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Research Paper

Comparison of Microbiological Sampling Methods for Inspection Verification Sampling of Raw Beef Trim Products

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ABSTRACT

In the United States, the N60 excision sampling method for beef trim products used by FSIS collects 60 thin slices of raw meat, which are then analyzed for bacteria. This destructive sampling method is time-consuming, results in inconsistent sample mass, and has a risk of worker injury. To address these limitations, FSIS conducted a series of laboratory and field studies to determine whether a surface sampling method could achieve equal or better bacterial recovery compared to N60 excision for sampling beef trim products. The first study compared, under laboratory conditions, sponge and cloth surface sampling methods against N60 excision using *Salmonella* and three strains of Shiga toxin-producing *Escherichia coli* (STEC), which led to the selection of the cloth-based method. An extensive field trial comparing the cloth and N60 excision sampling methods found that the dry cloth method was inferior for the detection of *Salmonella* and aerobic bacteria counts ($p = 0.05$). FSIS hypothesized that bacterial die-off during sample shipping to the laboratory may have reduced dry cloth recovery. FSIS completed two additional laboratory studies to compare cloth with the addition of two types of Buffered Peptone Water to the dry cloth and found statistically significant improvements ($p = 0.012$ and 0.039) in all measures of bacterial recovery with added buffer. The final study repeated the original field trial, except that sample collectors added 25 mL of neutralizing Buffered Peptone Water (nBPW) to the cloth prior to shipping. This study found that the cloth, with buffer, recovered significantly higher counts of aerobic bacteria compared to the N60 excision sampling method, and there was no significant difference in the recovery of *Salmonella* ($p = 0.41$). FSIS concluded that the cloth sampling method is the regulatory equivalent to the N60 excision sampling method and replaced N60 excision sampling with the cloth sampling method with nBPW for domestic raw beef trim products on February 1, 2023.

The United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) is the public health agency authorized to ensure that the nation's commercial supply of meat, poultry, and egg products is safe, wholesome, and properly labeled. Under the Federal Meat Inspection Act (FMIA), FSIS inspection program personnel (IPP) perform verification activities, including product sampling and testing, at establishments producing meat products for the commercial food supply (US Department of Agriculture Food Safety and Inspection Service, 2024a).

FSIS uses ongoing sampling and testing to verify that establishments' food safety systems function as intended. This includes sampling of raw beef trim products intended for nonintact use, such as beef manufacturing trimmings and bench trim. Beef manufacturing trimmings and bench trim include cuts, boneless beef, chucks, and pieces of meat remaining after primal, subprimal, and other cuts have been removed. Beef manufacturing trimmings are fabricated from cattle slaughtered onsite, while bench trim is fabricated from cattle that were slaughtered at another federally inspected establishment and sold for further processing. FSIS sampling of raw beef trim products verifies that establishments have controlled Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella*. Both STEC and *Salmonella* are human foodborne pathogens (Scallan et al., 2015) that can contaminate beef during slaughter and processing. STEC that contain an *stx* and an *eae* gene, and one of seven serogroups (*E. coli* O157, O26, O45, O103, O111, O121, and O145), are adulterants in nonintact raw beef products and raw beef components intended for nonintact use and, under the FMIA, cannot be sold to consumers unless treated to achieve lethality (Federal Meat Inspection Act, 1906; US Department of Agriculture Food Safety and Inspection Service, 2012). If *Salmonella* is detected in raw nonintact beef products, the

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product is not adulterated under the FMIA. Establishments may monitor and control the presence of *Salmonella* in raw beef products as part of their Hazard Analysis and Critical Control Point (HACCP) system, while FSIS verifies establishment controls through inspection.

FSIS began using the N60 excision sampling method (hereafter, "N60 excision") in 2007 (US Department of Agriculture Food Safety and Inspection Service, 2011), analyzing beef trim product samples for STEC as an inspection verification tool of food safety controls in establishments. In 2014, FSIS began coanalyzing N60 beef trim product samples for *Salmonella* (US Department of Agriculture Food Safety and Inspection Service, 2014). IPP use a knife and hook to cut 60 thin slices from the external surface of the beef manufacturing trimmings, increasing the likelihood of finding STEC and *Salmonella* if present (US Department of Agriculture Food Safety and Inspection Service, 2024b). From 2016 to 2022, FSIS IPP collected an average of 5,000 beef manufacturing trimmings and bench trim verification samples per year using N60 excision (US Department of Agriculture Food Safety and Inspection Service, 2011, 2016b, 2017, 2018, 2019, 2020b, 2020c, 2021b, 2021c, 2022a, 2022b, 2023a, 2023c). However, N60 excision has disadvantages. Sample weights may vary due to differences in slice thickness or size, requiring the three FSIS Field Service Laboratories (laboratory) to divide N60 samples with weight greater than 325 g ± 32.5 g into two subsamples to ensure all 60 slices are tested and reduce the risk of false negative sample results (US Department of Agriculture Food Safety and Inspection Service, 2023d; US Department of Agriculture Office of Inspector General, 2012). Each subsample FSIS process requires additional laboratory supplies and analyst time to achieve a single sample result. It is time-consuming, leads to food product waste, and has a risk of injury to IPP through extensive knife work (US Department of Agriculture Office of Inspector General, 2012).

In recent years, FSIS and other agencies have evaluated different methods for collecting samples from beef manufacturing trimmings that are less destructive, safer for IPP to collect, yet still produce comparable results to N60 excision. Other agencies evaluated the N60 Plus™ as an alternative sampling method. The N60 Plus™ is similar to N60 excision, except it uses a stainless-steel sampling device on a drill to collect surface tissue in large containers (such as combo bins) of beef manufacturing trimmings. The N60 Plus™ is not feasible to use in small and very small establishments that use smaller containers or in establishments without infrastructure (e.g., electrical connections) to support this sampling method; therefore, it is not feasible for FSIS.

Surface sampling methods may be alternatives to N60 excision. During surface sampling, the sample collection device is used to wipe or massage the product surface. Surface sampling methods are nondestructive, time-efficient, and do not require the use of hooks or knives, as no excision of product occurs. The sponge sampling method is a surface method that has been previously used to sample products, product contact surfaces, and environmental surfaces (Dorsa et al., 1997; Gill et al., 2001; Ware et al., 1999). Studies have shown that the sponge sampling method successfully recovers *E. coli* O157:H7, *Salmonella*, and *Enterobacteriaceae* on beef carcasses (Barkocy-Gallagher et al., 2002; Martinez-Chavez et al., 2015). Studies comparing the sponge sampling method to excision sampling methods concluded that the sponge sampling was as effective as using an excision sampling method and successfully recovered pathogens from beef carcasses (Byrne et al., 2005; Pearce & Bolton, 2005).

The USDA Agricultural Research Service (ARS) developed the MicroTally® swab ("cloth"), a newer sampling method used to sample beef manufacturing trimmings and bench trim (Wheeler & Arthur, 2018). ARS developed two procedures for using the cloth to sample raw beef products: a continuous and a manual sampling procedure. The continuous procedure mounts the cloth on a cartridge at the end of a moving conveyor belt and samples beef manufacturing trimmings as they pass over the cloth. The continuous procedure is not feasible for small and very small establishments, which lack infrastructure (e.g. conveyor belts) to support continuous sampling. In the manual procedure, a sample collector massages the cloth over the surface of beef manufacturing trimmings held in containers like boxes or combo bins. Wheeler and Arthur compared the ability of N60 excision, N60 Plus™, and continuous and manual cloth sampling procedures to recover naturally occurring *E. coli* O157:H7 and *Salmonella,* inoculated surrogates (green fluorescent protein-labeled *E. coli*), and indicator organisms in beef trim products (Wheeler & Arthur, 2018). They concluded that sampling beef trim products using the cloth sampling method (using either a continuous sampling device or manual sampling device) provides organism recovery that is similar to, comparable to, or better than N60 excision. In later studies, ARS compared the ability of N60 excision, N60 Plus™, and the cloth in both continuous and manual sampling to recover index targets in beef trim products. These studies used the recovery of index targets (hemolysin, intimin, adhesion siderophore, nonadulterant O serogroups, *tetA*, and *tetB*), virulence genes that are indicative of STEC and *Salmonella* but not necessarily confined to those pathogens, to compare pathogen recovery by the sampling methods (Arthur & Wheeler, 2021). The studies concluded that the cloth sampling methods demonstrated robust pathogen recovery across a variety of production applications (Arthur & Wheeler, 2021).

The findings from these studies provide scientific support for the use of a sponge or cloth-based sampling method as a potential alternative to N60 sampling of beef trim. However, these studies only had limited comparisons to N60 sampling and further did not evaluate the performance of the sampling methods in high-capacity laboratories, under nationwide logistical demands, and against individual establishment sampling program variations and production practices.

To address the concerns of N60 excision, FSIS sought alternative sampling methods that would support the agency's high-throughput laboratory system, allow nationwide distribution of supplies to IPP, allow sampling from a variety of systems and containers (boxes, totes, combo bins), allow for ease of IPP training, and be able to recover foodborne pathogens to achieve public health protection. After reviewing all available technologies, FSIS identified sponge and cloth as candidate methods and conducted studies to evaluate each sampling method on beef trim products.

Materials and methods

FSIS designed five studies (three laboratory and two field) to confirm proof of concept and evaluate the feasibility of adopting a surface sampling method for beef trim in the field and in a high-throughput laboratory setting (Table 1). These studies evaluated the methods by examining recovery of a variety of analytes – either *Salmonella*, STEC, enumeration of aerobic count (AC), or a combination of those analytes. Study 1 evaluated *Salmonella* and STEC recovery from inoculated beef trim using the sponge and cloth (ARS manual procedure) sampling methods against N60 excision in the laboratory. Based on the results of Study 1, FSIS selected the cloth sampling method for additional evaluation in the field. In Study 2, FSIS conducted a field study to directly compare the cloth sampling method to N60 excision for beef trim products, as completed by Wheeler and Arthur (2018), but this time considering all establishments of various sizes nationwide, instead of the limited number of large-volume establishments previously studied. This led to Study 3, where the laboratory inoculated the cloth with *Salmonella,* then added a transport buffer to evaluate recovery under simulated shipping conditions. In Study 4, the laboratory evaluated the effect of two transport buffers on STEC recovery by inoculating the cloth with low levels of *E. coli* O157:H7. In Study 5, FSIS conducted a field study to directly compare the cloth sampling method, with the addition of 25 mL of neutralizing Buffered Peptone Water (nBPW) as a transport buffer, to N60 excision for beef trim products.

Table 1
Summary of study designs

Study	Setting	Sampling methods	Analyte (MLG chapter)	Aerobic count (AC) dilutions
1	Laboratory Nov 20, 2019 – Dec 12, 2019	N60 excision (n = 20) Sponge, moistened (n = 20) Cloth, dry (n = 20)	*Salmonella* (4.10) STEC (5C.00)	No AC enumeration of samples
2	Field Jan 4, 2021 – Sept 30, 2021	N60 excision (n = 2,634) Cloth, dry (n = 2,634)	AC (3.02) *Salmonella* (4.10, 4.11) STEC[a] (5C.00, 5C.01, 5C.02)	1:16 and 1:160
3	Laboratory Aug 2021	Cloth, dry (n = 30) Cloth, BPW (n = 30) Cloth, nBPW (n = 30)	AC (3.02)	1:4
4	Laboratory Aug 2021	Cloth, dry (n = 20) Cloth, BPW (n = 20) Cloth, nBPW (n = 20)	STEC (5C.01)	No AC enumeration of samples
5	Field Oct 1, 2021 – Jan 31, 2023[b]	N60 excision (AC n = 1,569; *Salmonella* n = 4,590) Cloth, nBPW (AC n = 1,569; *Salmonella* n = 4,590)	*Salmonella* (4.11, 4.12) AC (3.02) STEC[a] (5C.02)	1:16 and 1:1600

[a] N60 excision methods only.
[b] AC testing suspended April 2022.

Study 1 (Laboratory): Surface sampling of laboratory-inoculated raw beef trim

Study 1 compared alternative sampling methods in the laboratory, evaluating the N60 against two surface sampling devices. The purpose of this study was to identify the surface sampling device most suitable for evaluation in a field setting. The first surface sampling device was a polyurethane EZ Reach™ Large Sponge with 80 mL of HiCap™ Neutralizing Broth (sponge) (World Bioproducts, Bothell, WA), connected to a polypropylene twist-off handle that is designed to be removed from the sponge once sampling is completed. The second surface sampling device was the MicroTally® Swab (dry cloth) (FREMONTA Corporation, Fremont, CA) made of a nonwoven spun polymer measuring 610 by 200 mm (24 by 8 inches). The study evaluated whether the surface sampling devices recovered STEC and *Salmonella* from inoculated raw beef trim as well as a simulated N60 excision sample. To create study samples, the laboratory mixed beef trim slices from recent screen-negative beef sample reserves to ensure a homogeneous background microflora, then verified the matrix as negative for target analytes. The laboratory divided the beef trim slices into 907 ± 18 g (approximately two pounds) tubs of product spread into a thin layer across the 250 by 280 mm (10 by 11 inches) surface area, simulating combo bin lotting practices at inspected establishments. The laboratory prepared and analyzed two sets of tubs on different days as replicates, with each set containing 10 tubs per sampling method to ensure reproducibility. This provided a total of 20 samples per method: N60 excision, sponge, or dry cloth sampling device (Table 1).

Prepared beef samples were inoculated with a cocktail of three *E. coli* strains representing adulterant STEC serogroups (*E. coli* O157:H7, *E. coli* O103:H2, and *E. coli* O121:H19) and a *Salmonella* strain (*Salmonella* Typhimurium) (Table 2). The laboratory prepared cultures on trypticase soy agar with 5% sheep blood and incubated the cultures overnight at 35 ± 2 °C. The laboratory then diluted the cultures tenfold in 0.85% saline to a target inoculum of approximately 5 CFU/mL. The laboratory inoculated each 907 g (two pounds) tub of beef trim slices with 1 mL of each culture. The average pathogen inoculum level for each sample ranged from an estimated 3.5 to 7.5 CFU per 907 g (two pounds) of beef trim slices. The laboratory then refrigerated the tubs at 2−8 °C for 48 ± 4 h to simulate carcass chilling time in establishments after slaughter. The laboratory then randomly assigned a sampling method to each tub.

To simulate N60 collection, the laboratory randomly chose a 325 g portion of raw beef trim slices from each tub. For the sponge collection method, the laboratory held the plastic handle (stick) attached to the sponge and used slight pressure to rub the sponge across the top surface of the raw beef slices in a back-and-forth motion across the surface for two minutes. Following collection, the sponge was placed into the provided sample collection bag and twisted it to detach the handle. For dry cloth samples, sterile gloves were worn while massaging the raw beef slices with the dry cloth for one minute per side of the cloth, for a total sample time of two minutes. All samples (N60, sponge, and dry cloth) were packed in FSIS insulated sample collection boxes with frozen gel packs and stored at room temperature to simulate shipping conditions. The samples were removed 18–20 h later, and each sample was checked to verify an acceptable temperature of <15 °C. Each sample was analyzed for *Salmonella* using MLG Chapter 4 and STEC using MLG Chapter 5C to compare recovery (US Department of Agriculture Food Safety and Inspection Service, 2023d). The N60 samples consisted of 325 ± 32.5 g beef manufacturing trimmings enriched with 975 ± 19.5 mL modified Tryptone Soya Broth (mTSB). The sponge samples were enriched with 400 ± 8 mL mTSB to maintain the 1:6 buffer-to-mTSB ratio described in MLG Chapter 5C, and the dry cloth samples were enriched with 200 ± 4 mL mTSB following the manufacturer's instructions. The

Table 2
Bacterial strains used in Studies 1, 3, and 4 to inoculate samples (raw beef slices or cloth)

Species and serotype	Strain number	Source	Reference
Salmonella enterica Typhimurium	Microbiologics Sal54 01223 UV-V FDA SAL5694	Microbiologics: Derived from FDA SAL5694	https://www.microbiologics.com/uv-biotag-swab-salmonella-enterica-subsp-enterica-serovar-typhimurium-sal54-fda-sal5694
E. coli O157:H7	Microbiologics EC43 01227 UV-V FDA ESC1177	Microbiologics: Derived from FDA ESC1177	https://www.microbiologics.com/uv-biotag-vial-escherichia-coli-o157-h7-ec43-fda-esc1177
E. coli O103:H2	USDA ARS #Tarr Strain; ELC36	USDA Agricultural Research Service (ARS); FSIS Eastern Laboratory	(Tarr et al., 1996; Wasilenko et al., 2012)
E. coli O121:H19	USDA ARS #96-1585 Strain	USDA ARS;	(Wasilenko et al., 2012)

samples were stomached after the addition of enrichment broth for 2 min ± 2 s. The laboratory culturally confirmed all samples to verify the results. Based on the results of this study, FSIS selected the cloth sampling device (dry) for additional evaluation in the field, as described in Study 2.

Study 2 (Field): Paired study of the cloth sampling device (dry) with N60 excision of beef trim from cattle slaughtered on-site

Study 2 used paired samples in a field study to evaluate the cloth sampling device (dry) against N60 excision in establishments that produce beef manufacturing trimmings from cattle slaughtered on-site that are eligible for FSIS verification sampling under FSIS project code MT60 per FSIS Directive 10,010.1 "Sampling Verification Activities for Shiga Toxin-Producing *Escherichia coli* (STEC) in Raw Beef Products" (US Department of Agriculture Food Safety and Inspection Service, 2024b). FSIS assigned cloth sampling tasks as paired samples with N60 excision under FSIS sampling project code MT60_CLOTH for establishments that received the routine verification tasks (US Department of Agriculture Food Safety and Inspection Service, 2020a). FSIS IPP collected beef trim product samples and then shipped the paired N60 excision and cloth samples overnight with prefrozen gel packs in an insulated sample box to one of the three FSIS laboratories for AC and *Salmonella* analysis. The laboratory discarded cloth samples received above 15 °C, leaking, or without a paired sample (i.e., missing N60 excision sample) upon receipt. The N60 excision and cloth samples were prepared by the laboratory as described in Study 1, but if excess beef manufacturing trimmings remained after processing the first N60 excision sample, a second subsample was prepared using between 63 g and 357.5 g of beef manufacturing trimmings to ensure analysis of all 60 pieces. The second subsample was enriched with mTSB to maintain a 1:4 dilution per MLG Chapter 5C sample preparation instructions. The AC analysis was performed using a 1 mL aliquot of mTSB enrichment broth from the N60 excision samples after stomaching but before incubation. Each sample was analyzed for the presence of *Salmonella* using MLG Chapter 4 and AC using MLG Chapter 3 – TEMPO® (US Department of Agriculture Food Safety and Inspection Service, 2023d). The N60 excision samples were analyzed for STEC as part of routine verification, but the cloth samples did not receive STEC analysis. Based on the results of this study, FSIS performed additional studies (Study 3 and Study 4) to determine whether adding a transport buffer to the dry cloth, after sample collection and before shipping, would improve bacterial recovery.

Study 3 (Laboratory): Effect of transport buffers on bacterial recovery from cloth

Study 3 evaluated the addition of a transport buffer to the cloth after sample collection and before shipping to improve recovery of *Salmonella*. FSIS identified two transport buffers routinely used to increase bacterial survival during shipping: neutralizing buffered peptone water (nBPW, Culture Media Concepts®, Anaheim, CA) and buffered peptone water (BPW, Neogen®, Lansing, MI). FSIS compared AC recovery in two treatments for the cloth sampling device: 1) the addition of 25 mL nBPW (cloth with nBPW), 2) the addition of 25 mL BPW (cloth with BPW), with a control of dry cloth with no additional buffers added. *Salmonella* was the representative organism used to inoculate the cloth for AC analysis to compare recovery among treatments. The laboratory inoculated 90 dry cloth samples with approximately 5×10^4 CFU of *Salmonella* per cloth, then used 30 of the inoculated cloths for each treatment. After refrigerating samples for 24 ± 4 h to simulate shipping conditions, 200 mL of mTSB was added to the dry cloths and 175 mL of mTSB was added to all cloths receiving 25 mL of transport buffer to maintain the 200 mL final volume for enrichment. After the addition of enrichment broth, each sample was stomached for 2 min ± 2 s and each cloth was analyzed in triplicate for AC using MLG Chapter 3 – TEMPO® (US Department of Agriculture Food Safety and Inspection Service, 2023d). The triplicate results were averaged for each sample to give a final AC result.

Study 4 (Laboratory): Effect of transport buffers on STEC recovery from inoculated cloth

In previous studies, nBPW has been suspected to inhibit the recovery of some species of bacteria (Berrang et al., 2018; Williams et al., 2018). So, after examining AC and *Salmonella* recovery on cloth using BPW and nBPW as transport buffers, Study 4 evaluated whether either transport buffer inhibited STEC recovery using the same three cloth treatments as Study 3: 1) cloth with 25 mL nBPW, 2) cloth with 25 mL BPW, and 3) a dry cloth with no additional buffers added.

The target for the inoculum was approximately 5 CFU/mL of *E. coli* O157 per cloth. FSIS split the study into two separate experiments to ensure reproducibility. The estimated average concentrations for the two experiments were 4 and 5.5 CFU/mL. These concentrations represent the lowest practical concentration that ensures that nearly all cloth samples would have at least one viable *E. coli* O157 per cloth (i.e., using the Poisson distribution, the probability of observing 1 or more organisms in a 1 mL aliquot drawn from an inoculum with an average concentration of 4 CFU/mL is 0.98).

The laboratory inoculated 30 dry cloth sampling devices using the estimated 4 and 5.5 CFU/mL inocula of *E. coli* O157 per cloth and then used 10 inoculated cloths per treatment. All cloth samples were refrigerated for 24 ± 4 h, and then 200 mL of mTSB was added to dry cloths, and 175 mL of mTSB was added to the cloths that received nBPW or BPW as a transport buffer. After the addition of enrichment broth, each sample was stomached for 2 min ± 2 s before proceeding with the STEC analysis detailed in MLG Chapter 5C for cultural confirmation (US Department of Agriculture Food Safety and Inspection Service, 2023d). Based on the results of Study 3 and Study 4, FSIS evaluated the use of nBPW as a transport medium with the cloth in the field, as described in Study 5.

Study 5 (Field): Paired study of cloth sampling device with nBPW with N60 excision of beef trim from cattle slaughtered on-site

In Study 5, FSIS assigned and collected paired N60 excision and cloth samples as described in Study 2, except that after sample collection, IPP added 25 mL of prechilled nBPW to the cloth sampling device before shipping to the laboratory, per FSIS Notice 36–21: Continued In-Field Study to Test the Cloth Sample Collection Method for Beef Manufacturing Trimmings (US Department of Agriculture Food Safety and Inspection Service, 2021a). Upon receipt, the samples were prepared as described in Study 2, but 175 mL of mTSB was added to the cloth samples with nBPW to maintain a final volume of 200 mL. The N60 excision and cloth samples were analyzed for the presence of *Salmonella* and AC using MLG Chapter 4 and MLG Chapter 3 (US Department of Agriculture Food Safety and Inspection Service, 2023d). The N60 excision samples were analyzed for STEC as part of routine verification. FSIS did not analyze the cloth samples for STEC.

Sample collection for field studies: N60 excision and cloth

Study 2 and Study 5 required FSIS IPP to collect both N60 excision and MicroTally® cloth samples. IPP collected the paired samples on the same day and from the same production lot of beef manufacturing trimmings. IPP collected N60 excision samples following procedures in FSIS Directive 10,010.1 rev 4, selecting a random production lot for sampling, then aseptically excising and collecting 60 slices each measuring approximately 76 mm long, 25 mm wide, and 3 mm thick (3 inches long, 1 inch wide, and 1/8th inch thick) from the lot (US Department of Agriculture Food Safety and Inspection Service,

2024b). If the production lot constituted multiple containers (e.g., combo-bins or boxes), IPP chose equal amounts of beef manufacturing trimmings from up to five production containers. IPP then placed the 60 slices in two roll-top bags with approximately 30 slices per bag. IPP also collected additional trim reserve in a third sterile roll-top bag for a total sample weight of approximately 907 g (two pounds). IPP did not cut reserve pieces to a certain dimension. The laboratory only used the trim reserve if the 60 slices did not meet the required weight, as specified in the sample preparation section of the FSIS Microbiology Laboratory Guidebook (MLG) Chapter 5C.

After collecting N60 excision samples, IPP swabbed the same production lot using a cloth following published manual sampling device procedures (Wheeler & Arthur, 2018) with modifications as follows. If the production lot constituted multiple combo bins, then IPP selected a single combo bin for cloth sampling. If boxes or totes held the production lot, IPP sampled up to five boxes or totes by cloth sampling, which has approximately the same surface area as the top of a combo bin. FSIS IPP massaged the beef manufacturing trimmings for a minimum of 45–60 s per side of the cloth, for a total of 1.5 to 2 min per sample. In Study 2, IPP refolded the cloth sampling device following the original fold lines and sealed it in a roll-top bag without the addition of buffer (dry cloth) (Wheeler & Arthur, 2018). In Study 5, IPP refolded the cloth sampling device following the original fold lines and folded the cloth two additional times before placing it into a roll-top bag. Then IPP poured 25 mL prechilled nBPW into the bag (cloth with nBPW). The additional folds allowed the cloth sampling device to fit into the sample collection bag and to ensure full submersion of the cloth in transport buffer. IPP then shipped both the paired N60 excision and cloth samples overnight together with prefrozen gel packs, in the same insulated sample box to the designated laboratory. IPP refrigerated late collections and shipped the following day.

Bacterial enumeration

The laboratory studies (Study 1, Study 3, and Study 4) verified the average inoculum level for each pathogen using MLG 3 – Aerobic Plate Count (APC) Petrifilm™ (US Department of Agriculture Food Safety and Inspection Service, 2023d). At the low inoculum levels used in Studies 1 and 4 (approximately 4–5.5 CFU/cloth), classical enumeration falls below the LOQ of Petrifilm™ APC, so inoculum values are reported as estimated. Because Poisson variation governs how many viable cells adhere to each cloth, some samples will contain zero organisms by chance. For this reason, pathogen recovery in these studies was evaluated solely as presence/absence after enrichment, and statistical comparisons appropriately focus on detection probability rather than CFU counts.

Study 2, Study 3, and Study 5 used the MLG 3 – TEMPO® Method to enumerate AC in the sample enrichment broth before incubation (US Department of Agriculture Food Safety and Inspection Service, 2023d). Distinct dilutions were applied for each study. To calculate cloth results in CFU/sample, FSIS multiplied TEMPO® MPN/mL results by 200 to account for the 200 mL volume of the initial enrichment broth.

FSIS prepared N60 excision samples in parallel to the cloth samples using a 1 mL aliquot of the mTSB enrichment broth after stomaching. If a second subsample was available from N60 excision, an aliquot was prepared by combining the enrichment of each subsample prior to TEMPO® analysis. Specifically, 1 mL of homogenized enriched mTSB from the first subsample was combined with 0.1 mL for every 32.5 g of meat in the second subsample to maintain the same proportion of product. For example, if the second subsample weighed 162.5 g, then 0.5 mL from the second subsample was combined with the 1 mL aliquot from the first subsample, vortexed, and 1 mL of that combined aliquot was analyzed for AC.

The TEMPO® software is designed to report results on a per-gram basis, assuming a homogeneous distribution of bacteria throughout the matrix. However, bacterial contamination is largely confined to the surface of the meat, introduced during slaughter and processing. Therefore, adding broth does not dilute the bacteria evenly throughout the total weight (975 mL mTSB + 325 g raw beef slices); it only transfers the bacteria location from the meat surface into the broth (975 mL) like a rinse. The TEMPO® MPN/g automated output was divided by four to account for the lack of meat dilution, then multiplied by the total volume of mTSB enrichment broth which had been added to the N60 excision sample, to report a CFU/sample result consistent with recommendations from the National Advisory Committee on Microbiological Criteria for Foods (National Advisory Committee On Microbiological Criteria For Foods, 2010).

Statistical analysis

The choice of statistical analysis techniques used for each study was dependent on whether the samples were paired observations. Samples were unpaired for the laboratory studies (Study 1, Study 3, and Study 4) and paired for the field studies (Study 2 and Study 5). For the pathogen testing, a standard contingency table analysis was used to test differences in the performance of the different sampling methods. Fisher's exact test was used because of the smaller sample sizes in the laboratory studies. P values were adjusted to account for the possibility of false discovery using the Benjamini-Hochberg adjustment factor (Benjamini & Hochberg, 1995). To compare the efficiency of the different methods at recovering AC values, the concentrations were $\log_{10}$ transformed before calculating the mean and variance of the distribution. Using the difference in the means of these distributions ($\Delta\mu$), estimates of the difference in the fraction of bacteria recovered can be estimated by $10^{\Delta\mu}$. Among the five studies, Study 4 is unique because the very low average concentrations in the samples and the singular focus on die-off during shipping make it possible to estimate the probability of survival for each *E. coli* O157 organism during transport. This is done using a Poisson-binomial mixture distribution and making the simplifying assumptions of 5 CFU in each sample and assuming the laboratory method will successfully grow any viable organism (Hong, 2013).

The field studies (Study 2 and Study 5) are unique because samples are paired in the sense that the same combo bin was sampled using each method. Analyses of AC concentrations used a multivariate normal distribution to account for a) the paired sampling design, b) some observations being censored at the lower or upper limits of quantification, and c) the limits of quantification changing during the study (Williams & Ebel, 2014). For the paired sampling data, the confidence intervals for the difference in observed average $\log_{10}$AC are determined by Eq. (1). Similarly, the confidence intervals for the difference in the proportion of positive tests for *Salmonella* are given by Eq. (2). A McNemar's Chi-square test was used to test the hypothesis of no significant differences in *Salmonella* detection frequency between the sampling methods. To examine the degree of agreement in *Salmonella* results between the methods, we also estimated Kappa statistics. Kappa values range from –1 to 1, with 1 indicating perfect agreement. Values of 0.4–0.5 imply a moderate level of agreement.

Equation (1). Confidence interval for the difference in estimated mean contamination levels between the cloth-based sampling method and the N60 sampling method, derived under a paired-sample *t*-distribution and incorporating sample variances and covariance to account for within-sample correlation.

$$(\hat{\mu}_{cloth} - \hat{\mu}_{N60}) \pm t_{1-\alpha/2}^{df=n-1} \left(\sqrt{\frac{\hat{\sigma}^2_{cloth} + \hat{\sigma}^2_{N60} - 2cov(x_{cloth}, x_{N60})}{n}} \right) \quad (1)$$

Equation (2). Confidence interval for the difference in estimated contamination proportions between the cloth-based and N60 sampling methods, constructed under a paired-sample *t*-distribution and incorporating binomial variance terms and covariance to account for within-sample dependence.

$$(\hat{p}_{cloth} - \hat{p}_{N60}) \pm t_{1-\alpha/2}^{df=n-1}\left(\sqrt{\frac{\hat{p}_{cloth}(1-\hat{p}_{cloth}) + \hat{p}_{N60}(1-\hat{p}_{N60}) - 2cov(x_{cloth}, x_{N60})}{n}}\right) \quad (2)$$

Results

Study 1 (Laboratory): Cloth (dry) and sponge sampling methods compared to simulated N60 for the recovery of pathogens from inoculated artificially contaminated raw beef

Study 1 analyzed twenty samples for each sampling method using four pathogens in the laboratory. The simulated N60 excision had the highest recovery of all pathogens combined (74%), followed by the dry cloth (64%), and the sponge (56%) methods (Table 3). Comparing the aggregated results of the three sampling methods, using Fisher's exact test, the results were not significantly different ($p = 0.073$).

The proportion of the positive samples for each pathogen was compared between the alternative sampling methods using the simulated N60 sampling as the baseline for comparison (Fig. 1). Recovery for the dry cloth and sponge was higher than the simulated N60 excision method only for *E. coli* O157, though the difference was not significant ($p = 0.752$). The only significant difference was that the laboratory recovered less *E. coli* O121 from the dry cloth ($p = 0.047$). After adjusting for the chance of false discovery using the Benjamini and Hochberg correction, all p values were greater than 0.225.

In summary, the alternative sampling methods performed similarly to the simulated N60 excision method, with the dry cloth performing slightly better than the sponge. The laboratory also reported additional operational and handling benefits for the cloth method; therefore, FSIS proceeded to field test the cloth.

Study 2: Comparison of the cloth sampling method (dry) and N60 excision for the recovery of AC and Salmonella from beef trim from cattle slaughtered on-site

Study 1 determined that alternative sampling methods performed comparably to the N60 excision method under controlled laboratory conditions, leading to Study 2 to evaluate an alternative sampling method in a field setting. Study 2 collected a total of 2,634 paired samples in the field, comparing AC and *Salmonella* recovery between N60 excision and dry cloth sampling methods.

AC results. FSIS used AC to compare the quantitative recovery between the N60 excision and dry cloth sampling methods. As shown in Figure 2, the median concentration of AC per sample was stable for each method over the course of the study and did not reflect any detectable trends over time. The median AC concentration per sample for the dry cloth sampling device was less than N60 excision for each month of Study 2. The dry cloth sampling method recovered significantly fewer AC on average than N60 excision at a p value of 0.05 (4.36 $\log_{10}$ and 4.58 $\log_{10}$ for dry cloth and N60 excision, respectively). The dry cloth obtained -0.22 $\log_{10}$ (CI_{95}: -0.27 to -0.16), or 60% ($10^{-0.22}$) of AC relative to N60, on average.

***Salmonella* results.** Monthly *Salmonella* results demonstrate an increasing pattern from January through September for both methods (Fig. 3). Except for February and March 2021, the proportion of positive samples was larger for N60 excision than for dry cloth. McNemar's Chi-square test indicates significant differences in the proportion of positive samples. Table 4 shows that the dry cloth sampling method detected significantly fewer *Salmonella*-positive samples than the N60 excision method ($p = 0.003$). Overall, the dry cloth sampling method found 62% of *Salmonella*-positive samples compared to the N60 excision method (31/50). N60 excision detected 28 samples that the dry cloth did not detect, while the dry cloth sampling method only detected nine samples that N60 excision did not detect. The Kappa statistic calculated for Table 4 (Kappa = 0.54) suggests a moderate degree of agreement in results between the sampling methods.

In summary, the dry cloth sampling method performed significantly worse for both AC and *Salmonella* recovery when compared to N60 excision.

Study 3 (Laboratory): Effect of transport buffers on bacterial recovery from cloth

Study 2 led to a second laboratory study, Study 3, that evaluated adding different transport buffers to increase recovery by reducing perceived die-off of *Salmonella* on the cloth sampling device during shipping. Although *Salmonella* was the representative organism used to inoculate the cloth for AC analysis to compare recovery among treatments, these results are presented as AC because the laboratory enumerated the bacterial count using the AC method in MLG Chapter 3 – TEMPO®.

The dry cloth recovered a mean of 4.57 $\log_{10}$ AC, the cloth with BPW recovered a mean of 4.75 $\log_{10}$ AC, and the cloth with nBPW recovered a mean of 4.73 $\log_{10}$AC (Fig. 4). Adding the transport buffer resulted in mean recoveries that were not significantly different ($p = 0.244$ and 0.245) from the average inoculum concentration, suggesting no detectable die-off when either buffer was added. The difference in recovery between BPW and nBPW was also not significant ($p = 0.241$). In both cases, using either BPW or nBPW as a transport buffer significantly increased the recovery compared to a dry cloth. BPW recovered 0.18 $\log_{10}$ AC (CI_{95}: 0.12 to 0.25) more than dry cloth, while nBPW recovered, on average, 0.16 $\log_{10}$ AC (CI_{95}: 0.09 to 0.23) more. The dry cloth recovered between 70% and 66% ($10^{-0.18}$ and $10^{-0.16}$) of AC relative to the cloth with BPW and nBPW, respectively.

In summary, adding either buffer significantly increased the AC recovery of the cloth. Note that the increased recovery between the dry cloth and adding the buffer is similar in magnitude for the AC recovery of N60 and the dry cloth (Study 2).

Table 3
Study 1 results from laboratory-inoculated raw beef slices sampled with three different methods

Organism	N60, Simulated (Positive analyses/Total samples)	Sponge (Positive analyses/Total samples)	Cloth, dry (Positive analyses/Total samples)
Salmonella	80% (16/20)	55% (11/20)	80% (16/20)
E. coli O157	45% (9/20)	55% (11/20)	55% (11/20)
E. coli O103	70% (14/20)	35% (7/20)	45% (9/20)
E. coli O121	100% (20/20)	80% (16/20)	75% (15/20)
Combined	74% (59/80)	56% (45/80)	64% (51/80)

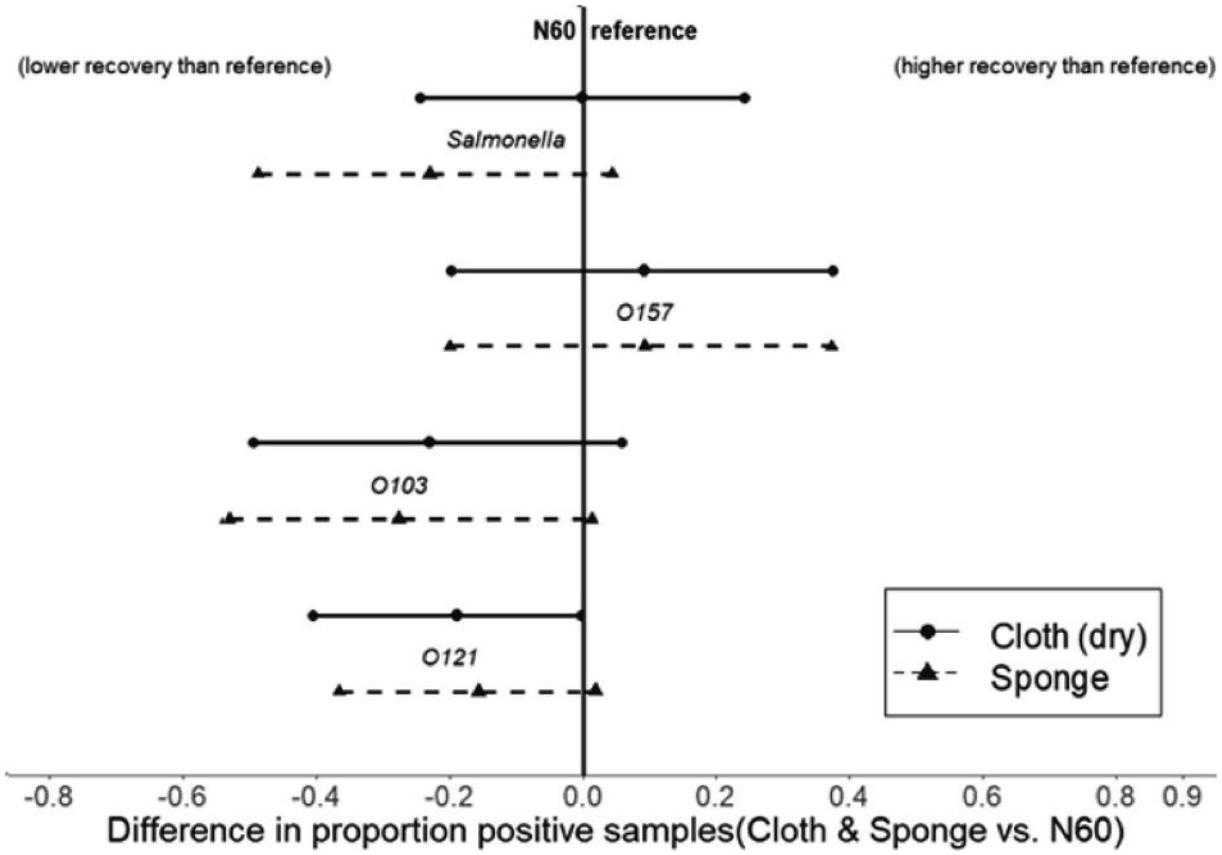

Figure 1. Results of Study 1, comparison of the dry cloth and moistened sponge sampling method with the simulated N60 excision sampling method. The vertical solid black line represents equivalence of the proportion of positive samples (i.e., no difference) between the dry cloth and sponge methods compared to N60 excision. The dots (dry cloth) and triangles (sponge) on each horizontal line reflect the proportional difference (median and 95 percent confidence limits) compared to the N60 excision reference method. A difference is significant at the 0.05 level if the confidence interval does not intersect the vertical line. The Benjamini-Hochberg adjustments for a false discovery rate are not included.

Study 4 (Laboratory): Addition of transport buffer did not inhibit STEC recovery

Study 4 evaluated whether added buffer (BPW and nBPW) affected *E. coli* O157 recovery from cloth with added buffer (BPW and nBPW) compared to dry cloth controls, after simulated shipping conditions. Recovery was poor for the dry cloth, with only 45% of tests at both the estimated 4 CFU and 5.5 CFU concentrations (Table 5). In contrast, the addition of the buffers resulted in 90% and 95% of tests being positive using the BPW and nBPW buffers, respectively. Both BPW- and nBPW-treated cloths recovered significantly more STEC-positive samples than the dry cloth (Fisher's exact test, $p = 0.0003$). However, recovery did not differ significantly between BPW and nBPW at either inoculum concentration ($p > 0.05$; Fisher's exact test).

Pathogen presence for each treatment was compared to the dry cloth using Fisher's Exact Test, analyzing the two sets of samples separately (Fig. 5). In the first set of ten samples for each method, the inoculum concentration was estimated at 4 CFU/cloth. The cloths with the transport buffer recovered more *E. coli* O157 (80% for BPW and 100% for nBPW) than just using the dry cloth samples (60%), but this finding was not significant at the 0.05 level ($p = 0.628$ and 0.087 for BPW and nBPW, respectively). In the second set of ten samples for each method, the inoculum concentration was estimated at 5.5 CFU/cloth. Both transport buffers recovered significantly more *E. coli* O157 (100% for BPW and 90% for nBPW) than the dry cloth samples ($p = 0.003$ and 0.020 for BPW and nBPW, respectively). After adjusting for the false discovery rate (Benjamini & Hochberg, 1995), both buffers recovered significantly more *E. coli* O157 ($p = 0.012$ and 0.039).

Using the Poisson binomial distribution to estimate the probability of survival for each organism during transport resulted in estimates of 0.16, 0.46, and 0.60 for the dry cloth, cloth with BPW, and cloth with nBPW, respectively. In summary, this study suggests that the dry cloth samples exhibited significant die-off during simulated shipping conditions. In contrast, the survival of *E. coli* O157 was improved, and there was no differential effect between using BPW or nBPW.

Study 5: Comparison of the cloth with nBPW transport buffer and N60 excision for the recovery of AC and Salmonella from beef trim from cattle slaughtered on-site

Study 3 and Study 4, performed under controlled laboratory conditions, showed that the addition of transport buffer to the cloth increased the bacterial recovery compared to dry cloth and did not inhibit STEC recovery, leading to Study 5 to evaluate the cloth with nBPW transport buffer in a field setting.

AC results. Study 5 collected 1,569 paired samples in the field, N60 excision samples and cloth samples with nBPW, for AC analysis. FSIS suspended AC analysis in April 2022 after collecting sufficient samples to quantify statistically significant differences in AC recovery by cloth with nBPW and N60. As shown in Figure 2, the median concentration of AC per sample was stable for each method over the course of the study and did not reflect any detectable trends. The median AC concentration per sample for the cloth sampling device

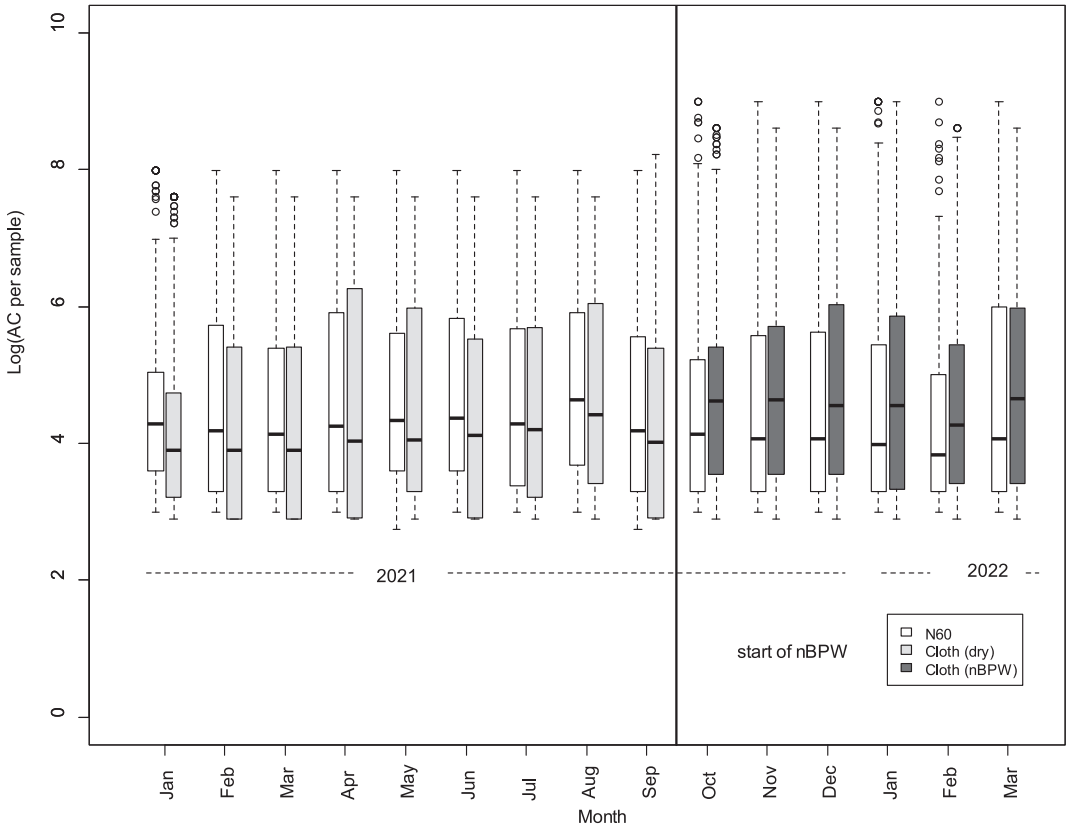

Figure 2. Monthly comparison of mean AC (log_{10}) between the N60 excision, cloth (dry), and cloth (nBPW) sampling methods from paired samples collected by IPP in federal establishments. In Study 2 (the first 9 months), the dry cloth obtained -0.22 log_{10} (CI_{95}: -0.27 to -0.16), less than the mean log_{10}AC recovered by N60 excision. In Study 5 (last 6 months), the cloth with nBPW transport buffer recovered 0.38 log_{10} (CI_{95}: 0.32 to 0.45) more AC relative to N60 excision on average (cloth with nBPW: 4.72 log_{10}; N60: 4.34 log_{10}). The solid vertical line represents the beginning of cloth + nBPW implementation.

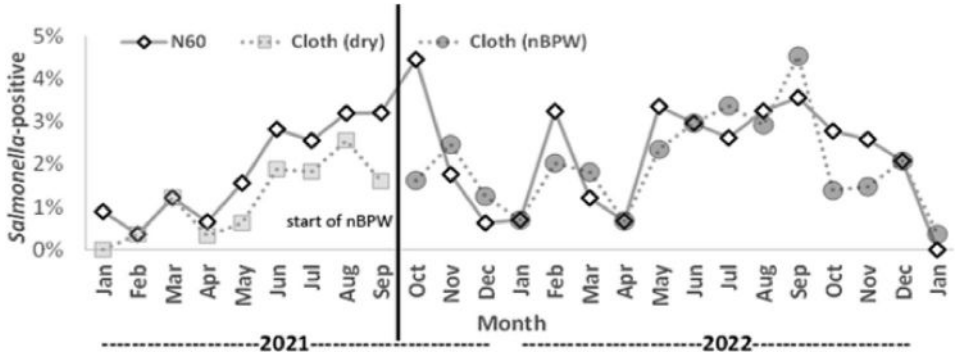

Figure 3. Monthly *Salmonella*-positive proportions for Study 2 and Study 5 using each sampling method: N60 excision (diamonds), dry cloth (squares), and cloth + nBPW (circles), from paired samples collected by IPP in federal establishments. The solid vertical line represents the beginning of cloth + nBPW implementation.

Table 4
Study 2 results from *Salmonella* analysis of 2,634 paired samples for dry cloth and N60 excision sampling methods

	N60 Excision		
Dry cloth	Positive	Negative	Total
Positive	22	9	31
Negative	28	2575	2603
Total	**50**	**2584**	**2634**

with nBPW was consistently greater than N60 excision for each month of the study. The cloth sampling method with nBPW transport buffer recovered 0.38 log_{10} (CI_{95}: 0.32–0.45), while the N60 excision method recovered, on average, 42% ($10^{-0.38}$) as many AC relative to the cloth with nBPW (cloth + nBPW: 4.72 log_{10}; N60 excision: 4.34 log_{10}).

***Salmonella* results.** Study 5 collected 4,590 paired samples in the field for *Salmonella* analysis. Table 6 shows that the frequency of *Salmonella* recovery is not significantly different between the cloth sampling method with nBPW and N60 excision ($p = 0.41$). Overall, the cloth sampling method with nBPW detected *Salmonella* in 2.0% of

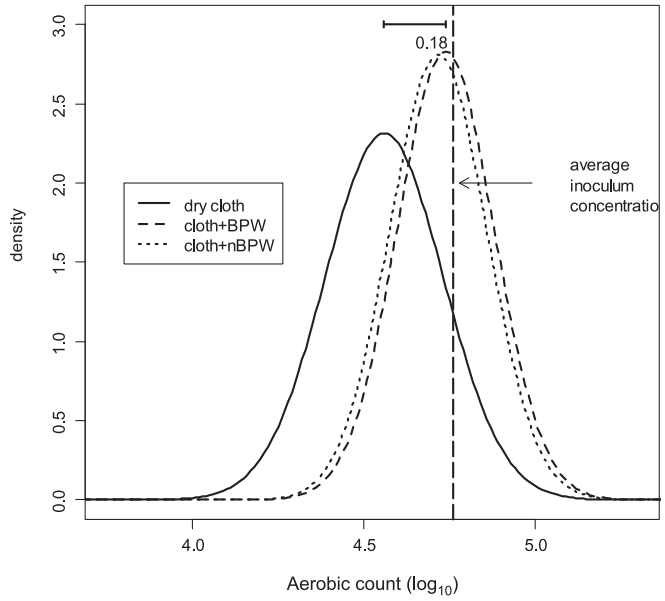

Figure 4. Comparison of the concentrations of AC recovered using the dry cloth and the cloth with either BPW or nBPW (Study 3). The units on the y-axis are probability densities that are calculated for normal distributions with mean and standard error (se) values as shown. Probability density—or density—can be interpreted as the relative likelihood of the x-axis values. The distribution of AC concentrations was significantly lower for the dry cloth sampling method. On average, the dry cloth recovered about 70 and 66% as many organisms compared to BPW and nBPW, respectively.

Table 5
Study 4 results for three cloth treatments and percent of samples confirmed positive for *E. coli* O157

Treatment	Confirmed positive		
	Set 1 (estimated 4 cfu)	Set 2 (estimated 5.5 cfu)	Combined
Cloth, dry	60% (6/10)	30% (3/10)	45% (9/20)
Cloth, BPW	80% (8/10)	100% (10/10)	90% (18/20)
Cloth, nBPW	100% (10/10)	90% (9/10)	95% (19/20)

the samples (92/4590), while N60 excision detected *Salmonella* in 2.2% (101/4590) of the samples. The 95% confidence interval for the observed difference of −0.2 percent is −0.61% to 0.22%, indicating no significant difference in the methods' ability to detect *Salmonella*. N60 excision detected 52 samples that the cloth sampling method did not detect, while the cloth sampling method detected 43 samples that N60 excision did not detect (Table 6). The Kappa statistic calculated for Table 6 (Kappa = 0.50) suggests a moderate degree of agreement in results between the sampling methods.

In summary, the cloth with nBPW recovered slightly more AC, and there was no difference in *Salmonella* recovery compared to N60 excision.

Discussion

Study 1 compared alternative sampling methods in the laboratory, evaluating the N60 excision against two surface sampling devices for proof-of-concept and suitability in a high-throughput laboratory environment. The cloth sampling method easily adapted to the existing laboratory workflow, with identified benefits. Specifically, it used less media, occupied less physical space in incubators, and was easily disposed of after incubation. Additionally, the cloth sampling device design resisted tearing and lacked removable parts, decreasing the risk that the device would shed extraneous material into the product during sample collection. The cloth sampling method was statistically equivalent to the simulated N60 excision method in recovery of three of the four pathogens, and it provided significant operational benefits; therefore, FSIS chose the cloth sampling device for further field evaluation.

In Study 2, the dry cloth recovered less AC (−0.22 $\log_{10}$) than N60 excision (Fig. 2) from samples collected in the field from federal inspected establishments with cattle slaughtered on-site. Table 4 shows the results of 2,634 paired *Salmonella* analyses where the dry cloth detected 9 *Salmonella*-positive lots that the N60 missed, while N60 detected *Salmonella* in 28 lots of beef manufacturing trimmings that the dry cloth missed (Table 4). When the cloth sampling device recovered significantly fewer AC and *Salmonella* than N60 excision in Study 2 (Fig. 2 and Table 4), FSIS determined dry cloth was not an adequate replacement for N60 excision. Instead, FSIS considered which factors in the sampling program and procedures could be contributing to the difference in results compared with previous research (Wheeler & Arthur, 2018).

One factor FSIS considered was the samples' hold times before shipment and receipt by the laboratory. As part of routine verification sampling procedures, including those used during Study 2, FSIS IPP collects samples and ships them overnight to the laboratories for analysis. To verify compliance and food safety during all hours of establishment operation, FSIS also collects samples during establishment second shifts after common carrier daily pickup hours. These second shift samples are subjected to extended hold times until the samples are picked up the following day and transported to the laboratories. Additionally, earlier investigations by FSIS and ARS identified the importance of antimicrobial (e.g., peroxyacetic acid (PAA) and other organic acids) carryover and its effects on *Salmonella* and *Campylobacter* in raw poultry samples shipped to the laboratories (Berrang et al., 2018; Gamble et al., 2017). Like poultry, some raw beef trim products are subjected to antimicrobial treatment (including organic acids) prior to FSIS sample collection (US Department of Agriculture Food Safety and Inspection Service, 2024b). Antimicrobial carryover may have been less impactful for N60 excision samples due to the inherent buffering capacity of the proteins in the beef trim. However, dry cloth has no buffering capacity; therefore, antimicrobial carryover may injure or inactivate bacteria during shipping. As a result, FSIS hypothesized that the addition of a transport buffer by FSIS IPP prior to shipping could increase AC and *Salmonella* recovery.

FSIS has used different buffers to collect samples in inspected establishments as resources have changed and methods have improved. FSIS previously used BPW as the transport buffer in other FSIS sampling projects to sample raw poultry products. On July 1, 2016, FSIS began using nBPW to incorporate neutralizing agents to reduce the effect of potential carryover of antimicrobial interventions on *Salmonella* in raw poultry product samples (US Department of Agriculture Food Safety and Inspection Service, 2016a; US Department of Agriculture Food Safety and Inspection Service, 2021d). FSIS developed Study 3 to evaluate the addition of a transport buffer, BPW or nBPW, to the cloth sampling device before shipping to potentially improve AC and *Salmonella* recovery. In this study, FSIS evaluated cloth samples in the laboratory using both the BPW and nBPW transport buffers during simulated shipping and compared the cloth samples to a simulated N60 excision method. Study 3 demonstrated that adding nBPW buffer increased AC and *Salmonella* recovery in a laboratory setting that simulated shipping (Fig. 4). Although a 0.16 $\log_{10}$ increase in AC recovery is not a large difference, FSIS hypothesized that the increase in AC recovery from field samples would be even greater since laboratory samples were not subjected to stress from antimicrobial treatments that are used during beef slaughter and processing. A transport buffer can also help stabilize and recover sublethally injured pathogens that may otherwise be inactivated by antimicrobial carryover during shipping.

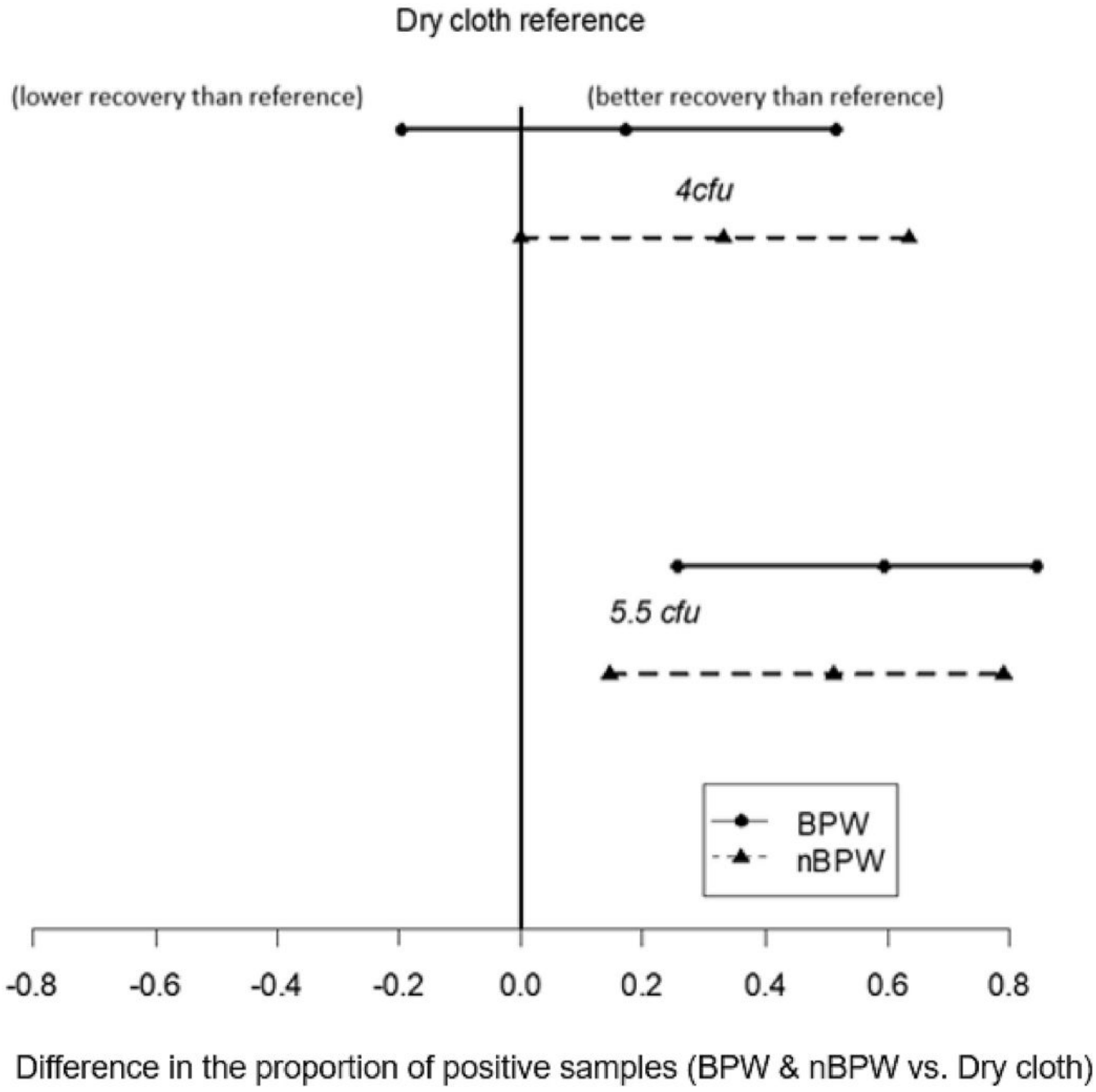

Figure 5. Summary of the effect on the recovery of *E. coli* O157 of adding either BPW or nBPW to the cloth prior to simulated shipping in comparison to the dry cloth (Study 4). The vertical dashed black line represents the equivalence of the proportion of positive samples (i.e., no difference) between adding either BPW or nBPW to the cloth compared to the dry cloth alone. The triangles (nBPW cloth) and dots (BPW cloth) reflect the proportional changes associated with adding the buffers (median and 95 percent confidence limits) compared to dry cloth. A difference is significant at the 0.05 level if the confidence interval does not intersect the dashed vertical line. Adjustments for false discovery rate are not included.

Table 6
Study 5 results from *Salmonella* analysis of 4,590 paired samples for the cloth with nBPW and N60 excision sampling methods

Cloth, nBPW	N60 Excision		
	Positive	Negative	Total
Positive	49	43	92
Negative	52	4,446	4498
Total	**101**	**4489**	**4590**

The seven adulterant STEC serogroups are important analytes for FSIS verification sampling in raw beef intended for nonintact use. Since FSIS had not used a transport buffer as part of a beef sample analysis method previously, FSIS conducted Study 4 to ensure that the transport buffer options did not inhibit adulterant STEC recovery when using MLG Chapter 5C. The results from Study 4 show that both BPW and nBPW recovered as much or more STEC compared to the dry cloth (Table 5; Fig. 5). Because these STEC inoculation levels fall below the formal LOQ, the appropriate statistical endpoint is detection of at least one viable cell. Fisher's exact test and the Beta-binomial

posterior simulations used in these analyses rely solely on binomial presence/absence evidence and do not assume quantitative accuracy above LOQ. These methods directly incorporate Poisson variability in the number of organisms per cloth, producing valid and robust inference for low-dose enrichment-based detection.

For Study 5, FSIS selected nBPW for its broad antimicrobial neutralizing capability and the operational benefit of using one transport buffer for numerous meat and poultry sampling programs.

FSIS conducted Study 5 using the nBPW transport buffer to evaluate the efficacy of adding the transport buffer to cloth samples collected in the field by IPP before shipping to the laboratories. By April 2022, FSIS had collected sufficient samples to determine that the cloth with nBPW recovered significantly more AC ($+0.38$ $\log_{10}$) than N60 excision; therefore, FSIS suspended AC analysis (Fig. 2; Table 1). However, FSIS continued to test samples for *Salmonella*; continuing this testing was twofold. First, *Salmonella,* like STEC, is an enteric pathogen that colonizes the gut and can contaminate meat products through contact with gastrointestinal tract contents. Therefore, FSIS considered *Salmonella* to be a better indicator of potential STEC recovery from raw beef trim products than AC. Second, more samples were needed to determine statistically significant results for *Salmonella* recovery due to the low percent positive in raw beef trim products (approximately 2%) compared to AC. Table 6 shows the results of 4,590 paired *Salmonella* analyses that cloth with nBPW recovers *Salmonella* similarly to recovery of *Salmonella* with N60 excision. Cloth detected 43 *Salmonella*-positive lots that the N60 excision missed, while N60 excision detected *Salmonella* in 52 lots of beef manufacturing trimmings that cloth missed (Table 6). Based on these results, FSIS concluded that the low prevalence and nonhomogeneous distribution of *Salmonella* in raw beef trim products increases the risk of false negative sampling results by both methods. However, N60 excision has been accepted as an industry and inspection standard sampling method for many years.

FSIS also considered changes to the *Salmonella* recovery by month (Fig. 3). The greatest difference in *Salmonella*-positive samples between methods occurred in October 2021, when N60 excision detected 11 positives in 243 samples, and the cloth with nBPW only detected four positives in 243 samples. FSIS hypothesized that the reduced cloth sampling efficacy in October 2021 may be because it was the first month new instructions were provided for IPP to add two additional folds to the cloth before returning it to the bag and adding nBPW, which was not included in the training video used at the time. After the first month, the laboratory observed receiving more cloth samples with the additional folds needed to submerge the cloth in the nBPW, which is also supported by the observed increased recovery of *Salmonella* by cloth with nBPW (Fig. 3).

For Study 2 and Study 5, FSIS plotted analyte recovery by month to visualize the difference in recovery before and after adding nBPW before shipping samples from the field to the laboratories. Figure 2 shows the monthly mean $\log_{10}$ AC results, while Figure 3 shows monthly *Salmonella* results. Cloth with nBPW appears to generally recover higher mean $\log_{10}$ AC than the dry cloth. However, FSIS conducted Studies 2 and 5 over several seasons, and external factors such as weather may have impacted cattle conditions entering slaughter and therefore impacted the microbial load on the finished raw beef trim products (Barkocy-Gallagher et al., 2003). A more appropriate comparison is to consider the three months of the year in which both dry cloth and cloth with nBPW were evaluated: January to March (2021 and 2022). In these three months, the data show that cloth with nBPW consistently recovered a higher monthly mean AC (2022) compared to dry cloth than it had in the previous year (2021) (Fig. 2). When considering *Salmonella* recovery, the dry cloth did not recover more *Salmonella* than the N60 sampling. However, the cloth with nBPW recovered more *Salmonella* in six of the 16 months in Study 5 (Fig. 3; Nov. 2021, Dec. 2021, Mar. 2022, July 2022, Sept. 2022, Jan. 2023). This is another metric FSIS used to support that adding nBPW to the cloth after sampling increased bacterial recovery and pathogen detection. Across the 5 studies described in this paper, FSIS completed more than 23,000 laboratory tests to evaluate the effectiveness of surface sampling methods to recover *Salmonella* and to enumerate AC from raw beef trim products. These data provide support for FSIS replacing N60 excision with a surface sampling method.

A limitation of these studies is that although STEC is a key analyte for FSIS' verification sampling program in raw beef trim products, STEC was only included in Studies 1 and 4 and was not evaluated in-field. FSIS conducted Study 1 to evaluate the respective ability of a cloth sampling method and a sponge sampling method to recover STEC inoculated at low levels onto raw beef trim slices compared to a simulated N60 excision sampling method. FSIS selected the cloth sampling method for additional review in Study 2 because there was no difference in *E. coli* O157:H7 or O103 recovery, although the cloth sampling device recovered significantly less O121. The limited sample size in Study 1 would not, on its own, have been enough to support STEC recovery. Study 4 did not consider the cloth's ability to recover STEC, but only that the presence of a transport buffer did not inhibit STEC recovery. In Study 2 and Study 5, FSIS did not test the cloth sampling device for STEC because industry has made great improvements in reducing STEC, making its isolation from raw beef manufacturing trimmings a rare event, and the low number of detections would not have provided sufficient statistical power with the planned sample sizes in each study (US Department of Agriculture Food Safety and Inspection Service, 2020a). Instead focusing a large amount of agency resources in these studies on looking for low numbers of STEC, FSIS relied on research conducted by ARS to evaluate the cloth's effectiveness to recovery STEC in paired method studies and in the reviewing FSIS noncompliance reports from establishments that may have already adopted cloth sampling in their own verification sampling programs (US Department of Agriculture Food Safety and Inspection Service, 2022).

In several experiments, ARS scientists collected 1,650 paired (cloth sampling and N60 excision) samples from beef processing establishments, then analyzed them for the presence of select virulence associated genes (hemolysin, five nonadulterant O serogroups (O55, O113, O117, O126, and O146), intimin, heme receptor, adhesion siderophore, tetA, and tetB) to act as index targets—measures that would correlate with the percent positive of STEC and *Salmonella* (Arthur & Wheeler, 2021). First, the authors observed no difference in the percent positive for pathogen index targets from product at two lean types, between the cloth manual sampling device and N60 excision method ($n = 185$) (Wheeler & Arthur, 2018). When compared to N60 Plus™, on combo bins with a smaller surface area, the cloth manual sampling device had a higher percent positive for the heme receptor gene target (52.5 versus 25 percent) and recovered 0.3 $\log_{10}$ more APC/sample ($p < 0.05$; $n = 40$) (Arthur & Wheeler, 2021). In a further experiment on smaller surface area combo bins, the cloth manual sampling device method recovered more O serogroup-positive samples than the N60 Plus™ (86% and 64%, respectively; $p < 0.05$), and 0.2 $\log_{10}$ more *Enterobacteriaceae* than N60 Plus™ ($n = 80$). There was no difference between the cloth manual sampling device and N60 Plus™ recovery of five other pathogen index target genes and APC. In one final experiment, there were no significant differences among the three sample collection methods (continuous cloth sampling device, manual cloth sampling device, and N60 Plus™) for any of the pathogen index gene targets ($n = 80$). As a result, ARS concluded that their study supports the cloth sampling method for robust pathogen detection, including STEC.

In addition to reviewing published research, FSIS conducted a qualitative review of noncompliance reports (NRs) for establishments failing to detect STEC when FSIS verification sampling detected an STEC-positive sample result (US Department of Agriculture Food Safety and Inspection Service, 2022). Some establishments began using the cloth sampling method in 2017, after FSIS issued a letter of no objection to

allow industry to use cloth sampling methods for microbiological sampling of raw beef trim product based on data later published (Wheeler & Arthur, 2018). Yet, industry more widely adopted cloth sampling after March 2020, when FSIS issued a second letter of no objection for in-plant validation procedures for cloth sampling, publishing the submitted data in 2021 (Arthur & Wheeler, 2021). Large volume beef processing establishments adopting cloth sampling methods for their ongoing verification sampling presented an opportunity to compare FSIS' ongoing N60 excision sampling with establishments' cloth sampling for STEC. An analysis of NRs among 44 large volume raw beef establishments (each producing >250,001 lbs. beef trim/day) showed that industry adopting cloth sampling did not increase NRs due to missed STEC-positive lots (before cloth implementation = 8 NRs from 6 large volume raw beef establishments; after establishments began cloth sampling = 4 NRs from 4 large volume raw beef establishments). Most of the NRs issued after cloth sampling implementation were due to the establishments only testing for *E. coli* O157:H7 and failing to detect non-O157 adulterant STEC-positive product (US Department of Agriculture Food Safety and Inspection Service, 2022).

Careful consideration of these studies and the analyses detailed in this paper led FSIS to conclude that there is no difference in microbial recovery between the cloth sampling method with nBPW and N60 excision. Further, the cloth sampling method with nBPW is the regulatory equivalent to N60 excision. On November 22, 2022, FSIS announced it intended to use the cloth sampling method in place of N60 excision to sample domestic beef manufacturing trimmings and bench trim for adulterant STEC and *Salmonella* beginning February 1, 2023 (US Department of Agriculture Food Safety and Inspection Service, 2022). FSIS did not implement any changes to the sample collection method for frozen imported products or any domestic raw beef products other than beef manufacturing trimmings and bench trim. At the time of writing, the ability of the cloth sampling device to recover bacteria from frozen beef products had not been evaluated. Further, ARS scientists recommended not to sample frozen beef trim products with the cloth since there is no liquid for the cloth sampling device to absorb and collect (personal communication), and these authors agree. FSIS will continue to review scientific literature and work with research partners to evaluate sampling improvements for imported raw beef products.

FSIS also based its decision to replace N60 excision with cloth sampling with nBPW on the practical operational impacts of cloth sampling with nBPW. In Fiscal Year (FY) 2022, FSIS planned to collect 20,023 verification samples and conduct 52,824 tests on raw beef products (US Department of Agriculture Food Safety and Inspection Service, 2023b). After adopting cloth sampling during FY2023, for FY2024, FSIS planned to collect the same number of beef trim product verification samples as FY2022, but allocated 15,552 fewer tests due to reduced need for subsamples by switching to the cloth sampling method (US Department of Agriculture Food Safety and Inspection Service, 2023b). Eliminating subsamples saves FSIS money on laboratory supplies and reduces the analyst time required to obtain sample results. Moreover, the cloth sampling method allows FSIS to sample without destroying product, which reduces food waste by about 907 g (2 pounds) per sample (US Department of Agriculture Food Safety and Inspection Service, 2022). If FSIS had been using the cloth sampling method instead of N60 excision in FY2022, the agency would have avoided removing approximately 4,700 kg (10,340 pounds) of raw beef from commerce for microbiological verification testing (907 g or 2 pounds per sample times 3,713 beef manufacturing trimmings and 1,459 bench trim samples; US Department of Agriculture Food Safety and Inspection Service, 2023c).

The cloth sampling method may also reduce IPP injuries, as they will no longer be using knives to sample product. Cloth sampling is safe without personal protective equipment (PPE) like the cut-resistant gloves required for N60 excision sampling (US Department of Agriculture Food Safety and Inspection Service, 2024b), and aligns with August 1, 2022, FSIS Memorandum of Understanding with the Department of Labor (US Department of Agriculture Food Safety and Inspection Service, 2022c). Cloth sampling also decreases sample collection time. FSIS allocates 30 min of direct inspection time and 54 min of indirect time for conducting all sampling tasks, except the N60 excision task, which is allotted 60 and 108 min for direct and indirect time, respectively (US Department of Agriculture Food Safety and Inspection Service, 2020d). By switching to cloth sampling, FSIS reduced the total inspection time for each beef trim product sample by 84 min and reallocated approximately 7,240 inspection hours per year to other food safety tasks.

Conclusions

In conclusion, the cloth sampling procedure with an nBPW transport buffer performed equally well for FSIS inspection verification sampling as N60 excision. The cloth with nBPW sampling method has replaced the N60 excision sampling method for FSIS' domestic verification sampling of raw beef manufacturing trimmings and bench trim, allowing the agency to more efficiently serve the American people while ensuring food safety, wholesomeness, proper labeling, and humane slaughter in the production of meat under the FMIA and Humane Methods of Slaughter Act. Overall, adopting the cloth sampling procedure with nBPW has enabled FSIS to optimize the allocation of resources, including supplies, shipping costs, analysis time, and inspection verification activities. This method change is also an important step forward to reduce food waste caused by removing products from commerce for FSIS verification testing and efficiently optimizes government resources while protecting public health.

CRediT authorship contribution statement

Susan R. Hammons: Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Conceptualization. **Natalie Baker:** Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Eric D. Ebel:** Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Lorenza Rozier:** Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Lisa Jacobsen:** Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Hannah Anderson:** Writing – original draft, Validation, Resources, Methodology, Investigation, Data curation, Conceptualization. **Sabrina Wiggins:** Writing – original draft, Visualization, Methodology. **Michael S. Williams:** Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Selena Kremer-Caldwell:** Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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