Penetration of surface-inoculated bacteria as a result of hydrodynamic shockwave treatment of beef steaks

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Abstract

The top surface of raw eye of round steaks was inoculated with either GFP (Green Fluorescent Protein) labeled *Escherichia coli (E. coli*-GFP) or rifampicin-resistant *E. coli (E. coli*-rif). Cryostat sampling in concert with laser scanning confocal microscopy (LSCM) and plating onto antibiotic selective agar was used to determine if hydrodynamic shockwave (HSW) treatment resulted in the movement of the inoculated bacteria from the outer inoculated surface to the interior of intact beef steaks. HSW treatment induced the movement of both marker bacteria into the steaks to a maximum depth of 300 µm (0.3 mm). Because popular steak cooking techniques involve application of heat from the exterior surface of the steak to achieve internal temperatures ranging from 55-82°C, the extent of bacterial penetration observed in HSW treated steaks does not appear to pose a safety hazard to consumers.

Keywords: Hydrodynamic shockwave; Bacterial penetration; Beef; Laser Scanning Confocal Microscopy

Introduction

The production of a safe and wholesome whole-muscle meat product with a reduced aging time and acceptable tenderness has been an objective for the muscle foods industry for decades. Researchers have explored the use of many technologies for improving tenderness, including application of enzymes, acids, blade tenderization, and hydrostatic pressure. Investigators have demonstrated the ability of HSW treatment to improve tenderness in pork and beef (18,22). HSW processing, also known as the Hydrodyne process (11,12), involves the passage of an explosive-generated shock wave through a vacuum packaged raw muscle food and its surrounding medium (water). The wave passes through substances with a mechanical impedence that matches that of water, rupturing specific cellular components thus producing a more tender product (10,12). Tenderization of the product is therefore achieved by the mechanical force of the shockwave on the cellular components.

The bulk muscle in healthy animals is essentially sterile prior to slaughter (17). Contamination of outer surfaces occurs during processing, where blades, knives, or gloves may cross-contaminate the cut surfaces with gut or environmental flora while leaving the interior of the steaks essentially sterile. In the past, researchers have performed studies on microbial dislocation to detect the natural movement of surface bacteria into the sterile inner tissues of muscle foods (7,13). The majority of studies have shown migration of bacteria does not occur to a significant extent (7). The very slow and selective nature of this movement allows the public to safely consume intact meats prepared “medium” and “medium rare” since the outer non-sterile surfaces of the meats are cooked to a temperature above the desired internal temperatures of 71

and 63°C, respectively (1). *Escherichia coli* O157:H7 is an adulterant of raw ground beef, and is deposited on the surfaces of carcasses as a natural component of fecal matter. The pathogen can cause hemorrhagic diarrhea and hemolytic uremic syndrome (HUS) in susceptible populations. Because of the severity of the illnesses and the low infective dose of the pathogen (<100 cells), regulatory agencies have been spurred to evaluate tenderization processes which may contribute to the survival of the pathogen in beef products, including ground beef, intact beef steaks, and products formed from beef trimmings (3,4,8). As stated by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) Meat and Poultry Subcommittee in 1997, steaks should be safe to consume "...if the external surfaces are exposed to temperatures sufficient to effect a cooked color change…" (4). External surface temperatures encountered in routine steak cookery techniques produce a cooked color change (1).

It has been suggested that HSW treatment of raw muscle foods may destroy spoilage and potentially pathogenic microorganisms while producing a product with improved tenderness (23). Because HSW treatment destroys portions of muscle fibers via penetration of a large force from the outer surface into the muscle (25), HSW treatment may produce a non-intact cut of beef. Such a product would thus fall under proposed FSIS sampling and testing programs for non-intact cuts of beef (3). If the process forces or permits the dislocation of surface bacteria deep into the beef muscle, normal cooking may not kill microorganisms now protected within the tissue, creating a microbial safety hazard in HSW-treated beef. USDA/FSIS regulations may consider non-fully cooked HSW treated beef as a microbial safety hazard if scientific evidence shows significant penetration of surface bacteria occurs.

In this study, the top outer surface of raw intact eye of round steaks was inoculated with one of two marker organisms (*E. coli*-GFP or *E. coli*-Rif) prior to vacuum packaging, subjected to HSW treatment, and then examined for the dislocation of the marker organisms from the surface into the interior of the muscle. Laser scanning confocal microscopy (LSCM), a useful tool for the non-invasive examination of thick biological samples, was used to visualize the dislocation of *E. coli*-GFP into the steaks (20,21). Traditional microbiological plating techniques were used to determine the extent of dislocation of viable *E. coli*-Rif into the tissue. The objectives of the study were twofold. The first objective was to determine if hydrodynamic pressure (HSW) treatment of intact raw beef steaks pushed marker bacteria from the outer inoculated surface to the interior of the steaks. The second objective was to determine whether the penetration occurred to an extent that the treated steak could pose a potential microbial safety hazard to consumers.

Materials and Methods

Preparation of marker microorganisms

E. coli-GFP:

A plasmid encoding the Green Fluorescent Protein (GFP) gene (PGFP conA vector lambda plasmid) and *E. coli* strain BLD21D3Plys were obtained from Novagen (Madison, WI). GFP expression was introduced into the *E. coli* strain following published methods (2). Twenty-five ml log phase stocks were generated in SOC medium following published methods (2). One ml aliquots were dispensed into sterile graduated 1.5 mL polypropylene flat top microcentrifuge tubes and centrifuged 3 min (IEC Centra-M Centrifuge, model 2399 s/n:0370, International Equipment Co., Needham Hts., MA, 13,300 rpm) to pellet cells. A 0.6 ml aliquot of the supernatant (SOC medium, (2) was discarded and replaced with 0.5 ml 7 % (v/v) dimethyl sulfoxide DMSO (Fisher, Pittsburgh, PA) and stored as stock cultures at -80°C prior to use (2). Fourteen hours prior to use, stock cultures were thawed on ice, placed into 25 ml LB medium containing 100 ug/ml ampicillin (Sigma, St. Louis, MO) + 0.25mM Isopropyl-Beta-D-thiogalactopyranoside (IPTG, Pittsburg, PA) and grown aerobically at 37°C in a shaking incubator (4L, Precision Scientific, Chicago, IL) for 4 hr to log phase (2). Logarithmic growth of inocula was confirmed previously in the laboratory using growth curve experiments. A0.1 ml volume was spread-plated onto individual LB agar plates containing 100ug/ml ampicillin and

0.25mM IPTG (LBAI; [2]) and the plates were incubated for 9 hr at 37°C to allow the growth of lush bacterial lawns.

E. coli-Rif

Rifampicin resistance was introduced into a laboratory strain of *E. coli* following the methods of Kaspar and Tamplin (9). A frozen culture aliquot was cultivated in 25 ml Trypticase Soy Broth (TSB, Becton Dickinson, Cockeysville, MD) with 100ug/ml rifampicin (Sigma) and grown to log phase at 37°C in a shaking incubator. Logarithmic growth of inocula was confirmed previously in the laboratory using growth curve experiments. A0.1 ml volume was spread-plated onto individual TSA agar plates containing 100ug/ml rifampicin (TSA-rif; [9]) and the plates were incubated for 9 hr at 37°C to allow the growth of lush bacterial lawns.

Preparation of steak samples

Fresh eye of round beef steaks (approx. 2.54 cm in thickness) purchased from a local grocery store were hand trimmed in the laboratory to form disks 6.4 cm in diameter and 2.54 cm in thickness, and then stored at 4°C for 24 hrs. Steaks were randomly designated as "uninoculated control", "*E. coli*-GFP" or "*E. coli*-Rif" and treated accordingly. The top surface was inoculated by placing individual steaks onto a bacterial lawn of *E. coli*-GFP or *E. coli*-Rif in

petridishes for 30 min. at room temperature. Inoculated steaks and uninoculated controls were individually vacuum-packaged (Ultravac model UV-250, Koch, Kansas City, MO) in Reduced Temperature Shrink bags (Cryovac, Duncan, NC), with "top surface" and steak identity code (GFP, Rif, or uninoculated control) clearly marked on package. Packaged steaks were dipped into 100°C water bath for 2-3 s, further vacuum packaged into individual Bone-Guard bags (Cryovac), dipped into 100°C water bath 3-5 s. The steak samples were transported in a Styrofoam cooler on ice to the Buena Vista Hydrodyne prototype (Dynawave, Inc., Buena Vista, VA) for HSW treatment. Hot water dipping of packaged samples was performed in order to "shrink fit" the package to the steak sample, reducing the void spaces between the steaks and the package which could contribute to failure of the packages during HSW treatment. Double bagging of samples was performed to reduce the failure rate of the packages during HSW treatment (11,12,15,22,23,25).

HSW treatment

Samples were treated in the carousel unit of the Buena Vista Hydrodyne prototype. This unit consisted of a large stainless steel tank with a dome top holding 1060 L cold tap water (7-10°C). Two GFP steaks and two Rif steaks were secured with plastic cable ties to the inner stainless steel basket of the commercial carousel unit so that the surface labeled "top" faced the explosive in each detonation. One DCI-10 molecular explosive (Pentaerythritol Tetranitrate, (PETN), product no. BOB010, 360 g, Donovan Commercial Industries, Nortonville, KY) was

molded into a shaped charge (hemishell) and was suspended with wire 48.3 cm above the steaks to induce a supersonic shockwave upon detonation. The basket was lowered into the tank, then the tank was sealed and flooded with cold water to remove all void spaces in the tank. An electronic detonation device located outside the tanks was utilized to detonate the explosive within the tank. The samples were removed from the tank after treatment, inspected for package integrity, and placed on dry ice for transport to the food microbiology laboratory at Virginia Tech for analysis. Unopened steak samples were held at -20°C until sampling within 24 hrs of receipt. In total, 16 steaks were treated per experiment (8 *E. coli*-Rif , 8 *E. coli*-GFP), with each steak receiving only one HSW treatment. Those steaks not subjected to the Hydrodyne process were labeled inoculated controls and held on ice during HSW treatment and then frozen with the HSW treated steaks. The experiment was repeated a total of two times, providing 32 HSW-treated steaks (16 *E. coli*-Rif , 16 *E. coli*-GFP).

Sampling of frozen steaks

Frozen steaks were aseptically removed from their packages under a laminar flow hood. The uninoculated side of each steak was seared on a preheated (5 min, approx. 200°C) electric non-stick skillet (West Bend 28 cm, model no. 72020). Searing for 2-3 s. was done to achieve a cooked color appearance. Searing was performed to inactivate any bacteria that may have been transferred from the "top" inoculated surface to the "bottom" surface during treatment. A nickel burnished steel cork borer (0.5 cm x 2.2 cm; d x l; Boekel, Fisher Scientific, Pittsburg, PA) was flame-sterilized prior to use in removing core samples. The hot sterile cork borer was twisted

and pushed from the seared uninoculated surface of the steak to the inoculated surface and pushed into the non-tapered end of a sterile 1000 ul pipette tip (approx. 7.5 mm diameter) by aseptically and gently pushing the core through the borer with a sterile stick. Hot coring from the seared to the non-seared end ensured no bacteria were transmitted into the sample during the coring step. Five core samples were removed from each steak and labeled. Cores were kept at -20°C until Cryostat sampling (approx. 8 days).

Cryostat sampling of cores

The uninoculated end of each frozen core was mounted onto the center of a microtome die with fixing compound (Tissuetek O.C. T. Compound, Miles Inc., Elkhart, IN). The inoculated en was exposed to be aseptically sliced and sampled at -20°C with a Cryostat (Cryocut 1800, Reichert-Jung; Cambridge Instruments GMBH, Heidelberg, Germany; microtome blades Shandon Lipshaw High Profile Disposable blades No. 1001259, Pittsburgh, PA, autoclave sterilized). Each core was sampled at 50 µm intervals. Each 50µm-wide segment was made by slicing the core with a sterile portion of the blade then the slice retrieved and treated according to the marker bacterium each core contained. The blade was then shifted 2 cm to the left to expose a sterile portion of the blade to eliminate cross contamination. The entire procedure was repeated until the entire core was sampled. Only the blade and sterile retrieving elements made contact with the cores or the slices. For cores with *E. coli*-Rif, each slice was removed from the blade by touching it with a sterile cotton-tipped swab pre-moistened in sterile 0.1 % peptone. The swab was then placed into a labeled sterile tube containing 1 ml 0.1 % peptone blank, and the tip was

broken off into the tube. Two *E. coli*-Rif slices were placed into each volume (1 ml) of peptone to represent a sampling depth of 100 µm. For cores marked with *E. coli*-GFP, each slice was removed from the blade by touching it with a sterile pre-warmed microscope slide for the period of time needed for the slice to adhere to the microscope slide. The slide was then shifted approximately 1 mm to the left, and then the process was repeated in order to place five to six slices onto each slide.

Laser scanning confocal microscopy examination

To determine the extent of Hydrodyne-assisted dislocation of bacterial cells into the HSW-treated inoculated steaks, GFP HSW-treated core slices, cores slices removed from GFP non-HSW treated controls, and uninoculated core slices were viewed by LSCM (Zeiss LSM 510, Thornwood, NY). Slices were viewed under a 40x objective (Apochromat lens with 1.2 w correction), subjected to 488 nm excitation, and the emitted light filtered with a narrow band filter (FITC setting). The settings allowed for light transmitted imagery collected as a red image (red setting for transmitted light) of the tissue in concert with green fluorescent viewing of the marker bacteria. Slices from inoculated HSW-treated steaks in which GFP expressing bacteria were present beyond the maximum depth in the inoculated control steaks were designated positive for Hydrodyne-assisted microbial penetration. Because the beef was not stained prior to LSCM examination, autofluorescence from the raw beef was encountered in initial studies. This was reduced by utilizing a narrow band path filter (FITC, 488 nm), which allowed the photodetector to collect only the light emitted by GFP.

Microbiological examination

To determine the extent of Hydrodyne-assisted penetration of viable bacterial cells into the HSW-treated inoculated steaks, each blank containing two 50µm thick core slices (representing a sampling depth of 100 µm) were removed from Rif HSW-treated core, core slices removed from Rif non-HSW treated controls, and uninoculated core slices were placed into individual 1.0 ml 0.1 % peptone blanks. The blanks were vortexed for 20 s (Variable Speed Touch Mixer, model 232, Fisher Scientific, Pittsburgh, PA; setting 10), 1ml aliquots were pour plated in TSA-Rif (100ug rifampicin/ml) and examined for growth after aerobic incubation for 48 hrs. at 37°C. Sampling depths of 100 µm were designated positive upon visual confirmation of microbiological growth on TSA-Rif.

Results

No differences were observed in the growth characteristics of the parental strain *E. coli* and the two genetically modified strains, *E. coli*-Rif and *E. coli*-GFP. Growth curves did not significantly differ among the strains (data not shown, $p > 0.05$).

HSW treatment induced dislocation of *E. coli*-GFP from the outer inoculated surface towards the interior of the intact steaks (Table 1). Penetration occurred only to a maximum depth of 250 µm from the outer surface. HSW treatment increased the depth at which *E. coli*-GFP was found by 150 µm beyond the depth seen in untreated inoculated control samples. In slices removed from HSW-treated steak samples, the marker bacteria appeared as plump rods displaying a bright green fluorescence, contrasted with the dark red and black background of the unstained beef tissue. No bright green fluorescent plump rods were observed in slices removed from uninoculated steak samples nor in slices representing a depth beyond 250 µm towards the interior of HSW-treated steaks inoculated with *E. coli*-GFP. In most instances, *E. coli*-GFP cells were visually found to be associated with the crevices between muscle bundle fibers. Prachaiyo and McLandsborough (20) found a similar association, finding their GFP-expressing *E. coli* strains in the spaces between and on the surface of muscle fibers.

E. coli-Rif marker bacteria also exhibited dislocation from the outer inoculated surface towards the interior of the intact steak as a consequence of HSW treatment (Table 2). Visual examination of bacterial growth on TSA-Rif plates revealed viable *E. coli*-Rif cells within the

first three 100 µm segments removed from HSW-treated inoculated steaks, corresponding to a depth of 300 µm from the outer surface. Viable *E. coli*-Rif cells were detected within the first 100 µm segment of non-HSW treated inoculated control steaks, most likely reflecting surface inoculum (*E. coli*-Rif) and minimal dislocation into the tissue. Bacterial growth was not detected in plates reflecting a depth beyond 300 µm from the outer surface towards the interior of HSW treated steaks nor was it detected in plates reflecting a distance beyond 100 µm from the outer surface towards the interior of the non-HSW treated inoculated control steaks. Slices removed from the uninoculated control samples failed to produce visible growth on TSA-Rif plates, as expected.

The extent of which bacterial dislocation occurred in the non-HSW treated controls is consistent with the observations of Prachaiyo and McLandsborough (20), who reported that most bacteria in intact steaks (beef top round roasts) were observed between 0 – 30 µm from the surface of the meat. The distance range found by the researchers (20) was encompassed within the first slices obtained from all steaks, with the first slice representing a distance of 50 µm for those inoculated with *E. coli*-GFP and 100 µm for those inoculated with *E. coli*-Rif.

Discussion

Green Fluorescent Protein (GFP) was originally extracted from the jellyfish *Aequorea victoria*. It is a fluorescent protein with absorption and emission peaks 395 nm and 509 nm, respectively (5). Bacterial strains genetically modified to express GFP have gained use by food microbiology researchers to study the interaction of *E. coli* with lettuce leaves and meats at the structural level (14,20,21,24). Researchers tout the power of employing LSCM for the study of complex food matrices (6,21). Linking LSCM with genetically modified luminescent strains of pathogens and spoilage bacteria allows one to observe their behaviors in the foodstuff without drastically changing the environment of the bacteria as do electron microscopy and traditional light microscopy (14,20). In our studies, linking LSCM and GFP proved to be a powerful technique, allowing for visualization of individual cells among structures of the meat. This was consistent with the work of Prachaiyo and McLandsborough (20) who indicated *E. coli*-GFP associated itself within the surface or sarcolema of individual muscle fibers or within crevices of raw beef muscle.

An “Intact Beef Steak is defined as a “cut of whole muscle that has not been injected, mechanically tenderized, or reconstructed” (4). The mechanical action of the shockwave generated by HSW processing on the cellular components of the raw steak produces a non-intact product by definition. Not only is the steak mechanically tenderized, but it may also be considered to be “injected” when one considers the strict definition of injected as “manipulating a meat so that infectious or toxigenic microorganisms may be introduced from its surface to its

interior through tenderizing with deep penetration..." (4). The fact that bacterial dislocation occurred at all may pose regulatory concerns with HSW treatment of raw steaks if proposed FSIS regulations are enacted. FSIS must first define the threshold distance from the outer surface to the interior which it considers as "deep" for penetration purposes. If this depth exceeds 1/4 mm, then HSW-assisted bacterial dislocation would not fit within the threshold distance and the treated steak would not be defined as an "injected" steak by the proposed FSIS rule. Other tenderization techniques have been shown to produce non-intact steaks but not pose a bacterial safety hazard to consumers. For example, blade tenderization of raw intact muscles produce non-intact products as well. This technique has been shown to transfer surface bacteria to the interior of the muscle where the bulk of the muscle may protect bacteria from thermal inactivation during traditional cookery techniques (19). Phebus (19) suggested blade-tenderized steaks posed no additional safety hazard to consumers, observing that although the technique transferred 3-4% of the artificially inoculated surface bacteria into the interior of the muscle, cooking the steaks in an oven broiler to a target internal temperature of as low as 54°C (rare) produced a 5.0 log reduction (CFU/g) in treated steaks as compared to a 5.6 log reduction (CFU/g) in non-blade tenderized steaks. The 0.6 log difference in the two treatments was not statistically significant.

Typical steak cookery techniques involve application of heat from the exterior surface of the steak to achieve an internal endpoint temperature range of 55-82°C (very rare to very well done, respectively) (1). Vegetative bacterial cells which may be on the outer surface of raw beef are typically inactivated by the high temperatures used in such cookery. In 1999, Moeller and

others found that Hydrodyne processing had no significant effect on the natural flora of pork loins, though the effect of the process on pathogenic bacteria has not been published to date (16). Future research should focus on the effects of current and future modifications of HSW processing on bacterial populations and bacterial penetration.

HSW treatment caused penetration of both marker bacteria into the steaks to a maximum depth of 300 µm (0.3 mm) in all steak samples. Although HSW-induced bacterial penetration was observed in treated steaks, the extent of penetration would not appear to pose a safety hazard to consumers if proper cookery techniques are followed.

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Table 1. Hydrodynamic Shockwave (HSW) induced penetration of *E. coli*-GFP cells in intact beef eye of round steaks.

	Percentage of samples found positive within each depth from the surface to the interior of the steak (μm) [b]						
Steak Sample	0 - 50	50 - 100	100 - 150	150 - 200	200 – 250	250 - 300	300 - 350 [c]
HSW-treated	100 [e]	100	94[g]	93	79	75	0 [h]
Control	90	0 [h]	0 [h]	0 [h]	0 [h]	0 [h]	0 [h]

[a] Depth measured in μm from the surface to the interior of the steak

[b] Percentage of slices in which marker bacterium was detected. 16 HSW treated steaks, 8 non-treated controls with five core samples removed per steak, yielding 80 slices from HSW treated steaks and 40 slices from non-HSW treated controls

[c] Two core slices lost via laboratory accident

[d] No *E.* coli-GFP was detected in any slices beyond this depth

Table 2. Hydrodynamic Shockwave (HSW) induced penetration of *E. coli*-Rif cells in intact beef eye of round steaks. [a]

Steak Sample	Percentage of samples found positive within each depth from the surface to the interior of the steak (µm) [a,b]			
	0 - 100	100 - 200	200 - 300	300 - 400 [c]
HSW	100	62	90	093
Control	100	0 [c]	0 [c]	0 [c]

[a] Depth measured in µm from the surface to the interior of the steak

[b] Percentage of slices in which marker bacterium was detected. 16 HSW treated steaks, 8 non-treated controls with five core samples removed per steak, yielding 80 slices from HSW treated steaks and 40 slices from non-HSW treated controls

[c] No *E.* coli-GFP was detected in any slices beyond this depth