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Effects of bacteriophages and peroxyacetic acid applications on beef contaminated with *Salmonella* during different grinding stages

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ABSTRACT

Research has suggested that the incidence of *Salmonella* in ground beef may be associated with contaminated lymph nodes that are not removed from trimmings destined for grinding. In this study, we tested the application of bacteriophages and peroxyacetic acid solutions on trimmings and on coarse and fine ground beef to simulate different scenarios of contamination. Overall, peroxyacetic acid applications did not reduce *Salmonella* loads on ground beef when applied on trimmings or at any stage of grinding. When applied on contaminated trim, bacteriophage solutions at 1×10^8 PFU/g and 1×10^9 PFU/g reduced more than 1 log cfu/g of *Salmonella*. When applied directly on contaminated coarse or fine ground beef, bacteriophage solutions at 1×10^9 PFU/g reduced approximately 1.6 log cfu/g. Results of this study suggest that bacteriophage applications on contaminated, comminuted beef may be used as an aid to decrease *Salmonella* loads.

1. Introduction

Salmonella contamination during beef processing is still an important concern for the meat industry in the United States. Over the last 10 years, meat processors have made significant efforts at reducing *Salmonella* by following strategies proposed by the USDA-FSIS (United States Department of Agriculture – Food Safety and Inspection Service) *Salmonella* Action Plan (USDA-FSIS, 2013). For ground beef, major concerns addressed by the plan were related to the presence of contaminated lymph nodes (LNs) in trimmings destined for grinding. The first FSIS report from 2014 suggested that there was no conclusive association between positive ground beef samples and contaminated LNs (USDA-FSIS, 2015). However, the report from 2015 (two-year update) recommended the removal of major LNs from beef carcasses (USDA-FSIS, 2016a). A survey evaluating 5450 peripheral LNs from healthy cattle collected from 12 different commercial processing plants in the United States showed that 289 nodes (5.6%) were contaminated with *Salmonella* (Webb et al., 2017). This suggests that LNs still may be a potential source of contamination if they are ground, releasing *Salmonella* and leading to cross contamination of ground beef. In the United States, there is zero tolerance for *Salmonella* in ground beef supplied to the National School Lunch Program - NSLP (USDA-ERS, 2014). The NSLP is a federally assisted meal program that operates in public and nonprofit private schools and residential child care institutions. The NSLP supplies meat products to a vulnerable population since children 5 years of age and younger are more likely than others to get salmonellosis after consuming contaminated food. Therefore, it is imperative that ground beef distributed by the NSLP is free of *Salmonella*. However, Vial et al. (2019) reported that 1.4% of 23,475 sublots of ground beef supplied to the NSLP from 2015 to 2018 were positive for *Salmonella*. The Centers for Disease Control and Prevention (CDC) reported *Salmonella enterica* outbreaks in 2018 and 2019 that led to 416 cases, 126 hospitalizations, and 1 death in 30 states in the United States (CDC, 2019). The *Salmonella enterica* species is comprised of six subspecies with 2659 serovars. In the United States, Enteritidis and Newport seemed to be the most prevalent serovars involved in foodborne illness outbreaks (Ferrari et al., 2019). Thus, developing *Salmonella* control strategies in meat processing is important to not only ensure public health, but also decrease economic losses caused by recalls and medical costs. In this study, we evaluated the effects of the application of bacteriophages and peroxyacetic acid solutions on trimmings, and coarse and fine ground beef, simulating contamination that may occur during each grinding step such as the possible presence of *Salmonella* from ground LNs.

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2. Materials and methods

2.1. Salmonella strains and inoculum preparation

Four strains of *Salmonella* including *S*. Infantis (ATCC 51741), *S*. Heidelberg (ATCC 8326), *S*. Newport (ATCC 27869), and a Streptomycin resistant *S*. Enteritidis (SE13) obtained from Micreos Food Safety B.V. (MICREOS Food Safety, Inc., Wageningen, The Netherlands) were used in this study. The inoculum was prepared by transferring 1 mL of overnight pure culture into 20 mL of Luria-Bertani broth and incubating overnight at 37 °C with shaking. Subsequently, absorbance was measured at OD_{600} to calculate concentration (CFU/mL) by using the Agilent *E. coli* cell culture biocalculator (AGILENT, 2019). Each culture was diluted to 1×10^7 CFU/mL with sterile 0.1% Buffered Peptone Water (BPW, 10% Peptone, 5% Sodium chloride, 3.5% Sodium phosphate, and 1.5% Potassium phosphate; Thermo Fisher Scientific, Waltham, MA, United States) and then combined in equal amounts to create the final inoculum.

2.2. Sample preparation, treatments, and experiment design

Fresh beef, flank, rose meat (individual muscle) (*Cutaneous trunci*, IMPS 194) (USDA-AMS, 2014) was used as the source for beef trimmings. Samples were processed into 120 g of trim-like pieces, coarse ground (7 mm), and fine ground (3 mm), and randomly assigned to a 3 × 4 factorial design including fixed effects of grinding stage (trim, coarse, fine) and antimicrobial treatment (Control inoculated - Control, Peroxyacetic acid - PAA, and PhageGuard S™ at 1×10^8 PFU/g – Phage 10^8 or PhageGuard S™ at 1×10^9 PFU/g – Phage 10^9). The PAA (Ethaneperoxoic acid, stabilized, < 43%, composition = acetic acid 40–50%, hydrogen peroxide 5–7%, and peroxyacetic acid 10–19%; Xgenx®) was applied at 400 ppm at room temperature.

2.3. Sample inoculation, antimicrobial applications, and bacterial enumeration

Beef samples were inoculated with 1×10^7 CFU/mL to achieve 2×10^4 CFU/g of *Salmonella* contamination. All samples were kept under refrigeration prior to inoculation and during the trial (5 ± 2 °C). A volume of 240 μL of the inoculum was uniformly pipetted onto the

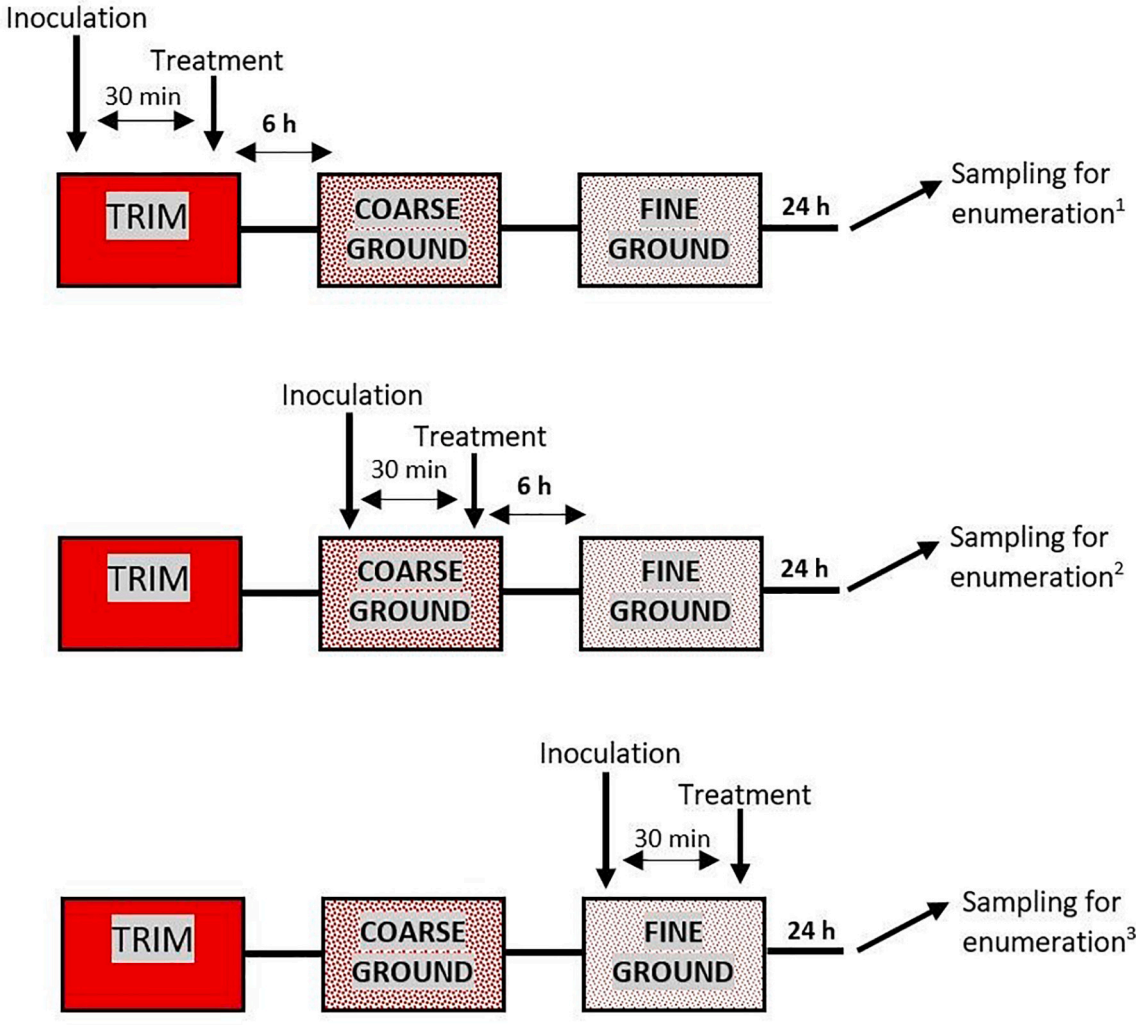

Fig. 1. Inoculation and treatment application steps for trimmings and coarse and fine ground beef.
[1] Beef trimmings were inoculated and after 30 min, antimicrobial treatment was applied. After 6 h sample was coarsely ground and subsequently finely ground. Sampling was performed 24 h after the sample was finely ground.
[2] Coarse ground beef was inoculated and after 30 min, antimicrobial treatment was applied. After 6 h sample was finely ground. Sampling was performed 24 h after the sample was finely ground.
[3] Fine ground beef was inoculated and after 30 min, antimicrobial treatment was applied. Sampling was performed 24 h after the treatment was applied.

samples and homogenized by mixing for 10 s prior to bacterial attachment at 5 °C for 30 min. Subsequently, samples were treated by pipetting on the surface 1200 μL of Buffered Peptone Water (CI), PAA 400 ppm, Phage 10^8 or Phage 10^9. Samples were homogenized and allowed to dwell at 5 °C. The inoculation and treatment application steps are demonstrated in Fig. 1. Subsequently, an aliquot of 10 g of individual samples were stomached for 2 min at 230 rpm in 900 mL of BPW. The homogenate was serially diluted and plated onto XLD agar at 35 °C for 24 h.

2.4. Statistical analysis

The experiment was conducted as a completely randomized design (CRD) with 3 repetitions in each of 3 replications ($n = 108$ total, 9 observations per fixed effect combination). The following model was used: $Yijkl = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk$, where Yijkl was *Salmonella* count, μ was the grand mean across the treatments included in the experiment, αi was the fixed effect of antimicrobial (Control, PAA, Phage 10^8, and Phage 10^9) and βj was the fixed effect of grinding stage (intact trim, coarse ground, and fine ground). In addition, three nested models of interest within grinding stage (β) were identified. The following model was used: $Yij = \mu + \alpha i + \mathcal{E}ij$ to analyze individual data obtained from fine ground beef after treatments were applied on intact trim, coarse ground and fine ground beef. Data were analyzed using the GLIMMIX procedure of SAS® 9.3 package (SAS Institute, Inc., USA). When significance ($P \leq .05$) was indicated, means separations were performed by using the LSMEANS and DIFF functions.

3. Results

When analyzing the two-way factorial 4 × 3, no interaction between the fixed effects of antimicrobial treatment and grinding stage was observed ($P = .36$). For individual effects, grinding stage was not significant at $P = .29$, but there was a significant effect of antimicrobial treatment ($P < .001$; Fig. 2). Overall, a reduction of 1.5 log CFU/g was observed when applying Phage 10^9. Applications of PAA did not lower bacteria loads. Results for each individual nested model are shown in Fig. 3. When applied on contaminated trim, Phage 10^8 and 10^9 significantly reduced *Salmonella* in fine ground beef by 1.12 and 1.29 log, respectively ($P < .001$; Control = 3.48 log, Phage 10^8 = 2.36 log, Phage 10^9 = 2.19 log). When applied on contaminated coarse ground beef, only Phage 10^9 significantly reduced *Salmonella* in fine ground beef ($P < .001$; Control = 3.61 log, Phage 10^9 = 2.06 log; reduction =1.55 log). When applied directly on contaminated fine ground beef, Phage 10^8 reduced *Salmonella* by 0.78 log ($P < .001$; Control = 3.70 log, Phage 10^8 = 2.92) whereas Phage 10^9 reduced 1.67 log ($P < .001$; Control = 3.70 log, Phage 10^9 = 2.03). In all contamination scenarios, PAA did not reduce *Salmonella* contamination in fine ground beef.

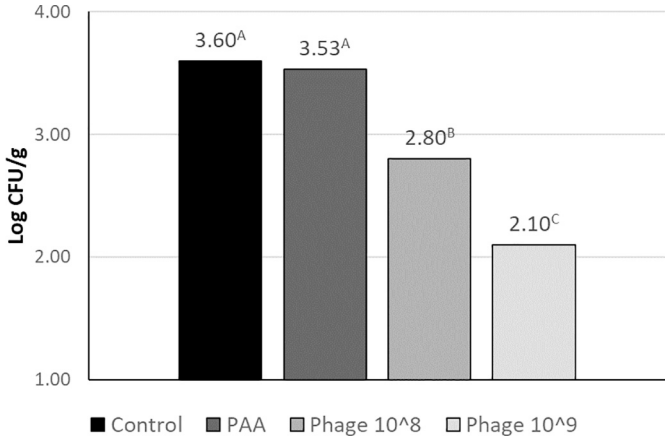

Fig. 2. Effects of PAA and bacteriophage applications on *Salmonella* counts in fine ground beef. Average of treatments applied directly on trimmings, coarse or on fine ground beef.
[A,B,C] Different superscripts indicate significant diffrences within antimicrobial treatment ($P < .001$). STD error: 0.20.
Control = treated with BPW; PAA = 400 ppm; Phage $10^8 = 1 \times 10^8$ PFU/g; and Phage $10^9 = 1 \times 10^9$ PFU/g.

4. Discussion

Ellebracht et al. (2005) observed a reduction of approximately 1 log of *Salmonella* by submerging contaminated beef trimmings in solutions containing 200 to 500 ppm of PAA. Submerging trimmings in PAA solutions provides an abundant distribution of the organic acid throughout the surface and seems to be more effective against *Salmonella* when compared to current methods used by the meat industry in the United States, such as spraying for example. Our previous research has demonstrated that PAA is not efficient in decreasing *Salmonella* loads when applied by pipetting on contaminated samples (Yeh et al., 2017; Yeh, de Moura, Van Den Broek, & de Mello, 2018). PAA is a highly reactive compound and reacts with several organic materials including proteins (Chino, Nukui, Morishita, & Moriya, 2017), which may decrease its effects on microorganisms when applied directly to proteinaceous surfaces. In addition, *Salmonella* may develop resistance against PAA by upregulating genes encoding catalases and reductases that are able to degrade hydrogen peroxide upon bacterial exposure to Reactive Oxygen Species (ROS) (Buchmeier et al., 1997; Hébrard, Viala, Méresse, Barras, & Aussel, 2009). In the United States, beef trimmings are treated with antimicrobial solutions by spraying to prevent additional moisture pick-up as it may happen when using immersion techniques. Based on the FSIS compliance guidelines for retained water, products should not retain more than 0.49% water (USDA-FSIS, 2001). Therefore, ground beef processors must carefully control how much of an antimicrobial solution can be applied on trimmings or ground beef. Immersion techniques may result in excessive water pick-up when compared to spraying.

Previous studies reported that phage applications reduced *Salmonella* in different meat matrices (Sharma, Dhakal, & Nannapaneni, 2015; Sukumaran, Nannapaneni, Kiess, & Sharma, 2015; Yeh et al., 2017). In this study, bacteriophage applications reduced *Salmonella* in fine ground beef when applied on trimmings and at all grinding stages. The solution used in our experiment was comprised of two phages, the S16 and the FO1a. The S16 has an efficient mechanism of attachment that broadens its host range (Guenther, Herzig, Fieseler, Klumpp, & Loessner, 2012; Marti et al., 2013). Both phages belong to the *Myoviridae* family (Lavigne et al., 2009; Marti et al., 2013) and have an advanced structural design including a complex contractile tail structure (Comeau et al., 2012). The tail tube penetrates through the bacteria cell wall and transfers the phage genome into the host cytoplasm (Leiman & Shneider, 2011; Novacek et al., 2016). Phages attach to the bacteria using complementary receptors on the surface of the host cell. The S16 attaches to the outer membrane protein C (OmpC) (Marti et al., 2013). Since OmpC is present on all *Salmonella*, this bacteriophage lyses a broad range of strains.

This study was designed to evaluate the effects of phage applications on coarse and fine ground beef inoculated with a cocktail of *Salmonella* to simulate potential contamination by LNs harboring this microorganism. Both lower and higher concentrations of bacteriophages led to a similar decrease of *Salmonella* in fine ground beef when solutions were applied on trimmings. When comparing the effects of both concentrations (Phage 10^8 and Phage 10^9), results suggest that encounters between phage and host were possibly similar, independently of which concentration was applied.

The process of grinding a single trimming piece generates multiple smaller particles (USDA-FSIS, 2016b) increasing the surface area that needs to be populated by bacteriophages. When bacteriophages were applied on comminuted beef (coarse and fine), a higher concentration of

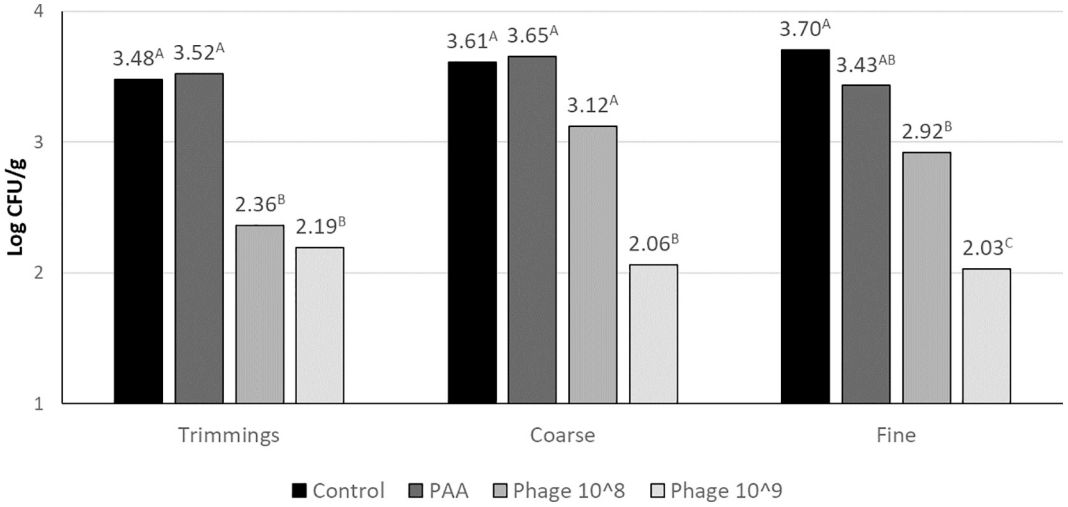

Fig. 3. Effects of PAA and bacteriophage applications at different grinding stages on *Salmonella* counts of fine ground beef.
[A,B,C] Different superscripts indicate significant diffrences within grinding stages ($P < .001$).
STD errors: 0.18, 0.20, and 0.21, for Trimmings and Coarse and Fine ground beef, respectively.
Control = treated with BPW; PAA = 400 ppm; Phage $10^8 = 1 \times 10^8$ PFU/g; and Phage $10^9 = 1 \times 10^9$ PFU/g.

bacteriophage (Phage 10^9) was required to statistically lower *Salmonella* contamination. These results suggest that as surface area increases, higher concentrations of bacteriophages are needed to increase encounters between phage and host and reduce bacterial contamination. Some research suggested that T4 phages such as the S16 may show a subdiffusive (dispersive) motion in a mucus matrix resulting in increased host encounters (Barr et al., 2015). However, bacteriophages are known to be non-motile microorganisms and depend on Brownian motion to reach their target cells. When applied on meat surfaces, phages do not move towards the host. In larger surface areas, applications of higher concentrations provide improved distribution of bacteriophages facilitating their encounters with hosts.

5. Conclusion

Applications of phage solutions into contaminated ground beef lowered *Salmonella* loads, suggesting that it is possible to address possible cross-contamination caused by grinding lymph nodes harboring *Salmonella*. When applying directly on ground beef, a higher concentration of bacteriophages is required for application on pieces bearing a larger surface area (i.e., comminuted samples have larger surface area than intact samples). The application of bacteriophage solutions into ground beef provides an additional food safety measure, especially for establishments that produce ground beef for the USDA NSLP.

Declaration of Competing Interest

None.

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