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Recovery of associated and internalized *Salmonella* in broiler skin by stomaching and grinding

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ABSTRACT

The objective of this study was to evaluate the recovery of associated and internalized *Salmonella* by stomaching and grinding broiler skin during exposure at 4 °C and at room temperature, using a two-strain green fluorescent protein (GFP) labeled cocktail of *Salmonella* Enteritidis. In the first experiment, broiler skins were immediately taken from eviscerated carcasses and exposed to a *Salmonella* cocktail containing ~1 × 10^9 CFU/ml for 0.5, 6, 12, 24, and 48 h at 4 °C. After each exposure, two 1-min stomachings and subsequent grinding of the stomached skin were conducted to quantify loosely associated (from two stomachings) and tightly associated (from grinding) *Salmonella* on the skin, respectively. Broiler skins exposed to *Salmonella* for 24 and 48 h were also examined by confocal microscopy before and after the two stomachings. The 1st and 2nd stomachings recovered an average of 71 and 17% of the *Salmonella* population, respectively, with an additional 12% of the cells recovered after subsequent grinding, regardless of incubation time. Based on the confocal images, most *Salmonella* were removed after two stomachings, however a few cells further penetrated from 9 to 29 μm into the skin. In the second experiment, broiler skins were immersed in the same two-strain *Salmonella* cocktail (~1 × 10^8 cells/ml) and dip-inoculated for 2 min with/without stomaching at room temperature. Based on the confocal images, *Salmonella* penetrated the flat skin surfaces and crevices up to 10 and 68 μm without stomaching, respectively, and up to 62 and 132 μm with stomaching. The presence of free-floating *Salmonella* cells in the skin crevices indicates that entrapped water is important for bacterial translocation in poultry skin. These findings indicated that extent of observable *Salmonella* association, penetration, and subsequent recovery from poultry skin is related to both surface topography of poultry skin and method of sample processing.

1. Introduction

Many studies have been conducted to evaluate the effects of various physical and chemical sanitizing agents on broiler carcasses for improvement of microbiological quality during processing (Lee, Park, Kang, & Ha, 2014; Nassar, Al-Mashhadi, Fawal, & Shalhat, 1997; Oh, Kang, Oh, & Ha, 2014; Zhang, Singh, Lee, & Kang, 2013). However, few studies have been conducted to elucidate the extent of bacterial association, penetration, and recovery from the broiler skin after inoculation. Given the right conditions, bacteria can attach tightly within a few minutes, with no difference in bacterial populations seen between 0.5 and 10 min of exposure (Arritt, Eifert, Pierson, & Sumner, 2002). Direct visual observation using scanning electron microscopy has been used to study *Salmonella* attachment and detachment on broiler skin (Nayak, Kenney, & Bissonnette, 2001), however most of these studies have not been conducted with the comparison of bacterial enumeration using stomaching for loose attachment and grinding for tight attachment.

The advent of green fluorescent protein (GFP)-labeled bacteria has greatly aided researchers in assessing bacterial attachment, penetration and movement in biological systems (Buchholz, Davidson, Marks, Todd and Ryser, 2012; Burnett & Beuchat, 2002; Ling, Wang, Xie, Lim, & Leung, 2000). A major advantage of using GFP-tagged includes the visualization of the organism on agar plates under ultraviolet light and simpler sample preparation for fluorescence and confocal microscopy.

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Since fluorescence does not depend on access to a substrate, these cells can be visualized and counted when internalized in poultry skin and various types of tissue, using z-stacking procedures of confocal microscopy (Chantarapanont, Berrang, & Frank, 2003). However, some disadvantages of GFP-tagged cells include possible loss of the plasmid over time and denaturation of the fluorescent protein upon exposer to sanitizers (Burnett & Beuchat, 2002). As a result, such GFP-labeled bacteria are best suited for short term or slow growth studies that do not use sanitizers or disinfectants. Using this technique, survival of *Campylobacter jejuni* cells at specific sites and various depths in chicken skin during storage was investigated (Chantarapanont et al., 2003).

In this study, *Salmonella* association, penetration, and recovery from broiler skins was assessed at 4 °C and at room temperature using stomaching and grinding as well as confocal scanning laser microscopy for visualization.

2. Materials and methods

2.1. Salmonella strains

Two GFP labeled, ampicillin resistant *Salmonella* Enteritidis strains (ME18 and H4717) were obtained from the Center for Food Safety, University of Georgia (Athens, GA). All strains had been preserved at −80 °C in trypticase soy broth (TSB) containing 0.6% (wt/vol) yeast extract (YE) (BD, Sparks, MD) and 20% glycerol.

2.2. Experiment I: Salmonella attachment and recovery from broiler skin using stomaching and grinding

2.2.1. Preliminary attachment comparison

A modified microtiter plate assay was conducted in triplicate to compare attachment abilities of two GFP-labeled *Salmonella* Enteritidis strains (ME 18 and H4717) with one non-GFP-labeled *Salmonella* Thompson strain (FSIS 120) isolated from chicken (Stepanovic, Vukovic, Dakic, Savic, & Svabic-Vlahovic, 2000). Stock cultures were streaked onto trypticase soy agar (TSA) plates that contained 100 μg/ml of ampicillin (Sigma-Aldrich Corp., St. Louis, MO) for the two GFP-labeled strains and no ampicillin for the non-GFP labeled strain, prior to incubation at 37 °C for 24 h. A single colony of each strain was transferred to 9 ml of TSB with/without 100 μg/ml of ampicillin for the GFP labeled and unlabeled strains, respectively, followed by overnight incubation at 37 °C. The resulting strains were serially diluted to 10^7 CFU/ml in TSB. After vortexing, the diluted bacterial suspensions (200 μl) were transferred to three wells of a 96 well non-pyrogenic polystyrene microtiter tissue culture plate (Corning Inc., NY). Three wells per plate containing 200 μl of sterile TSB served as negative controls.

After 48 h of incubation under refrigeration (4 °C), the microtiter plates were emptied, rinsed three times with 250 μl of sterile phosphate buffer, and air dried. The remaining cells were fixed to the well by adding 200 μl of 99% methanol (Fisher Chemicals, Fair Lawn, NJ). The methanol in the wells was decanted after 15 min, and the plates were air dried, again. After drying, the wells were stained for 5 min by adding 200 μl of 2% crystal violet. Excess stain was removed by placing the plates under running sterile deionized water. After the plates were air dried, 160 μl of 33% (vol/vol) glacial acetic acid (Sigma-Aldrich Corp., St. Louis, MO) was added to solubilize the dye bound to cells on the wells. Optical density (OD) of each well was measured at 570 nm using an EnSpire® Multimode Plate Reader (PerkinElmer, MA).

2.2.2. Broiler skin model

Fresh broiler skin (5 g/carcass) was excised from the breast area immediately after evisceration and before chemical spraying at a commercial broiler processing plant. Thereafter, the broiler skin method of Kim, Lillard, Frank, and Craven (1996) was used with a slight modification. Briefly, the bottom end of a 50-ml Nalgene conical-shaped centrifuge tube was cut for inoculum addition. Broiler skin was then tightly mounted on the top open mouth of the tube using a rubber band with the outer skin surface facing inside the tube. Finally, the top of the tube was covered with sterile aluminum to minimize drying and contamination of the skin.

2.2.3. Storage of broiler skin after Salmonella inoculation

The two frozen GFP-labeled strains of *Salmonella* Enteritidis (ME 18 and H4717) were transferred to TSB containing 100 μg/ml ampicillin (Sigma-Aldrich, MO), incubated for 24 h at 37 °C, pelleted by centrifugation at 3100*g* for 15 min at 4 °C, and re-suspended in sterile phosphate buffer saline (PBS, pH 7.4). After measuring the OD at 600 nm, both suspensions were adjusted to have a same OD value. The resulting suspensions were then combined in equal volumes to obtain a two-strain *Salmonella* Enteritidis cocktail containing ~1×10^9 CFU/ml. The *Salmonella* population in the inoculum cocktail was confirmed by plating appropriate dilutions on trypticase soy agar (TSA; Difco, BD), containing 100 μg/ml ampicillin followed by 24 h of incubation at 37 °C.

For microbial analysis in each of the two replications, eight skin samples were prepared and inoculated with the cocktail containing ~1×10^9 CFU/2 cm^2. The samples were then randomly subjected (1–2 samples/treatment) to five storage durations (0.5, 6, 12, 24, and 48 h) at 4 °C. For confocal imaging in each of the two replications, six skin samples were similarly prepared and inoculated as before. The samples were then randomly subjected (3 samples/treatment) to two different storage durations (24 and 48 h) at 4 °C.

2.2.4. Skin sampling for microbiological analysis and confocal microscopy

At the end of each storage time, the tubes containing the *Salmonella* cocktail were decanted and the skins were gently rinsed 3 times with 4 ml of sterile water to remove unattached *Salmonella* cells. The resulting skin was cut (1 g) from the centrifuge tube, transferred to a stomacher bag containing 9 ml of sterile PBS, and stomached for 1 min. The 1 min stomaching was then repeated with new PBS. After the two 1-min stomachings, the skin was ground for 1 min with sterile PBS (9 ml) to enumerate *Salmonellae*, using a sterile homogenizer (Polytron, Model PT10/35, Brinkmann Instruments Inc., Westbury, NY), having 2 1-cm blades.

The *Salmonella* cells recovered from the skin after two consecutive stomachings and grinding were deemed to be loosely and tightly associated, respectively, as previously defined by Singh, Lee, Chin, Ha, and Kang (2015). Before and after the two stomachings, skin samples were also collected for confocal microscopy.

2.2.5. Microbiological analysis

Serial 10-fold dilutions of the stomached and ground broiler skins were plated on TSA supplemented with ampicillin (100 μg/ml). All colonies fluorescing green under ultraviolet light were counted as *Salmonella* after 24 h of incubation at 37 °C.

2.2.6. Confocal microscopy

The broiler skin surfaces were examined using a confocal Laser