

Use of Negative Air Ionization for Reducing Bacterial Pathogens and Spores on Stainless Steel Surfaces

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Primary Audience: Poultry Processing Management, Quality Assurance Personnel, Researchers, Poultry Extension Specialists

SUMMARY

The use of chemicals in food plant sanitation for removing and killing microorganisms could be reduced by the use of alternative nonchemical interventions. Supercharged negative air ionizers have shown potential to effectively reduce airborne and surface microorganisms. In our earlier studies, a small chamber, controlled at 85% relative humidity and supercharged with a -25 kV electrostatic space charge system (ESCS), was used to transfer a strong negative charge to bacteria on stainless steel surfaces. The ESCS treatment caused the levels of biofilm bacteria from chicken carcass rinses to be significantly decreased with 99.8% efficiency at a distance of 18 cm with ion densities of 10^6 negative ions/cm³ or more. In the present study, effects of the -25 kV charge of ESCS on specific pathogens important for food safety were studied. Treatment of *Campylobacter jejuni*, *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes*, and *Staphylococcus aureus* achieved up to a 4 log reduction with 99.9% reduction efficiency in 3 h. Treatment of bacterial spores of *Bacillus stearothermophilus* achieved up to a 3 log reduction with a 99.8% reduction efficiency in 3 h. These significant reductions for 5 species of pathogenic bacteria and bacterial spores suggest that the ESCS is a promising alternative treatment for reduction of microbial load in a food-processing facility with the potential to reduce the amount of antimicrobials used.

Key words: biofilm, negative air ionization, pathogen, poultry, stainless steel

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DESCRIPTION OF PROBLEM

Reduction of bacterial contamination of poultry products during processing is of major concern among processors and those concerned with food safety because of the frequent incrimination of these products in outbreaks of food-borne illness [1, 2]. Mechanical equipment has vastly increased the number of carcasses pro-

cessed by a single plant each day. The addition of equipment to increase automation has resulted in the presentation of new surface areas for carcasses to contact repeatedly, thus, new opportunities for bacterial attachment and cross-contamination. Contamination of raw meat may result from normal intestinal flora released from live birds as a result of the processing steps or from bacteria already present in the processing plant

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on equipment surfaces [3]. The reduction of microbial pathogens, such as *Campylobacter jejuni* and *Listeria monocytogenes*, in food products is the most pressing food safety problem today.

The use of spores of *Bacillus anthracis* (causative agent for anthrax) as a weapon for bioterrorism has brought the attention of the world to the need for new methods of detection of and killing bacterial spores. Unfortunately, bacterial spores are generally known to be extremely resistant to processes that kill bacterial vegetative cells, and bactericidal agents usually have little effect on bacterial spores [4, 5].

A promising new technology, the electrostatic space charge system (ESCS), has been shown to provide highly effective, nontoxic, and nonchemical reduction of dust and pathogens in the air and on surfaces. The ESCS was developed by Mitchell and Stone [6] for use in poultry areas to reduce airborne dust and microorganisms, but it can also be applied to any enclosed space. The ESCS generates very high negative ion density levels even in large spaces and does not require air to move through it in order to be charged. The ESCS was intentionally designed to minimize ozone production, with levels of detection below 0.01 ppm. Ozone is a strong oxidizing agent whose threshold limit value has been set at 0.1 ppm for safe human exposure. Because of the potential for ozone to cause lung problems, the Environmental Protection Agency and the American Lung Association recommend that devices designed to generate ozone not be used in spaces occupied by humans.

The ESCS reduced airborne levels of *Salmonella enteritidis* by 95% in caged layer rooms and eliminated airborne transmission in controlled environment cabinets [7, 8]. Use of the ESCS has reduced *Salmonella* transmission to chicks in experimental hatching cabinets by 3.4 logs [9] and pathogenic bacteria in commercial hatcheries up to 96% [10]. The mechanism, by which these reductions were achieved, was attributed primarily to the reduction in airborne dust levels.

In a further study, a smaller, bench-top version of the ESCS was developed to test the killing effects of electrostatic space charge on bacteria in biofilms. When used at close range, 18 cm or less, the ESCS significantly ($P < 0.01$) reduced plate counts from mixed populations of

bacteria from poultry carcass rinses on stainless steel surfaces by 99.8 % compared with untreated controls [11]. However, 2 usually non-pathogenic species of gram-positive bacteria, *Enterococcus faecalis* and *Brochothrix thermosphacta*, were identified from the few colonies that were not killed. This indicated that bacterial species could have variable responses to the system.

Although the mixed population of bacteria used in the initial ESCS biofilm study probably included several potentially pathogenic species of bacteria, further study was needed to show ESCS effects on specific pathogenic bacteria important for food safety and on bacterial spores. The objective of the present study was to test the specific effects of the ESCS as an alternative intervention strategy against 5 bacterial pathogens and on bacterial spores on stainless steel surfaces.

MATERIALS AND METHODS

Apparatus Setup

A miniature ESCS was custom-built for this application (Figure 1). The ionizer consisted of an array of 4 ionizer bars 13 cm long, with 7 sharp-pointed electrodes each and an expanded metal ground plane 7.6 cm above the electrode points. The bars were suspended such that the electrode points were 18 cm above the samples inside the closed 40.5- × 20.0- × 29.5-cm plastic chamber. The samples were placed on a metal ground plate on the floor of the chamber. Ion densities at this distance were over $10^6/\text{cm}^3$ (maximum measurable density by our instrumentation). Ionizer voltage was maintained at -25 kV by a high-voltage power supply during sample treatment. Control samples were placed in a second chamber without ionizer bars. Air flow into the chambers was generated on 1 side by a Penn-Plax Silent-Air- X6 air pump [12] at 2 L/min. Humidity was maintained inside the chambers by bubbling the air from the pump through water inside a 2-L Erlenmeyer flask, then through tubing (0.5 mm i.d. Tygon) in the top of the flask to an opening in the chambers 9 cm above the samples.

Experimental Design

The steel used in this study was 11-ga (3.04 mm thick) stainless steel plate with a 2B mill

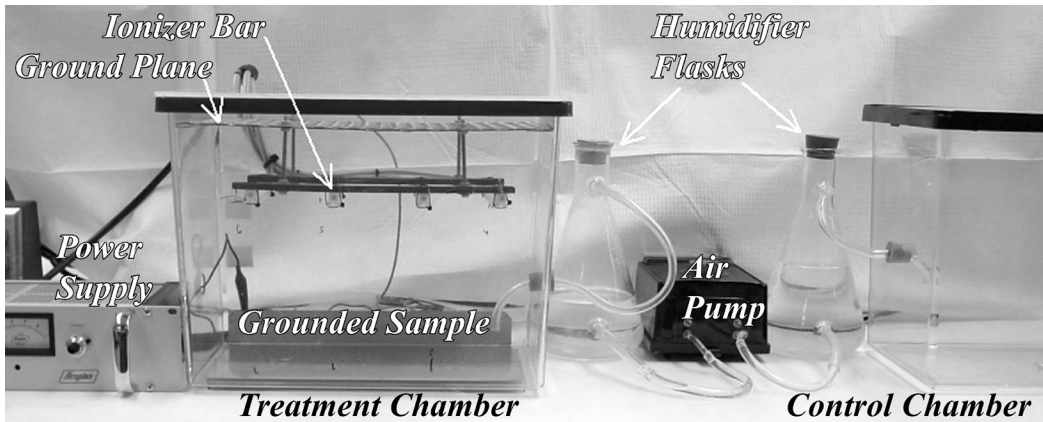


FIGURE 1. Equipment setup for negative air ionization with dual chamber system. The samples were placed on a grounded plate on the bottom of the test chamber for ionization. Control samples were placed in control chamber without ionizer bars. Air flow into the chambers was generated on 1 side, and humidity was maintained inside the chambers by bubbling the air from the pump through water inside the flask, then through tubing in the top of the flask to the opening in the chambers above the samples.

finish [13]. Coupons (4×1 cm) were sheared from the original sheet using a 14-ft LVD shear [14]. Prior to use, coupons were washed briefly in 1% Micro [15], rinsed in distilled water, sonicated for 30 min, and air-dried. Each new coupon was used once. Samples were divided into 3 groups for treatment by air ionization: 1) treated coupons with bacteria (or spores), 2) untreated coupons with bacteria (or spores), and 3) negative controls (untreated coupons).

Biofilms were developed on the stainless steel coupons using pure cultures of the following pathogens: *Escherichia coli* American Type Culture Collection (ATCC) 25922, *L. monocytogenes* ATCC 15313, *S. enteritidis* ATCC 13076, and *Staphylococcus aureus* ATCC 25923. Each pathogen was first grown in tryptic soy broth (TSB) for 18 h at 37°C , and subsamples (1 mL) of the respective culture were frozen in 2-mL cryovials with an equal volume of 20% glycerol. Subsequently, aliquots (200 μL) of a thawed culture were resuscitated by inoculation into 9 mL of TSB, which was incubated for 18 h at 37°C . To establish bacterial biofilms, 1 mL of an 18-h culture was transferred to a tube with 9 mL TSB, a stainless steel coupon was added, and the tube was incubated at 37°C for 4 h (for gram-negative bacteria) or 24 h (for gram-positive bacteria). For negative controls, stainless steel coupons were added to tubes containing only TSB, and no growth was observed after the treatment time.

Coupons with attached bacteria were removed from the tubes and treated at room temperature for 3 h, in the presence or absence of ionization. Afterwards, 100 μL of sterile water was added to the coupon surface, the surface was swabbed, and the swab was used to inoculate 2 mL of TSB. Serial dilutions (10^{-1} to 10^{-8}) of the inoculated TSB were prepared in TSB, and 0.1 mL of each dilution was spread on aerobic plate count agar. The number of colony-forming units per milliliter of original solution was determined from the counts on the agar plates incubated at 37°C for 24 or 48 h (for *L. monocytogenes* only). The colony forming units for each set was analyzed for statistical differences between the treated and untreated controls [16]. Results were compared with the colony forming units for each of the pathogens (Table 1) during separate experiments, including 2 trials each. The data for each trial were the means from triplicate tests.

Biofilms of *C. jejuni* ATCC 49943 were prepared on stainless steel coupons as follows. *C. jejuni* was grown on Campy-Cefex medium at 42°C for 24 h in an atmosphere of 5% O_2 :10% CO_2 :85% N_2 . Cells were harvested from plates and suspended in Bolton broth without blood [17] to an optical density of approximately 1.0. A 1:5 dilution of the inoculum was prepared in test tubes containing fresh Bolton broth. Stainless-steel coupons were added to tubes, which were incubated for 48 h at 42°C . Coupons were removed and

TABLE 1. Effect of negative air ionization for 3 h on biofilms of bacterial pathogens

Bacterium	Trial ^A	Bacterial counts		Reduction efficiency ^{B,C}	Log reduction	Humidity ^D
		Ionized	Not ionized			
<i>Campylobacter jejuni</i>	1	8.00 × 10 ¹	9.04 × 10 ⁴	99.9	3	ND ^E
<i>Campylobacter jejuni</i>	2	3.11 × 10 ¹	2.20 × 10 ⁵	99.9	4	ND
	Average	5.56 × 10 ¹	1.55 × 10 ⁵	99.9†	4	ND
<i>Escherichia coli</i>	1	2.61 × 10 ⁵	1.35 × 10 ⁷	98.1	2	70.1
<i>Escherichia coli</i>	2	5.74 × 10 ⁵	1.19 × 10 ⁷	95.2	1	65.0
	Average	4.18 × 10 ⁵	1.27 × 10 ⁷	96.7***	1	67.6
<i>Salmonella enteritidis</i>	1	1.34 × 10 ²	2.30 × 10 ⁶	99.9	4	73.0
<i>Salmonella enteritidis</i>	2	1.90 × 10 ⁴	4.11 × 10 ⁶	99.7	2	77.0
	Average	5.55 × 10 ³	3.15 × 10 ⁶	99.8*	3	75.0
<i>Listeria monocytogenes</i>	1	1.78 × 10 ¹	1.32 × 10 ⁵	99.9	4	75.2
<i>Listeria monocytogenes</i>	2	1.89 × 10 ¹	5.12 × 10 ⁴	99.9	3	64.5
	Average	1.83 × 10 ¹	9.18 × 10 ⁴	99.9***	4	69.9
<i>Staphylococcus aureus</i>	1	2.31 × 10 ⁴	1.52 × 10 ⁶	99.2	2	64.0
<i>Staphylococcus aureus</i>	2	2.10 × 10 ⁴	2.75 × 10 ⁶	99.2	2	70.5
	Average	2.21 × 10 ⁴	2.13 × 10 ⁶	98.9***	2	67.3

^AEach trial represents the mean of triplicate samples.

^BThe percentage of bacterial reduction efficiency was calculated with the following formula:

$$\frac{[(\text{mean number of bacteria not ionized}) - (\text{mean number of bacteria ionized})] \times 100}{(\text{mean number of bacteria})}$$

^CSignificance of reduction efficiencies as a result of negative air ionization (†*P* ≤ 0.10; **P* < 0.05; ****P* < 0.001).

^DThe percentage of humidity was measured inside the test chamber.

^ENot determined.

treated according to the protocol for the other pathogens, except that dilutions were prepared in Bolton broth and plated on Campy-Cefex medium, which was incubated at 42°C for 48 h in an atmosphere of 5% O₂:10% CO₂:85% N₂. For negative controls, stainless steel pieces were added to tubes containing only Bolton broth, and no growth was observed after the treatment time.

For testing the ESCS on bacterial spores, spore suspensions of *Bacillus stearothermophilus* ATCC 7953 [18] were used. An aliquot of the spore suspension (0.25 mL) was added to each of 2 coupons per sample, which were subsequently placed in the treatment chamber for ionization or control chamber without ionization. Negative controls were treated similarly without ionization, using 0.25 mL of sterile water per coupon instead of spore suspension, and no growth was observed after the treatment time. Treatments were performed at room temperature for 3 or 6 h. After treatment, coupons were swabbed, and swabs from each set of 2 coupons were incubated in 2 mL of TSB for 36 h at 55°C to initiate germination of the spores and allow time for cell growth. Serial dilutions were prepared from the heat-shocked cultures (1 mL of culture in 9 mL of TSB). Sub-

samples (100 μL) of the respective serial dilutions were plated in duplicate on plate count agar and incubated at 55°C for 24 h to obtain vegetative cell growth. The colony-forming units per milliliter of original solution were determined from the counts on the plates and analyzed for statistical differences between the ionized and nonionized samples [16]. Results were compared for the colony forming units for the growth from the bacterial spores (Table 2) during separate experiments, including 2 trials each. The data for each trial were the means from triplicate tests.

Germination of the bacterial spores and growth of the vegetative cells were confirmed by spectrophotometry. Aliquots of the initial inoculum of spores that were added to the coupons were serially diluted and added to cuvettes. Aliquots of the serial dilutions to obtain vegetative cell growth were also placed in cuvettes for testing. Absorbance values of the spore or cell suspensions in TSB were measured by a Beckman DU640 spectrophotometer equipped with a temperature controller and auto cell holder [19]. The absorbance values were measured at 410 nm, 55°C, and every 15 min for 24 h to follow cell growth. Data were evaluated to compare the differences

TABLE 2. Effect of negative air ionization on bacterial spores of *Bacillus stearothermophilus*

Exposure time (h)	Trial ^A	Bacterial counts		Reduction efficiency (%) ^{B,C}	Log reduction	Humidity ^D
		Ionized	Not ionized			
3	1	2.50×10^2	2.39×10^5	99.9	3	85.0
	2	4.84×10^3	1.18×10^6	99.6	2	82.9
	Average	3.01×10^3	8.02×10^5	99.8†	2	84.0
6	1	7.55×10^4	2.15×10^5	64.9	1	69.8
	2	2.84×10^4	1.36×10^5	79.1	1	68.9
	Average	4.72×10^4	1.67×10^5	72.0	1	69.4

^AEach trial represents the mean of triplicate samples from lots 255 or 257.

^BThe percentage of bacterial reduction efficiency was calculated with the following formula:

$$\frac{[(\text{mean number of bacteria not ionized}) - (\text{mean number of bacteria ionized})] \times 100}{(\text{mean number of bacteria})}$$

^CSignificance of reduction efficiencies († $P \leq 0.10$) as a result of negative air ionization.

^DThe percentage of relative humidity was measured inside the test chamber.

observed in growth kinetics between spores and cells [16].

RESULTS AND DISCUSSION

In previous work, we developed methods to measure growth and attachment of bacteria to stainless steel [20], and a miniature ESCS was built to determine its effects against mixed populations of bacteria growing in biofilms [11]. The ESCS transferred a strong negative electrostatic charge to microorganisms on the surfaces of stainless steel. In our initial study [11], mixed populations of bacteria in biofilms were killed with 99.5 to 99.9% reduction efficiency. In the present study, the ESCS dramatically reduced levels of 5 species of pathogenic bacteria and 1 bacterial spore former.

The effects of negative air ionization on biofilms of bacterial pathogens are shown in Table 1. Coupons inoculated with each pathogen were exposed separately to the ionizer for 3 h under the same conditions as the initial experiment [11]. Data from the 2 trials for each pathogen indicated that the relative average reduction efficiency followed the order of *C. jejuni* and *L. monocytogenes* (99.9%) > *S. enteritidis* (99.8%) > *Staph. aureus* (98.9%) > *E. coli* (96.7%). For comparison, Massachusetts Institute of Technology recently reported a 94% reduction of *Staph. aureus* cells sprayed on treated surfaces that had been coated with various alkyl bromides [21]. Although the log kill and reduction efficiency was greatest for *C. jejuni* and *L. monocytogenes*, the probability for statistical difference was less than the other

pathogens ($P = 0.06$ and $P = 0.11$, respectively) because of their instability during growth and culture. The range in plate counts for individual samples of *L. monocytogenes* was 3 to 5 logs cfu for nonionized controls and 0 to 1 log cfu after ionization. The range for *C. jejuni* was 3 to 5 logs cfu for nonionized controls and 0 to 2 logs cfu after ionization. The range for *E. coli*, *S. enteritidis*, and *Staph. aureus* were within 1 log for individual samples before or after ionization. Because nearly all trials resulted in nearly total kill, the data for *C. jejuni* and *L. monocytogenes* bear reporting. Only 18 to 80 cells remained for these 2 pathogens after ESCS treatment of 51,200 to 220,000 cells.

Gram-positive (*L. monocytogenes*, *Staph. aureus*) and gram-negative (*C. jejuni*, *E. coli*, *S. enteritidis*) pathogens were tested to determine whether structural differences in cell walls may affect killing. Among the 5 species tested, the system was effective against both types of bacteria; therefore, the gram reaction did not appear to be an indicator of effects. The size and shape of the target cells did not appear to determine the effects either. For example, the rod-shaped cells of *L. monocytogenes* were the smallest tested, and the reduction efficiency was the highest achieved. The rod-shaped cells of SE were larger than the cocci of *Staph. aureus* or the rods of *E. coli*, but its reduction efficiency was greater. These results support the indication in our previous study [11] that the effect of the ESCS varies according to species. Other important physiological factors may be determined with further study.

The viability of the bacterial spores of *Bacillus stearothermophilus* was tested prior to ionization

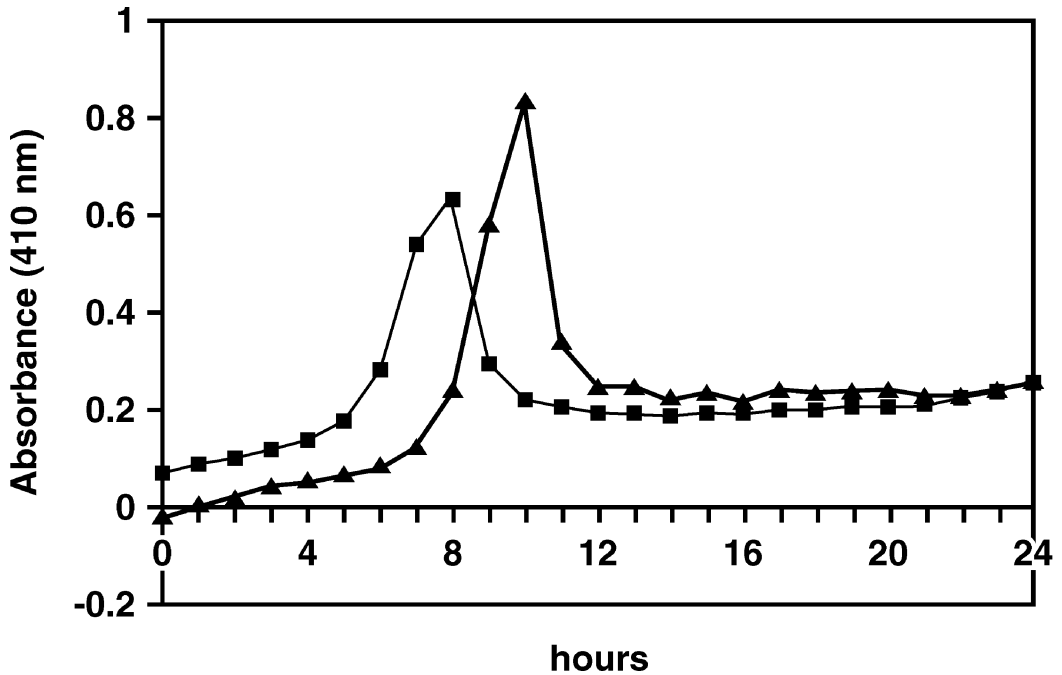


FIGURE 2. Germination of the bacterial spores of *Bacillus stearothermophilus* and growth of its vegetative cells. Turbidimetric assays by spectrophotometry were performed at 55°C with aliquots of the initial inoculum of spores (▲) added to the coupons. Assays with aliquots of serial dilutions, made from swabs of the coupons, to obtain growth of vegetative cells (■) were performed at the time of plating (after 36-h incubation in tryptic soy broth (TSB) at 55°C).

to ensure consistent germination of the spores and growth of the vegetative cells. Spores for research are produced in batches that can have variation in germination and viability. Each batch of spores grown and collected on a specific date is assigned a lot number. Composite data for 2 trials of lot 266 are represented in Figure 2. Thirty-six hours were required to obtain visible turbidity in test tubes after spores were removed from coupons. The maximum turbidity for untreated spores measured directly by spectrophotometry occurred at 9.75 h during heat shock at 55°C. Vegetative cells grown from spores reached maximum turbidity at 43.5 h, 7.5 h following the 36-h incubation of spore inoculum. These data confirmed the viability of the initial spore inoculum and determined the times necessary for incubation after treatment of spore samples.

The effects of negative air ionization on bacterial spores are shown in Table 2. Coupons were inoculated with spores of *B. stearothermophilus* and exposed to the ionizer. The average reduction efficiency for samples exposed for 3 h was 99.8%.

A longer, 6-h exposure was expected to increase the efficiency. However, there were fewer spores killed at 6 h than at 3 h, therefore the plate counts were larger after ESCS treatment for 6 h. The reduction efficiency at 3 h (99.8%) was greater than that for samples exposed for 6 h (72.0%). It is possible that some of the loss in reduction efficiency seen after 6 h of exposure may have been caused by an average 4-fold loss of viable spores as was seen in the nonionized control samples during the 6-h incubation (Table 2).

Because this is the first report of successful research with killing of bacterial spores by alternative strategies, some of the inherent problems with the research should be mentioned. Differences in reduction efficiency were noted between spore suspensions generated from different lot numbers and from the same lot number used in the experiments. In fact, the results of subsequent trials have been inconsistent, the number of spores reduced by treatment ranged from less than 1 log to 3 logs. In a preliminary trial, the plate distance between the discharge points and the stainless steel

coupons was decreased from 18 cm to 4 cm in an attempt to increase the charge level, but less than 1 log kill was achieved. The proximity of the bars and ground plate may have negated the charge. Future trials are planned with closer proximity of the discharge points to the biofilm. The spore samples in Table 2 contained the indicator dye, crystal violet. Because of a concern that the dye may cause some clumping of spores or otherwise interfere with the charge, spores with certi-

fied counts by the manufacturer and no dye were used in separate trials (data not shown). There was no increase in kill. In separate trials, spore inoculum density was varied by sequential dilution (data not shown), but there was no increase in kill. Supplemental humidity was removed to increase the electrostatic charge (power supply output current increased from 0.08 to 0.1 mA), but the kill was not increased.

CONCLUSIONS AND APPLICATIONS

1. Treatment of the bacterial pathogens *C. jejuni*, *E. coli*, *S. enteritidis*, *L. monocytogenes*, and *Staph. aureus* by high levels of negative air ionization achieved up to a 4 log reduction with averages of 96.7 to 99.9% reduction efficiency.
 2. Negative air ionization reduced surface contamination from bacterial spores of *B. stearothersophilus* up to 3 logs with an average 99.8% reduction efficiency.
 3. The ESCS can significantly reduce the microbial load on stainless steel surfaces without the use of chemical antimicrobials and potentially could be used in many processing plant areas as an alternative or supplemental treatment. More research is needed to determine the effect of the ESCS on biofilms for other materials, such as plastic conveyor belts, and to determine effectiveness in open areas, as contrasted to the treatment chamber used in the present study.
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