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Inactivation of *Listeria monocytogenes* on ready-to-eat food processing equipment by chlorine dioxide gas

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ABSTRACT

The effectiveness of chlorine dioxide (ClO_2) gas to control *Listeria* contamination on food contact and environmental surfaces was investigated in order to comply with the zero tolerance policy. Different ClO_2 gas concentrations (0.3, 0.5, 1 and 2 mg/l) were evaluated in order to inactivate *Listeria monocytogenes* (planktonic cells and biofilms) on stainless steel coupons. An ideal condition was then selected, and its applicability was studied on a commercial meat slicer and an industrial hot-dog peeler (using a surrogate organism). *L. monocytogenes* biofilm cells initially showed more sensitivity to ClO_2 treatments, as compared to planktonic cells, but after 10 min, ~4 log CFU/cm^2 reduction was observed for all the concentrations used. The treatment at 2 mg/l for 30 min was selected for the further validation study. Complete pathogen inactivation, >5 log CFU/cm^2, was obtained on both the meat slicer and peeler, demonstrating the potential applicability of ClO_2 gas as a sanitizing agent for RTE meat processing equipment.

1. Introduction

In the early 1980s, the United States Department of Agriculture and Food Safety Inspection Service (USDA-FSIS) implemented a "zero-tolerance" policy for *Listeria monocytogenes* (USDA, 1989). Within the last two decades, this pathogen has been associated with several outbreaks and more information about its prevalence in food-manufacturing plants and products has become available (Gombas, Chen, Clavero, & Scott, 2003). Foods considered at high-risk for *L. monocytogenes* are Ready-To-Eat products (RTE), especially those that require long-term refrigeration. Moreover, the final rule, issued by USDA-FSIS in 2003, requires manufacturers to address the risk of recontamination of RTE meat and/or poultry products that support pathogen growth and are exposed to the environment following thermal processing. Options listed in the final rule include adding antimicrobials or using processes to inhibit, reduce, or eliminate the growth of *L. monocytogenes* (FSIS, 2003). Therefore, efficient sanitary practices are needed in food processing facilities.

Sanitation of food processing equipment is a regular practice, but cleaning and disinfection eliminates only some microorganisms from surfaces (Rossoni & Gaylarde, 2000). Bacteria can show different susceptibility to the sanitizer, due to their growth and nutrient status, surface attachment and resistance (Langsrud, Sidhu, Heir, & Holck, 2003). Microbial attachment and biofilm formation can occur due to complex physical–chemical interactions between bacteria and surfaces, such as charge, hydrophobicity, Van der Waals forces, and acid–base interactions. Surface heterogeneity (polished or unpolished), surface-conditioning by organic soil and presence of irregularities such as pits, cracks, welds, and crevices also affect the attachment of bacteria and biofilm formation (Faille & Carpentier, 2009).

Biofilms are defined as a "microbial-derived sessile community, where cells are irreversibility attached to a substratum, interface, or to each other and are embebbed in a matrix of extracellular polymeric substrate" (Donlan & Costerton, 2002). This exopolysaccharide matrix is a hurdle for the interaction of biocides, since it can protect the cells from the action of sanitizers, and in some instances promote antimicrobial resistance (Vaid, Linton, & Morgan, 2010). All food processing surfaces are potential sites for biofilm formation and *L. monocytogenes* has the ability to grow on several materials frequently used in processing plants (Herald & Zottola, 1988; Mafu, Roy, Goulet, & Magny, 1990; Vaid et al., 2010). Cells in biofilms have higher probability to survive after sanitation procedures, as compared to planktonic (biofilm-free) cells, and may detach and contaminate the processed food products. Moreover, the resistance to sanitizers increases with the maturity of the biofilm, as reported by Lee and Frank (1991); consequently, the choice of a sanitizer agent must be made carefully, taking in account the

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physiology of the target microorganisms, the possibility of biofilm formation, and the type of surfaces.

In United States, the most frequently employed chemical agents for sanitation procedures are: hypochlorous acid, chlorine, iodine, ozone, hydrogen peroxide, peroxyacetic acid, quaternary ammonium chloride and anionic acids (Grinstead, 2009). Among innovative sanitation procedures, chlorine dioxide (ClO_2) gas, a strong oxidizing agent with a broad antimicrobial spectrum, shows potential to be used to decontaminate food contact surfaces. Most applications of ClO_2 refer to aqueous solutions. Only few researchers have investigated ClO_2 gas applicability as a biocide agent on hard food contact surfaces. Han, Guentert, Smith, Linton, and Nelson (1999) reported the use of ClO_2 gas to sanitize tanks for aseptic juice storage, while Vaid et al. (2010) showed its effectiveness for the inactivation of *L. monocytogenes* biofilms on stainless steel coupons. The direct application of ClO_2 gas, within a gas tight chamber, can be more effective than aqueous solutions and minimize operator exposure.

The overall objective of this study was to propose and validate a ClO_2 gas treatment that completely inactivates *L. monocytogenes* on food processing equipment. In order to achieve this goal, the effectiveness of different gas concentration (0.1, 0.3, 0.5, 1 and 2 mg/l) was evaluated and compared on stainless steel (SS) coupons inoculated with *L. monocytogenes* planktonic cells and biofilms. The treatment that led to the greatest CFU/g log reduction was selected to verify complete pathogen inactivation on meat processing equipment (e.g. commercial meat slicer and industrial hot dog peeler).

2. Material and methods

2.1. Bacterial cultures

Three strains of *L. monocytogenes* (FSL 103-M, FSL N1-227, Scott A) and one strain of *L. innocua* were selected for this study and obtained from the Purdue Bacterial Collection (Purdue University, IN, USA). For biofilm experiments, two more strains (strong biofilm formers), *L. monocytogenes* 311 and 82, were added to the three strain mixture resulting in a total of five strains (Russell Research Center, Athens, GA). Cultures were grown twice in Tryptic Soy Broth (TSB) (Difco Laboratories, MD, USA) with 0.6% yeast extract (Bacto, MD) at 37 °C before experiments. The virulent strains were previously isolated from food products associated with *Listeria* outbreak episodes and used to inoculate SS coupons and the meat slicer. The non-virulent strain was used as a surrogate for experiments with the hot dog peeler, since the peeler tests were conducted in a pilot plant where the use of pathogens was not permitted (Food Science Department, Purdue University).

2.2. Preparation of coupons

Stainless steel (SS) coupons (type 304, 2B finish) 1.4 cm by 5 cm (total area 7 cm^2) were obtained from Enerfab, Inc. (Cincinnati, OH, USA). The coupons were treated at 60 ± 5 °C for 1 h with 100 ml/l solution of Micro-90® soap (International Products Corporation, NJ, USA) in an ultrasonic bath (model 3510R-MT, CT, USA), rinsed with deionized water and sonicated again with 1.5% phosphoric acid solution for 20 min (Vaid et al., 2010). The cleaned coupons were rinsed with deionized water, air-dried and autoclaved at 121 °C for 20 min.

2.3. Bacterial suspension inoculation and biofilm development on SS coupons

Equal volumes of *L. monocytogenes* overnight cultures were mixed (initial total population ~ 3 x 10^9 CFU/ml) and 100 μl of the cocktail was spotted onto SS coupon surfaces. Samples were kept in a biosafety hood for 2 h to allow for cell attachment before chlorine dioxide treatment.

Biofilms were developed following the procedure described by Vaid et al. (2010). Breifly, the five strains of *L. monocytogenes* were mixed in equal volumes (initial population ~ 1×10^9 CFU/ml) and 100 μl of the solution was spread on each coupon surface using a sterile inoculation loop. Coupons were kept for 3 h in a desiccator with 100% relative humidity and then washed with 20 ml of potassium buffer (PPB, 50 mM, pH 7), in order to remove unattached cells. 100 μl of TSB was added on top of each coupon and samples were returned to the desiccator. Each day for 4 days, coupons were washed with PPB, and topped with TSB. Before inactivation experiments, surfaces with 4 day old biofilms were rinsed with PPB and dried for 2 h in a biosafety hood.

2.4. ClO_2 gas treatments

ClO_2 gas was generated based on the Cl_2 gas - $NaClO_2$ solid method described by Simpson (2005) and reported by Trinetta, Morgan, and Linton (2010). ClO_2 gas (0.3, 0.5, 1 and 2 mg/l) was used to treat SS coupons inoculated with *L. monocytogenes* planktonic cells and biofilms for 2, 4, 6, 8 and 10 min inside a closed gas chamber. Samples were also exposed to a 10 min pre-conditioning treatment at 25 °C and 75% relative humidity prior to gas exposure.

2.5. Microbiological analysis

After each treatment, coupons were removed from the chamber, aseptically transferred into 20 ml neutralizing buffer (DIFCO, MD, USA) and vortexed for 1 min to remove surviving cells. A one ml sample was then used to prepare serial decimal dilutions. Aliquots of 100 μl were spread plated onto Tryptic Soy Agar (DOT Scientific Inc, MI, USA), +0.6% yeast extract, +1% sodium pyruvate (Sigma, MO, USA) (TSAYEP) for the planktonic cells experiments; instead aliquots of 1 ml were pour plated to recover biofilms. All plates were incubated for 24–48 h at 37 °C, colonies were counted and results expressed as log CFU/cm^2. Preliminary experiments showed less clumped cells in pour plated Petri dishes and better repeatability, therefore this recovery method was used for biofilms enumeration.

Afterward, an end-point determination method was used to determine the required time to completely inactivate *L. monocytogenes* at 2 mg/l ClO_2 gas (Han, Applegate, Linton, & Nelson, 2003). After treatments, each coupon was aseptically transferred to 50 ml TSBYEP and incubated at 37 °C for 5 days. Turbid samples were considered positive, indicating bacterial growth, while clear samples were considered negative suggesting complete inactivation. All tubes were verified for presence or absence of *L. monocytogenes* by streaking the broth after 5 days on MOX agar plates (Modified Oxford Agar, MI, USA).

2.6. Validation study on meat processing equipment

Based on the data obtained from the inactivation experiments on SS coupons, 2 mg/l 30 min treatment was selected for the further validation study. A commercial meat slicer (2000 series, Hobart, Corporation, OH, USA) and an industrial hot-dog peeler (Ranger Apollo Model, Townsend Engineering, IA, USA) were treated with 2 mg/l ClO_2 gas for 30 min. Before treatment, different parts of the equipment were dismantled and autoclaved (Fig. 1 and Fig. 2). The target areas (1 cm^2), chosen based on the equipment use, product preparation, and handling procedures, were inoculated with 10 μl of *Listeria* suspension as previously described. The different inoculated parts were placed inside the chamber and treated at the selected conditions.

Fig. 1. Different selected sites inoculated with *L. monocytogenes* cocktail on the commercial meat slicer. A: SS base; B: blade; C: SS grip; D: plastic handle; E: plastic handle; F: plastic handle; G: SS removable carriage.

The mixture of virulent strains was used to inoculate the meat slicer, while the hot-dog peeler was inoculated with *L. innocua* as a surrogate (experiments carried out in the pilot plant of Food Science Department at Purdue University).

2.7. Enumeration of Listeria on equipment surfaces

After treatment, the remaining bacterial population on equipment surfaces was recovered following the surface sampling procedure described by Vanderzant and Splittstoesser (1992). Briefly, the selected points were swabbed with a cotton swab, previously moistened with Letheen Broth (Difco Laboratories, MD, USA); after sampling, swabs were moistened again and 1 ml of suspension was used to prepare serial decimal dilutions. Appropriate aliquots were spread plated onto MOX agar plates (Modified Oxford Agar, MI, USA) to enumerate remaining population. Plates were incubated up to24–48 h at 37 °C, black colonies were counted and results expressed as log CFU/cm^2.

Also for the validation study, an end-point determination method was used to verify the complete inactivation of microorganisms by ClO_2 gas on treated equipment surfaces. Equipment parts were aseptically transferred into 250 ml sterile LEBM media (Listeria Enrichment Broth Modified, Becton-Dickson, NJ, USA) and left at room temperature for 5 days, following the procedure previously described.

2.8. Statistical analysis

All experiments, for each surface and treatment type, were performed in triplicate. Significant differences and comparison among means were determined using a Tukey multiple comparison test (Minitab 15v, State College, PA). Data were considered to be significantly different when $p < 0.05$. Bacterial populations were converted from CFU/ml to log CFU/cm^2. Following conversion, data were pooled; means with standard deviations were calculated and presented in the figures. Inoculated and untreated samples were considered as controls and used to monitor pathogen population over time.

3. Results

The initial population of *L. monocytogenes* planktonic cells recovered from SS coupons before treatment was 5.85 ± 0.15 log CFU/cm^2, and remained constant over time (10 min) for untreated control samples (Fig. 3). During the first 2 min treatment, no significant difference ($p > 0.05$) was observed between control and treated cells, except for the experiment at 2 mg/l, where a reduction of more than 1 log CFU/cm^2 was detected. For increasing treatment concentrations and times, significant and progressive log reductions were reported ($p < 0.05$). After 10 min gas exposure, 0.3 and 0.5 mg/l gas concentrations showed similar effects, 1.17 ± 0.22 log CFU/cm^2 and 1.45 ± 0.15 log CFU/cm^2 reduction, respectively. Treatments with 1 and 2 mg/l ClO_2 gas led to greater inactivation: 2.5 ± 0.22 log CFU/cm^2 and 3.8 ± 0.19 log CFU/cm^2 were reported after 10 min, respectively.

For comparison purpose, a four day old biofilms (five strains cocktail of *L. monocytogenes*) were developed on SS coupons. In our preliminary experiments we observed that biofilms developed using three *L. monocytogenes* strains mixture (FSL 103-M, FSL N1-227 and Scott A) were reduced more than 3 log CFU/cm^2 after

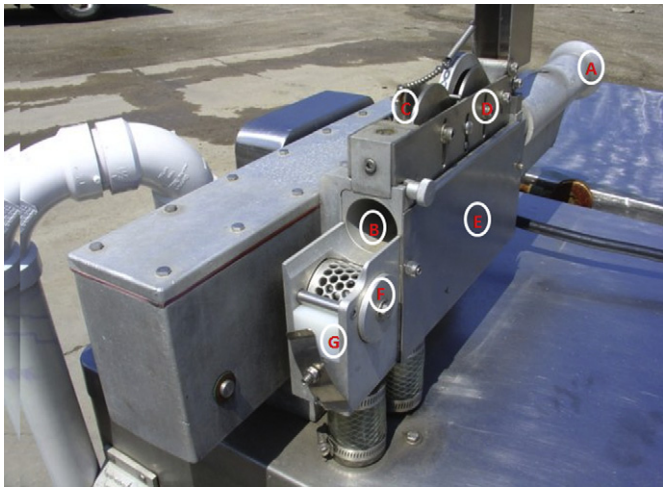

Fig. 2. Different selected sites inoculated with *L. innocua* on the industrial hot dog peeler. A: SS inlet; B: SS point of physical removal of casings; C: rollers; D: SS housing for rollers and knives; E: SS panel; F: SS vacuum roller for casings removal; G: plastic block to maintain separation of split casing from peeled frankfurter.

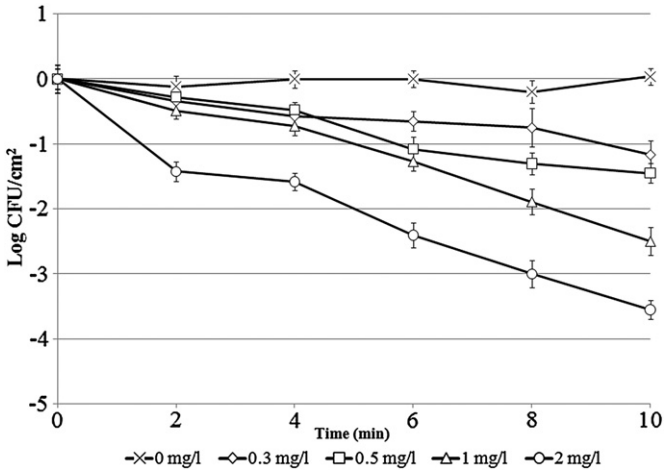

Fig. 3. Remaining population of *Listeria monocytogenes* planktonic cells observed in contaminated SS coupons treated with different ClO_2 gas concentrations: 0 mg/l (×), 0.3 mg/l (◇), 0.5 mg/l (□), 1 mg/l (△) and 2 mg/l (○). Mean values of three replicates ± standard deviation are presented.

only 2 min treatment (data not shown); therefore two strong biofilm forming strains (311 and 82) were added in this set of experiments. The initial biofilm population on SS surfaces was 5.13 ± 0.13 log CFU/cm^2. As shown in Fig. 4, it was observed that even a low concentration of ClO_2 gas (0.3 mg/l) significantly reduced ($p < 0.05$) the population of *L. monocytogenes* in a biofilm matrix: a 3.21 ± 0.19 log CFU/cm^2 reduction was observed after 10 min of treatment. Independently from the gas concentration applied, *Listeria* biofilms always presented the same inactivation trend. A rapid decrease was observed after 2 min, the population remained constant at 4 min treatment and again a decrease was reported after 6 min. At the end of the experiment (10 min), a 3.2 ± 0.19 log CFU/cm^2 reduction was observed in treated samples with 0.3, 0.5 and 1 mg/l ClO_2 gas concentrations, while 3.8 ± 0.23 log CFU/cm^2 reduction was reported after 10 min treatment at 2 mg/l. Initially *L. monocytogenes* biofilms showed more sensitivity to ClO_2 treatments, as compared to planktonic cells, but the total log reductions after 10 min with a concentration of 2 mg/l were similar.

Table 1 shows the results obtained from the end-point determination experiments on inoculated SS coupons. Time was a significant factor for *L. monocytogenes* inactivation: data showed that no growth (i.e. no turbidity after 5 days) was observed in triplicate samples after 30 min and 70 min of treatment with 2 mg/l for planktonic cells and biofilms, respectively. Conversely to the observations previously reported for a 10 min period, over time biofilms showed more resistant to treatments: longer exposure was required for complete inactivation using the end-point determination. Probably, the external layers of the extracellular substrate were easily and quickly eliminated by the sanitizer (10 min), then more time was necessary to deeply penetrate into the matrix (70 min), as compared to the time required to completely inactivate the free living cells (30 min).

For the final scale-up study on RTE meat processing equipment, the treatment at 2 mg/l for 30 min was selected. In this part of the research, only *Listeria* planktonic cells were considered, based on the assumption that cleaning and sanitizing are procedures performed daily (or more frequently) with the purpose of reducing the risk of pathogen contamination and preventing microorganism persistence

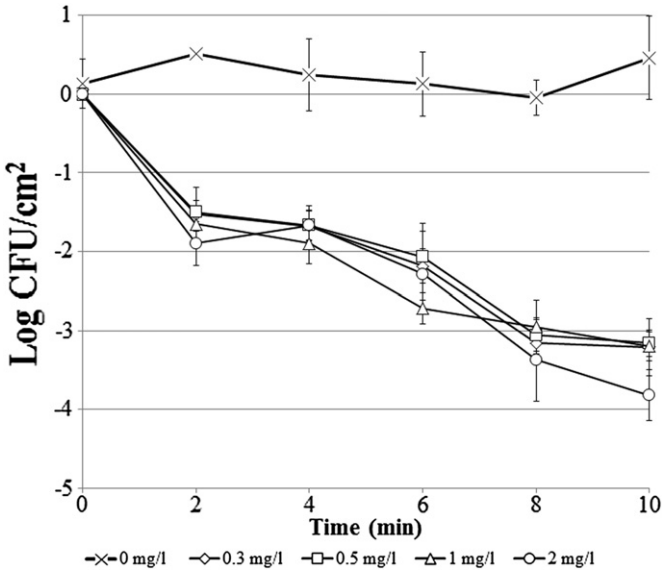

Fig. 4. Remaining population of *Listeria monocytogenes* biofilms observed in contaminated SS coupons treated with different ClO_2 gas concentrations: 0 mg/l (×), 0.3 mg/l (◇), 0.5 mg/l (□), 1 mg/l (△) and 2 mg/l (○).Mean values of three replicates ± standard deviation are presented.

Table 1
End-point method results of *L. monocytogenes* planktonic cells (initial population recovered 5.85 ± 0.15 log CFU/cm^2) and biofilm (initial population recovered 5.13 ± 0.13 log CFU/cm^2) on stainless steel coupon surfaces treated with 2 mg/l ClO_2 gas at 25 °C and 75% relative humidity for different treatment time.

ClO_2 treatment time (min)	Post-treatment growth of *Lm* (number of positives out of 3 replicate samples)	
	Planktonic cells	Biofilm
0	3/3	3/3
10	3/3	3/3
20	1/3	3/3
30	0/3	3/3
40	0/3	3/3
50	0/3	2/3
60	0/3	2/3
70	0/3	0/3

(i.e. biofilms). Different inoculation sites were selected on the meat slicer and on the hot dog peeler (Figs. 1 and 2), since a predisposition of cells to accumulate in metal surface irregularities was observed by Rossoni and Gaylarde (2000). The initial *L. monocytogenes* population recovered on meat slicer surfaces before treatment was 5.78 ± 0.19 log CFU/cm^2, while the number of *L. innocua* cells enumerated on hot-dog peeler surfaces was 6.15 ± 0.18 log CFU/cm^2. Table 2 reports the ability of ClO_2 gas to inactivate microorganisms on the equipment surfaces: no growth (turbidity) was observed after 5 days of incubation, indicating that complete microbial inactivation was achieved on RTE processing equipment at the selected condition of 2 mg/l ClO_2 for 30 min. ClO_2 gas showed the same effectiveness previously observed on experimentally inoculated SS coupons, validating its potential use as an antimicrobial sanitizer on commercial and/or industrial hard surfaces.

4. Discussion

ClO_2 gas has been previously applied to hard surfaces, such as stainless steel, paper, wood, epoxy and plastic, to decontaminate pathogens and spoilage microorganism (Han et al., 1999; Han et al., 2003). However this is the first study that validates the applicability of the gas on ready-to-eat meat processing equipment. In general, long contact times were required to completely inactivate microorganisms on hard surfaces: Han et al. (1999) reported more than 6 log CFU/strip reduction of *Lactobacillus buchneri* and *L. mesenteroides* after a treatment with 10 mg/l ClO_2 gas for 30 min, while 12 h were required to completely inactivate spores of *Bacillus thuringensis* (Han et al., 2003). Spores in general have greater resistance to environmental stresses, as compared to free-living cells (Ryu & Beuchat, 2005). The influence of treatment time on

Table 2
End-point method results of *L. monocytogenes* (initial population 5.78 ± 0.19 log CFU/cm^2) inoculated on meat slicer and *L. innocua* (initial population 6.15 ± 0.18 log CFU/cm^2) inoculated hot dog peeler treated with 2 mg/l ClO_2 gas at 25 °C and 75% relative humidity for 30 min.

Inoculation site[a]	Post-treatment growth of *Listeria* (number of positive out of 3 replicate samples)	
	Meat slicer	Hot-dog peeler
A	0/3	0/3
B	0/3	0/3
C	0/3	0/3
D	0/3	0/3
E	0/3	0/3
F	0/3	0/3
G	0/3	0/3

[a] for inoculation site details see Figs. 3 and 4.

ClO_2 effectiveness was also reported in other research works, where longer exposure time led to greater pathogen reduction (Mahmoud & Linton, 2008; Trinetta et al., 2010).

Conversely, fewer researchers have investigated the abilities of the gas toward biofilms. Vaid et al. (2010) compared the effectiveness of ClO_2 gas and aqueous solutions to reduce *L. monocytogenes* biofilms on SS coupons, and reported a 3 log CFU/cm^2 reduction after 10 min treatment both with 0.3 mg/l gas or 7 mg/l aqueous. Our results are comparable with those previously reported: more than 3 log CFU/cm^2 reduction in *L. monocytogenes* biofilm population was reported after 10 min treatment at all the gas concentrations tested. Interestingly, after 2 min injection, independently to the concentration used, all biofilm cells were reduced by about 2 log CFU/cm^2, while only a 1.5 log CFU/cm^2 reduction was reported in planktonic cells with the highest gas concentration at the same sampling time. A progressive decrease was observed over time in planktonic cells, while biofilms reached a plateau of inactivation between 4 and 6 min of exposure, and then decreased again. Similar inactivation patterns were reported by other researchers during the treatment of biofilms with antibiotics (Ashby, Neale, Knott, & Critchley, 1994; Brooun, Liu, & Lewis, 2000; Muli & Struthers, 1998). Our results indicated that biofilms were more sensitive to ClO_2 antimicrobial action initially, but the remaining population was essentially insensitive to increased gas concentrations. This may represent the reason why longer time (70 min) was necessary to completely inactivate biofilms, as compared to free cells living (30 min). Spoering and Lewis (2001) observed similar behaviors in *Pseudomonas aeruginosa* biofilms treated with antibiotics and explained the phenomena of rapid decline followed by a "stationary phase" to the presence of "super-resistant cell fraction" in the biofilm matrix.

Other sanitizers have also been applied to reduce the population of *L. monocytogenes* on hard surfaces. Deza, Araujo, and Garrido (2005) rinsed SS and glass surfaces inoculated with *L. monocytogenes* for 1 min in neutral electrolyzed water solutions (63 mg/l of active chlorine). After treatment, pathogen population was decreased by more than 5.3 log/cm^2 (6 log CFU/50 cm^2), as compared to untreated control samples. The difference with our results can be referred to the type of sanitizer used, but also to the protocol followed for surfaces preparation and inoculation. In the study of Deza et al. (2005) coupons were immersed for 20 min in the microbial suspension and then dried for 15 min. In our work, cells were allowed to attach and dry for 2 h before treatment, increasing physical bonding to the surfaces (Bower, McGuire, & Daeschel, 1996), and therefore simulating the worst case-scenario.

The overall goal of this study was to suggest and validate a ClO_2 gas treatment to completely inactivate *Listeria* on meat processing equipment, such as a meat slicer and hot dog peeler. In the document written by FSIS-USDA (FSIS, 2003) three possible ways to address *L. monocytogenes* contamination are illustrated: 1) expose the product to a post-lethality treatment and use an antimicrobial agent to prevent the growth of the pathogen; 2) use either a post-lethality treatment or an antimicrobial agent/process; 3) have a sanitation program that controls and prevents *Listeria* contamination in the processing environment and on the product. ClO_2 can be included in the latter category and may represent an effective sanitizing procedure to control *Listeria* contamination on food contact and environmental surfaces. An ideal sanitizer should be approved for food contact surface applications, inactivate microorganisms rapidly, be stable and tolerant to different environmental condition, have some detergent characteristics, low toxicity and corrosivity (Schmidt, 1997). ClO_2 gas meets some of the above criteria: it is approved for food-contact surfaces, possesses a broad antimicrobial spectrum, does not produce carcinogenic by-products, its antimicrobial properties are not significantly affected by pH, it is more convenient and cost effective than liquid sanitizers (especially for large scale equipment, such as million gallon aseptic bulk tanks), and saves water use and operational costs (Morgan & Burke, 2010). Currently several manufacturers' supply ClO_2 in solution form, but relatively few gaseous applications are used in the food industry. Consequently, it was important validate its effectiveness on process equipment surfaces and show that complete inactivation of *Listeria* was obtained with a treatment at 2 mg/l for 30 min on the treated meat processing equipment.

5. Conclusions

This study demonstrated the efficacy of ClO_2 gas to reduce the growth of *L. monocytogenes* planktonic cell and biofilms at room temperature on SS coupons. Two mg/l ClO_2 for 30 min (at 25 °C and 75% RH) was tested and used to validate the applicability of the gas as an effective strategy to completely eliminate *Listeria* on RTE meat processing equipment, as requested by the "zero-tolerance" policy (FSIS, 2003). No microbial growth was detected on the surfaces of a treated meat slicer and hot-dog peeler, supporting the direct use of ClO_2 gas as effective sanitizer agent.

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