AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
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TITLE: Efficacy of traditional and non-traditional eco-friendly "green" sanitizers on *Listeria monocytogenes* strains recovered from diverse food production environments.

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EXECUTIVE SUMMARY: *Listeria monocytogenes* (*Lm*), a microorganism causing a wide range of infections in humans, continues to be a recurring issue in food production environments (FPEs) and ready-to-eat products even after a decade of interventions and increased efforts to improve food safety. The challenge of keeping these bacteria out of the food chain comes from the ability of *Lm* to form biofilms and survive a wide range of stress conditions, such as refrigeration temperatures, and also their ability to resist a variety of antimicrobials used in food production. In addition to widely used chemical sanitizers in food industry, alternatives with ingredients deemed to be eco-friendly, also referred to as "green sanitizers", are increasingly emerging. The ability of these sanitizers to effectively control *Lm* when applied to different food-contact surfaces found in food processing facilities, and the impact of temperature on their efficacy is lacking, and not well documented. The aim of the proposed research project is to investigate the effectiveness of traditional chemical sanitizers (e.g. quaternary ammonium compounds [QAC]) and non-traditional eco-friendly sanitizers at different temperatures and organic loads, against diverse *Listeria* isolates grown in broth and on surfaces.

OBJECTIVES:
Evaluate the performance of eco-friendly and traditional sanitizers against *Listeria* spp. at different temperatures and organic loads, grown in planktonic and attached states.

PROCEDURES:

Minimum inhibitory (MICs) and minimum bactericidal concentrations (MBCs). MICs were determined using a slightly modified agar dilution method (e.g., for QACs) described by Elhanafi et al. (2010), and MBCs were determined using a broth microdilution protocol described by Kovacevic et al. (2013). Briefly, for the agar dilution method, strains (6 *Lm*) were grown on Mueller-Hinton agar (MHA-B) (1.2% agar; Difco) supplemented with 5% defibrinated sheep blood and incubated at 37°C overnight. Two colonies were transferred to 200 µl of Mueller-Hinton broth (MHB; Difco), and 5 µl of suspension was spotted in duplicate onto MHA-B plates containing appropriate concentrations of sanitizer compounds, and incubated at 4, 15, and 30°C. Following 24 h (30^{o}C) and

48 h (15°C) or 2 weeks (4°C) of incubation, MICs were determined as the lowest assessed concentrations that prevented confluent growth. For the broth microdilution method, a collection of tested strains was expanded to include additional *Listeria* spp., such as *L. innocua, L. seeligeri* and *L. welshimeri*, as well as additional *Lm* strains from produce, meat, and seafood environments (16 *Lm*, 2 *L. innocua*; 2 *L. seeligeri*; 2 *L. welshimeri*). Five microliters of overnight culture (tryptic soy broth [TSB] at 37°C; final concentration corresponding to 5-6 log CFU [colony forming units]/ml)) was added to 96-well microtiter plates containing 95 µl of TSB and 100 µl of appropriate sanitizer. Microtiter plates were incubated at 4 and 30°C for 24 h. Cultures from each well of the microtiter plate were applied with a sterile loop onto trypticase soy agar with 0.6% yeast extract (TSAYE) plates and incubated overnight at 37°C to confirm the presence or absence of bacterial growth. In each microtiter plate, sterility (TSB only; TSB and sanitizer) and positive controls (cultures in TSB without sanitizers) were included. All assays were run in duplicate and performed at least three times.

Sanitizer efficacy against adhered cells. To determine the efficacy of sanitizers (n=3; 2 QAC-based [Benzalkonium chloride and Lysol], and 1 [Purell] with ingredients listed in EPA's Design for the Environment program's list) against attached cells, five *Lm* strains with the highest MBCs (assessed at 4 and 30°C; *Lm* 2, *Lm* 113, *Lm* 345, *Lm* 354, *Lm* 360), were grown in TSB at 37°C overnight, serially-diluted in 0.1% peptone water (5-6 log CFU/ml), inoculated (1 ml) onto coupons (d=8 mm; stainless steel and plastic) in sterile 24-well microtiter plates, and allowed to adhere for 24 h at 30°C. Following incubation, coupons were rinsed three times, and exposed to temperature adjusted (30 min exposure to 30°C prior to use) sanitizers at manufacturer recommended concentrations and exposure times (MRC/ET). Following sanitizer treatment, coupons were rinsed with sterile deionized water, transferred with sterile forceps into Dey/Engley neutralizing broth (DEB) to neutralize sanitizers, and incubated at 35°C for 24 h. Growth was confirmed by color change (from purple to yellow), and also by streaking cultures onto TSAYE plates (35°C for 24h). In each microtiter plate, sterility and positive controls were included. All assays were run in duplicate and performed at least two times.

Sanitizer efficacy in the presence of organic material. Sanitizer (n=2; a traditional type chlorine-based sanitizer [SparCHLOR] and an eco-friendly alcohol-based sanitizer [Purell]) efficacy against *Listeria* spp. isolates (n=2; 1 *Lm,* 1 *L. innocua*) attached to stainless steel coupons coated with organic material was determined by soaking stainless steel (SS) coupons (d=8 mm) in a produce rinsate (beet, lettuce, and red cabbage) for 24 h at 23°C in 12-well microtiter plates. SS coupons were then moved to new 12-well microtiter plates using sterile forceps and inoculated with 100 µl of overnight culture (grown in TSB at 37°C and 200 rpm). Isolates were allowed to attach for 5 min, 24 h, or 48 h before SS coupons were triple-washed with sterile deionized water and exposed to sanitizer at MRC/ET. Following exposure to sanitizer, coupons were moved using sterile forceps to DEB tubes, and incubated at 35°C for 24 h. Growth was assessed by color

change (from purple to yellow) and confirmed by streaking onto TSAYE plates (35°C for 24 h). All assays were run in duplicate and performed at least three times.

SIGNIFICANT ACCOMPLISHMENTS:

Even though start of the project was postponed by approximately six months due to delays in the construction and certification of the new biosafety level 2 laboratory at the Food Innovation Center, significant data have been collected for each objective. Results have been summarized in abstracts submitted for presentation at the International Association for Food Protection Annual Conference in 2018 (poster presented; Salt Lake City, Utah) and 2019 (awaiting decision on acceptance; Louisville, Kentucky).

Sanitizer selection. Review of existing literature and EPA standards for sanitizers approved for use in food industry was performed. A total of five sanitizers were selected for use in the study, including two sanitizers traditionally used in the food industry: a QAC (Lysol) and a chlorine-based sanitizer (SparCHLOR). A second QAC, benzalkonium chloride (BAC), which has been commonly used in studies that investigated susceptibility of strains to QACs, and is frequently used to compare sanitizer susceptibilities of strains among different studies was also included. The "green" sanitizers were chosen based on their active ingredients found on the EPA's Design for the Environment program's list of approved ingredients. These included a citric acid-based (Pro-San L), and an alcohol-based sanitizer (Purell Food Service Surface sanitizer).

MICs of BAC against dairy *Lm* strains. The MICs of BAC against six *Lm* strains isolated from dairy processing environments were evaluated at 4, 15 and 30°C. Tolerance to BAC is commonly described as strains with MIC values greater than 10 μg/ml. With the highest MIC value 3 μg/ml, all isolates evaluated were determined to be susceptible to BAC. MIC values for two of the isolates were unchanged by temperature (remaining at <1 μg/ml for all three temperatures tested). However, the other four had MIC values that increased slightly as temperature increased, with the MIC value for the most tolerant strain seen at 30°C (3 μg/ml) and the lowest for the same strain found at 4°C (1 μg/ml).

MBCs of diverse *Listeria* spp. strains. The MBCs of four sanitizers (BAC, Lysol, Purell, Pro San L) against 22 *Listeria* spp. isolates were evaluated at 4 and 30°C. MBCs for all sanitizers were lower than the manufacturer recommended concentrations, indicating that when these sanitizers are used following the manufacturer specifications, they are effective against diverse *Listeria* spp. With MBCs 17-38 times (5.21-11.45 μl/l) lower than MRCs, Lysol was the strongest of the sanitizers tested. Pro San L (2-16x; 413-3,300 μl/l) and Purell (4-8x; 36750-73500 μl/l) were determined to be the least effective of sanitizers tested. Temperature had a statistically significant ($P < 0.05$; t-test) impact on MBCs for all sanitizers tested. MBCs for Purell (20/22

isolates) and Pro San L (18/22) were two times lower at 30°C than at 4°C. Similarly, MBC values were approximately 1.5 times lower at 30°C than at 4°C for Lysol (11/22) and BAC (13/22).

Sanitizer efficacy against adhered cells. Adherence to stainless steel and plastic surfaces affected sanitizer efficacy. Following treatment with QACs at MRC/ET, consistent growth was observed for all tested isolates (n=5) on both stainless steel and plastic surfaces, whereas growth was observed for only 2/5 isolates (*Lm* 354, *Lm* 360) when exposed to Purell at MRC/ET on stainless steel surfaces, and 3/5 isolates (*Lm* 2, *Lm* 354, *Lm* 360) on plastic surfaces.

Efficacy of chlorine- and alcohol-based sanitizers against adhered *Listeria* spp. and in the presence of organic load. The ability of a chlorine-based sanitizer (SparCHLOR) and alcohol-based sanitizer (Purell) to control *Listeria* spp. in the presence of organic material was greatly impacted by the time between inoculation and sanitizer treatment. Both sanitizers eliminated cells when adherence time was low (5 min); however, these sanitizers were less effective with extended times between inoculation and sanitizer treatment (24 and 48 h). Purell was unable to eradicate all viable cells after 24 h of adherence time, while SparCHLOR became ineffective after 48 h of adherence.

BENEFITS & IMPACT:

This study confirmed that eco-friendly and traditional sanitizers are effective against *Listeria* spp. when following manufacturer recommendations. However, their efficacy may be reduced under conditions relevant to FPEs (i.e. temperature, presence of organic material, extended times between sanitation treatments). These findings highlight variables that should be considered when determining suitable sanitizers and sanitation schedules in FPEs. Future work will expand upon the data presented here to include more temperatures and sanitizers. Additionally, we will explore how these isolates behave when exposed to sub-lethal sanitizer concentrations, mimicking situations of unintentional sanitizer dilution that may occur in FPEs.

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FUTURE FUNDING POSSIBILITIES: Center for Produce Safety.