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Evaluation of Household Sanitizers for Reducing Levels of *Salmonella typhimurium* on Iceberg Lettuce and Rocket Leaves

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ABSTRACT

The effect of various household sanitizers including water, water acidified with white vinegar (1.5%), vinegar and lemon juice were evaluated for their effectiveness in reducing counts of *Salmonella typhimurium* on rocket and lettuce leaves. Treating rocket and lettuce samples with water containing 1.5% vinegar for 30 min or washing with water (at 28°C) for 5 min showed negligible reduction in pathogen counts. Sanitizing rocket leaves with vinegar or lemon juice alone caused significant reductions in pathogen count of 2.3 and 2.5 $\log_{10}$ CFU/g respectively. A maximum reduction ranged from 3.1 $\log_{10}$ CFU/g to an undetectable level was recorded after dipping rocket leaves with high (5.8 $\log_{10}$ CFU/g) and low (4.0 $\log_{10}$ CFU/g) inoculums in a mixture of lemon juice and vinegar for 30 min exposure time respectively. A reduction of 2.4 was obtained when lettuce leaves with high load (5.1 $\log_{10}$ CFU/g) were dipped in vinegar-lemon mixture for 30 min. However all treatments; vinegar, lemon and their mixture significantly reduced the bacterium load on lettuce with low inoculum (4.0 $\log_{10}$ CFU/gm) to an undetectable level. Rocket and lettuce samples treated with vinegar, lemon or their mixture were analyzed for consumer acceptability by sensory evaluation using a 9-point hedonic scale. The results suggested that the use of these disinfecting treatments reduced pathogen loads on rocket and lettuce more effectively and did not negatively affect the important sensory characteristics of the plants.

INTRODUCTION

Vegetables are essential for good health, and they are a major source of vitamins, minerals, and dietary fiber and other phytonutrients including flavonoids, carotenoids and phenolic compounds that may lower the risk of cancer, heart disease and others illnesses [1,2]. Therefore, their consumption is encouraged in many countries by government health agencies. However, the number of gastroenteritis outbreaks caused by foodborne pathogens after consumption of raw vegetable salads and sprouts has increased worldwide due to the fact that fresh produce such as fruit and salads are often consumed as raw, putting consumers at risk of infection by contaminating organisms [3,4, 5]. Even though *Salmonella* is the most common cause of disease outbreaks associated with lettuce and sprouts [5, 6, 7, 8, 9, 10]. Other pathogens (Shiga toxin, producing *E. coli* O157, Norovirus) that have been described as relevant microbial hazards [11,12]. Fresh vegetables can become contaminated by pathogens as *Salmonella* at any point of production process. During preharvest, contact with contaminated irrigation water, soil, manure, or fecal matter of wild animals may occur. Also, asymptomatic human carriers might contaminate the products at the postharvest level and products may contaminate by other production process environments. Human pathogens can both bind to plant leaves and/or be internalized via the leaves or the endophytic root system [13,14]. The wash processes one of the first processing operations for ready-to-eat salads. Wash water containing 50–100 mg/L) of free chlorine is usually used to reduce microbial contamination in commercial procedures [15,16]. However, the limited efficacy of chlorine in reducing bacterial populations [17,18, 19] and its adverse effects such as formation of trihalomethanes have raised concerns by the consumers against chlorine [20]. Most of other sanitizers used in food industry would not be suitable for application at the household level [21] and in addition, consumers are increasingly avoiding to consume foods treated with preservatives of chemical origin and therefore natural alternatives are needed to achieve high degree of safety with respect to foodborne pathogenic microorganisms [22]. In this concept, researchers are trying new alternatives to disinfect fresh vegetables and fruits. Organic acids are naturally found in a variety of fruits and fermented foods and known to have bactericidal activity and additionally, they are generally recognized as safe (GRAS) [23]. Antimicrobial activity of acetic acid was shown against *Salmonella typhimurium* and *L. monocytogenes*[23, 24] whereas citric acid in the form of lemon juice has been demonstrated to reduce *S. typhimurium* populations on some fresh vegetables [25]. However, reports related to natural products namely lemon juice and vinegar are very limited [23, 24, 26]. In Libya, there is no

ready salad sold in supermarket but instead, leafy salads are prepared at home from raw vegetables and subjected to a simple and inexpensive sanitizing treatment that can be performed in consumer's homes directly prior to consumption. These treatments include washing with water acidified with vinegar or fresh lemon juice. To our knowledge, no previous study was conducted locally to assess these treatments. The objective of this study was to investigate the efficacy of household sanitizers usually used at homes such as acidified water, vinegar or lemon juice for inactivating *Salmonella typhimurium* on lettuce and rocket leaves and to determine any effects of such treatments on the consumer acceptability of these salad plants.

MATERIALS AND METHODS

Bacterial cultures and Preparation of inoculum

Salmonella typhimurium (local isolate) were kindly supplied by Biotechnological Researches Center, Tripoli-Libya. Suspensions of *S. typhimurium* was prepared by transferring stock culture of the tested bacteria to 1L-Erlenmeyer flasks containing 500 ml of brain heart infusion broth (Oxoid, Basingstoke, England). Cultures were left to grow aerobically at 37^0C with agitation (200 r/m). The cells were harvested by centrifugation (3550 X g for 10 min at 4^0C) and washed once in sterile maximum recovery diluent (MRD; Oxoid). The cell pellet was finally resuspended in MRD to give a suspension containing *ca.*10^6 or 10^3colony forming unit/ml (CFU/ml).

Inoculation of rocket and lettuce leaves

Rocket (*Eruca vesicaria* L.*)* and Iceberg lettuce (*Lactuca sativa* L.) were purchased from a local supermarket on the day of experiment. The outer leaves and the core were removed from the lettuce heads. The remaining leaves were then rinsed with sterilized water for 1 min (*ca.* 28^0C). Intact and unwilted lettuce leaves were sliced (*ca*. 5.2 cm) using a sterile knife whereas rocket leaves were used as whole leave forms. Lettuce or whole rocket leaves were dipped into a suspension of *S. typhimurium* (10^7CFU/ml) for 20 min (in first experiment as described below) and placed on sterile cheese cloth for removing excess liquid then placed into an open container and allowed to dry for 2 hrs (ca. 28^0C) in a biosafety cabinet. The inoculated leaves were transferred to sterile container. In second experiment two levels of inocula were examined. For this, suspensions of *S. typhimurium* containing approximately 10^6 or 10^3CFU/ml for high and low level of inoculum respectively were used. Rocket or lettuce leaves was dipped into culture suspensions for 20 min. and after removing of excess

liquid, leaves were dried at room temperature (ca. 28^0C) for 2 hrs then transferred to sterile containers.

Preparation of sanitizers

Two experiments were conducted to evaluate the effect of disinfecting methods using natural disinfectants applied at household level, on removing *S. typhimurium* artificially attached to rocket and lettuce leaves. In first experiment; the efficacy of washing with water (sterilized distilled water at 28^0C), water acidified with vinegar (ca. 1.5%) or 200ppm NaOCl were examined. Soaking leafy salad vegetables in acidified water containing approximately 1.5% vinegar for 15 or 30 min is a common home treatment to clean and disinfect them before conception. Chlorinated water was prepared by adding sodium hypochlorite (NaOCl) solution containing ≥13% active chlorine (Merck, Darmstadt, Germany) to deionized water to obtain a solution with a concentration of 200 mg l^{-1} free chlorine. In second experiment; fresh lemon juice or vinegar alone or mixture of lemon juice and vinegar (1:1) were used as treatment solutions. Lemons (Citrus Lemon; *Eureka*) were purchased from a local supermarket and washed with tap water. After cutting lemons with a sterile knife, a household juice machine was used to squeeze lemons. Commercial pasteurized grape vinegar (Al-Tadamon brand, Benghazi, Libya) was used directly. Lemon juice and vinegar mixture was prepared at a ratio of 1:1 under aseptic conditions. Acetic acid (titratable acidity) of vinegar and acidity of lemon juice (as citric acid) or mixture of lemon juice and vinegar (as acetic acid) were determined by using the volumetric method as described by Kirk and Sawyer [27].

Treatment of vegetables

In first experiment, to mimic house treatments, inoculated lettuce or rocket leaves were dipped into 1.5 l of distilled water for 30 min or distilled water containing vinegar (1.5% for 30 min) or into a solution of NaOCl (200 ppm for 2min; ca. 28^0C). The latter treatment was used for comparison as it is the common treatment used in commercial fresh vegetable salad industry. In second experiment; for each treatment, inoculated leaves of rocket or lettuce were dipped into appropriate amounts of treatment solution including vinegar or lemon juice alone or mixture of vinegar and lemon for 15 or 30 min (at ambient temperature: ca. 28^0C).In all experiments, after dipping, samples were removed aseptically and washed thoroughly three times with sterilized distilled water to remove the disinfectants and drained on sterile cheese cloth for 5 min. Inoculated untreated rocket and lettuce leaves samples were used as control.

Microbiological analysis

A 25g of sample from each dipping treatment was aseptically transferred into a sterile stomacher bag then macerated through a stomacher (VWR Star-Blender.LB400- UK). Serial dilutions of each homogenized sample were made in MRD. Duplicate 0.1-ml samples were spread plated on Bismuth Sulphite Agar (BSA, Merck, Germany) and plates were incubated at 37^0C for 48 hrs. Randomly selected presumptive *S. typhimurium* colonies were confirmed using biochemical tests [triple sugar iron agar (TSI, Oxoid) and lysine iron agar (LI, Oxoid) reactions and serological tests [28]. To determine the possible presence of *Salmonella* spp. on uninoculated rocket and lettuce leaves samples, 25 g of each plant was transferred to 225 ml of buffered peptone water (BPW, Oxoid) in a stomacher bag and homogenized in a stomacher for 1 min at medium speed. Homogenates of each sample in BPW were incubated at 37 ^{0}C for 24 hrs. and then 1 ml was transferred to selenite cysteine broth (SC, Oxoid) and tetrathionate brilliant green broth (TT, Oxoid) and incubated at 37^0C (SC) and 43^0C (TT) for 24 hrs. Cultures were streaked onto bismuth sulfite agar (BSA, Oxoid) and xylose lysine desoxycholate agar (XLD, Oxoid) then incubated at 37^0C for 24hrs and examined for presumptive *Salmonella* colonies [28]. Pre-enrichment step for 4 hrs [29] in BPW was conducted before plating the sample on BSA for the recovery of acid injured *Salmonella* cells and no significant differences between the enriched and non-enriched vegetable samples was found therefore enrichment steps have been ignored in the further experiments.

Sensory analysis

Sensory analyses of sanitizing treatments were performed to evaluate the consumer acceptability of treated rocket and lettuce leaves. The lettuce and rockets sanitization treatments used for the sensory analysis were as follows: vinegar alone, lemon juice alone or mixture of vinegar and lemon juice (1:1) and each treatment was conducted for 15 and 30 min. and untreated samples was used as control. Lettuce and rocket samples from each treatment were rinsed three times with sterilized distilled water and served in small bowls labeled with random codes. Testers (30 persons) were received an explanation of the study and evaluated the appearance, texture, taste, and overall acceptability of treated lettuce or rocket leaves. Testers were given one sample at a time along with drinking water and encouraged to rinse their mouths between samples. All treatments were carried at room temperature (ca. 28^0C). A nine-point hedonic scale was used for evaluation (1 = dislike extremely, 5 = neither like nor dislike (midpoint), and 9 = like extremely).

Statistical analysis

Data were subjected to analysis of variance and Duncan's multiple tests (SAS Institute, Inc.) to determine if significant differences in populations of *S. typhimurium* existed between mean values. Statistical analysis of sensory evaluation was conducted for each sensory attribute and scores for the treated samples were subjected to analysis of variance and the least significant difference test. Level of significance at $P \leq 0.05$ significance level was used for comparisons.

RESULTS AND DISCUSSION

Antimicrobial effect of washing using acidified water (vinegar 1.5%) or chlorine (200 ppm) against *S.typhimurium* on lettuce and rocket leaves was investigated. The results are presented in Table 1. The initial population of *S.typhimurium* on rocket and lettuce leaves was 5.8 and 6.8 $\log_{10}$CFU/gm respectively. Dipping of rocket and lettuce into 200ppm NaOCl solution for 2 min reduced the number of viable *S.typhimurium* on plant samples by 0.1 $\log_{10}$CFU/gm although this reduction was not significant. However, no significant reduction was recorded when inoculated plants were subjected to dipping into water containing 1.5% vinegar for 30min or soaking in water (ca. 28^0C) for the same period. These results are consistent with the data reported by Akbas and Olmez [23] and others [15,21] who found that water containing 50–200 ppm of chlorine resulted in a reduction in bacterial populations of less than 2 $\log_{10}$CFU/gm for fruits and vegetables.

Table 1: Efficacy of household and chlorine dip washing treatments on reducing *S.typhimurium* populations on artificially inoculated rocket and lettuce samples

*Treatment	Contact time (min)	*S.typhimurium* on rocket		*S.typhimurium* on lettuce	
		Log_{10} CFU/g		Log_{10} CFU/g	
			Reduction		Reduction
Control (untreated)	-	5.80 A ± 0.7		6.81A ±0.08	
Sterilized distilled water	30	5.30 A ± 0.7	0.50	6.05 A ±0.05	0.67
Vinegar (1.5%) (Acetic acid: 0.086%)	30	5.67 A ± 0.3	0.13	7.41A ±0.02	NR
200 ppm NaOCl	2	4.80 A ± 0.3	1.0	5.80 A ±0.00	1.0

*All disinfectants at around 28^oC. Values are the mean of three replicates and reported as $\log_{10}$ CFU/g ± standard deviation. NR: no reduction. Values with the same letter in the same column are not significantly different ($P>0.05$).

Effectiveness of vinegar and fresh lemon juice on *S. typhimurium* attached to rocket and lettuce

Inoculated rocket and lettuce samples were washed with sterile water in order to differentiate washing effect of treatment solution, and minor reduction (~0.5 log reduction) in the population of *S. typhimurium* was reported. *Salmonella* was not detected in uninoculated plant samples. The efficacy of treatments with lemon juice (7.78% citric acid), vinegar (5.79% acetic acid) and their mixture (8.57% acetic acid) on *S. typhimurium* attached to whole rocket leaves, is illustrated in Table 2. All treatments were carried out at room temperature and at different exposure times (15 or 30 min). Regarding the effect of vinegar containing (5.79% v/v) acetic acid on *S. typhimurium* contaminated rocket leaves, results showed that there was a significant ($P \leq 0.05$) reduction in pathogen count of 2.3 and 2.1 $\log_{10}$CFU/g at high inoculum level after 15 and 30 min exposure respectively compared to control (untreated rocket) (Table 2).In low inoculum level, reductions of 0.51 and 1.82 $\log_{10}$ CFU/g were achieved after dipping rocket in vinegar for 15 and 30 min treatment respectively. A significant reduction (3.03 $\log_{10}$ CFU/g) was reported by dipping rocket leaves (with high inoculums) in vinegar for 30 min. However, maximum antimicrobial effect (significant; $P \leq 0.05$) was obtained by treating rocket leaves (with low inoculums) with lemon juice and vinegar mixture for either 15 min (reduction; 3.44 $\log_{10}$ CFU/g) or 30 min as the latter reduced pathogen number to an undetectable level(<0.7$\log_{10}$CFU/g; for low inoculums;). Although the count of S. *typhimurium* on low inoculated rocket samples was reduced to an undetectable level after 30 min treatment with lemon juice vinegar combination, no significant difference was observed between 15 and 30 min treatment times ($P>0.05$) (Table 2).

For lettuce leaves, Table 3 summarizes the most effective tested sanitizers. Dipping lettuce samples (with high inoculum) in vinegar for 15 or 30 min caused a population reduction of around 1 $\log_{10}$CFU/g. However, treating samples with fresh lemon juice for 15 or 30 min decreased population to 4.1 ±0.10 and 3.4 ±0.70 $\log_{10}$CFU/g respectively compared to control. When lettuce samples (at high inoculums) were disinfected by a mixture of lemon juice and vinegar (1:1), the initial populations were reduced significantly ($P \leq 0.05$) to 3.3 and 2.7 $\log_{10}$CFU/g after 15 and 30min treatment respectively. Interestingly, at low inoculums, all applied treatments (except distilled water) significantly dropped population of *S. typhimurium* on lettuce leaves to undetectable levels (<0.7 $\log_{10}$ CFU/g) (Table 3).

Table 2: Effectiveness of fresh lemon juice (7.78% v/v, citric acid), vinegar (5.79% acetic acid) in removing *S. typhimurium* on rocket leaves

*Sanitizing treatment	Contact time(min)	High inoculum		Low inoculum	
		Population Log_{10} CFU/g	Reduction Log CFU/g	Population Log_{10} CFU/g	Reduction Log_{10} CFU/g
Control (untreated)	-	5.80 A ± 0.00		4.03 A± 0.00	-
Distilled water	30	5.30 A ± 0.01	0.50	3.54 A ±0.02	0.49
Vinegar only	15	3.51 B ± 0.40	2.3	3.87 B ±0.09	0.51
	30	3.73 B ± 0.60	2.1	2.56 B ±0.19	1.82
Lemon only	15	3.61 B ± 0.20	2.2	3.50 B ±0.00	0.88
	30	3.27 B ± 0.40	2.5	1.35 B ±0.20	3.03
Vinegar + Lemon (1:1)	15	3.36 B ± 0.60	2.4	0.59B ± 0.01	3.44
	30	2.73 B ± 0.50	3.1	ND B	-

*Rocket leaves were dipped in culture suspensions containing 6 or 3 log CFU/ml at high and low inoculum levels. Attached cell numbers of *S. typhimurium* were 5.80 and 4.03 log_{10} CFU/g at high and low inoculum levels, respectively, ±Standard deviations. Rocket leaves were treated with vinegar, lemon or mixed of vinegar and lemon for either 15 or 30 min.ND: not detected.Values with common letters within the same column for the same treatment are not significantly different (P>0.05).

Table 3: Effectiveness of fresh lemon juice (7.78% v/v, citric acid), vinegar (5.79% acetic acid) in removing *S. typhimurium* on lettuce leaves

Treatment	Contact time (min)	High inoculum		Low inoculum
		Log CFU/g	Reduction	Log CFU/g
Control (untreated)	-	5.1 A ± 0.30	-	4.0 A ± 0.80
Distilled water	30	4.4 A ± 0.07	0.66	3.4 A ± 0.06
Vinegar alone	15	4.0 B ± 0.20	1.1	NDB
	30	4.1 B ± 0.10	1.0	ND
Lemon only	15	4.1 B ± 0.10	1.0	ND
	30	3.4 B ± 0.70	1.7	ND
Vinegar + Lemon (1:1)	15	3.3 B ± 1.30	1.8	ND
	30	2.7 B ± 0.30	2.4	ND

*Lettuce samples were dipped in culture suspensions containing 6.0 or 3.02 log_{10} CFU /ml cells at high and low inoculum level respectively. Initially attached cells were 5.1 and 4.0 CFU/g for high and low inoculum levels respectively. Inoculated samples were treated with lemon, vinegar or mixed with vinegar and lemon for either 15 or 30 min ND: not detected. ± Standard deviations. ND: not detected. Values with the same letters within the same column for the same treatment are not significantly different (P>0.05).

Similar finding was obtained by Sengun and Karapinar [25] who showed that treating rocket leaves with fresh lemon juice and vinegar caused a significant reduction in *S.typhimurium* population while the number of pathogens dropped to an undetectable level by using lemon juice–vinegar mixture for 15 min. Another study [26] showed that treatment of carrot samples with lemon juice or vinegar alone for different exposure times caused significant reduction in *S. typhimurium* reached up to 3.58 $\log_{10}$ CFU/g while the number of pathogens was reduced to an undetectable level after 30-min treatment by combined used of lemon juice vinegar. Other workers [30] obtained a 6-$\log_{10}$ reduction in *S. sonnei* when inoculated parsley leaves were treated with vinegar containing 5.2% acetic acid. Also, these results are consistent with results of Karapinar and Gonul [31] who achieved a 5-$\log_{10}$ reduction in *Yersinia enterocolitica* when inoculated parsley leaves were dipped in 2% acetic acid or 40% vinegar. Vijayakumar and Wolf-Hall [32] reported that 5-log reduction in *E. coli* counts was obtained on lettuce leaves treated with 35% white vinegar. This variation may be due to differences in the type and quantities of test microorganisms, the types of produce, and the methods of treatment.

Sensory evaluation

The sensory ratings of panelists are presented in Table 4. It is evident from the results that all treatments resulted in consumer ratings above the midpoint, indicating acceptability. The results revealed that the evaluated sanitizing treatments reduced microbial loads on rocket and lettuce more effectively than water alone (control) without affecting the sensory attributes of these plants as no significant differences between samples were reported.

Table 4: Sensory responses for seven rocket or lettuce samples subjected to different sanitization treatments

Treatment	*Score for attribute							
	Appearance		Taste		Texture		Overall acceptability	
	R	L	R	L	R	L	R	L
Control	8.3A±0.55	7.3A ±1.4	8.0A±0.47	7.4A±0.62	8.3A±0.39	8.3A±0.5	8.0A±0.60	7.6A ±1.2
Vinegar								
15	7.3A±0.94	6.3A ±1.2	7.4A±0.95	7.2A ±1.2	6.9A±0.79	7.4A±0.6	6.6A±0.82	7.3A ±1.1
30	6.7A±0.48	6.3 A±1.0	7.2A±0.93	6.8A ±1.4	6.8A±0.46	6.6A±1.1	6.4A±0.62	6.8A ±1.4
Lemon								
15	7.7A±0.48	8.1A ±0.5	6.8A±0.53	6.4A ±1.1	7.4A±0.49	7.0A±1.3	7.7A±0.38	6.8A ±1.3
30	6.5A±1.16	6.9A ±0.6	6.6A±1.29	6.3A ±1.0	7.0A ± 0.8	7.0A ±0.6	6.7A ±1.18	6.5A±0.95
Mixture: vinegar and lemon (1:1)								
15	7.3A±1.19	7.0A ±0.9	6.5A ±1.1	5.4A ±1.2	6.9A±1.20	6.5A ±1.2	6.9A±1.26	5.6A ±1.4
30	7.4A±0.73	6.8A ±1.2	5.9A±1.2	4.9A ±1.6	7.4A±0.78	6.5A ±1.0	7.4A ±1.16	5.5A ±1.5

*Hedonic values are based on a nine-point scale (9 = like extremely, 5 = neither like nor dislike, and 1 = dislike extremely) for each attribute. Values presented are means for 30 responses. Values with the same letter in the same column are not significantly different. R: rocket. L: lettuce.

CONCLUSION

It is evident from the results of this study that the household sanitizing agents such as vinegar and fresh lemon can be an effective method for eliminating viable *S. typhimurium* on fresh rocket and lettuce effectively without affecting the sensory attributes of the lettuce severely. Therefore, these effective sanitization methods could be recommended to consumers as a step to reduce the risk of foodborne illness.

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