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Efficacy of citrus-based disinfectants to inhibit growth, swarming, and biofilm formation of *Salmonella* and decontaminate parsley

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Abstract

Biofilms allow bacteria to adhere to biological or nonbiological surfaces and are difficult to remove, whereas swarming enables the rapid colonization of nutrient-rich environments. In this study, the efficacy of six commercial citric-based antimicrobial formulations to control growth, biofilm production, and swarming of *Salmonella* was determined. Furthermore, the efficacy of Citrik Agro® to disinfect contaminated parsley was established. Minimum bactericidal concentrations (MBCs) of the disinfectants against five *Salmonell*a strains were evaluated by the microplate-dilution method. For the swarm motility test, subinhibitory concentrations of the disinfectants were mixed with Luria–Bertani agar. Biofilm formation was quantified in microplates with broth after staining with safranin. Parsley was artificially contaminated with *Salmonella*, then washed with Citrik Agro and the presence of *Salmonella* was determined after several days. The MBCs of the disinfectants ranged from 81 to 922 µg/ml. Citrik AB was the most effective inhibitor of *Salmonella* growth (MBC: 81–105 µg/ml). Most disinfectants inhibited biofilm formation at 75% of the MBC, and a reduction was observed at lower concentrations. However, Citrik AB inhibited biofilm formation even at 25% of the MBC, and it also produced a higher ($P<0.05$) swarming reduction (75%) when 75% of the MBC was used, compared to the other disinfectants. In addition, Citrik Agro reduced more than 2 logs of *Salmonella* in parsley. This reduction was higher ($P<0.05$) than that observed by the chlorine treatment. In conclusion, citric extract-based products could be a natural alternative to reduce the risk of *Salmonella* contamination in fresh produce.

Key words: *Salmonella*, fresh-greens, parsley, citric extracts, disinfectants.

Introduction

Salmonella is one of the most prominent bacterial pathogens that causes substantial morbidity, mortality, and disease burden globally. In the US, *Salmonella* is responsible for an estimated one million cases of disease and 19,586 hospitalizations annually[1].

Biofilms are structured surface-associated communities of growing bacteria enclosed in an amorphous, extracellular matrix that adhere to biological or nonbiological surfaces and are difficult to remove [2, 3]. Attachment and biofilm formation by *Salmonella* on different food surfaces, such as plastic, glass, stainless steel, silicone, and polycarbonate, increase resistance to environmental stresses, and some cells may not be eliminated during cleaning and disinfection procedures [2, 3]. For this reason, the most effective disinfectants against bacterial cells in suspension may not be as effective when treating bacterial cells embedded in a biofilm [4].

Swarming is the fastest known bacterial mode of surface translocation and enables the rapid colonization of nutrient-rich environments [5]. These motilities contribute to the formation of biofilms [6]. In the laboratory, when *Salmonella* is propagated on glucose-supplemented nutrient-rich semisolid medium, it undergoes morphological differentiation into swarmer cells, rendering them physically capable of active surface migration [7].

Several outbreaks associated with consumption of *Salmonella*-contaminated leafy greens have been documented [8]. Fresh parsley (*Petroselinum crispum*) has been identified as a vehicle of transmission of enteropathogenic bacteria [9, 10]. Biofilm formation also plays a role in the persistence of *Salmonella* after chlorination treatment of parsley [11]. Since leafy greens are regularly consumed fresh, and current decontamination methods are not always effective, alternative strategies for their decontamination are required.

Washing procedures for produce are devised to remove soil and to decrease acquired microbial contamination in the field during growth and harvesting. They also help to prevent cross-contamination between batches. Synthetic compounds added to the washing solution have been widely used by the food industry to reduce microbial contamination; however, in recent years, consumer trends have shifted toward using fewer synthetic food additives so that products can be consumed in a more natural or all-natural state [12].

Various natural antimicrobials based on citric extracts are commercially available, but little information is available regarding their effect on biofilm formation and swarming of *Salmonella*. In this work, the efficacy of six commercial citric-based disinfectants used in agriculture, livestock, plastics, and food industries against growth, biofilm formation, and swarming of *Salmonella* was determined. Furthermore, the efficacy of one of these citric-based

formulations to disinfect parsley that was artificially contaminated with *Salmonella* was established.

Material and Methods

Bacterial cultures: *Salmonella* Typhi ATCC 19430 and *Salmonella* Typhimurium ATCC 14028 were originally obtained from the American Type Culture Collection (ATCC; Manassas, VA). The following bacterial strains were isolated in our laboratory: *Salmonella* Muenchen M59410 (from chicken feces), *Salmonella* E1 monophasic (from parsley), and *Salmonella* spp. (from raw chicken). These strains were identified using the Biolog System (Hayward, CA), confirmed by polymerase chain reaction (PCR), and serotyped at Mexico's National Institute of Epidemiological Diagnosis and Reference (INDRE). All strains were stored at -80°C in Brain Heart Infusion (BHI) broth (Difco) containing 20% (v/v) glycerol. Active cultures were prepared by inoculating an aliquot in BHI broth and were incubated at 37°C for 48 h. An aliquot of this was spread onto Mueller-Hinton (MH) agar (Difco) and incubated at 37°C for 24 h. Colonies were suspended in saline solution and adjusted to 1.5×10^8 CFU/ml.

Citric-based antimicrobials used: In this work, six commercial disinfectants were used. Citrik AB liquid and Citrik AB powder are used for general use in the food industry, Citrodex is used for surface disinfection, Citrol K Ultra is used for general use in the food industry, Citrik Max is used for livestock use, and Citrik Agro is used for disinfection of produce and agricultural devices and facilities. All disinfectants were obtained from Corpo Citrik, S.A. de C.V., Mexico. According to the technical specifications, these formulations are based on citric extracts with "generally recognized as safe" (GRAS) status.

Minimum bactericidal concentration (MBC): Sterile 96-well polystyrene U-microtiter plates (BD Falcon, San José, CA) were filled with 50 µl of 2× MH broth (Difco) plus 50 µl of different concentrations of the commercial products (diluted in distilled water) to be tested [13, 14]. The plates were inoculated with 1 µl (1% v/v) of the activated culture of each strain or a pool of five *Salmonella* strains (1.5×10^8 CFU/ml) and incubated at 37°C for 24 h. After incubation, the content of each well was plated on MH agar and incubated at 37°C for 48 h. The MBC was regarded as the lowest concentration of the extract that prevented any visible bacterial colony growth (total absence of colonies) on the MH agar plate after the 48 h incubation period. Separate experiments were conducted to determine the effect of disinfectants (at their MBC) on the pH of MH broth.

Quantitative biofilm assay: Sterile 96-well polystyrene U-microtiter plates (BD Falcon) were filled with 150 µl of commercial products at various concentrations diluted in distilled water (100, 75, 50, 25 or 0% of the MBC, final concentration) plus 150 µl of 2× trypticase soy broth containing 2% glucose and 2% sodium chloride. The media was inoculated with 3 µl of an active *Salmonella* culture (1.5×10^8 CFU/ml). The plates were incubated for 24 h at 37°C. After that, the culture was removed, and the plate was washed twice with distilled water and left to dry for 24 h. Then, 200 µl of 0.1% safranine was added to the wells and incubated for 3 min. The plate was washed twice, 200 µl of 95% ethanol was added, and the absorbance (492 nm) was recorded [15]. Medium with each disinfectant at various concentrations and medium alone were used as controls.

Biofilm formation was quantitated by determining the biofilm formation index (BFI) obtained from the formula: BFI = (AB – CW)/G [16], where AB is the optical density of the stained attached microorganism, CW is the optical density of the stained control wells containing microorganism-free medium only, and G is the optical density of the cell growth in suspended culture. According to the BFI values, biofilm formation was considered strong (>1.10), moderate (0.70–1.09), weak (0.35–0.69), and no biofilm formed (<0.35).

Effect on swarming: Swarming plates were used to assay the ability of the disinfectants to inhibit cells to spread over the agar surface. Disinfectants at various concentrations (75, 50, 25 and 0% of their respective MBCs) were mixed with presterilized liquid Luria Beltrani agar (0.5% agar). To promote swarming motility, glucose was added to a final concentration of 0.5% and poured into a Petri plate. After sitting at room temperature, 5 µl of an active culture (1.5×10^8 CFU/ ml) was added to the center of the solidified plate and incubated for 18–24 h at 37°C. Finally, the diameter of the colony was measured, compared with the control (0% disinfectant) and the percent of reduction determined.

Decontamination of parsley: From the disinfectants analyzed, Citrik Agro was chosen in this assay because its intended use is to disinfect vegetables. To assess the effectiveness of Citrik Agro at decontaminating parsley, the method described by Foley *et al.* [17] was performed with minor modifications.

Preparation of inocula : A loopful of each active strain grown on BHI agar was inoculated into separate tubes with 10 ml of tryptic soy broth (TSB, Difco) and was incubated at 37°C for 24 h. Cells were collected by centrifugation (3000 × *g*, 10 min at room temperature), the pellet was resuspended in 1% peptone water, and the concentration was adjusted to 1×10^5 CFU/ml. An aliquot (1 ml) of each strain suspension was combined, and the suspension was used to inoculate parsley.

Inoculation of parsley: Parsley was purchased from local markets and stored at 4°C for a maximum of 24 h before use. To determine the initial *Salmonella* spp. content of parsley, samples were subjected to microbiological analyses (presence of *Salmonella* spp.) according to US-FDA Bacteriological Analytical Manual protocols [18]. If *Salmonella* spp. was detected in the samples, the assays were discarded.

For the assays, parsley samples were cleaned with gently washing using running tap water followed by gently rinsing in sterile distilled water and drying for 2 h in a class II biosafety cabinet (Labconco, Kansas City, MO) at room temperature. Cleaned parsley (60 g) was submerged into 60 ml of strain suspension (1×10^6 CFU/ml) for 10 min at room temperature in a biosafety cabinet with occasional mixing using a sanitized magnetic stirring bar. The inoculated parsley was then allowed to dry for 2 h at room temperature in a biosafety cabinet.

Effectiveness of Citrik Agro as a decontamination agent: Inoculated parsley samples were submerged for 15 min in a container with 90 ml of Citrik Agro at the MBC (928 ± 41.3 µg/ml).

Chlorine (200 ppm) served as the positive control. Unrinsed and water-rinsed parsley were also used as controls. The entire parsley sample was transferred to sterile bags and stored at 4°C for 7 days.

Parsley samples were removed at days 0, 1, 5, and 7, and the presence of *Salmonella* was determined. For this, a 10-g sample was placed in a container with 90 ml of 0.1% peptone water. After homogenization, decimal dilutions were made, and the dilutions were spread plated in duplicate on XLD agar (for *Salmonella,* Difco). These plates were incubated at 37°C for 24–48 h. Pink colonies with or without black centers in XLD agar were enumerated.

Statistical analysis: All experiments were done twice, and all samples were performed at least in triplicate. Efficacy and comparisons between treatments were analyzed by SPSS 17.0 (SPSS Inc.; Chicago, IL).

Results

In the experiments conducted here, all the *Salmonella* strains had similar susceptibilities to the natural disinfectants tested (Table 1). Although at different concentrations, all the disinfectants decreased the viability of *Salmonella* to undetectable levels. The MBC of the various disinfectants ranged from 81 to 922 µg/ml (Table 1). Citrik AB powder was the most effective at inhibiting growth of the five *Salmonella* strains (MBC: 81–105 µg/ml), and Citrodex had the highest MBC (855–995 µg/ml). All disinfectants (at concentrations lower than the MBC) inhibited biofilm formation. Most disinfectants completely inhibited biofilm formation at 75% of the MBC (Table 2), and a reduction was observed at lower concentrations. However, Citrik AB liquid inhibited biofilm formation even at 25% of the MBC.

Swarming was reduced by all disinfectants at concentrations lower than the MBC (Table 2). Citrik AB also produced the highest swarming reduction when 75% of the MBC was added to the treatments. At that concentration, the other disinfectants showed less swarming reduction, but it was always higher than 50%.

Citrik Agro is intended to disinfect vegetables, seafood, and fresh meats. The MBC of Citrik Agro against a pool of five *Salmonella* strains was 928 ± 41.3 µg/ml. This concentration was slightly lower than the recommended dose suggested by the manufacturer to disinfect produce (1000 µg/ml). This compound reduced more than 2 logs of *Salmonella* in parsley (Table 3). This reduction was higher ($P<0.05$) that that observed by the chlorine treatments.

Discussion

Many chemicals and intervention measures have been examined for their effectiveness to eliminate pathogenic bacteria from different food products. Chlorine is the most common chemical used in the food industry for disinfecting fresh produce [19]; however, a chlorine wash cannot completely remove or inactivate

Table 1. Minimum bactericidal concentration (MBC) of various disinfectants against five strains of *Salmonella.*

Disinfectant	MBC (µg/ml)				
	Salmonella E1 monofasica	*Salmonella* Typhi ATCC 19430	*Salmonella* Muenchen M59410	*Salmonella spp.*	*Salmonella* Typhimurium ATCC 14028
Citrik AB® Liquid	81.2 ± 23.9	102.5 ± 40.9	122.5 ± 38.4	116.2 ± 22.8	106.2 ± 4.7
Citrik AB® Powder	81.6 ± 24.6	81.6 ± 24.6	95.5 ± 0.5	105.3 ± 21.9	103.5 ± 12.7
Citrodex®	855.0 ± 63.6	900.0 ± 0.0	995.0 ± 7.0	990.0 ± 14.1	900.0 ± 141.4
Citrol K Ultra®	147.5 ± 2.8	150.0 ± 7.0	148.3 ± 2.5	148.3 ± 12.9	136.6 ± 19.4
Citrik Max®	154.0 ± 23.7	137.7 ± 32.0	152.7 ± 25.3	161.1 ± 18.2	173.0 ± 21.9
Citrik Agro®	917.5 ± 35.0	885.0 ± 131.2	877.5 ± 126.5	900 .0 ± 81.6	922.5 ± 93.2

± standard deviation.

Table 2. Biofilm formation index (BFI) and % swarming reduction (SR) of *Salmonella* strains as a result of exposure to disinfectants.

Disinfectant	% MBC	*Salmonella E1* monophasic		*Salmonella* Typhi ATCC 19430		*Salmonella* Muenchen M59410		*Salmonella* spp.		*Salmonella* Typhimurium ATCC 14028	
		BFI	% SR	BFI	% SR	BFI	% SR	BFI	% SR	BFI	% SR
Citrik AB®	75%	0.00	75.7	0.02	75.7	0.05	67.1	0.02	66.2	0.01	70.1
Liquid	50%	0.00	69.2	0.01	75.6	0.01	64.2	0.00	55.9	0.03	69.3
	25%	0.04	59.2	0.28	75.2	0.04	53.7	0.05	54.1	0.06	46.6
Citril AB®	75%	0.00	74.4	0.00	74.5	0.00	62.4	0.00	67.5	0.00	73.2
Powder	50%	0.00	72.6	0.00	74.2	0.00	60.0	0.03	60.6	0.00	57.8
	25%	0.29	63.1	0.82	68.2	0.74	59.2	0.53	64.8	0.60	48.9
Citrodex®	75%	0.00	72.0	0.00	72.3	0.00	69.9	0.00	56.8	0.10	72.3
	50%	0.00	70.5	0.00	66.9	0.00	60.0	0.30	52.1	0.40	63.1
	25%	1.50	66.2	0.40	71.7	0.40	68.5	0.50	48.3	0.50	67.6
Citrol K	75%	0.30	72.7	0.00	65.0	0.40	69.3	0.50	55.0	0.20	64.4
Ultra®	50%	0.50	69.6	0.00	63.2	0.30	58.7	0.4	56.4	1.40	51.4
	25%	0.90	37.7	0.70	59.7	0.80	64.6	0.80	51.1	0.42	54.5
Citrik Max®	75%	0.20	73.7	0.20	69.7	0.30	65.9	0.40	72.2	0.40	67.9
	50%	0.30	68.5	0.40	68.0	0.60	62.2	0.30	68.5	0.60	66.4
	25%	0.30	62.0	0.60	63.6	1.20	58.5	0.20	64.2	0.00	56.0
Citrik Agro®	75%	0.10	73.8	0.20	72.1	0.70	61.4	0.40	69.6	0.00	69.6
	50%	0.00	58.5	0.00	65.2	0.00	58.6	0.00	45.0	0.20	50.5
	25%	0.40	59.3	0.40	67.7	0.20	58.1	0.40	55.0	0.60	54.2

MBC, minimum bactericidal concentration.

Table 3. Efficacy of Citrik Agro on decontamination of parsley that was artificially contaminated with a pool of *Salmonella*.

Treatment	Day 0	Day 1	Day 3	Day 5	Day 7
	LOG CFU/g				
Not washed	5.8 ± 0.1*	5.1 ± 0.9	5.6 ± 0.1	5.5 ± 0.7	5.6 ± 0.4
Water	4.9 ± 0.3	5.2 ± 0.7	5.5 ± 0.6	5.3 ± 0.3	5.2 ± 0.1
Chlorine	4.1 ± 0.7	4.0 ± 0.9	4.0 ± 0.8	4.1 ± 0.2	3.1 ± 1.6
Citrik Agro®	3.1 ± 0.6	3.2 ± 0.7	3.2 ± 0.3	3.1 ± 0.1	2.6 ± 0.3

* Standard deviation.

microorganisms on fresh produce. Furthermore, it can generate chlorinated organic compounds, and the impact of these chemicals on humans and the environment has raised numerous safety concerns [17].

Produce has been a significant source of outbreaks of salmonellosis worldwide. Fresh produce may become contaminated with foodborne pathogens at various points along the production, handling, and packing processes. Sources of *Salmonella* contamination may include contaminated soil, poor water quality, animal fecal droppings, poor worker hygiene, sewage, inadequately composting, and raw animal manures, among others. Once *Salmonella* is on the vegetable surface, it can rapidly attach and colonize plant tissues and can reach large populations, similar to plant-associated bacteria [20, 21]. In addition, biofilm formation has been involved in colonization and persistence of *Salmonella* in leafy greens [21]. Swarming motility also appears to be involved in plant colonization [20].

The antimicrobial activity of citrus constituents is well known. Citrus species are a rich source of flavonoids, especially naringenin, quercetin, sinensetin, and apigenin. These compounds are capable of suppressing bacterial cell-cell signaling, biofilm formation, and the type III secretion system in several microorganisms [22-24]. Tetranortriterpenes such as limonoids, which are important constituents of grapefruit and other citrus fruits, are also inhibitors of these physiological processes. The application of these components into formulations to reduce microbial contamination has been recorded previously. For example, citrus essential oils have been shown to reduce the levels of microorganisms on salad products [25]. Of the disinfectants analyzed in this study, Citrik AB liquid and powder are made using grapefruit and orange extracts, while the other disinfectants are based on grapefruit, orange, and lime extracts.

Although the farm industry is adopting various practices to reduce the likelihood of contamination, the number of reported illnesses linked to contaminated produce has increased [26]. Therefore, it is essential to control or inhibit the initial stages of *Salmonella* attachment to plant tissues so that effective intervention and mitigation strategies can be utilized to abolish plant attachment and prevent produce outbreaks.

Although washing procedures are able to decrease the level of pathogens, these procedures must be applied in conjunction with good agricultural and handling practices so that the risk of contamination is decreased. The disinfectants analyzed in this study are examples of the various commercial citric extract-based products available in the market. The ability of these disinfectants to reduce the levels of *Salmonella* and interfere with biofilm formation and swarming motility could be a natural alternative to reduce the risk of *Salmonella* contamination. In this study, the efficacy of one disinfectant to reduce *Salmonella* in parsley at levels higher than chlorine is of particular significance. Studies are under way to determine the efficacy of these and other natural disinfectants to reduce *Salmonella* biofilm formation in leafy greens and other commodities.

Conclusions

Citrus-based disinfectants were able to inhibit growth, swarming, and biofilm formation of *Salmonella* and decontaminate parsley. These products could be a natural alternative to reduce the risk of *Salmonella* contamination in fresh produce.

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