant values are indicated in blue text. Honest Significant Difference (HSD): used to determine if the relationship between two sets of data is statistically significant. T1: Bleach (100ppm); T2: 0.2% cinnamaldehyde; T3: 0.5% cinnamaldehyde; T4: 1.0% cinnamaldehyde.

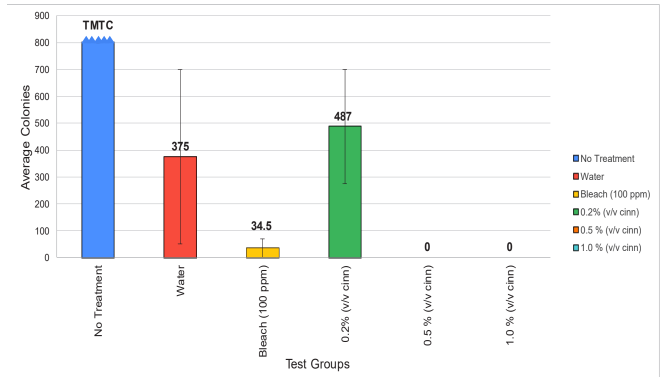

Figure 2: Average *E. coli* colony count isolated from inoculated lettuce leaves treated with cinnamaldehyde or controls. 0.5% and 1.0% cinnamaldehyde were the most effective sanitizers. Columns sharing letters are not statistically different (column 2: a, c, and column 3: b, c are not statistically different). The broken line on the first column denotes a CFU that was too high to reliably count based on the dilution series performed.

Samples treated with both 0.5% and 1.0% cinnamaldehyde yielded no colonies, which was significantly fewer than the other groups (F = 7.81205, p = 0.000395, **Table 3**). The bleach group had a significantly lower colony number than the 0.2% cinnamaldehyde group (p = 0.0077, **Table 4**). In addition, the results from the 0.5% and 1.0% cinnamaldehyde groups were significantly lower than the groups treated with water (p = 0.3385, **Table 4**) and groups treated with 0.2% cinnamaldehyde (p = 0.00387, **Table 4**). There was no meaningful difference between colony counts of the water and bleach groups, showing that higher concentrations of cinnamaldehyde are necessary to decontaminate produce effectively, but lower concentrations could be used to reduce microorganisms in the wash water. This data reinforces the conclusion that higher concentrations of cinnamaldehyde prove to be more effective sanitizers.

DISCUSSION

Serious problems for consumers and the food industry alike come from food-borne illnesses caused by leafy greens. Millions of cases of illness are caused by foodborne pathogens, with contaminated produce being the main

Source	*SS*	*df*	*MS*	
Between Groups	1240561.179	4	310140.2946	*F = 7.81205*
Within Groups	913105.5	23	39700.2391	*p = .000395*
Total	2153666.679	27		

Table 3: Swab samples one-way ANOVA. Swabs from the surface of each treated lettuce sample were evaluated to determine the presence of E. coli. There was a significant difference found between the results of the test groups studied. Significant values are indicated in blue text.

Trials	Mean Values	HSD	Q Values
T1:T2	M1 = 34.50 M2 = 375.00	340.5	Q = 4.03 (p = .06271)
T1:T3	M1 = 34.50 M3 = 487.00	452.5	Q = 5.35 (p = .00770)
T1:T4	M1 = 34.50 M4 = 0.00	34.5	Q = 0.41 (p = .99837)
T1:T5	M1 = 34.50 M5 = 0.00	34.5	Q = 0.41 (p = .99837)
T2:T3	M2 = 375.00 M3 = 487.00	112	Q = 1.32 (p = .87955)
T2:T4	M2 = 375.00 M4 = 0.00	375	Q = 4.44 (p = .03385)
T2:T5	M2 = 375.00 M5 = 0.00	375	Q = 4.44 (p = .03385)
T3:T4	M3 = 487.00 M4 = 0.00	487	Q = 5.76 (p = .00387)
T3:T5	M3 = 487.00 M5 = 0.00	487	Q = 5.76 (p = .00387)
T4:T5	M4 = 0.00 M5 = 0.00	0	Q = 0.00 (p < .00001)

Table 4: Swab samples Tukey post-hoc. Swabs from the surface of each treated lettuce sample were evaluated to determine the presence of *E. coli*. 0.5% and 1.0% cinnamaldehyde were the most effective sanitizers. Significant values are indicated in blue text.

contributor. Effective washing procedures minimize the risk of transmission of food-born illness to the consumer. The cinnamaldehyde produce wash tested in this research could be applied similarly to chemical sanitizing agents currently used in the produce processing industry. Treatment with chemical sanitizers typically includes mechanical washing while immersed in a sanitizing solution (12). After washing, excess moisture from the produce is removed through air or centrifugal drying, and the produce continues to the next step in processing (25). While the bacteria do need to be removed from the surface of the lettuce sample, high concentrations of surviving bacteria in the wash water pose a risk for cross-contamination when multiple items of produce are being washed in the same vat. Cinnamaldehyde has the potential as a post-harvest sanitizing wash because it is an effective sanitizer and is approved by the FDA for human consumption, making it a promising alternative to harmful solutions such as bleach. Based on the results of this experiment, we can draw three conclusions. Cinnamaldehyde effectively prevents cross-contamination by removing bacteria in wash water better than bleach. Cinnamaldehyde treatment has a significant effect on the survivability of *E. coli* on lettuce after washing. The concentration of cinnamaldehyde used in the solution significantly affects the success of the washing treatment against *E. coli*.

These conclusions support our original hypothesis that cinnamaldehyde is a successful inhibitor against *E. coli* when used in a produce wash. The wash solution tests suggest that cinnamaldehyde significantly affects the surviving number of *E. coli* on lettuce. Our results agree with previous studies examining the effects of cinnamon oil and cinnamaldehyde on microorganisms grown in a standard medium (28, 29). Tests using the agar well diffusion method have shown both cinnamon extract and oil to be effective against a variety of gram-positive and gram-negative bacteria, along with numerous species of fungi (28). In addition, cinnamon extract has been shown to have similarly effective results against *E. coli* in agar well studies (27). The data presented in this research study is in agreement with other current studies, but further studies with larger test groups are required to confirm this data.

Due to time and resource constraints, experiments performed in this research could only be repeated twice with a limited sample group. As a result of low experimental repetitions, leading to a small sample size, the results from the swab samples, in particular, may have been skewed. However, our small study indicates that cinnamaldehyde is an effective antibacterial agent at high concentrations as a produce wash for leafy greens.

MATERIALS AND METHODS

Preparation of Bacterial Culture and Lettuce Inoculation

Escherichia coli (strain K-12, Flinn Scientific) stock culture was transferred from the agar slant to tryptic soy broth and incubated at 37°C for 24 hours before use.

Romaine lettuce (*Lactuca sativa var. longifolia*) was purchased from a local grocery store within one day of the experiment. Individual lettuce leaves were separated from the head and washed with deionized water before being cut into 2.5 cm x 2.5 cm squares (average weight 0.36 g). Cut samples were then sprayed with ethyl alcohol and dried to eliminate background microorganisms. After being sterilized with alcohol, each lettuce sample was placed in a sterile 4 oz Whirl-Pak sample bag (Millipore-Sigma) and soaked in 15 mL of *E. coli* culture broth for 5 minutes. The lettuce squares were then dried in a sterile container for one hour to promote cell attachment and mimic real-world contamination.

Cinnamaldehyde Preparation

Cinnamaldehyde (Millipore-Sigma) was tested in concentrations of 0.2% v/v, 0.5% v/v, and 1.0% v/v. Treatment solutions were prepared by mixing the desired concentration of cinnamaldehyde and sterile water containing 0.5% Polysorbate 80 (as an emulsifier) via vortexing for 2 minutes. Polysorbate 80 is a non-ionic surfactant and emulsifier commonly used in food and cosmetics. Previous research on the antimicrobial effects of essential oil emulsions, including cinnamaldehyde, has shown that the addition of an emulsifier, namely Polysorbate 80, has no significant effect on antimicrobial action (29,30).

Washing Procedures

Inoculated lettuce samples were placed in sterile Whirl-Pak sample bags containing 15 mL of cinnamaldehyde treatment solution at a concentration of 0.2%, 0.5%, or 1.0% and soaked for 5 minutes. Each treatment concentration was tested twice. In addition, water and bleach (concentration of 100 ppm) were tested on two samples, each as positive controls. Both bleach and water have varying degrees of effectiveness at removing *E. coli* from solid surfaces. Two samples of lettuce were not treated with any solution and were used to determine the original cell count. Lettuce samples were dried in a sterile container for five minutes after washing.

Data was collected from both the lettuce samples and treatment solutions after the washing procedures took place. After washing, the surface of each lettuce sample was swabbed five times and transferred to a Petri plate of RAPID *E. coli* 2 growth medium (Bio-Rad), a chromogenic media used for enumeration of *E. coli* and other coliforms. The treatment solution leftover from washing was serially diluted up to 10^{-2} in tryptic soy broth, and each dilution series was plated onto the growth medium. All plates were incubated at 37°C for 24 hours before colony counting.

Graphs and data tables were all created through Google Sheets. Statistical analysis was done with a one-way Analysis of Variance (ANOVA) test. Post-hoc analysis was performed using Tukey's HSD test. An alpha level of 0.05 was used to determine significance.

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