

Microbiological Analysis of Bovine Lymph Nodes for the Detection of *Salmonella enterica*[†]

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ABSTRACT

Bovine peripheral lymph nodes (LNs) have been identified as a potential source of *Salmonella* when trim containing these nodes is incorporated into ground beef. Studies examining the prevalence of *Salmonella* in peripheral LNs of cattle are few in number, and the microbiological methods used for these analyses have not been validated. Given that *Salmonella* contamination may be found on postintervention carcasses, it is important to understand the extent to which *Salmonella* contamination from surrounding adipose tissue is transferred to LN samples during sample preparation. To better understand the potential for cross-contamination, 906 LN samples were collected from postintervention carcasses and these, along with the corresponding adipose trim (AT), were analyzed for the presence of *Salmonella*. The results showed that the *Salmonella* prevalence in LNs and on AT was 0.8 and 5%, respectively, but that it was possible to find AT positive for *Salmonella* contamination while the corresponding LNs were negative and vice versa. In order to examine the dynamics of cross-contamination between surface adipose tissue and LNs in the trimming process, inoculation studies were performed. The efficacy of LN submersion in boiling water as a means of surface sterilization and the effect of boiling on the viability of *Salmonella* contained within LN samples were also examined. The results showed that, on average, 23 to 43% of the inoculated LN samples in this study were cross-contaminated by *Salmonella* on surrounding adipose tissue when present in the range of 10¹ to 10² CFU per sample; however, surface decontamination methods were very effective at removing *Salmonella* cross-contaminants in this range.

Bacterial contamination is responsible for vast numbers of foodborne illnesses each year in the United States. *Salmonella enterica* is one of the leading bacterial agents of foodborne disease, causing approximately 40,000 documented cases in the United States each year (6, 15). While poultry products and, more recently, contaminated fresh produce are well-established vectors for *S. enterica*, several foodborne disease case studies have shown undercooked ground beef and beef products to be sources of sporadic and outbreak cases of salmonellosis (8, 17, 19). In order to mitigate contamination of food products and aid epidemiological investigation, it is necessary to identify sources of contamination.

According to the Centers for Disease Control and Prevention, approximately 1 in 10 foodborne disease outbreaks attributed to beef as a single commodity is due to *S. enterica* contamination (7). One potential source of *S. enterica* in the beef food chain is contaminated ground beef

that is produced from beef trim, including adipose tissue that may contain lymph nodes (LNs). LNs function as a filtering mechanism to sequester bacteria, viruses, and other infectious agents for eventual destruction by lymphocytes. However, certain bacteria (such as some members of the genus *Salmonella*) are able to evade the host immune response by invading and surviving within immune cells, such as macrophages (12). A number of studies have reported on the isolation of *S. enterica* from cattle LNs (2, 9, 13, 16). Most of these studies have focused on *S. enterica* contamination of mesenteric LNs that normally would not be included in ground beef, as they are discarded during the evisceration process. However, the superficial cervical and the subiliac LNs are located within the adipose tissue of muscle cuts (such as the flank and chuck), and it is these tissues that are of concern as a potential pathogen source for ground beef (2).

Studies examining the prevalence of *Salmonella* in peripheral LNs of cattle are few in number (2, 13, 14), and the microbiological methods used for these analyses have not, to our knowledge, been validated. Given that *Salmonella* contamination may be present on the surface of postintervention carcasses, it is important to ensure, when *Salmonella* is detected from LN samples, that it originated from within the LN and was not present as the result of

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[†] Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. The USDA is an equal opportunity provider and employer.

cross-contamination from the surrounding adipose tissue. Thus the objectives of this study were to examine (i) the potential for cross-contamination to confound microbiological analysis of LNs for *Salmonella* contamination, (ii) the dynamics of cross-contamination between surface adipose tissue and the LN in the trimming process, and (iii) the efficacy of a previously described method (13) for the removal of bacterial contamination on the LN sample surface.

MATERIALS AND METHODS

Bacterial inoculum. All inoculation experiments were performed using a fresh, overnight culture of *Salmonella enterica* serovar Typhimurium previously isolated from cattle (5). *Salmonella* Typhimurium was routinely cultivated in 10 ml of Difco tryptic soy broth (TSB; BD, Sparks, MD) for 18 h at 37°C. Inocula were prepared by diluting overnight cultures through a series of 10-fold dilutions in sterile normal saline (0.85% NaCl), and the CFU in each dilution level used were quantified by spread plating 100- μ l aliquots onto tryptic soy agar plates (BD) in duplicate.

Subiliac LN sample collection. Subiliac LNs (also known as the prefemoral LN) are located on the ventral side of the carcass between the round break and the sirloin break in an area of the carcass termed the flank. LNs were excised from individual animals postintervention (after application of carcass interventions) but prior to carcass chilling and fabrication. LNs were harvested by cutting into the adipose tissue surrounding the node. The resulting adipose tissue-encased LN (AELN) samples were placed into individual Whirl-Pak sample bags (Nasco, Fort Atkinson, WI) and shipped in coolers containing ice packs to the U.S. Meat Animal Research Center, Clay Center, NE. Samples were analyzed within 24 h of collection or were stored at -20°C until further use in inoculation studies.

Culture media, enrichment, and confirmation of *Salmonella* isolation. *Salmonella* contamination was examined in 906 LNs and the corresponding surrounding adipose trim (AT). LN samples were collected and processed as previously described (2), with modifications as follows. LN samples were aseptically trimmed of excess fat and fascia, and if the LN was received cut (i.e., cut during the process of LN removal from the carcass), this was noted. For every five or six LN samples, the corresponding AT was collected and pooled into a large Whirl-Pak filter bag (Nasco). The resulting AT pools were weighed (average weight was ~300 g) and 500 ml of TSB was added, resulting in an AT/TSB ratio ranging from about 1:2 to 1:6. The AT pool samples were then massaged by hand for approximately 10 to 15 s. Trimmed LN samples were surface sterilized by submersion in a boiling water bath for 3 to 5 s. After boiling, each LN was placed into a filtered stomacher sample bag (Nasco), weighed, and pulverized using a rubber mallet. TSB (80 ml) was added to each sample bag, and the samples were homogenized for 30 s with a laboratory blender (BagMixer 400VW, Interscience Laboratories, Inc., Weymouth, MA) at medium speed (7 strokes per s). The homogenized LN and AT pool samples were then incubated at 25°C for 2 h and at 42°C for 12 h and held at 4°C for no more than 4 to 6 h until further processed.

After incubation, 1 ml from each enrichment culture was subjected to anti-*Salmonella* immunomagnetic separation with 20 μ l of anti-*Salmonella* beads (Invitrogen, Carlsbad, CA). The bead-culture mixture was incubated with shaking on an orbital shaker (900 rpm) at room temperature for 15 min. The beads were then extracted from the enrichment samples and washed twice in

phosphate-buffered saline-Tween 20 (Sigma, St. Louis, MO) with an automated magnetic particle processor (KingFisher 96, Thermo Fisher Scientific, Inc., Waltham, MA) as described previously (3). The beads were transferred to 3 ml of Rappaport Vassiliadis soya (Remel, St. Louis, MO) broth and incubated at 42°C overnight. *Salmonella* organisms present in these secondary enrichments were detected by swabbing the Rappaport Vassiliadis soya enrichment culture onto (i) XLDtnc agar (4) composed of XLD medium (Remel) with 4.6 ml/liter Tergitol, 15 mg/liter novobiocin, and 10 mg/liter cefesulodin (Sigma) and (ii) brilliant green agar with sulfadiazine (80 mg/liter) (BD). All plates were incubated at 37°C for 18 to 20 h. After incubation, one or two suspect colonies (if different colony morphologies were present) were picked for confirmation. Suspect *Salmonella* isolates were confirmed by PCR assay using primers for the *Salmonella*-specific portion of the *invA* gene (10, 11). All confirmed *Salmonella* isolates were serogrouped using Wellcolex Colour *Salmonella* tests (Remel) according to the manufacturer's recommendations. *Salmonella* isolates were further serotyped using antisera for the identification of somatic and flagellar antigens (Remel). Slide agglutination was used to confirm or refine the results of the Wellcolex Colour test O-group results, and tube agglutination was used to identify flagellar H-antigens (1).

Salmonella contamination present in LNs and corresponding AT pool samples was compared. For the 906 LNs examined, there were 180 resultant AT pool samples (AT from five or six LNs per AT pool). Possible outcomes included the following: LNs and AT pool sample negative for *Salmonella* (LN-/AT-), LN samples positive but the corresponding AT pool negative (LN+/AT-), LN samples negative but the AT pool sample positive (LN-/AT+), and both LN and AT samples positive (LN+/AT+). The results were grouped by outcome and are depicted as a histogram.

Examination of LN surface decontamination methods.

Cross-contamination of *Salmonella* bacteria from the surrounding AT to the corresponding LN in the trimming process and the efficacy of submerging the LN in boiling water for 3 to 5 s for removing this contamination were examined by inoculating the surface of 56 AELN samples with *Salmonella* Typhimurium bacteria at three different concentrations, 10¹, 10², and 10³ CFU/ml. AELN samples, contained within individual Whirl-Pak sample bags, were weighed prior to the addition of 1 ml of inoculum. Inoculated AELN samples were then massaged by hand in order to distribute the inoculum and held at 4°C (~18 h) prior to further processing. The following day, each AELN sample was trimmed, with care being taken in order to prevent cross-contamination between independent AELN samples. The AT from each LN sample was placed into a sample bag, while the corresponding LN was cut in half with a sterile razor blade. Immediately after cutting, one LN half was placed directly into a Whirl-Pak sample bag and the other was submerged in boiling water for 3 to 5 s prior to being placed into a sample bag. All samples were weighed, LN samples were pulverized and enriched, and the presence of *Salmonella* determined as described above, with the modification that the AT samples received 250 ml as opposed to 500 ml of TSB prior to enrichment. The percentages of AT, LN, and boiled LN (BLN) samples in each range that were found contaminated with *Salmonella* are reported. Calculating the CFU per gram inoculated resulted in three ranges of inoculation levels: >0 to <2 CFU/g, 2 to <10 CFU/g, and 10 to <200 CFU/g. The average AELN weight and standard deviation were calculated and reported for each level.

Viability and enumeration of *Salmonella* from LNs inoculated via injection. The effect of the surface decontamination process on the viability of *Salmonella* inoculated within LN

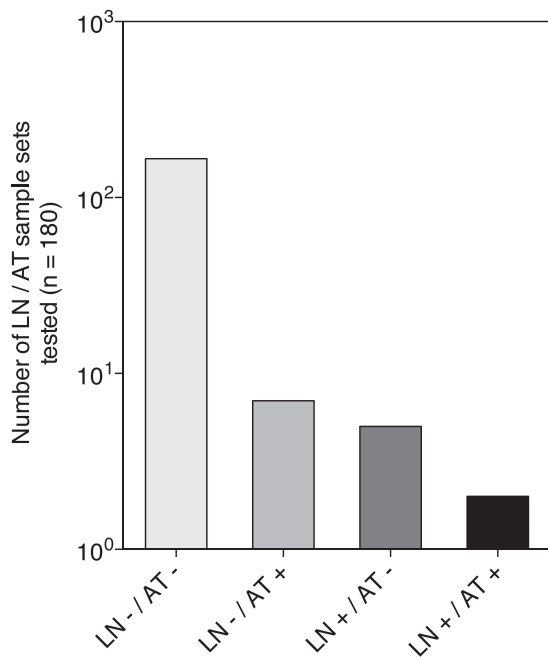


FIGURE 1. Histogram of lymph node (LN) and corresponding adipose trim (AT) pool *Salmonella* contamination status, grouped by outcome. Outcomes listed on the x axis include LN and AT pool samples both negative for *Salmonella* (LN-/AT-), LNs negative but AT pool positive (LN-/AT+), LN positive but AT pool negative (LN+/AT-), and both LN and AT samples positive (LN+/AT+).

samples was examined. This was accomplished by injecting trimmed LN samples with an inoculum of approximately 0.25 ml of *Salmonella*. Three concentrations were used, including 10^1 , 10^2 , and 10^3 CFU/ml, with eight LN samples inoculated per level, for a total of 24 LN samples. Prior to injection, the weight of each LN sample was recorded, and after injection, samples were incubated at 4°C for 30 to 60 min. Each injected LN sample was submerged in boiling water for 3 to 5 s and placed into a sample bag, pulverized, and homogenized in 80 ml of TSB as described above. *Salmonella* Typhimurium viability within inoculated LN samples was examined by plating 1-ml aliquots of LN-TSB homogenate in quadruplicate on EB Petrifilm (3M Microbiology, St. Paul, MN), which was incubated at 37°C for 18 to 22 h. Following incubation, colonies were counted and 15 to 100% of those present per Petrifilm (up to five colonies per plate) were picked and plated to XLDtnc to confirm the presence of the *Salmonella* phenotype. Isolates with the correct phenotype were further serogrouped using the Wellcolex Colour serogrouping kit (Oxoid, Remel, Basingstoke, UK) in order to confirm that they were the same serogroup as the inoculated strain. Once confirmed, the CFU per gram enumerated was calculated and compared with the theoretical CFU per gram, based on the initial inoculum level and weight of the LN sample. These data were plotted, and a linear regression analysis to examine goodness of fit (r^2) between the theoretical and observed CFU per gram values was performed. All data were analyzed and plots constructed using Prism 5.0, GraphPad Software, Inc. (www.graphpad.com, San Diego, CA).

RESULTS AND DISCUSSION

In this study, we examined 906 LN samples and 180 corresponding AT pool samples for the presence of *Salmonella*. The results showed the mean prevalence of

TABLE 1. Microbiological analysis of LN and corresponding AT pool samples for the presence of *Salmonella*^a

Sample event outcome (no.)	<i>Salmonella</i> isolated	
	LN positive	AT positive
LN-/AT+ (7)	—	Newport
	—	O3,10:R1H
	—	Montevideo
	—	Typhimurium
	—	Newport
	—	Mbandaka
LN+/AT- (5)	—	Typhimurium
	Anatum	—
	Anatum	—
	Dublin	—
	Cubana	—
LN+/AT+ (2)	Typhimurium	—
	Montevideo	Typhimurium
	Montevideo	Montevideo

^a LN, lymph node; AT, adipose trim pool; —, *Salmonella* not detected.

Salmonella to be 0.8 and 5.0% for LN and AT pools, respectively. It should be noted that the observed prevalence for *Salmonella* in the AT pools is the result of analyzing five or six AT samples per pool. Accordingly, the observed prevalence value is somewhat inflated, given the likelihood that only one of the AT samples in any given pool was actually positive. With that in mind, these observations are generally in keeping with previous reports of *Salmonella* prevalence in peripheral LNs of cattle (1.6% for $n = 1,140$ LNs examined (2)) and on postintervention carcasses (0.8% for $n = 3,040$ carcasses sampled, 95% confidence interval, 0.18 to 1.42% (5)) and with the results of a beef trim baseline study conducted from 2005 to 2007, which estimated the prevalence of *Salmonella* in U.S. beef trim to be 0.78% (95% confidence interval, 0.29 to 1.27%) (18).

Examination of the *Salmonella* serotypes isolated from LN and/or AT pool samples revealed that results from corresponding samples rarely matched. *Salmonella* contamination was detected in 14 of 180 LN/AT paired samples (Fig. 1). In seven cases, AT pools were found to be positive for *Salmonella*, but all corresponding LNs tested were found to be negative. In five cases, LN samples positive for *Salmonella* contamination were observed, but the corresponding AT pools were negative. Finding both LNs and corresponding AT pools positive for *Salmonella* contamination was rare and occurred in two cases. In one case, an AT pool was found positive for *Salmonella* Typhimurium while an LN corresponding to that AT pool was positive for *Salmonella* Montevideo. In only one case did the serotype isolated from an LN and AT pair match, and the strain isolated in this instance was *Salmonella* Montevideo (Table 1).

These results revealed the potential to have surrounding AT positive for *Salmonella* contamination but the concomitant LN negative. To better understand the dynamics of *Salmonella* cross-contamination between surface adipose tissue and the residing LN, we performed a surface adipose

TABLE 2. *Salmonella* cross-contamination and surface decontamination method efficacy of inoculated AELN^a

Calculated inoculation level	AELN (no.)	Avg wt (g)	SD ^b	% <i>Salmonella</i> positive ^c		
				AT	LN	BLN
>0-<2 CFU/g	17	117.5	52.8	100	23.5	0.0
2-<10 CFU/g	19	108.9	77.6	100	42.1	0.0
10-<200 CFU/g	20	71.5	61.4	100	95.0	35.0
Total	56	98.1	67.1	100	53.5	11.7

^a AELN, adipose tissue-encased lymph node.

^b SD, standard deviation of the LN average weight in grams.

^c AT, adipose trim; LN, lymph node; BLN, boiled lymph node.

tissue inoculation study. To that end, we inoculated the surface of AELN samples with three concentrations of *Salmonella*, resulting in three levels of surface contamination, 10⁰ (n = 17), 10¹ (n = 19), and 10² (n = 20) CFU/g. *Salmonella* detection from the resulting AT, LN, and BLN samples showed that *Salmonella* cross-contamination had occurred in the trimming process (Table 2). As expected, cross-contamination increased with increasing levels of AT contamination, as 42.1 and 95% of LN samples from AELN samples inoculated at 10¹ and 10² CFU/g, respectively, were found to be contaminated with *Salmonella*. However, for AELN inoculated at 10⁰ CFU/g, only 23.5% of the resulting LN samples were found contaminated with *Salmonella*. The Food Safety and Inspection Service (U.S. Department of Agriculture) reported in its Beef Trim Baseline study (18) that *Salmonella* levels, when detected, are quite low (geometric mean of 0.7 CFU/g trim). This is in keeping with reports of *Salmonella* levels on postintervention carcasses in the range of 0.01 to 0.07 CFU/100 cm² (4) and suggests that the low end of the AELN inoculation study (10⁰ CFU/g) more likely reflects what might occur with naturally contaminated samples.

With results confirming that *Salmonella* cross-contamination of LN surfaces from surrounding AT could occur

even if present at very low levels, studies were undertaken to determine if submersion of LNs in boiling water was an adequate mitigation procedure. Inoculation studies showed this method to be most effective when AELN surface contamination was in the range of 10⁰ to 10¹ CFU/g (~75 to ~800 CFU per AELN), as all BLN from these samples were negative for *Salmonella* contamination. For AELN inoculated at greater concentrations (>800 to ~6,000 CFU per AELN), the surface decontamination method worked 65% of the time. However, 35% of BLN at this inoculation level were found contaminated with *Salmonella* (Table 2). While it is possible that some of these LNs could have been contaminated with *Salmonella* prior to inoculation, it should be noted that five of the seven BLNs in this category originated from AELN samples where the residing LNs were noted as having been cut during removal from the carcass, thus providing a possible infiltration route for the inoculated *Salmonella*.

Finally, the effect of thermal exposure during the surface decontamination process on the viability of *Salmonella* contained within LN samples was examined using LN samples that were inoculated via injection. Trimmed LNs were injected with three different levels of *Salmonella* and then subjected to surface decontamination methods as described above. The LN-TSB homogenate of each inoculated sample was enumerated and the observed CFU per gram calculated and plotted against the theoretical CFU per gram value (Fig. 2). This analysis showed that for LNs inoculated in the 10¹ to 10² CFU/g range (~75 to 1,500 CFU per LN), the observed and theoretical CFU per gram values were in general agreement, as more than 50% of the values fell within the same log range. LN samples that were inoculated in the lowest range (10⁰ CFU/g or less than 20 CFU per LN) approached the limit of detection for the enumeration assay, so it was difficult to conclude if thermal exposure negatively impacted the *Salmonella* inoculum in this range or if the levels were simply too low to be reliably enumerated using the described method.

In conclusion, when surface adipose tissue contamination levels were in range with those observed for postintervention carcasses and trim, we found the methods described for LN surface decontamination to be very effective for removing cross-contamination incurred during the trimming process. It is noteworthy that while levels of *Salmonella* prevalence on postintervention beef carcasses

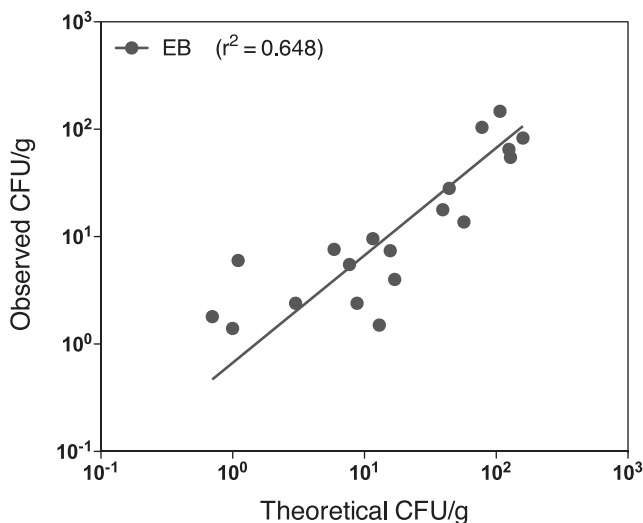


FIGURE 2. Comparison of the observed CFU per gram of *Salmonella Typhimurium* enumerated from LN samples inoculated via injection and the calculated theoretical CFU per gram inoculated, as a measure of *Salmonella* viability.

and trim have been found in the range of 0.8%, prevalence in ground beef has been observed to be somewhat higher, 4.2% on average (3). A possible explanation for this disparity may be that carcass and trim surface sampling will not likely detect *Salmonella* located within trim harboring contaminated LNs. Accordingly, to fill an important knowledge gap, it is necessary to assess the level of *Salmonella* present in contaminated peripheral LNs. With this knowledge, we can begin to understand the contribution of this potential pathogen source when AT containing LNs is incorporated into ground beef. The methods described here will be useful in experiments designed to address this question.

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