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Bacteriophage application on red meats and poultry: Effects on *Salmonella* population in final ground products

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ABSTRACT

This research was conducted to study the effects of bacteriophage application during tumbling on *Salmonella* populations in ground meat and poultry. Red meat trim and poultry were inoculated with a *Salmonella* cocktail to result in a contamination level of 7 log CFU/g in ground products. A commercial preparation containing bacteriophages S16 and Felix-O1a (FO1a) was applied during tumbling at 10^7 and 10^8 PFU/ml. Samples were held at 4 °C for 6 h and 18 h (red meat) and 30 min and 6 h (poultry). Overall, bacteriophage application on trim reduced 1 and 0.8 log CFU/g of *Salmonell*a in ground beef and ground pork, respectively. For ground chicken and ground turkey, *Salmonella* was reduced by 1.1 and 0.9 log CFU/g, respectively. This study shows that bacteriophage application during tumbling of red meat trim and poultry can provide additional *Salmonella* control in ground products.

1. Introduction

Salmonella is a major food-borne pathogen that leads to a high number of hospitalizations in the U.S. (CDC, 2016a). In 2014, 7452 laboratory-confirmed infections of *Salmonella* led to 2141 hospitalizations and more than $3.6 billion of medical care costs (CDC, 2014; USDA-ERS, 2014). Common symptoms include nausea, vomiting, abdominal cramps, diarrhea, fever, and headache (FDA, 2012). Animal products that can be contaminated with *Salmonella* include red meats, poultry, and eggs (FDA, 2012). Although the U.S. Centers for Disease Control and Prevention (CDC) data represents total illnesses and not only cases attributable to meat consumption, *Salmonella* is often detected in meat and meat products, which are usually linked to outbreaks (Doménech, Jiménez-Belenguer, Pérez, Ferrús, & Escriche, 2015).

From 2011 to 2015, the CDC reported *Salmonella* outbreaks involving beef, chicken, turkey, and pork. Investigations suggested that contamination of products possibly happened during production (CDC, 2016b). The intestinal tract of animals is a common reservoir of *Salmonella*, which can be transferred to meats during slaughter and processing (Doménech et al., 2015). Poor hygienic conditions during processing and improper product handling seem to be major causes of *Salmonella* contamination of meat products (Manios & Skandamis, 2015).

Recently in the U.S., the Food Safety and Inspection Service (FSIS) received requests from non-profit organizations to declare antibiotic-resistant (ABR) *Salmonella* to be adulterant in raw ground meat and ground poultry. Although FSIS responded the requests by concluding that available data did not support giving ABR strains an adulterant status, the FSIS developed a strategic plan to decrease *Salmonella* contamination in processing facilities and reassessed sampling procedures (USDA-FSIS, 2014).

Using bacteriophages for specific pathogen biocontrol offers an additional hurdle for robust food safety management systems (Endersen et al., 2014). Bacteriophages attach to specific receptors present on bacteria cell wall including protein, lipopolysaccharides, teichoic acids, pili and flagella (Rakhuba, Kolomiets, Szwajcer Dey, & Novik, 2010). Due to this specificity, bacteriophages lyse only targeted bacteria without disrupting other non-targeted microbiota (Meaden & Koskella, 2013; Sulakvelidze, Alavidze, & Morris, 2001).

No previous studies were conducted to test bacteriophage application on ground products during tumbling of red meat trim and poultry. Tumbling is a common method adopted by the meat industry to incorporate antimicrobials (most commonly organic acids) into meats prior to grinding. During tumbling, trim or intact cuts are placed into a rotating drum to optimize the distribution of solutions on meat surface. The objectives of this study were to: i) determine the efficacy of bacteriophage application during tumbling of red meat trim and intact poultry on *Salmonella* populations in ground products, and ii) verify the effectiveness of different holding times prior to grinding.

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2. Materials and methods

2.1. Salmonella strains and inoculum preparation

Four strains of *Salmonella* were used in this study. *S. enterica* (ATCC 51741), *S.* Heidelberg (ATCC 8326), *S.* Newport (ATCC 27869) were obtained from the ATCC and a streptomycin resistant strain (*S.* Enteritidis C, Se 13) was obtained from Micreos Food Safety B.V. (MICREOS Food Safety, Inc., Wageningen, The Netherlands). ATCC strains were recovered by following the ATCC bacterial culture guidelines (ATCC, 2015). Briefly, individual freeze-dried pellets were thawed for approximately 2 min in water bath at 37 °C. The entire content of each vial was aseptically transferred to a sterile test tube containing 5 ml of brain heart infusion (BHI) broth and incubated overnight at 37 °C. Each enriched broth containing individual strains was streaked on xylose lysine deoxycholate (XLD) agar. The *S.* Enteritidis C strain was streaked from a glycerol stock onto XLD agar supplemented with 500 μg/ml of streptomycin. Plates were incubated at 37 °C for 24 h to ensure cultures were live and viable. For each individual strain, the inoculum was prepared by suspending a single colony in independent sterile tubes containing 10 ml of tryptic soy broth (TSB), which were incubated at 37 °C for 16–18 h. Subsequently, 0.1 ml of each culture was inoculated into 40 ml of TSB and incubated for 16–18 h at 37 °C with stirring to achieve the concentration of 10^8 CFU/ml. Individual cultures were centrifuged at 3400 × *g* for 10 min to discard metabolic products, diluted in 0.1% BPW, combined into a single cocktail, and inoculated into red meat trim and poultry thighs. Infectious dose for salmonellosis range from 5 to 7 log (Doyle & Mazzotta, 2000). However, lower levels of 1 log may also cause illness based on age and health of the host, and strain virulence (FDA, 2012). In this study, we aimed to achieve bacterial concentration of 7 log CFU/g after grinding.

2.2. Phage preparation and stock titer

The commercial bacteriophage preparation Salmonelex™ containing two non-temperate phages S16 and Felix-O1a (FO1a) was obtained from MICREOS Food Safety, Inc. (Wageningen, The Netherlands). Concentration of phage in the stock solution was determined by following the double-layer agar method described by Adams (1959) with some modifications. Briefly, the solution titer was determined by plating 100 μl from 10-fold dilutions in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl, pH 7.2) with a 100 μl of a fresh log-phase cocktail comprising all 4 *Salmonella* strains in the soft LB agar (0.6% agar) tempered to 45 °C. The mixtures were vortexed twice and evenly distributed over the surface of the hard LB agar (1.6% agar). Soft agar was allowed to solidify at room temperature and plates were inverted and incubated at 37 °C for 24 h. Bacteriophage concentration in the stock solution was determined to be 10^{11} PFU/ml.

2.3. Killing efficiency of bacteriophage preparation on all 4 Salmonella strains

The killing efficacy of the bacteriophage preparation for each *Salmonella* strain was determined by plating in quadruplicate a volume of 100 μl of diluted overnight cultures of individual strains on LB agar. A volume of 100 μl of phage solution (10^9 PFU/ml) was applied onto the lawn and allowed to be fully dried before incubating. Plates were incubated at 37 °C for 24 h to observe plaque formation.

2.4. Sample preparation and treatment design

One batch weighing approximately 5 kg of beef trim (80% lean), pork trim (72% lean), chicken thighs, and turkey thighs were procured from federally inspected meat facilities and transported under refrigeration (4 °C) to the University of Nevada, Reno's Meat Quality Laboratory. An aliquot weighing approximately 500 g of each meat type was obtained collecting random pieces from each batch. Aliquots were screened for background *Salmonella* spp. contamination. A total of thirty ($n = 30$) samples containing 100 g of each meat type were randomly assigned to a 3 × 2 factorial design. Fixed effects were bacteriophage application (3 levels) and holding time (2 levels). Bacteriophage levels include control (no phage, 5 ml of sterile double-distilled deionized water), 10^7 PFU/ml, and 10^8 PFU/ml; whereas holding time levels included 30 min and 6 h for poultry and 6 h and 18 h for red meat. In order to simulate industry application, phage stock solution was diluted in potable water as recommended by the manufacturer. Potable water was previously tested to ensure it was free of *Salmonella* spp. and chlorine.

2.5. Sample inoculation, bacteriophage application, and bacteria enumeration

Intact red meat trim and turkey and chicken thighs were inoculated with a diluted cocktail comprising all four *Salmonella* strains to yield approximately 7 to 7.3 log CFU/g after grinding. Briefly, 5 ml of the inoculum was uniformly pipetted onto surfaces of meat and poultry. Samples were kept for 30 min at room temperature to allow bacteria attachment. Samples were then placed into a tumbler (model VTM-15, Chard, China) and treated with sterile double-distilled deionized water (control) or bacteriophage solutions (10^7 or 10^8 PFU/ml). Samples were tumbled for 2 min at 4 rpm while solutions were uniformly pipetted onto the meat during the first minute. Subsequently, samples were removed from the tumbler and held under refrigeration (4 °C) for two different periods, 30 min and 6 h for poultry, and 6 h and 18 h for red meat prior to grinding. Samples were ground using a table top electric grinder (model 33-0201-w, Weston, China) and a 10 g aliquot was collected from each sample and placed in sterile bags. Samples were stomached for 2 min in 110 ml of sterile 0.1% BPW. For quantitative determination of *Salmonella* counts, 10 ml of the homogenate was centrifuged at 10,000 × *g* for 5 min and supernatant was discarded to avoid plating phages. Pellets were resuspended in 10 ml of sterile saline solution, vortexed, serially diluted in BPW, and plated onto XLD plates in duplicate. Plates were incubated overnight at 37 °C and typical *Salmonella* colonies were counted (CFU/g).

2.6. Statistical analysis

Data were analyzed using the SAS® 9.3 package, SAS Institute, Inc., USA. For each meat type, as described in item 2.4, a completely randomized design (CRD) with a 3 × 2 factorial was used. Data were analyzed by using the GLIMMIX procedure and when significance ($P \leq 0.05$) was indicated by ANOVA, means separations were performed by using the LSMEANS and DIFF functions.

3. Results

The results of killing efficacy for all 4 *Salmonella* strains are presented in Table 1. Bacteriophage preparation reduced approximately 99% of all strains in vitro.

Table 1
Killing efficiency of bacteriophage preparation (phages S16 and FO1a) for four *Salmonella* strains.

Strain	Bacteriophage application	Average CFU (4 plates)	Killing efficiency (%)
ATCC 51741	[a]Control	126.75	99.0
	10^9 PFU/ml	1.25	
ATCC 8326	Control	334.75	99.1
	10^9 PFU/ml	3	
ATCC 27869	Control	160	99.2
	10^9 PFU/ml	1.25	
Se 13	Control	398.50	98.6
	10^9 PFU/ml	5.25	

[a] No bacteriophage applied.

Interaction of fixed effects of bacteriophage application and holding time was not significant for ground beef ($P = 0.06$) (Table 2). Holding time did not affect *Salmonella* counts ($P = 0.29$). Bacteriophage application on trim significantly reduced bacteria populations in ground beef ($P < 0.0001$). As phage concentration increased during trim application, a greater decrease of *Salmonella* was observed in ground beef. Overall, phage application at 10^8 PFU/ml reduced *Salmonella* by 1 log CFU/g (control = 7.1 log CFU/g and 10^8 PFU/ml = 6.1 log CFU/g), whereas, applications at 10^7 PFU/ml reduced 0.7 log CFU/g (control = 7.1 log CFU/g and 10^7 PFU/ml = 6.1 log CFU/g).

For ground pork, no significant interaction of bacteriophage application and holding time was observed on final bacteria counts ($P = 0.83$) (Table 2). Holding time did not affect populations of *Salmonella* when samples were treated with different phage concentrations ($P = 0.24$). Bacteriophage applications at 10^7 and 10^8 PFU/ml led to similar reduction when compared to control group ($P < 0.0001$).

For ground chicken, a significant interaction was observed between both fixed effects (bacteriophage application and holding time, $P = 0.04$). No significant reduction was observed when samples were held for 30 min and bacteriophage was applied at 10^7 PFU/ml (Table 2) However, when samples were held for 6 h and bacteriophage was applied at 10^8, *Salmonella* populations decreased by 0.8 and 1.1 log CFU/g respectively.

For turkey, both concentrations (10^7 and 10^8 PFU/ml) led to significant reductions of *Salmonella* population in ground products ($P < 0.0001$). The treatment with 10^8 PFU/ml led to higher bactericidal activity by decreasing 0.9 log CFU/g. When samples were treated with 10^7 PFU/ml, *Salmonell*a was reduced by 0.7 log CFU/g. As we noted previously for beef, pork, and chicken, holding time did not affect bacteria counts in ground turkey ($P = 0.77$).

4. Discussion

To our knowledge, this is the first study that has tested the efficacy of bacteriophage on ground meat by applying this intervention during tumbling. In order to provide additional safety of ground meat products, meat trim or intact cuts are introduced in rotating drums and antimicrobials are usually sprayed during tumbling (Sofos, 2014). When performed for long periods or associated with vacuum, tumbling leads to a disruption of muscle structure and degradation of sarcomere I-bands and *Z*-lines, thus facilitating the penetration of solutions into meat and leading to internalization of surface pathogens (Gao et al., 2015; Pietrasik & Shand, 2004; Pokharel et al., 2016). However, meat processors utilize short periods of tumbling (1–2 min) as an efficient technique to uniformly distribute antimicrobial solutions on trim surface. Short tumbling periods neither promote physical damage on intact pieces nor adulterate final meat products by increasing final weight. Moreover, it ensures that all meat surfaces are treated, enhancing decontamination and resulting in growth suppression of pathogenic bacteria in ground meat (Castillo et al., 2001). In this study, we have demonstrated that application of bacteriophages by using a recognized meat industry technique is able to reduce *Salmonella* contamination in ground meat and poultry. Our data have also demonstrated that preparations containing S16 and FO1a phages are able to reduce the population of different *Salmonella* strains. Both phages belong to the order *Caudovirales* and *Myoviridae* family (Lavigne et al., 2009; Marti et al., 2013). Bacteriophages of the *Myoviridae* family have a sophisticated and unique structural design that includes a complex contractile tail structure (Comeau et al., 2012). The contraction of these tails facilitates the penetration of the tail tube through the bacteria cell wall resembling the action of a syringe that transfers the phage genome into the bacteria cytoplasm (Leiman & Shneider, 2011; Novacek et al., 2016). Phage attachment is a highly specific process that requires complementary receptors on the surface of the host cell. The S16 uses as primary binding site the outer membrane protein C (ompC) (Marti et al., 2013). Since ompC is present on all *Salmonella*, this bacteriophage is able to lyse a broad range of strains including the ones that have rough mutations. Marti et al. (2013), tested the efficiency of S16 on 46 *Salmonella* strains and observed that this bacteriophage was able to lyse 45. Additionally, S16 has complex mechanisms for packaging host DNA and transduction of genetic material. In order to successfully control bacteria, phages should not be able to perform generalized transduction (Marti et al., 2013). Therefore, S16 characteristics meet this specific requirement, which makes this virus an efficient biocontrol agent. The FO1a bacteriophage uses as primary binding site the terminal *N*-acetylglucosamine residue, which is present on the outer LPS core (Lindenberg & Holme, 1969). Previous research also described FO1a as a broad-host-range bacteriophage for *Salmonella* although its characteristics differ from those attributed to S16 (Guenther, Herzig, Fieseler, Klimpp, & Loessner, 2012; Whichard, Sriranganathan, & Pierson, 2003). In this study, the combination of both bacteriophages was able to reduce *Salmonella* populations including 4 different strains.

Regarding holding time, host inactivation starts occurring within the first few hours after phage application (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003; Leverentz et al., 2001). Goode, Allen, and Barrow (2003) reported that bacteriophage activity on samples held for 24 and 48 h led to 0.6 log CFU/cm^2 and 1 log CFU/cm^2 reductions of *Salmonella* populations on chicken skin. According to Fiorentin, Vieira, and Barioni (2005) chicken thighs held for 6 days after bacteriophage application showed higher decrease of *Salmonella* when compared with thighs held for 3 days. Zinno, Devirgiliis, Ercolini, Ongeng, and Mauriello (2014) reported higher reductions of *Salmonella*

Table 2
Least square means (log CFU/g) of *Salmonella* in ground beef, pork, chicken, and turkey followed by application of bacteriophages on intact trim (beef and pork) and thighs (chicken and turkey).

Meat matrix	Holding time	Bacteriophage application			SEM[2]	*P* value		
		Control[1]	10^7 PFU/ml	10^8 PFU/ml		BA[3]	HT[4]	BA * HT[5]
Beef	6 h	7.0	6.6	5.9	0.13	<0.0001	0.29	0.06
	18 h	7.2	6.3	6.3				
Pork	6 h	7.3	6.5	6.4	0.13	<0.0001	0.24	0.83
	18 h	7.4	6.7	6.6				
Chicken	30 min	7.2[a]	7.1[A,a]	6.3[b]	0.15	<0.0001	0.98	0.04
	6 h	7.5[a]	6.7[B,b]	6.4[b]				
Turkey	30 min	7.2	6.6	6.4	0.13	<0.0001	0.77	0.65
	6 h	7.3	6.7	6.3				

[A,B] Means in the same column within meat matrix having different superscripts are significant at $P = 0.04$. [a,b] Means in the same row within meat matrix having different superscripts are significant at $P = 0.04$.

[1] Not treated with bacteriophage, ground 30 min after inoculation.
[2] Standard error of the mean.
[3] Fixed effect of bacteriophage application.
[4] Fixed effect of holding time.
[5] Interaction of bacteriophage application and holding time.

population on chicken breast held for 48 h when compared with breasts held for 24 h. Results of this study showed that extending holding time from 30 min to 6 h for poultry and from 6 h to 18 h for red meats was not enough to maximize bacteriophage activity.

Bigwood, Hudson, Billington, Carey-Smith, and Heinemann (2008) and Shao and Wang (2008) reported that inactivation of the host by bacteriophages can also be related to optimal temperature. Although better phage activity is observed at temperatures varying from 25 °C to 37 °C (Hungaro, Mendonça, Gouvea, Vanetti, & de Oliveira Pinto, 2013), previous studies reported that phages were found to be effective in reducing *Salmonella* in chicken and ready-to-eat foods stored at temperatures ranging from 4 °C to 8 °C (Fiorentin et al., 2005; Guenther et al., 2012; Zinno et al., 2014). In our study, bacteriophage application led to *Salmonella* reduction in all meat matrixes stored at 4 °C. Additionally, levels of 10^7 and 10^8 PFU/ml were able to reduce high counts of *Salmonella* (7 log CFU/g) in ground products by 1 log CFU/g when applied on intact meats prior to grinding. Our data is consistent with studies in which phages were used to control *Salmonella* in meats (Bielke et al., 2007; Goode et al., 2003; Higgins et al., 2005; Sharma, Dhakal, & Nannapaneni, 2015; and Sukumaran, Nannapaneni, Kiess, & Sharma, 2015), however, in those studies, bacteriophages were applied at higher concentrations (10^9 PFU/ml or higher). Similar *Salmonella* reduction caused by the application of S16 in lower concentration (10^8 PFU/ml) when compared to application of other phages in higher concentrations may be associated with its particular mode of action. Additionally, contamination of meats during processing usually ranges from 2 to 3 log CFU/cm^2 (Jay, 1996). Possibly, the activity of S16 on lower levels of *Salmonella* populations (2 to 3 log CFU) may lead to a higher reduction in the number of bacteria.

Regarding industrial application, only Hungaro et al. (2013) and Sukumaran et al. (2015) examined phage intervention by simulating bacteriophage application during water chilling for poultry. Our data demonstrated that application of bacteriophages during tumbling can also function as an additional hurdle to improve the control of *Salmonella*.

5. Conclusion

Bacteriophages can be used as an additional hurdle in robust food safety systems to improve the control of *Salmonella* in ground meat and poultry when applied on red meat and poultry prior to grinding. Bacteriophage solutions can be applied during meat tumbling in existent meat processing machinery commonly used to apply other antimicrobials. The combination of both bacteriophages S16 and FO1a reduced *Salmonella* in all meat matrixes under processing temperatures. Results of this study can be used as support documentation for HACCP plans that adopt bacteriophage application as part of their pathogen control pre-requisite programs.

Conflict of interest

The authors declare no conflict of interest associated with this research.

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