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2900 100th Street, Suite 309, Des Moines, IA 50322-3855, USA

Travis Sananikone,[1,2] Derico Setyabrata,[2] Karina Desiree,[1,3] Peter Rubinelli,[1,3] and Jennifer C. Acuff[1,3]*

[1]2650 N Young Ave., Department of Food Science, University of Arkansas System Division of Agriculture, Fayetteville, AR 72704, USA

[2]Department of Animal Science, University of Arkansas, Fayetteville, AR 72701, USA

[3]Department of Food Science, Center for Food Safety, University of Arkansas System Division of Agriculture, Fayetteville, AR 72704, USA

Interventions for Dry-Aged Beef Crusts Inoculated with *Salmonella* Heidelberg, *Escherichia coli* O157:H7, and *Listeria monocytogenes* 4b

ABSTRACT

Dry aging enhances unique steak flavors but may allow pathogenic bacteria and fungi to grow on the crust, which is discarded before sale. This study evaluated six interventions for reducing *Salmonella enterica*, Shiga toxin-producing *Escherichia coli*, and *Listeria monocytogenes* populations on dry-aged beef crusts to reduce the risk of incorporating crusts into other products for product enhancements. Crusts were spot-inoculated with rifampicin-resistant *Salmonella* Heidelberg, *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b at 7.0 log CFU/mL. Six pre- and post-treatment crusts and negative and positive controls were sampled in triplicate (N=24). Treatments were hot-air drying (6 h, 60°C), *sous-vide* (2 h, 60°C), ambient water spray (1 min, 15°C), warm water spray (1 min, 50°C), 2% lactic acid spray (1 min), and UV-C light (30 min, 253.7 nm). Two cores per crust (25-mm diameter, total of 9.81 cm² per crust) were excised, diluted, and spiral-plated onto selective and differential agar with 200 ug/mL rifampicin to determine pathogen log reductions. Sous-vide exhibited the highest log reductions of 5.4, 5.0, and 4.3 log CFU/cm² for S. Heidelberg, *E. coli*, and *L. monocytogenes*, respectively. Other treatments' reductions ranged from 0.1-4.9 log CFU/cm², underscoring the variability of treatments in reducing foodborne pathogens on dry-aged beef crusts.

INTRODUCTION

Dry aging is a traditional value-adding process in which raw meat is held under controlled temperature and humidity for 28 to 55 days without any protective barrier. Optimal parameters are suggested to be 0-4°C at 75% relative humidity and with an airflow of 0.5 to 2 m/s within the dry aging chamber to obtain the best product *(6)*. The aging process significantly enhances the perceived palatability of the steak through a multitude of processes, such as dehydration, proteolytic enzyme degradation and microbial activity *(26)*. This leads to increased availability of flavor precursors, such as amino acids, reducing sugars and fatty acids, which promotes Maillard reaction during cooking to develop unique dry-aging flavors desired by consumers *(16, 19, 27, 38)*. These pro-

*Author for correspondence: Phone: +1.479.575.2108; Email: jcacuff@uark.edu

cesses, particularly the proteolytic enzyme activity, also aid in tenderizing the steak and reducing the toughness by breaking down muscle structure *(9)*.

During the dry aging process, the product can lose up to 23% of its total weight from dehydration as the product is exposed to the environment without any protective barrier *(6)*. The process will lead to the formation of crusts (dehydrated meat surface) that require to be trimmed before being sold due to palatability and safety concerns, making the dry-aging process expensive and wasteful. However, several studies have shown that the discarded crust has significant flavor potential and functionality, and it could be used as a novel ingredient to recover some of the losses if quality is not degraded too much *(15, 17, 26, 36)*.

Despite these potential reductions, bacteria can still be present on the outer trimmings and crusts of dry-aged beef. One study of beef aged for 21–28 days at 0.5°C and 75% relative humidity revealed significant levels of indicator organisms, including aerobic mesophiles (9.11 ± 9.10 log CFU/g), *Pseudomonas* spp. (8.60 ± 8.60 log CFU/g), *Lactobacillus* (4.44 ± 4.25 log CFU/g), and *Enterobacteriaceae* (5.22 ± 4.94 log CFU/g) on the trimmings *(33, 35)*. While these microbial population levels would normally indicate product spoilage, applying interventions to reduce the microbial load may allow for incorporation or further processing of the trimmings to avoid waste. However, there is a potential for the growth and persistence of pathogens like *Listeria monocytogenes*, Shiga toxin-producing *E. coli*, and *Salmonella* spp., in these conditions due to contamination that occurs during processing *(14, 30)*, so it would be prudent to identify interventions to reduce pathogen and other microbial populations before repurposing.

This study focused on the interventions for dry-aged beef crusts inoculated with *Listeria monocytogenes* 4b, *Salmonella* Heidelberg, and *Escherichia coli* O157:H7. *Salmonella* Heidelberg (ATCC 8326) was used in this study since 6.2% of salmonellosis cases are directly attributable to beef products *(10)*, and because this strain displayed ability to exhibit rifampicin resistance and was associated past outbreaks involving dairy calves and cattle *(34)*. *Escherichia coli* O157:H7. *Escherichia coli* O157:H7 is commonly found in the gastrointestinal tract of cattle and may contaminate beef products during the fabrication process or post-processing, as was the case for the strain in this study (USDA-FSIS-380-94), which isolated from a salami outbreak in Washington and California *(5)*. *Listeria monocytogenes* is associated with many raw foods, including beef products through surface and environmental contact contamination from improper sanitation. Its ability to survive at refrigeration temperatures allows it to remain present in contaminated stored beef, making it a significant food safety concern in the meat industry, as well as the dry aging industry, so *Listeria monocytogenes* 4b (ATCC 43257), isolated from a Mexican-style cheese outbreak in California was selected for the present study *(4)*.

The treatment methods (hot-air drying *sous-vide* cooking, ambient water spray, warm water spray, 2% lactic acid spray, and UV-C light) and parameters in this study were based on the requests of the funding partner and supplier of the trimmed crusts, as well as other studies that have used similar treatment parameters to reduce microbial populations. Other studies have found positive results for decreasing surface populations on beef using UV-C treatments *(32, 35)*, with some noting minimal changes to color and oxidation *(11)*. Hot air drying and *sous-vide* cooking are increasingly used methods for at-home and restaurant preparation since equipment has become smaller and more affordable *(13, 17, 19, 21)*. Finally, antimicrobial washes have been frequently investigated to inactivate foodborne pathogens from meat and poultry products *(7, 23)*. While the interest in dry-aged crust usage has been increasing, information on microbial safety and potential intervention processes of dry-aged crusts is limited. The objective of this study is, therefore, to determine the efficacy of different intervention methods to increase the safety of dry-aged beef crusts.

MATERIALS AND METHODS

Dry-aged crust storage and testing

Dry-aged crust samples were obtained from a commercial dry-aging facility and stored in a freezer (-20°C) until use. Samples were thawed in a 4°C refrigerator overnight and background microbial populations were enumerated prior to inoculation via plating on tryptic soy agar, *Listeria* Petrifilm™ (3M Petrifilm™ , Maplewood, MN), ECC Petrifilm™ (3M Petrifilm™, Maplewood, MN) for the detection of *E. coli* and coliforms, and Xylose Lysine Tergitol-4 (XLT-4, Hardy Diagnostic, Santa Maria, CA) for detection of *Salmonella* spp. Growth was found on all selective and differential media except for the environmental *Listeria*, signifying the presence of all organisms tested besides *Listeria* on the uninoculated samples. These populations were not recovered on media supplemented with rifampicin, which justified the use of rifampicin-resistant strains in the study.

Organisms

To prepare rifampicin-resistant strains, a single bead for each bacterial strain (non-rifampicin-resistant) was transferred from frozen stock cultures maintained on glycerol beads into individual 5 mL of Tryptic Soy Broth (TSB; Difco, BD, Sparks, MD) and incubated at 37°C for 24 h. Following this, 5 mL of TSB containing 5 ug/ml rifampicin were inoculated with 200 µL culture from the 24-hour culture and then incubated for another 24 h. After incubation, 200 µL from this medium was then transferred into 5 mL TSB containing 10 ug/ml rifampicin and incubated at 37°C for 24 h. This was repeated in increments of 10 ug/ml rifampicin until the final rifampicin concentration was 200 ug/ml

and culture growth were observed (turbidity) for all three strains *(18)*. The rifampicin resistance was verified again by enumerating from tubes to TSA plates containing 200 ug/mL rifampicin added. The rifampicin-resistant strains were maintained at -80°C on protectant beads in TSB with glycerol (Microbank™, Pro-Lab Diagnostics, Ontario, Canada) and working cultures were maintained on Tryptic Soy Agar (TSA; Difco, BD, Sparks, MD) plates with 200 ug/ml rifampicin at 4°C. Working cultures were transferred monthly to maintain their viability.

One isolated colony from each strain's working cultures were selected and transferred separately to a tube containing 10 mL TSB with 200 ug/ml rifampicin at 37°C for 24 h. Following incubation, the broth cultures were centrifuged at 3,000 g for 5 minutes to obtain a pellet. After discarding the supernatant, the pellet was washed by resuspending in 10 mL of 0.1% sterile peptone water. The culture was then centrifuged using the same parameters as before. Following the second centrifuge and discarding the liquid supernatant, the pellet was then resuspended in 10 mL of 0.1% sterile peptone water to obtain a stock cell suspension for the culture. This was repeated for each culture. Each culture was then combined into one cocktail for a total volume of 30 mL at a concentration of 9.0 log CFU/mL. The concentration of each pathogen was confirmed via plating on selective agar prior to conducting each replication.

Sample preparation and inoculation

Crust samples were cut into five-by-five cm pieces for 24 sub-primal samples (25 cm^2 each). Next, the samples were placed on a sanitized tray lined with aluminum foil. One mL of the inoculum cocktail was dispensed onto each sample of crust samples and then spread using a sterile spreader to ensure even distribution across the surface of the beef crust for 21 of the samples. Three of the samples were left uninoculated to serve as the negative controls. The 21 inoculated samples were allowed to dry for 30 minutes under the biosafety cabinet *(3)*. After drying, three of the crust samples were placed on a separate, aluminum lined tray to serve as positive controls (inoculated but not treated).

Treatments

Six treatment methods were explored to investigate the efficacy of inactivating *Listeria*, *Salmonella*, and *E. coli* present in dry-aged crust samples. These methods were chosen since they had significance in treating non-dry-aged beef products in other studies. Each treatment was performed in triplicate for a total of three samples per treatment per repetition.

Dehydration was performed in a commercial dehydrator (CFD-N051-WUS, Cosori). Three samples were placed onto the lowest level of the dehydrator at 60°C for six hours in line with machine instructions.

Dry-aged crust samples were vacuumed sealed by a packaging machine (FM2000, FoodSaver) and individually and placed in a hot water bath (APW Wyott 3D-W43V, American Permanant Wars Co., LLC, Allen, TX) at 60°C with an immersion circulator (SmartVide 7, Sammic Corp., Evanston, IL) for two hours. To halt the cooking process, the vacuum-sealed bags were immediately placed in an ice bath following completion of treatment.

The UV treatment was done using a countertop UV sanitizer box (Philips Hue UV Light Sanitizer Box, Signify N.V., Netherlands). The samples were placed with the dried portion on top within the UV chamber and set to 15 minutes, as the device had a maximum time limit of 20 minutes. This was repeated for a total of 30 minutes of UV-C (253.7 nm wavelength) treatment *(2)*.

Three rinse treatments were tested in the present study. An ambient temperature water rinse (15°C) was sprayed onto three meat samples suspended on a hook. The samples were sprayed for one minute and then allowed to drip for an additional minute immediately after. This rinse was conducted using a sprayer (Flo-Master, Hudson, Lowell, MI) and filled with 1-L sterile deionized water. To maintain consistency, the pump was compressed 30 times before each rinse and sprayed 5 cm from the nozzle to the meat's surface *(37)*. A warm temperature (50°C) rinse was conducted on three different samples after the ambient temperature rinse. The warm temperature rinse utilized the same 2-L hand pump (sanitized with 70% ethanol solution between uses) and was filled with 1-L of preheated and sterile deionized water. When spraying, the water temperature dropped due to the 5-cm nozzle-to-surface spraying distance. Therefore, the water was heated to 55°C before being transferred to the pump. This accounted for the heat loss and allowed the water to reach the desired temperature of 50°C when contacting the crust, which was verified with a T-Type thermocouple (52-260HZCAL 52-2 Dual Input Digital Thermometer; Fluke; Everett, WA). Finally, a 2% lactic acid rinse (lactic acid 88% F.G., Birko Corp., Henderson, CO) was conducted following the warm temperature rinse on three new, untreated samples. The 2-L hand pump was again sterilized and filled with 1-L of 2% lactic acid. This solution was then sprayed onto the samples following the previous procedure.

Sampling

From each of the controls and treated five-by-five cm samples (N=24), two cores with a surface area each of 4.92 cm^2 (total 9.84 cm^2) were taken using a Burkle BeefSteaker (BeefSteaker, Burkle, Bohemia, NY) from the surface of the crusts. Each two-core samples were placed into a sterile bag (Whirl-Pak® 710-mL Filter Sterilized Bags, Whirl-Pak, Pleasant Prairie, WI) filled with 20 mL of 0.1% peptone and stomached at 360 rpm for 30 s. The samples were then serially diluted with 0.1% peptone and spiral plated via Whitley WASP Touch® (Whitley WASP Touch®, Don Whitley Scientific, West Yorkshire, BD16 2NH, UK) onto Modified Oxford agar (MOX, Difco, BD, Sparks, MD) made

using Modified Oxford Antimicrobial supplement (Oxford *Listeria* selective supplement, Millipore, Burlington, MA) and MacConkey Agar with sorbitol (SMAC, Difco, BD, Sparks, MD), both containing 200 ug/mL of rifampicin additive. The SMAC plates were incubated at 35°C for 24 h while the MOX plates were incubated for 48 h.

Statistical analysis

Each experiment consisted of inoculating with a new cocktail and testing each treatment. The experiment was performed in triplicate on different days with a new cocktail and materials. Log reductions were calculated by converting bacterial counts from the treated samples into log CFU/cm^2 and then compared against the initial log CFU/cm^2 value of the untreated, inoculated sample. The data were analyzed using R (version 4.4.2) by running ANOVA and pairwise comparisons (Tukey's HSD) to determine if the differences in log reductions of treated samples were statistically significant compared to those of the untreated samples.

RESULTS AND DISCUSSION

The dry aging process requires a lengthened amount of time in controlled environmental conditions to achieve desirable qualities and enhanced flavors such as a more tender mouthfeel *(14, 27)*. As a result, dry-aged beef is usually more costly (up to 25% more) compared to conventional steaks and beef products *(6)*. However, as the crust is trimmed off and not consumed, there is a possibility of profit loss for producers and an increase in costs for consumers. Recognizing the potential value in repurposing dry-aged crusts, researchers and industry professionals have explored the possibility of rendering these crusts into safe-to-consume meat products such as ground dry-aged beef or dry-aged sausages, allowing for lower production costs and consequently lower consumer costs of purchasing dry-aged beef products. However, the main challenge in repurposing these trimmings is the presence of harmful bacteria on the products. Although consumers actively seek out dry-aged products for the unique flavor profile, safety should be the main priority of both consumers and producers *(31)*.

Initial pathogen populations were obtained from inoculating the culture cocktail onto dry-aged beef crusts and subsequent enumeration following the drying period. Initial mean bacterial populations were 7.2 ± 1.3 log CFU/cm^2, 7.6 ± 0.4 log CFU/cm^2, and 6.9 ± 1.2 log CFU/cm^2 for *Escherichia coli* O157:H7 *(Fig. 1)*, S. Heidelberg *(Fig.2)*, and *L. monocytogenes* 4b *(Fig. 3)*, respectively. *Sous-vide* cooking at 60°C for 2 hours observed a 5-log reduction for *E. coli* O157:H7 (5.0 ± 0.8 log CFU/cm^2) and S. Heidelberg (5.4 ± 1.0 log CFU/cm^2), but not *L. monocytogenes* (4.3 ± 0.9 log CFU/cm^2). The decimal reduction time suggested that extended the cooking time to 2.5 or 3 h would have achieved the target 5-log reduction. Log reductions from *sous-vide* treatments were also significantly different compared to those of the control ($p < 0.05$). Another study *(21)* examined the efficacy of *sous-vide* treatments targeting *Salmonella* Montevideo and *E. coli* O157:H7. Patil et al. (2024) found that significant average log reductions of over 5-log CFU/g can be observed for *Salmonella* and STEC at a temperature of 62.5°C and time of 2 hours for ground beef products. Similarly, the crust samples in this study were observed to have significant average log reductions of 5-log for *Salmonella* and STEC at a similar temperature (60°C) for 2 hours. However, Patil et al. (2024) also found that prolonged exposure to sublethal *sous-vide* temperatures (4–60°C) may cause challenges in the ability for the treatment to reduce pathogen populations to a safe level *(21)*. As observed in the current study, *sous-vide* was unable to achieve significant 5-log reductions for *L. monocytogenes* 4b – emphasizing the need to explore higher temperatures before considering the use of *sous-vide* as a viable treatment method for dry-aged crusts. The funding source requested this treatment's investigation, but applications for *sous-vide*-cooked crusts are not entirely clear, since rancidity and other quality characteristics would need to be investigated to understand if the process produced off-flavors.

When crusts were dehydrated via air dryer at 60°C for 6 hours, mean log reductions of 4.7 ± 2.2 log CFU/cm^2, 5.8 ± 0.8 log CFU/cm^2, and 2.7 ± 0.9 log CFU/cm^2 were observed for *E. coli* O157:H7, S. Heidelberg, and *L. monocytogenes*, respectively. Although the reductions of *L. monocytogenes* did not reach the 5-log reduction goal like those of *Salmonella* and STEC O157:H7, they were all significantly higher reductions than the other non-thermal treatments and higher than the nontreated, control samples ($p < 0.05$). However, a limitation worth noting is that in the present study, injury-recovery methods were not performed, so it is possible that the means overestimate the reductions if some populations were sublethally injured. Other studies *(12, 24)* have also shown hot-air heating with similar tools as being insufficient for 5-log reductions of *L. monocytogenes*, though the experiments were conducted on high-moisture fruit and vegetable items and are not directly comparable. Other studies examining jerky products show mixed results of pathogen reduction, but methods and product composition have been deemed to play significant factors *(19)*. Similarly, dry-aged crusts subjected to dehydration at 60°C in the present were also unable to present a log reduction greater than 5-log. This treatment temperature may account for the inability of warm air dehydration and *sous-vide* to achieve the desired 5-log CFU/cm^2 reduction for *L. monocytogenes*. Another explanation may be that *Listeria* can produce heat shock proteins in response to temperatures above 45°C *(20)*. Besides increased production of heat shock proteins, studies have shown that *Listeria* spp. are able to exhibit other heat-resistant traits while under stress and when subjected to non-lethal temperatures *(8, 28, 29)*. While it was unexpected that the strain of *L. monocytogenes* 4b exhibited

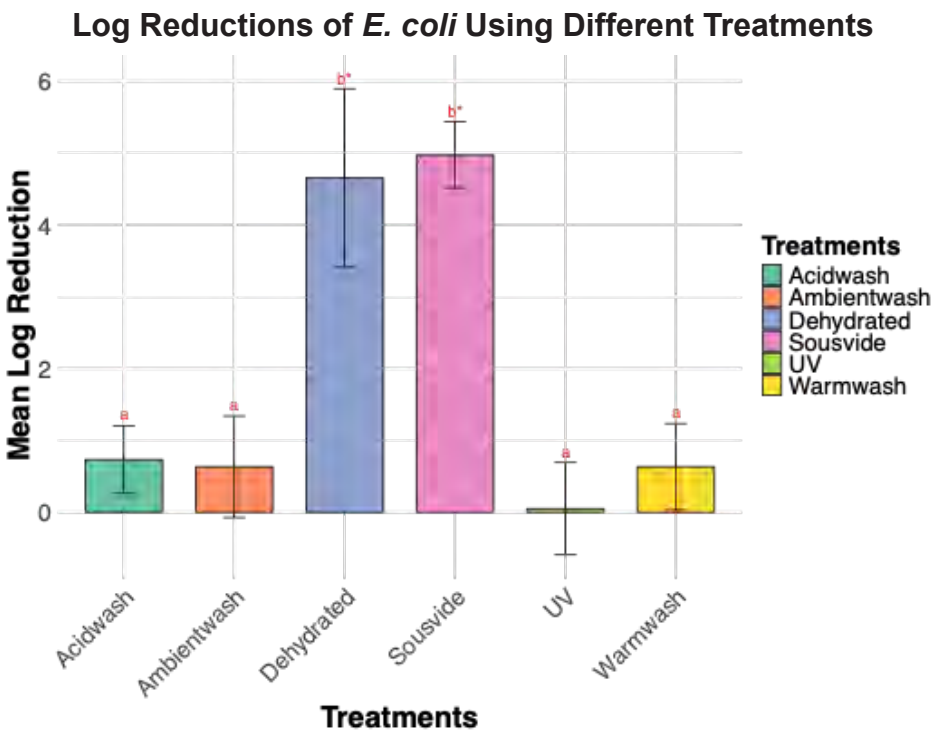

Figure 1. Log Reductions of *E. coli* O157:H7 on dry-aged beef crusts when subjected to various treatments. The asterisk indicates that the mean log reduction was significantly different from the nontreated. Lowercase letters indicate treatments that resulted in significantly reduced populations compared to the initial (nontreated) population, which was 7.2 ± 1.3 log CFU/cm².

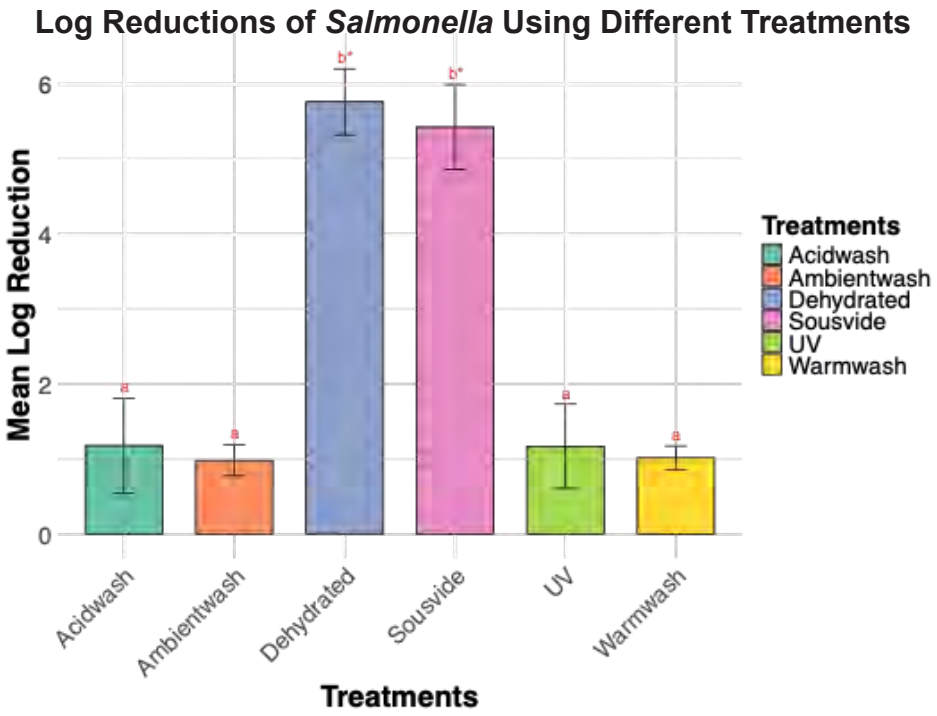

Figure 2. Log Reductions of S. Heidelberg on dry-aged beef crusts when subjected to various treatments. The asterisk indicates that the mean log reduction was significantly different from the nontreated. Lowercase letters indicate treatments that resulted in significantly reduced populations compared to the initial (nontreated) population, which was 7.6 ± 0.4 log CFU/cm².

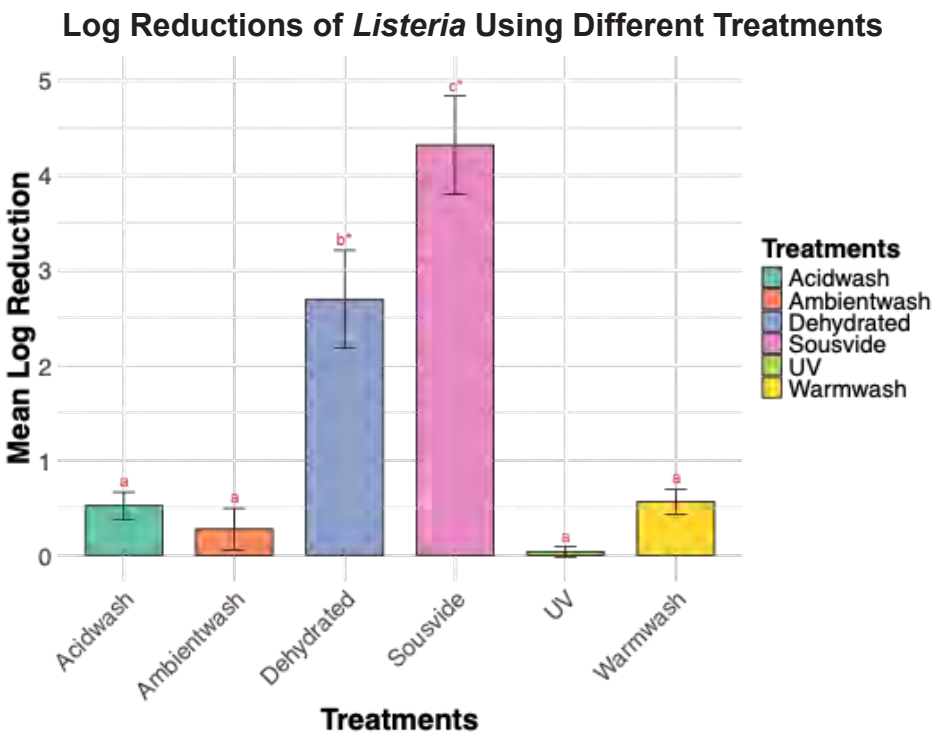

Figure 3. Log Reductions of *L. monocytogenes* on dry-aged beef crusts when subjected to various treatments. The asterisk indicates that the mean log reduction was significantly different from the nontreated. Lowercase letters indicate treatments that resulted in significantly reduced populations compared to the initial (nontreated) population, which was 6.9 ± 1.2 log CFU/cm².

greater resistance to thermal treatments than the other pathogens, it has been noted previously that the response of 4b strains can be variable (*27*). The same strain used in the present study was categorized as having low heat resistance by Shen et al. (2014), but inoculation procedure and time subjected to sublethal temperatures are known to impact survival of microorganisms, which could have contributed to the differences observed between the studies. Regardless, temperature and duration positively and significantly affected mean log reductions of *L. monocytogenes* populations. Subsequently, a combination of multiple treatments or a higher treatment temperature may deliver higher log reductions of *Listeria* in addition to *Salmonella* and *E. coli*.

The other four treatments were unable to achieve 5-log CFU/cm² reductions for *E. coli* O157:H7 *S.* Heidelberg, and *L. monocytogenes*. Ultraviolet light treatment resulted in reductions of 0.1 ± 1.1 log CFU/cm² for *E. coli* O157:H7, 1.2 ± 1.0 log CFU/cm² for *S.* Heidelberg, and 0.0 ± 0.1 log CFU/cm² for *L. monocytogenes*. The 2% lactic acid spray treatment resulted in mean reductions of 0.7 ± 0.8-log CFU/cm² for *E. coli* O157:H7, 1.2 ± 1.1-log CFU/cm² for *S.* Heidelberg, and 0.5 ± 0.3-log CFU/cm² for *L. monocytogenes*. The ambient temperature water rinse provided mean reductions of 0.6 ± 1.0-log CFU/cm² for *E. coli* O157:H7, 1.0 ± 0.3-log CFU/cm² for *S.* Heidelberg, and 0.3 ± 0.4-log CFU/cm² for *L. monocytogenes*. The final treatment, warm water rinsing (50°C), resulted in mean reductions of 0.6 ± 1.0-log CFU/cm² for *E. coli* O157:H7, 1.0 ± 0.3-log CFU/cm² for *S.* Heidelberg, and 0.6 ± 0.2-log CFU/cm² for *L. monocytogenes*. None of the reductions that resulted from the washes in the present study were significantly different from the controls or from one another ($p > 0.05$). Despite the limited microbial reductions observed for these treatments, they may be useful in combination with others in a multi-hurdle approach.

Although water rinses were unable to show significant reduction in pathogen levels, one study by Yoder et al. (2010) that tested a range of temperatures similar to the presented study found that water temperature is a principal factor in reducing pathogen populations from beef carcasses. Yoder et al. (2010) compared three different water temperatures: hot (77°C), warm (54°C) and cold (15°C); and found hot water to be the most effective at achieving significant reduction in pathogens (3.8 log CFU/cm²), while a warm wash only reduced pathogens by 1.1 log CFU/cm² (*37*). This study, along with the findings of the present study, highlights the need to use hot water to achieve pathogen reduction. Both dehydration and *sous-vide* were thermal-based treatments (60°C) that were performed for a couple of hours (six hours for dehydration and two hours for *sous-vide*). The warm water rinse was also a thermal based treatment (50°C) but occurred

under a lower temperature and shorter duration than the two previously mentioned treatment methods. The remaining nonthermal treatment methods were also performed for shorter durations and were significantly less effective.

CONCLUSIONS

Due to the possible presence of yeast, mold, fungi, and pathogenic bacteria, adequate interventions for dry-aged beef crusts should be developed. Dehydration in a hot air dryer and *sous-vide* cooking at 60°C for six hours were sufficient for achieving 5-log reductions for *Salmonella* Heidelberg and *Escherichia coli* O157:H7. Despite these reductions, the investigated treatments would still only be recommended to reduce microbial load on products meant for further processing. Further study of other forms of thermal and/or non-thermal treatments should be conducted to increase the safety of this product to make use of the unique flavors and properties that dry-aging produces and reduce waste. Additionally, a future study examining treatment methods on dry aged steaks inoculated prior to the aging process may give a better understanding of the effectiveness of interventions against pathogen populations that survive initial fabrication processing. Achieving proper treatment of dry-aged beef crusts will allow for producers to safely reincorporate or sell products with unique flavors and qualities that might otherwise be discarded.

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