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Antimicrobial Effect of Chlorine on *Listeria monocytogenes*

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ABSTRACT

The antimicrobial effect of reagent-grade sodium hypochlorite (SH) and household bleach (HB) on 2 strains of *Listeria monocytogenes* (Scott A and LCDC 81-861, both serotype 4a) was determined. After 24 h of growth in tryptic soy broth, cells were centrifuged, and pellets resuspended in potassium phosphate buffer (pH 7.0). Three-milliliter portions of the cell suspensions were then added to 27 ml of phosphate buffer containing about 0, 5, 10, 50, 100, or 200 ppm free residual chlorine. Cells were exposed to the chlorine for 20, 60, 120 and 300 s, at which time the chlorine was neutralized with 0.01 *M* sodium thiosulfate. Populations of surviving cells were determined by plating samples of the neutralized solution on tryptic soy agar and incubating the plates for 48 h at 30°C before counting. Chlorine concentrations less than about 50 ppm showed no antimicrobial effect but exposure to 50 ppm or greater chlorine resulted in no viable cells being recovered. Results for both SH and HB were similar. Dipping Brussels sprouts containing about 6 $\log_{10}$ colony forming units (CFU) *L. monocytogenes*/g into a 200-ppm chlorine solution for 10 s reduced viable cells recovered on McBrides agar by about 2 $\log_{10}$ CFU/g. Dipping Brussels sprouts in water alone reduced populations by about 1 $\log_{10}$ CFU/g.

Listeria monocytogenes is a foodborne pathogenic bacterium which has caused several disease outbreaks resulting in illness and death *(5,9,16)*. One of the best known of these outbreaks was traced to contaminated cole slaw *(17)*. In another instance, lettuce, tomatoes, and celery were implicated in cases of listeriosis *(8)*. The close relationship of *L. monocytogenes* to plants and soil *(19)* suggests that vegetables have a good chance of becoming contaminated with this organism. Thus it would be valuable to know what methods could be used to eliminate or control any listeriae which might be present on fruits or vegetables.

Use of chlorine dips or sprays is sometimes mentioned as being effective for controlling bacterial contamination of fruits and vegetables. Sommers *(18)* suggested that about 5 mg (ppm) of residual chlorine/L was adequate to reduce plate counts of several vegetables. Hobbs and Gilbert *(7)* recommend washing fruits and vegetables in solutions containing 60-80 mg of hypochlorite/L to remove enteric pathogens which might be present. However, it is unknown how effective similar treatments might be with *L. monocytogenes*.

The purpose of this research was twofold. The first goal was to determine the lethal effects of both reagent and household grade hypochlorite on *L. monocytogenes*. Secondly, the potential use of hypochlorite for removing *L. monocytogenes* from a vegetable, Brussels sprouts, was also evaluated.

MATERIALS AND METHODS

Bacterial strains

Two strains of *Listeria monocytogenes* were used in these studies. These strains included Scott A (serotype 4a, clinical isolate) and LCDC 81-861 (serotype 4a, cabbage isolate). Cultures were maintained on tryptic soy agar (TSA, pH 7.3; Difco) at 5°C and activated for 24 h at 30°C in tryptic soy broth (TSB, pH 7.3; Difco).

Before and after experiments, cultures were confirmed as *L. monocytogenes*. Confirmation tests included gram stain, catalase, umbrella growth in motility agar tubes, tumbling motility when wet mounts were viewed with a phase contrast microscope, and characteristic blue colony morphology when viewed with Henry's oblique lighting *(6)*. Confirmations were considered positive when reactions to the confirmation tests by isolates matched those of the pure stock cultures.

Preparation of test solutions

All glassware used in experiments were thoroughly cleaned and then rinsed with distilled water before use. Chlorine demand-free water was prepared as described by Blaser et al. *(1)*. Such water has no appreciable chlorine demand. Therefore, all added chlorine remains as free available chlorine *(4)*. Potassium phosphate buffer (0.5 *M*, pH 7.0) used in all experiments was prepared from chlorine demand-free water.

Stock chlorine solutions were prepared from both household bleach (HB) and from a 5% solution of sodium hypochlorite (SH; Aldrich Chemical Co., Milwaukee, WI). Both solutions were first filter-sterilized using a 0.22 μm filter (Millipore Corp., Bedford, Mass.) after which the free residual chlorine was quantified. Stock solutions were prepared by adding sufficient HB or SH to 500 ml of potassium phosphate buffer to give target chlorine concentrations of 0, 5, 10, 50, 100, and 200 μg/ml. Amounts of HB or SH used to arrive at these concentrations were calculated based on the initial concentrations of free residual chlorine. The actual chlorine concentration in each solution was again determined at the time each experiment was done.

Sodium thiosulfate (0.1 *N*) neutralizer stock solution was prepared by dissolving 25 g of $Na_2S_2O_3 \cdot 5\ H_2O$ in 1 L of freshly boiled distilled water. This solution was standardized with 0.01 *N* potassium dichromate *(15)*.

Chlorine determination

The concentration of free residual chlorine in all solutions was determined by the iodometric method; all reagents used in these tests were prepared as described for this method *(14)*.

Experimental procedure

One loopful of a 24-h-old culture grown in TSB was inoculated into each of duplicate 25-ml centrifuge tubes containing 10 ml of TSB. These tubes were incubated for 24 h at 30°C, centrifuged at about 2000 × *g* for 5 min, and the supernatant fluid was decanted. Five ml of buffer was added to each tube and the cell pellet was resuspended. The contents of both tubes were then combined and the resulting cell suspension, referred to as the test suspension, was used in all survival tests.

Effect of chlorine and neutralizer on L. monocytogenes *in buffer*

Test suspensions were prepared as described above and concentrations of viable *L. monocytogenes* present in each suspension were determined. Three ml of this suspension was added to the 27 ml of each of the stock solutions HB or SH in 50-ml test tubes. Samples were immediately mixed and allowed to react for 20, 60, 120, and 300 seconds. At each reaction time, 1 ml of each sample was withdrawn and added to 9 ml of neutralizing solution. After about 30 s, populations of surviving cells were determined. Viable *L. monocytogenes* cells were enumerated by making duplicate serial dilutions of each neutralized test suspension. From the neutralizer solution and each dilution, 1.0-ml samples were pour-plated in tryptic soy agar plates and the plates were incubated for 48 h at 30°C before colonies were counted.

The effect of thiosulfate neutralizer of *L. monocytogenes* was also determined to be sure that the neutralizing solution did not adversely affect *L. monocytogenes* cells. One ml of the test suspension was added to each of triplicate test tubes containing 9 ml of neutralizer. Viable populations of *L. monocytogenes* in the thiosulfate solutions were determined initially and after 5 and 15 min. Results were compared to those from a similar procedure using buffer in place of the thiosulfate solution.

Experiments with fresh Brussels sprouts

Fresh Brussels sprouts *(Brussica oleracea,* type *germifera)* were purchased at a local wholesale produce market. All Brussels sprouts used in experiments were rinsed in tap water and allowed to dry for 30 min. At that time, viable populations of *L. monocytogenes* on each of two Brussels sprouts was determined. Immediately before the start of the experiment, an inoculation solution was prepared by mixing 5 ml of a 24-h-old culture with 495 ml of chlorine demand-free potassium phosphate buffer in a sterile 500-ml beaker. Eight Brussels sprouts were dipped into the inoculation solution for 10 s and then again dried for 30 min. Drying of contaminated Brussels sprouts was always done in a laminar flow hood.

The eight inoculated Brussels sprouts and two uninoculated Brussels sprouts were sequentially dipped into a chlorine (200 mg/L) solution for 10 s. The uninoculated Brussels sprouts were third and ninth in the dipping sequence. This was done to determine if the chlorine solution would serve to spread *L. monocytogenes* cells from contaminated to uncontaminated Brussels sprouts. After removal from the chlorine solution, all Brussels sprouts were allowed to dry for 30 min and surviving populations of *L. monocytogenes* were determined as described below.

A parallel experiment was also done to determine what effect washing alone would have on the population of *L. monocytogenes* on Brussels sprouts. These experiments were done as above except that no chlorine was added to the dip water and only 2 Brussels sprouts were dipped rather than 10.

Enumeration of L. monocytogenes *in Brussels sprouts*

Populations of *L. monocytogenes* on Brussels sprouts were determined by first placing individual Brussels sprouts into preweighed, sterile plastic bags. The bags and contents were then weighed and the mass was recorded. Ninety ml of buffer was then added to the bag and the bag was pummeled for 15 s in a stomacher (Dynatech Laboratories, Inc., Alexandria, VA). After pummelling, the mixed samples were allowed to stand for about 15 min to allow the solid portion of the Brussels sprouts to settle. The liquid portion of the mixture was then serially diluted, in duplicate, in phosphate buffer. From each dilution, 0.1-ml samples were deposited on the surface of each of duplicate series of 10 McBride Listeria Agar (MLA; Difco) plates. A sterile bent glass rod was used to evenly distribute the inocula on the plates. At the same time, reference plates were also prepared by inoculating pure cultures of the particular strain on MLA plates. Both reference and sample plates were incubated for 48 h at 30°C before suspect *L. monocytogenes* colonies were counted. Colonies presumed to be *L. monocytogenes* and counted were those with the same colony morphology as colonies on reference plates. Typical presumptive colonies from sample plates were further confirmed to be *L. monocytogenes* as described previously.

Effect of Brussels sprouts and L. monocytogenes *cells on free residual chlorine*

Tests were done to determine how much contaminated Brussels sprouts or *L. monocytogenes* cells would lower the free residual chlorine of the chlorine dips. A 24-h-old culture of *L. monocytogenes* was steamed (100°C) for 30 min to inactivate cells. This was done to avoid contamination of laboratory personnel and equipment during subsequent analyses. The steamed culture was used to prepare an inoculation dip with which Brussels sprouts were inoculated as described above for live cells. Ten Brussels sprouts were sequentially dipped in a 200-ppm chlorine dip; another 10 Brussels sprouts were dipped all at once in a different chlorine dip. In both instances, Brussels sprouts were allowed to soak in the chlorine dip for 10 s. In addition, 0.2 ml of the steamed cell suspension was added to duplicate 80-ml solution samples of 0, 5, 10, 50, 100, and 200 mg of SH/L. Free residual chlorine in both chlorine dips was determined before Brussels sprouts were dipped, after 5, and after 10 Brussels sprouts were dipped. Free residual chlorine was likewise determined in 80-ml chlorine samples with and without added *L. monocytogenes* cells.

Statistical analysis

Results from all experiments were subjected to Analysis of Variance (ANOVA) to determine statistical differences ($p <= .05$) *(16)*. All experiments were replicated twice.

TABLE 1. *Populations of* L. monocytogenes *strain* LCDC 81-861 *exposed to sodium hypochlorite in phosphate buffer.*

Target chlorine conc. (mg/L)	Free residual chlorine (mg/L)	Mean $\log_{10}$ CFU/ml				
		Contact time (s)				
		0[a]	20	60	120	300
0	0	8.33	8.41	8.41	8.40	8.39
5	5.5	8.53	8.34	8.32	8.35	8.32
10	8.5	8.37	8.38	8.42	8.37	8.33
50	49	8.42	NR[b]	NR	NR	NR
100	93.2	8.48	NR	NR	NR	NR
200	190	8.40	NR	NR	NR	NR
NR						

[a]Calculated based on populations in inoculum.
[b]None recovered.

TABLE 2. *Populations of* L. monocytogenes *strain* LCDC 81-861 *exposed to household chlorine bleach in phosphate buffer.*

Target chlorine conc. (mg/L)	Free residual chlorine (mg/L)	Mean $\log_{10}$ CFU/ml				
		Contact time (s)				
		0[a]	20	60	120	300
0	0	8.38	8.37	8.36	8.41	8.36
5	5.8	8.25	8.14	8.10	8.02	8.18
10	9.5	8.36	8.35	8.40	8.32	8.28
50	39.5	8.36	2.70	3.60	3.45	2.97
100	98.3	8.14	NR[b]	NR	NR	NR
200	193	8.14	NR	NR	NR	NR

[a]Calculated based on populations in inoculum.
[b]None recovered.

RESULTS

Effects of thiosulfate on cells

Populations of viable *L. monocytogenes* cells exposed to the thiosulfate neutralizer for up to 15 min did not differ significantly from cells in phosphate buffers (data not shown). Therefore, the neutralizer was assumed to have no effect on results.

Effects of chlorine on cells in vitro

About 50 mg/L (Tables 1-4) was the limiting chlorine concentration which significantly decreased viable *L. monocytogenes*. In two instances (Tables 2 and 3), some cells appeared to survive at the 50 mg/L (ppm) target concentration. However, the actual free residual chlorine level in these instances was closer to about 40 mg/L. This would suggest that the actual limiting chlorine concentration for destroying *L. monocytogenes* cells is somewhere between 10 and about 40 mg/L.

Analysis of variance of combined data from all experiments revealed that SH and HB did not significantly differ from each other in antimicrobial effect. Likewise, there was no significant difference in the lethal effects of chlorine on the two strains used.

The length of time to which *L. monocytogenes* was exposed to chlorine in these experiments did not have a significant effect on survival of the cells. Complete inactivation occurred in less than 20 s in those instances where chlorine had an effect on survival (50 mg/L or greater).

Effects of chlorine on L. monocytogenes *in Brussels sprouts*

Dipping 10 Brussels sprouts in a SH solution decreased the free residual chlorine by less than 10 mg/L (Table 5). Likewise, the presence of about 5×10^6 *L. monocytogenes* cells/ml decreased the free residual chlorine less than 3 mg/L (Table 6).

Dipping contaminated Brussels sprouts into a 200 mg/L chlorine solution decreased viable *L. monocytogenes* cells on the sprouts by about 2 $\log_{10}$ CFU/g (Table 7). However, dipping contaminated Brussels sprouts in sterile, chlorine demand-free water reduced the number of viable cells by about 1 $\log_{10}$ CFU/g (Table 7). The decreases in viable cells from the chlorine dip were significantly different from that of the water dip. Reductions in viable populations found on any of the Brussels sprouts dipped in the chlorine solution were not significantly different from each other in any series.

No viable *L. monocytogenes* were isolated from uninoculated Brussels sprouts which had been dipped into the chlorine solution immediately after contaminated Brussels sprouts. However, *L. monocytogenes* cells may have been present on uninoculated Brussels sprouts at populations less than the level of detection (about 10 cells/g).

TABLE 3. *Populations of* L. monocytogenes *strain* Scott A *exposed to sodium hypochlorite in phosphate buffer.*

Target chlorine conc. (mg/L)	Free residual chlorine (mg/L)	Mean $\log_{10}$ CFU/ml				
		Contact time (s)				
		0[a]	20	60	120	300
0	0	8.61	8.46	8.41	8.46	8.49
5	5.1	8.44	8.26	8.27	8.26	8.23
10	8.3	8.50	8.20	8.24	8.18	8.13
50	43	8.54	NR[b]	0.70	0.25	0.33
100	83	8.44	NR	NR	NR	NR
200	190	8.40	NR	NR	NR	NR

[a]Calculated based on populations in inoculum.
[b]None recovered.

TABLE 4. *Populations of* L. monocytogenes *strain* Scott A *exposed to household chlorine bleach in phosphate buffer.*

Target chlorine conc. (mg/L)	Free residual chlorine (mg/L)	Mean $\log_{10}$ CFU/ml				
		Contact time (s)				
		0[a]	20	60	120	300
0	0	8.42	8.33	8.59	8.33	8.18
5	5.6	8.71	8.59	8.62	8.55	8.58
10	9.7	8.61	8.35	8.37	8.36	8.31
50	49	8.71	NR[b]	NR	NR	NR
100	99	8.75	NR	NR	NR	NR
200	193	8.45	NR	NR	NR	NR

[a]Calculated based on populations in inoculum.
[b]None recovered.

TABLE 5. *Effect of contaminated*[a] *Brussels sprouts on the concentrations of free residual chlorine.*

Sample	Free available chlorine (mg/L)	
	Sequentially dipped	All at once
Before dipping	177.5	178.8
After 5 Brussels sprouts	174.8	–
After 10 Brussels sprouts	170.4	169.3

[a]Brussels sprouts were dipped into about 10^7 killed cells.

TABLE 6. *Effect of* L. monocytogenes *cells on concentrations of free residual chlorine in distilled water.*

Target concentration (mg/L)	No cells		Cells added[a]	
	pH	FRC[b] (mg/L)	pH	FRC (mg/L)
0	7.00	ND	7.00	ND
5	7.00	2.22	6.95	1.77
10	7.05	7.99	7.00	7.99
50	7.04	38.16	7.00	37.18
100	7.04	79.88	7.00	78.35
200	6.98	153.98	7.00	151.38

[a]About 5×10^6 cells/ml.
[b]Free residual chlorine.

DISCUSSION

Chlorine compounds are very effective at killing a wide variety of microorganisms *(4)*. Results presented here indicate that *L. monocytogenes* appears to be in the same general range of sensitivity as other non-sporulating bacteria *(3)*. Chambers *(2)* specified that a sanitizer should reduce bacterial populations by 99.999% (i.e. 5 $\log_{10}$) in 30-s exposure to be considered effective. Although the Chambers method was not used here, all concentrations of either SH or HB at or above 50 mg/L met Chamber's criterion. Thus either form of hypochlorite should be considered effective at destroying *L. monocytogenes* in solutions. Moreover, recent results of similar experiments in our laboratory (unpublished) suggest that as little as 5 mg of free residual chlorine/L may be sufficient to inactivate most viable *L. monocytogenes* cells.

Although *L. monocytogenes* appears to be sensitive to rather low concentrations of chlorine, it would be prudent to use more than minimum concentrations in practical situations. One reason is that free available chlorine is affected by pH, presence of organic matter, water hardness, and temperature *(4)*. Hence, the actual active chlorine may be less than the user assumes is present. Second, use of sanitizers on dry stainless steel surfaces is less effective than in solution *(14)*. Results of Lopes *(11)* indicate that 100 mg of available chlorine/L is sufficient to sanitize non-pourous food-contact surfaces.

Although hypochlorite performed well in decreasing populations of *L. monocytogenes* in vitro, the same cannot be said for use in removing *L. monocytogenes* from fresh Brussels sprouts. Dipping contaminated Brussels sprouts into 200 mg/L solution of hypochlorite only reduced the populations of viable cells by about 2 $\log_{10}$ CFU/g (about 99%). This was much less than the com-

TABLE 7. *Survival of* L. monocytogenes *LCDC 81-861 on Brussels sprouts dipped in 200 mg/L chlorine solution for 30 sec.*

Sample	Dipped in chlorine		Dipped in water	
	Population (Log_{10} CFU/g)	Decrease (Log_{10})	Population (Log_{10} CFU/g)	Decrease (Log_{10})
Inoculum	9.17	–	8.68	–
Viable cells in dip	7.01	–	6.77	–
Inoculated Brussels sprouts	6.12	–	5.80	–
Brussels sprouts #1[a]	3.86	2.26	4.44	1.36
Brussels sprouts #2	3.97	2.16	–	–
Brussels sprouts #3	N.R.[c]	–	–	–
Brussels sprouts #8	4.00	2.04	–	–
Brussels sprouts #9	N.R.	–	–	–
Brussels sprouts #10[b]	3.63	2.51	–	–

[a]1st Brussels sprouts in a series of 10.
[b]10th Brussels sprouts in a series of 10.
[c]None recovered.

plete destruction found for the same hypochlorite concentration in buffer. The observation that water alone reduced the populations by over one order of magnitude makes the hyochlorite appear even less effective. Apparently, much of the reduction in viable cells caused by the chlorine wash resulted from loose cells being simply washed off the Brussels sprouts.

It is possible that addition of the Brussels sprouts to the wash solution could have reduced the free residual chlorine in the chlorine washes. This would have consequently also reduced the effectiveness of the sanitizer. However, this possibility is unlikely because addition of 10 Brussels sprouts or culture to a wash did not appreciably reduce the free residual chlorine (Tables 6 and 7). It is possible that surviving cells were located in the microenvironment on the sprouts that had reduced hypochlorite levels.

Although the dipped Brussels sprouts appeared wet, the possibility that the sprouts were not thoroughly wetted cannot be discounted. This possibility might be confirmed or rejected by further studies designed to see if wetting agents would enhance effectiveness of chlorine.

The small decrease in viable cell populations observed for Brussels sprouts is similar to that found in other foods. Treatment of beef *(12)*, chicken *(13)* and lamb *(10)* with at least 200 mg of chlorine/L all resulted in less than two orders of magnitude reduction in microbial populations. Therefore, the limited value of chlorine is not restricted to Brussels sprouts alone.

Based on the results of this work, hypochlorite appears to have value in removing *L. monocytogenes* from water supplies and perhaps, clean equipment. However, it does not appear effective for removing *L. monocytogenes* from contaminated vegetables.

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