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Evaluation of STEC on Beef Intended for Non-intact Use After Treatment with Microwaves and Low Temperature Storage

ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is a preeminent concern for the beef industry because of its ability to produce life-threatening complications. Thus, the objective of this study was to evaluate the effectiveness of microwaves, in combination with either refrigeration or frozen storage, as a post-harvest, post-packaging intervention to reduce STEC (serogroups O157:H7, O26, O103, O111, O121, O45, and O145) on vacuum-packaged beef. Beef striploins samples inoculated with STEC, were treated with microwaves (MW, 472.6 kW/s), or assigned for control (CTR, not subjected to microwaves). All samples were stored either at refrigeration (0–4°C) or frozen storage temperature (-18°C or below). The microbial analyses were performed at day 1, 5, 10 (cold storage) or at 7, 14, and 21 days (frozen storage) by surface swab sampling. The bacterial count results showed that samples under frozen storage had a greater reduction of inoculated STEC than that of samples subjected to refrigeration temperature ($P < 0.001$); however, microwave-treated samples did not undergo significant STEC reductions compared with controls ($P < 0.05$). These results suggest that the microwave treatment used in this study did not contribute to STEC reduction; rather, the effects observed were the product of temperature storage causing significant bacterial reduction ($P < 0.01$).

INTRODUCTION

Escherichia coli, a Gram-negative facultative anaerobe naturally present in the human gastrointestinal tract, can remain in the intestinal lumen without causing harm. However, some *E. coli* strains can cause illness and death in immunocompromised hosts *(8, 21)*. The Shiga toxin-producing *Escherichia coli* (STEC) comprise a group of enteric pathogens that can cause severe gastrointestinal disease that ranges from mild diarrhea to the most severe complications, including hemorrhagic colitis and hemolytic uremic syndrome (HUS). Majowicz et al. (2014) reported that STEC annually causes 2,801,000 acute illnesses, leads to 3890 cases of HUS, and causes 230 deaths worldwide *(20)*. *E. coli* O157:H7 is the strain most often associated with severe illness complications. However, non-O157 strains

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have also been linked to similar outbreaks *(5)*. The Centers for Disease Control and Prevention (CDC) has reported that six O groups of non-O157 STEC (serogroups O26, O45, O103, O111, O121, and O145) cause 71% of Shiga toxin-producing *E. coli*-related illnesses. Currently, non-O157 STEC have little public health surveillance, and many cases may go undiagnosed or unreported *(7)*.

According to the United States Department of Agriculture (USDA) Draft Risk Profile for Pathogenic Non-O157 Shiga Toxin-Producing *E. coli*, only small numbers of STEC cells are required on the food product to cause life-threatening damage to organ systems and to cause illness in children, the elderly, and other susceptible populations *(29)*.

Because of the high risk associated with STEC, under the Federal Meat Inspection Act (21 U.S.C. 601(m)(1)), the Food Safety and Inspection Service (FSIS) considers all raw non-intact beef and raw intact beef intended for use in raw non-intact product as contaminated and adulterated if these pathogens are present on these products *(26)*. One of the most common non-intact products is mechanically tenderized beef, a beef product that has been subjected to a process in which a set of blades or needles penetrate the meat, piercing and cutting through the muscle fibers and connective tissue of primal or subprimal cuts, thus improving tenderness of tough beef cuts *(9, 13, 14)*. If beef primal and subprimal cuts are intended for use in raw non-intact products or the intended use is unclear, the FSIS considers these products subject to the FSIS sampling for Raw Beef Manufacturing Trimmings (MT60) STEC testing and sampling program *(27)*.

Mechanical tenderization of beef has the inherent risk of potentially transferring pathogenic bacteria located on the beef surface into the previously sterile interior of the cut *(9, 13)*. For that reason, development of antimicrobial interventions is necessary, so that such interventions applied to the surface of the subprimal prior to tenderization, reduce or prevent the transfer of bacteria to inside the meat cut, thus decreasing the risk posed by foodborne pathogens such as STEC. The meat industry has been very proactive in developing interventions, best practices, and guidance for pathogen control for operations producing beef subprimals and whole cuts subjected to mechanically tenderization/enhancement *(10, 13, 14, 16, 31)*. However, there is a potential risk to consumers if food retailers, stores, restaurants and/or institutional settings obtain whole cuts (not intended for non-intact products) that have not been tested for STEC and subject these cuts to mechanical tenderization or enhancement at their establishments *(30)*. That practice can lead to pathogen translocation during the mechanical process and survival of the pathogen as the result of undercooking and consumer preferences *(15, 29)*.

Previous studies in our laboratory have shown that microwave technology has the capability to reduce or eliminate common bacterial pathogens and quality-affecting organisms in foods. Published studies by our group include studies of the elimination of mold from bread (99.9%) *(19)* and reduction of *Salmonella* (99%) in shell eggs without cooking the egg or changing its quality attributes *(18)*. A few studies have evaluated the antimicrobial effect of microwaves on meat and meat products *(2, 3, 23, 24, 25, 32)*, but to our knowledge, no study has evaluated the effectiveness of microwave technology on STEC O157:H7 and non-O157 STEC serogroups on fresh beef. Because of that, the objective of this study was to evaluate the effectiveness of a unique microwave treatment, combined with cold storage conditions (refrigeration or frozen storage), as a post-packaging intervention to reduce Shiga toxin-producing *E. coli* on beef intended for use in both intact and non-intact beef products.

MATERIALS AND METHODS

Product procurement

Beef striploins (Institutional Meat Purchase Specifications IMPS 180) were procured from a federally inspected meat processing facility located in Lubbock, TX. Each beef striploin was portioned into three equal sections. The average weight of the striploin sections was 1.530 Kg ± 0.240 (SD). Two of the striploin sections (from the same striploin) were randomly assigned to the treatments, and the third was used for measurement of the initial concentration of inoculated STEC. Six striploins were used per STEC serogroup, with a total of 42 striploins per replication. The experiment was conducted in triplicate (three individual, separate trials).

The experiment had two treatments: control *(CTR)* and microwave *(MW)*. The treatment *CTR* was a negative control in which samples were inoculated with STEC, vacuum packed, and stored at 4°C or -18°C but not subjected to any microwaves. For the treatment *MW*, the samples were inoculated, vacuum packed, treated with microwaves, and stored at 4°C or -18°C.

Inoculum preparation

For the inoculum, three strains of each STEC serogroup (O26, O45, O103, O111, O121, O145, and serotype O157:H7) were used, and each serogroup was processed separately. The decision to include a combination of strains per serogroup was based on the need to "encompass the variability among organisms" *(22)* that we could potentially encounter in a real industrial setting. The STEC strains used for this study were obtained from the frozen microbial collection of the TTU Food Safety Laboratory. For the preparation of each STEC serotype inoculum, a 1µl loop was transferred from the frozen culture vial to a 10-ml tube of Tryptic Soy Broth (TSB; EMD Millipore Co., Billerica, MA), and the tubes were incubated at 37°C for 18 to 24 h. After incubation, 1 ml of each STEC inoculum was transferred into fresh 9-ml TSB tubes, and the fresh tubes were incubated for 18 to 24 h. The double enrichment was used to ensure the recovery of injured STEC cells. The 10-ml

inoculum tubes were then transferred into 90-ml TSB bottles in order to have the volume necessary for the experiment. The three bottles of 100 ml for the same STEC serogroup/serotype were combined, to obtain 300 ml of inoculum of each STEC. The 300-ml STEC inoculums were separately diluted with 2700 ml of BPW, to make 3 l of inoculum of each STEC type *(Table 1)*. The inoculum preparation was carried out using the appropriate aseptic techniques to avoid any cross-contamination between STEC serogroups and was conducted at the Biosafety Level 2 (BSL2) food microbiology laboratory of Texas Tech University.

Inoculation

Striploin samples were inoculated by dipping them for approximately 20 seconds into one of the 3-l STEC inoculums contained in a plastic tub. After inoculation, the samples were allowed to sit at 4°C for 30 min to facilitate bacterial attachment and allow dripping of excess inoculum from the samples. Bacterial concentration (attachment) at day zero was sampled via surface swabbing of the inoculated beef (to be further described). The 3-l inoculums were used for all the samples in a replication, and the same strains were used for the three individual trials (replications) of the study, for uniformity of the experiment. After inoculation, the samples were vacuum packed in plastic bags (Sealed Air Corporation, Cryovac® bags 8 in × 22 in, Charlotte, NC) using a vacuum packaging machine (UltraSource LLC., Ultravac® 250 Single Chamber Vacuum Packaging Machine, Kansas City, MO). The samples were labeled with the treatment type, the storage temperature, and the sampling day. Inoculation was conducted at the Texas Tech University Biosafety Level 2 (BSL2) laboratory. The samples were transported under refrigerated conditions to the Experimental Sciences Building at Texas Tech University, where the samples assigned to *MW* were treated with microwaves.

Treatment with microwaves

The samples assigned to *MW* were treated with microwaves using an industrial, custom-made conveyorized microwave. The microwave settings were six magnetrons at 95% power exposing each sample to a wattage of 472.5 kW/s, and the average surface temperature of samples right after microwave treatment was 34 ± 4°C. After MW treatment, the samples were stored either at 4°C (refrigeration) or -18°C (frozen storage). The microwave treatment settings were chosen on the basis of preliminary trials (data not shown), which demonstrated that those settings were non-destructive for the beef protein, causing no observable changes in color or texture ("cooked" appearance). Because the objective of the study did not include the evaluation of meat quality attributes, surface pH water activity or sensory characteristics were not monitored or measured.

Quantification of microbial reduction

The objective of this study was accomplished by quantifying and comparing the concentrations (log CFU/100 cm^2) of STEC on striploin sample surfaces at each sampling storage interval after an initial post-harvest, post-packaging microwave treatment. Surface populations

TABLE 1. Individual strain identification and concentration (log CFU/ml) of Shiga toxin-producing *Escherichia coli* serogroups used in the inoculum cocktail and their respective post-inoculation attachment (CFU/100 cm^2) on beef striploins

STEC Serogroup	Strain 1	Strain 2	Strain 3	Inoculum Concentration (log CFU/ml)	SD (±)	[1]Post-inoculation attachment (log CFU/100 cm^2)	SD (±)
O157:H7	EC 994	EC 922	EC 966	7.64	0.31	6.22	0.28
O145	ECRC 95.0187	ECRC 9.0538	E1-169	7.85	0.39	6.40	0.29
O121	ECRC 3.1064	E1-158	E1-159	7.92	0.11	6.07	0.40
O111	ECRC 4.0005	ECRC 3.1009	BAA 179	7.64	0.36	6.30	0.34
O103	ECRC 97.1377	ECRC 97.1241	ATCC 23982	7.74	0.23	6.00	0.28
O45	ECRC 92.0244	ECRC 2.1064	E1-138	7.63	0.31	5.87	0.34
O26	ECRC 0.1302	ECRC 7.1556	BAA 1653	7.61	0.21	6.12	0.27

[1]Beef striploins (n = 6/serogroup) were inoculated in plastic tubs containing 3 L of inoculum cocktail. The samples were immersed into the inoculum for 20 s and then allowed to drip for 30 min. Surface swabs (100 cm^2) were taken prior vacuum packaging for verification of STEC attachment.

were determined by obtaining a 100 cm^2 swab from the sample surface, using sterile templates (3M Cattle template USDA 100, St. Paul, MN) and sterile sponges pre-moistened in 10 ml of buffered peptone water (BPW; EMD Millipore Co., Billerica, MA). The samples under refrigeration were analyzed for STEC at day 1, 5, and 10; the samples at frozen storage were analyzed for STEC at day 7, 14, and 21 after inoculation.

Surface STEC populations sampling

The microbiologic analysis was based on the USDA FSIS Microbiology Laboratory Guidebook method recommended for testing for STEC on beef products *(28)*. Surface swabs were used to mimic the sampling method used by the USDA-FSIS in beef processing facilities and by companies as part of their verification activities to control the presence of these pathogens. Furthermore, the study presented by Alnajrani et al. 2018 *(1)* demonstrate that there is no difference between the sampling methods of surface swab and rinsing ($P < 0.01$), which supports the use of this convenient, non-destructive sampling methodology in our experiment. Surface swab sponges were homogenized in a Stomacher (Seward Stomacher®, England) set for two minutes at 230 rpm. Serial dilutions were prepared by transferring 1 ml of the homogenized swab sample into 9-ml BPW tubes. The appropriate serial dilutions were plated onto Petri dishes with MacConkey's agar (Becton, Dickinson, and Co., Sparks, MD) and a trypticase soy agar thin layer (overlay) (TSA; EMD Millipore Co., Billerica, MA) to allow for recovery of injured cells *(6)*. Inoculated Petri dishes were incubated for 18 to 24 h at 37°C; then characteristic colonies of STEC were counted and the results recorded in a laboratory notebook.

Statistical analysis

The data were analyzed using the statistical software R (©The R Foundation, The R Project for Statistical Computing). The experimental design was a completely randomized design with a split-plot arrangement. The data for each STEC serogroup as well as for each storage condition (refrigeration or freezing) were separated and analyzed independently. The bacterial counts (CFU/100 cm^2) were transformed to logarithms of colony forming units (log CFU/100 cm^2) to control and stabilize statistical variance and obtain a normal distribution. For interpretation of results, an analysis of variance (ANOVA) was performed using the general linear model function with $\alpha = 0.05$.

RESULTS

Statistical analysis showed that there were statistically significant differences among treatments (MW and CTR) for reduction of STEC O145, O121, O111, O103, O45, and O26, on day 1, day 5 and day 10 of cold storage (4°C) ($P < 0.05$). The bacterial counts were greater for samples treated with electromagnetic radiation (MW) than for non-treated samples (CTR); however, both of these bacterial counts were lower than the initial attachment concentration. Furthermore, significant differences were found among treatments on samples inoculated with STEC O45 under frozen storage (-18°C) ($P < 0.05$); samples treated with microwaves had significantly higher concentrations of STEC O45. Similar to samples stored under refrigeration, samples under frozen storage and treated with microwaves had less reduction of STEC O45 compared with the control. Nonetheless, the treatments did not result in statistical significance for bacterial counts on samples inoculated with STEC O145, O121, O111, O103, and O26 and stored at -18°C, or on samples inoculated with STEC O157:H7 at the storage conditions tested ($P > 0.05$).

The results suggest that under the conditions used, microwave treatment could promote tolerance to low temperature in specific STEC serogroups, with this effect being observed as higher bacterial counts of STEC in samples treated with microwaves. The effect was more obvious in samples stored at refrigeration (4°C) than in samples under frozen storage (-18°C).

Furthermore, the effects of the two storage conditions evaluated in this study, (refrigeration (4°C) and frozen storage (-18°C)), differed significantly ($P < 0.05$). Thus, results are presented separately for the two storage types. There were no significant differences among results for different sampling days for the storage conditions ($P > 0.05$).

The statistical significance for blocks (striploins) in samples inoculated with STEC O157:H7, O145, O103, and O45, and stored at 4°C ($P < 0.05$) might be attributed to variation in the beef composition (fat and muscle) or variation in the size of the striploin sections, which could affect the amount of microwave energy absorbed. Last, no statistical significance was found for the interaction of treatment and days of storage ($P > 0.05$), indicating that the effect of the treatments (MW and CTRL) are not affected by the days of storage.

Figure 1 shows the concentrations of STEC serogroups in *MW* and *CTR* samples stored under refrigeration (4°C). Both treatments (*MW* and *CTR*) present STEC concentrations lower than the initial concentration inoculated (Attachment). However, as previously mentioned, the reduction of STEC on samples treated with microwaves was significantly lower than the reduction observed for control samples. The STEC concentrations shown in *Fig. 1* are composites for all the sampling days of refrigerated storage on the premise that results did not differ significantly among days of storage. The STEC concentrations of samples under refrigerated storage are presented separated by sampling days (Day 1, Day 5, and Day 10) in *Fig. 2.* It can be observed that the concentration of STEC was similar for the three sampling days tested. *Figure 3* presents the concentration of STEC on refrigerated samples and its trend over time; in some cases, the concentration of

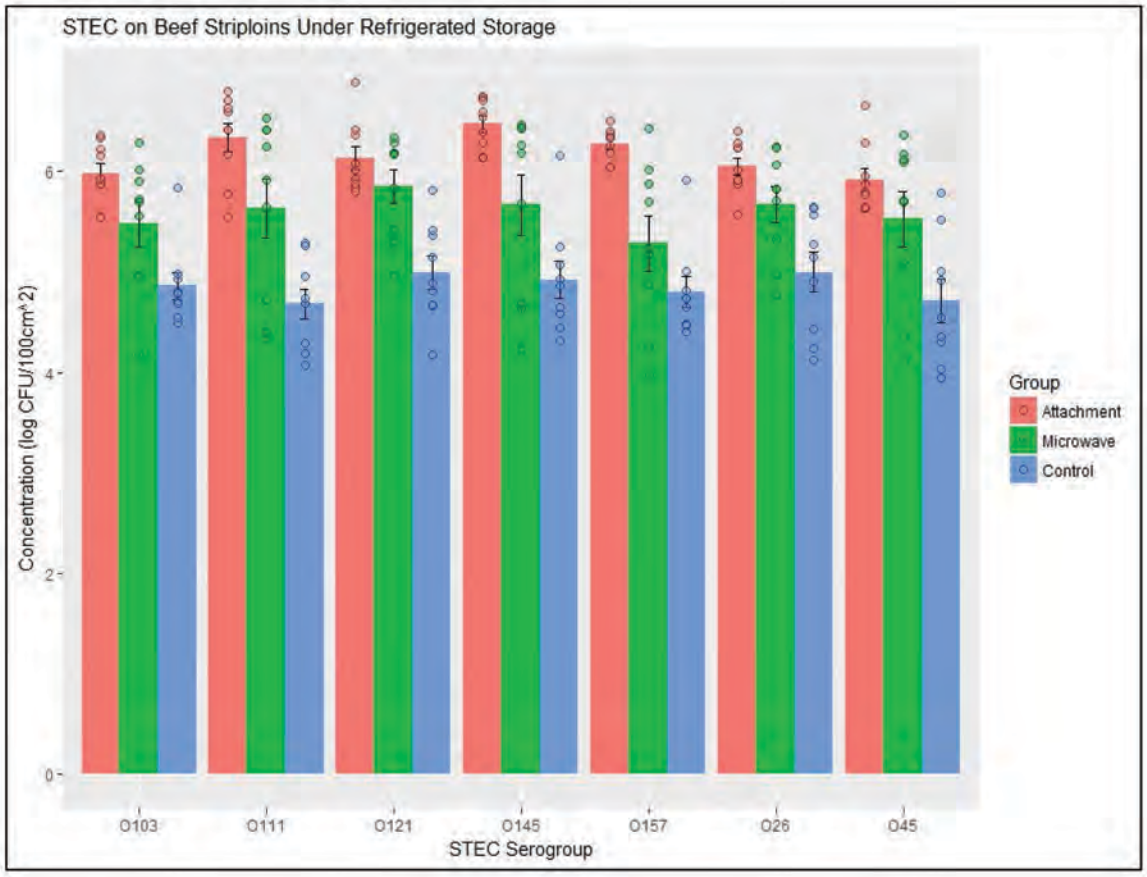

FIGURE 1

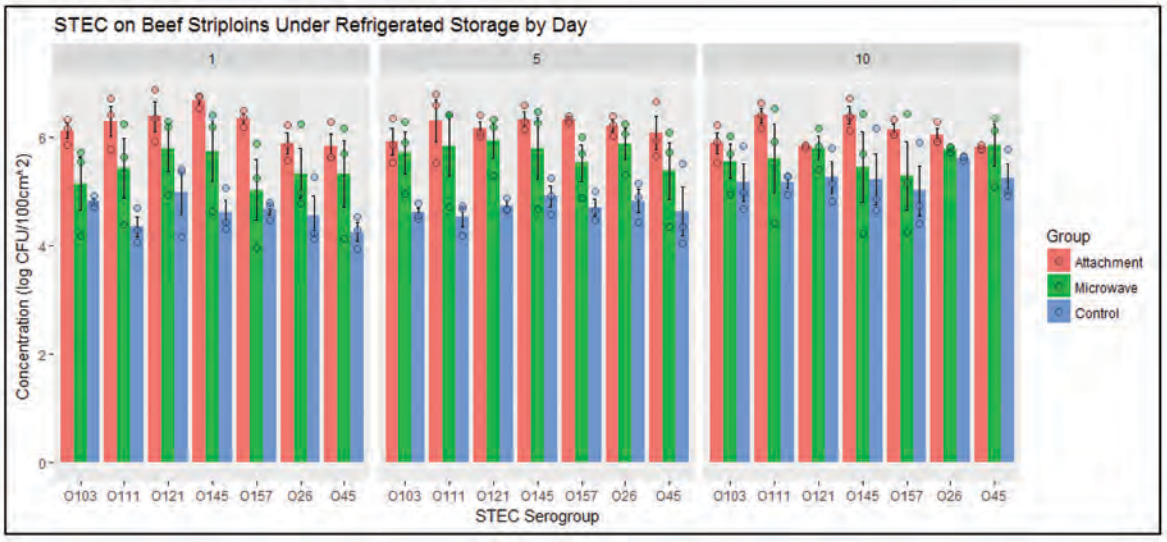

FIGURE 2

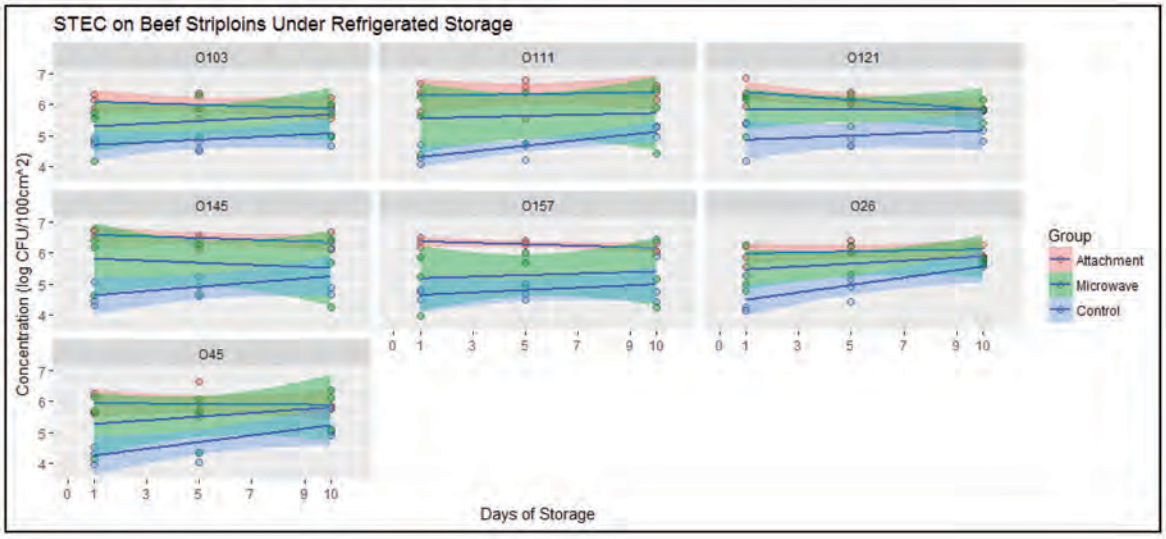

FIGURE 3

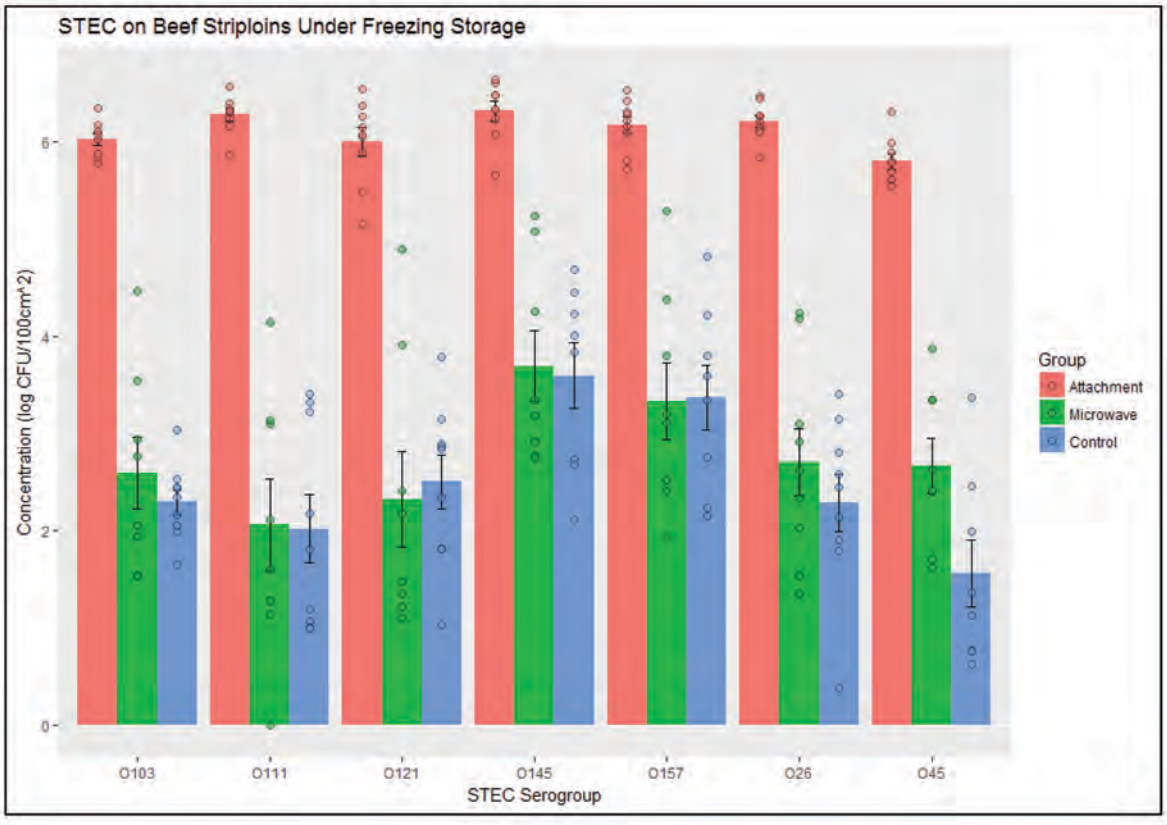

FIGURE 4

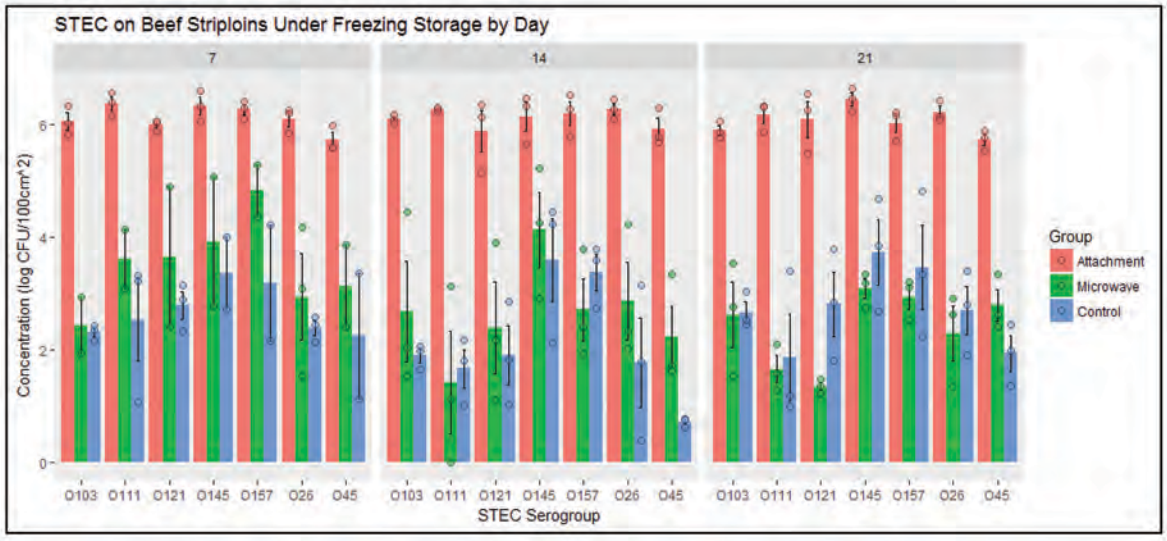

FIGURE 5

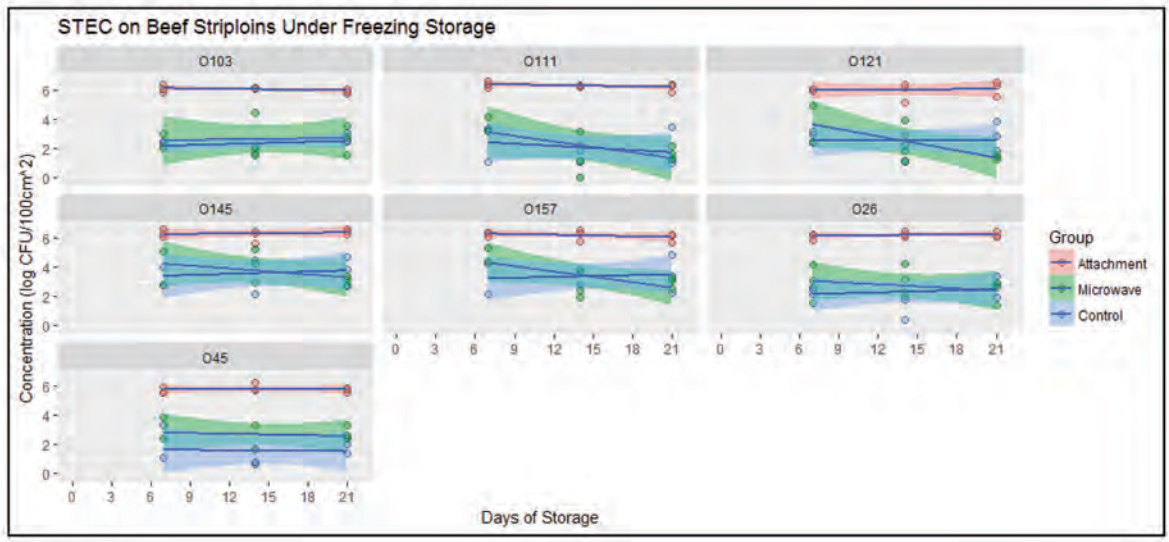

FIGURE 6

STEC appears to increase or decrease; however, statistical analysis did not reveal significant differences in the concentration of STEC between sampling days.

Figure 4 illustrates the concentrations of STEC serogroups in MW and CTR samples under frozen storage (-18°C). Both the samples treated with microwaves and the controls presented lower STEC concentrations after frozen storage for seven or more days. Concentrations of STEC in *Fig. 4* are composites of the three freezing storage periods, as no statistically significant differences were seen ($P > 0.05$). In *Fig. 5*, the concentration of STEC in samples under frozen storage is presented separated by sampling days (Day 7, Day 14, and Day 21), for illustration purposes. In *Fig. 6* the concentration of STEC in samples under frozen storage measured against time are presented; it can be observed that the concentration of STEC is unchanged over time. Last, the microbial reduction effect of low-temperature storage observed in this study was independent of treatment with microwaves, since all the STEC serogroups either did not differ significantly among treatments ($P > 0.05$) or presented a statistically significantly effect of a treatment, with less reduction of STEC in the samples treated with microwaves ($P < 0.05$).

DISCUSSION

Vacuum-packed subprimals and whole cuts subjected to mechanical tenderization/enhancement are a potential source of risk to consumers if the surface of these products is contaminated with pathogens that are translocated during the process. Moreover, boxed beef products that processors or retailers may divide into steaks or other types of cuts are not necessarily tested for pathogens by the Food Safety and Inspection Service (FSIS); therefore, interventions post-packaging are warranted such products to reduce the presence of harmful microorganisms. The use of microwaves as an intervention has been tested successfully by our group with other commodities, such as eggs and bread *(18, 19)*, and its use on fresh beef intended for non-intact products was therefore warranted. Studies have evaluated the efficacy of microwave technology in reducing the concentration of microorganisms, including *E. coli* O157:H7; however, those studies do not present enough evidence to permit drawing conclusions about the effectiveness of microwave technology applied at low temperatures. In studies conducted by other research groups evaluating the effectiveness of microwaves to decontaminate foods, reduction of inoculated *Salmonella* Typhimurium in fresh vegetables *(12)*, *E. coli* O157:H7 in chicken *(2)*, *Clostridium sporogenes* in plastic tray-packed meals *(25)*, *Clostridium difficile* in an aqueous suspension *(23)*, or *Salmonella, Yersinia, Campylobacter, Listeria, Aeromonas, Pseudomonas,* and *E. coli* in beef products was reported *(3)*. However, the settings were different and the samples reached temperatures of 63°C or above, which clearly indicates that the product was cooked after the process, which in our case was avoided. Moreover, only a few known studies have been conducted to evaluate the non-thermal effect of microwaves on animal protein. Shamis et al. (2008) evaluated the decontaminating effect of low-temperature microwaves in raw pork samples, obtaining one log reduction of generic *E. coli* and *Staphylococcus aureus* with the product temperature not exceeding 45°C. Unfortunately, although the results showed promise, their methodology was not suitable to be applied in the meat industry *(24)*.

The mechanisms of destruction of microorganisms through microwave are not well understood, and different hypotheses have been proposed. Some literature indicates that the microwave's antimicrobial effects are produced entirely by heat, which causes denaturalization of proteins, nucleic acids, and vital cell components as well as disruption of membranes *(32)*. Other authors indicate that there are as many as four different non-thermal effects of microwaves: the selective heating of microorganisms, electroporation, cell membrane rupture, and cell lysis due to electromagnetic energy coupling *(11)*.

The results suggest that the microwave treatment evaluated in this study as a potential antimicrobial intervention did not by itself significantly reduce Shiga toxin-producing *E. coli* serogroups O157, O145, O121, O111, O103, O45, and O26. However, a reduction of two to five log counts (2–5 $\log_{10}$ CFU/100 cm^2) of STEC was achieved in beef striploins vacuum packed and stored at -18°C for seven or more days. Furthermore, beef samples stored under refrigeration (4°C) showed about one to two log counts (1–2 $\log_{10}$ CFU/100 cm^2) reduction of STEC, a reduction that can be attributed to a storage temperature effect, rather than a microwave effect, as seen on *Fig. 1A–1G and 2A–2G*. Furthermore, the STEC serogroups O26, O45, O103, O111, O121, and O145 survived slightly better in the beef striploin samples treated with microwaves and stored at 4°C. For frozen storage (-18°), only STEC O45 had higher survival rates in samples treated with microwaves. This outcome was in contradiction to our initial hypothesis of a non-thermal decontaminating effect of microwaves.

Under the parameters used for this study, the use of microwave radiation was not effective in reducing STEC inoculated in beef striploins. However, we observed a significant reduction of STEC in *MW* and *CTR* samples stored under refrigeration and frozen storage ($P < 0.05$). This finding suggests that frozen storage could be applied as a post-packaging intervention by the beef industry to eliminate potentially 2–5 log CFU/100 cm^2 of STEC on beef surfaces and therefore must be considered, evaluated and validated by itself in such products with regard to its ability to reduce STEC *(4, 17, 33)*.

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CONFLICT OF INTEREST STATEMENT

Authors Echeverry, Sanchez and Cuellar report no conflict of interest. Authors Brashears reports being a member of MicroZap Inc.'s board of directors at the time of conducting the research. Authors Brashears and Brooks report owning MicroZap Inc.'s stock at the time of conducting the research.

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